Cellular and molecular studies on the mechanistic basis of clinical radioresistance in human glioma.

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A thesis presented for the degree of Doctor of Philosophy,
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Abstract

Human malignant glioma exhibits considerable clinical radioresistance. It has been postulated that the phenomenon of intrinsic cellular radioresistance might underlie treatment failure in these tumours.

The phenomena of cellular radioresistance has been investigated in this thesis, using as models both continuous human glioma-derived cell lines, and primary cultures.

The cellular radiosensitivity of five continuous glioma lines has been evaluated by clonogenic assay; this has been compared with results obtained in eight other human tumour cell lines, derived from a range of human tumour types of varying clinical radiocurability, including embryonic tumours and carcinomas. Parameters of cellular radiosensitivity were derived by fitting multtarget, and linear-quadratic models of radiation action to survival data; the model-free parameter of cellular radiosensitivity, the surviving fraction of clonogenic cells at the clinically relevant dose of 2Gy (SF2), was also measured.

Considerable heterogeneity of intrinsic cellular radiosensitivity was observed in the thirteen human tumour lines studied. All five continuous glioma lines displayed values of cellular radiosensitivity within the ranges previously observed in radioresistant human cell lines. By comparison, cell lines derived from three embryonic tumours exhibited marked cellular radiosensitivity. Considerable overlap in cellular radiosensitivity was noted between five carcinoma-derived lines, and the human glioma lines. Cellular radiosensitivity of 9 primary human glioma cultures was also assessed by soft agar cloning. The intrinsic cellular radiosensitivity of both primary cultures, and continuous cell lines derived from human malignant glioma were observed to fall within the range of values previously reported for radioresistant human tumours; it is therefore suggested that intrinsic cellular radioresistance might contribute to the profound clinical radioresistance observed in human glioma.

Cellular recovery from ionising radiation damage has been proposed as a determinant of intrinsic radiosensitivity. The ability of human tumour cell lines of differing intrinsic radiosensitivity to recover from sublethal radiation damage (SLD) was investigated by clonogenic cell survival following split dose irradiation. Five glioma lines exhibited an ability to recover from sublethal radiation damage, which increased with radiation dose, as predicted by the linear-quadratic model of radiation action. SLD recovery was assessed in three radiosensitive human cell lines, derived from a teratoma, SUSa, a neuroblastoma, IMR32, and an ovarian carcinoma A2780. All three lines exhibited cellular recovery, but the extent of
recovery at iso-effective radiation dose levels producing 90% cell kill were lower than observed in the glioma lines. Values for recovery half-times obtained in the glioma cell lines did not differ significantly from those obtained in the three radiosensitive cell lines. Proficient recovery from sublethal damage may therefore contribute to the cellular radioresistance observed in human glioma cell lines.

The molecular basis of the differences in radiosensitivity observed in human tumours remains unclear. A body of evidence supports the notion that DNA double strand breaks (dsb) are a class of radiation-induced lesions which can lead to both chromosomal aberrations, and cell death. An in vitro assay has been established to assess the ability of nuclear protein extracts derived from human cells to rejoin dsb. The model dsb substrate was plC20H, a 2.7Kb recombinant plasmid DNA molecule bearing two bacterial genes encoding ampicillin resistance and β-galactosidase activity; dsb of defined end-structure were produced by restriction endonuclease digestion to yield both cohesive and noncohesive termini for rejoining reactions. Rejoining activity was assessed both qualitatively, and quantitatively. Conformation of the plasmid substrate molecule was evaluated following rejoining reactions by electron microscopy, and Southern blotting. Accurate dsb rejoining enabled the plasmid molecule to confer both ampicillin resistance and β-galactosidase activity on JM83 E.coli transformants. A protein concentration-dependent increase in rejoining of cohesive dsb (EcoRI) was detected in nuclear extracts from 4 human tumour lines studied; no significant rejoining activity for a noncohesive terminus (NruI) was observed in any extracts. No significant difference has been detected in maximal rejoining or rejoin fidelity of cohesive dsb mediated by extracts derived from 2 radiosensitive and 2 radioresistant human tumour cell lines. Such an in vitro system provides a powerful tool for the further analysis of the molecular mechanisms involved in the recognition, and repair of ionising radiation-induced DNA lesions.
I would like to dedicate this thesis to the memory of two people who have
influenced my professional career significantly; my mother, Elizabeth Ridley Ross,
and Ralph Wright, Professor of Medicine, University of Southampton.
# TABLE OF CONTENTS

| Title page | 1 |
| Abstract   | 2 |
| Dedication | 4 |
| Table of contents | 5 |
| Index of tables | 6 |
| Index of figures | 8 |
| Abbreviations | 10 |
| Acknowledgements | 11 |
| Declaration | 12 |

**Chapter one:**
Human malignant glioma as a model for the study of clinical radioresistance. 13

**Chapter two:**
Materials and methods. 70

**Chapter three:**
Cellular biology of human glioma lines. 88

**Chapter four:**
Intrinsic cellular radiosensitivity of human glioma cell lines. 128

**Chapter five:**
Cellular radiation recovery potential of human glioma. 174

**Chapter six:**
Cellular radiosensitivity of primary cultures of human glioma. 201

**Chapter seven:**
DNA double strand break rejoining mediated by cell-free extracts of human tumours of differing radiosensitivity. 220

**Chapter eight:**
Summary of conclusions. 272

References 277
LIST OF TABLES

Chapter 1

1.1. Mechanisms influencing clinical radioresistance in human glioma 23
1.2. Molecular and chromosomal abnormalities in human glioma 35
1.3. Ranking of radiocurability of human tumours 40
1.4. Cellular radiosensitivity of human glioma 45
1.5. Human syndromes associated with hypersensitivity to DNA damage 48
1.6. Cloned human DNA repair genes 53

Chapter 2

2.1. Tissue culture requirements of continuous human tumour cell lines 72

Chapter 3

3.1. Review of characterised human glioma cell lines. 91
3.2. Origins of continuous lines and primary human glioma cultures. 93
3.3. Phenotypic and genetic characteristics of 5 continuous human glioma cell lines. 112

Chapter 4

4.1. Acute radiation survival curve parameters: human glioma cell lines. 153
4.2. Acute radiation survival curve parameters: human carcinoma cell lines. 154
4.3. Acute radiation survival curve parameters: human embryonal cell lines. 155
4.4. Theoretical radiocurability of human glioma as a function of in vitro estimates of intrinsic radiosensitivity, and tumour doubling times. 160
4.5. Theoretical radiocurability of human carcinomas as a function of in vitro estimates of intrinsic radiosensitivity, and tumour doubling times. 161
4.6. Theoretical radiocurability of human embryonic tumours as a function of in vitro estimates of intrinsic radiosensitivity, and tumour doubling times. 162
4.7. Analysis of variance of SF2Gy values 163

Chapter 5

5.1. Potentially lethal damage repair (PLD), in human tumour cell lines, in vitro. 179
5.2. Maximal recovery ratio at 3 radiation dose levels for 8 human cell lines. 186
5.3. Split dose recovery and recovery kinetics for 8 human tumour lines. 187
5.4. Comparison of clonogenic recovery achieved at isoeffective radiation
dose levels in 8 human cell lines.

Chapter 6
6.1 Surviving fraction at 2Gy in primary cultures of human glioma.

Chapter 7
7.2 Evaluation of background transformation by cut plC20H substrates, and suitability for religation.
7.3. Maximal ligation activity of human tumour extracts; EcoR1 and Nru1 cut plC20H substrates.
7.4. Proportion of white vs blue bacterial transformations following treatment of EcoR1-cut plasmid with human tumour nuclear extracts.
### List of figures.

#### Chapter 3

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Morphology of human glioma cell lines, SB18, and U251</td>
<td>102</td>
</tr>
<tr>
<td>3.2</td>
<td>Morphology of human glioma cell lines, UVW, and CCM</td>
<td>103</td>
</tr>
<tr>
<td>3.3</td>
<td>Morphology of human glioma cell line, IJK, and IJK multicellular spheroid</td>
<td>104</td>
</tr>
<tr>
<td>3.4</td>
<td>Morphology of early passage(1) BG 488.</td>
<td>105</td>
</tr>
<tr>
<td>3.5</td>
<td>Monolayer growth curves of 5 glioma cell lines</td>
<td>107</td>
</tr>
<tr>
<td>3.6</td>
<td>Fluorescence micrograph of cell line UVW, CD 44 staining.</td>
<td>109</td>
</tr>
<tr>
<td>3.7</td>
<td>Fluorescence micrograph of cell line CCM, GFAP staining.</td>
<td>110</td>
</tr>
<tr>
<td>3.8</td>
<td>Fluorescence micrograph of cell line SB18, GFAP staining.</td>
<td>111</td>
</tr>
<tr>
<td>3.9</td>
<td>Electron micrograph of IJK and G-MCN xenografts.</td>
<td>115</td>
</tr>
<tr>
<td>3.10</td>
<td>Gel electrophoresis, and staining for LDH isoenzymes in 6 human glioma lines</td>
<td>117</td>
</tr>
<tr>
<td>3.11</td>
<td>Flow cytometry profiles of 5 glioma cell lines</td>
<td>119</td>
</tr>
<tr>
<td>3.12</td>
<td>Karyotypes of cell lines UVW, CCM.</td>
<td>121</td>
</tr>
<tr>
<td>3.13</td>
<td>Karyotypes of cell lines U251, SB 18.</td>
<td>122</td>
</tr>
<tr>
<td>3.14</td>
<td>Criteria of malignancy and glial lineage in vitro.</td>
<td>124</td>
</tr>
</tbody>
</table>

#### Chapter 4

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Acute radiation survival data for human glioma cell line, U251</td>
<td>144</td>
</tr>
<tr>
<td>4.2</td>
<td>Acute radiation survival data for human glioma cell line, UVW</td>
<td>145</td>
</tr>
<tr>
<td>4.3</td>
<td>Acute radiation survival data for human glioma cell line, SB18</td>
<td>146</td>
</tr>
<tr>
<td>4.4</td>
<td>Acute radiation survival data for human glioma cell line, IJK</td>
<td>147</td>
</tr>
<tr>
<td>4.5</td>
<td>Acute radiation survival data for human glioma cell line, CCM</td>
<td>148</td>
</tr>
<tr>
<td>4.6</td>
<td>Acute radiation survival data for 3 human embryonal cell lines</td>
<td>149</td>
</tr>
<tr>
<td>4.7</td>
<td>Acute radiation survival data for 5 human carcinoma cell lines</td>
<td>150</td>
</tr>
<tr>
<td>4.8</td>
<td>Acute radiation survival data for 13 human tumour cell lines</td>
<td>152</td>
</tr>
<tr>
<td>4.9</td>
<td>Clonogenic surviving fraction at 2Gy for 13 human tumour cell lines</td>
<td>157</td>
</tr>
<tr>
<td>4.10</td>
<td>TCD-50 doses calculated for human tumours of a given histological type, based on in vitro SF2 data</td>
<td>159</td>
</tr>
</tbody>
</table>

#### Chapter 5

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>Relationship between acute radiation sensitivity and cellular recovery capacity measured at isoeffective radiation doses.</td>
<td>190</td>
</tr>
<tr>
<td>5.2</td>
<td>Split dose recovery achieved at 3 different dose levels, plotted as a function of 2d², in 5 human glioma lines.</td>
<td>192</td>
</tr>
<tr>
<td>5.3</td>
<td>Split dose recovery achieved at 3 different dose levels, plotted as a</td>
<td>192</td>
</tr>
</tbody>
</table>
function of 2d^2: cell lines A2780, IMR32, SuSa.

5.4. Relationship between recovery ratio, bRR, and recovery kinetics in 8 human tumour lines.

**Chapter 6.**

6.2. Relationship between colony forming efficiency and SF2Gy values

**Chapter 7**

7.1. Restriction map of the plasmid DNA molecule plC20H.
7.2. In vitro plasmid rejoining reaction protocol
7.3. Gel retard analysis of extract protein DNA binding activity.
7.4. Electron micrograph of plC20H test and control ligation products.
7.5. Maximal bacterial transformation mediated by plC20H rejoining reaction products.
7.6 Dose dependent increase in bacterial transformation: SB 18 extracts
7.7 Dose dependent increase in bacterial transformation: IUK extracts
7.8 Dose dependent increase in bacterial transformation: IMR 32 extracts
7.9 Dose dependent increase in bacterial transformation: A2780 extracts
7.10 Misrepair events in rejoining of EcoR1 cut plC20H by human cell extracts
7.11 Southern blot of plC20H DNA; effect of tumour extracts on uncut plasmid
7.12 Southern blot of plC20H DNA; linear and T4 controls, SB18 mediated ligation, and V79 concatemers.
7.13 Southern blot of plC20H DNA; protein concentration dependent increase in formation of rejoined, supercoiled plasmid; tumour extract SB18.
7.14 Southern blot of plC20H DNA; formation of supercoiled ligation products by IMR32 extract.
7.15 Proposed schema for processing of plC20H plasmid molecules by human extracts, and relationship to bacterial transformation events.
**Abbreviations.**

AT Ataxia telangectasia
A,T,C,G. Adenine, thymine, cytosine, guanine
ATP Adenosine triphosphate
DNA Deoxyribonucleic acid
DSB DNA double strand breaks
DTT Dithiothreitol
EDTA Ethylenediamine tetra-acetic acid
FCS Fetal calf serum
g,ng,mg. grams, nanograms, micrograms
GFAP Glial fibrillary acidic protein
Gy,cGy Gray, centiGray
irs ionising radiation-sensitive Chinese hamster ovary mutant cell lines
kb kilobase pairs
LQ Linear-quadratic model
MT Multitarget model
M,mM Molar, millimolar
NaHCO₃ Sodium bicarbonate
OD₂₆₀ optical density at 260 nanometers
PBS Phosphate buffered saline
PEG Polyethylene glycol
RE restriction endonucleases
RTOG Radiotherapy and Oncology Research Group, USA.
SDS Sodium dodecyl sulphate
SF₂Gy surviving fraction of clonogenic cells, after 2 Gray dose of radiation
TNM Tumour, Node, Metastasis staging system
UICC International Union against Cancer
UV ultraviolet radiation
XP xeroderma pigmentosum
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**Declaration.**

I declare that the work contained within this thesis is the result of my own observations, undertaken during the period of my supervised research training at the University of Glasgow.

Included in this thesis are some data obtained in collaboration with other workers, or for which substantial technical assistance was required.

1) Human glioma xenograft immunohistochemistry, and electron microscopy were kindly performed by the Department of Neuropathology, Institute of Neurological Sciences, Glasgow, in collaboration with Prof. D. Graham.

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5) The analysis of tumour control probability based on in vitro radiosensitivity data was carried out by Dr. Tom Wheldon.
Chapter 1

Human malignant glioma as a model for the study of clinical radioresistance.
"These are tumours which usually recur soon after surgical removal, even after extensive removal of the involved area of the brain en bloc. The lesion has infiltrating propensities, and from its behavior it is natural that it should have been looked upon as a 'gliosarcoma', and has been so designated in former days. When first exposed, the tumour may sometimes have the misleading appearance of enucleability, but when enucleation is attempted, a growth is found at the depth to spread into and merge with the normal cerebral tissue without recognizable demarcation......Operative procedures, howsoever radical (block extirpations repeated on signs of recurrence; saturation with the X-rays or radium emanations after wide decompression with or without surgical interference with the tumour), have done little more than to prolong life, save vision, and alleviate headache for an average of a few months."

1926 Bailey and Cushing
Human malignant glioma as a model for the study of clinical radioresistance. .................................................................................................. 13

1.1. Role of radiotherapy in the treatment of glioma

1.1.1. The concept of clinical radioresistance. ....................... 18
1.1.2. Development of radiotherapeutic management .......... 19
1.1.3. Prognostic factors influencing outcome following radiotherapy ................................................................. 20
1.1.4. Clinical evidence of a radiation dose-response in glioma ............................................................................... 20
1.1.5. Causes of clinical radioresistance in human glioma. ....... 22

1.2. In vitro models of human glioma ........................................ 29
1.2.1. Heterogeneity in glioma cell lines. .............................. 30
1.2.2. Markers of the glial phenotype. ................................ 30
   1.2.2.1. Glial fibrillary acidic protein expression ................. 31
   1.2.2.2. Cell surface ganglioside expression in human glioma ............................................................... 31
   1.2.2.3. Production of glutamine synthetase .................... 33
1.2.3. Cytogenetics of human glioma cell lines ...................... 33
1.2.4. Does clinical tumour grade influence establishment of cell lines? .............................................................. 36

1.3. How is cellular radiosensitivity measured? ...................... 36
1.3.1. Radiation cell killing and reproductive cell death .......... 36
1.3.2. The clonogenic cell survival assay .............................. 37
1.3.3. The radiation cell survival curve ................................ 37
1.3.4. The concept of intrinsic cellular radiosensitivity .......... 38
1.3.5. Predictive assays of tumour response ......................... 42
1.3.6. Predictive testing of normal tissue radiosensitivity ...... 43
1.3.8. Evidence for cellular radioresistance in human glioma .... 44
1.3.9. Inherent radiosensitivity of both normal tissue and tumour as predictors of radiotherapy response .................... 47

1.4. The study of DNA repair in mammalian and human cells and its role in determination of cellular radiosensitivity .......... 47
1.4.1. Human syndromes with hypersensitivity to DNA damaging agents .......................................................... 47
1.4.2. Ataxia telangiectasia as a model of human ionising radiation hypersensitivity ........................................ 49
1.4.3. DNA repair systems .................................................. 50
   1.4.4.1 DNA excision repair pathways ............................... 51
   1.4.4.2. Recombination ...................................................... 52
1.4.5. Identification of DNA repair genes ............................. 52
1.4.6. Rodent mutants selected for ionising radiation hypersensitivity ................................................................. 54
1.4.7. Evidence for the importance of the DNA double strand break in determining cellular radiosensitivity ..................... 55
1.4.8. Methods for the quantitative study of DNA repair .......... 57
1.4.9. The contribution of recombinant DNA techniques and in vitro systems to the study of DNA repair ....................... 59
  1.4.9.1. The use of exogenous plasmid or viral DNA as a repair substrate .......................................................... 59
  1.4.9.2. Restriction endonucleases as models of ionising radiation-induced DNA damage ........................................... 62
1.4.10. Concept of DNA repair fidelity ............................................. 62
1.4.11. Use of in vitro systems to study DNA repair in mammalian cells .................................................................... 63
  1.4.11.1. Excision repair in vitro ................................................. 64
  1.4.11.2. Application of in vitro repair systems to the study of DNA dsb rejoining .................................................... 65

1.5. Rationale for the work undertaken in this thesis ............... 67
1.1. Role of radiotherapy in the treatment of glioma: 
Development of practice

The therapy of malignant glioma continues to pose major challenges to tumour 
biologists and clinicians alike. Central nervous system tumours comprise 9% of all 
adult neoplasms; of these, 40% are glioma, of varying grade of malignancy. 
Hence an average regional neuroscience and neuro-oncology practice serving a 
population of 3 million might be expected to diagnose and treat 100 new cases of 
malignant glioma per annum.

Although considerably rarer than the major epithelial malignancies, such as 
carcinomas of the lung, colon, breast, and prostate, malignant glioma has a 
particularly difficult clinical course. It is often diagnosed when producing significant 
impairment of neurological function, in a younger cohort of patients than the 
aforementioned tumours. Moreover, there are as yet few clues as to the aetiology 
of these tumours to guide any form of "preventive" intervention, nor prospect of 
 diagnosis at a pre-malignant stage of the disease. It is likely that many cases will 
continue to present with overt neurological deficits, and bulky intracranial tumours.

There has recently been speculation that the actual incidence of these tumours 
may be increasing in the elderly, though the part played by altered diagnostic 
practice in determining estimates of prevalence is not clear.

Efforts have been made by many groups to improve the outcome for patients with 
malignant glioma, both by the investigation of its biology and by the adoption of 
empirical clinical strategies based on likely mechanisms of treatment failure.

The optimal therapy of malignant glioma has been the subject of investigation by 
large cooperative groups such as the Medical Research Council, EORTC, and 
Brain Tumour Cooperative Group (BTSG) since the early 1970's. During this time 
the management of these tumours has evolved, with corresponding modest 
increments in survival.

However, despite the frequent use of regimens employing cytoreductive surgery, 
"adjuvant" external beam radiotherapy, and adjuvant chemotherapy, the prognosis 
for patients with grade III and IV glioma remains poor. Whilst combined modality 
therapy offers prolongation of median survival, usually with symptomatic benefit, 
the median survival of grade IV glioma is 40 weeks, with no survivors at 3 years.
The outcome for grade III tumours is slightly better. It is this group from which the 16% of 5 year survivors are obtained [Nelson et al, 1985].

Malignant glioma is judged to be clinically radioresponsive, but not radiocurable. Moreover, it is often cited as an example of a radioresistant human tumour [Malaise et al, 1987]. The mechanistic basis of these clinical observations are not clear, and despite many empirical attempts to circumvent radioresistance in the clinic, usually based on theoretical assumptions, there has been remarkably little direct investigation of the radiobiology of human glia.

The object of this section is to review the in vitro and clinical evidence that human glioma is at an extreme of clinical radioresistance, compared with other human tumour types, and discuss the possible mechanistic basis of radioresistance in this disease.

1.1.1. The concept of clinical radioresistance.

"For any given set of circumstances there must be an optimal point or range of volume, time, and dose yielding the maximum probability of cure"

Patterson, 1952

The clinical term "radioresponsiveness" and its in vitro counterpart "radiosensitivity" are widely used in discussion of the radiation response of both normal and malignant human tissues. The clinical therapeutic ratio at a given anatomical site is determined by the relative radiosensitivity of tumour and dose-limiting normal tissue in the treatment volume.

The concept of the variability of radioresponsiveness in clinical radiotherapy was advanced by workers at the Paterson Institute, Manchester, where clinical radiation dosimetry was pioneered. Careful clinical observation led Ralston Patterson to classify human tumour response into 3 broad categories:
Tumour response | Tumour type
---|---
1) Radioresponsive - | lymphoma; seminoma
2) Limited radiosensitivity- | squamous and adeno-carcinomas
3) Radioresistant- | sarcoma; melanoma; G-I tract adenocarcinoma

The objective of clinical radiotherapy is the eradication of tumour at a specific site with the lowest rate of treatment complication. It therefore follows that the concept of clinical radioresistance in a tumour is both operational, relative and site-dependent.

Two specific aspects of the biology of human glioma render the tumour type of particular interest to radiotherapists. Firstly, the growth pattern and extension of glioma is essentially a localised and compartmental process, with few exceptions. Secondly, the close relationship between tumour and critical normal brain render surgical extirpation with wide excision margins, the classical "cancer" operation, inapplicable.[Kaplan, 1987]

1.1.2. Development of the radiotherapeutic management of malignant glioma

The use of radiotherapy to treat glioma is recorded in the earliest observations on the use of radiation in human tumours. In 1921 Ewing made observations on the relationship between histology and radiosensitivity of different CNS tumours.

The current conventional management of high grade glioma would be considered to be optimal debulking surgery, followed by external beam radiotherapy delivering a tumour dose of approximately 60Gy, in daily fractions, over 6 weeks.

There is clear evidence from both retrospective, uncontrolled and prospective, randomised studies that a combination of surgery, and postoperative radiotherapy increases the median survival of patients, compared with surgery alone.

The first randomised prospective study demonstrating a clear survival benefit was published by the Brain Tumour Study Group (BTSG) 6901, in 1978. Patients were randomised to receive radiotherapy or radiotherapy and adjuvant BCNU following surgery. A prolongation of median survival to 36 weeks was observed in the
group receiving radiotherapy, compared to 14 weeks in those receiving supportive therapy alone [Walker et al, 1978].

1.1.3. Prognostic factors influencing outcome following radiotherapy

The clinical trials in radiotherapy of glioma have identified several important clinical prognostic factors influencing outcome as independent variables [Nelson et al, 1985; MRC Brain Tumour working party, 1990]. Four positive prognostic factors have been identified in multiple studies:

1) Tumour histological grade
2) Clinical performance status
3) Patient age
4) Extent of surgery

The predictive power of tumour grade, age, and performance status is such that some consider it desirable to prospectively stratify the assignment of patients to experimental treatment arms in clinical studies by these factors. [Hamilton, 1987]

The biological basis of these prognostic factors is poorly understood. In particular, the effect of tumour grade on response to radiation or cytotoxic chemotherapy has not been explored in vitro. It is possible that variations in the radiosensitivity of individuals influences the outcome. No study has been undertaken to assess in vitro heterogeneity of radiosensitivity in glioma. One of the aims of this study was to assess the heterogeneity of radiosensitivity in primary culture or low passage cell lines derived from human glioma.


Although few cures are achieved by adjuvant postoperative radiotherapy, three bodies of evidence would suggest a radiation dose response relationship in human glioma. These include:

a) retrospective dose-effect analyses
b) prospective, randomised studies using several dose levels
c) recent experience with stereotactic, focal boost techniques
Salazar et al reported a significant increase both in the number of survivors at 1 year and median survival in grade IV glioma, for those patients receiving a total tumour dose of 70-80Gy, when compared with a cohort receiving "standard" therapy of 50-60Gy [Salazar et al, 1976].

Walker used the pooled data from three successive BTSG studies to examine the relationship between dose of radiation received and survival in 621 patients.

<table>
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<tr>
<th>Nominal dose(rad)</th>
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<th>Median survival(wks)</th>
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<tr>
<td>0</td>
<td>194</td>
<td>18</td>
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<tr>
<td>&lt;4500</td>
<td>61</td>
<td>13.5</td>
</tr>
<tr>
<td>5000</td>
<td>56</td>
<td>28</td>
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<tr>
<td>5500</td>
<td>33</td>
<td>36</td>
</tr>
<tr>
<td>6000</td>
<td>270</td>
<td>42</td>
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</table>

An increment in survival of 1.3 was noted with the increase in dose from 5000-6000 rads. However, it should be noted that the period over which patients were accrued to the three studies in this meta-analysis was 1966-1975, with most of those receiving the higher radiotherapy doses treated in the last years (BTSG 72-01). Despite these limitations, it was concluded that evidence of a dose-response relationship did exist [Walker et al, 1979].

The first study addressing dose-response in a prospective manner was conducted by the RTOG-ECOG, and reported by Chang in 1983. Two hundred and fifty three patients were randomised between two arms, to receive 60Gy in 6-7 weeks, or 70Gy in 7-8 weeks with conventional daily fractions. No statistical difference was found between the two arms, with a lower median survival in the higher dose cohort (8.2 months vs. 9.3 months)[Chang et al, 1983].

Within the last five years there has been a resurgence of interest in clinical dose-response relationships in the management of glioma, with the development of stereotactic technology, and major improvements in the radiological localisation of CNS tumours. Computerised tomography and magnetic resonance imaging can both be employed to construct 3D tumour volumes. Combined with stereotactic frames much more precise localisation of tumours is obtained. This approach has had a major impact on the accuracy of tumour biopsy, and offers the ability to define target volumes for either interstitial or external beam radiotherapy, using stereotactic localisation[Kelly, 1987].
The results are now awaited from those studies employing higher total tumour
doses by stereotactic techniquest, as primary treatment. This information should
contribute further to our understanding of radiation dose-response relationships in
normal CNS and glioma.

1.1.5. Causes of clinical radioresistance in human glioma.

On the basis of clinical experience human glioma exhibits considerable
radioresistance, relative to many human tumour types. The purpose of this section
is to review the theoretical basis of such resistance. The results of clinical studies
which have been designed to circumvent a particular mode of resistance are
presented, and the results of in vitro studies pertinent to specific mechanisms are
discussed.

Mechanisms of clinical radioresistance are usually grouped under three
categories:

1) Tumour related factors
2) Host related factors
3) Technical factors

These factors, and clinical strategies adopted to circumvent them are shown in
table:1.1.
Table 1.1. Mechanisms influencing clinical radioresistance in glioma

<table>
<thead>
<tr>
<th>1) TUMOUR FACTORS</th>
<th>CLINICAL STRATEGY</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Size</strong></td>
<td></td>
</tr>
<tr>
<td>total clonogen number</td>
<td>Combined cytoreduction</td>
</tr>
<tr>
<td></td>
<td>surgery,</td>
</tr>
<tr>
<td></td>
<td>radiation</td>
</tr>
<tr>
<td></td>
<td>chemotherapy</td>
</tr>
<tr>
<td>kinetics</td>
<td></td>
</tr>
<tr>
<td>cell contact effect</td>
<td>Low dose rate radiation</td>
</tr>
<tr>
<td>metabolism</td>
<td></td>
</tr>
<tr>
<td><strong>Kinetics</strong></td>
<td></td>
</tr>
<tr>
<td>% $G_0$ clonogens</td>
<td>Accelerated radiation</td>
</tr>
<tr>
<td>repopulation</td>
<td>Hyperbaric O2</td>
</tr>
<tr>
<td>cell cycle distribution</td>
<td>Electron-affinic drugs</td>
</tr>
<tr>
<td><strong>Hypoxia</strong></td>
<td></td>
</tr>
<tr>
<td>cellular radioresistance</td>
<td></td>
</tr>
<tr>
<td>Increase in $G_0$ clonogens</td>
<td></td>
</tr>
<tr>
<td><strong>Intrinsic cellular radioresistance</strong></td>
<td></td>
</tr>
<tr>
<td>DNA damage induction</td>
<td>Increase RT dose</td>
</tr>
<tr>
<td>repair</td>
<td>(hyperfractionation;</td>
</tr>
<tr>
<td></td>
<td>brachytherapy;external</td>
</tr>
<tr>
<td></td>
<td>stereotactic boost)</td>
</tr>
<tr>
<td></td>
<td>Halogenated pyrimidine</td>
</tr>
<tr>
<td></td>
<td>sensitisation</td>
</tr>
<tr>
<td>2) HOST FACTORS</td>
<td></td>
</tr>
<tr>
<td>Volume effect</td>
<td>Focal irradiation</td>
</tr>
<tr>
<td>Radiosensitivity of dose-limiting normal tissue</td>
<td>Hyperfractionation</td>
</tr>
<tr>
<td>Pathophysiology</td>
<td></td>
</tr>
<tr>
<td>Hb; vascular disease</td>
<td></td>
</tr>
<tr>
<td>3) TECHNICAL FACTORS</td>
<td></td>
</tr>
<tr>
<td>Tumour localisation</td>
<td>Improved imaging and</td>
</tr>
<tr>
<td>Quality control in planning, dose delivery</td>
<td>localisation(MRI, CT,</td>
</tr>
<tr>
<td></td>
<td>stereotactic biopsy)</td>
</tr>
</tbody>
</table>
**Tumour-related factors.**

Two related factors complicate the management of large tumours at any site. They require larger doses to be controlled, and the target volume and hence treatment volume increase. The biological basis of this close relationship between tumour size and local control probability is considered to be related to total clonogen number, and alterations in the tumour microenvironment, i.e., cell-cell contact, availability of metabolites, and changes in kinetics.

For clonogenic tumour cells of a given "intrinsic" radiosensitivity, the probability of overall tumour control is related to the number of such cells, and the Poisson statistics resulting from the randomness of radiation distribution in the tumour volume [Peters *et al.*, 1982].

In vitro radiation survival curves place great emphasis on the relevance of colony forming cells in measuring response of tumours. Whilst clinical experience reveals a good correlation between tumour size and local control by radiation at many tumour sites, there are no direct assays of the number of clonogenic cells in human tumours. (The relevance of colony-forming cells in vitro to "stem" cells, and the measurement of clonogenic cells in primary cultures of human glioma is discussed in chapter 5)

In human glioma, an analysis of the relationship between tumour size and local control has been complicated by two factors. Few cures have been obtained in high grade tumours by radiation, therefore it might be considered that currently employed radiation schedules are not delivering doses on the dose-response curve for glioma, where local control is the endpoint. In addition, the difficulties in imaging CNS tumours have delayed the development of a clinical prognostic staging system such as the widely employed UICC T.N.M. classification, in which tumour size is a major factor determining the predictive power of the classification. The wider availability of more sophisticated imaging techniques, such as magnetic resonance imaging and enhanced computerised tomographic resolution, together with a trend towards detection of smaller volume, low grade tumours, may facilitate the development and validation of a staging system for glioma. This would aid the analysis of dose-response relationships and further refine prognostic information.
It is estimated that optimal surgical debulking of glioma achieves at best a 90% reduction in overall tumour burden. [Moser, 1988] At the time of presentation most gliomas represent over 1gm of tumour, therefore the potential number of cells to be sterilised by radiation even after surgical cytoreduction is $10^9$ or greater. By contrast, some of the best results with radiotherapy are obtained from common epithelial tumours in which tumour has been debulked to leave "microscopic" disease, containing some $10^6-10^8$ cells (eg conservative breast cancer therapy; head and neck) [Dutreix et al, 1988].

An attempt to circumvent the "probabilistic radioresistance" [Peters, 1982] of human glioma has been made both by increasing total dose of irradiation, and attempting cytoreduction by adjuvant chemotherapy. Between 1973 and 1985 a total of 13 randomised studies containing over 1500 patients evaluated the role of chemotherapy combined with radiation in high grade glioma [Shapiro, 1986]. Agents used included CCNU, BCNU, MeCCNU, VM26, cisplatin, and AZQ. In 10 of these studies the chemotherapy given was a single agent nitrosourea. In a meta-analysis reported by Stenning for the MRC in 1987 only a 9% survival advantage could be detected at 1 year, by the addition of a nitrosourea to radiation, and this difference was reduced to 3.5% at 2 years [Stenning et al, 1987]. Although it was concluded that the benefit afforded by the addition of single agent chemotherapy was statistically valid, the real clinical gains have been disputed, and adjuvant chemotherapy does not form part of the routine management of glioma patients, outside the context of clinical trials.

**Glioma kinetics**

Both the population doubling time of a tumour, and variations in radiosensitivity throughout the cell cycle might influence the radioresponsiveness of a tumour [Tubiana, 1982]. Cell kinetic factors pertinent to the clinical outcome in human glioma have been thoroughly reviewed by Steel [Steel, 1980].

Early studies employed thymidine labelling to measure the labelling index (LI) of glioma, and derived typical values of 4-8% for grade III glioma and 9% for grade IV. Results have now been obtained for human glioma using both bromodeoxyuridine (BUdR) labelling, and the monoclonal antibody KI-67.

The values obtained for BUdR S phase labelling compare well with thymidine labelling, at 4-8% in high grade tumours. Calculated tumour potential doubling times (Tpot) ranged from 7 to 26 days, with a median of 11 days, in 6 gliomas examined [Danova et al, 1988]. These values are very similar to previous
estimations of potential doubling time obtained from thymidine incorporation[Hoshino, 1984].

The use of BUdR labelling in predictive testing on a wide range of human tumour samples suggests that glioma is relatively slow growing by comparison with common epithelial tumours. Over half the squamous carcinoma samples evaluated as part of the CHART study (continuous, hyperfractionated, accelerated radiotherapy) have Tpot values of less than 5 days [McNally, 1989].

Most studies examining cell kinetic parameters in glioma have contained too few samples to enable any assessment of the predictive value of glioma kinetics as an independent prognostic variable.

Clinical trials have evaluated the role of experimental fractionation schedules in the radiotherapy of glioma. Accelerated schedules shortening overall treatment time have been used to try to circumvent tumour repopulation during therapy. Hyperfractionated schedules giving multiple small daily fractions, with the aim of dose escalating but avoiding complications in late responding normal tissue, are still being fully evaluated.

In studies claiming a benefit for accelerated radiotherapy, the survival of patients in a conventionally treated arm was significantly lower than in other studies [Douglas, 1982]. Five further studies (1 nonrandomised, 4 randomised) incorporating a further 500 patients, have failed to show any benefit for accelerated radiotherapy in glioma [Davis, 1989]. The major benefit of accelerated fractionation in glioma would therefore appear to be a useful shortening of the overall treatment period for each patient. Small randomised studies of hyperfractionation have suggested little survival advantage to date, although an optimal total dose escalation may not have not yet been reached [Fulton et al, 1984; Ludgate et al, 1988].

**Tumour hypoxia in glioma.**

The potential of hypoxia to modify the radiosensitivity of human cells is accepted, but the degree to which the process might influence radiocurability in particular tumour sites remains unclear.

Compared with well-oxygenated tissue, hypoxic cells are more resistant to conventional irradiation by a dose factor of 2.5-3.0. The importance of the oxygen concentration at the time of radiotherapy has been clearly demonstrated in studies
rendering human skin hypoxic; radiation dose must be doubled to achieve similar results when compared with radiation under oxic conditions [Dische et al, 1976].

There is now little doubt that such hypoxic cells do occur in human tumours. Enhanced responses to radiation have been obtained using hyperbaric oxygen or chemical radiosensitisers in patients with multiple tumour nodules, giving indirect evidence of the existence of hypoxic cells [Thomlinson et al, 1976]. Estimates have also been made of the hypoxic fraction of human tumours using radiolabelled misonidazole.

Grade IV gliomata contain regions of coagulation necrosis, and it been presumed that there were likely to be regions of hypoxic but viable clonogens. Using human glioma cell lines, Millar demonstrated that the effect of hypoxia required a doubling of radiation dose to achieve one log of cell kill [Millar, 1985]. No estimates have been made of hypoxia occurring in transplanted human glioma.

No evidence has been found of hypoxia in vivo in high grade glioma, within the limits of the 1-1.5cm resolution imposed by current positron emission tomographic scanners, using $^{15}$O$_2$ as the tracer [Brooks, 1990]. However, a clinical study has measured oxygen tensions in 10 gliomas perioperatively. Comparisons were made between tumour, surrounding normal tissue and arterial oxygen levels under anaesthesia. The range of intratumoral oxygen levels was 6-38 mmHg, with a mean of 14mmHg, while mean normal brain levels were significantly higher at 112mmHg. No correlation was found between tumour oxygen levels, vascularity on angiography, or the presence of CT scan enhancement [Kayama, 1988].

There is therefore evidence in human glioma that hypoxia modifies the cellular response to radiation in vitro, and some clinical evidence that tumour hypoxia does occur in vivo.

Considerable clinical effort has been invested in studies attempting to overcome the presumed hypoxia problem in glioma, using strategies including hyperbaric oxygen, chemical radiosensitisers, and neutron therapy.

In conclusion, while there is both in vitro evidence that hypoxia might modify radiosensitivity of glioma, and some clinical evidence that gliomas have low oxygen tensions, survival benefits have not accrued in clinical studies designed to circumvent hypoxic radioresistance. An explanation of this might be that with currently fractionated radiotherapy, reoxygenation between treatments is
adequate, and the overall response is dominated by low intrinsic radiosensitivity of well-oxygenated cells.

**Host factors**

**Radiosensitivity of dose limiting normal tissues and the volume effect**

The correlation between the tumour volume and the dose required to obtain tumour control has been appreciated since the early days of clinical radiotherapy [Fletcher, 1986]. It was also rapidly noted that with increasing target volume size for a given dose the incidence of normal tissue damage increased. This so called 'volume effect' is not explained fully at the cellular level. The total biological dose of radiation that can be given to a tumour varies according to the dose-limiting critical tissue in the irradiated volume, therefore relative clinical radioresistance is closely dependent on the radiosensitivity of adjacent normal tissue. Given that the clinical circumstances most often dictate treating macroscopic residual glioma even after surgical debulking, the relative radiosensitivity of normal brain tissue is an important consideration.

Early technical difficulties in the tissue culture of normal cellular components of the human brain have dictated that much of the literature on the radiosensitivity of the normal CNS is clinically based. The earliest detailed attempts to suggest guidelines for avoidance of excess CNS toxicity were proposed by Lindgren [Lindgren, 1958]. The time-dose curves for the incidence of radionecrosis reported in this study formed the basis of knowledge of radiation tolerance of the normal CNS for years.

The more subtle neuro-psychological deficits attributed to irradiation of normal brain in long term survivors of glioma require the development of alternative models for analysing dose-response relationships in the CNS [Ross, 1988].

The single study comparing the intrinsic radiosensitivity of normal and malignant glia suggested much greater sensitivity in normal glia [Nilsson et al., 1980]. There is no human in vitro data examining the radiosensitivity of other potential target cells for late radiation damage, which might include oligodendroglia, neurons, and endothelium.
In summary, to date radiation 'tolerance' of the normal brain has been defined by identifying radiation dose schedules with low incidence of frank radionecrosis. With the routine use of protocols treating within these guidelines, it has become clear that this endpoint is inappropriate given the more complex functional deficits in glioma patients, and the likelihood of underestimating true toxicity due to a significant proportion of patients dying before expression of damage. Given the especially devastating consequences of late radiation damage in the CNS, the radiosensitivity of normal brain relative to glioma is critical. Further data on the comparative radiosensitivity of glioma and potential normal target cells for the expression of late damage is required.

1.2. In vitro models of human glioma

The major objectives of the work undertaken for this thesis were an evaluation of both cellular and molecular processes determining radiation resistance in human glioma. An important part of this work was therefore obtaining appropriate models for the study of human glioma in vitro, and reaching a conclusion on the relevance of such models to clinical disease.

Progress in tissue culture techniques has yielded an increasing number of human tumour cell lines. Since the first report of a successful culture of malignant glia by Fisher in 1925, over 85 different long term human glioma lines have been established, albeit with varying degrees of detail in reported biological characterisation [Collins, 1983; Studer et al, 1985; Jacobsen et al, 1987; Rutka et al, 1987; Knott et al, 1990].

In an ideal situation, continuous cell lines would be near-diploid, with little genetic drift, whilst maintaining unequivocal evidence of malignancy, and cell lineage. In practice few human continuous cell lines fulfill these criteria.
1.2.1. Heterogeneity in glioma cell lines.

An important feature of malignant glioma in the clinical setting is considerable heterogeneity of outcome between individual cases, managed apparently identically. A major clinical prognostic factor is the "grade" of malignancy established at biopsy by conventional histological and immunocytochemical techniques. It is clear that many of the phenotypic and genetic variables observed in fresh tumour specimens are also represented in the panels of established human glioma lines available.

Considerable cellular heterogeneity is observed in vitro, both between individual lines and occasionally within single cultures cloned at relatively high passage level [Bigner et al, 1981]. Several workers have classified morphological types of glioma lines, and in some cases correlated morphology with other phenotypic characteristics in vitro, in an attempt to identify a more differentiated phenotype in vitro. However, as our knowledge of human cells in culture increases, the strong dependency of such phenotypic characteristics on tissue culture conditions becomes apparent.

1.2.2. Markers of the glial phenotype.

The classification system of human CNS tumours proposed by Bailey and Cushing dominated the study of glial malignancy in the first half of this century [Bailey and Cushing, 1926]. It proposed that CNS cells of a particular lineage could be distinguished by careful morphological study. It was believed that the commitment of a cell to a particular differentiation pathway, the degree of differentiation, and "transitional" forms could be distinguished by similar light microscopic techniques [Russell and Rubinstein, 1977].

It is now recognised that our knowledge of the developmental biology of normal human glia is still rudimentary. It has become clear that much more detailed analysis of markers of cell lineage and "differentiation" are required. This is a prerequisite to begin to understand the mechanisms of glial transformation, and to facilitate better clinical prognostic grading. The development of cell-type specific antibodies has already refined the classification of glial cell populations, and permitted an analysis of developmental pathways in the rat CNS [Raff and Noble, 1983].
Expression of cytoplasmic glial fibrillary acidic protein (GFAP) is a specific marker of astrocytic lineage. Examination of GFAP expression and patterns of cell surface gangliosides have been used to attempt to define lineage in cultures of human glia, and to make correlations between the phenotypic profile of cell lines, and behavior in vitro.

1.2.2.1. Glial fibrillary acidic protein expression

Intermediate filaments are 8-10 nm fibres that form part of the cytoskeleton of virtually all cell types in higher eukaryotes. On the basis of their tissue-specific distribution, intermediate filaments have been divided into 5 classes: GFAP; desmin; vimentin; keratins; and neurofilaments. GFAP is considered to be a marker of the astrocytic lineage [Bignami et al, 1972]. However, a high proportion of cell lines derived from human malignant glioma do not express GFAP [Kennedy et al, 1987].

While there is limited experimental data suggesting that high levels of expression of GFAP might reduce the motility of malignant astrocytes, there is no consistent evidence that loss of this cytoskeletal protein defines a more malignant phenotype. [Duffy et al, 1982]

1.2.2.2. Cell surface ganglioside expression in human glioma.

Gangliosides are a family of acidic glycolipids characterised by the presence of sialic acid, which selectively localise in the plasma membrane. Their functional significance is poorly documented, though a role has been suggested in influencing both cell morphology and regulating myelination in the developing nervous system [Knott et al, 1990].

The potential value of gangliosides in elucidating cell lineage in the developing nervous system has been highlighted by studies using the monoclonal antibodies LB1 and A2B5. This latter antibody recognises several different cell surface gangliosides. In vitro studies on astrocytes derived from normal rat optic nerve have revealed two pathways of differentiation in astrocytes, on the basis of expression of gangliosides recognised by A2B5, together with other markers (GFAP, galactocerebroside, Gal C) [Miller and Raff, 1984; Raff and Noble, 1983]. These workers have identified a common progenitor cell (the O2A cell) capable of
differentiating into either an astrocyte (type II) or a myelin-producing oligodendrocyte, depending on growth factor stimulation conditions.

The ability of patterns of cell surface ganglioside and GFAP expression to define such cell populations representing varying degrees of astrocytic "differentiation" in the rat has led to similar studies in both normal and malignant human astrocytes.

Kennedy and Fok-Seang [1986] investigated the relationship between antigenic phenotype and function of normal human glia in vitro. Using dissociated cell cultures from fetal optic nerve, it was demonstrated that two populations of GFAP + astrocytes could be identified on the basis of A2B5 monoclonal positivity; the subsequent proportion of A2B5+ astrocytes (analogous to rat type II astrocytes?) or oligodendrocytes developing in culture was dependent on the presence of serum in culture medium. Cortical astrocytes were A2B5 negative. It is of interest that after 3 days in culture, up to half the astrocytes expressed no GFAP or the oligodendroglial marker recognised by Gal C.

It was not possible to define unequivocally a bipotential cell analogous to the rat O2A cell in this system. However, it is possible that using human fetal material at a specific time point in embryonic development (16-18 weeks) might not be representative. It seems more likely that the specific pattern of surface gangliosides recognised by the A2B5 monoclonal which help define a rat astrocyte progenitor cell are not shared by a similar human astrocyte.

