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E1B Attenuated Adenoviruses in Genetic Therapy for Cancer.

Ian Ganly

This thesis was submitted to the University of Glasgow for the degree of Doctor of Philosophy.

CRC Department of Medical Oncology, October,1998.

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CONTENTS

ABBREVIATIONS

A	adenine
bp	base pairs
BSA	bovine serum albumin
cdk	cyclin dependent kinase
cDNA	complementary DNA
C	cytosine
^{o}C	degrees centigrade
DAPI	4,6-diamidino-2-phenylindole
DEPC	diethylpyrocarbonate
DMEM	Dulbeccos modified Eagle medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleic acid triphosphate
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediamine tetra-acetic acid
EtBr	ethidium bromide
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde phosphate dehydrogenase
GTP	guanosine 5-triphosphate
G	guanine
Gy	Gray
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid

HRP	horseradish peroxidase
IgG	immunoglobulin G
kb	kilo base pairs
kD	kilodaltons
KBM	keratinocyte basal medium
Lac Z	Beta galactosidase
MOPS	3-(N-morpholino) propane sulphonic acid
mRNA	messenger RNA
ml	millilitre
mM	millimolar
mm	millimetres
mA	milliamps
NaCl	sodium chloride
NaOH	sodium hydroxide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBF	phosphate buffered formalin
PCR	polymerase chain reaction
RNA	ribonucleic acid
Rnase	ribonuclease
SDS	sodium dodecyl sulphate
SLM	special liquid medium
SSC	sodium chloride/sodium citrate(buffer)
TAE	Tris/acetate buffer
Taq	Thermus aquaticus DNA polymerase

TBE	Tris/borate buffer
TBS	Tris buffered saline
TE	Tris/EDTA buffer
TEMED	N,N,N,N-tetramethyl-ethylenediamine
Tris	tris(hydroxymethyl)aminomethane
T	thymine
UV	ultraviolet
uM	micromolar
ug	microgram
w/v	weight/volume
W	watts

TABLES AND FIGURES.

FIGURES.

TABLES.

ACKNOWLEDGMENTS

I would like to thank everyone at the CRC Beatson Institute and Department of Medical Oncology but especially groups O4 (previously R7) and O1 for all the assistance they have given me over the past 3 years. Special thanks are reserved for Spiros Linardopoulos, Sheelagh Frame, Francis Fee and Debbie Stuart for all their help. I thank my supervisors Allan Balmain, Bob Brown and Stan Kaye for being so inspirational in their ideas and I hope that I have learned from the experience. I also thank Onyx pharmaceuticals for supplying virus to enable this research to be carried out and David Kirn and Carla Heise for their ideas. Lastly I thank Adele for all her support over the past 3 years.

ABSTRACT.

The E1B deleted adenovirus, Onyx-015, has been shown to selectively replicate in and lyse cells with nonfunctional p53 (Bischoff et al,1996). This virus may be therapeutically useful in the treatment of a wide range of tumours since p53 abnormalities are very common in human cancer. The aims of this thesis were to develop an immunocompetent mouse model to study the role of the immune system in this form of therapy, to further examine the mechanism of selectivity of Onyx-015 for cells with non-functional p53, and lastly to determine whether or not Onyx-015 could increase the cytotoxicity to DNA damaging agents in functional and non-functional p53 cells.

We have shown that human adenoviruses will infect rodent cell lines but with variable infectivity. In tissues with high infectivity, productive virus infection only occurs in mouse epidermal cells but is 25 to 50 fold less efficient compared to the human ovarian adenocarcinoma cell line A2780Cp70. The efficiency of replication in mouse epidermal cells is dependent on the expression of the early gene E1A and this correlated with the expression of the nuclear factor φAP3 , a transcriptional repressor of the E1A promotor. Replication is shown to be 20 fold greater in squamous (well differentiated) epidermal cell lines in which there is a high expression of E1A and low φAP3 expression compared to clonally related spindle (poorly differentiated) epidermal cell lines. Using mouse epidermal cell lines of known p53 status and function, the selective replication of Onyx-015 for cells with non-functional p53 is in general agreement with that reported in human cell lines . However, some cell lines with wild type p53 function do allow replication of Onyx-015 and we postulate that this is determined by 2 factors, the expression of E1A and the ability for Onyx-015 to repress p21 levels. Using the squamous epidermal cell line PDVc57 , in-vivo studies in both nude mouse and syngeneic mouse tumour xenograft models showed decreased tumour growth with intratumoural virus injection compared to diluent injected tumours. However, viral replication is markedly reduced in

the syngeneic host suggesting limitation of viral replication by the immune system.

Using the paired cell lines A2780 (functional p53) and the cisplatin resistant variant A2780Cp70 (non-functional p53) we have shown that replication is dependent on S-phase entry of the host infected cell and this correlated with E2F induction. In the cell line A2780, S phase entry and viral replication is limited due to virus induced p53 mediated apoptosis. We suggest that this is mediated by E1A induction of $p19^{ARF}$. In contrast, A2780Cp70 allows a productive virus infection since apoptosis does not occur due to the absence of functional p53 and high expression of the anti-apoptotic factor E1B19kDa.

We have shown that in the cell line with nonfunctional p53 (A2780Cp70) , cytotoxicity to both cisplatin and radiation is increased by preinfecting cells with Onyx-015. In the cell line with functional p53 (A2780), preinfection with Onyx-015 for 72 hours also resulted in increased cytotoxicity to both cisplain and radiation. In contrast, preinfection of A2780 for 24 hours followed by cisplatin resulted in an antagonistic interaction leading to reduced drug sensitivity. It is suggested that this was due to p21 induction causing a reduction in the S-phase cell population.

Further in vivo and clinical studies investigating the role of the immune system in this form of therapy and in the use of combination therapies are therefore warranted.

CHAPTER 1
INTRODUCTION

1.1. GENERAL INTRODUCTION.

p53 is the most important tumour suppressor gene and plays a central role in the negative regulation of the cell cycle. Mutations in the p53 gene are present in over 50% of all cancers (Hollstein et al, 1991; Hollstein et al, 1996) and represent the single most important genetic alteration in human cancer. The incidence in individual cancers is 50% for colon, 40% for breast, 50% for lung, 50-70% for head and neck. Mutation leads to loss of function of p53 and this is one mechanism for resistance to chemotherapy and radiotherapy (Bergh et al,1995; Eliopoulos et al,1995). Therefore new effective therapies for tumours which lack functional p53 are needed. Gene replacement therapy, in which the p53 gene is reintroduced into tumour cells is one possible approach. The first clinical trial involving the reintroduction of p53 into tumours using a retroviral vector was carried out recently in patients with unresectable lung cancer (Roth et al,1996). This study showed that the therapy was safe but also produced regression of tumours in some patients. Another approach is to use therapies which selectively target cells which lack functional p53. The selectively replicating adenovirus, Onyx-015, has recently been shown to selectively replicate and lyse cells with non-functional p53 (Bischoff et al,1996). This virus is a chimeric human group C adenovirus formed from Ad2 and Ad5 (Barker et al,1987). It has an 800bp deletion in the E1B region which codes for the 55kDa protein of E1B. The normal function of this protein is to bind to and inactivate the p53 protein in infected cells (Kao et al,1990). Because Onyx-015 lacks this protein it is unable to replicate in cells with functional p53 since it cannot inactivate p53. However it will replicate effectively in cells with non-functional p53 (**Figure 1**). Since over 50% of human tumours have p53

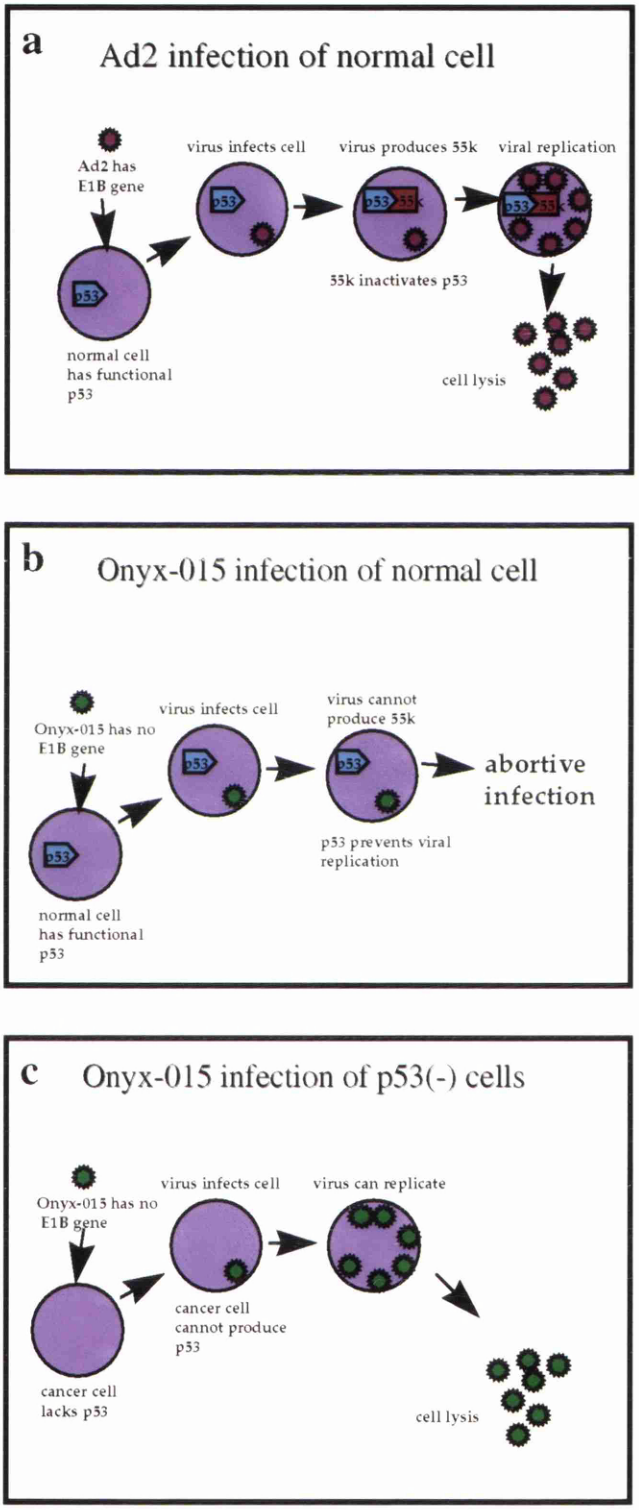


Figure 1. Schematic diagram showing the mechanism of action of the E1B55kDa deleted adenovirus.Onyx-015.

mutations, this virus may have an important therapeutic role in the treatment of a broad range of different cancers. Its main advantage lies in its ability to selectively destroy p53(-) tumours but leave surrounding normal tissue alone. Such targeted therapy at the molecular level cannot be achieved with conventional chemotherapy or radiotherapy. As such, phase I trials have begun in patients with pancreatic cancer, ovarian cancer and recurrent head and neck cancer. Results from the trial in patients with recurrent head and neck cancer are encouraging (Ganly et al,1997). In this study , 32 patients were treated with no significant toxicity and evidence of tumour necrosis occurred in some patients. However , despite these early results, several criticisms of this therapy have become evident.