These studies were extended to investigate the relationship between morphological and antigenic characteristics of human gliomata cultures [Kennedy et al. 1987]. Only 6 out of 60 cultures were found to express GFAP; none of the 11 cultures derived from oligodendrogial tumours expressed antigens recognised by monoclonals raised to the markers of the oligodendroglial lineage, including galactocerebrosides, and 04. By contrast, 80% of glioma-derived cultures were found to express fibronectin on their cell surface. This extracellular matrix protein usually forms characteristic fibrils on the surface of epithelial cells, fibroblasts, and meningeal cells, but not astrocytes. Fibronectin positive, GFAP negative cultures were demonstrated to be aneuploid (mean chromosome number 63), and to have lost contact inhibition of cell division, thus corroborating a malignant glial phenotype. Two important conclusions could be drawn from this work. Firstly, antigenic analysis can classify categories of glioma that can not be resolved by classical morphological criteria. Secondly, there may be considerable selection of cell populations inherent in the establishment of human glial cultures in vitro.
1.2.2.3. Production of glutamine synthetase.

The cytosol enzyme, glutamine synthetase, is produced by normal, reactive and malignant astrocytes, and has been regarded as a marker of the astrocytic lineage [Pilkington and Lantos, 1982]. Its production can be monitored by both biochemical and immunological methods. Together with other biochemical markers of astrocytic function, for example, high affinity γ amino butyric acid uptake, it has been cited as evidence of astrocytic differentiation in human glioma cultures in which GFAP has been lost [Frame et al, 1984].

1.2.3. Cytogenetics of human glioma cell lines

The value of studying cytogenetic abnormalities in a given tumour type is two-fold. Firstly, it offers the potential to identify nonrandom abnormalities which might be associated with a particular natural history or treated prognostic phenotype. Secondly, the approach may give clues to the involvement of specific, mapped oncogenes or tumour suppressor genes in tumour induction or progression.

Established human glioma lines differ in their cytogenetics from biopsies or low passage level cultures, and the extent to which tissue culture favours the selection of a particular genetic type is poorly understood. It is therefore important to consider how the genetic changes found in glioma cell lines might alter their relevance as models of human glial malignancy.

Cytogenetic changes in glioma have been examined both at the level of gross abnormality of ploidy, and specific chromosomes. Using flow cytometry, Hoshino [1984] has documented highly variable DNA content in high grade glioma, with populations of diploid and tetraploid cells even within regions of the same tumour biopsy. It must therefore be concluded that there is considerable genetic diversity within a high grade glioma at the time of clinical presentation.

At the karyotypic level, sufficient information has been gathered to recognise certain, non-random patterns of chromosomal abnormality. Bigner et al [1989] have developed a large database of chromosomal defects occurring in glioma of varying grade, and have monitored evolution of these abnormalities with sequential passage in vitro.

See table 1.2
Over 90% of malignant gliomata have abnormal chromosome number, and 40% of biopsies have multiple co-existing karyotypic patterns, identified on Giemsa staining without banding. This finding would be in keeping with the heterogeneity of ploidy documented by Hoshino. Some 10% of glioma karyotypes examined contain predominantly normal metaphase spreads, or show loss of 1 sex chromosome alone.

Cytogenetic and molecular changes noted in continuous glioma lines differ from those observed in fresh glioma material in two specific areas.

1) Permanent cell lines rapidly establish major ploidy changes in vitro, usually becoming tri- or tetraploid within the first 50 passages. There is no evidence that glioma material exhibiting marked abnormality of ploidy is more likely to give rise to continuous lines.

2) The phenomenon of double minutes, often representing major changes in the epidermal growth factor receptor gene, is common in fresh glioma material, but lost in tissue culture.
## Table 1.2  Molecular and Chromosomal Abnormalities in Human Glioma

<table>
<thead>
<tr>
<th>Tumour</th>
<th>Defect</th>
<th>Technique</th>
<th>Incidence (%)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glioma(IV)</td>
<td>+7</td>
<td>karyotype</td>
<td>80</td>
<td>Bigner,89</td>
</tr>
<tr>
<td></td>
<td>-10</td>
<td>RFLP</td>
<td>80-99</td>
<td>James,88</td>
</tr>
<tr>
<td></td>
<td>-10</td>
<td>karyotype</td>
<td>60</td>
<td>Bigner,89</td>
</tr>
<tr>
<td></td>
<td>dmins</td>
<td>karyotype</td>
<td>30-40</td>
<td>Mark,71</td>
</tr>
<tr>
<td></td>
<td>-9p13-24</td>
<td>&quot;</td>
<td>30</td>
<td>Bigner,89</td>
</tr>
<tr>
<td></td>
<td>-17p</td>
<td>RFLP</td>
<td>30</td>
<td>James,89</td>
</tr>
<tr>
<td></td>
<td>45,XO/normal</td>
<td>karyotype</td>
<td>15-20</td>
<td>Bigner,89</td>
</tr>
<tr>
<td></td>
<td>-22</td>
<td>&quot;</td>
<td>5-10</td>
<td>Bigner,89</td>
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<tr>
<td></td>
<td>EGFR gene +</td>
<td>Southern</td>
<td>30-40</td>
<td>Liberman,85</td>
</tr>
<tr>
<td></td>
<td>N-myc/Gli +</td>
<td>Southern</td>
<td>3-5</td>
<td>Bigner,89</td>
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<tr>
<td>Glioma(III)</td>
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<td>Bigner,89</td>
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<td></td>
<td>EGFR +</td>
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<td></td>
<td>-17p</td>
<td>RFLP</td>
<td>30</td>
<td>James,89</td>
</tr>
</tbody>
</table>

**Abbreviations:**
- RFLP: restriction fragment length polymorphisms
- Southern: Southern blotting
- dmins: double minutes
- EGFR+ : epidermal growth factor receptor amplification
1.2.4. Does clinical tumour grade influence establishment of cell lines?

Reviews of the human glioma lines available suggest a strong association between grade of malignancy and likelihood of establishment of cell lines [Collins, 1983; Darling, 1990]. All continuous lines to date have been established from Kernohan grade III or IV tumours, with no significant differences between these grades. Many technical factors can influence the likelihood of obtaining a primary culture, but the relative lack of success in generating continuous lines from low grade human glioma may reflect fundamental differences in the biology of such tumours, or at least a much greater fastidiousness of culture requirement.

The characterisation of genetic and phenotypic features of the continuous human glioma lines used in this thesis are described in Chapter 3, together with a critical evaluation of their use as models of human glioma.

1.3. How is cellular radiosensitivity measured?

1.3.1 Radiation cell killing and reproductive cell death

The killing of a cell by radiation is the result of the absorption of energy, followed by the production of molecular lesions, finally expressed biologically as cell death. A considerable body of data has documented the biophysical stages of the interaction of ionising radiation with cells. A wide range of damage can be detected within vital subcellular compartments including cell membranes, and nuclear DNA. A more detailed review of the lesions produced in DNA is presented in Chapter 4.

The most widely used endpoint in quantifying the response of cells to radiation to ionising radiation is that of reproductive or "clonogenic" cell death [Elkind and Whitmore, 1967]. The term reproductive cell death derives from early bacteriological studies, in which loss of colony forming ability was one of the few quantitative endpoints. It is defined as the loss of unlimited cellular proliferation potential. Following ionising radiation a damaged cell may proceed through a limited number of divisions, and appear morphologically and functionally intact. However, only a surviving cell which has retained reproductive integrity is able to proliferate to produce a colony or clone, and is said to be "clonogenic".
1.3.2. The clonogenic cell survival assay.

The clonogenic cell survival assay is the most widely employed endpoint in cellular radiation research, despite the lack of understanding of the molecular basis of reproductive cell death. The use of such an operationally defined experimental endpoint does not ignore the diversity of biological effects that follow cellular irradiation. It is argued that the eradication of potential clonogenic cells is the major objective of therapeutic irradiation, therefore the use of such an in vitro endpoint is particularly relevant to the measurement of tumour response to radiation.

The practical limitations of this experimental endpoint have been particularly highlighted with increasing interest in measuring the radiosensitivity of human cells in a "predictive" manner. Many normal human tissues have limited or no proliferative potential, and the low cloning efficiencies observed in primary tumour cultures are major obstacles to the routine measurement of cellular radiosensitivity [Peters, 1990].

Despite some conceptual and practical difficulties inherent in the clonogenic cell assay, it has generated most of the currently available data on mammalian cellular radiosensitivity. It has the advantage of allowing the measurement of cellular radiation dose-response over a logarithmic range. This is possible because relatively high concentrations of viable cells (10^5-10^6 / ml) can be readily prepared from continuous cell lines. With the use of dilution techniques the probability of cell survival, or surviving fraction, can be measured over 3 to 4 decades of survival, with fairly uniform levels of experimental error.

1.3.3. The radiation cell survival curve.

The generation of data over such extended ranges of cell survival has lead to the presentation of survival curves plotted on semilogarithmic co-ordinates. Radiation dose in conventionally plotted on a linear scale, and surviving fraction of clonogenic cells on a logarithmic scale. Surviving fraction is calculated from the relationship:

\[
\text{surviving fraction} = \frac{\text{colonies counted}}{\text{cells seeded} \times (\text{PE}/100)}
\]
where PE, or plating efficiency of the experiment is the percentage of cells forming colonies in control plates.

Given the large range of cell kill observed, statistical considerations require that test cell numbers are increased with dose to yield valid colony numbers across the experiment. This requires that the relationship between cell density and linearity of colony numbers obtained is established for each cell line examined.

The models of radiation action most commonly used to quantify cellular radiosensitivity are described in chapter 4.

1.3.4. The concept of intrinsic cellular radiosensitivity.

Until the early 1980's it was widely assumed that the differences in clinical radiocurability observed were likely to be attributable to differences in tumour kinetics or the presence of hypoxic cells. It was considered that the cellular radiosensitivity of most human cells was very similar, and did not contribute to the differences seen in clinical radiocurability.

The first mammalian radiation cell survival curve was reported by Puck and Marcus in 1956, using cells from a patient with cervical carcinoma. Many radiation clonogenic cell survival data have since been reported on rodent and human cell types of differing histology [Elkind and Whitmore, 1967]. Various biophysical models have been developed to try to explain the shapes of survival curves, and the theoretical basis of the models are discussed in more detail in Chapter 3. The apparently small differences noted comparing cell lines of differing histology, both in size of the shoulder, or final slope of the survival curve, were attributed to part to technical considerations: the passage level of the cell line; irradiation on glass versus plastic; variable plating efficiency; use of feeder layers; quality of X-ray and cloning technique [Weischelbaum et al, 1976].

In 1981 Fertil and Malaise published the results of their re-analysis of published human cell survival curves, which included 36 tumours, and 6 fibroblast lines. Values of radiation dose and surviving fraction of clonogenic cells were estimated from the published data. Where original data were available for direct comparison this method appeared to generate errors of approximately 4% for dose, and 10% of surviving fraction. They fitted data with both linear-quadratic and multitarget models of radiation action to derive numerical values describing the cellular radiosensitivity of the lines. Several important observations were made:
1) Differences in parameters of cellular radiosensitivity were found between individual cell lines, particularly in parameters describing the initial slope of the survival curve.

2) A positive correlation was found between the in vitro response of a given cell type at 2Gy, and the clinical radioresponsiveness of tumours of the same histology, as assessed by the TCD 95%, the dose estimated to give 95% probability of tumour control. Although it was acknowledged that technical factors varied considerably between laboratories, these were insufficient to mask the contribution of cell type to determination of radiation response at 2 Gy.

Fertil and Malaise have since extended their review of human cellular radiosensitivity to include over 70 separate tumour cell lines, and human fibroblast lines [Fertil and Malaise, 1985; Chavaudra et al., 1989]. Parameters of cellular radiosensitivity that are most influenced by the low dose region of the survival curve ie. 2Gy region, include the actual surviving fraction at 2Gy, and the a component of the linear-quadratic model. Considerable heterogeneity of radiosensitivity is observed between cell lines of a specific histological type. However, the relative ranking of these parameters for cell lines of a specific histological type correlates with clinical radioresponsiveness of tumours of the same histology.

A similar analysis of human tumour cellular radiosensitivity was performed by Deacon and Steel, using both original data on tumour cell lines, and published data from other workers [Deacon et al., 1984]. In contrast to the method of Fertil, they chose to examine the correlation between response of human cell lines at 2Gy, and a non-parametric ranking of tumour radiocurability. Cell lines were assigned to groups A to E as shown, on the basis of clinical radiocurability of the tumour of derivation. See table.1.3.
Table 1.3. Ranking of clinical radiocurability of human tumours

Deacon and Steel 1984

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean SF2 Gy</th>
<th>SF2$^{30}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>0.19 +/- 0.04</td>
<td>2.3x10^{-23}</td>
</tr>
<tr>
<td>Group B</td>
<td>0.22 +/- 0.03</td>
<td>1.8x10^{-20}</td>
</tr>
<tr>
<td>Group C</td>
<td>0.46 +/- 0.06</td>
<td>7.6x10^{-11}</td>
</tr>
<tr>
<td>Group D</td>
<td>0.43 +/- 0.08</td>
<td>2.3x10^{-11}</td>
</tr>
<tr>
<td>Group E</td>
<td>0.52 +/- 0.04</td>
<td>2.6x10^{-9}</td>
</tr>
</tbody>
</table>

**Group A:** neuroblastoma, lymphoma, myeloma (6 cell lines)

**Group B:** medulloblastoma, small-cell lung carcinoma (8 lines)

**Group C:** breast, bladder, cervix (4 lines)

**Group D:** pancreas, colo-rectal, squamous lung (8 lines)

**Group E:** melanoma, osteosarcoma, glioblastoma, renal carcinoma (25 lines)

$\text{SF2}^{30}$ denotes total cell kill achieved if intrinsic radiosensitivity measured at 2 Gy operates over a theoretical 30 treatment course of radiotherapy
They also concluded that considerable differences did exist in the radiation survival curves of human cell lines, and a correlation was noted between radiosensitivity at 2Gy (SF2), and clinical radioresponsiveness.

A statistical difference was observed in mean SF2 for cell lines derived from radiosensitive human tumours eg neuroblastoma, lymphoma, and cell lines derived from common epithelial cancers eg. breast, cervix, lung, colon. Group E contains cell lines derived from tumours that are usually considered to be more radioresistant in the clinic, including 4 glioma cell lines. It was not possible to demonstrate a statistically significant difference in the mean SF2Gy between groups D and E. ie On the basis of this analysis, lower 'intrinsic' cellular radiosensitivity would not account for the observed clinical radioresistance in tumour types contained in group E. However, the responses at 2Gy cited for glioma cell lines A3 and A7 (0.67, and 0.86 respectively) were significantly higher than the mean values for group D and E overall.

The relevance of the ranking of radiosensitivity at the dose of 2Gy was further emphasised in this paper. Estimates of total clonogen kill that could be theoretically obtained if the surviving fraction at 2 Gy operated over a 30 fraction course of treatment were derived by the formula:

\[
\text{Overall surviving fraction after 30x 2 Gy} = (\text{SF2})^{30}
\]

The differences in clonogenic cell survival observed between cell lines at a given dose, irradiated as a well-oxygenated, asynchronous population, have been attributed to differences in 'intrinsic cellular radiosensitivity'. This concept acknowledges the potential diversity of molecular mechanisms that might operate at the cellular level to influence response to ionising radiation. The mechanistic basis of this observation has been the subject of much radiobiological research in the last decade. Early studies have attempted to make correlations between clonogenic cell survival and the potential of cells to 'recover' from irradiation, in operationally defined 'repair' experiments [Weichselbaum and Little, 1984]. More recent approaches have tended to concentrate on the molecular nature of the lesions in DNA which correlate with radiation-induced cell death. These are discussed in chapter 5 of this thesis.
1.3.5. Predictive assays of tumour response.

The measurement of cellular radiosensitivity has served two functions; firstly, to ask mechanistic questions regarding an association between cellular radiosensitivity and subcellular events; secondly, to provide empirical measures of comparative radiosensitivity of possible clinical relevance.

Several laboratories are currently assessing the relationship between inherent radiosensitivity of human tumour cells measured in primary or early passage culture, and clinical radiation response. The two largest of these studies are based on pretreatment assays of radiosensitivity in primary cultures of cancer of the head and neck [Brock et al, 1990], and uterine cervix [Davidson et al, 1990]. Both studies have published interim data, showing a wide range of SF2Gy values, with coefficients of variation between individual tumour samples of approximately 40%. This degree of variation from tumour to tumour has been interpreted as encouraging for the future use of SF2GY as a predictive test, as the broader the distribution of values in a population, the greater probability of detecting real differences between individuals. In both studies, preliminary analyses indicate that in tumours that recurred locally, the mean pretreatment values of SF2GY are higher than in the overall patient population, although considerable overlap exists. Both are discussed in more detail in chapter 5.

Another approach to identifying the part played by differences in individual tumour response has been adopted by Weichselbaum et al[1988]. The radiosensitivity of early passage cell lines derived from recurrent head and neck tumours treated by radiotherapy has been defined relative to that of cultures from untreated tumours. The mean values of parameters describing radiosensitivity, Do, and SF2 were significantly higher in cultures derived from recurrent tumours. They compared both parameters with data previously obtained in the same laboratory for cultures of normal human diploid fibroblasts. The recurrent carcinoma lines were significantly more radioresistant in vitro. Unfortunately, no pretreatment assays were performed on these recurrent tumours to assess whether the radioresistance observed both clinically and at relapse in vitro, could have been detected predictively.

Technical aspects of the selection of an appropriate assay for predictive testing of tumour response are discussed in chapter 5.
1.3.7. Predictive testing of normal tissue radiosensitivity.

Human normal tissues are observed to vary in their radiosensitivity, both in the clinic and in vitro [Denekamp, 1988]. Variations in radiosensitivity can be demonstrated in different normal tissue cell types from the same individual, and in cells of the same lineage from different individuals [Weichselbaum et al, 1980].

The population distribution of inherent cellular radiosensitivity of normal tissues is of considerable importance. The concept of radiotherapy treatment to normal tissue tolerance requires that in order to maximise tumour control probability, radiotherapy regimens are designed to produce a defined probability of normal tissue injury. Tolerance doses are determined empirically from population averages without regard for individual variability of response. This must result in subtolerance doses being given to a proportion of patients.

This concept has been developed further by the notion that the distribution of normal tissue radiosensitivity in individuals in the cancer population may not be normal. The work of Swift et al [1987] has revealed that relatives of patients homozygous for the radiation hypersensitivity disorder, ataxia telangiectasia, exhibit an excess incidence of many common tumours.

It has been suggested that the association of human radiation hypersensitivity syndromes and cancer proneness could bias the definition of radiation tolerance in the treatment population [West et al, 1991]. The maximal radiation doses currently employed might be maintaining an acceptable level of late normal tissue damage in a cohort of individuals potentially hypersensitive to radiation.

There is currently little data addressing the issue of an association between radiosensitivity of normal tissue and tumour in the same individual. If genetically determined factors modulate both responses, a relationship might be expected between the severity of normal tissue reactions and probability of tumour control, when tumours of the same type are treated by a similar regimen. The only clinical data lending support to this hypothesis has been reported by the Institute Gustave-Roussy. In a series of 135 patients with T3 carcinoma of the cervix, there was a highly significant survival advantage was noted for those patients developing severe acute and late gastrointestinal treatment complications, compared with those developing only mild symptoms [Peters, 1990]. This suggested the possibility of a shared hypersensitivity to radiation of target cells in both tumour and normal bowel of an individual.
Several laboratory studies have attempted to demonstrate unusual cellular radiosensitivity in normal cells derived from patients exhibiting adverse treatment reactions. In most of these studies it was concluded that fibroblasts from such patients exhibited greater cellular radiosensitivity, defined relative to controls derived from healthy volunteers, or patients with 'normal' responses [Weichselbaum et al, 1976; Smith, et al, 1980; Loeffler et al, 1990].

1.3.8. Evidence for cellular radioresistance in human glioma

The role played by intrinsic cellular radioresistance in determining clinical radioresistance in human glioma has been unclear. Studies addressing this question form the subject of Chapter 4 of this thesis.

Human glioma cell lines have been available for in vitro studies since the late 1960's. Given the poor prognosis generally observed in high grade glioma even after optimal therapy, there has been surprisingly little direct investigation of the cellular radiobiology of glioma. See table 1.3.
Table 1.4. Cellular radiosensitivity of human glioma

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Multitarget</th>
<th>Linear-quadratic</th>
<th>SF2</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Do</td>
<td>n</td>
<td>α(Gy⁻¹)</td>
<td>β(Gy⁻²)</td>
</tr>
<tr>
<td>TX13</td>
<td>1.43</td>
<td>1.4</td>
<td>5.59</td>
<td>1.52</td>
</tr>
<tr>
<td>A2*</td>
<td>3.36</td>
<td>3.94</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>A3*</td>
<td>0.86</td>
<td>3.94</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>A7*</td>
<td>0.97</td>
<td>3.19</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>U251MG</td>
<td>2.25</td>
<td>3.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U118MG</td>
<td>1.5-2</td>
<td>4.5</td>
<td>0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>U622(normal glia)</td>
<td>1.3-1.5</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U787 &quot;</td>
<td>1.0-1.5</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U251MG</td>
<td>1.6</td>
<td>2.62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot; with hypoxia</td>
<td>3.79</td>
<td>3.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U118MG</td>
<td>1.30</td>
<td>8.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot; with hypoxia</td>
<td>3.55</td>
<td>7.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U87</td>
<td>1.02</td>
<td>38</td>
<td>0.02</td>
<td>0.06</td>
</tr>
<tr>
<td>U138</td>
<td>1.14</td>
<td>14</td>
<td>0.056</td>
<td>0.057</td>
</tr>
<tr>
<td>U373</td>
<td>1.38</td>
<td>5</td>
<td>0.21</td>
<td>0.035</td>
</tr>
<tr>
<td>IN 859*</td>
<td>0.31</td>
<td>0.033</td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td>IN1265*</td>
<td>0.042</td>
<td>0.053</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>SB</td>
<td>0.36</td>
<td>0.007</td>
<td>0.47</td>
<td></td>
</tr>
</tbody>
</table>

* denotes low passage line
Weichselbaum compared the cellular radiosensitivity of a panel of human tumour cell lines, including a glioma, TX13. Using clonogenic cell survival as the endpoint, survival curves were fitted by the multitarget model of radiation action. The values of $D_0$ representing the final slope of the curve, were found to lie within a range of 131-150 for the 7 lines studied. These included cell lines derived from both radiocurable tumour types, such as neuroblastoma, and radioresistant tumours, such as melanoma, and osteogenic sarcoma. The values for $D_0$ obtained were not statistically different between cell lines [Weichselbaum et al, 1976].

Gerweck et al [1977] investigated the radiosensitivity of malignant glioma at low passage level. Cultures were initiated from 9 astrocytomas, and 2 normal brain biopsies. Normal glia proliferated very slowly and growth ceased after 20-40 passages. Growth adequate to permit radiation clonogenic assay appears to have been acheived in only 3 of the gliomas. The experimental system used 250 kV X-rays, cells were cloned with feeder cells on plastic, and survival curves were fitted by eye. The only estimate of radiosensitivity cited was the surviving fraction of cells after a single dose of 6Gy (range 0.13-0.17). The survival curves were compared to those obtained from the human tumour cell lines published in the Weichselbaum study, as well as normal human fibroblasts. They concluded that 2 of the 3 glioma lines had large initial shoulders to the survival curve, suggesting a capacity to accumulate and repair radiation damage, and that the overall survival curve for these two gliomas was suggestive of greater radioresistance than the other tumour types.

Nilsson has reported the only comparative study of the radiosensitivity of both normal and malignant human glia [Nilsson et al, 1980]. A specialised technique was developed permitting assessment of clonal cell survival, with high density cultures. Palladium grids were used to limit the migration of cells on a 1% agarose base. The technique was validated by comparison of the radiation survival in chinese hamster cells using this procedure and classical agar cloning. Radiosensitivity was assessed over 1-2 logs of cell kill, and data fitted by both multitarget single hit and linear-quadratic models of radiation action. Significant differences were observed in the shape of the survival curves for the 2 gliomas, U118MG, and U251MG, when compared with the normal glia. It was concluded that malignant glioma cells were more radioresistant than normal glia.
1.3.9. Inherent radiosensitivity of both normal tissue and tumour as predictors of radiotherapy response.

On the basis of observations on human cells in culture, and variability of clinical outcome following tumour radiotherapy, it has been suggested that heterogeneity in tumour response might be determined by intrinsic cellular radiosensitivity. An extension of this hypothesis is that genetically determined variability might also occur in the radiosensitivity of normal tissues from individuals, with important considerations for possible dose escalation in radiotherapy treatments [Peters, 1990].

1.4. The study of DNA repair in mammalian and human cells and its role in determination of cellular radiosensitivity.

The molecular basis of the variability observed in intrinsic cellular sensitivity to cancer therapy remains one of the most important areas in cancer biology. A large body of evidence supports the idea that DNA is the most important biological target of radiation damage [Elkind, 1985]. There is speculation that repair of DNA damage is a critical process in determining biological outcome, through a spectrum of functional cellular events including mutation, carcinogenesis, and cell death in response to therapeutic DNA damage [Lindahl, 1987].

1.4.1. Human syndromes with hypersensitivity to DNA damaging agents

Evidence for the potential involvement of DNA repair disorders in determining response to radiation comes from the rare human genetic syndromes in which partially characterised defects in DNA repair mechanisms are part of a pleiotropic phenotype together with hypersensitivity to DNA damaging agents, and often cancer-proneness [Thacker, 1989].

The recognised syndromes are summarised in table: 1.5
<table>
<thead>
<tr>
<th>SYNDROME</th>
<th>PHENOTYPE</th>
<th>HYPERSENSITIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ataxia</td>
<td>Cerebellar dysfunction</td>
<td>Ionising radiation</td>
</tr>
<tr>
<td>Telangectasia</td>
<td>Skin telangectasia</td>
<td>Bleomycin</td>
</tr>
<tr>
<td></td>
<td>Increased incidence of epithelial and lymphoid tumours</td>
<td></td>
</tr>
<tr>
<td>Bloom's</td>
<td>Photosensitivity, Immuno-deficiency, Growth retardation</td>
<td>UV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bifunctional alkylators</td>
</tr>
<tr>
<td>Cockayne's</td>
<td>Photosensitivity</td>
<td>UV</td>
</tr>
<tr>
<td></td>
<td>Dwarfism, ataxia</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neural deafness</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Retinitis pigmentosum</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Progeria-like</td>
<td></td>
</tr>
<tr>
<td>Xeroderma Pigmentosa</td>
<td>Neurological defects</td>
<td>UV</td>
</tr>
<tr>
<td></td>
<td>Telangectasis</td>
<td>Bifunctional alkylators</td>
</tr>
<tr>
<td></td>
<td>Melanoma, squamous Ca</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Basal cell Ca</td>
<td></td>
</tr>
<tr>
<td>Fanconi's Anaemia</td>
<td>Hypoplastic anaemia, Skeletal defects</td>
<td>Bifunctional alkylators</td>
</tr>
<tr>
<td></td>
<td>Hypogonadism</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thrombocytopenia</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leukaemia</td>
<td></td>
</tr>
</tbody>
</table>
1.4.2. Ataxia telangiectasia as a model of human ionising radiation hypersensitivity.

The human paradigm of ionising radiation hypersensitivity is the disorder ataxia telangiectasia [Taylor et al, 1975]. It is a recessively inherited disorder in which patients show a progressive cerebellar ataxia presenting in infancy or early childhood. Common neurological features include diminished deep reflexes, choreoathetosis, oculomotor apraxia, and dysarthria. Laboratory abnormalities include a defect of cell mediated immunity, and increased levels of spontaneously occurring chromosome abnormalities in peripheral lymphocytes [Taylor et al, 1989]. Genetic differences exist between patients as evidenced by the identification of separate complementation groups by somatic cell fusion studies [Jaspers and Bootsma, 1982]. An excess of both lymphoid and epithelial malignancy has been documented in both AT homozygotes, and heterozygous relatives [Swift et al, 1987].

Patients with AT are of particular interest in studying the relationship between hypersensitivity to ionising radiation and DNA repair processes.

1) Their cultured cells exhibit hypersensitivity to radiation in vitro as measured by clonogenic cell survival [Arlett and Harcourt, 1980].

2) Cells fail to display the radiation-induced inhibition of DNA synthesis considered part of the normal cellular response to ionising radiation damage [Painter and Young, 1980].

3) Cellular and in vitro DNA repair systems suggest a defect in the fidelity of rejoining of DNA double strand breaks as a mechanistic basis of the cellular hypersensitivity to ionising radiation [Debenham et al, 1988; North et al, 1990].

Although AT is a rare condition, it is estimated that approximately 1% of the population are heterozygous for the gene. Such individuals may constitute a significant percentage of the cancer treatment population as previously discussed. If a defect could be measured in cellular radiosensitivity it might form the basis of a screening test for radiation hypersensitive individuals. Fibroblasts, and lymphocytes from such AT heterozygotes have been demonstrated to have a
cellular radiosensitivity intermediate between that defined for the normal population, and homozygous AT cases [Cole et al, 1988 ].

Data on AT and other human syndromes with less well-defined defects in cellular radiosensitivity lend credence to the idea that at least part of the observed variability in both tumour and normal tissue response to radiation is genetically determined.

Further support for this idea derives from data showing that human lymphocyte radiosensitivity measured by low dose rate irradiation in 260 healthy donors showed a greater variation than expected from a normal distribution [Gentner et al, 1990 ]. This study was also able to demonstrate a hypersensitivity to radiation in the lymphocytes derived from a cohort of cancer patients identified as displaying severe normal tissue reactions to radiotherapy. The control samples from cancer patients showing a normal reaction could not be differentiated from normal donors. They concluded that patients developing treatment-related complications from radiation are not "statistical outfalls" of the population presenting for treatment, but constitute a heterogeneous subset.

1.4.3. DNA repair systems.

DNA damage may be defined as the modification of DNA structure sufficient to alter coding or normal transcription. Its repairability is dependent on proteins recognising damage then initiating a sequence of biochemical changes to restore the intact duplex DNA molecule. DNA repair is therefore the cellular process of restoration of normal nucleotide sequence and stereochemistry of the DNA molecule. Study of prokaryotic, simple eukaryotic and mammalian cells suggest a remarkable degree of conservation of some aspects of the processes involved. They have served to counteract a wide spectrum of DNA damage, including heat hydrolysis, UV light, ionising radiation, and enviromental chemicals.

It is now recognised that a variety of both constitutive and inducible DNA repair pathways exist, with varying degrees of characterisation of genes and protein products involved [Lindahl, 1982 ; Sancar and Sancar, 1988 ].

The study of the excision repair pathway of UV induced DNA damage, and other repair mechanisms in bacterial systems has provided the basis for much fundamental knowledge, facilitated by the existence of a large number of repair mutants. Somatic genetic complementation approaches that have proved so successful in simpler organisms have been complicated in the study of human
DNA repair by absence of a large number of repair deficient mutants, and difficulties transfecting human cells with foreign DNA [Thompson, 1989].

Extrapolation from bacterial DNA repair systems to mammalian or human cells is complicated at a number of conceptual and practical levels.

1) In bacteria DNA is essentially a naked molecule, whilst in higher organisms it has a complex chromatin associated tertiary structure. This may impose significant constraints on repair activity requiring enzyme access [Sidik and Smerdon, 1990].

2) Mammalian systems demonstrate heterogeneity of repair within different regions of the genome [Bohr et al., 1987], with some evidence of preferential repair of actively transcribed genes.

3) Any mutation affecting DNA repair must be compatible with viability of the human fetus, thus the span of diversity of human mutant systems to facilitate the characterisation of repair pathways is inherently limited.

It is expected that DNA repair will show further levels of complexity related to effects of the cell cycle, induction by DNA damage, or even constitutive variations in different tissues dependent on cell lineage [Mitchell and Hartman, 1990].

1.4.4.1 DNA excision repair pathways.

The generic term “excision repair” has been superceded by the identification of three biochemically distinct pathways of removing damaged, or inappropriate bases from DNA. These are designated nucleotide excision repair, base excision repair, and mismatch repair. The nucleotide excision pathway was described following the classic studies on removal of cyclobutane pyrimidine dimers from DNA following UV irradiation, but has since been implicated in the repair of a wide range of DNA helix-distorting lesions [Epstein, 1990].

In contrast to prokaryotes, little is known about damage-specific recognition and incision of DNA in eukaryotic cells.

A role for the involvement of DNA base damage has not been directly demonstrated in radiation-induced cell killing. However, it is recognised in the spectrum of radiation-induced lesions, and undoubtedly plays a part in point mutation. Removal of base damage, as measured by endonuclease-sensitive
sites, has been shown to be prolonged in one cell line derived from a patient with ataxia telangectasia, [Paterson and Smith, 1979], and in fibroblasts derived from patients with Xeroderma pigmentosum. [Fornace et al, 1986]

1.4.4.2. Recombination.

Genetic recombination involves the exchange of DNA sequence between strands. The homologous pairing of DNA strands, strand exchange, and branch migration require a series of enzymic events. Recombination can occur extra-, inter- or intra-chromosomally. These processes are ubiquitous in nature, and can be demonstrated in both germ lines, and somatic cells [Bollag et al, 1989]. Different classes of DNA rearrangements, for example, crossing over (reciprocal exchange), gene conversion (non-reciprocal exchange), deletion, inversion, duplication etc are the end-products of such events.

The process of genetic recombination has been proposed as a possible mechanism for the repair of radiation-induced strand breakage [Resnick, 1976]. Recombination can be demonstrated to participate in the resolution of DNA double strand breaks in yeast. The repair of a double strand break involving damaged bases and sugar moieties on adjacent strands might involve this process.

1.4.5. Identification of DNA repair genes.

Considerable efforts are being made in many laboratories to identify and clone the genes involved in the inherited human disorders that exhibit hypersensitivity to DNA damaging agents, including ionising radiation. See table 1.6.
Table 1. 6 Cloned Human DNA repair genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosomal location</th>
<th>Gene size</th>
<th>No.of aa in protein</th>
<th>Activity of gene product</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERCC-1</td>
<td>19q13</td>
<td>15-17</td>
<td>297</td>
<td>?</td>
</tr>
<tr>
<td>ERCC-2 (XP-D)</td>
<td>19q13</td>
<td>20</td>
<td>760</td>
<td>DNA helicase&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>ERCC-3 (XP-B)</td>
<td>2q21</td>
<td>45</td>
<td>782</td>
<td>&quot; 1</td>
</tr>
<tr>
<td>ERCC-5</td>
<td>13q</td>
<td>32</td>
<td>&gt;1500</td>
<td>DNA helicase&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>ERCC-6</td>
<td>10q11</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XP-A</td>
<td>9q34</td>
<td>25</td>
<td>273</td>
<td>DNA ligase</td>
</tr>
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<td>DNA ligase I</td>
<td>19q13</td>
<td>919</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HHR6A</td>
<td>Xq24</td>
<td>15</td>
<td>152</td>
<td>Ubiquitin-conjugating enzyme</td>
</tr>
<tr>
<td>HHR6B</td>
<td>5q23</td>
<td>152</td>
<td></td>
<td></td>
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<sup>1</sup> = presumed from amino acid sequence  
aa = aminoacid
Somatic cell genetic techniques rely on the isolation of hypersensitive mutants of a "wild type" cell, identified by their abnormal sensitivity to the DNA damaging agent of interest. It is assumed that the mutation involved is in a gene comprising at least part of the relevant repair pathway. Genomic DNA or a cDNA expression library derived from cells exhibiting a normal phenotype is then transfected into the mutant cells in an attempt to restore or 'complement' the repair defect. This approach has led to the chromosomal mapping of 5 human genes involved in the excision repair pathway, ERCC 1-5, with the cloning of 3 human genes ERCC1, 2 and 6. Three genes involved in the repair of ionising radiation have been mapped, designated XRCC 1-3, with XRCC 1 successfully cloned [Thompson et al, 1990; Bootsma et al, 1988; Troelstra et al, 1990].

Cell fusion studies have also identified subclasses or 'complementation groups' of both human and mammalian cells exhibiting a hypersensitive phenotype to a given DNA damaging agent. The implication is that many different genes can be involved in regulation of a specific repair pathway, with different mutations leading to a similar hypersensitive phenotype [Jones et al, 1990].

The study of human DNA repair has been further facilitated by the development of immortalised cell lines from patients with recognised DNA repair defects, including Xeroderma pigmentosum, Blooms syndrome, Fanconi's anaemia, and ataxia telangetasia [Cox et al, 1986; Collins and Johnson, 1987].

1.4.6. Rodent mutants selected for ionising radiation hypersensitivity

A number of rodent cell lines have been mutated with the specific purpose of selecting for ionising radiation hypersensitive phenotypes, to permit further mechanistic studies.

The V79 -4 irs mutants cell lines were established by the Thacker group as models for the study of mechanism underlying ionising radiation hypersensitivity [Jones et al, 1987]. The mutant lines exhibit a 2-3 fold enhancement in radiosensitivity as measured by the Do, in comparison with the parental line. The molecular basis of the defect underlying this radiation hypersensitivity is unclear. In comparison with the parental line no alteration of dsb induction or repair kinetics can be demonstrated [Jones et al, 1990]. The irs 1 mutant has been demonstrated to show lack of fidelity of rejoining of a transfected plasmid containing a restriction endonuclease ds [Thacker, 1989].
The CHO ionising radiation sensitive mutant strains (xrs), are described in the following section.

1.4.7. Evidence for the importance of the DNA double strand break in determining cellular radiosensitivity.

The biochemical nature of the lesions induced in DNA by ionising radiation are diverse, and include alterations of bases, sugars, single strand breaks, double strand breaks, and DNA-protein cross-links. For many of these lesions the induction and kinetics of repair have been elucidated in biochemical assays, with the relative correlations with cellular radiosensitivity [George and Cramp, 1988; Frankenberg-Schwager, 1990; Ward, 1985].

The association of X-ray-induced cell death and chromosomal aberrations is well established [Grote and Revell 1972]. Far less is understood regarding the transition from the biochemical stage of DNA damage to such chromosome damage. Bender proposed a model for the formation of chromosome aberrations involving nonrepair or misrepair of DNA double strand breaks [Bender et al, 1974].

Experimental evidence for the relationship between dsb and chromosome aberrations was first provided by Natarajan. An increased level of chromosome aberrations was produced by the action of single strand specific endonucleases on cells already bearing radiation induced single strand breaks [Natarajan et al, 1980].

Bryant demonstrated the production of chromosome aberrations in mammalian cells by restriction endonuclease induced dsb. Using Sendai virus permeabilised V79 cells, an 10 fold increased level of chromosome aberration was noted following treatment with the restriction enzyme, Pvu II, which generates non-cohesive dsb; such an effect was not observed with the enzyme Bam H1, which produces cohesive dsb [Bryant, 1984].

Genetic mutants of bacteria [Krasin and Hutchinson, 1977], yeast [Ho, 1975], and mammalian cells deficient in repair of dsb demonstrate cellular hypersensitivity to ionising radiation [Jeggo and Kemp, 1983]. This is associated with an observed increase in the number of chromosome aberrations [Bryant et al, 1987].

It has been estimated that approximately 40 dsb are induced per mean lethal event in the mammalian genome following irradiation, with the implication that
cells must normally possess effective repair of such lesions [Blocher and Pohlit, 1982].

The strength of the association between dsb, chromosome aberrations, and cellular hypersensitivity to radiation in dsb repair deficient mutants is not disputed. However, it has proven much more difficult to demonstrate a direct causal relationship between dsb and cell death in mammalian cells. This is in part due to the biochemical multiplicity of DNA lesions induced when genomic DNA is irradiated, and the low level of resolution of assays for residual DNA damage when cells are irradiated at clinically relevant doses.

The difficulties are well-exemplified by the complex set of observations relating to one well characterised rodent mutant system. An association between a defect in rejoining of dsb and hypersensitivity to ionising radiation has been demonstrated in the xrs (X-ray sensitive) mutants of CHO K1 cells, using neutral elution [Kemp et al, 1984]. The strength of this association was consolidated by confirming that xrs lines 5, 6, and 7 exhibited higher than expected levels of chromosomal aberration in response to both X-irradiation, and restriction enzyme-induced damage [Kemp and Jeggo, 1986; Bryant et al, 1987]. Despite the compelling nature of these data, the precise defect leading to ionising radiation hypersensitivity in the xrs mutants has yet to be proven. The observation of a high frequency of chromosomal exchange aberrations has led to the speculation that an associated defect in recombination might increase misrejoining.

Neutral elution of DNA following cellular irradiation has also demonstrated an association between increased induction of dsb and enhanced cellular radiosensitivity, when conditions are modified to increase radiation toxicity eg hyperthermia, or hypoxia [Radford, 1986]. Further evidence for a relationship between initial induction of dsb and cellular radiosensitivity was the demonstration of an elevated level of dsb in radiosensitive murine mutant cell lines [Wlodek and Hittelman, 1987].

Several authors have, however, been unable to demonstrate a relationship between initial induction of dsb and cellular radiosensitivity [Iliakis and Okayasu, 1988; Van Ankeren et al, 1988]. Similarly, it has not proved possible to show a consistent relationship between the induction or removal of dsb in human tumour cells of widely differing radiosensitivity [McMillan et al, 1990].

It is of particular interest that techniques such as neutral elution, which measure overall genomic dsb after radiation, have not been able to reveal a defect in
several radiation hypersensitive mutants, including the irs hamster lines, [Jones et al, 1990], or VC 4, VE 5, VG 8 [Zdzienicka et al, 1990]. Similarly neutral elution has not revealed any overall defect in dsb repair in cell lines derived from patients with AT [Thacker, 1989].

1.4.8. Methods for the quantitative study of DNA repair.

The precise measurement of DNA damage by ionising radiation at clinically relevant doses has been the goal of much research.

General methods used for the study of DNA repair fall into 3 broad categories:

1) DNA “damage” assays, in which repair is inferred from the disappearance of damage in genomic DNA with time.
2) Quantification of unscheduled DNA “repair synthesis” following DNA damaging events.
3) Host cell reactivation assays, in which the fate of exogenous, damaged DNA is monitored after introduction to the “host cell” under study.

This section will concentrate on the methods relevant particularly to the study of DNA double strand breaks.

Two overall concepts are pertinent to the quantitative evaluation of DNA repair. Firstly, approaches concentrating on the disappearance of damage must take account of both rate and completeness of lesion clearance. Secondly, it is likely that the accuracy of repair is as significant as the extent in maintaining integrity of genetic information. A degree of residual damage might be tolerated at the cellular level, or expressed as an altered phenotype. The nature of the contribution of misrepair to determination of subsequent cell death is poorly understood in mammalian cells.

Studies of induction of dsb suggest that naked DNA suffers as much as 80 times the damage of genomic DNA, stressing the need to develop assays which evaluate the processing of DNA dsb in the cellular milieu [Ljungman et al, 1991].

The situation is further complicated by evidence that the fate of DNA double strand breaks vary throughout the cell cycle [Wlodek and Hittelman, 1987]. The usual cellular response to ionising radiation damage is delay of DNA synthesis.
Hence there is likely to be a complex relationship between the timing of damage in the cell cycle, and consequences of residual, unrepaired lesions. Whilst a defect in radiation-induced delay of DNA synthesis is part of the pleiotropic AT phenotype, a general relationship between extent of damage-induced cell cycle delay and radiosensitivity is lacking [Musk, 1991].

DNA damage assays which have shed light on the relationship between the dose-induction, and disappearance of DNA double strand breaks include velocity sedimentation, neutral filter elution, and more recently, pulsed field gel electrophoresis (PFGE). Conditions are established to study DNA fragmentation in non-denaturing conditions. Protein-free DNA fragments are physically separated by centrifugation, elution or gel electrophoresis.

The neutral elution assay is based on the principle that small (broken or "unrepaired") radiolabelled DNA fragments will elute through a filter more rapidly than large fragments. The technique has been widely used to measure the kinetics of dsb, and is more sensitive than velocity sedimentation at lower doses of radiation. Major concerns have been the lack of a linear radiation dose-DNA damage response, and the inability of the method to include molecular weight markers. These factors, together with a number of other methodological problems have resulted in increasing interest in alternative methods such as PFGE.

Radiolabelled cells are embedded as plugs in low temperature gelling agarose, irradiated, lysed, and the DNA subject to electrophoresis. Neutral conditions have been developed to favour study of double strand breaks. The technique offers the advantage of detecting induction of damage at doses as low as 1Gy, and residual damage after a "repair" period at 5Gy and above. This enables the study of lesions that are likely to be involved in determining cell fate at clinical doses [Blocher et al, 1989]. Resolution of fragments between 200 Kb and 6 megabases are obtained. In addition, it offers the potential of permitting study of specific areas of the genome, if used in conjunction with hybridisation techniques. Using this assay, estimates of 3-5x10^-9 induced dsb/ per base pair/ per Gy have been made for diploid mammalian cells.

A number of promising DNA damage assays are currently being evaluated, include the halo technique [Wynstra et al, 1990], and the comet assay [Olive et al, 1991].

The single cell microgel or "Comet" assay enables the study of DNA damage in individual cells. It has the theoretical advantage of requiring no radiolabelling of
cells (visualised by staining DNA with fluorescent dyes), and requiring relatively few cells. When run under neutral lysis conditions it is felt to be selective for dsb, and therefore holds the promise of potentially showing a correlation of DNA damage with cellular radiosensitivity.

1.4.9. The contribution of recombinant DNA techniques and in vitro systems to the study of DNA repair.

Modern recombinant DNA techniques have made a major impact on the experimental approach to the study of DNA repair over the last 5 years, though conclusions reached with regard to the role of DNA repair as a modulator of cellular radiosensitivity have been mixed. The use of defined DNA substrate molecules, together with the ability to 'model' specific classes of biochemical damage using restriction endonucleases, has proved the tools to examine DNA repair processes relevant to ionising radiation at a new level of precision [Thacker, 1986]. It has proven difficult in the complex mammalian genome to make simple quantitative correlations between overall genomic biochemical damage to DNA, and subsequent cellular events. There is therefore increasing interest in systems which study DNA repair at the level of specific genes within cellular systems, [Hanawalt, 1989], or permit more precise evaluation of the repair of specific classes of DNA damage in defined recombinant DNA substrate molecules.

1.4.9.1. The use of exogenous plasmid or viral DNA as a repair substrate.

The theoretical attraction of the necessity to study DNA damage within the cellular environment led to the development of "host cell reactivation" assays. Exogenous, damaged DNA, in the form of plasmid, or virus is introduced into the cell under study by standard transfection techniques.

The reactivation of damaged virus is mediated by host cell repair mechanisms [Defais and Hanawalt, 1983]. The "repair" is measured by viral plaque formation, or expression of viral antigens. Using such a system, cell lines from patients with XP have been shown to be defective in the reactivation of UV-irradiated virus. In contrast, using ionising radiation of viral DNA there have been conflicting conclusions as to the ability of the assay to detect a form of repair predictive of cellular sensitivity. Whilst Rainbow et al [1981] found decreased reactivation of
irradiated adenovirus by XP fibroblasts, no defect could be detected in reactivation of herpes simplex in host AT lymphoid lines [Henderson and Long, 1981]. As reactivation of viral sequences is largely the result of single strand break repair, the lack of correlation with cellular sensitivity is not surprising. This has lead to the more recent interest in plasmid DNA as a model for genomic DNA repair events.