Firstly, it was clear from the phase I trial that all patients treated developed a rising neutralising antibody to adenovirus after the first injection. The cell mediated response(Cytotoxic T lymphocytes) was not studied. We would expect that both the CTL and antibody responses to be detrimental to this therapy since the CTL response would cause the early elimination of virus limiting virus spread, and the antibody response would reduce the ability to reinfect host cells with adenovirus after the first inoculation. At present, there is no immunocompetent mouse model to test replicating adenoviruses since it is generally accepted that mouse tissue is not permissible to human adenoviruses. One of the aims of this thesis was to develop an immunocompetent mouse model by identifying a mouse tissue type which would support adenovirus replication . This would thus enable us to study the role of the immune system in this form of therapy and therefore potentially improve this type of therapy.

Secondly, several publications have disputed the p53 selectivity of the Onyx-015 virus. Goodrum and Ornelles,1997 showed that replication only occurred in cells which were in the S phase of the cell cycle and that this was independent of the p53 status of the cell lines studied. Hall et al,1998 recently suggested that adenovirus induced cell death was greater in cell lines which had wild type p53. Therefore another aim of this thesis was to further examine the mechanism of selectivity of Onyx-015 for p53(-) cells and to explain mechanistically any discrepancies to this selectivity.

Thirdly, most cancer therapies involve the use of multiple agents with different mechanisms of action in order to overcome any cellular resistance that tumour cells develop to any one particular cytotoxic agent. It is therefore unlikely that viral therapy with Onyx-015 would be efficacious as a single agent . We therefore wanted to determine whether or not combination therapy of Onyx-015 with chemotherapeutic agents or with radiation would be more effective than either agent alone and to explain any mechanistic interaction between these agents.

It is first necessary to describe the principles of cell cycle control and carcinogenesis, the role of p53 in human cancer , and the use of new forms of therapy such as gene therapy and adenovirus therapy in cancer therapy.

1.2. THE CELL CYCLE, ONCOGENES AND TUMOUR SUPPRESSOR GENES.

The cell cycle consists of 4 phases - S phase during which DNA synthesis occurs, M phase during which mitosis occurs, and temporal gaps in

between these two events termed G1 and G2 during which time the molecules required for DNA synthesis and mitosis are synthesised.

Between each of these phases there are checkpoints which are controlled by genes which switch on the cell cycle (e.g. Ras oncogene, myc oncogene) and genes which switch off the cell cycle (e.g. p53, p16 tumour suppressor genes). These genes act at the checkpoint through a family of protein kinases, called cyclin dependent kinases (cdks) (Pines et al, 1993). All the cdks require associated cyclin proteins for activity. The G1/S checkpoint is controlled by complexes between cdk4 and Cyclin D and cdk6 and Cyclin D (Sherr, 1993). Cyclin E in association with cdk2 is required for G1/S transition (Knoblich and Lehner, 1993) and cyclin A, in complex with cdk2 is essential for progression through S phase (Reviewed in Fisher, R. P. 1997). Both A and B type cyclins associate with cdc2 to promote entry into mitosis at the G2/M checkpoint (Knoblish et al, 1993).

At the G1/S checkpoint, the phosphorylation status of the Rb gene determines S phase entry (Weinberg,1995 for review). In its active form, Rb exists in a hypophosphorylated form complexed to the transcription factor E2F. When it is phosphorylated into the hyperphosphorylated form by either cdk4/cyclinD or cdk6/cyclin D, it becomes inactive and releases the E2F factor. E2F then activates genes essential for DNA replication such as dihydrofolate reductase (DHFR) (Slansky et al,1993), DNA polymerase α, thymidine kinase, thymidylate synthase (Johnson et al,1994) by binding to E2F sites in the promoters of these genes. This therefore allows S phase entry to occur(Hinds and Weinberg, 1994). (**Figure 2**)

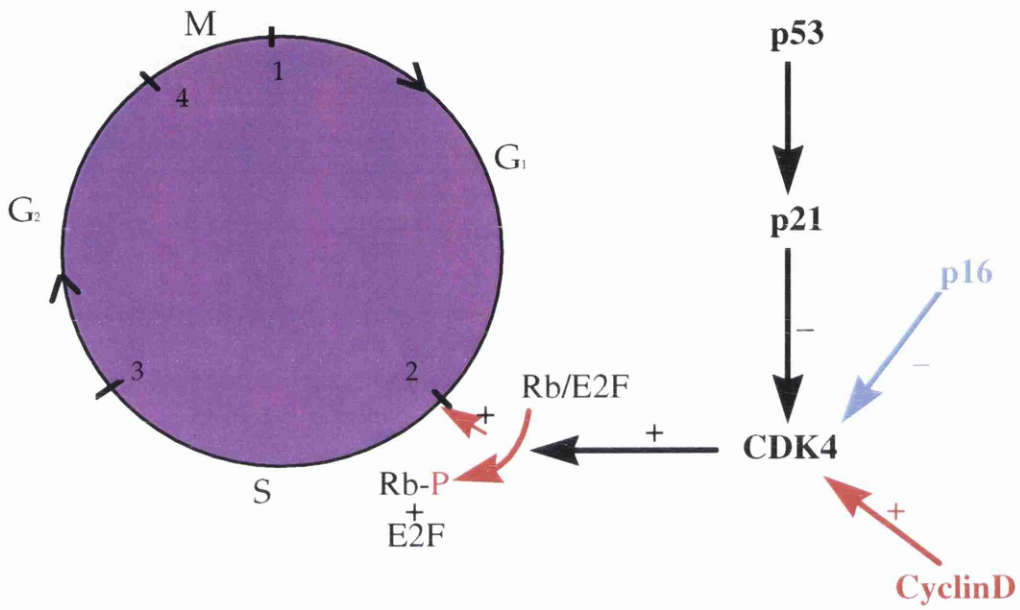


Figure 2. Diagram showing control of Rb phosphorylation and S phase entry.

S phase entry requires phosphorlation of Rb which causes release of E2F. Phosphorylation of Rb is by cyclin dependent kinases CDK4 and CDK6. These kinases are activated by Cyclins such as CyclinD and are inactivated by inhibitors p16 and p21(cdk inhibitors).

Cdk4 and cdk6 can be inactivated by cdk inhibitors. The 2 most important cdk inhibitors are p16 and p21. Thus, loss of either p16 or p21 causes activation of cdk4 and cdk6 and therefore phosphorylation of Rb causing S phase entry. p16 is a tumour suppressor gene and is frequently inactivated in tumours, particularly squamous cell cancer of the head and neck (Reed et al,1996). In addition, the main factor controlling p21 expression is p53. Mutation of p53 leads to loss of transcriptional activity causing loss of transcriptional activation of p21. This leads to activation of the cdk4/6 and S phase entry.

1.3. P53 AND HUMAN CANCER.

1.3.1.Molecular Structure of p53.

a) Domains of p53.

The p53 gene is located on chromosome 17p.21 and codes for a 393 amino acid nuclear phosphoprotein. p53 was originally discovered as a protein complexed to SV40 T antigen in SV40 transformed cells (Lane and Crawford, 1979). Its structure can be divided into 3 domains - the transcriptional activation domain at the N terminus, the sequence specific DNA binding domain in the centre, and the oligomerisation domain at the C terminus. There are 5 conserved regions (conserved through evolution) , 4 of which are located in the DNA binding domain.**(Figure 3)**

p53 is present predominantly as tetramers through the oligomerisation domain (Cho et al,1994; Jeffrey et al,1995). Dimers and monomers account for less than 5% of the total p53 molecules in a cell. The sequence specific DNA binding domain (Pavletich et al, 1993; Wang et al,1993) is very important as

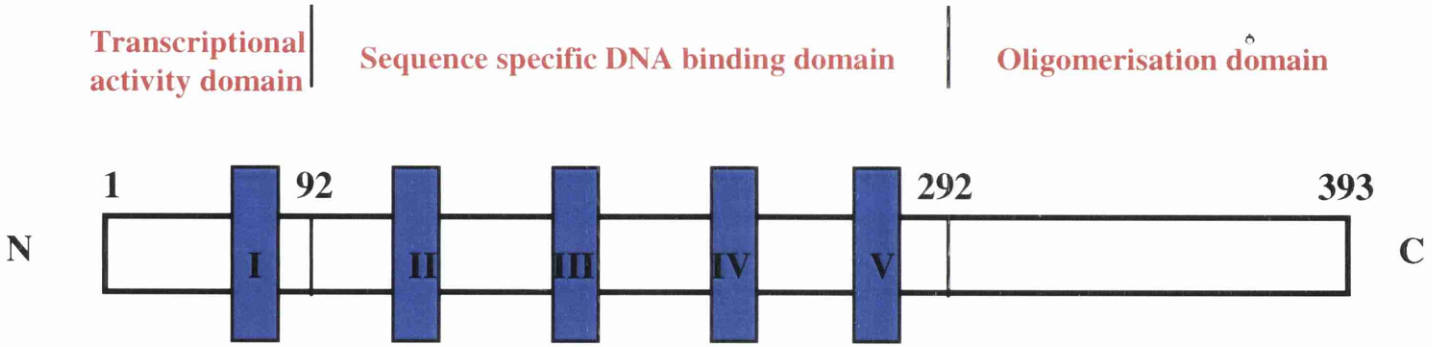


Figure 3.
Functional domains of the tumour suppressor p53. The 5 conserved domains are indicated in blue(I-V). 4 of the conserved regions are located at the DNA binding domain. p53 mutations occur mostly in these conserved domains

p53 regulates transcription through this region. p53 binds specifically to a p53 responsive element that contains repeats of the consensus binding sequence Pu-Pu-Pu-C-A/T-T/A-G-Py-Py-Py where Pu and Py are purine and pyrimidine nucleotides respectively (El-Deiry et al,1992).The N terminus of p53 is the transcriptional activation domain. This region can transcriptionally activate or repress other genes. p53 acts as a transcriptional activator of *mdm2, bax, IGF-BP3, p21,GADD45, Cyclin G, Thrombospondin* (Dameron et al, 1994) and acts as a transcriptional repressor for the genes *PCNA* (Proliferating Cell Nuclear Antigen), *c-fos ,c-jun, IL-6 and bcl2* (Horikoshi et al, 1995).

b) Phosphorylation of p53.

p53 protein is extensively post-translationally modified, mostly by phosphorylation and this is important in regulation of p53 function(Meek,1994). p53 is phosphorylated at sites within its N terminal and C terminal regions by several protein kinases. One such kinase is the double stranded DNA dependent protein kinase , DNA-PK(Lees-Miller et al, 1990). It is a nuclear serine/threonine protein kinase. Activation of DNA-PK requires DNA double strand breaks or other discontinuities in DNA (Gottlieo et al,1993). DNA-PK phosphorylates human p53 at serines 15 and 37(Lees-Miller,1992). The DNA-PK phosphorylation sites are localised within the transcriptional activation domain of p53. These sites also interact with transcription factors such as the TAFs and cellular coactivators p300/CBP (Lill et al,1997). These phosphorylation sites are also located next to the region where p53 binds to mdm2 (Kussie et al,1996). Recently it was shown that DNA damage induces phosphorylation of p53 at serine 15 via DNA-PK and

that this results in the disruption of the p53/mdm2 interaction (Shieh et al,1997) thus alleviating mdm2 inhibition of p53. Phosphorylation of serine 15 of p53 in response to DNA damage from ionising radiation has also recently been shown to occur by the ATM (Ataxia telangectasia) protein (Banin et al,1998; Canman et al,1998).