There is now a large body of data employing the "host cell reactivation" approach to the processing of plasmid DNA damage. The conclusions from the data up to 1989 were reviewed in detail by Vos and Hanawalt [1989].

Plasmid DNA has been used as a substrate to model the repair of genomic DNA in both cellular and in vitro, cell-free repair systems. The use of plasmid DNA to study radiation-induced DNA damage, repair and genetic change in mammalian cells has been reviewed in detail by Thacker [Thacker, 1986; 1989]. Substrate plasmid molecules have been produced by both UV and ionising radiation, restriction enzyme digestion, and cytotoxic drugs. Repair events can be inferred from a transient or stable alteration of phenotype of the host cell, conferred by the plasmid-encoded genes.

Experimental systems using plasmid DNA have yielded interesting and important information on two DNA repair events, namely recombination, and rejoining of DNA dsb.

Substrates for the analysis of recombination were constructed from pBR 322 sequences in SV 40 virus, subsequently transfected into CV-1 cells to give information on the sequence homology requirements for both homologous and non-homologous recombination [Roth et al, 1985]. A similar approach has revealed that damaged DNA in the form of a dsb can itself stimulate recombination occurring in a range of mammalian cells types [Rubnitz and Subramani, 1985; Brenner et al, 1986], raising the possibility that such a mechanism might be invoked in the cellular repair of radiation-induced dsb.

Difficulties associated with poor cell transfection efficiency were circumvented by Wong et al, using microinjection of plasmid DNA bearing dsb into mammalian cells, to confirm the recombinogenic effect of dsb [Wong and Capecchi, 1986].

Plasmid molecules have been used to assess cellular repair proficiency for both cytotoxic drug-induced damage, as well as radiation lesions. The technique of introducing drug damage within plasmid DNA eg platination, followed by
transfection into the cell type of interest has been extensively employed to monitor modification of DNA damage 'in vivo' [Chu and Berg, 1987].

Several studies have used irradiated plasmid molecules to assess DNA repair in the cellular environment. Model systems have been reported based on the megavoltage irradiation of recombinant plasmids bearing the bacterial genes neo or gpt, subsequently transfected into mammalian cells. Although the processes of cellular plasmid uptake, integration and expression are not fully understood, measurable frequencies of plasmid-mediated mammalian cell transformation can be obtained, with an approximate two-fold reproducibility. A radiation dose-dependent reduction in the number of successfully transformed CHO cells has been demonstrated in one such study [Thacker, 1986].

Spivak et al [1984] used transfected, UV irradiated pSV2-gpt plasmid to assess the repair proficiency of a cell line derived from a patient with the excision repair defect, xeroderma pigmentosa. Whilst the approach was interesting, the study illustrated two absolute requirements of this transfection / gene expression approach: 1) to show that the effects of gene transfer itself do not influence cell viability.; 2) the need for an expression time of the plasmid encoded genes prior to cell selection was not acknowledged.

Some of the most innovative studies have employed plasmid DNA as integrating vectors to study repair in intact human cells derived from AT patients, or rodent cells with presumed dsb rejoining deficiencies.

Recombinant plasmid molecules such as the pPM 17, pPMH16, and pSV vectors engineered by Mulligan and Berg [1980] encode small, well-characterised genes gpt and neo, which express in mammalian cells. Human cells containing these genes can be detected using selective growth media. Restriction endonucleases (RE) have been used to generate a dsb within one of the selectable genes, the second plasmid-encoded gene acting as a marker of transfection efficiency. Repair 'fidelity' of RE induced dsb has been assessed by comparing numbers of colonies integrating and expressing plasmid DNA encoded genes, following transfection of intact or linear plasmid.
1.4.9.2. Restriction endonucleases as models of ionising radiation-induced DNA damage.

The type II restriction endonucleases (RE) are bacterial enzymes. They are thought to serve a function in bacterial cells analogous to the immunological surveillance mechanisms of higher organisms, i.e., the elimination of foreign DNA by digestion. Many such enzymes are now isolated from bacteria, available commercially, and are crucial to modern recombinant DNA technology.

Their application in the study of DNA repair in mammalian cells has been reviewed in detail by Bryant [Bryant, 1988]. RE are of particular interest in the context of modelling ionising radiation DNA damage. They recognise specific base sequences in DNA to produce a double strand scission bearing either cohesive or blunt termini. The overlapping ends of the cohesive or 'sticky' termini can have either 5'-phosphoryl or 3'-hydroxyl structure. The property of RE of specific interest in the context of this thesis is their application to produce defined DNA repair substrates in 'simple' DNA molecules, such as plasmid DNA. The choice of RE can generate a variety of dsb configurations to mimic the diversity of such lesions following irradiation of genomic DNA.

A disadvantage of the use of RE to model radiation-induced dsb is the relative 'cleaness' of the termini generated, with intact 3' hydroxyl and 5' phosphoryl groups. In theory such termini might be rejoined by the action of a single enzyme, a DNA ligase. Analysis of the end-structure of single strand radiation breaks suggests that radiation dsb are likely to have 'dirty' ends requiring exonuclease action prior to religation. However, despite the apparent chemical differences, restriction enzymes have been shown to generate a similar spectrum of cellular endpoints to those observed to occur following true ionising radiation damage, namely both chromosome aberration, and cell death. This is suggestive that the cellular processing of dsb is similar despite the different biophysical origins of the lesions.

1.4.10. Concept of DNA repair fidelity.

Repair fidelity has been assessed in a number of radiation hypersensitive mutants, including transformed cells from AT patients [Thacker, 1989]. AT5BIVA cells showed a significant reduction in fidelity of rejoining of both cohesive and blunt dsb, when compared with a transformed normal human
fibroblast line, MRC5BIVA, with comparable transformation frequencies in both lines. A similar defect was demonstrated in only one of the ionising radiation-sensitive rodent lines, *irs* 1. It was of note that no reduction of fidelity could be demonstrated in this assay in the V79 *xrs* lines previously shown to be deficient in dsb rejoining in neutral elution studies. However, the *xrs* lines had a much lower overall transformation efficiency.

The approach of using integrating plasmid DNA vectors to assess repair fidelity has been employed to study human tumour lines of differing cellular radiosensitivity [Powell *et al.*, 1992]. Using a similar system to that described above, the fidelity of rejoining in a radioresistant human tumour cell line, RT112, was shown to have only a repair fidelity of 17%, ie 83% of successful plasmid integrations occurred with sufficient loss of information at the site of the dsb to inactivate expression of the *gpt* gene. The interpretation of these authors was the controversial suggestion that some human cell lines might be resistant to radiation induced cell kill by virtue, not of repair proficiency, but ability to ‘tolerate’ a higher level of residual genomic damage.

A discussion of some of the technical difficulties and conceptual compromises in the use of such integrating vector systems is pertinent. It can be argued that the validity of using plasmid DNA damaged prior to its introduction into mammalian cells as essentially foreign DNA is a limited model of genomic DNA repair. The presence of damage within the plasmid DNA is itself a stimulus to host chromosomal integration, thus making comparisons of transformation frequencies achieved with the control undamaged plasmid difficult. Comparisons of rejoin fidelity would need to be made ideally at 'equal integration" frequencies. The plasmid copy number within each transformed cell can vary, with only limited screening for this phenomenon by Southern analysis of genomic digests. The picture is further complicated in studies using transient plasmid transfection and gene expression, as a type of host cell reactivation assay. Such episomal plasmid is likely to be subject to quite different cellular processing than chromosomal DNA.

1.4.11. Use of in vitro systems to study DNA repair in mammalian cells

A cell-free system is a fractionated cell extract that maintains a biological function. The use of cell-free, in vitro systems has made a major contribution to the study of many complex cellular processes. The ultimate goal is to analyse and reconstruct a particular cellular process in a test-tube, from constituent parts.
Cell-free extracts can be prepared from whole cells, the cytoplasmic or nuclear protein fraction. A complex range of cellular functions have been reproduced in such cell-free systems.

1) The transcription of purified genes has been initiated in vitro by the RNA polymerase II activity of extracts of mammalian nuclei [Dignam et al, 1983 ].

2) Nuclear extracts derived from human cells have catalysed SV 40 DNA replication and supercoiling [Stillman and Gluzman, 1985 ].

3) Homologous recombination has been initiated using extracts derived from mammalian cells [ Lopez et al, 1987 ; Kucherlapati and Moore, 1988 ].

In vitro systems have been applied to the study of both excision repair events, and rejoining of DNA dsb.

1.4.11.1. Excision repair in vitro.

The in vitro approach to investigating excision repair of DNA has proved a valuable adjunct to the cloning of repair genes.

Excision repair of apurinic / apyrimidinic sites and excision of pyrimidine dimers has been demonstrated in Hela extracts, using UV treated SV 40 minichromosomes as substrates [Evans and Linn, 1984 ].

It has been possible to demonstrate that the likely defect in the group A complementation group of xeroderma pigmentosa (XP) resides in an inability of the cells to excise pyrimidine dimers in native chromatin, rather than a specific enzyme deficiency, by showing that extracts prepared from the cells could perform the required excision step in naked E.coli DNA [Kano and Fujiwara, 1983 ].

Wood et al[1988 ] have developed an in vitro repair synthesis assay to model the excision repair pathway using plasmid substrates, and human cell free extracts. Extract-mediated excision of UV or drug-induced adducts within the plasmid molecule is monitored by the subsequent incorporation of radiolabelled nucleotides to generate a 'repair patch' within the plasmid DNA.

One of the major attractions of using cell extract systems is the possibility of 'complementing' biochemically any defect revealed in extracts from a
hypersensitive cell line. This can be attempted in a mutant system by the use of extracts from the wild type cell, or alternatively, by addition of specific purified proteins or known enzymes.

Important results to date using this assay have shown that the defect in several complementation groups of XP lies in the incision step of excision repair. This was demonstrated by showing that extracts of these cells (complementation groups A,B,C,D, H, and V) could perform repair patch synthesis on UV irradiated plasmid, but only if the incisional step was first initiated by the M.luteus pyrimidine dimer DNA glycosylase.

Hansson has further demonstrated that the incision defect in these XP lines can be complemented in such a cell free assay by addition of the E.coli UVr ABC protein complex [Hansson et al, 1990 ].

1.4.11.2. Application of in vitro repair systems to the study of DNA dsb rejoining.

At the time of initiating this study, in vitro repair systems had been successfully applied to demonstrate DNA dsb rejoining in fertilised Xenopus eggs, [Pfeiffer and Vielmetter, 1988 ] but not mammalian cells.

Thacker et al have since reported the use of an in vitro repair assay to evaluate a possible defect in the rejoining of DNA dsb in cell extracts derived from the ataxia telangectasia fibroblast cell line, AT5BIVA [North et al, 1990 ].

The assay system used a plasmid DNA substrate, pUC18, bearing 2 bacterial genes, one encoding ampicillin resistance in a bacterial host, and the Lac Z gene, conferring b-galactosidase activity. The plasmid molecule was linearised by the generation of a single dsb in the circular molecule, by restriction enzymes. This molecule was then a substrate for rejoining mediated by the cell extracts, under appropriate ligation conditions.

The use of a range of restriction enzymes cutting once in the region of the multicloning site of pUC18 allowed the analysis of rejoining of 6 different biochemical forms of dsb termini. The plasmid products of the ligation reaction were evaluated by examination of the pattern of plasmid DNA migration on gel,
assessed by Southern blotting, and plasmid-mediated transformation of the phenotype of competent DH5 α E.coli.

Rejoin proficiency was assessed as the number of ampicillin resistant bacterial colonies per $10^5$ viable bacteria growing through ampicillin selection on agar. Rejoin fidelity was assessed as the percentage of these colonies which expressed β-galactosidase activity i.e. had rejoined the cut site with sufficient preservation of sequence to permit expression of the second selectable marker, the LacZ gene.

The rejoin activity in AT 5 extracts was compared with that of extracts of the human fibroblast line MRC5, which exhibits normal radiosensitivity. Efficiency of rejoicing mediated by both types of extract varied depending on the class of dsb, with far greater rejoining of the Sal I (5' TCGA ) and Hind III (5' AGCT ) sites. Enzymes generating cohesive termini were ligated much more readily than those producing blunt ends.

No significant difference in extent of rejoining was demonstrated between AT5 and MRC5 extracts at any of the 6 sites examined, although rejoin efficiency varied for different types of termini. However the main observation of the study was that the fidelity of the rejoining achieved by AT extracts was much lower than MRC5 using substrates generated by the enzyme EcoR1.

It was concluded that part of the explanation of the cellular radiation hypersensitivity of AT might reside in a reduction of dsb repair fidelity at sites of DNA damage which were less efficiently ligated. This might permit the degradation of ends prior to successful religation, with consequent loss of sequence. It was further postulated that a possible defect in AT might involve the lack of proteins which 'protect' broken DNA strands prior to religation, as identified in human HeLa cells [de Vries et al, 1989 ]. However, no data has been obtained to substantiate this hypothesis to date.
1.5. Rationale for the work undertaken in this thesis.

Given that human glioma exhibits considerable clinical radioresistance, it has been postulated that a significant determinant of treatment failure might be the intrinsic cellular radioresistance of this tumour.

1) At the time of undertaking this study no laboratory had conducted a detailed evaluation of the cellular radiosensitivity of human glioma. Although a limited amount of data existed to suggest that human glioma cell lines might lie at an extreme end of the spectrum of human cellular radiosensitivity, few of the cell lines cited had been well-characterised as models of human glioma. Chapter 3 describes the establishment of primary human glioma cultures, and the characterisation of 5 continuous cell lines.

2) No single worker had previously made a direct comparison of the cellular radiosensitivity of human glioma and cell lines derived from human tumours of different histological type, particularly the more radiocurable epithelial carcinomas.

The objectives of the work undertaken and described in chapter 4 of this thesis was therefore to measure the cellular radiosensitivity of a panel of 5 well-characterised, continuous cell lines derived from human glioma, using clonogenic cell survival as the experimental endpoint. Comparison has been made with the cellular radiosensitivity of 8 other human tumour cell lines, including both embryonic and epithelial cell types, as cell lines representing tumours of widely differing clinical radiocurability.

3) An extension of the idea of the "measurability" of cellular radiosensitivity is the concept of predictive testing of tumour radiosensitivity in vitro. There is at present little data on the prognostic value of measuring the cellular radiosensitivity of human tumour biopsies [West, 1990].

An objective of this study was, therefore, to assess the feasibility of using clonogenic cell survival to measure cellular radiosensitivity in primary cultures derived from human glioma biopsies, as a possible prognostic variable. The establishment of 17 of these cultures, and an evaluation of the radiosensitivity at the clinically relevant dose of 2Gy is described in chapter 6.
4) The ability of cells to recover from ionising radiation can be measured by enhancement of clonogenic cell survival in operationally defined systems which modify the delivery of radiation eg. low dose rate irradiation, or "split dose" irradiation, or by manipulation of the post irradiation cultures conditions eg. delayed subculture.

A comparative evaluation of the cellular recovery potential of human glioma cell lines has been made using the split dose technique, and the work is presented in chapter 5.

5) Whilst it is now accepted that human tumour cells do differ in their cellular radiosensitivity, the subcellular mechanisms that determine this variability are poorly understood. The relevance of the induction of specific classes of DNA damage by ionising radiation, and the role played by DNA repair processes in modulating the subsequent cytotoxicity of these lesions is one of the most important areas in molecular radiobiology. There is a relative paucity of information on the possible role played by differing DNA repair proficiency in determining the response of human cells to DNA damaging agents such as ionising radiation.

Chapter 7 describes the use of an in vitro system designed to measure the proficiency of DNA double strand break rejoining mediated by human tumour cell extracts. The assay was initially established in this laboratory to evaluate dsb rejoining efficiency in the ionising radiation hypersensitive mutants (irs) of the V79 chinese hamster cell line.

Part of the work undertaken for this thesis included an evaluation of this assay as a means of measuring dsb rejoining mediated by human tumour cell extracts. Data obtained on the dsb rejoining proficiency of extracts from 4 human tumour cell lines is presented.

A variety of experimental endpoints have been used to assess the dsb rejoining activity exhibited by nuclear protein extracts derived from these human tumour cells. The results obtained are discussed, and the validity of the system as a model of the repair of genomic DNA damage reviewed.
Chapter 2

General materials and methods
2.1 General tissue culture techniques
  2.1.1 Maintenance of cell lines 71
  2.1.2 Cell storage and handling of stock 73
  2.1.3. Mycoplasma testing 73
  2.1.4. Origins of human cell lines. 73
2.2. Clonogenic cell survival assays.
  2.2.1. Monolayer colony formation assay. 74
  2.2.2. Radiation protocol for cell survival assays 74
2.3. Plasmid DNA preparations
  2.3.1. Large scale plasmid preparation 75
  2.3.2. Restriction endonuclease digestion. 76
  2.3.3. Assessment of linearity of pIC20H substrates. 76
2.4. Preparation of human tumour nuclear extracts.
  2.4.1 Mass culture techniques 77
  2.4.2. Nuclear protein extract preparation 77
  2.4.3. Evaluation of nuclear protein extract activity 78
  2.4.4. Biorad extract protein estimation 79
2.5. Plasmid rejoining reaction conditions 80
  2.5.1. Assessment of plasmid DNA dsb rejoining qualitative procedures 81
    2.5.1.1. Gel electrophoresis and Southern analysis
    2.5.1.2. Transmission electron microscopy of plasmid DNA forms post-ligation reactions 82
  2.5.2. Assessment of extent and fidelity of plasmid dsb rejoining
    2.5.2.1. Bacterial host 82
    2.5.2.2. Bacterial transformation by pIC20H ligation reaction products. 83
    2.5.2.3. Use of pHSG 272 as an internal control for recovery of plasmid DNA, and intra-experimental variation in bacterial viability. 84
    2.5.2.4. Selective culture media for transformed JM83 E.coli. 85
2.6. Solutions and reagents 85
Chapter 2 General materials and methods

2.1 General tissue culture techniques

2.1.1 Maintenance of cell lines

All tissue culture was carried out in class 2 microbiological safety hoods, with vertical laminar airflow. Non-disposable equipment was sterilised by dry heat at 160° C for 1 hour. Heat stable solutions were sterilised by autoclaving at 120 °C for 40 minutes.

Cells were maintained subconfluent in appropriate tissue culture medium (see table 2.1.), incubated at 37° C, in an atmosphere of 5% CO2, 20% O2.

Human glioma lines, continuous and primary, were maintained in a 50:50 mixture of Ham's F10 (Flow Labs) and Dulbecco's modification of Eagle's Basal medium (Gibco, DMEM), supplemented with 10% foetal calf serum (Northumbria), 2mM glutamine, and buffered with sodium bicarbonate.

Cells were passaged as required by population doubling times, using 0.25% trypsin in 1mM EDTA, with phosphate buffered saline (P.E.T. solution)

Routine cell counting was performed on a Coulter counter, with settings calibrated separately for each cell line. A haemocytometer was used to count single cell suspensions for cloning assays. Cell viability was determined by exclusion of 0.1% trypan blue dye.

Morphological observations were usually made by phase-contrast microscopy of living cultures, or by bright field microscopy of monolayer cultures, after fixing in methanol, and staining with 0.1% crystal violet.
Table 2.1 Tissue culture requirements of continuous human cell lines

<table>
<thead>
<tr>
<th>Line</th>
<th>Primary Histology</th>
<th>Medium</th>
<th>Growth</th>
<th>Passage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human gliomas</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IJK</td>
<td>Glioma</td>
<td>Ham's F10</td>
<td>Adherent</td>
<td>3/week</td>
</tr>
<tr>
<td>CCM</td>
<td>Glioma</td>
<td>+DMEM (50:50)</td>
<td>monolayer</td>
<td>2/ week</td>
</tr>
<tr>
<td>UVW</td>
<td>Glioma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U251</td>
<td>Glioma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IPSB18</td>
<td>Glioma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human carcinomas</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A549</td>
<td>Lung, adenocarcinoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SKMES</td>
<td>&quot; squamous.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HT29</td>
<td>Colon, adenocarcinoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCF-7</td>
<td>Breast, adenocarcinoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2780</td>
<td>Ovary, adenocarcinoma</td>
<td>RPMI 1640</td>
<td></td>
<td>3/week</td>
</tr>
<tr>
<td>Human embryonal lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SKNSH</td>
<td>Neuroblastoma</td>
<td>Ham's F10</td>
<td>&quot;</td>
<td>2/ week</td>
</tr>
<tr>
<td>IMR32</td>
<td>Neuroblastoma</td>
<td>+DMEM (50:50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NB 1</td>
<td>Neuroblastoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Susa</td>
<td>Teratoma</td>
<td></td>
<td></td>
<td>3/ week</td>
</tr>
</tbody>
</table>

RPMI 1640
DMEM, Dulbecco's modification of Eagle's minimum essential medium
All lines were cultured with 10% fetal calf serum, 2mM glutamine, and buffered for 5% CO₂ gas phase.
2.1.2 Cell storage and handling of stock

All continuous cell lines were stored in liquid nitrogen, suspended in complete medium, with 10% DMSO. Cells were frozen at 1-2x10^6 per ml. with a cooling rate of 1°C per minute. Following thawing and maintenance in culture, lines were maintained for no more than 12 passages, before discarding, and use of fresh stock.

Stocks of each cell line were grown up at the start of this project, and frozen to provide material of similar passage level throughout the research, unless otherwise stated.

2.1.3. Mycoplasma testing

All lines were screened for mycoplasmal contamination monthly, and prior to freezing. Cells were fixed at 50% confluence in petri dishes, with 25% acetic acid in methanol, and incubated with the fluorescent DNA stain Hoechst 33258 at 0.05mg per ml, for 15 minutes at room temperature. Cells were then screened by fluorescence microscopy for the extra-nuclear DNA pattern indicative of mycoplasmal infection.

2.1.4. Origins of human cell lines.

Continuous lines

Three human glioma cell lines had been established in this laboratory by previous workers; G-CCM; G-IJK-T; and G-UVW. The clinical origins of these lines, and the primary glioma cultures established in this study are summarised in table 3.2

At the time of undertaking these studies all three lines were at passage levels in excess of 100, and had been donated to the ECACC at Porton Down. U251, a continuous human glioma cell line, was kindly donated by Dr. B. Westermark, Uppsala. IP-SB 18 was derived from a high grade human glioma, and provided at passage 23 by Dr. Jeff Pilkington, and Joanna Knott, Dept. of Neuropathology, Institute of Psychiatry, London.

Epithelial and embryonic human tumour lines were obtained from ATCC, Rockville and stocks were all in regular use as part of the research program of the Dept. of Medical Oncology, Glasgow University.
2.2. Clonogenic cell survival assays.

2.2.1. Monolayer colony formation assay.

The colony forming ability of tumour cells irradiated in vitro was examined according to the following protocol. Exponential cultures of adherent monolayer tumour cells were trypsinised using P.E.T, harvested in complete culture medium and mechanically disaggregated by pipetting, and finally passed through a 25 gauge hypodermic needle. The quality of the single cell suspension was checked by microscopy, and cell numbers counted using a haemocytometer. All 5 glioma cell lines yielded successful single cell suspensions by this method. All epithelial human tumour lines were subject to the same procedure. The 2 human neuroblastoma cell lines, IMR 32, and SKHSH required vigorous mechanical disaggregation to give acceptable single cell suspensions.

Cells were subcultured into T25cm² culture flasks at varying cell densities. A low density feeder layer was added to each separate flask, comprising $10^4$ heavily irradiated homologous cells (100Gy single dose irradiation). This procedure was shown in pilot experiments to enhance plating efficiency of all continuous cell lines examined, and maintain linearity of plating efficiency over a range of cell densities yielding between 50 and 200 colonies per T25cm² flask.

Flasks containing test and feeder cells were then returned to the incubator and maintained at 37°C with gassing, for a period of 4 hours, to minimise any potential interaction of trypsin and radiation lesions.

2.2.2. Radiation protocol for cell survival assays

Megavoltage irradiation was carried out using a Mobaltron cobalt-60 therapy source, at ambient room temperature. Doses of 1-12Gy were used, at a dose rate of 1.3-1.15Gy per minute, using full dose build-up, and backscatter. Dosimetry was checked regularly by the Dept. of Clinical Physics.

Cultures were returned to the incubator within minutes of irradiation, then left undisturbed in a designated cloning incubator for periods of 10-20 days, varying with the doubling time of the cell line. Cultures were washed with PBS, fixed for 15 minutes with methanol, then stained with 0.1% crystal violet. Colonies of greater than 50 cells were scored by eye using bright field microscopy, representing cells capable of a minimum of 6 doublings after irradiation.
The plating efficiency (PE) of the experiment was calculated as:

\[
PE = \frac{\text{Number of colonies obtained}}{\text{number of cells plated}}
\]

The surviving fraction (SF) of each experimental point was then calculated as:

\[
SF = \frac{\text{plating efficiency of treated cells}}{\text{plating efficiency of control cells}}
\]

The surviving fractions of cells at each dose point were plotted on logarithmic axes, against dose in Gray on a linear axis.

2.3. Molecular techniques

Plasmid DNA substrates.

The plasmid DNA substrate used for construction of substrates for DNA double strand break rejoining studies was the recombinant molecule, pIC20H, bearing the selectable gene encoding ampicillin resistance in bacteria, and the Lac Z sequence. See figure 7.1. The choice of this substrate is discussed in section 7.1.3.

2.3.1. Large scale plasmid preparation

An overnight culture of the E.coli strain JM 83 containing the pIC20H construct was established. 5mls was inoculated into 500mls of L-broth containing 50 mgs ampicillin and grown to stationary phase overnight. Bacteria were pelleted at 3000 rpm, and cell lysis achieved by resuspending in 10 mls of 50mM Tris pH 8.0 with 25% sucrose, followed by the addition of 5mls of 10mg/ml lysosome. Incubation in these reagents was carried out at 4 C for 3 minutes, then 5 mls of 0.2M EDTA added, prior to a further 25 minutes on ice. 600 µl of 10% NP-40 in Tris-sucrose was added and tubes inverted to promote bacterial lysis. Once the mixture had become viscous it was spun at 15,000 rpm for 30 minutes to clear the lysate. The supernatant was removed, and extracted with an equal volume of phenol, by mixing for 10 minutes. This was followed by a 3000 rpm spin, then the supernatant removed and extracted with an equal volume of chloroform:isoamyl alcohol (24:1). Ribonuclease A was added at 20µg/ml for 30 minutes at 37° C to remove RNA. The RNase was inactivated by a further phenol / chloroform extraction.
DNA was then precipitated by the addition of 1 volume of isopropanol, and 1/2 volume of 7.5 M ammonium acetate, left at room temperature for 30 minutes, and then spun at 3000 rpm for 20 minutes. The DNA pellet was washed in 100% ethanol, followed by 70% ethanol, and finally dried and resuspended in 1 ml TE.

2.3.2 Restriction endonuclease digestion

Single digests of pIC20H by both EcoR1 and Nru 1 (Boehringer) were carried out overnight at 37° C in a waterbath, using an excess of each enzyme, in buffers provided by the manufacturers. Enzymes were inactivated by phenol extraction, using an equal volume of phenol equilibrated with TE (10mM Tris-HCL pH 7.5). Organic and aqueous phases were separated by centrifugation, and the aqueous phase extracted using an equal volume of chloroform-isoamyl alcohol. (equilibrated with isoamyl alcohol, 24:1). Ether extraction was performed to remove residual chloroform, by adding an equal volume of ether to plasmid DNA in solution, mixed by inversion, and separated by centrifugation. The organic phase (top) was discarded, and residual ether allowed to evaporate in a fume hood.

DNA was then precipitated from solution by the addition of a tenth of a volume of 3M sodium acetate pH 5.2, followed by 2.5 volumes of 100% ice-cold ethanol. Precipitation was completed at -70° C for a minimum of 1 hour, followed by 15 minutes centrifugation at 13,000 rpm in a microfuge. The pellet was washed with 70% ethanol, and dried under vacuum in a Speedivac at 37° C for 5 minutes. Plasmid DNA was then resuspended in TE.

2.3.3 Assessment of linearity of pIC20H substrates.

Completion of restriction digestion was checked by both visualisation of the plasmid digestion products on gel electrophoresis, and by bacterial transformation.

A 0.8% TBE/agarose gel (10x stockTBE = 900mM Tris base; 889mM boric acid; 25mM EDTA) was made by dissolving the required amount of agarose (Boehringer electrophoresis grade) in 1xTBE, containing ethidium bromide at 0.5mg/ml to facilitate visualisation of the plasmid DNA under UV light. The final solution was microwaved, and a horizontal slab gel poured.

One mg aliquots of both uncut and each restriction plasmid digest were loaded onto the gel using loading buffer (0.25% bromophenol blue; 0.25% xylene cyanol; 20% Ficoll; 10mM Tris-HCL pH 7.5; 1.0 mM EDTA). The gel was run in
1xTBE containing 0.5mg/ml ethidium bromide, at 50-100 V, then the gel examined and photographed under UV light. Plasmid DNA was compared with the position of migration of Hind III restricted λDNA, which generated fragment markers of 23.6, 9.46, 6.72, 4.34, 2.26, 1.98, and 0.58 kb.

The DNA content of uncut and cut plasmid TE stocks were estimated by measuring the optical density, (OD) 260:280 ratio in a spectrophotometer (1 OD unit= 50mg./ml). Stocks were then diluted to the 10ng/ml concentration used for the dsb rejoining assay, and frozen at -20° C.

2.4. Preparation of human tumour nuclear extracts.

The method of preparation of extracts used was modified from Dignam.

[Dignam, 1983 ]

2.4.1 Mass culture techniques

Monolayer cultures of the human tumour lines studied were scaled up into 850cc roller bottles. Care was taken to ensure that cultures were maintained in exponential phase, and were harvested prior to confluence. Medium was changed every day to prevent significant pH changes, and encourage growth. Tissue culture media and gas phases were as described in section 2.1.

2.4.2. Nuclear protein extract preparation

Between $10^8$ and $10^9$ cells were harvested for each cell extract prepared, usually from 5 to 7 70% confluent roller bottles. Routine trypsinisation was carried out, then cells were pelleted at 2000rpm, and resuspended in full medium. Cells were pelleted, and then washed in ice-cold PBS.

Cell lysis was achieved by osmotic swelling and detergent, with care taken to ensure that all procedures, and solutions used were kept at 4° C. The cell pellets were suspended in 10mls of TMS at 4° C, containing protease inhibitors: 0.05M PMSF; aprotinin, 0.1mg/ml; chymostatin, 0.1mg/ml; leupeptin, 0.1mg/ml; 0.05M benzamidine. Cell were pelleted at 2000rpm, and resuspended in 10mls TMS with 0.25% Triton X100. Nuclei were pelleted at 2000rpm, and washed in TMS three times to clear excess detergent. An estimate of the total nuclear yield was made by determining the DNA concentration (OD 260nm) of an aliquot after sonication in 1M NaOH. The nuclei were then resuspended in TMS at 7.5mg/ml DNA, and genomic DNA salt extracted from the nuclear proteins by addition of 4M NaCL to a final concentration of 0.3M NaCl. This solution was left on ice for 30 minutes, then
cellular debris removed by a 15,000rpm spin. The supernatant was clarified further by a 1 hour ultracentrifugation step at 40,000rpm.

Nuclear proteins were then precipitated on ice for 30 minutes by the addition of ammonium sulphate at 0.35g/ml. Proteins were pelleted at 10,000rpm, and then dissolved in 1ml of E50 buffer.

Proteins were dialysed overnight at 4° C against storage buffer containing 1mM DTT. Extracts were finally clarified at 40,000rpm, and frozen in 100μl aliquots at -70° C.

2.4.3. Evaluation of nuclear protein extract activity

Prior to using any tumour nuclear extract in test ligation assays, it was necessary to demonstrate that proteins were generally functional, and to estimate total protein activity in each cell extract.

Gel retardation assay.

The synthetic oligonucleotide αP3A containing the recognition sequence, CCAAT, for the ubiquitously expressed mammalian CCAAT box binding protein [Plumb et al, 1989]. was synthesised to order. The DNA required radioactive end-labelling for protein binding studies.

Kinase P32 end-labelling of αP3A oligonucleotide

Two ml of 'cold' oligonucleotide (synthesised in Beatson Institute) were incubated with 4 μl of T4 Poly nucleotide kinase (PNK), 4μl γP32 ATP (Amersham), in low salt buffer, and reacted at 37°C for 45 minutes. The kinasing reaction was halted by the addition of 1μl 0.5M EDTA, and the oligonucleotide precipitated by routine sodium acetate and ethanol techniques. The pellet was resuspended in TE.

Klenow fill-in reaction.

The oligonucleotide was incubated for 30 minutes at room temperature with 1 μl Klenow enzyme, in Klenow buffer, with a final concentration of 2mM dNTP. This 'fill-in' reaction was halted by inactivation at 70°C for 5 minutes in a hot water bath.
Purification of the end-labelled oligonucleotide, and subsequent gel retardation analysis were carried out using non-denaturing polyacrylamide gel electrophoresis. Vertical gels were made by diluting a stock solution of 29:1 acrilamide:bis to 5% in TBE, and the solution degassed and polymerised using ammonium persulphate and TEMED.

The P$^{32}$ end labelled oligonucleotide was then separated from unincorporated P$^{32}$ by 0.5% TBE, 5% acrilamide gel electrophoresis. The position of the hot oligonucleotide was located by autoradiography, cut out, and the DNA eluted in 500μl of TE overnight. The DNA was recovered by centrifugation in Spinnex tubes, and the activity of the radiolabelled oligonucleotide determined.

The detection of specific CAATT binding activity in each tumour extract used in this study was carried out by incubation of 5μl of extract with 1μl of radiolabelled oligonucleotide (approximately $10^4$ cpm incorporated radioactivity), in a final volume of 20μl storage buffer, with an excess of a non-specific competitor, poly d(l).d(C).

The reaction was halted by the addition of loading buffer, and the binding reaction products were then separated on a non-denaturing 0.5% TBE, 5% polyacrilamide gel run as a vertical system (Atto kit.) for 2-3 hours at 4° C, at 150V, with 0.5x TBE circulating buffer. The gel was run until 'free' oligonucleotide had reached the lower part of the gel, then dried and autoradiographed at -70°C.

2.4.4. Biorad extract protein estimation

The total protein concentration of all cell extracts was determined by use of the Biorad kit employing the assay described by Bradford. On binding to basic and aromatic amino acid residues in protein, the absorbance spectrum of the dye Coomassie Brilliant Blue G 250 shifts from 465 to 595nm on spectrophotometry. This forms the basis of the assay.

Protein concentration of each cell extract was estimated by comparison with a standard curve generated by mixing of varying quantities of bovine serum albumin with the Biorad dye reagent, and reading the optical density of the solution at 595nm.
Cell extracts yielding less than 1mg/ml total protein concentration were usually noted to have insignificant CCAAT binding activity on gel retard analysis, and were not used in subsequent assays.

2.5 Plasmid rejoining reaction conditions

The reaction schema is depicted graphically in figure 7.2

Reactions were assessed using 100ng of plasmid substrate, in a final volume of 350μl of ligation buffer. All nuclear extracts were thawed on ice prior to the reaction. Test ligations containing varying quantities of the nuclear extract of interest were added last to the reaction mix containing 100ng cut or uncut plC20H DNA in ligation buffer.

Unless stated otherwise, dsb rejoining reactions were carried out at 14° C, overnight, with the following conditions:

A reaction was set up in a final volume 350μl, containing

1) plC20H plasmid DNA ; 100ng of cut or uncut substrate
2) 70 μl of 5x ligation buffer, containing 250mM Tris-HCL, 5mM ATP , 50mM MgCl₂, and 5mM DTT.

Varying amounts of nuclear extract were added in storage buffer, containing 50mM NaCl, 5mM MgCl₂,20% glycerol, at pH 7.9. The final reaction volume was taken to 350μl using distilled water, or storage buffer.

The reactions were carried out at 14° C in a heated waterbath within a 4°C cold room. Reactions were terminated by the addition of an equal volume of phenol/TE, spun, the aqueous compartment removed and an equal volume of chloroform added. The sample was again centrifuged, the aqueous layer removed, and plasmid DNA precipitated by the addition of tenth of a volume of 3M sodium acetate, two and a half volumes of ice cold 100% ethanol, and 1μl glycogen.

Precipitation of plasmid DNA was carried out at -70°C overnight. The DNA was pelleted at 13,000rpm, ethanol removed, and the sample lyophilised in a Speedivac at 37°C for 5 minutes. The plasmid DNA was resuspended in 100μl of TE, and stored at -20 °C prior to further analysis by bacterial transformation, Southern blotting or electron microscopy.
2.5.1 Assessment of plasmid DNA dsb rejoining: qualitative procedures

2.5.1.1. Gel electrophoresis, and Southern analysis

Ten ng of plasmid DNA reaction products were loaded onto a horizontal slab 0.8% TAE agarose gel, using loading buffer. Gel electrophoresis was carried out overnight at 30V, with buffer circulating. All gels were run with Hind III digested, P<sup>32</sup> end-labelled, λ DNA size markers. Gels were run both with and without ethidium bromide at 1μg /ml in gel and running buffer to aid visualisation of differently migrating plasmid DNA forms.

Plasmid DNA was then denatured in situ by the action of denaturing buffer, changed twice, then neutralised. Gels were washed in 1x Genescreen buffer, and DNA transferred to Genescreen nitrocellulose filters by capillary action overnight, using 1x genescreen buffer for transfer, as described in Maniatis[Maniatis, 1982]. Filters were then baked for 1 hour at 80° C, and stored dry prior to probing with nick translated pIC20H.

Nick translation of pIC20H for Southern analysis.

Radiolabelled pIC20H was used to probe the nitrocellulose filters carrying electrophoretically separated plasmid forms. Amersham nick translation kits were used to synthesise (α 32-P) dCTP labelled plasmid probe. See methods 2.6.

100ng of uncut pIC20H was incubated with solutions 1 and 2, together with 100mCi of (a 32-P) dCTP, at 14 °C for 2.5 hours. Unincorporated nucleotides were removed by nick column purification, using a Sephadex G-50 packed column. The eluate was collected into eppendorfs, and the activity of the probe checked in a scintillation counter. The probe was judged to be adequate for hybridisation if the activity was greater than 10<sup>7</sup> cpm.

Hybridisation.

Nitrocellulose filters were soaked briefly in 1x Genescreen, then placed in hybridisation boxes with a prehybridisation mix as in 6.2.7, and incubated in a shaking waterbath at 42° C for 4 hours. The radiolabelled pIC20H probe was then denatured by boiling for 10 minutes, then rapidly cooled. It was added carefully to the hybridisation box in a fume hood, within the radio-isotope suite, sealed then returned to the 42° C waterbath overnight.
Filters were washed in a solution of 2xSSC /0.1% SDS to remove unbound probe, with a final high astringency wash in 0.1% SSC / 0.1% SDS for 1.5 hours at 65° C in the shaking waterbath. Filters were monitored to check levels of non-specific radioactivity binding prior to sealing in polythene. Autoradiography was then carried out at -70° C.

2.5.1.2. Transmission electron microscopy of plasmid DNA forms post-ligation reactions

In order to corroborate the assumption that bacterial transformation by plC20 H was mediated by recircularised plC20H reaction products, samples of plasmid DNA were visualised by transmission electron microscopy. This was performed in collaboration with Dr. Lesley Coggins, Electron Microscopy Unit, Beatson Institute, Glasgow.

The 3D form of plasmid DNA in aqueous solution must be converted to a two dimensional form for EM. This was achieved without shearing forces, by mixing the nucleic acids with a mixture of ammonium acetate pH 7.5, and cytochrome C, and a monolayer generated at the air-fluid interface of the solution. The plasmid DNA diffused to the interface and was then 'blotted' on to a supporting film carried on an EM grid, for subsequent microscopy.

Samples screened included EcoR1 cut plC20H, T4 ligated EcoR1 substrates, and EcoR1 substrate which had been incubated with tumour nuclear extracts.

2.5.2. Assessment of extent and fidelity of plasmid dsb rejoining

A schematic summary of the bacterial selection method is shown in figure 7-2

2.5.2.1. Bacterial host; preparation of competent bacteria

The bacterial host used for these studies was the E.coli strain JM 83 [Yanisch-Perron et al, 1985]. The choice of this strain was based on its possession of the C-terminal portion of the lacZ gene; this was necessary for complementation of the LacZ gene encoded within plC20H.

All bacterial plating, culture, and transformation procedures were carried out in category 1 containment.
Bacteria were stored at -20°C in 50% sterile glycerol. Colonies were maintained on L-broth agar plates without antibiotics, at 4°C, sealed with parafilm, for up to 2 weeks before discarding.

Production of competent cells

Stock JM 83 E.coli were removed from storage at -20°C in 50% glycerol, and streaked onto L broth-agar petri dishes using a sterile iridium wire. They were incubated overnight at 37°C, then a single colony picked to propagate a suspension culture in 25ml L-broth. This was grown up overnight at 37°C in a shaking incubator. 100µl of this culture was inoculated into 25mls of L-broth, and incubated under conditions permitting exponential growth. The culture was sampled after 3-4 hours, and interrupted by placing on ice, when the optical density had reached 3.0 to 5.0 units at 600nm. Bacteria were pelleted at 3000 rpm at 4°C for 10 minutes, then resuspended in 12.5mls of ice-cold 100mM calcium chloride, Tris 10mM ph 7.5, and left on ice at 4°C for 1 hour. Bacteria were pelleted as before, then resuspended in a 2.5ml volume of the 100mM calcium chloride/Tris solution and left at 4°C overnight. A “test” transformation of 100µl of the bacterial culture was performed on the day of preparation, using 10ng of uncut plasmid DNA. Transformed bacteria were plated and incubated overnight. Competence was presumed to be adequate for experimentation if the preliminary transformation yielded a minimum of 5x10^4 bacterial colonies overnight. Following overnight incubation at 4°C in 100mM CaCl_2 bacterial competence was usually obtained at a transformation frequency of 5x10^5 to 2x10^6 ampicillin resistant bacterial colonies per µg uncut plC20H plasmid DNA. It was noted that optimal bacterial competence was only obtained if bacteria were maintained strictly at 4°C following the exponential growth phase, and fresh 100mM CaCl_2 10 mM Tris was made up for each batch of bacterial transformations.

2.5.2.2. Bacterial transformation by plC20H ligation reaction products.

Forty ng of each plC20H reaction, suspended in TE, were carefully added to 100µl aliquots of freshly made competent JM83 E.coli. The bacteria were left on ice at 4°C for 30 minutes, then heat shocked at 37°C in a waterbath for 5 minutes. One ml of L-broth was then added to each eppendorf, and cultures left at 37°C for one hour expression time. Cultures were briefly spun at 6500rpm to pellet bacteria, excess L-broth decanted, and bacteria carefully resuspended in 30µl of L-broth before plating onto preheated selective L-broth agar plates containing ampicillin (100µg/ml), and X-gal (250µg/ml). Plates were inverted, and
cultured overnight at 37°C. Plates were then examined for ampicillin resistant, and β-galactosidase positive colonies.

2.5.2.3. Use of pHSG 272 as an internal control for recovery of plasmid DNA, and intra-experimental variation in bacterial viability.

Replicate experiments using the reaction schema described, carried out using batches of the same tumour nuclear extract, suggested that considerable experimental variability was generated by errors in the recovery of the small amounts of substrate plasmid DNA used in this assay (100ng). Similarly, the possibility of variable bacterial transformation or viability across the experiment exists, due to differential toxicity of incomplete removal of nuclear proteins between samples, and handling. This latter problem has been addressed by Thacker, whose assay technique describes the success of plasmid transformation in terms of bacterial viability [North et al, 1990].

It was found that the addition of a second, uncut plasmid molecule, pHSG 272, at the time of nuclear extract protein extraction by phenol could allow results obtained subsequently on the bacterial transformation by plC20H to be "normalised" within an experiment, for variations in DNA recovery. At the end of each ligation incubation, an aliquot of reaction plasmid DNA (10ng) was removed for Southern analysis, and then a fixed quantity of uncut pHSG 272 was added to the eppendorf.

At the time of plating bacteria, a fixed aliquot of the mixture was removed from each sample, and plated onto agar plates containing 40 units/ml kanamycin. The number of bacterial colonies obtained per ng plC20H on the ampicillin plates for each test sample was then corrected across the experiment for the number of kanamycin resistant bacterial colonies obtained in the co-transformation.

2.5.2.4. Selective culture media for transformed JM83 E.coli.

Selective culture of transformed bacteria was carried out on L-broth agar plates containing ampicillin and X-gal, or kanamycin. The constituents of the antibiotic selection are detailed in section 2.6.
2.6. Solutions and reagents

Antibiotics: Sigma
Autoradiography: Kodak
Bacterial materials: L-broth from Gibco; Bacto-agar from Difco.
Dialysis systems: Sartorius
Enzymes: Boehringer Mannheim; Pharmacia; BRL
General chemicals: Sigma; BDH
Nick columns: Pharmacia
Radiochemicals: Amersham International
Tissue culture: plastics from Falcon, Nunc.
media and supplements from Gibco, Paisley, and Northumbria Biologicals
Transfer membranes: Du Pont

2.6.1. Plasmid DNA preparation, and electrophoresis

Buffers: 10x TBE
900mM Tris base
889mM boric acid
25mM EDTA

50x TAE
2.0M Tris base
1M acetic acid
0.2M EDTA

TBE gels
0.8% agarose (electrophoresis grade) with
TBEx1
0.5µg/ml ethidium bromide

TAE gels
0.8% agarose with TAEx1

Molecular weight markers
Hind III digested λ DNA, with or without P32 end
labelling.
( fragment sizes: 23.6; 9.46; 6.72; 4.34; 2.26; 1.98; 0.58 kb)

Gel loading buffer
20% Ficoll
0.25% bromophenol blue
0.25% xylene cyanol FF
10mM Tris-HCL pH 7.5
1.0 mM EDTA
85
Southern blotting of plasmid DNA:

Denaturing buffer: 1.5M NaCl
0.5M NaOH
Neutralisation buffer 3.0M NaCl
0.5M Tris-HCl pH 7.0
20x Gene Screen 0.5M Na₂PO₄
0.5M NaH₂PO₄ pH 6.5
20xSSC 3M NaCl
0.3M Na citrate

Hybridisation procedures:

Prehybridisation solution 25 mls formamide
(50mls) 2.5mls 100x Denhardts
12.5ml 20x SSC
5ml 20x Genescreen
0.5ml. salmon sperm DNA (10mg/ml)
0.5ml 10% SDS
4ml distilled H₂O

Nick translation of pIC20H plasmid DNA for Southern analysis:

Amersham kit:

Solution 1: 100mM dGTP,dATP,dTTP, in Tris-HCl pH 7.8
Solution 2: 5units per 5ml of DNA polymerase 1, 100pg DNase 1 in Tris-HCL pH 7.5 with MgCl₂, BSA, and glycerol.