Relatively little is known about the enzymes which catalyse the dephosphorylation of p53 *in vivo.* However protein phosphatase 2A has been shown to dephosphorylate several phosphorylated sites on p53 *in vitro* (Scheidmann, et al 1991).

1.3.2. Activation of p53.

p53 is activated by DNA damaging agents such as ionising radiation and cytotoxics, hypoxia and UV light (Kastan et al,1991). DNA damage from ionising radiation causes p53 protein levels to rise in cells with an increase in transcriptional activity (Kastan et al,1992; Kuerbitz et al, 1992). Ionising radiation causes DNA damage by causing strand breaks in the DNA and these strand breaks signal p53 activation (Hartwell et al, 1989). It has been suggested that as little as one double strand break per cell can initiate the p53 pathway (Huang et al 1996). It has recently been shown that activation of p53 in response to DNA damage involves phosphorylation of p53 at serine 15 causing a conformational change in p53. This phosphorylation involves kinases including DNAPK (Shieh et al,1997) and ATM (Banin et al,1998; Canman et al,1998) as described above.

Exposure of cells to hypoxic conditions results in increased p53 protein levels and can lead to p53 dependent apoptosis (Graeber et al, 1996). It is

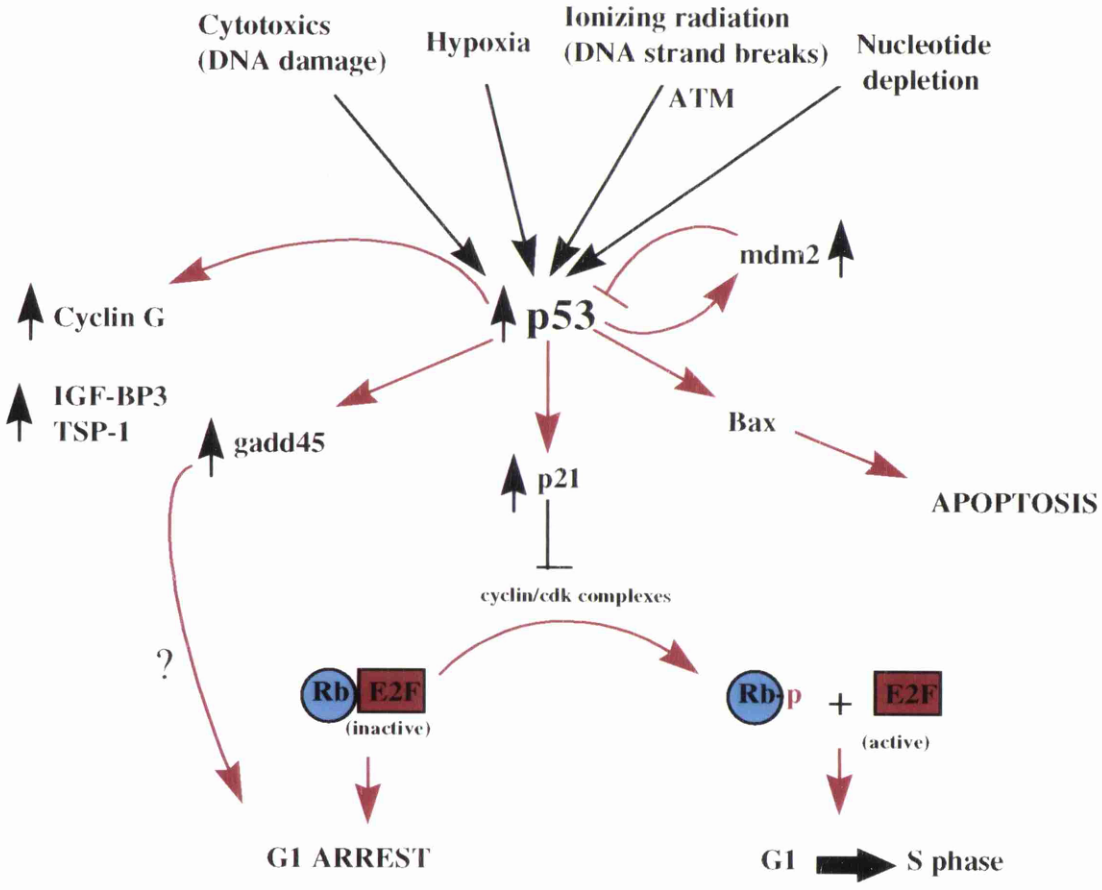


Figure 4.
Schematic diagram of p53 activation and p53 dependent G1 arrest and apoptosis.

widely believed that this induction of p53 occurs in the absence of DNA strand breaks. It has been shown that depletion of ribonucleotide triphosphates (rNTP) using inhibitors of purine and pyrimidine biosynthesis, is sufficient to induce p53 and p53 dependent G1 arrest (Linke, et al 1996). It may be the case that an inadequate supply of rNTPs results in changes in the RNA macromolecule population, analogous to the way that dNTP inhibitors lead to DNA strand beaks.

1.3.3. Normal function of p53.

p53 is the most important tumour suppressor gene and plays a central role in the negative regulation of the cell cycle (Lane,1992; Levine,1997 for review). In response to DNA damage or foreign DNA synthesis, wild type p53 is stimulated causing either a G1 arrest or apoptosis of the cells. **Figure 4** shows a schematic diagram of p53 activation and p53 dependent G1 arrest and apoptosis.

a) G1 arrest.

Ionising radiation induced DNA damage can result in a G1 arrest which is mediated mainly by the p21 protein (El-Diery et al, 1993; Harper el al 1993). p21 mRNA levels, as well as protein levels, increase following exposure of cells to ionising radiation in a p53 dependent manner (Xiong et al, 1993). p21 causes a G1 arrest by acting as a cyclin dependent kinase inhibitor preventing the phosphorylation of the Retinoblastoma gene product (pRb). In addition, p21 causes G1 arrest by inhibiting PCNA(proliferating cell nuclear antigen) ,a 36kDa protein capable of interacting with DNA polymerase α and which normally stimulates DNA replication and repair (Luo et al, 1995). The binding

of p21 to PCNA does not affect the DNA repair activity of PCNA but is thought to alter the availability of PCNA for DNA replication. p21 is the most important mediator of p53 dependent cell cycle arrest. However other genes transcriptionally activated by p53 may play a role in DNA damage arrest. These include Cyclin G (Okamoto et al, 1996) and GADD45 (Kastan et al, 1992). Like p21, GADD45 also contains a p53 binding site and has been shown to be induced by p53 (Lu et al, 1993).

b) Apoptosis.

Apoptosis, or programmed cell death, is the process by which a cell actively commits suicide under a tightly controlled cascade of events. Apoptosis is recognisable microscopically by characteristic condensed basophilic cytoplasm, darkly staining nuclei, and the presence of apoptotic cell bodies. DNA from cells undergoing apoptosis is cleaved into fragments of roughly 200 base pairs. The signals that trigger apoptosis include the presence of DNA damage, growth factor or nutrient withdrawal, disruption of cell matrix interactions, altered expression of potent cellular oncogenes such as *myc*, and viral infection.

Apoptosis is controlled by the Bcl2 group of proteins which include the apoptotic factors bax (Oltavi et al 1993) , bak and $bclx_s$ (Boise et al, 1993) and the antiapoptotic (survival) factors Bcl2 , bad and $bclx_L$. Bcl2 protein is an intracellular membrane protein that resides in the outer mitochondrial membrane, nuclear envelope and parts of the endoplasmic reticulum. The protein has been shown to block cell death induced by numerous stimuli including growth factor deprivation, Ca^{2+} ionophores, reactive oxygen species, some viruses, heat shock, and irradiation (Reviewed in White 1997).

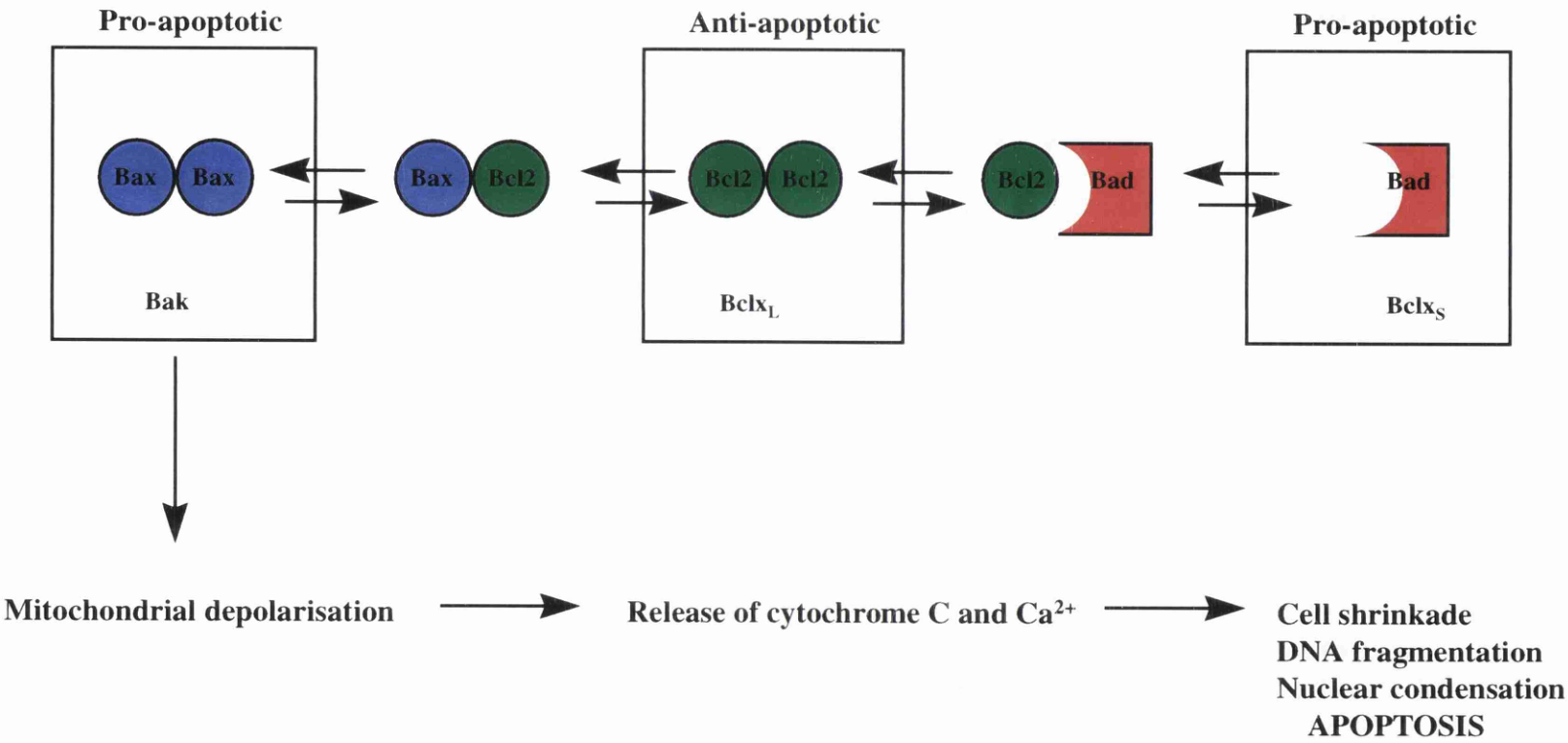


Figure5. Control of apoptosis by the Bcl2 group of proteins.

p53 can transactivate *bax* (Selvakumaran et al, 1994) which contrasts with its ability to repress transcription of *Bcl2.* (Miyashita et al, 1994) Thus in response to DNA damage, *p53* is activated causing activation of *bax* and repression of *Bcl2*. It has been proposed that when Bcl2 is in excess, Bcl2 homodimers predominate and cells are protected and when bax is in excess, bax homodimers predominate and cells are susceptible to apoptosis (Oltavi et al, 1993).**(Figure 5)**

Although bax is the main mediator of apoptosis, the insulin-like growth factor binding protein 3 gene (*IGF-BP3*) may also be important in apoptosis (Buckbinder et al, 1995). IGF-BP3 protein has the ability to inhibit the signalling of insulin- like growth factor receptors and so plays an antimitogenic role in the cell. It can cause both G1 arrest and also apoptosis.