Nick translation reaction:

100ng uncut pIC20H plasmid DNA
10μl solution1
10μl P³² dCTP (100mCi / 3.7 MBq)
5μl Solution2
Final reaction vol. 50 μl with dH₂O
Buffers, reagents and solutions used for nuclear protein preparations.

1) TMS
- 0.25M sucrose
- 5mM MgCl₂
- 10mM Tris pH 7.5

2) Storage buffer (SB)
- 50mM NaCl
- 20mM HEPES pH 7.9
- 0.1mM EDTA
- 5mM MgCl₂
- 20% glycerol
- 1mM DTT

3) E 50
- 50mM NH₄SO₄
- 20mM HEPES pH 7.9
- 5mM MgCl₂
- 20% glycerol
- 0.1mM EDTA
- 0.1% Brij 35

4) Protease inhibitors
- Leupeptin 0.1 mg/ml
- Benzamidine 50mM
- Aprotinin 0.1 mg/ml
- Pepstatin 0.1 mg/ml
- Chymostatin 0.1 mg/ml
- PMSF 50mM

Bacterial transformation procedures:

L-broth
- 1% w/v tryptone
- 0.5% w/v yeast extract
- 1.0% w/v NaCl

L-agar
- Selection
- L broth with 1.5% agar
- Ampicillin, 100μg /ml
- X-gal, 250μg / ml
- Kanamycin 40 units /ml

87
Chapter 3

Cellular biology of human glioma lines
Cellular biology of human glioma lines .......................................................... 88

3.1. Characterisation of human glioma continuous cell lines. ........ 90
   3.1.1. Origins and establishment of human glioma lines ....... 92
   3.2.1. Phenotypic characteristics ........................................... 94
      3.2.1.1. Morphology in monolayer .................................. 94
      3.2.1.2. Population doubling times in monolayer culture ........................................ 94
      3.2.1.3. Loss of anchorage- dependent growth .............. 95
      3.2.1.4. Expression of the intermediate filament, glial fibrillary acidic protein (GFAP) ........................................ 95
      3.2.1.5. Expression of the cytosol enzyme, glutamine synthetase (GS) ........................................ 96
      3.2.1.6. Cell surface ganglioside and glycoprotein expression ........................................ 96
      3.2.1.7. Immunostaining of glioma cell membrane glycoproteins by CD44 (P80) ......................... 96
      3.2.1.8. Tumorgenicity in nude mice ...................... 97
   3.2.2 Genetic characteristics ........................................... 97
      3.2.2.1. Lactic dehydrogenase isoenzyme expression .... 97
      3.2.2.2. Cytogenetics, ploidy, and cell cycle analysis ...... 98

3.3. Results ................................................................. 101
   3.3.1. Morphology of glioma cell lines ........................................ 101
   3.3.2. Population doubling times of continuous glioma lines in monolayer ........................................ 106
   3.3.3. Expression of biochemical and immunological markers. .... 108
   3.3.4. Tumorgenicity in nude mice ........................................ 113
   3.3.5. LDH isoenzyme analysis ................................ .......... 116
   3.3.6. Cell cycle analysis by flow cytometry ...................... 118
   3.3.7. Cytogenetics ................................................. 120

3.4. Discussion .................................................................. 123
3.1. Characterisation of human glioma continuous cell lines.

**Background and specific methods.**

Human glioma lines in regular experimental use include those established by Westermark and Ponten at the University of Uppsala, and those established by Bigner, at Duke University, USA [Collins, 1983; Bigner et al., 1981; Ponten, 1975]. The line U251, used in the work for this thesis, was initially described by Ponten.

Studies in which human glioma lines are reported since 1977, and for which a detailed account exists of characterisation of 4 or more individual lines, are summarised in Table 3.1.

The rationale for the characterisation of existing continuous human glioma lines, was two-fold.

1) Review of the literature on the characteristics of the human glioma lines available suggested that few met the criteria of an "ideal" model of human glioma. In particular, many lines fail to be tumorigenic in nude mice, which may be considered the most unequivocal criteria of the malignant phenotype; similarly, many "permanent" human glial cultures fail to maintain markers of astrocytic cell lineage, e.g., glial fibrillary acidic protein, and further evidence of glial lineage has often not been sought.

2) Three continuous human glioma cell lines had been established in this lab in the early 1980's as part of a study of the phenotypic differences between normal and malignant glia. These were designated G-UVW, G-CCM, and G-IJK-T. These lines had served as tumour models as part of the Medical Oncology research program on the mechanisms of human tumour chemo-resistance [Merry et al., 1984]. At the time of commencing my study all three lines had reached high passage level (>100), and a re-assessment of their biological characteristics was considered important.
Figure 3.1. Review of characterised human glioma cell lines.

<table>
<thead>
<tr>
<th>Cell line/passage</th>
<th>Origin</th>
<th>Tumorigenic</th>
<th>Chromosomes mean/Range</th>
<th>GFAP (%)</th>
<th>GS (%)</th>
<th>FN (%)</th>
<th>S100 (%)</th>
<th>PE (%)</th>
<th>PE in agar (%)</th>
<th>PDT (hrs)</th>
<th>Sat. density</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA 4/100</td>
<td>GM</td>
<td>90%</td>
<td>40/80</td>
<td>NA</td>
<td>NA</td>
<td>42</td>
<td>NA</td>
<td>24</td>
<td>5.5x10^5</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>SA44/90</td>
<td>GM</td>
<td>No</td>
<td>66</td>
<td>NA</td>
<td>NA</td>
<td>61</td>
<td>NA</td>
<td>24</td>
<td>3x10^5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SA45/60</td>
<td>GM</td>
<td>No</td>
<td>88</td>
<td>NA</td>
<td>NA</td>
<td>11</td>
<td>NA</td>
<td>24</td>
<td>2x10^5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SA101/50</td>
<td>AA</td>
<td>No</td>
<td>71</td>
<td>NA</td>
<td>NA</td>
<td>16</td>
<td>NA</td>
<td>96</td>
<td>8x10^4</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>D18MG /198</td>
<td>GM</td>
<td>No</td>
<td>NR</td>
<td>NA</td>
<td>+</td>
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<td></td>
<td>9</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>D32MG /114</td>
<td>GS</td>
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<td>NR</td>
<td>NA</td>
<td>+</td>
<td>-</td>
<td>0.27</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>D37MG /86</td>
<td>GM</td>
<td>No</td>
<td>NR</td>
<td>NA</td>
<td>+</td>
<td>-</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D54MG /135</td>
<td>AA</td>
<td>Yes</td>
<td>-</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td>19-40</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D65MG /54</td>
<td>GM</td>
<td>No</td>
<td>NR</td>
<td>NA</td>
<td>+</td>
<td>-</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN 308/26</td>
<td>GM</td>
<td>+</td>
<td>81</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>NA</td>
<td>0.47</td>
<td>&gt;96</td>
<td>6x10^5</td>
<td></td>
</tr>
<tr>
<td>LN992/45</td>
<td>GM</td>
<td>NA</td>
<td>84</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>NA</td>
<td>0.28</td>
<td>48</td>
<td>6x10^5</td>
<td></td>
</tr>
<tr>
<td>LN215/90</td>
<td>GM</td>
<td>NA</td>
<td>66</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>NA</td>
<td>0.58</td>
<td>48</td>
<td>1.9x10^6</td>
<td></td>
</tr>
<tr>
<td>LN 235/63</td>
<td>GM</td>
<td>NA</td>
<td>116</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>NA</td>
<td>0.46</td>
<td>48</td>
<td>1.5x10^6</td>
<td></td>
</tr>
<tr>
<td>SF126/54</td>
<td>GM</td>
<td>No</td>
<td>AN</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>NA</td>
<td>42</td>
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<td>29</td>
<td>6x10^6</td>
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</tr>
<tr>
<td>SF188/70</td>
<td>GM</td>
<td>No</td>
<td>AN</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>NA</td>
<td>40</td>
<td>10</td>
<td>28</td>
<td>7x10^6</td>
<td></td>
</tr>
<tr>
<td>SF210/45</td>
<td>GM</td>
<td>No</td>
<td>AN</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>NA</td>
<td>34</td>
<td>0.65</td>
<td>28</td>
<td>5x10^6</td>
<td></td>
</tr>
<tr>
<td>SF268/51</td>
<td>AA</td>
<td>No</td>
<td>AN</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>NA</td>
<td>43</td>
<td>0.85</td>
<td>32</td>
<td>5x10^6</td>
<td></td>
</tr>
<tr>
<td>SF295/42</td>
<td>GM</td>
<td>No</td>
<td>AN</td>
<td>50%</td>
<td>-</td>
<td>50</td>
<td>NA</td>
<td>30</td>
<td>4.4</td>
<td>34</td>
<td>6x10^6</td>
<td></td>
</tr>
<tr>
<td>Nu74/103</td>
<td>GS</td>
<td>No</td>
<td>77-114</td>
<td>-</td>
<td>NA</td>
<td>+</td>
<td>-</td>
<td>9</td>
<td>41</td>
<td>24</td>
<td>2x10^5</td>
<td></td>
</tr>
<tr>
<td>2607/52</td>
<td>AA</td>
<td>Yes</td>
<td>80-98</td>
<td>-</td>
<td>NA</td>
<td>+</td>
<td>-</td>
<td>2.6</td>
<td>4.6</td>
<td>32</td>
<td>2.5x10^5</td>
<td></td>
</tr>
<tr>
<td>2891/46</td>
<td>AA</td>
<td>Yes</td>
<td>75-111</td>
<td>-</td>
<td>NA</td>
<td>+</td>
<td>-</td>
<td>3.7</td>
<td>8.1</td>
<td>26</td>
<td>1.5x10^5</td>
<td></td>
</tr>
<tr>
<td>5737/51</td>
<td>GM</td>
<td>yes</td>
<td>74-98</td>
<td>-</td>
<td>NA</td>
<td>+++</td>
<td>-</td>
<td>7.3</td>
<td>13</td>
<td>29</td>
<td>8.5x10^5</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations:
- positive
- negative
NA not assessed
NR not reported
AN anaploid
GFAP (%) refers to positivity of cells in a given culture
GFAP: glial fibrillary protein
PS: Plating efficiency
S100: glial fibrillary protein
GFAP: glutamine synthetase
PS: population doubling time, monolayer
FN: Fibronectin
GM: glioblastoma
GS: gliosarcoma
AA: anaplastic astrocytoma

Saturation density: cells/cm²

Reference:
Maounoury 1977
Bigner 1981
Studer 1985
Rutka 1987
Jacobsen 1987
Although continuous cell lines may serve as useful in vitro models of human tumours, the possibility of in vitro selection highlights the need for complementary studies in primary or low passage cultures. Primary and short term cultures of human glioma were established to facilitate the studies described in detail in Chapter 6.

3.1.1. Origins and establishment of human glioma lines

Continuous lines

Three human glioma cell lines had been established in this laboratory by previous workers; G-CCM; G-IJK-T; and G-UVW. The clinical origins of these lines, the primary glioma cultures established in this study, and continuous human glioma lines contributed to this study by other workers, are summarised in table 3.2
Table 3.2. Origins of continuous and primary glioma cultures

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Passage level</th>
<th>Origin</th>
<th>Source/ comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CCM</td>
<td>&gt;100</td>
<td>GM</td>
<td>Med.Oncology, Glasgow</td>
</tr>
<tr>
<td>G-IJK-T</td>
<td>&gt;100</td>
<td>GM</td>
<td>&quot;</td>
</tr>
<tr>
<td>G-UVW</td>
<td>&gt;100</td>
<td>GM</td>
<td>&quot;</td>
</tr>
<tr>
<td>U251</td>
<td>&gt;100</td>
<td>GM</td>
<td>Dr.Westermark, Uppsala</td>
</tr>
<tr>
<td>SB18</td>
<td>35-40</td>
<td>GM</td>
<td>Dr.Pilkington, London</td>
</tr>
<tr>
<td>SB clone 1</td>
<td>&quot;</td>
<td>GM</td>
<td>&quot;</td>
</tr>
<tr>
<td>SB clone 12</td>
<td>&quot;</td>
<td>GM</td>
<td>&quot;</td>
</tr>
<tr>
<td>G-MCN/X*</td>
<td>3-6</td>
<td>GM</td>
<td>INS; xenografted at P3</td>
</tr>
<tr>
<td>G-ELL</td>
<td>10</td>
<td>GM</td>
<td>INS</td>
</tr>
<tr>
<td>G-MCF</td>
<td>9</td>
<td>GM</td>
<td>&quot;</td>
</tr>
<tr>
<td>BG 398*</td>
<td>1-7</td>
<td>AA</td>
<td>&quot;</td>
</tr>
<tr>
<td>BG 424*</td>
<td>1-4</td>
<td>GM</td>
<td>&quot;</td>
</tr>
<tr>
<td>BG 488*</td>
<td>1-6</td>
<td>AA</td>
<td>Maudsley Hospital</td>
</tr>
<tr>
<td>BG 500*</td>
<td>1-5</td>
<td>GM</td>
<td>Edinburgh</td>
</tr>
<tr>
<td>BG 535*</td>
<td>1-5</td>
<td>GM</td>
<td>&quot;</td>
</tr>
<tr>
<td>BG 550*</td>
<td>1</td>
<td>GM</td>
<td>&quot;</td>
</tr>
<tr>
<td>BG 551*</td>
<td>1-3</td>
<td>GM</td>
<td>INS</td>
</tr>
<tr>
<td>BG 555*</td>
<td>1-7</td>
<td>GM</td>
<td>&quot;</td>
</tr>
<tr>
<td>BG 560*</td>
<td>1-3</td>
<td>GM</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

GM = glioblastoma multiforme, grade IV glioma  
AA = anaplastic astrocytoma, grade III glioma  
* = low passage cell lines established in this study  
INS; Institute of Neurological Sciences, Glasgow
U251, a continuous human glioma cell line derived from a high grade human glioma, was kindly donated by Dr.B.Westermark, Uppsala. IPSB 18 was derived from a high grade human glioma, and provided for collaborative studies at passage 35 by Dr.Jeff Pilkington, and Joanna Knott, Dept. of Neuropathology, Institute of Psychiatry, London.

The methods used to establish and maintain human glioma cell lines are described in Chapter 2, section 2.

3.2.1. Phenotypic characteristics

3.2.1.1. Morphology in monolayer

Human glioma lines exhibit considerable cellular heterogeneity in early passage, and distinct morphological forms can persist in established lines. Several workers have assigned descriptive categories to the morphology of human glioma lines [Ponten, 1975]. Three broad groups can be distinguished:

1) Fasicular / fusiform / fibroblastic
2) Polygonal / epithelioid
3) Glial / multipolar

The cell lines used in this study grew as adherent monolayer cultures on tissue culture grade plastic. Assessment of morphology was made in mid-log phase growth, using both phase-contrast microscopy, or fixation, Giemsa staining, and bright field microscopy.

Typical morphology of each of the 5 continuous lines used most extensively in this study are shown in section 3.3., figures 3.1-3. The morphology of an early passage human glioma culture, BG 488, established from a grade 3 tumour, is shown in figure 3.4.

3.2.1.2. Population doubling times in monolayer culture.

An assessment of the rate of cell doubling in exponential phase growth, and terminal density was made for each of the five glioma cell lines. $10^4$ cells were seeded into 2 individual 24 multiwell plates, and allowed to adhere. Three wells were trypsinised per day and cell numbers counted using a Coulter counter.
calibrated for each cell line independently. Medium was gently removed and 1ml fresh medium added each day to maintain exponential growth. Terminal density was judged to have been achieved when cell counts showed a plateau on 3 consecutive readings.

Mean counts obtained in 3 experiments were plotted on semilogarithmic axes, and a curve fitted by eye. All lines except IJK-T exhibited a lag phase of 24-48 hours, followed by exponential growth. Population doubling times were calculated from the slope of the exponential part of the growth curve.

Results are shown in figure 3.5

3.2.1.3. Loss of anchorage-dependent growth; multicellular spheroids, and soft agar cloning

Evidence of anchorage-independent growth was sought as a marker of the malignant phenotype. The capacity to form multi cellular aggregates and colony formation in soft agar was evaluated in the 5 glioma lines, and all successful primary cultures. 10^6 cells were harvested from exponential monolayer cultures, and seeded into T25cm^2 flask base-coated with 1% agar, to prevent adherence. All 5 lines formed multicellular spheroids within 24 hours and were then transferred into spinner flasks containing 100ml medium, and cultured for a further 48 hours.

All 5 continuous glioma lines demonstrated colony formation in 0.3% agar. Eleven of seventeen primary glioma cultures showed colony formation in 0.3% agar. (See Chapter 6) The remaining 6 cultures exhibited multicellular spheroid formation only, without growth in soft agar.

See figure 3.4

3.2.1.4. Expression of the intermediate filament, glial fibrillary acidic protein (GFAP).

The significance of positivity for the astrocytic lineage marker, GFAP, was discussed in Chapter 1.

10^4 tumour cells were seeded in 100μl of medium onto polylysine-coated sterile coverslips in 24 well plates, and allowed to adhere. The wells were fed with 1ml of medium and incubated overnight. Cells were fixed with ice-cold methanol, and left
for 15 minutes, at -20°C, prior to brief rehydration with Hanks staining medium. Rabbit anti-human GFAP (Dako) was added in 50ml Hanks medium (1:200 dilution), and left for 45 minutes. Coverslips were then washed in Hanks medium, and a goat anti-rabbit GFAP polyclonal second antibody conjugated with fluorescein (Southern Biotechnology) added at 1:100 dilution. Coverslips were treated with Cityflor antifade, and mounted cell-down on microscope slides, and sealed with nail varnish to prevent movement, and drying. Cells were then examined for fluorescence using a Reikert Polyvar microscope, and an estimate of percentage total cells staining, as well as intensity. A negative control was included (human lung carcinoma line, A549).

3.2.1.5. Expression of the cytosol enzyme, glutamine synthetase (GS)

The production of GS was evaluated in the 5 continuous glioma lines, using immunocytochemical techniques. This work was carried out by Dr. G. Pilkington, using rabbit polyclonal antibody (Sigma) raised against ovine GS.

3.2.1.6. Cell surface ganglioside and glycoprotein expression

There is increasing interest in elucidating whether cell surface properties of transformed human glioma cells might determine a more aggressive phenotype. Both surface gangliosides and membrane glycoproteins have been suggested as molecules which might participate in determination of cell motility, and both cell-cell, or cell-extracellular matrix interactions [Knott et al., 1990]. An evaluation of the expression of gangliosides recognised by the monoclonal antibody, A2B5, and glycoproteins recognised by the anti-CD 44 monoclonal, 44D10, was undertaken on the 5 human glioma lines. This work was done in collaboration with Dr. G. Pilkington, Dept. of Neuropathology, Institute of Psychiatry.

Cells examined for A2B5 positivity were prepared as described for GFAP staining, but were stained unfixed with undiluted mouse monoclonal A2B5 antibody (gift of Dr. Pilkington). A biotinylated anti-mouse Ig (Amersham) was used as second layer, and staining visualised by the fluorochrome FITC.

3.2.1.7. Immunostaining of glioma cell membrane glycoproteins by CD44 (P80)

CD 44 (cluster of differentiation) is a polymorphic family of membrane glycoproteins associated with cell adhesion. The monoclonal antibody, 44D10 recognises a 80 kDa glycoprotein antigen thought to be part of the CD 44 cluster...
in the membrane of astrocytes [Girgrah et al, 1991]. Hyaluronic acid is an extracellular matrix component involved in the control of motility in developing and neoplastic cells. The 44D10 antibody also recognises the hyaluronic acid receptor (HA) on cell membranes.

The 5 glioma cell lines were examined for Mab 44D10 positivity, using the protocol as described for A2B5 staining, using unfixed cells adherent to glass cover slips. Cells treated with Mab 44D10 were stained with 1:10 dilution of FITC-conjugated goat anti-mouse IgG for 30 mins at 0° C prior to fluorescence microscopy.

3.2.1.8. Tumorgenicity in nude mice.

The tumorgenicity of the 5 continuous glioma lines was evaluated by inoculation of female nu/nu athymic mice at a flank site, using 10^6 tumour cells in 0.5ml sterile saline.

Animals were housed in negative pressure isolators, and fed Sprat's SDI diet with autoclaved water ad lib. They were sacrificed by cervical dislocation when tumours reached 2cm diameter.

Tumour tissue was removed for histology, immunohistochemistry, and electron microscopy. (performed by Prof.D.Graham, Institute of Neurosciences, Glasgow) See section 3.3.

3.2.2 Genetic characteristics

3.2.2.1. Lactic dehydrogenase isoenzyme expression.

Confirmation of the human origin of the glioma cell lines, and exclusion of interspecies contamination was performed by examination of lactate dehydrogenase (LDH) isoenzyme profiles. The species of origin can be determined for cell lines by a variety of tests, including isoenzymology and cytogenetics. The ability of isoenzyme analysis performed on homogenates of cell lines derived from over 25 separate species has been demonstrated [Hay, 1986], and the technique can be reliably used to monitor for cross-contamination in laboratories routinely handling cell lines of differing species. The cell line G-MCN had been propagated via a xenograft, therefore it was considered particularly important to confirm the human nature of the culture.
In man LDH production is controlled by 2 genes, each responsible for the synthesis of a unique peptide, which combines with itself to form a tetramer. Two tetrameric proteins are thus formed, i.e., LDH-1 and LDH-5, which migrate differently in an electrophoretic field. LDH-1 represents the most anodic form, and LDH-5 the most cathodic form. Isoenzymes of intermediate migratory position, designated LDH 2, 3, and 4, are hybrids formed by random association of LDH 1 and 5 subunits.

By contrast, well-documented isoenzyme profiles of LDH in other mammalian species, including mouse, rat, and hamster, reveal quite distinct zymograms, differing from human forms in both number and migratory position on gel electrophoresis.

LDH isoenzymes contained within whole cell extracts, prepared by rapid freeze/thawing of tumour cell pellets, were separated by electrophoresis on agarose gels, and the areas of enzyme activity visualised by histochemical procedures, according to the following reactions:

1) $\text{L (+) Lactate} + \text{NAD} \rightarrow \text{Pyruvate} + \text{NADH}$
2) $\text{NADH} + \text{PMS} \rightarrow \text{NAD} + \text{PMS-H}$
3) $\text{PMS-H} + \text{TNBT} \rightarrow \text{PMS} + \text{TNBT-formazan}$

Abbreviations:
NAD - nicotinamide adenine dinucleotide
NADH - "", reduced
PMS - phenazine methosulphate
TNBT - tetranitroblue tetrazolium

Staining reagents and protocol were obtained from Sigma chemicals.

Analysis of LDH isoenzyme pattern was performed on each of 5 continuous glioma lines (and G-MCN, p6) on two separate occasions. The results of the analysis were recorded, with respect to both the number, and electrophoretic position of the enzyme forms in the tumour lines under study, together with the human and mouse LDH standards.

3.2.2.2. Cytogenetics, ploidy, and cell cycle analysis.

The objectives of these studies were:
1) To document the cell cycle characteristics and DNA content of each line by flow cytometric analysis.
2) To document the heterogeneity of karyotype within each cell line and identify any marker chromosomes.

**Chromosome analysis**
(Adapted from Freshney, 1987)

Cell lines were grown to mid exponential phase in T175cc flasks, then exposed to colcemid (Sigma) at a final concentration of $10^{-7}$ M for periods of time varying from 1-8 hours. Considerable modification of this general protocol was required for each glioma cell line, to compensate for large differences in cell cycle time, and to avoid excessive colcemid-induced chromosome condensation.

Cells were then harvested by routine trypsinisation into 2 separate universals, pelleted at 100g for 5 minutes, and the supernatant containing trypsin removed. Each pellet was resuspended in 5mls of a hypotonic solution at one of two different molarities (0.04M KCL, 0.025M sodium citrate or half this molarity) and incubated at 37° C to produce cell swelling. Time to produce adequate swelling without undue lysis required individualisation for each line examined, but varied between 15-30 minutes.

An equal volume of freshly made, ice-cold fixative (1 part glacial acetic acid, 3 parts methanol) was added slowly on a vortex mixer, then cells pelleted, and resuspended in 5mls fresh fixative, and left on ice for 20 minutes. The suspension was finally pelleted, and carefully resuspended on a vortex mixer with 0.5mls of fixative.

The suspension was dropped onto cleaned, cold microscopy slides. Slides were rapidly air-dried, and checked under the microscope for adequate dilution of the sample. Preparations were dried completely before staining with neat Giemsa, or G-banding.

G-banding was performed by incubation of fixed spreads in phosphate buffer (0.025M KH2PO4, pH 6.8) at 60° C for 10 minutes, followed by addition of a freshly made staining solution. (6.5mls phosphate buffer, 0.55mls 1% trypsin, 2.5mls 100% methanol, 0.22mls stock Giemsa solution).

After 15 minutes cells were rinsed in distilled water and air-dried. Preparations were examined unmounted under bright-field microscopy using a Leitz
microscope, and the number of chromosomes counted for a minimum of 50 spreads per cell line.

Photographs of representative spreads were taken on high definition Kodak Panatomic X film rated at 12 ASA to obtain optimal definition. Considerable individualisation of each of the major steps in this method were required to obtain evaluable spreads for the 6 glioma lines. Evaluation of karyotype was enhanced by use of a dedicated Cytoscan image analysis system, in collaboration with the Department of Cytogenetics, Institute of Cancer Research, London.

**Cell cycle analysis.**

These analyses were performed with the kind assistance of Dr. Campbell, Dept. of Biochemistry, Glasgow University, using a Becton Dickenson FACS 440.

Nuclei were prepared for flow cytometry by a modified trypsin-NP 40 detergent method; it has been demonstrated to yield uniform staining, with minimal clumping of human tumour cells, and relies on detergent lysis of cell membranes, together with digestion of cytoplasmic proteins by trypsin. Nuclei are stabilised during this detergent-trypsin stage by spermine. Using this procedure for human tumour cells it has been possible to produce "clean" nuclei, and reduce the coefficient of variation in fluorescence obtained, by eliminating clumping and nonspecific cytoplasmic staining.

An exponential culture of cells (T175cc flask) was harvested for each cell line by trypsinisation, pelleted, and resuspended in 200 microlitres of a citrate buffer (sucrose 250 mM, trisodium citrate 40mM, 5% DMSO, pH 7.6). At this stage cells could be stored at -70° C or further processed for cytometry.

Clean nuclei were prepared by the addition of 1800μl of Solution A (trypsin 15mg /500mls, NP 40 0.1% v/v, spermine tetrachloride 1.5mM, Tris 0.5mM, trisodium citrate 3.4mM) for 10 minutes at room temperature. Trypsinisation was halted by the addition of 1500μl of Solution B (trypsin inhibitor 250mg/500mls, Sigma, ribonuclease A 50mg/500mls) for 10 minutes. Nuclei were finally stained by ice-cold Solution C (propidium iodide 208mgs/500mls, spermine tetrachloride 1.5mM, pH 7.6) for 15 minutes on ice, covered with foil.
Cytometry was performed on the Becton Dickenson FACS using controlled aliquots of the suspension of stained nuclei in sheath fluid. Both chick red blood cells and human lymphocytes were used as internal standards for determination of G0 / G1 cell DNA content.

Cell cycle analysis was performed using Becton Dickinson software, employing both sum of broadened rectangles and polynomial models. Each analysis was the result of 10,000 "nuclear" counts on each sample.

3.3. Results.

3.3.1. Morphology of glioma cell lines.

Typical phase contrast micrographs of each of the 5 human glioma cell lines growing in monolayer, are shown in figures 3.1-3

The morphological features of each of the 5 continuous glioma lines was compared with published reviews, and the descriptive class is summarised in table 3.3.

For comparison, a plate illustrating the morphology of one of the early passage glioma cultures established in this study, BG 488, is shown in figure 3.4. This culture has morphological features in keeping with the description of "glial / multipolar" cultures described by Ponten [Ponten, 1975].
Figure 3.1. Morphology of human glioma cell lines, a) SB18, and b) U251

Phase contrast micrographs, x150 magnification
Figure 3.2  Morphology of human glioma cell lines, a) UVW, and b) CCM

Phase contrast micrographs, x150 magnification
Figure 3.3  Morphology of human glioma cell line, IJK, and IJK multicellular spheroid

200mm diameter spheroid
Figure 3.4. Morphology of human glioma culture, BG 488, at passage 1.

Phase contrast micrograph, x150 magnification. All lines except UJK exhibited a lag phase of growth of 24-48 hours before achieving exponential growth. Population doubling times, calculated from the exponential slope of the curve, are contained in Table 3.2. All 5 lines achieved saturation cell densities at significantly higher cell numbers than non-transformed glia, that is, approximately 7x10^5 per cm^2.
3.3.2 Population doubling times of continuous glioma lines in monolayer.

Growth curves obtained for the 5 continuous cell lines are shown in figure 3.5. All lines except IJK exhibited a lag phase of growth of 24-48 hours before achieving exponential growth. Population doubling times, calculated from the exponential slope of the curve are contained in table 3.2. All 5 lines achieved saturation cell densities at significantly higher cell numbers than non-transformed glia, that is, approximately $7 \times 10^4$ per cm$^2$. 
Figure 3.5  Monolayer growth curves of 5 glioma cell lines

Data points for each cell line represent mean and standard error.
3.3.3. Expression of biochemical and immunological markers.

Results obtained by immunostaining for the intermediate filament GFAP, glutamine synthetase, CD 44, and A2B5 are shown in table 3.3

Colour micrographs of fluorescence images obtained are shown in figures 3.6-8. FITC conjugated antibody staining fluoresces green within cytoplasm (GFAP), or at the cell membrane (CD 44). Nuclei have been counterstained with propidium iodide, which is seen to fluoresce as a yellow/red stain.

Figs.3.6-8 follow on the next 3 pages

3.6. Fluorescence micrograph of cell line UVW. Cell surface fluorescence of FITC-conjugated Mab anti CD 44 antigens.

3.7. Fluorescence micrograph of cell line CCM. Cytoplasmic fluorescence of FITC-conjugated Mab anti-GFAP, the intermediate filament protein. Nuclei counterstained with propidium iodide

3.8. Fluorescence micrograph of cell line SB18, GFAP staining
Table 3.3. Phenotypic and genetic characteristics of human glioma cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>UVW</th>
<th>CCM</th>
<th>U251</th>
<th>IJK</th>
<th>SB 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Passage level</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>35</td>
</tr>
<tr>
<td>Morphology</td>
<td>fusiform</td>
<td>fusiform</td>
<td>epithelioid</td>
<td>fusiform</td>
<td>fusiform</td>
</tr>
<tr>
<td>Cloning efficiency</td>
<td>20</td>
<td>10</td>
<td>30</td>
<td>28</td>
<td>29</td>
</tr>
<tr>
<td>Doubling time (hrs)</td>
<td>48</td>
<td>70</td>
<td>26</td>
<td>16</td>
<td>45</td>
</tr>
<tr>
<td>Terminal cell density</td>
<td>3.5x10^5</td>
<td>3.6x10^5</td>
<td>8.87x10^5</td>
<td>9.9x10^5</td>
<td>4.23x10^5</td>
</tr>
<tr>
<td>(cells/cm^2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>monolayer</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>spheroid</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>xenograft</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>GFAP (% cells +ve)</td>
<td>1-5</td>
<td>20-90</td>
<td>90+</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>GS (% cells +ve)</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>A2B5 (% cells +ve)</td>
<td>0.1</td>
<td>1</td>
<td>5</td>
<td>-</td>
<td>70</td>
</tr>
<tr>
<td>CD 44 (% cells +ve)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>DNA index</td>
<td>-</td>
<td>1.3</td>
<td>1.36</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>S phase fraction</td>
<td>-</td>
<td>-</td>
<td>37</td>
<td>25</td>
<td>16</td>
</tr>
<tr>
<td>(% nuclei per cell cycle)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G0 / G1</td>
<td>&quot;</td>
<td>&quot;</td>
<td>59</td>
<td>66</td>
<td>67</td>
</tr>
<tr>
<td>G2 / M</td>
<td>&quot;</td>
<td>&quot;</td>
<td>4</td>
<td>9</td>
<td>18</td>
</tr>
</tbody>
</table>

1 cell cycle analysis on mid log phase, exponentially growing culture.
3.3.4 Tumorgenicity in nude mice.

G-IJK, and G-MCN (passage 6) proved highly tumorgenic, with 100% take rate in 6 mice per line. Three primary cultures which exhibited vigorous monolayer growth, requiring passage within a week of initiation, were also examined for tumorgenicity. (BG 424; BG 550; BG 566) All three failed to be tumorgenic.

The xenografts were excised following sacrifice of the animals and immunohistochemistry, and electron microscopy kindly performed by Prof. DI. Graham, Dept. of Neuropathology, Institute of Neurological Sciences, Glasgow.

Both xenografts exhibited highly cellular growth, with anaplastic small cell/storiform morphology. Both tumours had approximately 3-5 mitoses per high power field (x400). G-IJK showed both muscle and skin invasion, on haematoxylin and eosin staining.

Tumours were evaluated by routine immunohistochemical techniques for a panel of markers of astrocytic or neural crest origin including: GFAP, and S100 protein. Production of other intermediate filament proteins, vimentin, and fibronectin were examined. Epithelial specific markers included epithelial membrane antigen (EMA), and cytokeratin.

<table>
<thead>
<tr>
<th></th>
<th>IJK xenograft</th>
<th>MCN xenograft</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>cytokeratin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EMA</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>vimentin</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td>fibronectin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>s100</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Nuclear and cytoplasmic appearances on electron microscopy were assessed in collaboration with Dr. J. Pilkington, Institute of Psychiatry. Figure 3.9 contains 2 electron micrographs showing a) mouse muscle infiltration by IJK tumour cells; b) the highly cellular G-MCN xenograft. Both tumours types gave rise to cells with a large nuclear/cytoplasmic ratio, and very prominent nucleoli, and margined heterochromatin. Phagocytic activity is seen in some cells, a feature of both reactive and malignant glia. Evidence of 10nm intermediate filaments could be detected in the cytoplasm in both tumours, and probably represents vimentin,
rather than GFAP, in keeping with the immunohistochemistry results. Desmosomal intercellular junctions, features of epithelial cells, were not seen.

a) IJK: showing invasion of mouse flank musculature. Mag.x 3000
b) MCN: densely cellular tumour, high nuclear/cytoplasmic ratio, with margined heterochromatin. Mag.x3000
3.3.5. LDH isoenzyme analysis

On the basis of the LDH isoenzyme profiles all 6 glioma lines would be considered to be of human origin, and to have zymograms differing considerably from all published for other routine laboratory mammals, including mouse, rat and hamster.

Glioma cell lines UVW, CCM, IJK-T, and SB18 had LDH isoenzyme profiles compatible with human origin, showing prominent LDH 1, 2 and 3. (the most anodic forms). LDH 4 was present at a lower intensity, and LDH 5 not discernible by eye.

U251 showed reversal of the above pattern, with greatest activity in LDH 4 and 5. The early passage line propagated via xenograft, G-MCN, revealed activity in this system at LDH 1 alone.

The mouse standard gave rise to a prominent band intermediate in mobility between human LDH 2 and 3, and dissimilar to the enzyme profiles of the 6 glioma cell lines, or the human LDH standard.

The LDH enzyme profile of G-MCN is interesting, with apparently no activity in LDH 5, or intermediate forms requiring the LDH 5 subunits. This result would be compatible with significant mutation at the site of the second human gene encoding LDH 5, leading to the production of an inactive form of the enzyme incapable of the biochemical reduction of formazan required by this assay. An alternative explanation might be complete deletion of this gene as part of the transformation of this cell line.
Figure 3.10  Gel electrophoresis, and staining for LDH isoenzymes in 6 human glioma lines

- cathode
+ anode

lane 1= Sigma human standard, 2ml
lane 2= UVW extract, 2ml
lane 3= CCM extract, 2ml
lane 4= IJK extract, 2ml
lane 5= SB 18 extract, 2ml
lane 6= U251 extract, 2ml
lane 7= MCN extract, 2ml
lane 8= Balb C mouse serum, 2ml
3.3.6. Cell cycle analysis by flow cytometry

See figure 3.11

Representative cytofluorograms are displayed for each cell line, with the normal human diploid lymphocyte distribution for comparison. Chick red blood cell internal standard diploid peaks appear at "27" on the horizontal scale of fluorescence. Normal human diploid DNA content gives a single peak at '66' on the horizontal scale.

DNA index is expressed as the ratio of G0/G1 peak channel number for each cell line, relative to that of the normal diploid human lymphocytes.

Table 3.3 contains the data obtained on 5 cell lines, for DNA index relative to normal human lymphocytes, and cell cycle analysis. Cell cycle analysis failed for samples from UVW, and CCM, using both polynomial and sum of broadened squares models due to the large coefficient of variation on peaks of fluorescence.

G-IJK shows a well-defined, hypodiploid peak at channel position "60", with a significant population of s phase cells. SB 18 shows a normal diploid DNA content, with a large G2/ M peak.

U 251 was highly aneuploid, with a broad G0 / G1 peak at channel "90".

Both G-CCM and G-UVW showed heterogeneity of ploidy, with evidence in both cell lines of two distinct cell populations. G-UVW contained a small hypodiploid peak at '42'. A broad second peak at '84', suggested the presence of a second cell population with a near-triploid DNA content. Similarly G-CCM shows a near-triploid cell population with peaks at 85/176, and a second, near tetraploid population with peaks at 150/300 on this scale.
Figure 3.11 Flow cytometry profiles of 5 glioma cell lines

control peaks; channel 22= chick red cells, channel 66= normal human lymphocytes

119
3.3.7. Cytogenetics.

Results are depicted as a representative karyotype of 4 of the continuous cell lines.

All lines demonstrated heterogeneity of populations within an individual cell line. A range of chromosome numbers was observed within a given cell line.

The modal chromosome number in UVW was 45, with evidence of a smaller second population of cells with a triploid chromosomal complement. Consistent chromosomal abnormalities noted were monosomy of chromosomes 4, and 10.

CCM had a modal chromosome number of 66, with monosomies for chromosomes 10, 11, and 18. Trisomy was frequently noted in chromosomes 1,3,4,5,6,7,8,9, 19,20, and X.

SB 18 had a modal chromosome number of 49. Monosomy for chromosome 10 was a typical abnormality, with loss of the Y sex chromosome, in a line derived from a male patient.

U251 had a modal chromosome number of 81, with cells showing frequent trisomies, and tetrasomal chromosome complements.

In all these 4 lines chromosome morphology was human.

Formal karyotyping of these lines was not available until the work for this thesis was being completed. At this time evidence of a phenotypic change was evident in late frozen stocks of IJK, with reduced cell size, and rapid population doubling times. Rodent contamination of the cell line IJK was found, on the basis of a population of cells having typical rodent chromosomal morphology. This had not been evident on the basis of LDH isoenzyme analysis undertaken at the start of these studies.
Figure 3.12  Karyotypes of cell lines UVW, CCM.
Figure 3.13 Karyotypes of cell lines U251, SB 18.
3.4. Discussion

The continuous human glioma cell lines used in these studies display a heterogeneous range of phenotypic and genetic features, but fulfill criteria required to be considered valid models of the malignant glial phenotype. Their properties are discussed in reference to other human glioma cell lines, and in knowledge of the heterogeneity observed in biopsies of human glioma.

The purpose of characterising human glioma lines at relatively high passage level can be justified on a number of grounds. Human cell lines are recognised to undergo genetic drift in sequential passage, often leading to changes in the phenotypic features of a specific cell lineage. Those human tumour lines preserving near-diploid status, and persistent markers of a particular pathway of differentiation are of considerable value in research. The 3 continuous lines G-IJK; G-CCM; G-UVW had been established in this lab prior to commencing this study, but no details of their characterisation at high passage had been published.

The aim of undertaking both genetic and phenotypic studies in the 5 human glioma lines used throughout this thesis was therefore to permit a critical assessment of the value of this particular panel of lines as models of human glioma. Secondly, as an extension of the notion that in vitro conditions permit evolution of cell lines, it was considered necessary to examine intrinsic cellular radiosensitivity in both continuous and low passage glioma cultures, thus requiring the establishment of primary glioma cultures.

The ideal human glioma cell line would be near-diploid, but with unequivocal evidence of malignancy, for example, tumorgenicity in nude mice. It would show persistent expression of recognised markers of astrocytic lineage, the most accepted of which remains expression of the intermediate filament protein, glial fibrillary acidic protein. A summary of phenotypic and genetic characteristics often sought in the characterisation of human glioma cell lines is presented in figure 3.14.
Figure 3.14  Criteria of malignancy and glial lineage in vitro

Tumour formation in nude mice

Other features suggestive of malignancy

Genetic
Aneuploidy
Abnormal karyotype
Oncogene expression

Growth
Anchorage-independent growth
Loss of contact inhibition; high saturation density
High passage level; ?>70

Biochemical
Abnormal plasminogen activator activity
Endothelial cell mitogenesis

Markers of glial lineage

Antigenic
GFAP expression
Glutamine synthetase activity
Surface ganglioside pattern
Monoclonal antibody positive (raised to human glioma, or fetal glia)
(S100 positive; neural crest origin)

Biochemical
High affinity GABA uptake
(lack of epithelial or mesenchymal markers)
In practice, many human tumour lines at the time of establishment, by definition, passage 70 or above, fail to fulfill idealised criteria. Review of the literature available on human glioma lines suggested that few cultures had been well-documented at high passage levels, whilst remaining in use as models of human glioma [Rutka et al, 1987; Studer et al, 1985].

Although lineage markers are important in the assessment of the nature of cells maintained in vitro, variations in their expression, both qualitatively and quantitatively are observed. Indeed, these observations are themselves subject to variation depending on method employed. For example, detection of the presence of GFAP is variable; decreasingly low levels can be found by staining with monoclonals to GFAP using immunofluorescent techniques; immunoblots to the protein; or detection of mRNA transcripts from the GFAP gene [Bongcam-Rudloff et al, 1991].

Equally, patterns of such markers are progressively altered with serial passage. Whether this represents culture selection of more "aggressive", less "differentiated" clones, lacking GFAP expression as part of their successful malignant phenotype, or merely reflects the inadequacy of current tissue culture techniques to maintain clonal diversity representative of the initial culture, is debatable. Indeed, there is very little data obtained from sequential biopsy of human glioma to state that such clonal changes observed in vitro may not be part of a biological progression in vivo. The pattern of expression of lineage/differentiation markers such as GFAP, or surface gangliosides, is likely to be heavily influenced by culture conditions, making comparisons of results between labs difficult [Westphal et al, 1990].

Groups who regularly employ explant or mechanical disaggregation without enzymes, claim a higher percentage of cultures maintaining GFAP expression. (personal communication: Dr. J. Pilkington) The biological relevance of persistence of GFAP expression in early passage cell lines is not clear; many workers report loss of GFAP positivity within the first 3-5 passages of malignant glial culture [Kennedy et al, 1987].

All 5 cell lines used in these studies have been demonstrated to express one or both of the accepted markers of astrocytic lineage within the CNS, namely GFAP or glutamine synthetase. In the cases of CCM, U251, and SB 18 both markers were expressed in most cells of each line. IJK produced glutamine synthetase, but
failed to express GFAP at high passage level, having expressed GFAP at low passage level [Frame et al,1984].

Whilst only IJK proved frankly tumorgenic in nude mice, the 4 other lines displayed unequivocal evidence of the transformed, malignant phenotype: U251, CCM, and UVW were highly aneuploid, containing populations of cells with near-triploid complement of chromosomes; all lines displayed anchorage-independent growth, both in soft agar, and as multicellular spheroids; high saturation density was reached in monolayer culture, with failure of contact inhibition of growth.

Of the 5 human glioma lines used in these studies, SB 18 would be regarded as approximating to the most satisfactory in vitro model of human glioma. The line remains near-diploid, and expresses markers of the differentiated astrocytic phenotype, GFAP and glutamine synthetase in a high percentage of cells. While it grows readily in monolayer and as multicellular spheroids, it is not tumorgenic. Although the cell line was established by Dr.Pilkington, the evaluation of its cloning efficiency, karyotype, cell cycle analysis, tumorgenicity, and radiosensitivity have not been previously undertaken.

All 5 cell lines exhibited satisfactory colony formation in monolayer, giving plating efficiencies ranging from 10-30%. This was achieved using 10% fetal calf serum, without specific growth factors. Plating efficiency was maintained most consistently by the use of a low density feeder layer of homologous, heavily irradiated cells. This system provided the basis for the use of colony formation as an endpoint for the measurement of cellular radiosensitivity in these 5 glioma lines.

It was also observed that these 5 glioma cell lines grew readily as multicellular spheroids. These provide models of human glioma in a multicellular, non-vascularised environment, and might well prove useful given the generally low level of tumorgenicity in glioma cell lines. Such models could be used for both fundamental or applied studies.

How relevant are current "in vitro" models of human glioma to clinical disease?

The relative ease with which human glioma cultures are established (30-40% of short term lines go on to form permanent lines [Maunoury,1977]) is partly explained by the relative lack of stromal fibroblastic overgrowth which complicates the establishment of epithelial tumour lines. However, it is important to address
the issue of the relevance of both short-term and established glioma cell lines to clinical disease.

Human malignant glioma exhibits considerable heterogeneity of clinical outcome. The histopathological assessment of biopsy material assigns a "grade" of malignancy on a three or four point scale, which is partly predictive of this clinical diversity of outcome. However, intratumour heterogeneity can complicate grading.

Similar morphological variation is observed in successful short-term glioma cultures and continuous cell lines. This observation of biological heterogeneity extends to many phenotypic and genetic parameters. A major goal of neurooncology is the refinement of prognostic assessment for patients with malignant glioma, based on both clinical and biological parameters. There is therefore considerable value in the careful documentation of in vitro characteristics of human glioma cell lines at a variety of passage levels.

Given the acknowledged selection of cells in long term culture, there are, however, still considerable attractions to the use of well-defined continuous cell lines. The more widespread use of short-term human glioma cultures has revealed some difficulties in characterising the exact nature of cells grown. There is evidence that bona fide tumour cells lose expression of GFAP in culture, whilst predominantly GFAP positive low passage cultures may fail to show unequivocal evidence of malignancy, for example, tumorigenicity in nude mice, or aneuploidy.