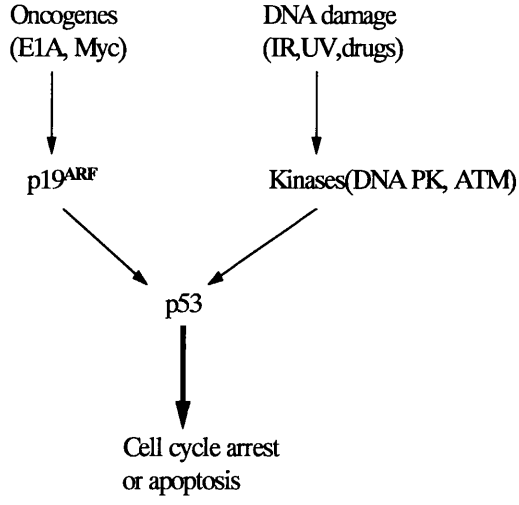
1.3.4. Regulation and stabilisation of p53.

p53 levels are controlled by the protein mdm2 (Barak et al, 1993). This protein has the ability to complex with p53 and inhibit its transcriptional activity. *Mdm2* is transcriptionally activated by wild type p53 in response to DNA damage. Thus an auto regulatory feedback loop exists between mdm2 and p53. Overexpression of mdm2 inhibits p53 dependent G1 arrest in response to irradiation (Barak et al, 1993; Perry et al, 1993). It has been shown that mdm2 also promotes the degradation of p53. This is thought to be an important mechanism in ensuring that the p53 signal is quickly terminated (Haupt, et al 1997).

All mutant p53 proteins are degraded in the presence of mdm2. It has been suggested that mutant p53 proteins have a longer half-life than wild type

because they are not degraded (Midgley et al,1997). This is because mutant p53 is non-functional and is therefore not capable of transcriptionally activating mdm2 leading to less mdm2 mediated degradation.

In response to DNA damage, p53 levels increase leading to increased transcription of mdm2. The auto regulatory feedback loop between mdm2 and p53 would then lead to the early degradation of p53 and loss of p53 transcriptional activity. This is undesirable in a situation of DNA damage where prolongation of p53 transcriptional activity is required. Shieh et al,1997 showed recently that after DNA damage, N terminal phosphorylation of p53 occurs by DNA-PK at serine 15 and this disrupts the p53/mdm2 interaction. Thus p53 is stabilised and transcriptional activity is prolonged. Another factor, $p19^{ARF}$-INK4a, has been reported to promote mdm2 degradation and therefore stabilise p53 (Zhang et al,1998; Pomerantz et al,1998). This mechanism of regulating p53 activity is distinct from that involving DNA damage since no phosphorylation of p53 occurs. It has been shown that oncogenes such as the adenoviral *E1A* and *Myc* regulate p53 dependent apoptosis by the induction of $p19^{ARF}$ (Zindy et al,1998; de Stanchina et al,1998). A schematic diagram showing the regulation of p53 is shown below.



1.3.5. Mutation of p53.

The most common somatic mutations in *p53* are single base substitutions (point mutations) in the conserved regions of the gene i.e. exons 5-8 (Hollstein et al, 1991) (**Figure 3**). Most point mutations result in missense mutations which cause a change in a single amino acid. However a few result in frameshifts and premature termination of translation (Stop codons). These mutations are termed nonsense mutations. As well as single base substitutions, complete or partial deletions can also occur but these are not common. As a consequence of mutation, mutated p53 cannot bind or regulate transcription through the specific DNA binding sequence. Therefore cells which sustain DNA damage are unable to respond by p53 mediated G1 arrest or apoptosis. Damaged DNA is retained leading to cellular transformation, genomic instability (Smith et al,1995) and the malignant phenotype. Germline mutations in p53 can also occur. Li-Fraumeni syndrome (LFS) is a rare autosomal dominant condition (Malkin et al,1990) in which patients are born with 1 mutated p53 allele and 1 wild type p53 allele. Mutation of the normal allele by somatic mutation results in p53 inactivation.These patients develop sarcomas in childhood (bone,soft tissues) and more rarely brain,lung, adrenocortical and bone marrow tumours.

1.3.6. Inactivation of p53.

As well as mutation and deletions, p53 may be inactivated by binding to cellular or viral proteins. The cellular factor mdm2 inhibits the transcriptional activity of p53 as previously described. Amplification or overexpression of mdm2 can therefore cause p53 inactivation mimicking p53

mutation . The mdm2 gene is frequently amplified in sarcomas (Oliner et al, 1992). Viral oncoproteins can also inactivate p53. Adenoviral E1B (55kDa) (Kao et al, 1990; Debbas et al, 1993; Sarnow et al, 1982) , HPV E6 (Lechner et al, 1992; Scheffner et al, 1990), and SV40 large T antigen (Gannon et al, 1987) all bind to p53 causing inactivation of the p53 protein. The same viruses also inactivate the other tumour suppressor gene Rb (retinoblastoma) by other viral proteins such as adenoviral E1A (Levine et al,1990), HPV E7 (Dyson et al,1989) and SV40 large T (Levine et al,1990).

1.3.7. Clinical importance of p53 inactivation.

p53 dependent apoptosis is one of the main mechanisms of cell death of many cytotoxic agents. Thus loss of p53 function results in reduced sensitivity to chemotherapy (Lowe et al,1993; Lowe et al,1994; Eliopoulos et al,1995) and radiotherapy (Lowe and Schmitt,1993). p53 inactivation is also correlated with poor prognosis and disease progression (Lowe et al,1994). Therefore p53 inactivation is very important clinically . Because of this, new agents which either restore p53 function or which selectively target cells which lack p53 are being developed. Such genetic based therapy represents an area which is rapidly expanding in cancer therapy. Indeed restoration of p53 function has recently been carried out in p53(-) lung cancer patients by bronchoscopic injection of a retroviral vector with the wild type p53 transgene (Roth et al,1996). This resulted in apoptosis in tumours causing regression of tumours in some patients.

1.4. GENE THERAPY.

1.4.1. Principles of gene therapy (Tolstoshev and Anderson ,1995 for review).

Gene therapy involves the introduction of foreign DNA into somatic cells to produce a therapeutic effect (Fujiwara and Roth,1994 for review). The therapeutic gene is transferred into the tumour cells using a vector. Transfer may either be *in-vivo* in which the DNA and vector are directly introduced into the body, or *ex-vivo* in which cells are removed from the body, transfected with DNA and then reintroduced into the patient. **Figure 6.**

1.4.2. Gene delivery systems.

The mode of gene transfer can be classified into chemical, physical and viral. In chemical transfection, DNA can be introduced into cells using calcium phosphate, liposomes or DNA/protein complexes. Although useful for laboratory transfections, these are inefficient in vivo due to their poor efficiency of transfection. In addition , liposomes and protein/DNA complexes are rapidly cleared from the body via the immune system. Physical methods of DNA transfection are by electroporation, microinjection and the use of ballistic particles. Electroporation guns for the introduction of chemotherapeutics, as well as genes, are currently being tested in various cancers. In addition, guns which fire small gold particles coated with DNA have recently been developed and are being tested. However, the most important method of transfer of DNA currently being used is viral vectors.

Viruses used include retroviruses, adenoviruses, adeno-associated viruses and herpes viruses. Retroviruses contain RNA genomes that are reverse

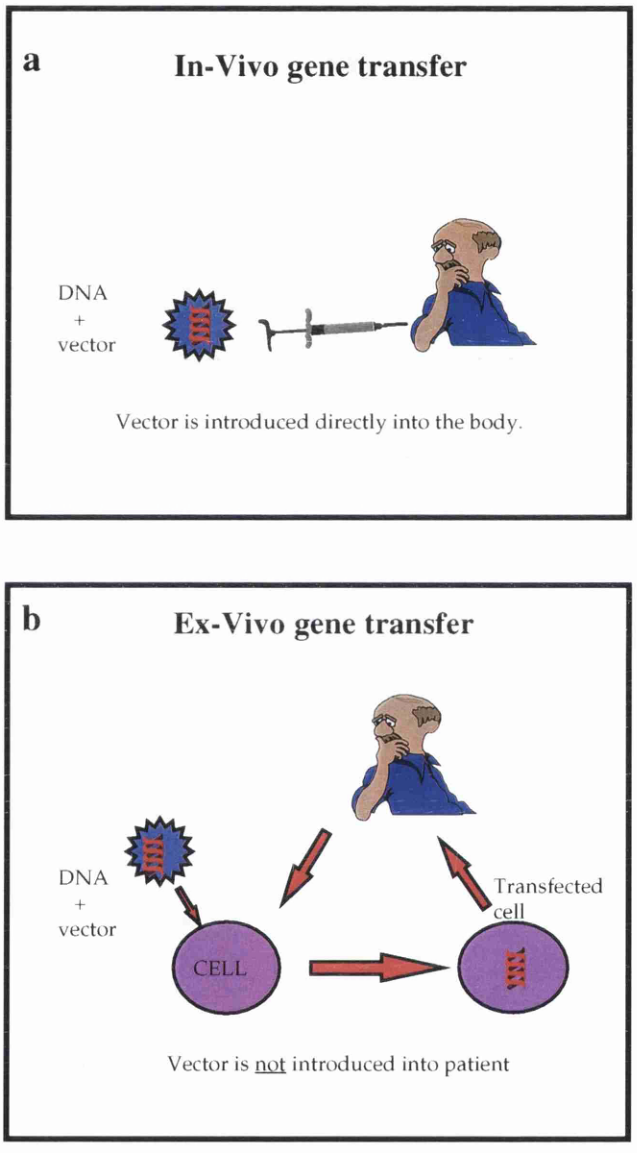


Figure 6.
Schematic diagram illustrating in vivo and ex vivo gene transfer.

transcribed after introduction to produce a double stranded cDNA. This integrates stably and heritably into random sites of the host genome. These viruses give a high efficiency of transfer. The host range of cells which they infect includes fibroblasts, epithelial cells and smooth muscle cells. Cells of lymphoid origin are more resistant. They give permanent gene expression which is advantageous in situations where long term expression of the transgene is required. This is the case in inherited conditions such as cystic fibrosis. They are currently limited due to the low production titre that can be produced (~10^{7-8} pfu) which reduces transduction efficiency into solid tumours. Other disadvantages are that they only infect dividing cells and that the stable random integration into the host genome may be dangerous if germ cell transfection occurs. They are also limited in the size of transgene which can be inserted (~7-8kb). Therefore, the use of retroviruses has recently been superceded by the use of adenoviral vectors (Wilson et al,1996; Ginsberg,1996).

Adenoviruses are double stranded DNA viruses. They can be made replication defective with deletion of the E1 and E3 regions of their genome. The transgene can then be introduced into the vacated site. Because they have a larger genome than retroviruses they can carry larger transgenes up to 36 kb DNA. They have a tropism for a broader range of cell types and because they can be produced at high titres (~10^{12}pfu) they have a higher transduction efficiency. Another advantage is that they can infect both nondividing as well as dividing cells. There is no integration into the host cell genome and therefore only transient gene expression occurs, usually lasting for 7-42 days. This is adequate for cancer therapy but would not be desired in situations where long

term gene expression is required. Other disadvantages are that they continue to express other viral gene products which are recognised by the immune system leading to an inflammatory response and short term gene expression, they may become replication competent by genetic recombination, and because 90% of humans have antibodies to human adenoviruses pre-existing immunity may reduce the transduction efficiency to very low levels particularly on second exposure to the adenoviral vector.

1.4.3. Classification of cancer gene therapy.

There are 4 main types of genes which can be introduced into tumour cells.These are

1. genes which suppress the expression of an oncogene i.e. anti-oncogenes.
2. genes which restore a defective tumour suppressor gene (replacement gene therapy).
3. genes which enhance immune surveillance (immunotherapy).
4. genes which activate prodrugs into active chemotherapeutic agents (gene directed enzyme prodrug therapy i.e. GDEPT).

1.4.4. Tumour suppressor gene replacement therapy.

There is currently one approved trial for tumour suppressor gene therapy (Clayman et al,1998). This utilises a replication deficient adenovirus for the transmission and overexpression of a wild type p53 gene. The hypothesis is that the wild type p53 gene would be dominant over its mutant gene and select against proliferation and induce a p53 mediated apoptosis (Neilson et al,1998). *In-vitro* and *in-vivo* studies using such a technique have

demonstrated growth suppression in a variety of cell types (Liu et al ,1995; Clayman et al, 1995; Ko et al,1996). Phase I/II trials are underway at the MD Anderson Cancer Center, Texas,USA in patients with advanced cancer of the head and neck and in patients with unresectable lung cancer.

1.4.5. Immunotherapy.

In immunotherapy, the aim is to either increase the immunogenicity of the tumour (Townsend et al, 1993) or increase the effectiveness of tumour infiltrating lymphocytes (Rosenberg et al, 1991). The immunogenicity of the tumour can be increased by transfecting into the tumour cells MHC class I and II genes to increase antigen presentation (Plautz et al,1993). Nabel et al,1993 reported the reduction of tumour size in a melanoma patient after gene transfer of MHC class I gene. The presence of antigen and MHC alone are insufficient for immune induction , and a class of molecules called costimulatory molecules are also required. These are expressed on antigen presenting cells and the best studied are called B7-1 and B7-2. If B7 is transfected into tumour cells then this should provide the necessary costimulation required for immune stimulation. Chen et al ,1994 showed that B7 transduction by a retroviral vector decreased the tumourgenicity and induced protective immunity .

The effectiveness of tumour infiltrating lymphocytes can be improved by cytokines interleukins 2 and 4 (Lin et al,1993), tumour necrosis factor , interferon alpha and gamma, and granulocyte-macrophage colony stimulating factor. Again clinical trials are underway.

1.4.6. GDEPT.

Adenovirus mediated gene transfer of the herpes simplex virus thymidine kinase gene has shown a reduced growth of squamous cell cancer in a nude mouse model (O'Malley BW Jr et al, 1995; O'Malley BW Jr et al, 1993) and also in lung (Esandi et al,1997), brain (Quillren et al,1997), prostate tumours. Herpes simplex virus thymidine kinase renders cells sensitive to the nucleoside analogue gancyclovir by converting gancyclovir into an active phosphorylated compound that terminates DNA synthesis. Thus tumour cells transduced with the gene will be killed when exposed to gancyclovir. The antitumour effect is dependent upon a biochemical bystander effect which is mediated by gap junctions between cells (Elshami et al,1996). The toxic metabolite is able to pass from a transduced cell into a nontransduced cell via gap junctions. Therefore only 10-20% of tumour cell transduction is required for efficacy. Other prodrug activating systems include the enzyme cytosine deaminase which converts the prodrug 5-flurocytosine to 5-flurouracil and the enzyme nitroreductase which converts the prodrug CB1954 into an active alkylating agent (Bridgewater et al,1997). Unlike HSVtk/gancyclovir, the active metabolite in these two systems is able to diffuse across cell membranes. Therefore the bystander effect is not dependent on gap junctions in these systems. Further selectivity can be achieved in GDEPT if the prodrug activating enzyme gene is linked to a tissue specific promoter. The gene erbB-2 is present in ~80% of pancreatic cancers and this is useful in GDEPT therapy since this promoter can be linked to the HSVtk gene to produce tissue selectivity (Ring et al,1997). This is the current area of research which is being explored in GDEPT at present.

1.5. HUMAN ADENOVIRIDAE.

1.5.1.Morphology and genome stucture.

There are 42 human serotypes. The first human adenoviruses were discovered by Rowe et al (1953) and Hilleman and Werner (1954) and are now classified into 6 subgroups, A-F, based on immunological, biological, morphological and biochemical criteria. They cause a number of diseases in humans including respiratory, ocular, urinary and gastrointestinal diseases and are important human pathogens, although they are not often associated with fatal diseases (White and Fenner,1986; Horwitz,1990).

a) Morphology.

Adenoviruses are non-enveloped icosahedral particles containing linear double stranded DNA and a protein capsid. The capsid consists of 252 capsomers (Horne et al,1959). Pentons lie at the 12 vertices and 240 hexons form the facets for each capsomere. Hexons contain 3 identical protein chains (protein II). This hexon trimer has a triangular top superimposed on a pseudohexagonal base. Hexons carry type, group, intrasubgroup and intersubgroup antigenic determinants (Norrby and Wadell,1969). Pentons consist of penton base and filamentous projections, the fibres. The penton base is a pentamer of protein III and the fibre is a trimer of protein IV (Van Oostrum and Burnett,1985).The fibre contains the sites responsible for interaction of the virus with cellular receptors (Philipson,1967).

b) Genome structure.

The adenovirus genome is a double stranded linear DNA molecule. The entire DNA sequence of Ad2 is known and is 35,937 nucleotides long. Gene expression can be divided into early and late phase, separated by the onset of DNA replication. Transcription is initiated from a number of promoters at early and intermediate times. All the late genes are transcribed from the same promoter, the major late promoter (MLP) (Gelinas and Roberts,1977).

1.5.2. The Lytic Cycle.

a) Virus entry into cells.

Adenoviruses bind to cells by the fibre capsid protein. The cellular receptor was recently discovered (Bergelson et al,1997; Bergelson et al,1998; Tomko et al,1997) and is called CAR (Coxsackie adenovirus receptor) as it is also the same receptor for coxsackie virus infection. This is followed by endosome-mediated internalisation of the virion via interaction between the penton capsid protein and $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (Wickham et al,1993). The virion is disassembled and extruded by lysis of the endosome and then the DNA-protein core enters the nucleus through nuclear pores (Greber et al, 1993).

b) Transcription

In the nucleus, the adenovirus genes are transcribed by the host DNA polymerase II. The immediate-early E1A proteins are expressed, and then they induce transcription of the delayed-early genes in the E1B, E2, E3, E4 and L1 (early) transcription units. Each of these early regions contains its own promoter (Berk and Sharp,1977a). These early proteins convert the cell into an

efficient factory for virus replication and counteract host antiviral defences. Viral DNA replication occurs at 7 hours postinfection. Finally, after the onset of DNA replication, the late genes (L1-L5) are transcribed. These late transcripts are processed from a common nuclear precursor RNA which is initiated from the major late promoter (MLP) (Ziff and Evans,1978).In this late phase we get the expression of virion proteins and proteins required for assembly of infectious virions. In addition the synthesis of cellular DNA, mRNA, protein is inhibited. Virions then assemble in the cell nucleus 1 day post infection and increase in number until 2-3 days post infection. They are then released from the cell.

c) Assembly of virions.

The initial step is the formation of capsomers, hexon, penton base and fibre, from their monomeric forms in the cytoplasm and the assembly of empty capsids. Viral DNA and core proteins are then inserted into the capsid (Philipson,1984). The L1 52/55kDa proteins have been shown to be required for virion assembly.

d) Cell lysis and release of virions.

The mechanism of cell lysis is not well understood. The L3 encoded protease cleaves cytokeratin, disrupting the cytokeratin network, rendering the cell susceptible to lysis (Chen et al,1993). This is enhanced by inhibition of host protein synthesis which prevents repair of the cytokeratin network (Zhang et al,1994). In addition, the E3 11.6kDa adenoviral death protein promotes cell lysis (Tollefson et al,1996) possibly by inhibiting the apoptotic factors Bcl2 and E1B 19kDa at the nuclear membrane or possibly by forming channels for e.g. Ca^{2+} ions.

1.5.3. Early region 1A

a) E1A transcription

The E1A DNA sequence is known for many serotypes and comparison shows 3 conserved domains termed CRI, CRII, CRIII (Moran and Mathews,1987). These domains determine the function of the E1A proteins. There are 5 mRNA species, 3 major termed 13S,12S and 9S and 2 minor termed 11S and 10S.The sizes of the mRNAs are shown in **Figure 7.** The 12S and 13S mRNAs are translated in the same reading frame but their products differ by a block of 46 aminoacids found only in the 13S product. This region is the CRIII domain found only in 13S. The E1A promoter consists of a TATA box and an ATF binding site.

The E1A region is the first region to be transcribed during viral infection (Nevins et al, 1979). The level of the 12S and 13S proteins in the cytoplasm peaks at 5 hours post infection and then declines through 12 hours post infection (Glenn and Ricciardi, 1988).The levels of the 9S, 10S and 11S proteins accumulates in the cytoplasm late in infection (Stephens and Harlow,1987).

b) E1A protein products.