Doubts over lineage and malignancy could be resolved in the future by routine use of modern molecular techniques. For example, probing for GFAP specific mRNA has been demonstrated to be a very sensitive technique for the non-immunological detection of GFAP expression in glia [Bongcam-Rudloff et al, 1990]. Similarly, probing for the presence of either activated oncogenes, or loss of heterozygosity at known tumour suppressor loci might provide evidence of a successful culture of malignant cells, where no change in ploidy can be detected [Bigner et al, 1989].
Chapter 4

Intrinsic cellular radiosensitivity of human glioma cell lines
Chapter 4 ........................................................................................................... 128

4.1. Measuring human cellular radiosensitivity ............................................. 131
  4.1.1. Models of radiation action and mathematical parameters of radiosensitivity. ................................................................. 131
  4.1.2. Multitarget models of radiation action. ........................................... 132
  4.1.3 The linear-quadratic model. ............................................................... 133
  4.1.4. Use of the surviving fraction at 2 Gy as a measure of cellular radiosensitivity. ................................................................. 134
  4.1.5. Experimental objectives ............................................................... 135

4.2. Materials and methods ......................................................................... 136
  4.2.1. General tissue culture techniques, and maintenance of cell lines. ......................................................................................... 136
  4.2.2. Determination of feeder cell requirements and linearity of plating efficiency ................................................................. 136
  4.2.3. Effect of experimental protocol ................................................... 136
  4.2.4. Acute radiosensitivity data analysis. ........................................... 136
  4.2.5 Statistical analysis of SF2 data ................................................... 137
  4.2.6 Tumour radiocurability calculations based on intrinsic cellular radiosensitivity, repopulation kinetics, and clonogen number. ......................................................................................... 139

4.3. Results .................................................................................................. 143
  4.3.1. Acute radiosensitivity of the 13 human tumour cell lines. ................................................................................................. 143
  4.3.2. Surviving fraction at 2 Gy as a measure of cellular radiosensitivity ................................................................. 156
  4.3.3. Analysis of tumour control probability as a function of radiosensitivity at 2Gy ................................................................. 158
  4.3.4. Analysis of variance on the surviving fraction at 2Gy, SF2 ................................................................................................. 163
  4.3.5 Multiple pairwise comparisons of mean SF2 values between cell lines. ......................................................................................... 164

4.4. Discussion ............................................................................................ 165
  4.4.1. Survival curve fitting. ................................................................. 166
  4.4.2. Linear-quadratic model ............................................................... 166
  4.4.3. Multitarget model ...................................................................... 168
  4.4.4. Cellular radiosensitivity of human glioma lines ......................... 169
  4.4.5. Relevance of in vitro cellular radiosensitivity to clinical practice ................................................................................................. 170
4.4.6. Do measurements of SF2Gy in human cell lines reflect clinical radiocurability? .............................................................. 171
4.4.7. Relationship between SF2Gy measurements and tumour control probability.......................................................... 171
4.4.8. Conclusions. ........................................................................... 173
4.1. Measuring human cellular radiosensitivity

4.1.1. Models of radiation action and mathematical parameters of radiosensitivity.

A major objective of this study was a comparison of the cellular radiosensitivity of various human cell lines. It was therefore necessary to review the methods of describing radiosensitivity in mathematical terms. Whilst it is possible to compare clonogenic cell survival at a given dose of radiation e.g. 2Gy, the routine measurement of radiation action over several logs of kill has led to the development of formal models of radiation action. Different models can be fitted to survival data to generate mathematical parameters of radiosensitivity [Alper, 1979].

A detailed review of the various models of radiation action is not appropriate to the work contained in this thesis. Broadly, the purpose of such biophysical models could be regarded as primarily phenomenological or mechanistic. The former approach has sought a mathematical description which 'fits' the data set well, with no specific need to be related to actual physical mechanisms or subcellular biological processes. The success of the model is judged predominantly by the accuracy of fit to data sets. Mechanistic models of radiation action have sought to establish a greater understanding of the biophysics involved, but have been criticised for their inability to distinguish alternative mechanistic assumptions [Goodhead, 1985].

Although there is no widespread agreement on which model of radiation action is most valid, it is acknowledged that models with a minimum of 2 components are necessary to obtain adequate fits to experimental data on radiation cell survival [Steel, 1989]. Cell survival curves from human tumours invariably have a finite initial slope, and can usually be fitted well by the 2 models used in this study, namely the multitarget (with initial slope) and linear-quadratic models [Stephens et al, 1987; Malaise et al, 1987]. These models have been used extensively to describe human tumour radiosensitivity. It therefore enabled a comparison of parameters obtained in this study with the published literature on human tumour radiosensitivity.
4.1.2. Multitarget models of radiation action.

Sparsely ionising radiation, such as the megavoltage X or gamma rays used in radiotherapy, generate survival curves of characteristic shape in mammalian cells in vitro. At low doses there is an initial shoulder, followed by a response which becomes almost straight on a semilogarithmic plot. This observation indicates that the effectiveness of cell killing is not uniform per unit dose, and that simple Poisson statistics based on the assumption of random radiation deposition cannot determine the overall shape of the survival curve.

The multitarget parameter, \( D_0 \), represents mathematically the dose of radiation which delivers on average, 1 lethal event per cell, but by virtue of the random nature of radiation deposition, described by Poisson statistics, reduces the cell population to 37% of the original number.

The dose-response characteristics of the 'simple' multitarget model require that 2 parameters must be specified:

a) the slope of the final near-exponential portion, expressed as the \( D_0 \) reciprocal slope

b) a measure of the extent of the initial shoulder region, expressed as the extrapolation number \( n \), determined by extrapolating the final slope back to the surviving fraction axis. An alternative measure of the initial shoulder region, where cell killing appears to be less effective per unit dose, is the quasithreshold dose, \( D_q \). This quantity is the dose at which the straight portion of the survival curve extrapolated backwards cuts the dose axis drawn through a surviving fraction of unity.

The 3 parameters \( D_0 \), \( D_q \), and \( n \) are related by the expression:

\[
\log_e n = D_q / D_0
\]

The mechanistic interpretation of the multitarget model postulates that the cell contains \( n \) sensitive targets. All of these must be inactivated or "hit" to produce cell sterilisation. Therefore the probability of hitting one target is given by:

\[
p_1 = (1 - e^{-d / D_0})
\]

where \( d \) is dose for multitarget expression.

Therefore the probability of a cell surviving a given dose of radiation is:
At high doses the survival curve predicted from this model approximates to a pure exponential, appearing as a straight line on a log-linear plot, with a slope of \(-1 / D_0\). In practice, much mammalian cell survival data does not appear to have a zero initial slope at zero dose, leading to the modification of the basic multitarget equation to include a single event component at low doses

\[ S(d) = e^{-d/Ds} \left[ 1 - \left( 1 - e^{-d/D_0} \right) \right]^n \]

where \(d\) is given dose, \(D_0\) is the final slope, \(D_s\) is the initial slope, and \(n\) the extrapolation number.

This model has been fitted to the cell survival data obtained in human tumour cells examined in this study, to obtain the parameters \(D_0\), \(n\), and \(D_s\) for each of the cell lines.

The "goodness of fit" of the model is discussed in the context of the results obtained.

4.1.3 The linear-quadratic model.

In recent years cellular radiosensitivity has been most commonly described by the parameters of the linear-quadratic model of radiation action. It was developed on the basis of a proposed relationship between dose-related induction of double strand breaks within DNA, popularised by the work of Keller and Rossi, the "dual radiation action theory". [1973], and the molecular theory of Chadwick and Leenhouts.[1973]

The model is specified by the equation:

\[ S(d) = e^{-\alpha d + \beta d^2} \]

where \(S(d)\) is the fraction of cells surviving after a dose \(d\), and \(\alpha\) and \(\beta\) are constants.
The linear term was considered to estimate the direct induction of lethal DNA double strand breaks, whilst the quadratic term was felt to represent the statistical probability that two adjacent single strand breaks, each arising by chance, could interact biologically to produce a lethal DNA lesion.

Although a mechanistic basis has been postulated for this model, its wide acceptance as a means of describing cellular radiosensitivity can be more readily attributed to the fact that as a simple, continuously bending survival equation it simulates much of the observed experimental data in mammalian systems [Steel and Peacock, 1989].

The linear-quadratic model has been fitted to experimental clonogenic survival data in this study, to obtain the parameters \( \alpha \) and \( \beta \) for each cell line examined. The statistical methods used to fit the survival curves are described in the methods section of this chapter.

### 4.1.4. Use of the surviving fraction at 2 Gy as a measure of cellular radiosensitivity.

In recent years there has been a trend towards the use of a model-free parameter of radiosensitivity, the surviving fraction of clonogenic cells after a single dose of 2Gy (SF2). There are several reasons for this development.

1) Doubts have been expressed concerning the need to describe radiosensitivity over a large dose range. Acute radiation survival curves are generated by measuring the response to varying single doses of radiation, usually between 1 and 12Gy. The models require replicate data points at each of a minimum of 4 different doses to permit acceptable statistical fits. In the case of the multitarget model, the most frequently cited parameter of response, the Do, is determined by the response beyond the initial shoulder region, and describes in the case of most tumours, the response to doses of radiation far in excess of those employed in clinical practice.

2) The work of Fertil and Malaise, followed by Deacon and Steel, drew attention to the variation between tumour types in clonogenic cell survival at doses of clinical relevance ie 2Gy. This is a single dose of radiation often given daily over a fractionated course of treatment. The large literature reviews of human tumour radiosensitivity carried out by these groups suggested that the variance in SF2...
estimation between human tumours of differing histology might be sufficient to explain the observed range of clinical radiocurability.

3) The original studies by Fertil and Deacon cited SF2 values obtained from published cell survival curves i.e., were obtained by model fitting then interpolation. The measurement of actual SF2Gy, as opposed to "fitted" SF2 values, is potentially more applicable to a primary cell culture. It requires fewer cell numbers, as response is assessed at only one dose. This is an obvious advantage in dealing with primary biopsy material in which clonogenicity is usually low.

4.1.5. Experimental objectives

The objectives of the experiments described in this chapter were:

1) To measure the response of 5 human glioma continuous lines to acute doses of megavoltage irradiation.

2) To describe the cellular radiosensitivity of the these lines in terms of the 2 major models of radiation action, and the model-free parameter, the surviving fraction of cells after a single dose of 2Gy. (SF2)

3) To compare the distribution of cellular radiosensitivity observed in the glioma lines with a panel of 8 other human tumour lines, derived from tumours of greater clinical radiocurability, including carcinoma and embryonal cell lines.

4) To conduct a detailed statistical comparison of the differences in intrinsic radiosensitivity measured in these 13 lines, using the model-free estimate of radiosensitivity, the SF2Gy. The purpose of this analysis was to examine the hypothesis that human glioma cell lines differed significantly in their radiosensitivity from cell lines derived from the more clinically radiocurable carcinomas.

5) To derive estimates of the radiation doses required to control human glioma, based on in vitro measurements of cellular radiosensitivity, by using Poisson statistics.
4.2. Materials and methods.

4.2.1. General tissue culture techniques, and maintenance of cell lines.

A summary of the lines used in this study and their culture requirements is detailed in table 2.1. Similarly, the protocol observed for clonogenic cell survival assays, and irradiation of cells is described in detail in section 2.3.

4.2.2. Determination of feeder cell requirements and linearity of plating efficiency

Pilot experiments were performed on each continuous cell line to establish the range of linearity of plating efficiency, ascertain feeder layer requirements, and determine the optimal incubation period to obtain colonies of greater than 50 cells. In subsequent experiments cell numbers were chosen to produce approximately 150 colonies per experimental data point.

4.2.3. Effect of experimental protocol: timing of subculture in relation to irradiation.

An alternative experimental protocol was evaluated, in which test cells were irradiated as exponentially growing monolayers, then subcultured at cloning densities immediately following irradiation. In all lines examined, this procedure resulted in a small, statistically insignificant fall in plating efficiency, but similar survival curves to those obtained by irradiation as preplated cultures.

4.2.4. Acute radiosensitivity data analysis.

Survival curves were fitted to the data plotted on semilogarithmic axes, using both multitarget (MT) and linear-quadratic (LQ) models of radiation action. A minimum of 5 different dose points were studied, to examine dose-response over at least 2 logs of cell kill. Within each experiment between 3 and 6 replicate flasks were treated per data point.
Both LQ, and MT models were fitted to the data by non-linear regression, using the error-minimising programme of Koschel, kindly provided by Prof. Gordon Steel [Millar, 1978]. The surviving fractions, weighted by the inverse of their variances, were fitted by the least-squares method to the linear-quadratic equation

$$S = \exp(- (\alpha d + \beta d^2))$$

where $S$ is the surviving fraction, $d$ is the dose of radiation, and $\alpha$ and $\beta$ are the constants describing the linear and quadratic components of the response.

The simple multitarget model was fitted according to the equation:

$$S = 1 - (1 - e^{-d/Do})^n$$

where $d$ is given dose of radiation, $S$ is surviving fraction measured, and $Do$ the slope of the exponential part of the response, and $n$ the extrapolation number, representing the derived parameters of radiosensitivity.

The multitarget model with initial slope was fitted by the equation:

$$S(d) = e^{-d/Ds} \left[ 1 - (1 - e^{-d/D0}) \right]^n$$

where $Ds$ is the initial slope.

Data was pooled for a minimum of 4 separate experiments on each cell line, to determine parameters of radiosensitivity according to the multitarget and LQ models.

### 4.2.5 Statistical analysis of SF2 data.

The variance of any single SF2 estimation on a cell line is made up of two components: a degree of variance is attributable to the experiment ($\sigma^2_{exp}$), and a further component contributed by replicates within the same experiment ($\sigma^2_{rep}$). The variance of the mean of replicates within experiments is given by:
\[ \sigma^2 \text{exp} + \sigma^2 \text{rep}/r \]

where \( r \) is the number of replicates

The mean SF2 calculated from the means of repeat experiments on the same cell line is used as an estimate of the "true" SF2 value for that line. The variance of this estimate for a theoretical study containing 3 independent experiments, each containing 3 replicates, is specified by:

\[ \sigma^2 \text{exp}/3 + \sigma^2 \text{rep}/9 \]

The standard error of this estimate is given by:

\[ \sqrt{\frac{\sigma^2 \text{exp}}{3} + \frac{\sigma^2 \text{rep}}{9}} \]

Estimates of all the above variances have been obtained by analysis of variance, using a nested design to specify these contributions to variance independently.

Data transformation.

Analysis of variance assumes that the variance of all the observations on different cell lines was similar. This was not valid for the SF2 observations on the 13 cell lines studied. They were therefore transformed by a log odds procedure to generate new observations, termed \( Z \) as follows:

\[ Z = \log \left( \frac{\text{SF2}}{1-\text{SF2}} \right) \]

The transformed observations were noted to have a normal distribution, required for analysis of variance. The analysis of variance was carried out on \( Z \) values of SF2 for each of the 13 cell lines, to assess the effect of both intra and interexperimental variation on estimates of SF2.
Multiple pairwise comparisons of mean SF2 values between cell lines.

The SF2 data on each of the 13 tumour lines was further assessed by use of the Tukey HSD test for post hoc pairwise comparisons of means. There are 78 pairwise comparisons of mean estimations of SF2, contained in the data set on the 13 cell lines. A comparison was made, specifying an overall minimum significance level of 5%.

4.2.6 Tumour radiocurability calculations based on intrinsic cellular radiosensitivity, repopulation kinetics, and clonogen number.

Tumour radiocurability calculations were based on a simple Poisson cure model. This incorporates the assumption that tumour cure requires that zero clonogens should survive treatment. If $S_k$ is the average surviving fraction following $k$ radiotherapy treatments, and $N_0$ is the average number of clonogens initially present, then $N$, the average final number of clonogens will be

$$N = S_k N_0$$

(1)

If $N$ is the average number of surviving clonogens per tumour, Poisson theory gives the probability that any particular tumour contains exactly zero clonogens at the end of treatment, i.e. is cured. The theory states that this probability is the first term of a Poisson series with a mean $N$. The cure probability $P_c$ is given by

$$P_c = e^{-N}$$

(2)

$$= e^{-S_k N_0}$$

(3)

The net surviving fraction $S_k$ after $k$ treatments depends on the balance of log cell kill achieved by each treatment, and the repopulation by surviving clonogens between treatments. If 2Gy treatment fractions are used throughout, and if $S_2$ is the surviving fraction after one treatment of 2Gy, and $T_D$ the tumour doubling time (assumed to be constant throughout treatment) then we have
\[ S_k = (S_2)^k \cdot e \]  

where \( T \) is the total treatment time for the administration of \( k \) fractions.

This gives

\[ \frac{0.693T}{TD} \cdot (S_2)^k \cdot e \cdot N_o \]

Equation (5) may be used to calculate the cure probability (\( P_c \)) as a function of total dose, for a given assumption regarding the intrinsic radiosensitivity (\( S_2 \)), and tumour doubling time (\( T_D \)). The results presented in section 4.3 have been calculated assuming conventional fractionation, i.e., daily single treatments of 2 Gy, five times per week.

The total dose required to give a tumour cure probability of 50% is known as the TCD-50 value. This parameter can be calculated from equation (5):

\[ -\ln P_c = (S_2)^k \cdot e \cdot N_o \]  

Equation (6)

\[ \frac{0.693 (1.4) k}{TD} \]

\[ = (S_2)^k \cdot e \cdot N_o \]  

Equation (7)

\[ \ln (-\ln P_c) = k \ln (S_2) + \frac{0.693 (1.4) \cdot k + \ln (N_o)}{TD} \]  

Equation (8)
or \[ K = \frac{\ln(-\ln Pc) - \ln(No)}{\ln(S2) + (0.693)(1.4)} \] \hspace{1cm} (9)

For 2 Gy fractions, the total dose \( D \) which corresponds to this is

\[ D = 2 \frac{\ln(-\ln Pc) - \ln(No)}{\ln(S2) + (0.693)(1.4)} \] \hspace{1cm} (10)

The dose required to achieve any level of cure probability may be calculated from this equation.

In the case of the dose required to give a 50% chance of tumour control (the \( TCD-50 \))

\( TCD-50 = -0.367 - \ln(No) \) \hspace{1cm} (11)

\[ \frac{\ln(S2) + 0.97}{TD} \]

The \( TCD-50 \) values presented in section 4.3. based on the human glioma cell line intrinsic radiosensitivity were calculated from equation (11).

Equation (10) may also be rearranged to give \( \ln No \), the initial clonogenic cell number which, if treated with a particular regimen, would result in cure probability \( Pc \).

For a 50% cure probability \( \ln No \) is given

\[ \ln No = -0.367 - 0.5 D \left\{ \ln(S2) + 0.97 \right\} \] \hspace{1cm} (12)

\[ \frac{TD}{TD} \]

This is the curable cell number at the 50% cure level, for a regimen consisting of total dose \( D \), given as 2Gy fractions. For a conventional curative clinical treatment regimen (30 \( \times \) 2Gy fractions, or 60 Gy total dose), this becomes
\[
\ln (\text{CCN}) = -0.367 - 30 \left\{ \ln (S2) + \frac{0.97}{TD} \right\} \quad (13)
\]

Equation (13) was used to calculate the curable clonogenic cell number, CCN, for the human glioma cell lines, as a function of tumour doubling time. The CCN values are presented in tabular form, as logarithms to the base 10.
4.3. Results

4.3.1. Acute radiosensitivity of the 13 human tumour cell lines.

The acute radiosensitivity of the 13 human tumour cell lines studied are depicted in both graphical and tabular form in figures 4.1-9, and tables 4.1-3 respectively.

The acute response to megavoltage irradiation of each of the 5 glioma lines is depicted as the semilogarithmic plot of the data obtained on each cell line using the surviving fraction of colony forming cells as a function of radiation dose as the experimental endpoint.

For each of the five human glioma cell lines studied, survival curves have been fitted to the data using the multitarget (both simple, and initial slope) and linear-quadratic models of radiation action. Each data point represents the mean surviving fraction obtained in replicate experiments. Figures 4.1-5.

Figure 4.8 summates the data obtained for the 13 cell lines. The mean LQ fitted curves are depicted for each of the 5 glioma cell lines. The mean LQ curves for the 3 embryonal lines, and 5 carcinoma lines are each depicted as single dashed lines.
Data points represent values obtained in replicate experiments

linear-quadratic model
simple multitarget model
MT with initial slope
Figure 4.2. Acute radiation survival data for human glioma line, UVW.

Data points represent values obtained in replicate experiments

linear-quadratic model
simple multitarget model
MT with initial slope
Figure 4.3 Acute radiation survival data for human glioma line SB18.

Data points represent values obtained in replicate experiments.

- linear-quadratic model
- simple multitarget model
- MT with initial slope
Figure 4.4. Acute radiation survival data for human glioma line, IJK

Data points represent values obtained in replicate experiments

- linear-quadratic model
- simple multitarget model
- MT with initial slope
Figure 4.5. **Acute radiation survival data for human glioma line CCM.**

Data points represent values obtained in replicate experiments.

- Linear-quadratic model
- Simple multitarget model
- MT with initial slope
Figure 4.6. Acute radiation survival data for 3 human embryonal cell lines.

Data points represent values obtained in replicate experiments. Curves fitted by LQ model.
Figure 4.7a. Acute radiation survival data for human carcinoma cell lines.

Data points represent values obtained in replicate experiments. Curves fitted by LQ model.
Figure 4.7b. Acute radiation survival data for human carcinoma cell lines.

Data points represent values obtained in replicate experiments. Curve fitted by LQ model.
Figure 4.8. Pooled acute radiation survival curves for 13 human cell lines

Lines represent the mean LQ curves for each cell line. Individual data points omitted for clarity.
<table>
<thead>
<tr>
<th>Cell line</th>
<th>LQ model MT (Initial slope model)</th>
<th>SF 2Gy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha$ (Gy$^{-1}$)</td>
<td>$\beta$ (Gy$^{-2}$)</td>
</tr>
<tr>
<td>IJK</td>
<td>0.076 (0.03)</td>
<td>0.024 (0.003)</td>
</tr>
<tr>
<td>UVW</td>
<td>0.10 (0.03)</td>
<td>0.042 (0.004)</td>
</tr>
<tr>
<td>CCM</td>
<td>0.22 (0.03)</td>
<td>0.048 (0.005)</td>
</tr>
<tr>
<td>u251</td>
<td>0.21 (0.04)</td>
<td>0.051 (0.006)</td>
</tr>
<tr>
<td>SB18</td>
<td>0.10 (0.02)</td>
<td>0.026 (0.002)</td>
</tr>
<tr>
<td>mean</td>
<td>0.14 (0.03)</td>
<td>0.038 (0.006)</td>
</tr>
</tbody>
</table>

Figures represent mean value of parameter with standard error, determined on 4-6 independent experiments on each cell line.
Table 4.2 Acute radiation survival curve parameters: human carcinoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>LQ model</th>
<th>MT(+IS) model</th>
<th>SF 2Gy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \alpha ) (Gy (^{-1}))</td>
<td>( \beta ) (Gy (^{-2}))</td>
<td>( \alpha / \beta )</td>
</tr>
<tr>
<td>A2780 (ovary)</td>
<td>0.61</td>
<td>0.047</td>
<td>12.9</td>
</tr>
<tr>
<td>MCF 7 (breast)</td>
<td>0.19</td>
<td>0.021</td>
<td>9.04</td>
</tr>
<tr>
<td>HT 29 (colorectal)</td>
<td>0.086</td>
<td>0.035</td>
<td>2.45</td>
</tr>
<tr>
<td>SKMES (lung,squamous)</td>
<td>0.21</td>
<td>0.027</td>
<td>7.78</td>
</tr>
<tr>
<td>A549 (lung,adeno)</td>
<td>0.15</td>
<td>0.025</td>
<td>6.0</td>
</tr>
<tr>
<td>mean</td>
<td>0.24</td>
<td>0.031</td>
<td>7.63</td>
</tr>
</tbody>
</table>

Figures represent mean value of parameter with standard error, determined on 4 -6 independent experiments on each cell line.
Table 4.3. Acute radiation survival curve parameters: human embryonal tumour cell lines

<table>
<thead>
<tr>
<th>Cell line (tumour type)</th>
<th>LQ model</th>
<th>MT model</th>
<th>SF 2Gy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha$ (Gy$^{-1}$)</td>
<td>$\beta$ (Gy$^{-2}$)</td>
<td>$\alpha/\beta$</td>
</tr>
<tr>
<td>Susa (teratoma)</td>
<td>0.76</td>
<td>0.051</td>
<td>14.9</td>
</tr>
<tr>
<td></td>
<td>(0.072)</td>
<td>(0.014)</td>
<td></td>
</tr>
<tr>
<td>IMR 32 (neuroblastoma)</td>
<td>0.85</td>
<td>0.032</td>
<td>26.5</td>
</tr>
<tr>
<td></td>
<td>(0.079)</td>
<td>(0.018)</td>
<td></td>
</tr>
<tr>
<td>SKNSH (neuroblastoma)</td>
<td>0.71</td>
<td>0.039</td>
<td>18.2</td>
</tr>
<tr>
<td></td>
<td>(0.06)</td>
<td>(0.011)</td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>0.77</td>
<td>0.041</td>
<td>19.8</td>
</tr>
<tr>
<td></td>
<td>(0.04)</td>
<td>(0.006)</td>
<td>(3.4)</td>
</tr>
</tbody>
</table>

Figures represent mean value of parameters determined on 4-6 independent experiments, with standard error.
The numerical results derived from the fitting of both multitarget and LQ models are presented as tables 4.1-3. The mean and standard error is presented for the each of the survival curve parameters obtained from data pooled from a minimum of 4 independent experiments on each cell line. In the case of the 3 embryonal lines, depicted in table 4.3, the simple multitarget parameters are given, due to difficulties encountered fitting the MT with initial slope to these very radiosensitive lines.

Data has been grouped for presentation by overall histological category ie. human glioma data in table 4.1, human carcinoma line data in table 4.2, and embryonic line data in table 4.3.

4.3.2. Surviving fraction at 2 Gy as a measure of cellular radiosensitivity

The surviving fraction at 2 Gy (SF2) for each cell line was measured directly in the clonogenic assay, as a model-free parameter of radiosensitivity. Data is presented included in tables 4.1-3 as the mean and standard error for each cell line. Data has been pooled from a minimum of 4 independent clonogenic assays on each cell line.

The mean values of SF2 are also shown in graph form, as figure 4.9. The error bars represent the 95% confidence limits on the estimation of the mean for each cell line.

The mean values of SF2 for the three tumour groups reflected the parameters of the multitarget and linear-quadratic models. The mean values for the glioma cell lines showed considerable overlap with the carcinoma cell lines, with group means of 0.68 (SE 0.05) and 0.60 (SE 0.1) respectively. The 3 embryonal cell lines had considerably lower values of SF2, with a mean of 0.18 (SE 0.01)
Figure 4.9 Clonogenic surviving fraction at 2Gy for 13 human tumour cell lines.

1.0
0.8
0.6
0.4
0.2
0.0

SF2Gy

8
9
10
11
12
13

1=IMR32
2=SKNSH
3=SUSA
4=A2780
5=MCF7
6=A549
7=SKMES
8=HT29
9=U251
10=SB18
11=CCM
12=UVW
13=IJK

157
4.3.3. Analysis of tumour control probability as a function of radiosensitivity at 2Gy.

The mean surviving fraction at 2Gy obtained on each of the 13 human cell lines has been used to calculate the theoretical doses required to control 50% of tumours of the same histological type, the so-called TCD-50, based on Poisson statistics.

Results are presented as:

1) The TCD-50 in Gray derived from the mean values of SF2 obtained on each of the 13 human cell lines, grouped by histology. Fig.4.10

2) The variation in TCD-50 for each of the 3 histological groups, as a function of radiosensitivity at 2Gy, and with varying theoretical tumour doubling times, from 2 days to infinity. Tables 4.-4-6

The calculations are based on a theoretical tumour of $10^{10}$ cells, all of which are clonogenic. The radiotherapy fractionation schedule is daily fractions of 2Gy, as commonly used in clinical practice.

It can be seen that the range of TCD-50 doses for the 3 histological classes of tumours vary considerably. The lowest value of 27Gy was calculated for the embryonic tumours, based on the mean SF2Gy for the 3 cell lines, of 0.18. This value is based on the presumption that no tumour repopulation occurs during the course of therapy.

The TCD-50 for the carcinomas was based on the group mean SF2 Gy of 0.60, again assuming no repopulation. The TCD-50 value of 91.6Gy would be considered high on the basis of clinical dose-response.

The TCD-50 value for the glioma group, assuming no repopulation, is 122Gy. This value is approximately double the total dose usually given in clinical treatment of glioma post-operatively.
Figure 4.10 TCD-50 doses calculated for human tumours of a given histological type, based on in vitro SF2Gy data.

Each symbol represents the dose required to control 50% of tumours, based on the in vitro cellular radiosensitivity of each of the 13 human tumour cell lines studied, displayed by histological group.
Table 4.4. Theoretical radiocurability of human glioma as a function of in vitro estimates of intrinsic radiosensitivity, and tumour doubling times.

<table>
<thead>
<tr>
<th>Doubling time days</th>
<th>TCD 50% (Gy)</th>
<th>log (CCN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>3</td>
<td>750</td>
<td>0.65</td>
</tr>
<tr>
<td>4</td>
<td>327</td>
<td>1.71</td>
</tr>
<tr>
<td>5</td>
<td>244</td>
<td>2.34</td>
</tr>
<tr>
<td>10</td>
<td>162</td>
<td>3.60</td>
</tr>
<tr>
<td>20</td>
<td>138</td>
<td>4.23</td>
</tr>
<tr>
<td>∞</td>
<td>122</td>
<td>4.85</td>
</tr>
</tbody>
</table>

Notes:
Calculation based on the following:
1) Mean SF2Gy of 5 human glioma lines: 0.68
2) Theoretical radiation treatment schedule: 2Gy daily, 5 days per week.
3) TCD 50% is total dose required to give 50% tumour cure probability, for a theoretical tumour containing 10^{10} clonogenic cells, based on Poisson statistics.
4) CCN is the 'curable cell number' i.e. number of clonogenic cells for which a treatment course of 30x2Gy would give cure probability of 50%
Table 4.5 Theoretical radio-curability of human carcinomas as a function of in vitro estimates of intrinsic radiosensitivity and tumour doubling time.

<table>
<thead>
<tr>
<th>Doubling time (days)</th>
<th>TCD 50%</th>
<th>log CCN</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1811</td>
<td>0.18</td>
</tr>
<tr>
<td>3</td>
<td>250</td>
<td>2.28</td>
</tr>
<tr>
<td>4</td>
<td>174</td>
<td>3.34</td>
</tr>
<tr>
<td>5</td>
<td>148</td>
<td>3.97</td>
</tr>
<tr>
<td>10</td>
<td>113</td>
<td>5.23</td>
</tr>
<tr>
<td>20</td>
<td>101</td>
<td>5.86</td>
</tr>
<tr>
<td>∞</td>
<td>91.6</td>
<td>6.5</td>
</tr>
</tbody>
</table>

Notes.

Calculation based on the following:

1) Mean SF2Gy of 5 human carcinoma lines: 0.60
2) Theoretical radiation treatment schedule: 2Gy daily, 5 days per week.
3) TCD 50% is total dose required to give 50% tumour cure probability, for a theoretical tumour containing $10^{10}$ clonogenic cells, based on Poisson statistics.
4) CCN is the 'curable cell number' i.e. number of clonogenic cells for which a treatment course of 30x2Gy would give cure probability of 50%
Table 4.6. Theoretical radiocurability of human embryonic tumours as a function of in vitro estimates of intrinsic radiosensitivity and tumour doubling time.

<table>
<thead>
<tr>
<th>Doubling time (days)</th>
<th>TCD 50%</th>
<th>log CCN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>63</td>
<td>9.6</td>
</tr>
<tr>
<td>2</td>
<td>38</td>
<td>15.9</td>
</tr>
<tr>
<td>4</td>
<td>32</td>
<td>19.0</td>
</tr>
<tr>
<td>5</td>
<td>31</td>
<td>19.7</td>
</tr>
<tr>
<td>10</td>
<td>29</td>
<td>20.9</td>
</tr>
<tr>
<td>20</td>
<td>28</td>
<td>21.6</td>
</tr>
<tr>
<td>\infty</td>
<td>27</td>
<td>22</td>
</tr>
</tbody>
</table>

Notes.

Calculation based on the following:

1) Mean SF2Gy of 5 human embryonic tumour lines: 0.18
2) Theoretical radiation treatment schedule: 2Gy daily, 5 days per week.
3) TCD 50% is total dose required to give 50% tumour cure probability, for a theoretical tumour containing 10^{10} clonogenic cells, based on Poisson statistics.
4) CCN is the 'curable cell number' i.e. number of clonogenic cells for which a treatment course of 30x2Gy would give cure probability of 50%
4.3.4. Analysis of variance on the surviving fraction at 2Gy, SF2

The tumour lines were grouped for analysis of variance by histological type as described in tables 4.1-3

1) Group 1  Embryonal lines  IMR32; SKNSH; SUSA
2) Group 2  Carcinoma lines  A549; MCF7; A2780; HT29; SKMES
3) Group3  Glioma lines  U251; cCCM; IJK; SB18; UVW

Table 4.7  Analysis of variance on SF2 values

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
<th>F-ratio</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lines</td>
<td>154.327</td>
<td>12</td>
<td>12.861 (1)</td>
<td>38.05 (4)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Experiment</td>
<td>8.782</td>
<td>26</td>
<td>0.338 (2)</td>
<td>5.506 (5)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Replicates</td>
<td>4.785</td>
<td>78</td>
<td>0.061 (3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean square value (3) represents $\sigma^2_{\text{rep}}$
Mean square value (2) represents $3\sigma^2_{\text{exp}} + \sigma^2_{\text{rep}}$

therefore $\sigma^2_{\text{exp}} = \frac{(0.338 - 0.061)}{3} = 0.0923$

The F ratio (5) = $\frac{(2)}{(3)}$

tested the hypothesis that repeat experiments on the same line generated no additional variability in SF2 estimations, ie $\sigma^2_{\text{exp}} = 0$.

This was rejected at the p<0.001 level.
The F ratio (4) = (1) / (2)

tested the hypothesis that there was no difference between the mean values of SF2 of any of the 13 cell lines. This was rejected at the p< 0.001 level.

The variance in the estimate of the "true" (transformed) SF2 value for each cell line is specified by:

\[ \sigma^2 \exp / 3 + \sigma^2 \text{rep} / 9 \]

= 0.038

therefore the standard error = \[ \sqrt{0.038} \]

= 0.194

The analysis of variance showed statistically significant differences between the mean values of SF2 for Group1 tumour lines, compared with group 2 or 3 (p value < 0.001).

Comparison of mean SF2 values in tumour group 2 with tumour group3 was also significant at a p value of 0.003. This difference was accounted for predominantly by the inclusion in group2 of the ovarian carcinoma line, A2780. When this line was excluded from the analysis no significant difference could be detected in the mean values for the remaining 4 carcinoma cell lines, and the 5 glioma cell lines.

4.3.5. Multiple pairwise comparisons of mean SF2 values between cell lines.

The SF2 data on each of the 13 tumour lines was further assessed by use of the Tukey HSD test for post hoc pairwise comparisons of means. There are 78 pairwise comparisons of mean estimations of SF2, contained in the data set on the 13 cell lines. A comparison was made, specifying a minimum significance level of 5%. The results of this procedure are shown:
The difference between means on any of two cell lines not joined by an underline was statistically significant at the 5% level.

4.4. Discussion

This is the largest study examining the radiosensitivity of human tumour lines, where the assays of clonogenic cell survival have all been carried out by one worker, using a consistent experimental protocol. These observations are particularly relevant to the objectives addressed in this study, namely to evaluate whether, allowing for intra- and interexperimental error, significant differences in cellular radiosensitivity could be detected between continuous human tumour cell lines of specific histological type.

The inclusion of carcinoma and embryonal cell lines served three functions in this study. Firstly, it provided a form of quality control for the clonogenic assays, as there is published data on the cellular radiosensitivity of both SuSA, the teratoma line, [Parris et al, 1988] and the colorectal adenocarcinoma line, HT29, [Fertil and Malaise, 1981] both of which have been previously determined to represent radiosensitive and resistant tumour lines respectively.

Secondly, it provided the opportunity to perform a limited analysis of the spread of human tumour cellular radiosensitivity by histological type. With the exception of one study using primary cultures, [Rofstad et al, 1987] this had previously only been conducted on data pooled from several laboratories, with limited quality control on the experimental techniques employed.

Within the limits imposed by data available on only 13 different lines, it would appear that the distribution of cellular radiosensitivity observed in these continuous
lines is bimodal, rather than normal. See figure 4.10. A group of radiation-hypersensitive lines, as represented by the 3 embryonal lines and A2780, can be separated quite distinctly in their response from the other 9 lines. Considerable heterogeneity was observed within the carcinoma and glioma lines, which appeared to reflect true biological variability rather than experimental error.

4.4.1. Survival curve fitting.

It was observed that both linear-quadratic and multitarget models of radiation action gave reasonable fits to the experimental data obtained for the 13 human tumour lines studied. The observed visual fit of the simple multitarget equation to data points appeared to be less satisfactory at higher doses, where the response of 8 of the 13 cell lines displayed varying degrees of continuous curvature, without an apparent true exponential final slope. This observation was corroborated by an improved statistical 'quality factor' for the nonlinear regression fits obtained for the linear-quadratic model.

4.4.2. Linear-quadratic model

The linear-quadratic equation was observed to give acceptable fits to the data sets obtained on all 13 cell lines, describing mathematically an observed trend towards continuous curvature of response with increasing single dose size. This was evident both by visual scrutiny, and reflected in small 'residual's on the least-squares non-linear regression used to fit curves and derive the parameters $\alpha$ and $\beta$.

The constant $\alpha$ is a measure of the initial slope of the survival curve, considered to estimate the linear (ie. exponential) component of cell kill thought to predominate over the dose range of clinical interest, 1-2 Gy. It is felt by some biomathematicians to represent mathematically the 'intrinsic' cellular radiosensitivity of the cell type, independent of any repair capacity [Steel and Peacock, 1989].

It has been observed in previous studies of human cell lines that at doses of 1-3 Gy, the initial slope, as measured by the parameter $\alpha$, differs considerably between cell lines, whilst little variation is noted in the quadratic parameter, $\beta$.

One interpretation of this observation is that at clinically relevant doses, recovery from acute radiation damage is virtually complete, and therefore cell death is
determined by the biological processes underlying the determination of the initial slope.

The range of values of $\alpha$ obtained for both the carcinoma lines and glioma lines overlapped considerably with a range of values between 0.07 - 0.61. The mean values of $\alpha$ for the 5 glioma lines was 0.14 (0.03), by comparison with values of 0.24 (0.09) and 0.77 (0.04) for the carcinomas and embryonal lines respectively. The mean value of $\alpha$ obtained for the 5 carcinoma lines was heavily influenced by the high value measured for the ovarian line, A2780. The trend towards lower $\alpha$ values in the glioma cell lines did not reach statistical significance when compared with the values obtained for the carcinoma lines. ($p=0.39$, Student's t test).

The values for $\alpha$ obtained in this study are comparable with the ranges of $\alpha$ values cited by Fertil et al as representative of radiosensitive and resistant human tumour lines, namely 0.59 (cv 44%), and 0.33 (cv 66%) respectively [Fertil and Malaise, 1985].

The parameter $\beta$ specifies the 'bending' component of the dose-response curve, and is proportional to $dose^2$, postulating an interaction of adjacent cellular radiation events at higher dose. Low values of $\beta$ are considered to signify little capacity to repair radiation damage, and generate survival curves that are almost exponential on a semilogarithmic plot, with the slope determined by the $\alpha$ value. By contrast, high $\beta$ values give considerable curvature to the dose-response. This may be not be obvious when examining the dose-response curves for lines with high $\alpha$ values ie. very radiosensitive lines, and has led to the erroneous assumption in the past that radiosensitive lines are "recovery-deficient" for radiation damage.

The mean $\beta$ values for the glioma and carcinoma cell lines were 0.038 (0.006) and 0.031 (0.005) respectively. These values are comparable with the mean values cited by Fertil and Malaise [Malaise et al, 1987] for moderate to poorly responsive tumour types, namely 0.039-0.045. A mean value of 0.041 (0.006) was obtained for the 3 embryonal lines. This would suggest little difference in the ability of this panel of 13 cell lines to recover from radiation damage on the basis the popular interpretation of the LQ model.

The $\alpha / \beta$ ratio represents the radiation dose at which the cell kill achieved by $\alpha$ and $\beta$ events is equal. As a ratio its numerical value is dependent on those obtained for both $\alpha$ and $\beta$. Human cells of high radiosensitivity are usually observed to have $\alpha / \beta$ ratios of greater than 8, whilst only the most resistant cells
have values of less than 5 [Williams et al, 1985]. The mean value of the \( \alpha / \beta \) ratio for the 5 glioma cell lines was 3.62 (SE 0.38), reflecting the low 'intrinsic' cellular radiosensitivity measured by the \( \alpha \) parameter, and the moderate capacity for cellular recovery measured by \( \beta \). The carcinoma lines gave a mean value of 7.63 (SE 1.72), again heavily influenced by the estimation on one cell line, A2780. The mean \( \alpha / \beta \) for the 3 embryonal lines was 19.8 (SE 3.4) and in keeping with the expected values for a highly responsive tumour group.

**4.4.3. Multitarget model**

It was observed that the 5 human glioma cell lines, and 4 of the 5 carcinoma lines appeared to exhibit continuous curvature of response with increasing radiation dose. Hence evidence for a final exponential slope as predicted by the multitarget model of radiation action was lacking. It was also found that the multitarget model with initial slope had difficulties achieving convergence in least-squares curve fitting, when applied to the most radiosensitive lines. Hence the simple multitarget model parameters are cited in table 4.3.

The mean value of the final multitarget slope, \( D_0 \), for the 5 glioma lines was 2.02 Gy (SE 0.14), with a range of 1.67 to 2.38 Gy. This compared with a mean value of 2.29 (SE 0.31) Gy for the 5 carcinoma lines, and 1.01 (SE 0.14) Gy for the embryonic cell lines. This parameter did not therefore appear to discriminate cellular radiosensitivity in human glial and carcinoma cell lines; both of these tumour types gave considerably higher values than the 3 embryonic lines examined.

Malaise et al [1987], in their meta-analysis of published data on 110 human tumour cell lines, reported mean values of \( D_0 \) of 1.15, 1.29, and 1.25 Gy for the groups of tumours ranked as highly, moderately, and poorly radiosensitive, respectively. The mean values of \( D_0 \) obtained in this study for the glioma and carcinoma lines are higher than cited by Fertil, but again within the range reported in the literature. It is of note that Fertil also found that the multitarget parameter, \( D_0 \) did not appear to differ significantly for cell lines derived from tumour types ranked moderately or highly resistant.

The multitarget parameter \( n \) is determined largely by the size of the so-called 'shoulder region' of the survival curve produced by fitting the MT model. The mean value of \( n \) obtained for the 5 glioma lines was 7.4 Gy (SE 1.22), compared with 2.6 Gy (SE 0.31) and 1.4 Gy (SE 0.45) for the carcinomas and embryonal lines respectively. The distribution of values for \( n \) obtained for the 3 histological
tumour groups again showed a lower mean value for the embryonic cell lines, but little difference between glioma and carcinoma lines, mean values for the carcinoma group being heavily influenced by the radiosensitive ovarian line, A2780.

According to the mechanistic assumptions underlying the multitarget model, the value of $n$ implies a larger number of critical "targets" within the glioma and carcinoma lines to be inactivated prior to induction of radiation cell death.

4.4.4. Cellular radiosensitivity of human glioma lines

A major objective of this set of experiments was to examine the hypothesis that human glioma might be clinically less radioresponsive than carcinomas by virtue of greater intrinsic cellular radioresistance. It had previously been observed by Fertil that human continuous cell lines of a given tumour type appeared to preserve in vitro a ranking of radiosensitivity that was comparable with the clinical radiocurability of the original tumour. It was therefore necessary to derive values for the radiosensitivity of human glioma lines alongside internal experimental controls representing cell lines of other histological types.

All parameters of cellular radiosensitivity obtained for the 5 human glioma lines used in this study lie within the ranges cited for radioresistant human tumours [Malaise et al, 1987; Steel, 1989].

Data obtained in this study show that all 5 glioma lines display a cellular radiosensitivity that lies at the most resistant end of the values observed for human cell lines, both normal and malignant.

The mean SF2 Gy value of 0.68 for the 5 glioma lines was significantly higher than that measured in 5 carcinoma lines, by analysis of variance. ($p = 0.003$). It is acknowledged that this result is biased significantly by the unusual radiosensitivity of the ovarian carcinoma A2780.

The results obtained on these 5 human glioma lines supported the observations made by Yang et al, who reported the relative radioresistance of 3 low passage human glioma cell lines. The SF2 values reported ranged from 0.47 to 0.73, with a mean value of 0.59. The relative radioresistance at low doses was also reflected in the low values for the LQ parameter, $\alpha$. Values ranged from 0.04 to 0.36, with a mean of 0.24. Although these values might suggest less extreme radioresistance than observed in this thesis, no other cell lines were included as internal.
standards in the Yang analysis. Similarly, the 3 glioma lines reported were not continuous, therefore direct comparisons are not applicable.

4.4.5. Relevance of in vitro cellular radiosensitivity to clinical practice.

The relevance of in vitro measurements of intrinsic cellular radiosensitivity on human continuous cell lines to clinical radiocurability must be addressed.

Attempts have been made to extrapolate from in vitro estimations of radiosensitivity on continuous human tumour lines, to the clinical experience of radioresponsiveness of the tumours of similar site and histology. Such analyses have been conducted with two objectives. Firstly, to assess whether such in vitro measurements have any parallels with clinical experience i.e. to what extent the observed ranking of radiosensitivity in human cell lines is 'validated' by clinical response. Secondly, to ask whether such in vitro measurements of tumour radiosensitivity might help guide clinical radiotherapy practice.

Several important factors must be acknowledged in assessing the relevance of in vitro measurements of radiosensitivity to clinical practice. Firstly, intrinsic cellular radiosensitivity is likely to be only one factor influencing overall cell kill. Other major variables include tumour repopulation, cycle progression, oxygenation, and intercellular contact.

The second major concern lies in the reproducibility of estimates of cellular radiosensitivity. It has been observed by both Malaise et al [1987] and Suit et al [1989] that measurements of radiosensitivity made using clonogenic cell survival on the same cell line in separate laboratories can yield different absolute values. Additionally, it is noted that a similar "ranking" of comparative radiosensitivity cannot always be achieved if groups of cell lines of different histological type are examined by the two commonly used experimental endpoints, namely growth assays or clonogenic assay. These are two technical problems more relevant to predictive applications of in vitro radiosensitivity assays, but clearly relate to the question of quality control in measuring cellular radiosensitivity generally. [West et al, 1991]

In this study a high degree of reproducibility was found for repeat experiments on each cell line. Interexperimental coefficients of variation for SF2Gy estimations of 8-20% were found for the 13 cell lines studied, with a mean of 13%. This degree of reproducibility may be attributable to several factors: all cell lines were continuous, therefore by definition were at high passage level, with possibly less
clonal heterogeneity than low passage cultures; repeat experiments were conducted on stock expanded and frozen at the outset of this study, there was therefore limited potential for biological drift in culture; care was taken to ensure that experiments were all performed on exponentially growing cultures in mid-log phase; similarly, the feeder requirements of each of the 13 lines was ascertained, and care taken to ensure linearity of controls and test cultures.

4.4.6. Do measurements of SF2Gy in human cell lines reflect clinical radiocurability?

Both Fertil et al. [1981] and Deacon et al. [1984] asked whether there is a relationship between predictions of overall tumour kill based on in vitro SF2 values for different tumour types, and clinical observations. They have tried to demonstrate that the apparently small differences in SF2 values for tumours of different type might be sufficient to account for the wide spectrum of clinical radiocurability observed, when sustained over a fractionated course of radiotherapy. This was modelled mathematically, by observing the differences in theoretical total cell kill that could be achieved by a conventional course of 30 treatments at 2 Gy per fraction. By raising the measured SF value for a given tumour type, to the power 30, they observed that the total cell kill achieved by clinically relevant doses of radiation could differ from $10^{-9}$ to $10^{-23}$.

Adopting this approach to the data obtained in these 5 human glioma cell lines, a fractionated course of 30 treatments of 2 Gy given over a typical period of 6 weeks, would achieve between $10^{-3}$ and $10^{-8}$ logs of cell kill. For a theoretical tumour of $10^{10}$ cells, this level of effect would be compatible with the varying degree of radioresponsiveness, but low cure probability observed in the clinic.

4.4.7. Relationship between SF2Gy measurements and tumour control probability.

Using Poisson statistics, a tumour control probability at a given total dose of radiation, can be calculated, based on measured SF2 values.

Using SF2 values obtained from the literature for a range of human tumour lines (adenocarcinomas; squamous carcinomas; melanoma; glioma), Suit calculated the doses needed to achieve a 50% tumour control probability (TCD $50$) for a theoretical tumour of $10^{10}$ cells. A good correlation with clinical experience was found for carcinoma cell lines, with a TCD 50 of 70-75 Gy. However, the TCD50 calculated for human glioma of 63Gy was clearly inappropriately low, and failed to
maintain the ranking of relative radiosensitivity usually observed between carcinomas, and glioma. The origin of SF2 data used for the glioma cell lines in this calculation was not clear [Suit et al, 1989].

Several observations should be made on the TCD 50 values calculated in this study. (Fig.4.3.10, and tables 4.3.5-7) The TCD 50 value calculated for the embryonal tumour group of 27Gy is a much lower dose than would be estimated from clinical experience of these tumours. When tumour repopulation is introduced as a variable the TCD 50 rises, and doses required to achieve control in 50% of cases become more plausible.

Conversely, the TCD 50 dose of 91.6Gy calculated for the carcinoma lines, assuming no repopulation, would appear high by comparison with the observed radiocurability of human carcinomas. However, this calculation is based on in vitro data from several adenocarcinoma lines, including lung adenocarcinoma, and colorectal carcinoma; clinical tumours of the same type would not be considered to be as responsive as other carcinomas eg. squamous head and neck or cervical tumours. In retrospect, the inclusion of more cell lines derived from squamous carcinomas, such as those established from carcinomas of the head and neck, or cervix, might have produced a more representative assessment of the radiosensitivity of the carcinoma group. These are human carcinomas of moderate radiocurability, and would have formed the ideal basis of a direct comparison of cellular radiosensitivity with human glioma lines. However, there are few well-characterised human squamous carcinoma lines available of passage level comparable to the human glioma lines used in this study. The lung adenocarcinoma line, A549, and a colorectal adenocarcinoma, HT29, used in this study, are derived from human tumours that are considered more radioresistant than most squamous tumours.

The mean TCD 50 value calculated by Poisson statistics for the human glioma lines used in this study was a dose of 122Gy. This value is approximately double the total dose of radiation currently employed in 'conventional' external beam radiotherapy for malignant glioma, and would be regarded as exceeding normal CNS tissue tolerance. However, using research techniques such as stereotactic external beam boosts or radioactive implants such doses can be delivered to very localised, small volume gliomas.
4.4.8. Conclusions.

1) There is considerable variability in cellular radiosensitivity of human tumour cell lines, even at high passage levels. This is particularly evident at low acute doses of clinical relevance ie. 2Gy.

2) Such differences would appear to be well discriminated by the model-free parameter of radiosensitivity, the surviving fraction at 2Gy. The distribution of SF2Gy values observed in this study suggests a bimodal distribution of human cellular radiosensitivity; there was considerable overlap of cellular radiosensitivity between the 5 human glioma and 5 human carcinoma cell lines studied, but the 3 embryonal cell lines displayed marked radiosensitivity.

3) The linear-quadratic model of radiation action was observed to give satisfactory fits to the data sets obtained on all 13 cell lines studied. The more radioresistant cell lines displayed continuous curvature of dose-response, less satisfactorily fitted by the multitarget model.

4) The intrinsic cellular radiosensitivity of the 5 human glioma lines studied appears to lie at the extreme of values observed for human tumours in vitro. Such cellular radioresistance may contribute to the marked clinical radioresistance observed in human glioma.

5) The molecular processes determining the intrinsic cellular radiosensitivity of human cells is uncertain. Great variability was observed in these 13 cell lines in $\alpha$, the parameter of the linear-quadratic equation considered to reflect differences in induction of non-repairable damage. By contrast, there was considerable overlap of values for the parameter $\beta$, considered to estimate the ability of cells to recover from radiation damage.

6) Further investigation of the relevance of intrinsic cellular radiosensitivity to clinical radiocurability is needed. This would be accomplished more satisfactorily by the use of primary human tumour lines, together with an estimate of tumour proliferation.
Chapter 5

Cellular radiation recovery potential of human glioma
Cellular radiation recovery potential of human glioma

5.1. Background and choice of experimental system

5.1.1. Cellular recovery phenomena.

5.1.2. Potentially lethal damage repair (PLD).

5.1.3. Sublethal damage repair (SLD).

5.1.4. Low dose rate sparing.

5.1.5. Cellular recovery in human cell lines.

5.1.6. Cellular recovery phenomena in human glioma.

5.1.7. Rationale for the choice of the split dose recovery assay to assess recovery potential in human glioma cell lines.

5.1.8. Experimental objectives.

5.2. Split dose irradiation protocol for estimation of sublethal damage repair.

5.2.2. Data analysis.

5.3. Results

5.3.1. Extent of cellular recovery observed in split dose irradiations.

5.3.2. Kinetics of cellular recovery in split dose irradiations.

5.3.3. Extent of cellular recovery at isoeffective radiation dose levels.

5.3.4. The parameter of cellular recovery capacity, βRR.

5.4. Discussion
5.1. Background and choice of experimental system.

5.1.1. Cellular recovery phenomena.

In clinical radiotherapy it is noted that fractionation of a given total radiation dose is less effective in producing tumour response, or normal tissue damage. It has been widely assumed that such so-called 'recovery' of cells from radiation damage determined the clinical effectiveness of radiotherapy. The molecular basis of recovery is poorly understood.

Tumour types displaying poor radioresponsiveness were those assumed to be able to accumulate and repair radiation damage proficiently. This later notion was further reinforced by the observation in vitro of a shouldered response at lower doses of radiation in the human cell survival curve [Hall, 1988].

The extent of cellular recovery from radiation damage has been measured using both in vitro and in vivo systems to study single cells, normal tissues and experimental tumours, or whole animals. However, the experimental endpoint most frequently used has been that of enhancement of clonogenic cell survival in operationally-defined 'cellular recovery' assays. The damage to mammalian cells produced by irradiation has been divided conceptually into three categories:

1) Lethal damage; irreversible and irreparable lesions leading inevitably to cell death.

2) Sublethal damage (SLD); lesions which can be modified and eliminated by cellular processes, given time, unless further damage is acquired eg by another radiation dose.

3) Potentially lethal (PLD); a component of radiation damage that can be influenced by post-irradiation conditions [Elkind and Whitmore, 1967].

These processes are operationally-defined, and the precise molecular nature of the lesions involved in each category of damage and their inter-relationship are still a subject of research. It is now felt appropriate to refer to these cellular observations as 'recovery phenomena', reserving the term 'repair' for the observation of post-radiation events at the DNA or chromosomal level. [Steel, 1991]. Much of the data available on the comparative ability of human tumours to recover from radiation damage at the cellular level has been obtained using such approaches.
Human cellular recovery from radiation damage has been commonly measured in three types of experimental system:

1) Post-irradiation plateau -phase holding prior to subculture (Potentially lethal damage repair or PLD) [Iliakis, 1988].

2) Split dose irradiation(Sublethal damage repair, or SLD) [Peacock et al, 1988].

3) Low dose-rate irradiation [Steel et al, 1987].

5.1.2. Potentially lethal damage repair (PLD).

Survival of cells exposed to radiation can be modified by post-treatment incubation with metabolic inhibitors or under conditions which affect progression through the cell cycle. Such modulation of cytotoxicity is considered to represent the induction of damage which is only potentially lethal. It is assumed that such PLD can be repaired by the cell if not fixed by subsequent cellular processes. Two types of experimental system have been used to simulate and measure this class of cellular processing: a) treatment with compounds which might 'fix' PLD induced by radiation, including DNA synthesis inhibitors, caffeine, anisotonic media; b) the use of post-irradiation conditions intended to permit recovery from radiation to proceed eg delayed subculture of plateau-phase cultures. This later approach has been claimed to simulate the density-inhibited conditions felt to prevail in tumours in vivo [Little, 1973].

5.1.3. Sublethal damage repair (SLD)

This class of damage and recovery has been defined by the observation of enhanced clonogenic cell survival when a total dose of radiation D is given as two equal fractions of D/2, separated by increasing time intervals. This process was first noted by Elkind, irradiating CHO cells. It was observed that the surviving fraction of irradiated cells increased with prolongation of the split dose interval from 0 to 6 hours, then reaching a plateau [Elkind and Whitmore, 1967]. The enhancement in cell survival was attributed to 'repair of sublethal damage' accumulated during the first fraction of radiation, and modified by the increasing time interval prior to a second dose. Recovery was expressed as the ratio of surviving fraction obtained at the plateau of recovery, to that observed when the two doses were administered consecutively, ie To.
5.1.4 Low dose rate sparing

As the acute radiation dose rate is reduced progressively from 2 down to 0.02 Gy/minute, a significant reduction in the final slope of the acute radiation survival curve is noted, until the response is virtually exponential on a semilogarithmic plot. The major difference between cell lines of differing acute radiosensitivity is then manifest by differences in the slope of this plot, which is thought to extrapolate the initial slope of the acute high dose-rate survival curve. Below 0.02Gy/min cell cycle progression events complicate analysis.

The enhancement in cell survival at a given radiation dose gained by a reduction in the dose-rate is referred to as low dose-rate sparing. The amount of this sparing varies between human cell lines, and is thought to reflect a capacity to repair radiation damage.

5.1.5 Cellular recovery in human cell lines.

A number of studies have attempted to evaluate a potential relationship between acute radiosensitivity in vitro, and cellular recovery capacity, using both human tumour cell lines, and cultures derived from fibroblasts of patients with ataxia telangectasia.

Astor *et al* [1989] have recently demonstrated deficient PLD recovery in 3 AT fibroblast cell lines. All three lines showed recovery from radiation damage, when subculture was delayed for 6 hours post-irradiation, when compared with the surviving fraction obtained following immediate subculture. However, when the extent of recovery was compared with that achieved by 2 normal human fibroblast cell lines, at a dose producing similar survival levels, a three-fold reduction was observed in the AT lines. This study also provided some evidence for two-component recovery kinetics, identifying a 'fast' recovery process, with a half-time of 5-7 minutes, in addition to a slower process, completed within 3 to 4 hours. The findings in this study are of note. It had previously been assumed that AT cells were deficient in cellular recovery. Secondly, this study employed very high dose rates, 10Gy/min, such that the time for irradiation would not obscure rapid recovery processes. This factor may explain difficulties observed in resolving separate kinetics of recovery from radiation damage in PLD experiments.

The radiation response of a panel of 12 human tumours studied by Steel *et al* [1987;1991] has demonstrated a seven fold difference in the final slope of the low dose-rate response, which appears to accentuate the differences in
radiosensitivity observed at high dose rate. In two radiosensitive human lines examined, HX143 (a human neuroblastoma), and WX 67 (a bladder carcinoma) no such sparing could be demonstrated. Analysis of a further 7 tumour lines gave estimates of recovery half-times of 0.1-0.85 hours.

By contrast, using split dose recovery as the endpoint, the same group obtained quite different values for 'repair' proficiency in 17 human tumour lines, including those studied by low dose rate. Half-time values varied from 0.36 to 2.3 hours, with a systematic tendency for split dose half-times to be longer than those derived from dose-rate analysis. It was noted that the confidence limits on the level of correlation between estimates of recovery proficiency on the same line by the two different methods was low (correlation coefficient 0.52)

PLD repair has been measured, using plateau-phase cultures and delayed subculture methodology, to investigate recovery potential in cell lines derived from a variety of human tumours. Guichard et al reviewed the data available on PLD recovery obtained in human tumour cell lines in vitro and in vivo [Guichard et al, 1984].

Table 5.1. Potentially lethal damage repair (PLD), in human tumour cell lines

<table>
<thead>
<tr>
<th>Tumour</th>
<th>SF at T0 (%)</th>
<th>Recovery ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>High curability</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCF7 (breast)</td>
<td>2.3</td>
<td>1.5</td>
</tr>
<tr>
<td>MB231</td>
<td>3.5</td>
<td>1.2</td>
</tr>
<tr>
<td>LAN-1 (neuroblastoma)</td>
<td>5.2</td>
<td>1.6</td>
</tr>
<tr>
<td><strong>Low curability</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAS (hypernephroma)</td>
<td>1.8</td>
<td>4</td>
</tr>
<tr>
<td>GBM (glioma)</td>
<td>1.3</td>
<td>2.8</td>
</tr>
<tr>
<td>TX4 (osteosarcoma)</td>
<td>0.5</td>
<td>24</td>
</tr>
<tr>
<td>C143 (melanoma)</td>
<td>2.6</td>
<td>11.5</td>
</tr>
<tr>
<td>C32 (melanoma)</td>
<td>4.7</td>
<td>8.2</td>
</tr>
<tr>
<td>MaLL (melanoma)</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>SAOS (osteosarcoma)</td>
<td>1.2</td>
<td>2.8</td>
</tr>
</tbody>
</table>

*SF at T0; surviving fraction of clonogenic cells obtained with no delay in plating.*

It was noted that a considerable range of recovery proficiency was exhibited by lines derived from radioresistant tumours, but that the median value (4) was
significantly higher than that of the radiosensitive lines. Data was cited on the recovery ratios measured in 4 radioresistant human tumour xenografts, irradiated in vivo, then subcultured at varying time intervals. A similar median recovery ratio was noted for the resistant tumours.

Marchese et al compared PLD repair, both in exponential and plateau phase cultures of 2 human tumour lines (2 melanomas, and an osteosarcoma) and a normal fibroblast line. Little evidence of any PLDR could be demonstrated in the three human tumours examined, either in exponential or plateau phase. By contrast, the normal fibroblasts displayed a 5.7 fold recovery in clonogenic survival with the six hour delayed plating post-irradiation. It was noted by these authors that the human tumours did not display a true plateau phase at high density, with 38-50 % of cells remaining in cycle, in contrast with only 19% of the normal fibroblasts [Marchese et al, 1985]

5.1.6. Cellular recovery phenomena in human glioma.

To date only 1 study has attempted to assess the cellular recovery proficiency of human glioma in any detail. Yang et al investigated both split dose recovery and low dose-rate sparing in three human gliomas cell lines [Yang et al, 1990] The glioma lines were all at relatively low passage levels (P2-26). All exhibited acute radioresistance relative to values obtained in other human tumours.

All three lines showed a definite dose-rate effect, as evidenced by a substantial increase in clonogenic survival with reduction in dose rate from 1.5 to 0.013Gy/min. Values for recovery half-times obtained by low dose rate analysis varied from 0.5 to 1 hour.

Using the split dose assay, recovery was assessed at 3 or 4 different dose levels per cell line. The values of βRR, the split dose recovery ratio, (see section 5.1.7.) were comparable with values obtained in previous studies on relatively resistant human tumour cell lines.

No attempt was made in this study to compare 'internally' the acute radiosensitivity, or recovery potential of these glioma lines, with other human tumour lines.
5.1.7. Rationale for the choice of the split dose recovery assay to assess recovery potential in human glioma cell lines.

The choice of the split dose system to assess comparative recovery potential in human glioma lines was based on both technical and theoretical considerations.

Firstly, both plateau-phase delayed plating and low dose-rate approaches have considerable operational problems. Human tumour cell lines frequently do not exhibit a plateau phase due to loss of contact inhibition, and the percentage of cells remaining in cycle can vary considerably between lines. Low dose rate sparing studies require the facility to maintain cells strictly at 37°C during protracted irradiation.

Secondly, it is recognised that no clear theoretical advantage has been demonstrated for any of the 3 operationally defined recovery assays, in terms of a precise correlation with acute cellular radiosensitivity.

The work of Peacock et al [1988] has suggested that a satisfactory method of comparing recovery potential between cell lines of differing acute radiosensitivity is the split dose technique. The linear-quadratic model of radiation action gives successful fits to much experimental data on the acute radiosensitivity of human cells. If it is assumed that full recovery from a split dose treatment gives a result that is the product of the single dose surviving fractions, then the LQ model defines the recovery ratio mathematically by the expression:

\[ \text{Recovery ratio (RR)} = \exp (2 \beta d^2) \quad 1) \]

where \( \beta \) is the coefficient of the dose squared term in the LQ equation, and \( d \) is the dose per fraction in split treatments [Thames, 1985].

This model predicts that recovery ratio should rise steeply with dose, with no finite limiting value, and therefore no single numerical value that describes the recovery potential of a given cell line. Peacock assessed the split dose recovery potential of 4 human tumour lines, and was able to validate the LQ prediction that such recovery does increase with dose. He defined a parameter \( \beta RR \), the split dose recovery ratio. This parameter is obtained from the definition of the recovery ratio in equation 1), whereby a plot of the logarithm of (RR) against dose\(^2\) should produce a straight line with a slope of \( \beta RR \).

It is currently held that values of recovery (\( \beta RR \)) obtained by such split dose analyses give a more robust estimate of the cellular recovery potential of human
cell lines than \( \beta \) values obtained in acute radiation experiments [Steel, 1989; 1991]

5.1.8. Experimental objectives.

The objectives of the experiments described in this chapter were:
1) To document the cellular recovery potential of human glioma, using as models the 5 human glioma cell lines described in chapter 2.

2) To examine the hypothesis that the difference in acute cellular radiosensitivity between the human glioma lines, and embryonic tumour lines might be attributable to enhanced cellular recovery from radiation damage.

5.2. Specific methods

5.2.1. Split dose irradiation protocol for estimation of sublethal damage repair.

The recovery potential of the 5 continous glioma lines has been examined, and compared with 3 radiosensitive human cell lines, A2780, SuSA, and IMR32.

Monolayer colony formation on plastic, as described in the acute radiation protocol, was used as the endpoint in these studies. Exponentially growing cell cultures were trypsinised, disaggregated to single cell suspensions, and subcultured into T 25 cm\(^3\) flasks at a range of cell densities. Low density heavily irradiated homologous feeders were used to maintain plating efficiency, and minimise the effect of varying cell density across the range of test cell numbers used. Flasks were gased at 37\(^\circ\) C for 4 hours prior to experimental irradiation.

Replicate flasks were irradiated with 2 equal doses of radiation, with the 2 doses given at time intervals ranging from 15 minutes to 24 hours apart. Flasks were returned to the incubator between fractions to permit cell cycle progression to occur. This procedure was carried out at 3 different dose levels per cell line. For the 5 glioma lines studied, split doses were administered as 2+2Gy, 3+3Gy, and 4+4Gy. Split doses were reduced for the three radiosensitive lines studied, to permit a comparison of recovery proficiency over an isoeffective radiation dose range. Doses were therefore used of 1+1Gy, 1.5+1.5Gy, and 2.5+2.5Gy for cell lines A2780, SUA, and IMR 32.
Colony formation was allowed to proceed over 10 to 20 days, depending on the cell line, then cultures fixed stained and colonies counted according to the previously defined criteria.

**5.2.2. Data analysis.**

The recovery ratio was calculated as the surviving fraction observed in the split dose flasks compared with the surviving fraction obtained when the total dose was given as a single fraction.

Three parameters were calculated to estimate the cellular recovery potential of each cell line examined;

1) The maximum recovery ratio obtained at 3 different dose levels, as a model-free measure of recovery proficiency.

2) The maximum recovery ratio obtained at the 10% isoeffect dose level.

3) $\beta RR$, an estimate of the recovery potential as a function of dose, derived from the linear-quadratic model.

Data was pooled for analysis from 3 separate experiments on each cell line. The maximum recovery ratio at each dose level examined was calculated as the mean of three values on the plateau of recovery plotted against time.

According to the linear-quadratic model, split dose recovery ratio should increase with dose, therefore an estimation of recovery potential at a single dose level would not be representative of the cell line. It is assumed that full recovery from a split-dose treatment gives a result that is the product of the single-dose surviving fractions. Therefore, according to the LQ model the recovery ratio is obtained by the relationship

$$\log_e (RR) = 2 \beta d^2$$

where $d$ is the single dose level, and $RR$ the recovery ratio.

The recovery ratio $\beta RR$ has been calculated from the linear regression slope obtained by plotting $(\ln(S2)-\ln(S1))$ against dose $^2$, being the difference in surviving fractions obtained in split dose compared with single dose, expressed in natural logs.
The recovery half-time was calculated at each dose level, assuming a simple rising exponential relationship. The data was fitted by the equation:

$$S = AR(1 - e^{-\theta})$$

where $A$ is the intercept on the cell survival axis, $R$ the recovery ratio, and $\theta$ the time-constant for recovery ($\theta = \log_e 2 / \text{half-time}$). The equation was fitted to the data using the MINSER optimising method, with algorithms kindly supplied by Prof. G. Steel, Radiotherapy Research Unit, Institute of Cancer Research.

5.3. Results

In all 8 lines examined, recovery of clonogenic cell survival was observed in split doses when compared with a single acute dose.

Results were obtained for split dose recovery capacity, described by the parameter $\beta_{RR}$, the maximal recovery ratios observed at isoeffective survival levels, and the kinetics of recovery, expressed as the recovery half-time. These values for each of the 5 human glioma tumour cell lines, and 3 radiosensitive human tumour lines, IMR 32, A2780, and SUSA, are summarised in Tables 5.2 to 5.4.

5.3.1. Extent of cellular recovery observed in split dose irradiations.

The dose levels used in the split dose protocol are shown in table 5.2. The recovery ratio at any given dose level was defined as the ratio:

$$\frac{\text{surviving fraction in split dose irradiation}}{\text{surviving fraction in single dose irradiation}}$$

The maximal recovery ratio measured at each of the 3 different total dose levels studied per cell line is shown as a mean value, and standard error, from 3 independent experiments per line. The choice of dose levels was determined by the acute radiosensitivity of each cell line, and chosen to study recovery proficiency over a similar survival range, i.e., surviving fraction 0.005 to 0.2.

5.3.2. Kinetics of cellular recovery in split dose irradiations.

A similar pattern of recovery was noted in all lines, namely, an increase in surviving fraction with increasing time intervals from 15 minutes, reaching a
plateau of recovery between 60 and 180 minutes in 7 of the 8 lines. The glioma UVW exhibited more protracted recovery kinetics.

In pilot experiments on each cell line studied, protraction of the split dose interval beyond 6 hours produced a reduction in surviving fraction, attributed to cell cycle progression, as cultures were maintained at 37°C between fractions. In all subsequent experiments split dose intervals were therefore limited to a maximum of 6 to 8 hours.

The kinetics of the recovery process have been calculated for each cell line by pooling the split dose survival data obtained at each of the 3 dose levels examined. It is expressed in hours as t 1/2 in table 5.2. The range of values observed in the 5 glioma lines was 0.53 to 2.6 hours, with a mean of 1.2 (SE 0.39). This value was influenced by the prolonged recovery in UVW; the median value for the 5 lines was 0.9 hours.

The recovery half-times calculated for the 3 radiosensitive tumour lines, A2780, SUSA, and IMR32 ranged from 0.64 to 1.25 hours, with a mean of 1 hour (SE 0.19). There appeared therefore to be no significant differences in the kinetics of split dose recovery in the radioresistant gliomas, and radiosensitive lines.
Table 5.2. **Maximal Recovery ratio at three dose levels per cell line**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Maximum recovery ratio at radiation dose:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2Gy</td>
</tr>
<tr>
<td>Gliomas</td>
<td></td>
</tr>
<tr>
<td>UVW</td>
<td></td>
</tr>
<tr>
<td>CCM</td>
<td></td>
</tr>
<tr>
<td>IJK</td>
<td></td>
</tr>
<tr>
<td>U251</td>
<td></td>
</tr>
<tr>
<td>SB 18</td>
<td></td>
</tr>
<tr>
<td>Others:</td>
<td></td>
</tr>
<tr>
<td>IMR32</td>
<td>1.13 (0.08)</td>
</tr>
<tr>
<td>SUSA</td>
<td>1.14 (0.15)</td>
</tr>
<tr>
<td>A2780</td>
<td>1.19 (0.15)</td>
</tr>
</tbody>
</table>

**Radiation dose represents total dose given in 2 fractions i.e. 2Gy = 1+1 Gy; 4Gy = 2+2 Gy etc**

**Values are mean of 3 separate experiments on each cell line, with standard error.**
Table 5.3. Split dose recovery ratio and recovery kinetics for 8 human tumour lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>$\beta$ RR (Gy$^{-2}$)</th>
<th>$t_{1/2}$ (hours)</th>
<th>$\beta$ (Gy$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gliomas:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UVW</td>
<td>0.024 (0.007)</td>
<td>2.6 (0.79)</td>
<td>0.042 (0.004)</td>
</tr>
<tr>
<td>CCM</td>
<td>0.015 (0.005)</td>
<td>0.57 (0.11)</td>
<td>0.048 (0.005)</td>
</tr>
<tr>
<td>IJK</td>
<td>0.019 (0.004)</td>
<td>0.9 (0.14)</td>
<td>0.024 (0.003)</td>
</tr>
<tr>
<td>U251</td>
<td>0.024 (0.008)</td>
<td>0.53 (0.17)</td>
<td>0.051 (0.006)</td>
</tr>
<tr>
<td>SB18</td>
<td>0.017 (0.005)</td>
<td>1.56 (0.9)</td>
<td>0.026 (0.002)</td>
</tr>
<tr>
<td>mean</td>
<td>0.0198</td>
<td>1.2</td>
<td>0.038</td>
</tr>
<tr>
<td>IMR 32</td>
<td>0.071 (0.019)</td>
<td>1.15 (0.39)</td>
<td>0.032 (0.018)</td>
</tr>
<tr>
<td>SUSA</td>
<td>0.094 (0.032)</td>
<td>1.25 (0.30)</td>
<td>0.051 (0.014)</td>
</tr>
<tr>
<td>A2780</td>
<td>0.037 (0.017)</td>
<td>0.64 (0.29)</td>
<td>0.047 (0.01)</td>
</tr>
<tr>
<td>mean</td>
<td>0.067</td>
<td>1.01</td>
<td>0.043</td>
</tr>
</tbody>
</table>

Values cited for $\beta$RR, $t_{1/2}$, and $\beta$ are are means with standard error.

$\beta$RR is recovery ratio measured in split dose studies.

$\beta$ is $\beta$ parameter calculated from LQ fits, acute radiation.

$t_{1/2} = \text{mean recovery half time calculated from 3 radiation dose levels}$
5.3.3. Extent of cellular recovery at isoeffective radiation dose levels.

Recovery proficiency has been compared for the 5 radioresistant glioma lines, and the 3 radiosensitive lines at a dose level producing approximately the same degree of cell kill, i.e., that reducing the surviving fraction to 0.1. These values are shown in Table 5.4.

This dose level varied between cell lines, depending on acute radiosensitivity. The difference between mean recovery ratio at this isoeffect level has been compared for the 5 glioma lines, and the 3 radiosensitive lines, using Student's t test. The differences are significant at the p < 0.05 level, suggesting that the radioresistant lines exhibit higher recovery from radiation damage than the 3 radiosensitive tumour lines, when compared at doses producing equivalent cell kill.

The relationship between potential to recover from radiation damage, and acute radiosensitivity is displayed in Figure 5.1. Recovery ratio measured at the 0.1 isoeffect dose level has been compared with the parameter of acute radiosensitivity for each cell line, namely, the surviving fraction at an acute dose of 2Gy. A positive correlation is noted, although the glioma cell line CCM exhibits a recovery ratio comparable with the most radiosensitive lines.
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Dose to achieve SF 0.1</th>
<th>Maximal Recovery ratio observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>UVW</td>
<td>6Gy (3+3)</td>
<td>1.79 (0.18)</td>
</tr>
<tr>
<td>CCM</td>
<td>6Gy (3+3)</td>
<td>1.22 (0.21)</td>
</tr>
<tr>
<td>IJK</td>
<td>8Gy (4+4)</td>
<td>1.89 (0.12)</td>
</tr>
<tr>
<td>U251</td>
<td>6Gy (3+3)</td>
<td>1.58 (0.21)</td>
</tr>
<tr>
<td>SB18</td>
<td>8Gy (4+4)</td>
<td>1.9 (0.12)</td>
</tr>
<tr>
<td><strong>mean</strong></td>
<td></td>
<td><strong>1.67 (0.13)</strong></td>
</tr>
<tr>
<td>IMR32</td>
<td>3Gy (1.5+1.5)</td>
<td>1.3 (0.29)</td>
</tr>
<tr>
<td>SUSA</td>
<td>2Gy (1+1)</td>
<td>1.14 (0.15)</td>
</tr>
<tr>
<td>A2780</td>
<td>3Gy (1.5+1.5)</td>
<td>1.42 (0.28)</td>
</tr>
<tr>
<td><strong>mean</strong></td>
<td></td>
<td><strong>1.28 (0.08)</strong></td>
</tr>
</tbody>
</table>

Values represent means of 3 independent experiments on each cell line.

Standard error in brackets.
Figure 5.1. Relationship between acute radiation sensitivity and cellular recovery capacity measured at isoeffective radiation doses.

Values are mean recovery ratio observed for each cell line at the total, single dose of radiation required to achieve 90% cell kill, i.e., surviving fraction of 0.1, plotted against the acute radiation sensitivity of the line, given by the surviving fraction at a dose of 2Gy (SF2Gy).
5.3.4. The parameter of cellular recovery capacity, βRR

Based on the assumptions underlying the linear-quadratic model, the parameter βRR was calculated for each cell line. The average value of recovery was calculated for three time points on the plateau region attained at each of three dose levels examined. The log of the maximal recovery ratio obtained at 3 dose levels was plotted against 2xdose². Least squares regression was used to calculate the slope, which represents βRR as defined by the linear-quadratic model. Plots are given in figures 5.2-3. Values are contained in table 5.3.

Values obtained for βRR in the 5 glioma lines were lower than the radiosensitive lines, with mean values of 0.019 and 0.067 respectively. Considerable differences in β are noted when the split dose values are compared with the β parameter calculated in acute radiation exposures.

There was no correlation between the kinetics of recovery, and the extent of recovery as measured by the βRR recovery parameter. The recovery half-time and βRR have been plotted for each of the 8 cell lines. Figure 5.4
Figure 5.2. Relationship between split dose recovery achieved at 3 different dose levels, plotted as a function of $2d^2$: in 5 glioma cell lines.

$d$ is size of radiation dose given in split fraction
slope of regression line is the value of parameter $\beta_{RR}$
values are means, with standard errors
Figure 5.3 Relationship between split dose recovery achieved at 3 different dose levels, plotted as a function of $2d^2$: cell lines A2780, IMR 32, SUSA.

$d$ is size of radiation dose given in split fraction
slope of regression line is the value of parameter $\beta$
RR values are means, with standard errors
Figure 5.4. Relationship between recovery ratio, βRR, and recovery kinetics, in 8 human tumour cell lines.
5.4. Discussion

The 5 human glioma cell lines examined in these experiments all displayed the capacity for split dose recovery from radiation damage. The values of $\beta_{RR}$, the parameter of recovery derived from the LQ model, were comparable with values obtained in 3 low passage, radioresistant glioma lines reported by Yang et al, [1990] and two radioresistant human bladder lines [Peacock et al, 1988].

This study has compared the recovery proficiency of these 5 radioresistant glioma lines with that of 3 radiosensitive human tumour lines, to examine the hypothesis that cellular recovery from radiation damage might explain the wide differences in cellular radiosensitivity seen in these lines. When split dose recovery proficiency was compared at doses of radiation producing equal levels of cell kill (10% surviving cells) it was found that the 5 glioma lines exhibited greater recovery than the 3 sensitive lines, as evidenced by higher recovery ratios. A positive correlation was noted between this recovery ratio value for each of the 8 lines, when plotted against a model-free parameter of acute radiosensitivity, the surviving fraction at 2Gy.(correlation coefficient 0.69) A similar relationship between split dose recovery proficiency and SF2Gy has been reported by Kelland, using 5 human cervical carcinoma lines, of differing acute radiosensitivity [Kelland et al, 1989].

The importance of making comparisons of cellular recovery between lines of differing intrinsic radiosensitivity at 'isoeffect' levels has been stressed by previous workers [Guichard et al, 1984]. Using this approach a trend towards higher recovery has been noted in cell lines derived from radioresistant human tumours. Summarising the published literature, Guichard observed plateau phase (PLDR) recovery ratio values ranging from 2.8 to 24 in 6 radioresistant human lines, by comparison with values of 1 to 1.6 in cell lines derived from human breast carcinomas, and a neuroblastoma. Although the experimental methodology in PLDR assays differs from this study, it corroborates the apparent association between cellular radiosensitivity and recovery proficiency in human tumour cell lines.

The kinetics of cellular recovery processes in mammalian and human tumours have been investigated by other groups. Using a very similar experimental protocol, and data analysis procedure, Stephens found recovery half-times of 0.16 to 0.97 hours in 5 tumour cell lines. The shortest times were recorded in 4 murine tumours, with the longest value of 0.97 hours being that of a human neuroblastoma line, and thus very similar to the half-time found for both sensitive and resistant lines in this study. These authors remarked on the fact that the
values obtained for recovery half-times were lower than previously recorded in split dose assays by a factor of 10 [Stephens et al, 1987]. In the same study recovery half-times were also measured on the same cell lines, using low dose rate irradiation, fitting data with the Incomplete repair model of Thames, and the Curtis Lethal-Potentially lethal model. The absolute half-times and ranking of values differed considerably for the 5 cell lines studied by the three methods.

Using a similar split dose protocol, and data analysis, Kelland reported recovery half-times in 5 human cervical carcinoma lines. Values ranged from 0.24 to 1.25 hours, with a mean of 0.81 hours. These estimates are comparable with the values obtained in the 8 human tumour lines assessed in this study. A similar lack of correlation was noted between half-times, and cellular radiosensitivity. It would therefore appear that no systematic relationship exists between the biological processes underlying cellular recovery kinetics, and the cellular radiosensitivity of mammalian cells.

It has previously been assumed 'shoulders' or shallow initial slopes to the acute radiation survival curves were a reflection of an ability to accumulate and repair radiation damage. It has been implied, conversely, that human cells with steep acute radiation survival curves, with little evidence of such a shoulder region, were deficient in cellular recovery capacity. The lower values of recovery proficiency obtained for radiosensitive cell lines in this study would tend to corroborate this notion.

This premise has been challenged in recent years by an alternative analysis of split dose recovery data. As described in the introduction to this chapter, the assumptions underlying the linear-quadratic model predict that cellular recovery should increase infinitely as a function of dose, with no appropriate value for recovery estimated at a single dose level. This hypothesis has been supported by the analysis of split dose data on 13 human tumour and fibroblast lines, which has shown that recovery ratio continues to rise with increasing dose, with no evidence of a plateau even at total doses as high as 16Gy [Peacock et al, 1988]. When an estimate of recovery is calculated from the regression slope of log RR against 2d2, the β RR parameter, it appeared that radiosensitive lines were not only recovery proficient, but apparently showed higher recovery capacities than radioresistant lines. This surprising result has been discussed by Steel [Steel, 1991]. It is postulated that cell survival following radiation might be the result of two processes, with a linear component due to non-repairable lesions, and a bending component due to repairable lesions. It is suggested that radiosensitive cells incur more of each of these classes of damage per unit dose. The steep slopes
observed on the acute radiation survival curve of radiosensitive cells is therefore considered to be the result of induction of irreparable lesions (the $\alpha$ component of the LQ model). This component dominates the response at all dose levels, and obscures the magnitude of repairable damage, represented by the $\beta$ component. The extent of recovery measured in split dose studies is therefore felt to reflect the amount of recoverable damage that is induced, rather than the overall recovery from all classes of radiation damage.

The data obtained in this study, analysed in a similar fashion, would tend to support the observations made by Peacock: recovery appeared to increase with dose for all 8 cell lines, although lower total doses were employed than in the Peacock study; secondly, the values of $\beta$RR were consistently higher in the 3 more radiosensitive cell lines than the radioresistant glioma lines. Nonetheless, it is usually considered that comparisons of radiobiological endpoints are most appropriately made at 'isoeffect' doses, and on this basis the 5 human glioma lines studied exhibited a significantly higher capacity to recover from a dose of radiation producing 90% cell kill, by comparison with the 3 radiosensitive cell lines.

The wide spectrum of cellular radiosensitivity observed in human tumours renders them potentially useful models for the study of the mechanistic basis of cellular radiosensitivity. However, the use of cell lines of differing lineage, differentiation, and probably quite different modes of malignant transformation has obvious difficulties, especially if the aim of such studies is the identification of genes involved in repair of radiation damage.

It has been suggested that the use of radiosensitive human tumour cells as recipients in genetic complementation studies might be inappropriate, in the light of failure to demonstrate absolute recovery deficiency. However, if the aim of such investigation is to complement a putative genetic 'defect' responsible for radiation hypersensitivity in its widest sense, rather than 'repair' genes, then some human tumour types may be very valuable models. Nonetheless, the approach might be more productive if a greater number of isogenic mutant systems were available for human tumours, differing only in cellular radiosensitivity. Conversely, the role of oncogenic transformation itself may well modify the cellular response to radiation. Many continuous human tumour lines exhibit a more pronounced cellular radioresistance than normal human fibroblasts, and this is true of the 5 human glioma lines used in this study. Systematic comparisons of cellular radiosensitivity, and recovery from radiation damage have not been made between normal and malignant human cells of the same histological type.
Speculation on the relationship between in vitro cellular recovery phenomena and in vivo repair of radiation damage must obviously be cautious. However, in vitro cellular recovery processes can be used for both mechanistic studies, and to screen the effects of agents with the potential to modify the radiation response.

Improvements in the outcome for human tumours managed routinely by radiotherapy, such as glioma, may depend upon the identification of agents inhibiting cellular recovery from radiation. Inhibition of recovery from sublethal damage, potentially lethal damage, and low dose rate sparing have been observed to various degrees using quite different classes of agents.

These have included chemotherapeutic drugs, putative DNA repair inhibitors, halogenated pyrimidines, caffeine, differentiating agents, and chemicals modifying intracellular thiol levels. Those agents acting via DNA repair pathways have been of particular theoretical interest. These include the DNA polymerase inhibitors, such as aphidicolin, dideoxythymidine (ddT), β arabinofuranosyladenine (β ara-A), and arabinofuranosylcytosine (β ara-C), the poly (ADP-ribose) transferase inhibitors derived from benzamide, and novobiocin. Many of the in vitro studies claiming enhancement of the radiation response using these agents have focused primarily on rodent lines, and UV damage rather than ionising radiation. Few of these agents have shown sufficient enhancement ratios in vitro to merit detailed in vivo studies, and even fewer are suitable for clinical evaluation [Kelland and Steel, 1988]. However, the potential approach of using cellular recovery as an experimental endpoint remains valid in the investigation of newer agents.

It is relevant to consider which recovery assay provides the most realistic model of the in vivo situation. It is commonly held that delayed post-irradiation sub-culture of plateau phase cells mimics some of the metabolic, kinetic and cell-cell contact phenomena likely to pertain in human tumours. However, whilst plateau phase cultures are often observed in non-malignant, transformed human cells, many tumour lines do not form such cultures.

A detailed review of the relative merits of particular cellular recovery protocols is beyond the remit of this thesis. It is clear that operationally defined recovery assays can produce quite different assessments of radiation recovery proficiency when the same biological material is studied with an alternative recovery experimental protocol. It would therefore appear that, even allowing for experimental error, quite different molecular processes are being studied when the
cellular recovery phenomena are evaluated by split dose, PLD, or low dose rate assays.

In general, positive correlations have been observed between extent of cellular recovery, and acute cellular radiosensitivity in all three experimental systems. By contrast, no such correlation has emerged between recovery kinetics and radiosensitivity.

**Summary.**

1) The 5 human glioma cell lines examined in this study all exhibit the capacity for split dose recovery from radiation damage. The extent of recovery of clonogenic survival increased with dose level, in accordance with the predictions of the linear-quadratic model.

2) Maximal recovery at a dose producing approximately 90% kill in all 5 glioma lines ranged from 1.2 to 1.9, with a mean of 1.67.

3) Recovery proficiency was assessed in 3 radiosensitive human tumour cell lines derived from a neuroblastoma, a teratoma, and an ovarian carcinoma. All exhibited split dose recovery, but values for maximal recovery at the dose level producing 90% cell kill were significantly lower than observed in the glioma lines.

4) This is the first in vitro study to evaluate the recovery proficiency of human glioma lines in a comparative manner. Results are suggestive that a factor determining relative radioresistance in human glioma may be a cellular recovery process.

5) When recovery capacity was calculated as the parameter $\beta_{RR}$, the slope of the regression of log recovery ratio against $2d^2$, the most radiosensitive lines gave higher values than the glioma lines, suggesting significant recovery capacity. This suggests that radiosensitive human cells are not deficient in recovery from radiation damage. The validity of this method of estimating and comparing the recovery proficiency of a given cell line is controversial.

6) A positive correlation was found in the 8 lines examined, between maximal recovery ratio at dose levels producing equivalent cell kill, and a measure of cellular radiosensitivity, the surviving fraction at 2 Gy.
7) Recovery half-times for the glioma lines ranged from 0.53 to 2.6 hours, with a mean of 1.2 hours. This did not differ significantly from recovery half-times obtained in the radiosensitive cell lines.

8) Several alternative systems including low dose rate sparing, and delayed plating procedures have demonstrated clonogenic cellular recovery following irradiation of human tumours. A correlation has been demonstrated between acute radiosensitivity and extent of 'repair' in recovery assays, with a trend for radiosensitive cell lines to show less recovery than resistant lines, when compared at doses producing equivalent cell kill.

8) Given that "recovery assays" are operationally defined, it is likely that each technique measures radiation-induced lesions that differ both qualitatively, and quantitatively. This should not preclude the use of cellular recovery as an experimental endpoint in the elucidation of basic mechanisms determining cellular radiosensitivity.
CHAPTER 6

Cellular radiosensitivity of primary cultures of human glioma
Cellular radiosensitivity of primary cultures of human glioma .................... 201

6.1. Intrinsic radiosensitivity of primary tumour cultures ................... 203
   6.1.1. Clinical applications for estimations of individual radiosensitivity. ................................................................. 203
   6.1.2. Rationale for studies of surviving fraction at 2 Gy in this thesis ........................................................................... 204
   6.1.3. Clonogenic vs nonclonogenic assays of cellular radiosensitivity
          rationale for the use of the clonogenic survival assay in primary glioma cultures. ................................................. 205

6.2. Specific methods ............................................................................. 209
   6.2.1. Establishment of primary malignant glioma cultures ....... 209
   6.2.2. Soft agar cloning technique for measurement of acute radiation survival in primary and low passage culture. ............ 210

6.3. Results .............................................................................................. 211
   6.3.1. Establishment of primary cultures and cloning ..................... 211
   6.3.2. Phenotypic characteristics of primary glioma cultures ..... 212
   6.3.3. Surviving fraction at 2 Gy. ................................................... 212
   6.3.4. Effect of variation in cloning efficiency on SF2 estimation. ................................................................................. 215

6.4. Discussion ........................................................................................ 217
6.1. Intrinsic radiosensitivity of primary tumour cultures.

6.1.1. Clinical applications for estimations of individual radiosensitivity.

Clinical experience clearly demonstrates that the radiation dose required to eradicate human tumours differs significantly between individual cases. These observations have given rise to the notion that the radiation response of a tumour might be determined at the level of an individual by genetic factors, as well as tumour histology [Suit et al., 1989].

There has been increasing interest in the development of tests that might be predictive of radiation response in the individual, both for tumour control, and normal tissue toxicity. Potential assays of relevance include tests of intrinsic cellular radiosensitivity, flow cytometric methods for estimation of tumour proliferation kinetics, and techniques to determine the proportion of hypoxic cells within a tumour.

Major factors influencing the predictive power of any single assay include the clinical relevance of the biological parameter, and the technical resolution of the assay. Sampling errors, reproducibility, and intratumour heterogeneity may all hinder the prognostic value of a test in which there is real inter-patient variation. It must be established that there does exist a distribution of the characteristic under scrutiny in the population, and that there is not significant variation between patients in other factors influencing response.

Tucker and Thames have mathematically modelled the interaction of several biological parameters currently being evaluated as predictive tests in radiotherapy, including tests of intrinsic tumour radiosensitivity, and tumour doubling times [Tucker and Thames, 1989]. They concluded that based on data available from in vitro studies to date, predictive tests based on measurement of intrinsic cellular radiosensitivity are most likely to be correlated with clinical outcome. They further suggested that the magnitude of influence of cellular radiosensitivity was so great, that evaluation of any other predictive test of clinical outcome after radiotherapy would need to be corrected for this factor.

Studies have already demonstrated that significant differences do exist in the distribution of cellular radiosensitivity measured in biopsies from tumours of a
given site and histology. The magnitude of interpatient variation was greater than experimental error. This would therefore suggest that measurement of intrinsic radiosensitivity might be technically feasible, and of clinical value.