E1A proteins are phosphoproteins predominantly localised in the nucleus of infected cells (Schmitt et al,1987). Specific functions have been assigned to 3 regions within E1A which are well conserved between different serotypes. CRI is present in the 289aa 13S protein and the 243aa 12S protein and is required for transcriptional repression, transformation and induction of DNA synthesis (Lillie et al,1987). CRII is present in all but the smallest E1A proteins and is required for transformation (Lillie et al,1986; Moran et

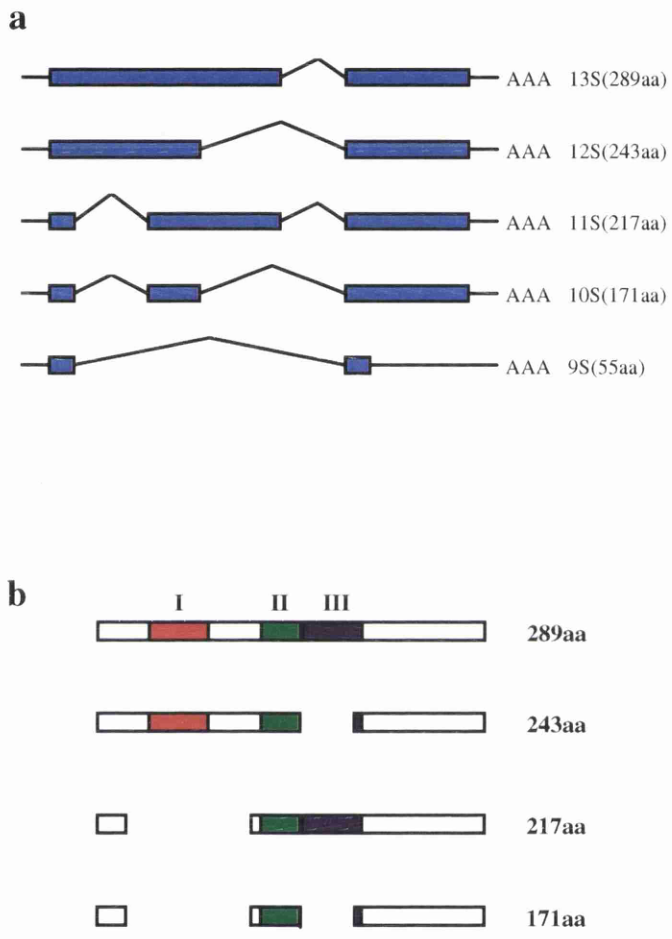


Figure7. Diagram of E1A mRNA's

Introns occur where the lines peak upward. Conserved domains are indicated as I,II and III. The 2 most important mRNAs are the 12S and 13S. These differ only in the CRIII domain which is absent in the 12S mRNA.

al,1986a) as well as induction of DNA synthesis (Howe et al,1986). CRIII is unique to the 289aa 13S protein and is essential for the transactivating function of E1A (Moran et al, 1986b; Lillie et al,1987).

c) Transactivation function of E1A.

The CRIII region is able to transactivate the E1B, E2, E3, E4 and the L1(early) transcription units. The mechanism of E1A transactivation appears to be indirect, not involving an interaction between E1A proteins and specific DNA sequences within the activated promoter, but through cellular components. It activates the E1B, E2, E3, MLP(major late promoter) via a TATA box thus implicating the TATA transcription factor (TFIID) in the process (Wu et al, 1987; Manohar et al,1990; Garcia et al, 1987; Nevins et al,1981). The E2 promoter also has two binding sites for E2F and E1A regulates the E2 promoter mainly through E2F rather than TFIID. The binding of two E2F molecules to their binding sites is assisted by the 19.5kDa E4orf6/7 protein which binds E2F (Huang and Hearing et al,1989a; Reichel et al,1989).

The E4 promoter has ATF binding sites which are activated by E1A . This regulation is via ATF factors, a factor termed EIVF, and E4F.

d) Transcriptional repression by E1A.

E1A protein products can repress transcription of viral or cellular enhancer sequences (Borelli et al,1984; Hen et al,1985; Velich and Ziff,1985; Hen et al,1986; Stein and Whelan,1989). Sequences within the CRI region are essential for repression (Lillie et al,1987; Jelsma et al,1989; Stein et al,1990).

e) Oncogenic transformation by E1A.

E1A proteins can immortalise primary rodent cells in culture and cooperate with other oncogenes, such as the E1B gene products or an activated

ras gene, to produce fully transformed cells. They do this by inactivating proteins Rb, p107 and p300 (Yee and Branton,1985; Harlow et al,1986). The Rb protein complexes to E1A causing release of E2F transcription factor from the Rb/E2F complex (Shenk and Flint,1991). This leads to S phase entry in the cell cycle as discussed before. Another domain of E1A binds to the transcriptional coactivator p300 (Samuelson and Lowe,1997). Binding of both E1A domains to Rb and p300 is required for transformation (Shenk and Flint,1991).

1.5.4. Early region 1B

a) General organisation of the E1B region.

The E1B region is located immediately downstream of the E1A region. The E1B sequence is known for many serotypes (e.g. Ad2,Gingeras et al,1982; Ad5,Bos et al,1981) and there is considerable sequence homology between serotypes. The 2 major transcripts are 22S and 13S . There are 2 minor species at 14S and 14.5S. The 2 major proteins are a 19kDa protein , encoded by the first open reading frame (ORF), and a 55kDa protein encoded by the second ORF (Bos et al,1981; Anderson et al, 1984).

b) E1B transcription.

E1B gene expression is controlled in a complex manner. The 22S mRNA accumulates first in the cytoplasm at 3.5-9hrs post infection, while the 13S mRNA remains low until 8 hrs post infection rising to a level comparable to the 22S mRNA at 12 hrs post infection (Glenn and Ricciardi,1988).

c) The E1B promoter.

The E1B promoter is activated by E1A. It consists of a TATA box and a binding site for the cellular transcription factor Sp1 (Wu et al,1987). These

elements are conserved in all adenovirus serotypes. E1A transactivation of E1B is mediated through the TATA box and is not affected by Sp1 binding. It does this by increasing the expression of the TATA binding factor TFIID.

d) The 19kDa protein.

This protein is important for viral infection as it inhibits E1A mediated apoptosis by inactivating the proapoptotic bax protein (Han and White,1996 for review). This is because the E1B 19kDa protein is homologous to the survival factor Bcl2. In addition, it prevents E1A induced cytolysis by Tumour necrosis factor (TNF), an inflammatory cytokine secreted by activated macrophages (White et al,1992).

e) The 55kDa protein.

The 55kDa protein is translated from the 22S mRNA (Bos et al,1981; Anderson et al,1984). The 55kDa protein acts within the nucleus by increasing the stability of late viral mRNA and transporting it from the nucleus to the cytoplasm. It does this in a complex with the E4ORF6 protein (Ornelles and Shenk,1991).

Transformation by viral infection requires the entire E1 region including the 55kDa protein (Babiss et al,1984; Barker and Berk,1987; Byrd et al,1988). The role of the 55kDa protein in transformation is due to its interaction with the cellular protein p53 (Sarnow et al,1982b). It binds directly to the N terminus of the p53 tumour suppressor gene and causes transcriptional inactivation of p53 (Dobner and Shenk et al,1996; Teodoro et al,1997). In addition, E1B 55kDa also mediates the degradation of p53. This degradation of p53 is believed to be mediated by complex formation between p53, E1B and

E4ORF6 (Dobner and Shenk ,1996; Steeganga et al,1997) which targets p53 for degradation via the ubiquitin degradation pathway.

1.5.5. The selectively replicating adenovirus, Onyx-015, as an anticancer agent.

The E1B deficient adenovirus, Onyx-015, has recently been shown to selectively replicate in and lyse p53(-) tumour cells both in vitro and in vivo (Bischoff et al,1996; Heise et al, 1997). This virus is a chimeric human group C adenovirus (Ad2 and Ad5) which has a deletion between nucleotides 2496 and 3323 in the E1B region encoding the 55kDa protein. In addition, there is a C to T transition at position 2022 in E1B which generates a stop codon at the third codon position of the protein. These alterations eliminate the expression of the 55kDa protein in Onyx-015 infected cells. The normal function of this protein is to bind to and inactivate the p53 protein in infected cells (Kao et al,1990). Because Onyx-015 lacks this protein it should replicate less efficiently in cells with functional p53 since it cannot inactivate p53. However it should replicate efficiently in cells with non-functional p53 as previously described. Such a virus is potentially very important clinically since over 50% of human cancers have inactivation of the p53 gene. This virus would allow us to target tumours at a molecular level thus limiting any damage to surrounding normal tissue.

1.6. OBJECTIVES OF PROJECT.

1.6.1. To develop an immunocompetent mouse model to test oncolytic adenoviruses(Onyx-015)

At present , there is no immunocompetent mouse model to test replicating adenoviruses as it is generally accepted that the infectivity and productive replication of adenoviruses in mouse cells is poor. A cotton rat model Sigmadon hispidus was described by Pacini et al, 1984 to be permissible to human adenovirus and enabled studies to be done on the mechanism of action of adenoviral replication in the pathogenesis of pneumonia. A mouse model for studying adenoviral pneumonia pathogenesis was later described by Ginsberg et al , 1991. In this model it was shown that pneumonia was dependent on the expression of early viral genes since replication did not occur in the mouse lung. Evidence that replication could occur in mouse tissue but at a low level was shown by Eloit et al , 1994 in which he looked at the dissemination of wild type, E3 deleted, and E1A deleted adenoviruses in both mice and cotton rats following intranasal inoculation of adenovirus. It was found that the dissemination of both the wild type and E3 deleted virus was wider than E1A deleted virus providing evidence that there was a low level of replication in the mouse species. Willcox et al , 1978 also showed that a low level of replication was possible in mouse liver. Kelly et al,1978 also showed mouse teratocarcinoma cells were permissive to adenoviral infection. However, all these studies are not useful for testing oncolytic adenoviruses since the level of productive infection is too low in the mouse tissues tested.

We decided to examine a wider range of rodent tissue types for adenoviral infectivity and replication. The aim of this was to identify a mouse tissue type which could support a productive adenoviral infection and which

could therefore be exploited for use in immunocompetent mouse models to examine the use of oncolytic adenoviruses in cancer therapy.

To develop a mouse model it was necessary to

1. Determine which mouse tissue types were permissible to adenoviral infection.

2. Determine whether or not a productive infection could be produced in mouse cells.

3. Determine whether or not Onyx-015 had the same p53 selectivity in mouse cells as was found in human cells.

4. Determine whether or not Onyx-015 had efficacy in vivo in nude mouse p53(-) xenografts.

5. Determine whether or not Onyx-015 had efficacy in vivo in syngeneic mouse p53(-) xenografts and to determine the effect of the immune system on efficacy.

1.6.2. To carry out mechanistic studies to explain the mechanism for selective replication of Onyx-015 for p53(-) cell lines.

Several publications have disputed the p53 selectivity of the Onyx-015 virus. Goodrum and Ornelles,1997 showed that replication only occurred in cells which were in the S phase of the cell cycle and that this was independent of the p53 status of the cell lines studied. Hall et al,1998 recently suggested that adenovirus induced cell death was greater in cell lines which had wild type p53. Therefore another aim of this thesis was to further examine the mechanism of selectivity of Onyx-015 for p53(-) cells. To study this we utilised the human ovarian adenocarcinoma cell lines A2780 (functional p53) and A2780Cp70 (non-functional p53 cisplatin resistant variant). To do this it was necessary to

1. Determine whether the infectivities of A2780 and Cp70 to adenoviruses were comparable.

2. Determine whether replication of adenovirus was greater in Cp70 compared to A2780.

3. Explain mechanistically the differences in replication by cell cycle analysis, p53 protein changes, and by examining the mode of cell death i.e. virus induced cytolysis or apoptosis.

1.6.3. To determine the effect of Onyx-015 on cytotoxicity induced by DNA damaging agents cisplatin and ionising radiation.

The adenoviral E1A gene is able to induce apoptosis in cells by p53 dependent and p53 independent mechanisms(Teodoro et al,1995; Debbas and White,1993; Lowe and Ruley,1993). The E1B(55kDa) gene can inhibit p53 dependent apoptosis by binding to the N terminus of p53 causing transcriptional repression and promoting p53 degradation via the ubiquitin degradation pathway (Debbas and White,1993; Querido et al,1997; Steeganga et al,1997). The E1B(19kDa) gene can inhibit both the p53 dependent and independent apoptosis due to its Bcl2 like properties(Han et al,1993; Huang et al,1997). Studies have shown that transfecting in the E1A gene into tumour cells which are either wild type or mutant p53 induces apoptosis (Sanchez-Prieto et al,1998). Moreover this apoptosis is enhanced when combined with DNA damaging agents (Sanchez-Prieto et al,1996). Rather than transfecting E1A into tumour cells , it would be simpler to infect with an adenovirus which retains the E1A region such as with Onyx-015. Our objective was therefore to determine whether or not Onyx-015 could increase cytotoxicity to DNA

damaging agents in non-functional p53 and also functional p53 cell lines. To study this we utilised the human ovarian adenocarcinoma cell lines A2780 (functional p53) and A2780Cp70 (non-functional p53 cisplatin resistant variant).

CHAPTER 2
METHODS AND MATERIALS

2.1 MATERIALS

2.1.1 Chemicals

All chemicals were of AnalaR grade and were obtained from BDH Chemicals Ltd,Poole,Dorset or Sigma Chemicals. Ltd, Poole,Dorset except those obtained from the suppliers listed below.

Advanced Biotechnologies Ltd,Surrey

Taq polymerase

Advanced Protein Products

Foetal calf serum

Amersham International PLC,Amersham,Buckinghamshire

α^{32}P dCTP, α^{32}P dGTP

rainbow protein markers

ECL Western blotting detection kit

Beatson Institute Central Services

sterile PBS(phosphate buffered saline)

Biogenesis

RNazol B

Boehringer Mannheim UK Ltd,Lewes,East Sussex

Insitu cell death kit

BRL(UK), Gibco Ltd, Paisley

all restriction enzymes

J.Burrough(FAD) Ltd,Witham,Essex

ethanol

FMC,Rockland,ME

Nuseive agarose

Gateway plc,Glasgow

Marvel dried non-fat milk powder

Gibco Europe, Life Technologies Ltd, Paisley

Special Liquid Medium(SLM)

10 x DMEM

10 x RPMI

200mM glutamine

2.5% trypsin

penicillin

streptomycin

RNA ladder

DNA ladders

Promega, Madison,WI

Taq DNA polymerase

dNTPs

Rathburn Chemicals Ltd, Walkerburn,Peebleshire

phenol(water saturated)

Severn Biotech, Kidderminster

acrylamide(30%, 40%)

bis-acrylamide 2%

Sigma Chemical Co.Ltd,Poole,Dorset

agarose

Tween 20

ethidium bromide

TEMED

β- mercaptoethanol

orange G

bromophenol blue

xylene cyanol

proteinase K

Rnase

Pepsin

Propidium iodide

Stratagene, La Jolla, CA

Prime- It RmT Random Primer Labelling Kit

Vector Laboratories Inc, Burlington,USA

Vectashield antifade mounting medium

BCIP/NBT substate

DAB substrate

Nuclear fast red

ABC Mouse peroxidase kit

ABC Rabbit peroxidase kit

2.1.2. Equipment and plasticware

Amersham International PLC,Amersham,Buckinghamshire

Hybond-N membrane

Applied Biosystems Ltd

PCR reaction tubes(thin walled)

Becton Dickinson Labware, Plymouth, Devon

tissue culture dishes(90mm,60mm)

Eastman Kodak Co, Rochester, New York

Ektachrome colour slide film

Fuji Photo Co.Ltd, Japan

x-ray film

Gibco Europe, Life Technologies Ltd,Paisley

Nunc 1ml cryotubes

Nunc 8-well chamber slides(permanox)

Nunc 25cm^2, 75cm^2, 175cm^2 tissue culture flasks

Griener Labortechnik Ltd, Dursley

eppendorf tubes

Hybaid Ltd, Teddington, Middlesex

Omnislide in situ system

Labsystems, Basingstoke

pipette tips

Molecular Bioproducts, San Diego, CA

aerosol- resistant tips

Pharmacia Ltd, Milton Keynes,Buckinghamshire

Nick columns

Spin columns

Whatman International Ltd, Maidstone

3MM paper

2.1.3. Antibodies

Amersham

Bak (mouse monoclonal)

DAKO

Bcl2 (mouse monoclonal)

mdm2 SMP14(mouse monoclonal)

Oncogene Science

p53 DO-1(mouse monoclonal)

E1A M73(mouse monoclonal)

E1B19kDa(rat monoclonal)

Pharmingen

Rb (mouse monoclonal)

Bax(rabbit polyclonal)

Santa Cruz

p21 sc-397 (rabbit polyclonal)

Sigma Chemical Co.Ltd,Poole,Dorset

vinculin VIN-11-1(mouse monoclonal)

Fluorescein(FITC) conjugated AffinitiPure Goat Anti-Mouse IgG

Gifts

RSA3 anti- E4orf6 and E4orf6/7 proteins(mouse monoclonal) - T.Shenk. (1990. J. Virology.64:2345-2359).

CM-5 murine p53(rabbit polyclonal) - D Lane (Dept of Biochemistry, University of Dundee).

mdm2 2A10(mouse monoclonal) - A Levine(Princeton University,USA)

PARP (mouse monoclonal)- K Caldecott

2.1.4. Probes used for Northern blot hybridisation.

a) Preparation of φAP3 mouse probe for Northern blotting.

RNA was made from mouse liver using the RNAzol method. cDNA was made from RNA using the Perkin Elmar RTPCR kit. Reverse transcription was done with 1μg of RNA. The amplification primers for mouse φAP3 were designed using the primer programme Designer PCR using the cDNA sequence for mouse φAP3 published by Fognani and Babiss,1993. The primers used were as follows:

5'- AGATGCCAAGGGTACTGTCAT-'3 (forward)

5'- AGGTGTGTCCGGAAGTGTACT-'3 (reverse)

and this produced a fragment of length 651bp.

Amplification was carried out at 95^0C for 3 minutes, then 30 cycles of 95^0C for 30s, 60^0C for 30s, 72^0C for 30s, followed by a final extension time of 10 minutes at 72^0C. An aliquot was run on a 1% agarose gel(Nu-Sieve) containing 0.5μg/ml ethidium bromide in TBE buffer and then photographed under UV light to check for correct amplification. The whole PCR product was then run on a 1% agarose gel(low melting point seakem agarose) containing 0.5μg/ml ethidium bromide in TAE buffer at low voltage of 40V for 2 hours, the correct band cut from the gel, and then the PCR product extracted from the gel using the Qiagen gel extraction kit(QIAquick gel extraction kit cat#28704).

b) Preparation of p21 mouse probe for Northern blotting.

As above. The amplification primers for mouse p21 were designed using the primer programme Designer PCR using the cDNA sequence for mouse p21 published by Huppi et al,1994.

The primers used were as follows:

5'-TTGTCTCTTCGGTCCCGTGGA -'3 (forward)

5'-TGGTCTGCCTCCGTTTTCGAC -'3 (reverse)

and this produced a fragment of length 368bp.

2.1.5. Plasmids and restriction digests.

a) Human p21.

The human p21 plasmid used was pCEP-WAF-1(Sense) and was donated by Dr Wafik S El-Diery (El-Diery et al,1992). The 2.1kb cDNA of human p21 was cut out of the plasmid by NotI restriction digest at 37^0C in React 3 buffer for 90 minutes. The whole restriction product was run on a 1% agarose gel (low melting point seakem agarose) containing 0.5μg/ml ethidium bromide in TAE buffer at low voltage of 40V for 2 hours, the correct band cut from the gel, and then the product extracted from the gel using the Qiagen gel extraction kit(QIAquick gel extraction kit cat#28704). This product was then further digested with EcoRI at 37^0C in React 3 buffer to produce a 1kb product which was extracted in the same way. This was used in the Northern blots.

b) Human mdm2.

The human mdm2 plasmid used was pCHDMIA and was donated by Dr A Levine (Chen et al,1993). The 1.8kb cDNA of human mdm2 was cut out of

the plasmid by EcoRI restriction digest at 37^0C in React 3 buffer for 90 minutes. The product was then extracted as described above.

c) Human bax.

The human bax plasmid used was donated by Clare Bradshaw (Beatson Research Institute - unpublished data). A 600bp fragment of human bax DNA was cut out of the plasmid by a double digest with EcoRI and NotI restriction enzymes at 37^0C in React 3 buffer for 90 minutes. The product was then extracted as described above.

2.2. MOUSE IN VITRO STUDIES.

2.2.1. Cell culture

a) Cell lines.

Mouse epidermal cell lines C5N, CarB, B9, A5, SN161, P1, P6 were grown in Special Liquid Medium (GibcoBRL) with 10%FCS and 10%L-Glutamine.

C5N.....non-tumourigenic, immortalised keratinocyte cell line isolated by single cell cloning of MCA3D cells derived from Balb/c mouse Kulesz- Martin et al;1983).

CarB.....highly aggressive spindle cell line isolated from carcinoma produced in NIH mice following DMBA/TPA treatment. (Diaz-Guerra et al;1992)

P1,P6.....cell lines derived from papillomas induced by treatment of *mus spretus/ mus musculus* F1 hybrid mice with DMBA/TPA (Haddow et al;1991)

B9.....squamous explant from the MSC11 carcinoma produced by multiple DMBA treatment of a *mus spretus/mus musculus* F1 hybrid mouse (single cell clones) (Burns et al;1991)

A5..... spindle explants from the MSC11 carcinoma produced by multiple DMBA treatment of a *mus spretus/mus musculus* F1 hybrid mouse (single cell clones) (Burns et al;1991)

SN161.....squamous/spindle explant derived from a carcinoma produced by DMBA/TPA treatment of 129/NIH hybrid mouse (Haddow et al,1991).

E4.....squamous clone from SN161 cell line.

H11.....spindle clone from SN161 cell line.