Brock et al [1990] have examined the variation in cellular radiosensitivity at 2 Gy in a cohort of 140 patients with squamous carcinoma of the head and neck. Using a non-clonogenic, cell growth assay (CAM assay), they obtained an average SF2Gy of 0.33 (range 0.11-0.91). Intra-experimental errors, and repeat estimations on the same tumour (multiple biopsies) gave much smaller variations than interpatient values. Coefficients of variation cited were 11%, 23%, and 44% respectively. This data set has been criticised for the use of a non-clonogenic endpoint to measure response to radiation, and failure to identify the malignant nature of the cells grown.

A similar degree of interpatient heterogeneity of response at 2Gy has been demonstrated in biopsies of human cervical carcinoma. Clonogenic cell survival after 2 Gy was measured in 86 biopsies. A wide range of values was found, 0.13-0.97, with a mean SF2 of 0.47. The interpatient coefficient of variation was again noted to be significantly greater than experimental error. (38% vs 19%)

In both these studies the in vitro estimations of radiosensitivity are to be correlated with clinical outcome.

Only one study to date has attempted to assess the variability in radiosensitivity of tumour primary cultures of different histological groups. Rofstad et al [1987] have measured clonogenic cell survival in 36 human tumour biopsies spanning 7 different tumour sites, including melanoma, breast, cervix, ovary, bladder, seminoma, and head/neck tumours. Although numbers varied between groups, no significant difference in mean SF2 values was detected when tumours were grouped by clinical site. They concluded that there was considerable heterogeneity of response at 2Gy between individual tumours, but no correlation with histology. This result would appear to be in direct conflict with the original conclusions of Fertil and Malaise that histology is a major determinant of tumour response.

6.1.2. Rationale for studies of surviving fraction at 2 Gy in this thesis

The measurement of surviving fraction at 2Gy was attempted in this study in 17 primary human glioma cultures. The purpose of these experiments was twofold:
1) To compare the measured SF2 values obtained in primary culture with the values in the 5 continuous glioma lines.

Inherent in the conclusions of Fertil et al is the concept that cellular radiosensitivity might be genetically determined by cell lineage or differentiation. This would supervene over evolution of relative radioresistance in vitro to maintain real differences in cellular radiosensitivity. However, it is usually the case that a number of significant phenotypic properties change in the establishment of a cell in culture eg plating efficiency, doubling times, ploidy.

It was therefore considered relevant to compare the SF2 measurements obtained in primary and continuous cultures of human glioma to exclude the possibility that the observed relative radioresistance seen at high passage level was the result of prolonged in vitro selection.

2) An objective of the study was to assess the feasibility of routine estimations of intrinsic radiosensitivity in primary glioma cultures. If sufficient interpatient variation was observed, compared with experimental error this might provide additional prognostic information.

6.1.3. Clonogenic vs nonclonogenic assays of cellular radiosensitivity: rationale for the use of the clonogenic survival assay in primary glioma cultures.

Clonogenic cell survival remains the most widely accepted experimental endpoint of cellular radiosensitivity. Alternative cellular endpoints include cell growth assays, in which a response is measured by the change in a parameter proportional to total cell number eg MTT assay or staining by dye [Brock et al, 1989; Price and McMillan, 1990]. The major objections to these systems are that they fail to select against growth of non-malignant cells, such as fibroblasts, and require careful calibration of control estimations to ensure linearity of measurement.

Promising techniques include those based on scoring chromosomal or DNA damage. However, extensive correlation of such endpoints with in vitro clonogenic cell survival or clinical outcome is awaited.
The major impetus to develop such assays has been difficulties applying the clonogenic assay technique to primary culture material, and the interest in developing assays which might give a ranking of cellular radiosensitivity within a matter of days rather than weeks.

The conceptual and practical limitations of using clonogenic assays of human tumours to measure response to therapy have been exhaustively reviewed[Selby et al, 1983].

Practical problems encountered include:
1) Small amounts of biopsy material, therefore limited cell numbers for assay.
2) Low percentage of biopsies exhibiting adequate growth
3) Infection of primary cultures
4) Problems achieving good single cell suspensions to assess true clonal growth
5) Low cloning efficiency
6) Long duration of assay-weeks
7) Technical artefacts in measuring response; cell clumps; poor linearity of cloning

Despite these problems there are good reasons to use the assay, with efforts to minimise these problems as far as possible.

1) Agar cloning appears to select for the growth of anchorage-independent, malignant cells.

In the few studies which have tried to establish clonal growth from malignant glioma propagation of either haemopoietic or normal glial growth was not felt to occur. Deliberate attempts to clone normal glia resulted in cloning efficiencies of less than 0.001%, and full characterisation of those clones was not undertaken[Rosenblum et al, 1983].

2) Clonal growth is a well-established cellular endpoint in radiation and tumour research.

The properties of neoplastic cell populations are consistent with a model in which cells with proliferative potential form a spectrum, from those with the capacity to renew the entire population, including themselves, to apparently more 'differentiated' cells. These self and population renewing cells, which may constitute only a small proportion of the total population are considered stem cells. The model presumes that stem cells are responsible for tumour repopulation
after treatment, and are therefore a primary target for cancer therapy. [Steel, 1977]

The validity of the stem cell model is supported by several lines of evidence.
1) There appears to be an inverse relationship between indices of tumour proliferation, such as mitotic rate, and the degree of tumour differentiation [Steel, 1977].

2) Cloning assays of human tumours are usually observed to yield low colony numbers, of the order of 0.001-5% [Courtney and Mills, 1978; Davidson et al, 1990].

3) Fractionation of suspensions of cells from human tumours has demonstrated that proliferative activity, clonogenic potential, and cell differentiation features are restricted to separate subpopulations with defined physical properties [MacKillop et al, 1982].

4) The availability of clonal markers eg the Philadelphia chromosome in CML, have shown that tumours may arise from a multipotent stem cell, even if the majority of cells are differentiated progeny [Selby et al, 1983].

There are therefore good reasons to believe that any system aiming to assess the radiosensitivity in an individual patient should be aiming to identify such stem cells, or in the case of normal tissue, tissue-renewing units.

In this study specific measures were undertaken to facilitate agar cloning of human glioma primary cultures, whilst trying to avoid some recognised pitfalls in the assay.

1) A semisolid agar cloning system was employed, as described by both Salmon, and Courtney, to select for malignant growth [Salmon and Hamburger, 1977; Courtney and Mills, 1978]. A further advantage of this approach relates to restricting the marked motility of malignant astrocytes in monolayer culture systems. Pilot studies using a monolayer cloning assay similar to that used in the assessment of the continuous lines achieved higher cloning efficiencies, but scoring of individual colonies was complicated by poor resolution of individual colonies.

2) Previous studies using the agar cloning system have emphasised the problems of low growth rate and cloning efficiency. Attempts to use such a system to quantify response of human glioma to cytotoxic drugs was significantly hampered...
by cloning efficiencies averaging 0.06% [Rosenblum et al, 1983]. Three specific manoeuvres designed to enhance cloning efficiency were adopted in the Courtney-Mills assay: routine inclusion of autologous heavily irradiated feeder cells; a reduction in oxygen tension from 20 to 5% during cloning; the addition of August rat red blood cells as additional "feeder" cells.

In this project the effect of all 3 of these procedures was assessed, and is discussed.

3) Particular attention was paid to obtaining a good single cell suspension prior to assay. Excessive processing may contribute to the low cloning efficiencies found in primary cultures, but this must be balanced against the need to avoid multiplicity. In accordance with recommendations of other workers, the quality of the single cell suspension used was carefully checked by haemocytometer, and filtration using a sterile mesh employed to remove cell clumps. Heavily irradiated controls (50 Gy) were examined for false positive colony formation.

4) Both monolayer and agar cloning systems require that response to radiation is only estimated over a cloning range where linearity can be demonstrated between cells plated and colony numbers. Linearity declines at low colony number, possibly due to inadequate paracrine stimulation, and at high colony number due to medium depletion. In this study control and irradiated test samples were seeded at a range of cell densities. As only limited glioma biopsy material was available, cell numbers were low. Therefore a modification of the usual protocols estimating radiosensitivity in immediately disaggregated biopsy was employed.

Primary cultures were established for several days prior to test irradiation to permit expansion of cell number, and facilitate disaggregation of cell clumps. Whilst this period may have allowed for a degree of in vitro selection, it could be argued that limited propagation of the more aggressive tumour elements might enable a more relevant cell population to be studied. The procedure is analogous to the use of xenografts to establish primary biopsy cultures used by other workers [Smith et al, 1976]. A further validation of this approach is that where primary tumours samples have been assayed after several passages no significant change has been noted in radiosensitivity [Davidson et al, 1990].
Similarly, the use of mass culture growth assays in a predictive manner requires such expansion of primary cultures, and results obtained on SF2 appear to correlate well with clonogenic assays [Price and McMillan, 1990].

6.2. Specific methods.

6.2.1. Establishment of primary malignant glioma cultures.

Human glioma material for this study was provided by the Departments of Neurosciences in Glasgow, Edinburgh and The Institute of Psychiatry, Maudsley Hospital.

Human glioma tissue was collected at the time of craniotomy into complete medium with antibiotics (penicillin, 200 units/ml; streptomycin, 200 ug/ml; gentamycin, 100 ug/ml; amphotericin B, 2.5 ug/ml). Tissue was usually transferred to the lab at 4 °C within 4-6 hours. Biopsies were received by post from both Edinburgh and the Maudsley within 24 hours of surgery, and were usually viable.

The sample was first dissected free of obvious non-tumour or necrotic material. Mechanical disaggregation was performed by mincing in a petri dish, using crossed scalpel blades, to a size of 1-2 mm. The tumour material was spun down at 600 rpm for 2 minutes, then resuspended in PBS containing antibiotics as above, and this washing repeated twice. Disaggregated tumour was finally resuspended in medium with antibiotics, and collagenase (Sigma), to give a final concentration of 200 units/ml. The material was incubated at 37 °C, usually overnight, with occasional pipetting of the solution under sterile conditions to aid disaggregation. The tissue was then spun out of collagenase, and resuspended in complete medium with penicillin and streptomycin, to permit adherent culture in a T25 cm² flask. The culture was examined daily, and turbid medium containing cellular debris removed, and replaced with fresh medium.

When the quantity of biopsy material permitted, the tissue was cut in half prior to mincing, to permit assessment of intratumor heterogeneity of radiosensitivity.

As most tissue was obtained from open craniotomy, it was usually possible to establish 2-4 separate T25 cm² cultures de novo.

Primary culture was also established without collagenase, allowing small pieces of tissue to adhere in μl quantities of medium in a T25 cm² flask, and monitored regularly for explant outgrowth. Using this latter procedure, outgrowth was regularly observed, but never proved adequate for further passage.
The collagenase technique provided successful short-term cultures in 80% of samples processed. All antibiotics were discontinued after 1 week in culture. Passage and radiosensitivity testing was undertaken when cultures reached 80% confluence, and usually a T25cm² flask would be subcultured into a T75cm². Less vigorous growth was split 50:50. Time to first passage varied from 3 days to 4 weeks. Cultures were passaged to level 3, then frozen in liquid nitrogen. Successful recovery following freezing was 90%.

6.2.2. Soft agar cloning technique for measurement of acute radiation survival in primary and low passage culture.

The growth of clonogenic tumour cells in soft agar was used to measure radiosensitivity in vitro, according to a modified Courtney-Mills technique [Courtney and Mills, 1978].

Primary cultures, and cultures at passage 1-3 were trypsinised using PET solution, and mechanically disaggregated to yield a single cell suspension. The final suspension was filtered through a sterile 30μ mesh to remove cell clumps. Cells were counted and the quality of the suspension checked, using a haemocytometer. Cells were irradiated at ambient room temperature as a single cell suspension, as per the acute radiation protocol previously described.

35mm petri dishes were precoated with 1% agar (Difco). 2% agar was sterilised by boiling, then maintained at 45°C in a water bath to prevent setting. Aliquots of the single cell suspension at varying dilutions were mixed with agar, and 5 x 10⁴ heavily irradiated (100Gy) feeder cells, to give a variable test cell number in a final 2 ml volume of 0.3% agar. Pilot experiments compared the enhancement of cloning forming efficiency achieved by the addition of homologous or heterologous feeders. Control cultures contained feeder cells only. The dishes were placed on ice at 4°C for 5 minutes to promote setting of the agar, then transferred to a 37°C incubator and gased at reduced oxygen tension, according to the Courtney-Mills protocol (5% O₂, 8% CO₂.)

Test cell numbers were chosen to try to obtain 100 to 150 colonies per dish, allowing for a range of possible plating efficiencies of 0.001-10%, with an anticipated range of surviving fractions at 2 Gy of 0.1-0.9.
Dishes were examined for evidence of cell clumps on day 3, and discarded if positive. This was noted to be a rare event after proper cell filtration. Individual dishes were fed 0.5ml fresh medium after 10 days. Cultures were examined for colony formation at 2-5 weeks. Identification of viable colonies was facilitated by incubation for 4 hours with the tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. This dye is reduced to a purple coloured formazan product by the mitochondria of live cells.

Dishes were incubated at 37° C wrapped in aluminium foil to prevent degradation of MTT, following the addition of 500μl of MTT (Sigma) at a concentration of 1mg/ml in PBS.

Colonies of greater than 50 cells were counted by eye using an inverted phase microscope. Validation of the criteria used to identify colonies of appropriate size included preliminary screening of several dishes and scoring of mean colony diameter of 50 colonies using an eye piece graticule micrometer scale. Colonies greater than 50μm diameter were scored as representing colonies of more than 50 cells. Several colonies were "picked" from the agar and mechanically disaggregated in another petri dish to allow approximate scoring of cell numbers.

6.3. Results.

6.3.1. Establishment of primary cultures and cloning.

The primary cultures established in this study are noted in table 3.2. Other early passage human glioma cell lines previously established within the lab but used in this study are included.

The success rate in establishing a primary monolayer culture from human malignant glioma biopsies was 80%. Two low grade tumours were established, and remained viable with no evidence of growth over a period of 6-8 weeks.

Of 17 primary cultures studied, growth adequate to estimate surviving fraction at 2 Gy was achieved in only 9 cases (52%).

The semisolid agar cloning system was found to yield efficiencies of 0.001-5%. Pilot experiments on the first 3 biopsies received evaluated the effect on cloning of a) oxygen tension; b) the effect of adding both homologous and heterologous (G-UVW continuous glioma) heavily irradiated feeder cells; c) stimulation of cloning efficiency by addition of irradiated August rat red blood cells.
The effect of a reducing oxygen tension during cloning, from 20% to 5% and the presence of heavily irradiated homologous cells was found to be critical to obtaining a cloning efficiency above 0.01%.

The effect of adding heavily irradiated August rat red blood cells, as employed in the original Courtney-Mills protocol, was tested in pilot experiments in 3 low passage cultures. No enhancement of cloning efficiency was noted, therefore this procedure was not routinely employed.

6.3.2. Phenotypic characteristics of primary glioma cultures.

Limited evaluation of the nature of the primary and subsequent early passage lines was undertaken. All of the 17 primary cultures established exhibited glial morphology in monolayer culture, with a range of predominant patterns. Cells were not allowed to achieve complete confluence, but nevertheless loss of contact inhibition was usually observed.

At first passage $10^4$ cells were seeded onto sterile glass coverslips in 24 well plates for evaluation of GFAP staining, as evidence of glial lineage. The protocol is described in chapter 3. The 9 primary cultures giving positive clonal growth in agar were all GFAP positive at the time of first passage, with more than 70% cells positive. Four of these cultures were retested at passage 5 by which time less than 5% of all cells were positive, despite little discernible change in culture morphology.

The morphology of BG 488 is shown in figure 3.5.

One cell line established from a biopsy subsequently reviewed as non-malignant gave rise to a culture that permitted 3 passages prior to senescence. GFAP staining was noted to remain positive in these normal glia, in comparison with cultures derived from malignant tissue.

6.3.3. Surviving fraction at 2Gy.

Results are presented in graphical and tabular form for the 9 cell lines. Figure 6.1 shows the mean surviving fractions obtained at 2GY on each glioma primary culture, with standard deviations. Table 6.1 contains the tabular data.
A range of mean SF2 was found of 0.37-0.75, with a mean of 0.56. The mean intra-experimental coefficient for the parameter was 21%, with a range of 9-26%.

A mean repeat experimental coefficient of variation of 15% was measured by repeating the SF 2Gy assay on 4 cell lines at passage 2. (BG 424; BG 535; BG 550; BG 566) Analysis of variance showed no significant differences in either colony forming efficiency or SF2GY values in the repeat experiments on these 4 cell lines. Material was insufficient to permit adequate assessment of intra-tumour heterogeneity by measuring SF2 GY in separate parts of the same culture.

The mean coefficient of variation in SF2 Gy across the 9 primary cultures was 25%. 
Figure 6.1 Surviving fraction at 2 Gy in primary cultures of human malignant glioma

Values are means obtained for each primary culture, with 1 standard deviation.
6.3.4. Effect of variation in cloning efficiency on SF2 estimation.

The lack of correlation between cloning efficiency and mean SF2Gy is shown as a plot of SF2 for each cell line against its mean cloning efficiency. Figure 6.2.

Figure 6.2. Relationship between colony forming efficiency and SF 2Gy values

Values for each primary culture represent mean
Table 6.1. Surviving fraction at 2 Gy in primary cultures of human malignant glioma

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CFE (%)</th>
<th>mean</th>
<th>1SD</th>
<th>1SEM</th>
<th>SF2&lt;sup&gt;30&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>BG 398</td>
<td>0.01</td>
<td>0.57</td>
<td>0.15</td>
<td>0.07</td>
<td>4.7 x 10&lt;sup&gt;-10&lt;/sup&gt;</td>
</tr>
<tr>
<td>BG 424</td>
<td>1.2</td>
<td>0.63</td>
<td>0.16</td>
<td>0.07</td>
<td>9 x 10&lt;sup&gt;-7&lt;/sup&gt;</td>
</tr>
<tr>
<td>BG 488</td>
<td>0.02</td>
<td>0.40</td>
<td>0.07</td>
<td>0.03</td>
<td>1 x 10&lt;sup&gt;-12&lt;/sup&gt;</td>
</tr>
<tr>
<td>BG 500</td>
<td>0.1</td>
<td>0.44</td>
<td>0.08</td>
<td>0.03</td>
<td>2 x 10&lt;sup&gt;-11&lt;/sup&gt;</td>
</tr>
<tr>
<td>BG 535</td>
<td>0.09</td>
<td>0.75</td>
<td>0.07</td>
<td>0.02</td>
<td>1.7 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>BG 550</td>
<td>4</td>
<td>0.71</td>
<td>0.09</td>
<td>0.03</td>
<td>3.4 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>BG 551</td>
<td>0.04</td>
<td>0.37</td>
<td>0.08</td>
<td>0.03</td>
<td>1.1 x 10&lt;sup&gt;-13&lt;/sup&gt;</td>
</tr>
<tr>
<td>BG 555</td>
<td>0.08</td>
<td>0.67</td>
<td>0.13</td>
<td>0.05</td>
<td>6 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
</tr>
<tr>
<td>BG 566</td>
<td>0.2</td>
<td>0.52</td>
<td>0.11</td>
<td>0.04</td>
<td>3 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
</tr>
<tr>
<td>mean</td>
<td>0.6</td>
<td>0.56</td>
<td></td>
<td></td>
<td>4.7 x 10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Mean intra-experimental coefficient of variation for SF2 = 21%*

*Mean repeat experimental coefficient of variation for SF2 = 15%*

*Coefficient of variation for glioma group SF2 = 25%*
6.4. Discussion.

The objective of measuring radiosensitivity at 2Gy in primary glioma cultures was twofold: firstly, to compare the values obtained with those measured in continuous glioma lines in this study; secondly, to assess the feasibility and value of routinely measuring SF2 Gy in human glioma biopsies as a possible additional prognostic factor.

It was considered that the SF2 estimations in primary and continuous cultures should be compared by identical endpoints. Technical problems were encountered in attempting to use the monolayer cloning technique as the experimental endpoint in primary culture, namely difficulties in distinguishing true clonal growth due to motility of the astrocytes on plastic. For this reason a semisolid agar cloning system was adopted. This obviously raises the question as to the direct comparibility of SF2 values using different experimental systems.

The mean SF 2 value observed in the 9 primary glioma cultures of 0.56 (SD 0.12) compared with a value of 0.68 (SD0.11) for the 5 continuous lines. Ideally, a direct comparison of glioma cultures at different passage level would have used the same experimental system. An alternative approach would have been to assess the ranking of SF2 GY obtained in primary culture of a variety of human tumour cultures as well as glioma. West et al have compared the values obtained for their primary cervix cultures with primary colorectal and lymphocyte cultures to show that the mean values are significantly different for these 3 cell lineages. Given these limitations on this data, the values of SF2 for glioma appears to be higher than those observed in human cervix tumours (mean SF2 0.47, SD 0.18), which is considered to be a more radiocurable tumour [Davidson et al, 1990].

A full technical evaluation of any proposed predictive test of response to radiation is required prior to attempts at clinical correlations. The stringency of this requirement is such that as yet only two laboratories have attempted to make any tentative correlations of in vitro radiosensitivity and clinical factors [West; Brock; unpublished data, Proceedings of 25th Paterson Symposium on Predictive Radiosensitivity testing, 1990].

These technical factors include sampling errors, reproducibility on repeat estimations, intra-tumour heterogeneity and interpatient variation. This study
coincided with a trend in clinical practice towards limited biopsy of human glioma, thus reducing numbers of samples available and quantities of tumour. Within these constraints it has been possible to demonstrate that successful culture of primary glioma could be achieved in 80% of cases, and that viable cultures could usually be established in material stored in holding medium for up to 36 hours.

The cloning efficiencies (CFE) achieved in this study precluded generation of full radiation survival curves, with the emphasis on measurement of radiosensitivity at 2 Gy. The CFE range of 0.01-4% are clearly much lower than continuous cultures but are comparable with those achieved by other groups working with primary tumour cloning systems. They represent a log increase on the mean values cited for primary glioma cultures in other labs, which may reflect the use of reduced oxygen tension. It is probable that the use of serum-free cloning with growth factor supplementation determined specifically for human glioma might significantly enhance cloning efficiency.

The intra-experimental variation in SF2 of 21% was considerably higher than observed in the continuous glioma cell lines but is comparable to that observed in other primary tumours. Of greater concern is that the inter-patient variation of 25% observed in SF2 in this study would suggest that the predictive power of measuring SF2 in glioma is likely to be obscured by intra-experimental error using this system.

It is recognised that the potential for considerable selection occurs once a primary culture is established. Similarly, the time taken to achieve measurable clonal growth in these cell lines varied from 3-5 weeks. The ideal predictive radiosensitivity test would yield an answer enabling a ranking of relative radiosensitivity in an individual patient within days of biopsy, in order to guide choice of therapy or dose of radiation.

There is therefore increasing interest in developing assays such as MTT, using limited cell growth over periods such as a week [Price and McMillan, 1990], or subcellular endpoints such as DNA damage. The major objection to the use of growth assays has been the problem of fibroblast overgrowth in non-selective growth conditions. This is not considered to be a problem in primary monolayer cultures of glia. Combined with a more detailed evaluation of the growth requirements of human glioma, this approach might permit assessment of radiosensitivity more readily. A further theoretical attraction of this type of 'mass
culture' system is the potential for cell-cell interaction more closely akin to a tumour. Whilst clonogenic growth has both practical and theoretical attractions as an experimental endpoint, the true relationship between tumour stem cells and cultured clonogenic cells is far from clear [Potten and Loeffler, 1990].

There is an increasing trend towards reporting human cellular radiosensitivity in terms of response to doses of radiation of clinical interest, namely, surviving fraction at 2 Gy. There are now a number studies addressing the predictive value of SF2 obtained in primary tumour cultures. Similarly, studies evaluating the population distribution of normal tissue radiosensitivity are also using the SF2 parameter. A number of concerns have been expressed regarding the means by which such a measurement could be employed prospectively to individualise radiotherapy.

It is of concern that values of SF2 for tumours of a given histology are clearly dependent upon the passage level of culture studied, and the assay employed. This would matter less if the relative ranking of radiosensitivity was preserved irrespective of technique. There is evidence that this does not happen. Similarly, they appear to generate tumour control probability curves that are not in keeping with clinical experience.

It is hoped that refinement of primary culture requirements for cells of a specific lineage, together with development of assay systems based on subcellular endpoints, might permit a fuller evaluation of the true clinical value of prospective estimations of intrinsic radiosensitivity in individual patients.
CHAPTER 7

DNA double strand break rejoining mediated by cell-free extracts of human tumours of differing radiosensitivity
CHAPTER 7 .......................................................................................................220

7.1. Establishment of an in vitro assay to assess DNA dsb rejoining in cell-free extracts

Introduction and specific methods................................................. 223
7.1.1. Use of in vitro plasmid dsb rejoining to investigate mechanisms determining cellular radiosensitivity in the V79 irs mutants. ................................................................. 223
7.1.2. Choice of cell fractionation for in vitro studies..................... 224
7.1.3. Choice of plasmid substrates for DNA repair studies .......... 225
7.1.4. Choice of specific restriction endonuclease-induced substrates...................................................................................... 227
7.1.5. Choice of plasmid DNA dsb rejoining reaction conditions..................................................................................... 228
7.1.6. Use of bacterial genetics to assess plasmid DNA rejoining ...................................................................................... 233
7.1.7. Choice of bacterial host for transformation studies ............. 234
7.1.8. Experimental objectives....................................................... 234

7.2 Results ..............................................................................................235
7.2.1. DNA binding activity of human tumour extracts .............. 235
7.2.2. Electron microscopy of plasmid DNA ligation products .... 238
7.2.3. Bacterial transformation by plC20H plasmid, post incubation with nuclear extracts..................................................... 240
7.2.3.1. Control rejoining reactions............................................. 240
7.2.3.2. Extent of rejoining of endonuclease induced DSB by human tumour extracts. ......................................................... 242
7.2.3.3. Protein concentration dependence of bacterial transformation. ................................................................. 244
7.2.3.4. Fidelity of rejoining of EcoR1 cut plC20H substrate.................................................................................. 250
7.2.4. Visualisation of plC20H ligation products by Southern analysis. .................................................................................. 253
7.2.4.1. Effect on tumour nuclear extracts on uncut plasmid. ................................................................................ 254
7.2.4.2. Effect of tumour nuclear extracts on EcoR1 cut plC20H. .................................................................................. 256

7.3. Discussion ........................................................................................262
7.3.1. In vitro DNA dsb rejoining activity of human tumour extracts.................................................................................. 262
7.3.2 Processing of plasmid substrates by human extracts ......... 262
7.3.3 Fidelity of dsb rejoining in human extracts ......................... 265
7.3.4. Effect of class of dsb termini on rejoining proficiency ........ 266
7.3.5 Relevance of in vitro model to cellular DNA repair
processes .......................................................................................... 267
7.3.6. Further protein activities participating in dsb rejoining. .... 268
7.3.7. Conclusions ........................................................................... 269
7.1. Establishment of an in vitro assay to assess DNA dsb rejoining in cell-free extracts: introduction and specific methods

There is considerable interest in the development of techniques refining the measurement of DNA dsb induction and repair. This is critical to the further evaluation of this class of lesions, and their precise relationship to the determination of cellular radiosensitivity.

An in vitro, cell-free assay has been established within this laboratory to evaluate the possibility that the molecular defect underlying the cellular radiosensitivity of mammalian cells expressing a radiosensitive phenotype might be a defect in the proficiency of DNA double strand break rejoining.

The development of this assay for the study of in vitro dsb rejoining proficiency in human tumour cell extracts and a comparison of the results obtained in extracts derived from radioresistant and radiosensitive cell lines was the final part of this studies undertaken for this thesis.

The detailed methodology of nuclear extract preparations, bacterial and plasmid DNA manipulations used in these studies is contained in Chapter 2.

7.1.1. Use of in vitro plasmid dsb rejoining to investigate mechanisms determining cellular radiosensitivity in the V79 irs mutants.

In vitro conditions favouring the rejoining of 2 types of cohesive termini of restriction digested plC20H had been established within the laboratory, using extracts derived from V79 and the ionising radiation sensitive mutant lines irs 1 and 2. The work formed the Ph.D thesis of Jane Parker [Parker, 1991]. The main findings of the work were:

1) Nuclear extracts from the parental V79 line, and the mutant irs 1 were able to catalyse the rejoining of the dsb with equal efficiency and fidelity, as assessed by bacterial transformation by the plasmid DNA products of the in vitro rejoining reaction.
2) Extracts of the *irs* 2 mutant were able to catalyse the formation of linear plasmid DNA to high molecular weight concatemers, as evidenced by Southern analysis, and examination of reaction products by electron microscopy, but appeared unable to mediate the formation of circular plasmid molecules. These *irs* 2 extracts did not therefore appear to be generally deficient in DNA ligation activity. This was confirmed by assay of the activity of DNA ligase I, and II in these extracts.

3) Reactions attempting to restore the *irs* 2 defect by mixing with extract from the parental V79 line gave evidence of biochemical complementation *in vitro*.

4) It was concluded that the ionising radiation-sensitive mutant of V79, *irs* 2, was able to rejoin cohesive dsb in plasmid DNA, but appeared to be deficient in a protein activity necessary for the recircularisation and rejoining of 3' or 5' termini in double stranded plasmid DNA.

Pilot experiments using similar assay conditions to assess ligation activity in extracts of human cell lines had met with technical difficulties. The cell lines used were MRC5, an SV40 transformed 'normal' human fibroblast line, and AT5BIVA, an SV40 transformed fibroblast line derived from a homozygous ataxia telangiectasia patient. Both plasmid Southern analysis and bacterial transformation suggested significant extract-mediated degradation of both uncut and linear plasmid substrates. It was postulated that excessive nuclease activity in both these cell lines might be attributed to SV 40 transformation.

Thacker (personal communication) had encountered similar difficulties in a similar in vitro plasmid dsb rejoining study, using MRC5 and AT5 cell extracts. This had been circumvented by using an excess of linear plasmid DNA substrate to 'quench' excess nuclease activities in extracts, in order to assess plasmid rejoining fidelity in the remaining linear molecules. In adopting this approach, it is acknowledged that the 'effective' substrate available for rejoining might vary between cell extracts. However, as the primary objective was assessment of plasmid rejoin fidelity rather than extent, then this was considered to be acceptable.

7.1.2. Choice of cell fractionation for in vitro studies.

It was considered that the use of nuclear extracts rather than crude whole cell extracts might selectively examine the activity of proteins likely to be most immediately involved in DNA repair activities.
The human tumour cell lines selected for study were:
1) Two radioresistant lines, the glioma lines SB18, and IJK.
2) Two radiosensitive lines, the ovarian carcinoma A2780, and the neuroblastoma, SKHSH.

Given that the 4 cell lines under evaluation were non-isogenic, a system for the comparison of protein activities between lines was required. Nuclear proteins were evaluated by both biochemical estimation of total protein levels, and seeking evidence of preservation of a specified DNA binding function.

The synthetic oligonucleotide aP3A contains the mammalian consensus binding site recognition sequence, CCAAT, for the ubiquitously expressed mammalian CCAAT box binding protein [Plumb et al, 1989] Evidence of preservation of this specific mammalian DNA-protein binding activity was sought in extract preparations by use of a gel retardation assay.

Only cell-free extracts demonstrating binding activity in this assay were used in plasmid rejoining studies. It provided confirmatory evidence that nuclear extract preparations were yielding functional, non-degraded proteins. The details of nuclear extract preparation, and evaluation of protein activities are described fully in chapter 2.

7.1.3. Choice of plasmid substrates for DNA repair studies

The plasmid DNA molecule chosen for this assay was pIC20H, a 2.7 kb recombinant DNA molecule containing 1 selectable bacterial gene, encoding ampicillin resistance, and the Lac Z gene [Marsh et al, 1984]. A restriction map of the plasmid is shown in figure 7.1
Figure 7.1. Restriction map of plasmid pIC20H.

Restriction map of pIC20H simplified from Marsh (1984)

Ori= origin of replication
Lac Z= lac Z gene with direction of transcription marked
ApR= ampicillin resistance gene
Selected restriction sites in multicloning site shown, with recognition sequences
The polylinker region of this plasmid contains a number of single-cutting restriction sites enabling the production of a variety of dsb of differing structure eg. the restriction enzyme EcoR\textsubscript{1} is a 6 base cutter, generating 5' phosphoryl breaks with 4 overhanging bases, with the recognition sequence 5' AATT; Nru\textsubscript{1} generates blunt termini, with the recognition sequence TCGCGA.

The plasmid contains both the regulatory sequences and coding information of the Lac Z gene. This encodes the production of the first 146 amino acids of the enzyme, β-galactosidase. The bacterial host chosen for this assay, the E.coli strain JM 83, possesses the carboxy terminus of β-galactosidase, and therefore complementation can occur by association of the two portions of the β-galactosidase protein. This renders the bacteria "Lac Z+", and capable of hydrolysing the chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactoside). If degradation or loss of sequence occurs in the plasmid portion of the LacZ gene as part of the ligation reaction, then no complementation can occur, and the bacterial colonies are white rather than blue. The integrity of the Lac Z sequence and bacterial β-galactosidase activity forms the basis of the assessment of dsb 'repair fidelity' in this assay.

7.1.4. Choice of specific restriction endonuclease-induced substrates.

The plasmid DNA substrate for rejoining reactions was pIC20H, digested by the restriction enzyme EcoR\textsubscript{1} to produce linear plasmid molecules with cohesive termini. This type of cohesive terminus was chosen based on the observation of significant mis-rejoining of this substrate by extracts of AT cells in Thacker's study. Similarly linear plasmid substrates with non-cohesive termini were produced by linearisation of the plasmid using the enzyme Nru\textsubscript{1}.

Both restriction enzymes have a single recognition site in the plasmid pIC20H, situated within the multicloning site of the Lac Z gene, as indicated in figure 7.1.

Similar termini are likely to arise by the action of ionising radiation, either by direct action, or the interaction of two adjacent single stranded events. Such enzymatic DNA strand breaks might be "cleaner" biochemically than those resulting from ionising radiation damage, with intact 5' phosphate, and 3' hydroxyl groups at termini, suitable for religation without further enzymatic modification.
Restriction endonuclease-induced DNA dsb are, however, capable of producing a similar spectrum of cell events consequent upon DNA damage, when used in permeabilised cells. These include chromosomal aberrations, and subsequent cell death, as discussed in Chapter 1.

7.1.5. Choice of plasmid DNA dsb rejoining reaction conditions.

A preliminary part of this study was to establish the ligation conditions in which plasmid dsb rejoining could be observed using human tumour cell extracts. Variables to be considered included:

1) Total linear plasmid DNA concentration per reaction.
2) Reaction total volume to favour intra-molecular plasmid rejoining activity.
3) Ligation buffer cation and energy requirements.
4) Reaction temperature.
5) Reaction time course.

The reaction protocol is depicted graphically in figure 7.2.

A major objective of the assays was to enable an assessment of the fidelity of rejoining of the double strand break. This was to be evaluated by the expression of the Lac Z gene in transformed bacteria. Reaction conditions were therefore required which favoured recircularisation of each linear plasmid molecule, rather than end to end rejoining of adjacent molecules to form high molecular weight concatemers, as the latter molecular forms are not efficient in bacterial transformation.

The total plasmid concentration used was a two-fold increase over that used in studies with the V79 lines, to 100ng in a final volume of 350μl. This represents a final concentration of DNA likely to favour intra-molecular end rejoining, rather than intermolecular ligation.

The equation of Dugaiczyk was used to derive these conditions, which enables a concentration of 'free' linear ends to be calculated which favours a significantly higher probability of intra-molecular recircularisation, rather than formation of concatemers. The ratio of cyclized to concatemeric ligation products depends on two parameters, $j$, and $i$. $J$ is the effective concentration of one end of a DNA molecule in the neighbourhood of the other end of the same molecule. $J$ is therefore inversely proportional to the length of the
molecule, a constant for a given DNA molecule, and independent of concentration.

\[
j = \frac{3}{2k lb}
\]

where \( l \) is the length of DNA in centimeters, and \( b \) the length of a randomly coiled segment of DNA. \( b \) is dependent on the ionic strength of the buffer, determining DNA rigidity.

\( i \) is a measure of complementary termini in the solution and is specified by

\[
i = 2 N_0 M \times 10^{-3} \text{ ends/ml}
\]

where \( N_0 \) is Avogadro’s number and \( M \) is the molar concentration of DNA. When \( j > i \) intramolecular recircularisation is favoured over linear concatenemisation.

Using this formula, a \( j/i \) ratio was calculated for the plC20H plasmid DNA which determined that a DNA concentration of less than 14\( \mu \text{g} \) per 350\( \mu \text{l} \) should favour such a rejoining activity [Dugaiczyk et al, 1975].
Figure 7.2. In vitro plasmid rejoining protocol

100ng linear pIC20H
eg. EcoR1 or Nru1 digest

Ligation buffer
1mM ATP
No PEG
Mg2+

Variables
Tumour protein concentration
Time
Temp (14°C)

+ / - pHSG 272 uncut
internal control

phenol / chloroform extraction

Na acetate + ethanol precipitation

Resuspension of plasmid DNA

accurate rejoining in Lac Z gene

inaccurate rejoining in Lac Z gene

No rejoining
A further measure taken to minimise end to end rejoining was the specific omission of polyethylene glycol (PEG) from the ligation buffer. This is used to promote T4 ligase reactions, especially of blunt termini, as it increases nonenzymic cohesion of termini by macromolecular crowding. Even at such low plasmid DNA concentrations it may have favoured intermolecular rejoining events [Zimmerman and Harrison, 1985].

The ionic conditions were adopted empirically from the observations using V79 extracts in this type of assay [Parker, 1991]. In choosing buffer conditions the requirements of many DNA ligase systems, including T4, E.coli, and mammalian DNA ligase, for divalent cations such as Mg$^{2+}$ and ATP were observed [Lindahl, 1982].

Unless stated otherwise, dsb rejoining reactions were carried out at 14°C, overnight, with the following conditions:

A reaction was set up in a final volume 350μl, containing

1) pIC20H plasmid DNA; 100ng of cut or uncut substrate
2) 70 μl of 5x ligation buffer, containing 250mM Tris-HCL, 5mM ATP, 50mM MgCl$_2$, and 5mM DTT.

Varying amounts of nuclear extract were added in storage buffer, containing 50mM NaCl, 5mM MgCl$_2$,20% glycerol, at pH 7.9. The final reaction volume was taken to 350μl using storage buffer, to ensure that the final plasmid DNA substrate concentration was constant.

The reaction was halted by the addition of an equal volume of phenol and plasmid DNA was then recovered as described in Chapter 2 , section 2.5.
The following controls reactions were included in each experiment:

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Uncut plC20H</td>
<td>+ tumour nuclear extract</td>
</tr>
<tr>
<td>2) Uncut plC20H + tumour nuclear extract.</td>
<td>+ tumour nuclear extract, boiled for 15 mins.</td>
</tr>
<tr>
<td>3) Uncut plC20H +1μl T4 ligase</td>
<td></td>
</tr>
<tr>
<td>4) Uncut plC20H</td>
<td>+1μl T4 ligase</td>
</tr>
<tr>
<td>5) EcoR1 or Nru 1 cut plC20H</td>
<td></td>
</tr>
<tr>
<td>6) EcoR1 or Nru 1 cut plC20H</td>
<td>+ boiled extract</td>
</tr>
<tr>
<td>7) EcoR1 or Nru 1 cut plC20H</td>
<td></td>
</tr>
</tbody>
</table>

The temperature conditions selected necessitated a balance between that required for optimal rejoining activity, and the possibility of thermal destabilisation of any pre-ligation complexes formed by proteins and plasmid DNA in aqueous solution. Both mammalian DNA ligase I and II are active at 14°C, and T4 ligase reactions are frequently conducted at 14°C, therefore this temperature was routinely used.

A preliminary assessment of comparative plasmid rejoining activity by an extract of the glioma cell line, IJK, was conducted at 4, 14 and 37°C. Significant rejoining over background was noted at 14°C. No activity over background was observed at 4°C, and a reduction in activity was noted at 37°C, by comparison with the 14°C samples. Further reactions were therefore carried out at 14°C.

The time course of plasmid rejoining activity of all 4 human tumour cell line extracts was examined in pilot studies. (data not presented). All exhibited a similar pattern of activity. Maximal rejoining activity was obtained by overnight ligation reactions at 14°C, though 90% of the maximal activity had been established by 4 hours.
7.1.6. Use of bacterial genetics to assess plasmid DNA rejoining.

The objective of the in vitro rejoining assay was to permit an assessment of both the extent and accuracy of rejoining of plasmid molecules mediated by the nuclear extracts derived from human tumour cells. The choice of the plasmid pIC20 H, containing 2 bacterial genes, permitted the use of bacterial genetics to achieve these objectives.

Following appropriate temperature and ionic manipulations, strains of E.coli can be made 'competent' to take up plasmid DNA, with a preference for circular molecules rather than linear DNA forms. After an appropriate expression time, the plasmid encoded genes are transcribed within the bacteria, altering its phenotype, a process known as bacterial transformation.

Selective bacterial growth media containing ampicillin then permit only bacterial colony formation in those bacteria transformed by the plasmid-encoded ampicillin resistance gene.

In the context of this assay, the expression of plasmid-encoded ampicillin resistance was interpreted as a marker of bacterial transformation by a recircularised plasmid molecule ie. the dsb forming the termini of restriction digested plasmid had been rejoined. The number of such ampicillin resistant E.coli per unit plasmid was therefore a measure of the extent of linear pIC20H rejoining.

The EcoR1 and NruI plasmid restriction digests used in this study were sited within the second bacterial gene, the Lac Z gene as displayed in fig.7.1 Hence expression of the Lac Z gene within ampicillin resistant colonies, detected by the generation of blue colonies on X-gal media, signified preservation of sequence at the restriction site during rejoining. The appearance of blue, LacZ+, Amp+ colonies was then interpreted as evidence of accurate plasmid DSB rejoining. White bacterial colonies resulted from expression of an Amp+, LacZ- phenotype in bacteria having incorporated rejoined plasmid in which the Lac Z was inactivated, ie. rejoining of termini located within this gene had occurred with loss of sequence.

Bacterial transformation procedures, and use of selective culture techniques is described in Chapter 2, section 2.5.
7.1.7. Choice of bacterial host for transformation studies.

The bacterial host used for these studies was the E.coli strain JM 83 [Yanisch-Perron et al, 1985 ]. The choice of this strain was based on its possession of the C-terminal portion of the lacZ gene; this was necessary for complementation of the Lac Z gene encoded within pIC20H. The Rec A recombination pathway has been inactivated in this strain, therefore it is also partially lacking in recombinational activity.

7.1.8. Experimental objectives.

The experimental objectives of this part of the study were:

1) To prepare nuclear protein extracts from 4 human tumour cell lines. Two radioresistant tumours were selected, the glioma lines SB18, and IJK. The two radiosensitive lines were A2780, the ovarian carcinoma line, and the neuroblastoma SKNSH.

2) To assess the functional protein activity of these extracts, and decide a means of comparison of activity in extracts derived from non-isogenic cell lines.

3) To establish the conditions needed to observe ligation activity in these extracts, specifically the time course of rejoining activity, and temperature dependence of the reaction.

4) To establish and minimise the major sources of experimental error, and thus modify the assay to permit quantitative assessment of DNA dsb rejoining events.

5) To compare both the extent and fidelity of rejoining of endonuclease-induced plasmid dsb in nuclear extracts prepared from the 4 human tumour cell lines.
7.2 Results

7.2.1. DNA binding activity of human tumour extracts

The DNA binding activity of ubiquitously expressed CCAAT binding protein was used to check that extracts had preserved protein function during preparation. Specific binding by functional proteins was observed in nuclear extracts derived from all 4 human tumour lines examined. These included two radioresistant human glioma cell lines, SB18, and IJK, together with two radiosensitive lines, A2780, and IMR 32.

A gel retard analysis of the nuclear extracts used in this study is shown in figure 7.-3. The protein activity of the extract produces a complex with the CCAAT binding sequence in the αP3A radiolabelled molecule. It is shown that these complexes are slowed or 'retarded' in their migration through the gel by comparison with uncomplexed radiolabelled αP3A. The specific nature of the binding is demonstrated by the ability of excess unlabelled αP3A to abolish the formation of these complexes.

The white arrow shows the position of migration of free radiolabelled αP3a, unretarded by complexing with tumour proteins.

Lanes 1, 3, and 5 contain αP3a/ tumour protein complexes, slowed in migration relative to free αP3a. Lanes are labelled in the figure to indicate the tumour origin of each extract.

Lanes 2, 4, and 6 illustrate the ability of an excess of cold αP3a, present in the pre-electrophoresis incubation, to compete for specific protein binding activity in each of the tumour extracts, thus reducing or eliminating the extent of formation of specific complexes with αP3a. It is noted that the IJK extract shown in this plate had high αP3a binding activity that was not completely eliminated by the amount of cold αP3a added. (lane 6)
The intensity of the highest molecular weight oligonucleotide/tumour protein complex formed was found to correlate well with the total protein concentration measured in each extract by Biorad assay.

Such activity was demonstrated in all extracts prior to use in ligation reactions; those not active by this criteria were discarded.
Figure 7.3 Gel retard analysis of specific binding of cell extract proteins and the CCAAT consensus sequence in the $\alpha$ P3A oligonucleotide.

GATCCAAACCAG CCAAT GAGAACTGCGCCA

Black arrows=position of migration of radiolabelled oligonucleotide/tumour protein complexes
White arrow=position of migration of free radiolabelled oligonucleotide

lane 1= $\alpha$ P3A+ A2780 tumour extract
lane 2= $\alpha$ P3A+ A2780 tumour extract + excess “cold” $\alpha$ P3A
lane 3= $\alpha$ P3A+ IMR32 tumour extract
lane 4= $\alpha$ P3A+ IMR32 tumour extract + excess “cold” $\alpha$ P3A
lane 5= $\alpha$ P3A+ IJK tumour extract
lane 6= $\alpha$ P3A+ IJK tumour extract + excess “cold” $\alpha$ P3A
Evidence for the rejoining of plasmid DNA double strand breaks by human cell extracts.

7.2.2. Electron microscopy of plasmid DNA ligation products.

Figure 7.4 demonstrates a transmission electron micrograph of

a) EcoR1 digested linear plC20H substrate, untreated by extract.
b) EcoR1 digested plC20H treated by T4 ligase ie a 'positive' control sample;
c) +d) EcoR1 digested plC20H, incubated with 5μg of nuclear extract, SB18.

It demonstrates that restriction digested substrate was formed predominantly of linear forms, and that such a substrate was a suitable molecule for rejoining of cohesive dsb by either purified T4 ligase activity, or the protein activity of nuclear extracts.

Extract-mediated formation of circular plasmid molecules as depicted in panels (c), and (d), gave visual evidence of the capacity of extracts to rejoin the restriction-induced dsb by the conversion of linear substrate to circular plasmid forms.

The size of the plasmid molecules was assessed by digitaliser, and confirmed to be compatible with a 2.7Kb molecule ie. no measurable loss of DNA appeared to result from treatment of linear plC20H by incubation with extract. This observation is in keeping with bacterial transformation data, in which less than 1% of most bacterial colonies failed to express the LacZ gene, ie rejoining of ends had occurred accurately, without loss of sequence.
Figure 7.4. Electron micrograph of pIC20H test and control ligation products.

a) EcoRI cut pIC20H plasmid DNA  b) EcoRI cut pIC20H treated with T4 ligase

c,d) EcoRI cut pIC20H following incubation with 5μg SB18 tumour proteins.
Table 7.1 shows a representative count of the relative numbers of linear and circular plasmid forms, performed on 1000 molecules in 3 independent preparations.

Table 7.1 Electron microscopy of plC20H ligation products

<table>
<thead>
<tr>
<th>Plasmid form</th>
<th>EcoRI cut</th>
<th>T4 ligase +ve C</th>
<th>+Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer circles</td>
<td>2%</td>
<td>98%</td>
<td>87.5%</td>
</tr>
<tr>
<td>Monomer linears</td>
<td>98%</td>
<td>1%</td>
<td>11%</td>
</tr>
<tr>
<td>Dimer circles</td>
<td>---</td>
<td>0.5%</td>
<td>0.4%</td>
</tr>
<tr>
<td>Dimer linears</td>
<td>---</td>
<td>0.5%</td>
<td>0.9%</td>
</tr>
</tbody>
</table>

Extract= SB 18 ligation products
Percentages are based on counts of 1000 molecules per reaction, on three independent samples.

7.2.3. Bacterial transformation by plC20H plasmid, post incubation with nuclear extracts.

7.2.3.1 Control rejoining reactions

Prior to experimental ligation reactions, two specific procedures were carried out. Firstly, the ability of the substrate to be religated by phage T4 ligase was examined. Secondly, the 'background' level of non-linearised plC20H molecules following restriction digests was assessed. 100ng aliquots of cohesive and blunt plC20H digests were incubated with 1μl of phage T4 ligase for 1 hour at 37°C, in a final volume of 350μl, using the ligation buffer without PEGi. Aliquots of this ligation reaction were then used to transform competent JM83 E.coli as described in section 2.2.6.2.

Control, uncut plC20H transformations yielded ampicillin-resistant, X-gal positive bacterial colonies on agar plates containing both ampicillin and X-gal.
This provided an estimate of the efficiency of bacterial transformation per unit uncut plasmid DNA, as a result of incorporation of intact, circular plasmid DNA by bacteria. This was compared with the 'background' number of such colonies resulting from a residual, undigested population of plasmid molecules in the EcoR1 and Nru I substrates.

Table 7.2. Evaluation of background transformation by "cut" plC20H substrates, and suitability for religation.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Treatment</th>
<th>λ exonuclease digestion</th>
<th>Ampicillin resistant bacterial colonies per ng DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncut plC20H</td>
<td>+ 1μl T4 ligase</td>
<td>+ 1μl λ exonuclease</td>
<td>1157 +/- 228 (4)</td>
</tr>
<tr>
<td>Uncut plC20H</td>
<td>+ 1μl T4 ligase</td>
<td>+ 1μl λ exonuclease</td>
<td>1297 +/- 323 (4)</td>
</tr>
<tr>
<td>EcoR1 digested</td>
<td>+ 1μl T4 ligase</td>
<td>+ 1μl λ exonuclease</td>
<td>2 +/- 1.46 (4)</td>
</tr>
<tr>
<td>EcoR1 digested</td>
<td>+ 1μl T4 ligase</td>
<td>+ 1μl λ exonuclease</td>
<td>115 +/- 18 (4)</td>
</tr>
<tr>
<td>EcoR1</td>
<td>+ 1μl T4 ligase</td>
<td>+ 1μl λ exonuclease</td>
<td>109 +/- 12 (4)</td>
</tr>
<tr>
<td>EcoR1</td>
<td>+ 1μl T4 ligase</td>
<td>+ 1μl λ exonuclease</td>
<td>9.6 +/- 4.2 (3)</td>
</tr>
<tr>
<td>Nru 1</td>
<td>+ 1μl T4 ligase</td>
<td>+ 1μl λ exonuclease</td>
<td>0.8 +/- 0.3 (3)</td>
</tr>
<tr>
<td>Nru 1</td>
<td>+ 1μl T4 ligase</td>
<td>+ 1μl λ exonuclease</td>
<td>61 +/- 18 (3)</td>
</tr>
<tr>
<td>Nru 1</td>
<td>+ 1μl T4 ligase</td>
<td>+ 1μl λ exonuclease</td>
<td>77 +/- 21 (3)</td>
</tr>
</tbody>
</table>

The bacterial transformation arising from the EcoR1 plC20H substrate could represent either a proportion of uncut molecules following restriction digestion, or alternatively, the rejoining activity of the JM 83 E.coli.

To test this hypothesis aliquots of uncut, EcoR1 cut, and Nru 1 cut plC20H were incubated with 1μl of lambda exonuclease with appropriate buffer, prior to
bacterial transformation. This enzyme digests linear plasmid DNA therefore removes all linear molecules, but does not cut circular plasmid DNA.

It is shown that both EcoRI and Nru I substrates have a reduced bacterial transformation frequency following λ exonuclease treatment, but there was no reduction in the transformation by uncut or T4 ligated products. It was therefore likely that the a finite level of recircularisation of the cut plasmid substrate was achieved by the bacterial host. However, it was decided that this activity would be unlikely to obscure significant rejoining activity in cell extracts. Lamda exonuclease treatment of test ligation products was not therefore routinely undertaken, to minimise plasmid DNA handling after ligation reactions.

In conclusion it was observed that:
1) Both EcoRI and Nru I digests of pIC20H were suitable substrates for ligation to circular forms capable of transforming JM83 bacteria, with expression of the Lac Z gene.

2) A background level of uncut molecules in both substrates or bacterial rejoining activity would not preclude their use for test ligation assays.

7.2.3.2. Extent of rejoining of endonuclease induced DSB by human tumour extracts.

Quantitative evidence of rejoining of endonuclease induced dsb was assessed by the ability of rejoined pIC20H to transform competent JM83 E.coli, thus producing an ampicillin resistant phenotype in the bacteria, due to incorporation and expression of the plasmid encoded ampicillin resistance gene. The extent of rejoining was expressed as the number of ampicillin resistant colonies growing on agar containing ampicillin, per ng of initial cut plasmid. The ability of plasmid DNA to transform bacterial phenotype was interpreted as evidence of ligation of cut plasmid molecules to regenerate circular forms.

Data has been pooled from ligation experiments using 3 independent tumour extracts on each of the 4 human tumours studied, to obtain values for maximal rejoining activity. Results were normalised within and between each experiment for variations in plasmid DNA recovery, bacterial viability and competence. This was achieved by correcting the number of ampicillin resistant colonies in each test plate for the number of kanamycin resistant colonies produced by the internal control pHSG 272 co-transformation.
Table 7.3 Maximal bacterial transformation per 40ng plC20H substrate

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Treatment</th>
<th>Bacterial colonies/40ng plasmid DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>plC20H</td>
<td>uncut</td>
<td>53,260 +/- 11,680 (12)a</td>
</tr>
<tr>
<td>plC20H EcoR1 cut</td>
<td></td>
<td>13 +/- 9.8 (12)</td>
</tr>
<tr>
<td>plC20H EcoR1 cut</td>
<td>+ T4 ligase</td>
<td>4720 +/- 760 (12)</td>
</tr>
<tr>
<td>plC20H EcoR1 cut</td>
<td>+ SB 18 extract (3)b</td>
<td>781 +/- 341</td>
</tr>
<tr>
<td>plC20H EcoR1 cut</td>
<td>+ IJK extract (3)</td>
<td>1760 +/- 695</td>
</tr>
<tr>
<td>plC20H EcoR1 cut</td>
<td>+ IMR32 extract (3)</td>
<td>1002 +/- 760</td>
</tr>
<tr>
<td>plC20H EcoR1 cut</td>
<td>+ A2780 extract (3)</td>
<td>804 +/- 427</td>
</tr>
<tr>
<td>plC20H Nru 1 cut</td>
<td></td>
<td>247 +/- 53</td>
</tr>
<tr>
<td>plC20H Nru 1 cut</td>
<td>+ T4 ligase</td>
<td>2659 +/- 606</td>
</tr>
<tr>
<td>plC20H Nru 1 cut</td>
<td>+ IJK extract (1)</td>
<td>217 +/- 42</td>
</tr>
<tr>
<td>plC20H Nru 1 cut</td>
<td>+ IMR 32 extract (1)</td>
<td>254 +/- 51</td>
</tr>
</tbody>
</table>

Bacterial colonies are expressed as the number of ampicillin resistant bacterial colonies per 40 ng plasmid DNA; this aliquot of the initial 100ng plasmid DNA used in the rejoining reaction was routinely used for bacterial transformations.

Values for EcoR1 cut plasmid are means of the maximal activity observed in 3 independent extract preparations on each cell line, with standard error of means.

Values for Nru 1 cut plasmid are mean and SEM for 3 independent ligations using 1 extract preparation of each cell line.

a) = number of independent ligations
b) = number of independent nuclear extract preparations
Nuclear extracts prepared from the four human tumours examined were able to catalyse the conversion of EcoR1 cut plasmid to molecules capable of transforming JM 83 to an ampicillin resistant phenotype. Significant activity over background (i.e. EcoR1 cut plasmid) was obtained in extracts from all 4 cell lines. The maximal number of ampicillin resistant colonies obtained following all nuclear extract treatments was substantially reduced when compared with the results of simple ligation by T4 ligase. Boiling nuclear extracts abolished the formation of plasmid molecules capable of bacterial transformation, with no significant increase in bacterial colonies compared with that obtained by EcoR1 cut plasmid alone.

Maximal bacterial colony numbers were corrected for variation in bacterial competence between experiments. Substantial variation in maximal transforming activity was observed between replicate extract preparations on the same cell line. Efficiency of maximal rejoining achieved by replicate extracts on each of the 4 cell lines was compared by analysis of variance. No significant difference was observed in maximal bacterial transformation by the products of EcoR1 cut plasmid ligations obtained in extracts of the 4 tumour lines.

By contrast, no significant bacterial transformation over background was detected when the ligation reaction products of Nru 1 cut plasmid (blunt termini) treated by an extract of either the radioresistant glioma, IJK, or the radiosensitive neuroblastoma, IMR32 were used to transform JM83 E.coli. The extracts used had significant activity as evidenced by the production of transformed bacteria after incubation with EcoR1 cut plasmid. The lack of bacterial transformation by the reaction products of Nru 1 cut plasmid and both IJK and IMR32 extracts was suggestive of reduced ability of these extracts to rejoin blunt termini to yield a circular plasmid form capable of bacterial transformation.

7.2.3.3. Protein concentration dependence of bacterial transformation.

The ability of nuclear protein extracts of each of the 4 human tumours to rejoin EcoR1 cut plC20H in a concentration dependent manner was examined, by varying the total extract protein added to a ligation reaction. The activity of
each extract was examined over the range of 0.1-200 µgs, ie approximately 3 logs of variation in total protein concentration. When plasmid DNA recovered from ligation reactions was used to transform competent JM 83 E.coli, a 'dose-response' was observed, as evidenced by increasing numbers of transformed, ampicillin-resistant bacteria per 40ng plasmid.

Data obtained on 3 independent extracts from each of the 4 human tumour lines is shown in figures 7-6-9.

Values represent the mean and standard deviation of the number of ampicillin resistant bacteria obtained per 40 ng plasmid substrate DNA. Values were corrected within an experiment on each extract for relative numbers of kanamycin resistant bacteria per replicate.

In all 4 human tumour extracts the number of ampicillin resistant colonies obtained increased over background with the amount of protein extract added, finally reaching a plateau. In the case of SB18 extract 2, and IMR32 extract 2, an initial increase in bacterial transformation with increasing extract protein was followed by a subsequent decrease in transformation, rather than a plateau.

Replicate extract preparations on the same cell line displayed activity over differing total protein ranges.
Figure 7.5. Maximal bacterial transformation for pIC20 H substrates

Bars=SEM
Figure 7.6  Dose dependent increase in bacterial transformation: SB 18 extracts

SB18 ext. 1

SB18 ext. 2

SB18 ext. 3

246
Figure 7.7  Dose dependent increase in bacterial transformation: IJK extracts
Figure 7.8  Dose dependent increase in bacterial transformation: IMR 32 extracts

**IMR extract 1:**
- Protein in micrograms
- Bacterial colonies/40ng plasmid DNA

**IMR extract 2:**
- Protein in micrograms
- Bacterial colonies/40ng plasmid DNA

**IMR extract 3:**
- Protein in micrograms
- Bacterial colonies/40ng plasmid DNA
Figure 7.9  Dose dependent increase in bacterial transformation: A2780 extracts

**A2780 ext. 1**

**A2780 ext. 2**

**A2780 ext. 3**
7.2.3.4. Fidelity of rejoining of EcoR1 cut plC20H substrate.

The fidelity of the plasmid rejoining process was assessed by the ability of transformed bacteria to express the Lac Z gene, as evidenced by β-galactosidase activity. This was detected by the production of blue bacterial colonies on agar plates containing X-gal. Only those EcoR1 cut plasmid molecules rejoined accurately would be capable of expressing the Lac Z gene. Hence the proportion of total ampicillin resistant bacterial colonies that were blue was a measure of the fidelity of the rejoining of the endonuclease-induced double strand break. Those endonuclease dsb rejoined with loss of information would result in in activation of the Lac Z gene, and the production of white colonies.

The fidelity of plasmid rejoining was high in tumour extracts of all 4 cell lines, with the proportion of white colonies varying between 0.75-0.94% of the total bacterial colony number. The results obtained for rejoining of the EcoR1 cut plasmid are shown in table 7.4, and figure 7.10. A Students t-test was then used to test the hypothesis that fidelity of rejoining did not differ significantly between radiosensitive (IMR 32, A2780), and radioresistant (SB18, IJK) cell lines. Differences between means for the 2 groups of cell lines did not reach statistical significance (p=0.5).
Table 7.4. EcoR1-cut plasmid misrejoining following treatment with human tumour nuclear extracts.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Extract</th>
<th>Total white colonies</th>
<th>Total blue colonies</th>
<th>% misrejoined</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td>1</td>
<td>43</td>
<td>6246</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>29</td>
<td>2367</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>27</td>
<td>7647</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>mean+SE</td>
<td></td>
<td></td>
<td>0.75 (0.24)</td>
</tr>
<tr>
<td>IMR32</td>
<td>1</td>
<td>58</td>
<td>9030</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>81</td>
<td>9323</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>93</td>
<td>10349</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>mean+SE</td>
<td></td>
<td></td>
<td>0.79 (0.08)</td>
</tr>
<tr>
<td>SB 18</td>
<td>1</td>
<td>107</td>
<td>12685</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>22</td>
<td>4682</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>48</td>
<td>3351</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>mean+SE</td>
<td></td>
<td></td>
<td>0.9 (0.27)</td>
</tr>
<tr>
<td>IJK</td>
<td>1</td>
<td>63</td>
<td>3534</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>34</td>
<td>4964</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>181</td>
<td>39905</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>mean+SE</td>
<td></td>
<td></td>
<td>0.94 (0.38)</td>
</tr>
</tbody>
</table>

Number of colonies for each independent extract represent crude numbers pooled from multiple experiments, uncorrected for inter-experimental variations in bacterial competence.
Results are depicted for 3 independent extract preparations on each of the 4 human tumour lines studied.

Total = number of ampicillin resistant bacterial colonies obtained in multiple experiments on given extract preparation

white = number of ampicillin resistant, Lac Z- bacterial colonies obtained for given extract preparation
7.2.4 Visualisation of pIC20H ligation products by Southern analysis.

The plasmid products of control and test ligation reactions were separated by gel electrophoresis, and blotted to nitrocellulose filters, prior to probing with radiolabelled pIC20H, as described in section 2.2.5.1.

7.2.4.1 Effect on tumour nuclear extracts on uncut plasmid.

Figure 7.11 shows a Southern blot containing the reaction products of incubating 100ng uncut pIC20H with extracts derived from the glioma line, SB18, and the neuroblastoma line, IMR32.

Lane M contains the 1 Kb ladder markers. Lanes 2-7 were loaded with 10ng of plasmid DNA.

Lane 1 shows the positions of migration of the 3 predominant forms contained within uncut pIC20H. The fastest form is labelled SCM, and represents the position of migration of monomeric supercoiled, closed circular plasmid. The second major form is labelled 'rc+lin'; this position marked the migration of both relaxed circular plasmid, and linear plasmid following digestion. The third band is marked 'dsc' and contains dimeric supercoiled circular forms. Further multimeric circular forms are seen as high molecular weight bands.

Lanes 2 and 3 illustrates the action of an extract derived from SB 18; extract was added to a 100ng uncut plasmid incubation at 2 protein levels, 10μg and 20μg total protein in lane 2 and 3 respectively. The major findings were that new bands appeared, labelled with small arrows, thought to represent topoisomerase-like activity in the tumour extract, causing unwinding of supercoiled monomer and dimer circles. No obvious degradation of plasmid DNA had occurred in overnight incubations with extract.
Figure 7.11. Southern blot of plC20H DNA; effect of tumour extracts on uncut plasmid

Filter has been probed with nick-translated $^{32}$P labelled plC20H plasmid DNA.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>1 Kb ladder</td>
</tr>
<tr>
<td>SCM</td>
<td>position of migration of monomeric, supercoiled, circular plC20H DNA</td>
</tr>
<tr>
<td>RC+LIN</td>
<td>position of migration of linear and relaxed circular plC20H</td>
</tr>
<tr>
<td>DSC</td>
<td>dimeric circular plC20H</td>
</tr>
<tr>
<td>4</td>
<td>positions of migration of partially unwound supercoiled plasmid</td>
</tr>
</tbody>
</table>

Lanes 1-7 were each loaded with 10ng of plC20H plasmid, following incubation of plasmid with extracts:

1 = 10ng of uncut plC20H alone
2 = " + 10µg SB18 extract protein"
3 = " + 20µg SB18"
4 = " + 10µg IMR 32"
5 = " + 20µg IMR32"
6 = " + 10µg boiled SB18"
7 = " + 10µg boiled IMR32"
Lanes 4 and 5 demonstrate the effect of 10 and 20μg protein extract from IMR32 on uncut plasmid. In this extract the topoisomerase-like action was sufficient to cause disappearance of the dimeric supercoiled form, and a significant reduction in the monomer supercoiled molecules. Again, no smearing of DNA was detected, suggesting little degradation of the plasmid molecule by extract.

Lane 6 and 7 illustrate that boiling of both SB18 and IMR32 extracts prior to incubation with uncut plasmid abolished the unwinding activity on supercoiled forms, suggesting that the activity was an active protein function.

Control incubations of uncut plasmid with extracts derived from all 4 cell lines, SB18, IJK, IMR32, and A2780 were all noted to produce similar results, namely, no evidence of plasmid degradation, and variable topoisomerase-like unwinding of supercoiled plasmid. Lack of plasmid degradation by these extracts enabled the use of relatively low plasmid concentrations in experimental ligations.

**Effect of tumour nuclear extracts on EcoR1 cut plC20H.**

Tumour extracts prepared from all 4 human cell lines studied were able to catalyse the rejoining of linear plC20H molecules, as evidenced by the appearance of bands migrating in the same position as supercoiled circular control DNA.

Figure 7.12 shows the position of migration of

1) Uncut plC20H
2) Linear, EcoR1 cut plC20H substrate
3) Positive ligation control; T4 ligase treated linear plC20H
4) EcoR1 cut plC20H, incubated with 20μg of SB18 nuclear proteins
5,6,7) Replicates of EcoR1 cut plC20H, incubated with 20μg of V79 hamster nuclear proteins
Figure 7.12 Southern blot of plC20H DNA; linear plasmid, T4 positive control, and products of human glioma SB18, and hamster V79 reaction products.

Filter has been probed with nick-translated $^{32}P$ labelled plC20H plasmid DNA.

- **W** = position of wells
- **SCM** = supercoiled monomeric circular plasmid
- **RC+LIN** = relaxed circular, and linear plasmid
- **DSC** = dimer supercoiled circular plasmid
- **HC** = high molecular weight concatamers

1. 10ng of uncut plC20H
2. 10ng of linear, EcoR1 cut plC20H substrate
3. 10ng of positive ligation control, T4 ligase treated linear plC20H
4. 10ng of EcoR1 cut plC20H, incubated with 20ug of SB18 nuclear proteins
5, 6, 7. 10ng replicates of EcoR1 cut plC20H, incubated with 20ug of V79 hamster nuclear proteins
This figure demonstrates that the EcoR1 digested substrate appeared to contain no residual supercoiled, circular molecules (lane 2), and therefore the reappearance of a plasmid DNA form migrating at this position must represent the effect of tumour extract mediated rejoining activity.

Lane 3 illustrates the effect of T4 ligase on EcoR1 cut plasmid, namely, the conversion of linear molecules to supercoiled circular forms. This finding is in keeping with electron microscopy and bacterial transformation data.

Lanes 4 by comparison with 5, 6, and 7 illustrate the quite dissimilar effects on linear plC20H, of extracts derived from human and V79 hamster lines. Lane 4 contains a 10ng aliquot of the plasmid products of an overnight incubation of 100ng of EcoR1 cut plC20H, with 20μg of SB18 (human glioma) extract. Lanes 5, 6, and 7 each contain a similar quantity of the ligation products of incubating 100ng EcoR1 digested plasmid with 20μg of V79 extract. The human tumour extract mediated the formation of a plasmid form migrating at the position of supercoiled circular DNA, suggestive of rejoining of the linear plasmid to a circular form. By contrast, the V79 hamster extract has converted the linear substrate to high molecular weight forms, with very little evidence of hybridisation at the position of supercoiled circular DNA. None of the 4 different human tumour extracts examined in ligation reactions showed any evidence of formation of high molecular weight forms.

As the relaxed circular form of plC20H migrates at a similar position to its linear form, it was not possible to monitor the formation of this type of product by Southern analysis. Running gels with ethidium bromide did not aid separation of these forms.

A dose-response relationship was sought for each tumour extract investigated by examining the effect of adding variable amounts of extract protein to a fixed amount of linear plC20H substrate (100ng). Total extract protein concentrations were varied over the range 100ng to 200μg per 350ml reaction volume, ie a range in excess of 3 logs. At the maximum protein concentrations examined, in which a plateau of bacterial transformation had been reached, some residual DNA was always observed migrating in the same position of linear/relaxed circular plasmid. It is probable that residual plasmid DNA migrating in this position represented a combination of successfully ligated, relaxed circular DNA, as well as some linear molecules which had been degraded by extract activity, and were not suitable for ligation.
Figure 7.13 illustrates the effect of incubating varying amounts of nuclear protein derived from the glioma SB18, with a fixed amount (100ng) of EcoR1 cut pUC20H linear substrate. Lanes 5 to 10 contain the plasmid products of reactions between EcoR1 cut plasmid and added nuclear protein levels varying from 100ng to 100μg. This blot demonstrates the ability of active extract proteins to catalyze the formation of a fast migrating plasmid form. This is compatible with rejoining of linear plasmid to reconstitute a supercoiled, circular molecule. The effect of boiling extracts prior to addition to the ligation reaction can be seen to produce no fast forms (lanes 2-4).
Southern blot of plC20H DNA; protein concentration dependent increase in formation of rejoined plasmid by tumour extract SB18.

M = size markers
SCM = position of migration of monomeric, supercoiled plC20H
LIN = position of migration of linear plC20H (and relaxed circular DNA)

lanes 1-10 were loaded with 10ng of plC20H, following incubation with extracts

1= EcoR1 cut plC20H alone
2= EcoR1 cut plC20H + 500ng boiled SB18 protein
3= EcoR1 cut plC20H + 1µg boiled SB18 protein
4= EcoR1 cut plC20H + 10µg boiled SB18 protein
5= EcoR1 cut plC20H + 100ng SB18 protein
6= EcoR1 cut plC20H + 200ng SB18 protein
7= EcoR1 cut plC20H + 500ng SB18 protein
8= EcoR1 cut plC20H + 1µg SB18 protein
9= EcoR1 cut plC20H + 10µg SB18 protein
10= EcoR1 cut plC20H + 100µg SB18 protein
Figure 7.14. Southern blot of pIC20H reaction products; effect of incubation with tumour extract IMR32

Filter has been probed with nick-translated, $^{32}$P labelled pIC20H plasmid DNA.

Each lane is loaded with 10ng pIC20H plasmid ligation reaction products

<table>
<thead>
<tr>
<th>Lane 1</th>
<th>Lane 2</th>
<th>Lane 3</th>
<th>Lane 4</th>
<th>Lane 5</th>
<th>Lane 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>sc= 10ng uncut pIC20H</td>
<td>Lane 2= EcoR1 cut pIC20H</td>
<td>Lane 3= EcoR1 cut pIC20H</td>
<td>Lane 4= +1ml T4 ligase (pos.control)</td>
<td>Lane 5= +500ng IMR protein extract</td>
<td>Lane 6= +10ug IMR protein extract</td>
</tr>
</tbody>
</table>
Figure 7.14. illustrates the ability of nuclear proteins from the human neuroblastoma line, IMR32, to mediate the production of fast migrating plasmid molecules from linear EcoR1 cut substrates.

Lane 1 contains the uncut plasmid alone.

Lane 2 shows positive control, i.e., EcoR1 cut DNA after incubation with 1μl T4 ligase. It shows the reappearance of the fast migrating plasmid form (SC), by comparison with the linear control in lane 1.

Lane 3 shows the single band of EcoR1 digested plC20H.

Lanes 4, 5, and 6 show the reappearance of a fast-migrating plasmid form (SC), following incubation of cut plasmid with 500ng, 1μg, and 10μg of IMR extract proteins respectively.

Plasmid Southern blots of ligation products were not directly comparable between hybridisations, due to variation in activity in the nick translated probes, and small variations in plasmid DNA loading. They were used to corroborate bacterial transformation data, namely, that the 4 human tumour extracts were capable of catalysing the production of plasmid forms that migrated in the same position as supercoiled circular plasmid DNA.

A consistent relationship was found between the ability of ligation reaction plasmid DNA to transform bacteria to ampicillin resistant, Lac Z+ phenotypes, and the appearance of supercoiled plC20H forms on Southern analysis.

A further observation made by Southern analysis was the apparent lack of activity catalysing the formation of high molecular weight concatemers in the extracts of the 4 human tumour lines examined. By contrast, this type of rejoining activity was the preferential mode of rejoining of linear plasmid molecules mediated by extracts from the hamster V79 and irs mutants.

The formation of high molecular weight ligation products was not observed to be an activity in any of 3 independent extract preparations on 4 human tumour extracts examined, namely, SB18 (glioma), IJK (glioma), IMR32 (neuroblastoma), and A2780 (ovarian carcinoma).
7.3. Discussion

7.3.1. In vitro DNA dsb rejoining activity of human tumour extracts.

These results demonstrate that cell-free extracts can be successfully prepared from human tumour cells, retaining in vitro some of the nuclear protein activities that may be involved in the processing of DNA strand breaks in vivo.

The principal objective in pursuing the in vitro studies of DNA dsb rejoining was to evaluate the possibility that human cell lines of differing intrinsic radiosensitivity might exhibit differences in their ability to rejoin DNA dsb.

These results have shown no significant quantitative differences in the cohesive dsb rejoining activity of the nuclear extracts of the 4 human cell lines examined, both in terms of maximal rejoining observed, or the fidelity of the process. A more limited study using extracts from 2 cell lines, the radioresistant glioma UK, and the radiosensitive neuroblastoma IMR 32, showed an inability to rejoin non-cohesive dsb in vitro.

7.3.2 Processing of plasmid substrates by human extracts

It is pertinent to consider the activities demonstrated by the human cell extracts examined in these studies, both in terms of the relevance of this in vitro model to cellular DNA repair processes, and to postulate methods by which the system might be developed further.

A schematic representation of the protein activities proposed for these studies is presented in figure 7.15.

Nuclear protein fractions were used in this study, in an attempt to enrich for the protein activities likely to be involved in the processing of genomic DNA damage. However, no further fractionation of extracts was undertaken. Extracts obtained could therefore contain a multiplicity of enzymes acting on DNA, including ligases, polymerases, phosphatases, nucleases, and kinases.
Figure 7.15 Proposed schema for processing of pIC20H plasmid molecules by human extracts, and relationship to bacterial transformation events.

For explanation of figure see section 7.3.2.
Thacker has shown that the comparative efficiency of rejoining in 7 different restriction-induced termini ranks very differently when mediated by human cell nuclear protein fractions, compared to purified T4 ligase activity. This is suggestive that these in vitro assays are evaluating several competing protein activities. [North, 1990]

The activities of DNA ligases are presumed in the rejoining of the plasmid dsb modelled in this system. Three forms of ligase activity have been identified in mammalian cells. They catalyse phosphodiester bond formation at single strand nicks with adjacent 3'OH and 5' PO4 termini in double stranded DNA. Theoretically, no other enzymic activity would be required to rejoin endonuclease-induced dsb. This scenario is depicted in pathway (1). Human DNA ligase activities within the extracts are proposed to be capable of rejoining the EcoRI cohesive termini, leading to recircularisation of the plasmid substrate, and accurate restitution of Lac Z gene function. The circular plasmid form is taken up by JM 83 E.coli, leading to an ampicillin-resistant phenotype, expressed as clonal growth on ampicillin-containing agar plates. The Lac Z gene product, the enzyme β-galactosidase, confers blue coloration on the colonies.

However, degradation of termini by nucleases or phosphatases present in the extracts could have rendered the plasmid substrate unsuitable for ligase activity without further modification. (2) Such modification might have included kinase-like restitution of phosphate groups, or polymerisation to produce "fill-in" of base loss using the undamaged strand as a template. (3)

It is proposed that loss of information within the coding region of the Lac Z gene leads to "misrepair" in this model. The plasmid molecule is eventually recircularised by ligation, after possibly incomplete modification of the degraded termini (4) by kinase or polymerase activities. Within the context of this model, misrepair results in the formation of a plasmid molecule capable of bacterial transformation to the ampicillin-resistant phenotype, but with a non-transcribing Lac Z gene. This produces clonal growth with no blue coloration.

The extent to which such enzymic modification of degraded termini could have been expected in these reactions is uncertain. The finding of significant amounts of residual linear substrate molecules on Southern analysis, (see fig. 7.11-14) and the observation of a "plateau" or fall in bacterial transformation with increasing protein concentrations (figs.7.6-9) is suggestive of degree of
degradation of plasmid substrate. However, a residual level of linear plasmid form is also observed following treatment of cut plasmid with purified T4 ligase. This later observation would suggest that endonuclease digestion by EcoR1 produced a proportion of substrate molecules unsuitable for religation.

It is envisaged that a proportion of the EcoR1 digested molecules are unsuitable for ligation de novo, but that a further fraction of the available substrate is degraded by enzymic activity within the extracts prior to successful ligation. (5) This molecule is incapable of bacterial transformation.

It is probable that the technique used in this study to produce nuclear protein fractions would have preserved significant DNA ligase II. This 70 kDa enzyme is tightly associated with chromatin, therefore retained in nuclear preparations. It represents 80% of the ligase activity of non-dividing cells. By comparison, DNA ligase I, the major enzymic form present in rapidly dividing cells, readily leaks from the nucleus even under isotonic preparations. There is emerging evidence that DNA ligase II might be DNA damage inducible.

### 7.3.3 Fidelity of dsb rejoining in human extracts

Several explanations can be proposed for the low level of 'misrepair' observed in this system. It is possible that the extracts did not preserve significant levels of DNA polymerases or kinases to render degraded termini suitable for ligation. Alternatively, the reaction conditions may have been biased to the observation of a finite level of ligase activity, but suboptimal for other enzyme activities. However, the use of a similar in vitro system by Thacker has proven capable of showing mis-rejoining of such linear plasmid substrates by extracts from human AT cells. Extracts from AT5BIVA mis-rejoined up to 13.8 % of EcoR1 cohesive termini, by comparison with a maximum rate of 0.5% in extracts from the human fibroblast line, MRC 5. Hence the cohesive, 5' 4-base overhang substrate generated by EcoR1 has proven capable of revealing a defect in the fidelity of AT cell extracts, and therefore was an appropriate substrate for these studies.

The major technical difference in his protocol was the use of a forty-fold increase in final plasmid DNA concentration. This was adopted to circumvent substrate degradation initially observed in SV40 transformed cell lines. It is possible that such a relative "excess" of plasmid substrate served to quench excessive nuclease or phosphatase activities in extracts, leaving a proportion of termini suitable for ligation after minor modification only.
All white bacterial colonies were judged to have arisen as the result of loss of sequence at the termini created by scission through the Lac Z gene. However, it is conceivable that inactivation of the gene could have resulted from a variable extent of degradation by extracts, with a range of deletion sizes. Thacker et al evaluated the range of deletion sizes leading to LacZ gene inactivation in the plasmid pUC18, following treatment by MRC5 and AT5 extracts. Plasmid DNA minipreps from 150 individual white colonies showed a range of deletion sizes from 20-500 base pairs. However, approximately 60% of these mis-rejoining events were the result of the loss of 50 base pairs or less. Perhaps more importantly, there appeared to be no difference in the distribution of deletion sizes when mis-rejoining was mediated by extracts of MRC5 or AT5 cells.

Such an analysis of the distribution of deletion events arising from mis-rejoining events was not undertaken in this study.

7.3.4. Effect of class of dsb termini on rejoining proficiency

The products of the incubation of non-cohesive, Nru I digested plasmid substrate with extracts from 2 cell lines, IJK and IMR32, failed to show any increase in bacterial transformation over background. This observation was made in extracts known to be active in rejoining activity of the cohesive EcoR I substrate. A similar lack of rejoining of non-cohesive termini was noted for Hinc II digests by Thacker et al.

North et al observed that the extent of overall rejoining mediated by human cell extracts was closely related to both the structure and chemistry of the breaks. A 5-fold difference was observed in rejoin efficiency for Sal I (5' TCGA overhang, 10 hydrogen bonds) compared to EcoR I (5' AATT overhang, 8 hydrogen bonds). This was associated with a 98-fold increase in misrejoining of the less efficiently rejoined EcoR I break [North et al, 1990].

Similarly, the Acc I substrate (5' CG, 2 base overhang) showed a significantly lower rejoin rate, with reduced fidelity. Thus it was suggested that those termini rejoined least efficiently were more likely to suffer loss of fidelity. However, no systematic relationship existed between the size of base overhang, and likelihood of rejoining. It is possible that specific base chemistry is as important as the extent of base overhang at the site of strand breaks.
The results obtained in this study, suggesting accurate rejoining of EcoR1 type strand breaks in 4 human cell lines, are comparable with the results obtained for MRC5 extracts by Thacker. It is also in keeping with the suggestion that EcoR1-induced termini are efficiently rejoined, when the kinetics of dsb are observed in permeabilised cell systems.

Similarly, the observed inability of these human cell extracts to rejoin non-cohesive substrates in vitro is of interest. The rejoining of non-cohesive ends is usually mediated by DNA ligase I. It is possible that the method of cell fractionation and nuclear protein extraction used in these studies preserves little of this activity, known to be readily leaked from the nucleus even under isotonic conditions.

Studies with permeabilised rodent cells have shown that restriction enzymes generating blunt termini (eg Pvu II) are effective in generating chromosomal aberrations, by comparison with enzymes introducing cohesive breaks [Bryant, 1988]. One interpretation of such observations is that blunt dsb might comprise a subset of lesions less effectively rejoined after DNA damaging events, leading to subsequent cell death.

An extension of these studies would include a more comprehensive evaluation of both a range of cohesive and non-cohesive termini, in extracts from several radioresistant and radiosensitive lines.

7.3.5 Relevance of in vitro model to cellular DNA repair processes

A number of methodological compromises were accepted in the design of this in vitro assay. A naked plasmid DNA molecule in aqueous solution can provide only a limited approximation to the complexity of genomic DNA.

A further consideration was the use of the non-physiological temperature of 14° C to study protein activities. These conditions were chosen in the knowledge of the activity of mammalian ligases, and were thought likely to minimise thermal destabilisation of ligation complexes, and excess nuclease activity.
In pilot studies examining the effect of running the reaction at 37 °C, overnight, substantial degradation of plasmid was found, with a fall in bacterial transformations. (data not presented) A possible modification of the system might include the use of physiological temperatures, in conjunction with a significant increase in the plasmid DNA concentration. This would tend to favour the formation of linear concatemers and other high molecular weight forms of ligation products. The former appear not to be transforming molecules, therefore could not contribute to an evaluation of extent or fidelity of rejoining using bacterial genetics. The formation of circular multimers could introduce multiplicity problems in transformations.

The relevance of the endonuclease-induced DNA double strand break to those induced by ionising radiation lesions has been discussed in section 7.1. It is likely that enzymes such as phosphatases and nucleases, present within the extracts, may themselves modify the 'clean' restriction-induced termini. Rejoining activity detected therefore might represent a competition between end degradation and rejoining. Evidence in support of this notion lies in the noted difference between maximal bacterial transformation mediated by pure T4 ligase activity, and all extracts examined.

Considerable variability was observed in the dsb rejoining activity of replicate extracts on individual cell lines, despite standardisation of both the preparation of extracts, and ligation reactions.

7.3.6. Further protein activities participating in dsb rejoining.

Several classes of proteins not conventionally considered to be frank DNA repair enzymes might participate in the rejoining of DNA dsb. These include proteins initiating strand exchange, or altering DNA conformation eg topoisomerases. Other classes of activity include the alignment of strand breaks prior to the formation of ligation complexes, or the protection of termini prior to rejoining [Malynn et al, 1988 ; de Vries et al, 1989 ]

By inference, deficiencies or enhanced induction of some of these activities might help determine the proficiency of DNA repair pathways.

In Southern analyses of uncut plasmid DNA incubated with human extracts the production of plasmid forms intermediate in migratory position between closed
and relaxed circular DNA was noted. It is postulated that this represents a topoisomerase-like activity within extracts, altering the degree of supercoiling of plasmid DNA.

It was noted that V79 hamster extracts, prepared under identical conditions to extracts of the 4 human cell lines studied, catalysed the formation of high molecular weight concatemers in ligation conditions specifically designed to favour only intra-molecular rejoining. This activity was not observed in the 4 human extracts examined. This could not be attributed to any systematic difference in the overall protein levels yielded by extract preparation, as measured by Biorad assay.

One interpretation of this data is that the V79 cell extracts might possess a protein activity that participates in the alignment of termini prior to rejoining of termini, analogous to the end-alignment activity detected in fertilised Xenopus eggs [Pfeiffer and Veilmetter, 1988].

It suggests that caution should also be exercised in extrapolating from rodent models of DNA repair events, to human systems.

7.3.7. Conclusions

1) Cell-free extracts prepared from 4 human tumour extracts have demonstrated the capacity to rejoin cohesive endonuclease digests of the plasmid molecule pIC20H in vitro.

2) This activity was evidenced by recircularisation of the substrate plasmid molecules on electron microscopy, the detection of a form migrating in the same position as closed circular DNA on Southern analysis, and the ability of plasmid to transform the phenotype of JM 83 E.coli.

3) Significant rejoining activity over background was detected for the cohesive substrate produced by EcoR1 digestion in extracts from all 4 cell lines.

4) By contrast, no rejoining activity in excess of background was observed for the non-cohesive substrate generated by Nru1 digestion in extracts from the 2 cell lines studied.
5) It is proposed that this dsb rejoining predominantly represents activity of human DNA ligases.

6) No difference in proficiency of rejoining of cohesive dsb was detected in extracts prepared from 2 radioresistant human tumour lines, by comparison with 2 radiosensitive human tumour lines.

7) The fidelity of rejoining of cohesive dsb was high in extracts from all 4 cell lines. The incidence of "misrepair", as evidenced by the proportion of ampicillin-resistant, Lac Z- bacterial colonies was 0.75-0.94% of total bacterial colonies. Rejoin fidelity of the EcoR1 cut plasmid substrate studied did not differ significantly between extracts derived from radiosensitive or resistant cell lines.
CHAPTER 8

Conclusions
Summary of conclusions.

1) Human malignant glioma appears to lie at an extreme of clinical radioresistance, defined relative to the radiocurability of other human tumours commonly managed by radiotherapy given with curative intent.

Empirical attempts to circumvent such resistance, based on theoretical assumptions regarding the mechanistic basis of radioresistance have met with little therapeutic gain.

2) Human glioma continuous cell lines provide useful models for the study of the in vitro behaviour of human malignant glioma. The five cell lines evaluated in this thesis exhibited phenotypic heterogeneity, whilst preserving evidence of both astrocytic lineage, and the transformed, malignant phenotype.

3) All five human glioma lines exhibited monolayer colony formation, thus permitting the use of clonogenic cell survival as an endpoint for studies of intrinsic cellular radiosensitivity. Each line also exhibited the capacity for anchorage-independent growth, readily forming multicellular spheroid aggregates, which could be used for further fundamental or applied studies.

4) The establishment of further human glioma cell lines, ideally of near-diploid status, with persistent markers of astrocytic differentiation, but unequivocal malignancy, would aid further studies on the biological behaviour of human glioma. This might be facilitated by a more rigorous evaluation of the specific growth requirements of primary cultures of human malignant glioma.

5) The intrinsic radiosensitivity of the five human glioma cell lines was measured by clonogenic cell survival after high dose-rate, acute radiation and compared with eight other human tumour cell lines; these comprised five carcinoma-derived lines, and three embryonal tumour lines. Considerable variability was found in the intrinsic cellular radiosensitivity of these thirteen cell lines, despite high passage level.
6) The intrinsic cellular radiosensitivity of these five human glioma lines lies at the extreme of values published for human tumour cell lines. Considerable overlap of cellular radiosensitivity was noted between the glioma lines, and carcinoma-derived lines. Intrinsic cellular radiosensitivity might contribute to the marked clinical radioresistance observed in high grade human glioma.

7) It has been postulated that the radiocurability of human tumours might be determined by the relative capacity of tumour clonogens to ‘recover’ from ionising radiation-induced damage. The 5 human glioma cell lines examined in this study all exhibited the capacity for cellular recovery, as operationally defined in a split-dose irradiation protocol. Recovery was assessed at three radiation dose levels, and was found to increase with dose, in accordance with the predictions of the linear-quadratic model of radiation action.

8) Cellular recovery was similarly evaluated in 3 radiosensitive human tumour cell lines, derived from a neuroblastoma, IMR32, a teratoma, SuSa, and an ovarian carcinoma, A2780. These lines also exhibited clonogenic cellular recovery.

9) The linear-quadratic model predicts that cellular recovery should increase with radiation dose, hence recovery extent cannot be adequately defined at a single radiation dose level. $pRR$, calculated as the slope of the regression of the recovery ratio obtained at different radiation dose levels, expressed in logs, against $2d^2$ where $d$ was the radiation dose given, has been suggested as a method of quantifying cellular recovery potential. Using this parameter, the 3 radiosensitive cell lines studied exhibited greater recovery potential than the 5 human glioma lines. However, a more conventional comparison of extent of recovery at an ‘iso-effective’ radiation dose level producing 90% cell kill gave higher values in the human glioma lines, than in the radiosensitive lines.

The evaluation of split dose cellular recovery data remains one of the most controversial areas of human cellular radiobiology. However, these results suggest that there is no systematic deficiency in cellular recovery from radiation damage in radiosensitive human tumour cell lines.

10) The intrinsic cellular radiosensitivity of human glioma primary cultures was evaluated in a semi-solid agar cloning system. Of 17 biopsies established,
growth adequate to evaluate clonogenic cell survival was only obtained in 9 cultures (52%). A mean SF2Gy value of 0.56 (SD 0.12) was observed in this group of tumours. Whilst this value was lower than observed in the continuous glioma lines (0.68), it remains within the range of SF2Gy values considered to define 'relative' radioresistance in human tumours.

Both the number of evaluable cultures, and the cloning efficiency might be improved by the optimisation of culture conditions for human glial cells.

11) The molecular determinants of cellular radiosensitivity remain poorly understood. DNA double strand breaks (dsb) are a class of radiation-induced lesions whose fate correlates with post-irradiation cell death in a number of systems. It has been proposed that differences in proficiency of DNA dsb rejoining might underlie variability in intrinsic cellular radiosensitivity.

Studies in this thesis included the establishment of conditions for the observation of DNA dsb rejoining by human cell extracts in an in vitro system. The ability of nuclear protein extracts derived from 4 human tumour cell lines of differing intrinsic cellular radiosensitivity was assessed, using restriction-induced dsb within plasmid DNA. Evidence of dsb rejoining was sought by monitoring changes in physical plasmid conformation (Southern blotting; electron microscopy), and bacterial phenotypic transformation by plasmid-encoded "reporter" genes.

12) Nuclear protein extracts from 2 radioresistant glioma cell lines, and 2 radiosensitive human tumour lines demonstrated the ability to rejoin cohesive dsb under defined in vitro conditions. The process was observed to be energy-dependent, and could be abolished by pretreatment of proteins by boiling or proteinase K. A protein concentration-dependent increase in cohesive dsb rejoining was observed in extracts of all 4 cell lines, with no systematic differences in proficiency of rejoining between extracts of radioresistant or radiosensitive cell lines.

13) Extracts proficient in rejoining the cohesive termini were unable to ligate blunt termini generated by Nru 1 digestion. Further investigation of this phenomena is warranted.
14) Fidelity of dsb rejoining was monitored by the expression of the ampicillin-resistant, LacZ+ phenotype in bacteria transformed by successfully rejoined plasmid molecules. "Misrepair" events were operationally defined in this system as the percentage of ampicillin-resistant bacterial colonies failing to express the Lac Z gene product. Such events were observed in 0.75-0.94% of transformations, with no statistical differences in rejoin fidelity between extracts derived from radioresistant or radiosensitive cell lines for the cohesive EcoR1 cut plasmid substrate.
References


Fletcher, G. H. (1986). Radiation therapy of cancers of the head and neck.


Natarajan, A., Obe, G., van Zeeland, A., & Pallitti, F. et al. (1980). Molecular mechanisms involved in the production of chromosomal aberrations. II Utilisation of Neurospora endonuclease for the study of aberration production by X-rays in G1 and G2 stages of the cell cycle. Mutation Res. 71, 133-.