Mouse melanoma B16F1, mouse kidney adenocarcinoma TCMK1, mouse Lewis lung carcinoma, rat thyroid carcinoma VH1 VRS2, rat colon carcinoma K12/TrB were also grown in Special Liquid Medium (GibcoBRL) with 10%FCS and 10%L-Glutamine. Rat glioblastoma 9L-82 was grown in Dulbecco's modified Eagle's medium (DMEM) with 10%FCS and 10%L-Glutamine. Rat Morris hepatoma was grown in 75%DMEM/F12 (1:1), 5%FBS, 20% horse serum.

b) Long term storage of cells

Subconfluent F175 flasks (surface area 175cm^2) were trypsinised and the cells resuspended in serum containing medium. The cells were then pelleted by centrifugation and resuspended in 2.5ml fresh medium and then an equal volume of frrezing medium (50% SLM, 30%FCS, 20%DMSO) was added

dropwise and 1ml aliquots were transferred to -70^{0}C in cell freezing container overnight . This permitted slow cooling of the cells to -70^{0}C before the vials were transferred to liquid nitrogen for long term storage.

c) Transfection of cell lines by electroporation

Transfection was carried out as described by Baum et al,1994. To determine the optimum conditions for transfection by electroporation for each cell line, transfection was done at a range of voltages and volumes and efficiency of transfection determined using a luciferase reporter construct PGL3 (Promega). Exponentially growing cells were trypsinised and centrifuged to a pellet at 1600rpm for 5 minutes. The cells were then resuspended in a volume of medium at a concentration of 2 x 10^6 cells/400µl, 2 x 10^6 cells/300µl and 2 x 10^6 cells/200µl. To each of 6 cuvettes was added the 2 x 10^6 cells in the appropriate volume of medium. 1µg of PGL3 luciferase reporter DNA was added to each cuvette and then each sample was electroporated at a range of voltages from 0.2 to 1.0 Volts. 1ml of medium was added to each cuvette and the mixture then added to a 6 well plate with 5ml of medium in each well. The cells were incubated at 37^{0}C/5%CO_2 for 24 hours and the cells harvested using a Universal cell scraper into a 20 ml universal container. Cells were centrifuged to a pellet at 1600rpm, medium removed , 1ml PBS added and the cells transferred to a 1.5ml eppendorf. The cells were centrifuged to a pellet at 13000rpm for 10seconds, PBS removed and then 50µl of cell lysis buffer (Promega) added and the mixture briefly vortexed before incubating on ice for 10 minutes. Cell debris was pelleted at 13000rpm for 1 minute and then 5µl of supernatant transferred to a 0.5ml eppendorf. 50µl of luciferase assay solution

(Promega) was added to each eppendorf and the fluorescence measured using a scintillation counter. The sample with the maximum fluorescence value indicated the optimum conditions of volume and voltage for electroporation.

2.2.2. Viruses

Onyx-015 is a chimeric human group C adenovirus (Ad2 and Ad5) which has a deletion between nucleotides 2496 and 3323 in the E1B region encoding the 55kDa protein. In addition, there is a C to T transition at position 2022 in E1B which generates a stop codon at the third codon position of the protein. These alterations eliminate the expression of the 55kDa protein in Onyx-015 infected cells (Barker et al,1987).

Ad5CMVlacZ is a nonreplicating E1 deleted adenovirus in which the reporter construct lacZ has been inserted into the E1 region under the control of the CMV promoter (Kolls et al , 1994).

All adenoviruses were grown on the human embryonic kidney cell line HEK293 which expresses the E1 region of Ad2 (Graham et al , 1977) and band purified on a CsCl gradient. Stocks were stored at -70^{0}C after addition of glycerol to a concentration of 50%vol/vol.

2.2.3. Plaque assays

Plaque assays were performed as described by Graham and Prevec,1991 to determine the quantity of infectious viral particles in viral stocks and burst assays. Briefly, plates were scraped into 0.5ml of media and frozen. Lysates were prepared by 3 cycles of freezing and thawing. Serial dilutions on the lysates were titred on HEK293 cells.

2.2.4. Burst assays

Burst assays were used to determine the replication efficiency of Onyx-015 and wild type Ad2 in infected cells. Cell monolayers were infected at a multiplicity of infection (MOI) of 10pfu/cell with either wtAd2 or Onyx-015. After 90 minutes incubation, nonabsorbed virus was washed off with PBS, and then medium with 2%FCS added. At 4 hours and 72 hours post infection, cells were scraped into 0.5ml of medium and frozen. Lysates were prepared by 3 cycles of freezing and thawing. Serial dilutions on the lysates were titred on HEK293 cells. The burst ratio was expressed as the concentration of virus at 72 hrs divided by the concentration of virus at 4 hrs post infection.

2.2.5. Infectivity assay- Ad5LacZ assay

Infectivity of cell lines to adenovirus was determined using the nonreplicating E1 deleted adenovirus with a lacZ reporter construct under control of the CMV promoter. Cell monolayers on 60mm plates were grown to 70-90% confluence and then infected with Ad5CMVlacZ adenovirus at 10 pfu/cell and 100pfu/cell for 90 minutes. Nonabsorbed virus was removed after 90 minutes and then medium with 2% FCS added. After 24 hours and 48 hours incubation, medium was removed, the cells washed once with cold PBS, and then fixed with ice cold formaldehyde in PBS for 30 minutes. Monolayers were then washed 3 times with PBS , then stained with X-gal solution in the dark for 24 hours at 37^0C. Cells were washed with PBS and then examined using a light microscope.The number of blue staining cells to total cells was measured in 5

separate random high power fields and the mean determined and expressed as a percentage.

2.2.6. Cytopathic effect(CPE) assay

Briefly, cells were grown to 70-90% confluence and then infected with either Onyx-015 or Ad2 for 90 minutes at MOI of 1 and 10pfu/cell. Plates were monitored for CPE and the assay terminated when total cytolysis was observed at MOI of 1 pfu/cell with wild type adenovirus.

2.2.7. Immunofluorescence for adenovirus hexon protein

Cells were grown to 70-90% confluence and then infected with either Onyx-015 or Ad2 for 90 minutes at MOI of 10pfu/cell. After 72 hours, the cell sheet was scraped into the medium and pelleted by centrifugation at 2000revs/min for 10 minutes.The cell pellet was washed in PBS and then resuspended in a small volume of PBS. 25 μl aliquots were pipetted onto 6mm wells on teflon coated microscope slides (DAKO.Product code No S6114), air dried and fixed in acetone at room temperature for 10 minutes.Cells were then stained with 25 μl of IMAGEN^{TM} adenovirus reagent containing an FITC labelled mouse monoclonal antibody to adenovirus hexon protein for 15 minutes at 37^0C in a moist chamber, washed with PBS, air dried, then one drop of IMAGEN mounting fluid added and then a cover slip added.The 6mm well was then scanned using a confocal fluorescent microscope. Positive hexon staining was indicated by bright green fluorescence in the cytoplasm and/or nucleus of the cells.

2.2.8. p53 Functional Assays

a) Radiation induced G_1 arrest.

Cells were grown to 10% confluence in SLM medium with 10%FCS on 90mm culture plates . 5 plates were irradiated at a dosage of 2Gy using a cobalt source. 5 other plates acted as controls. Cells were harvested from the irradiated and control plates at time points 4, 8, 12, 24 and 48 hours by trypsinisation. Cell pellets were obtained by centrifugation at 2,000revs/min and discarding the supernatant. The cell pellet was fixed in 70% ethanol on ice for at least 1 hour. After washing with PBS, the cells were resuspended in 0.4-1.0ml of propidium iodide solution (10 μg/ml in PBS with Rnase A) for 30 minutes at room temperature and then examined by flow cytometry.

b) Luciferase reporter assay

This assay was carried out by transfection by electroporation. Electroporation conditions were determined for each cell line as described before. The constructs used for transfection were tkluc-p53 (p53 consensus binding sequence with luciferase reporter) and the control construct tkluc (no p53 consensus binding sequence). These constructs were kindly donated by Dr Jinde Zhu. 1μg of each constuct was used for transfection. The luciferase control construct PGL3 (Promega) was used to check efficiency of transfection. Each experiment was carried out 3 times and the mean value and standard error determined for each value.

2.2.9.Northern Blot analysis.

a) Extraction of Total RNA from adherent cells

Total RNA was isolated from actively growing cells by a commercial version (Rnazol) of the guanidinium-phenol method (Chomczynski and Sacchi;1987). Cells were grown in a $75cm^2$ flask until subconfluent. The medium was aspirated and the cells washed twice in PBS. 3ml of RNazol was then added directly to the flask and the lysate was transferred to a polypropylene tube. 350µl chloroform was added and after vortexing, the lysates were left on ice for 15 minutes, then centrifuged at 10,000rpm for 20 minutes at 4^0C. The upper aqueous phase was then transferred into a new tube, and an equal volume of isopropanol was added to precipitate the RNA. The mixture was left at 4^0C for 3-4 hours. To obtain the RNA , the lysates were centrifuged at 10,000rpm for 30 minutes at 4 ^0C, then the pellet was washed in 75% ethanol, and centrifuged again at 7,000rpm for 20 minutes at 4 ^0C. The pellet was left to air-dry , dissolved in 100µl DEPC treated H_20 and then stored at -20^0C.

b) Extraction of PolyA RNA from cells.

PolyA mRNA was isolated using the Ambion PolyA RNA isolation kit(cat#1915) from the cell lines A5, B9, E4, H11. 5µg of RNA was used for the detection of φAP3 on Northern blots.

c) Northern blot transfer of RNA

20µg of Total RNA (5µg of Poly A RNA) was fractionated on 1.1% agarose gels (6% formaldehyde, 0.2M MOPS, 0.5M sodium acetate, 0.01M EDTA) in 1x MOPS buffer at 100V for 4 hours. The RNA was transferred to nylon membranes (Hybond N+) using 20x SSC as described by Sambrook et

al;1989 and fixed by UV crosslinking using a Stratagene UV souce at 1800 Joules.

d) Radioactive ladelling of probes by the random priming method

Labelled probes were made by the random priming method. Approximately 50-100ng of purified insert DNA was added to a reaction mixture (Stratagene Prime-It RMT cCTP kit) containing oligonucleotide labelling buffer and dNTPs. The mixture was then boiled for 5-10 minutes to ensure denaturation, cooled and 1.85mbq of α^{32}P dCTP and 5 units of Klenow enzyme was added. The reaction was incubated at 37^0C for 1hr 15mins. The unincorporated nucleotides were removed by running the probe through a Nick column (Pharmacia) and its activity measured using a scintillation counter. The probe was denatured by boiling for 5-10 minutes, and then placed on ice for 5 minutes before it was added to the pre-hybridisation buffer.

e) Northern blot hybridisation

Northern blots were prehybridised in Ambion prehyb/hybridisation solution(cat#8677) for 3 hours at the temperature of best hybridisation for each probe (60^0C for φAP3 , 60^0C for p21). ^{32}P labelled probe was added and the membrane incubated overnight at 60^0C. The membrane was washed in low stringency wash solution(Ambion cat#8673) x2 at room temp for 10 minutes each followed by high stringency wash(Ambion cat#8674) at 42^0C x2 for 10 minutes each before being exposed to X-Ray film. Equal loading of RNA was checked by using a GAPDH probe at a hybridisation temperature of 42^0C.

2.2.10. Mouse p53 sequencing

a) RTPCR Analysis.

cDNA was made from RNA using the Perkin Elmar RTPCR kit. Reverse transcription was done with 1μg of RNA. The amplification primers for mouse p53 were designed from Zakut-Houri et al(1983) and Bientz et al(1984). Amplification was performed using amplimers U3 and D5.1. The reaction was carried out at 95^0C for 3 minutes, then 30 cycles of 95^0C for 30s, 55^0C for 30s, 72^0C for 30s, followed by a final extension time of 10 minutes at 72^0C. An aliquot was run on a 1% agarose gel(Nu-Sieve) containing 0.5μg/ml ethidium bromide in TBE buffer and then photographed under UV light to check for correct amplification. Removal of primers and deoxynucleoside triphosphates of the PCR products was done using Pharmacia spin columns.

b) Sequencing analysis.

Direct sequencing of the double stranded PCR product was performed by a modification of the standard dideoxynucleotide chain terminating method. PCR products were directly sequenced with fluorescent dye-labeled dideoxynucleotides using *Taq* polymerase and cycle sequencing. The primers used for p53 sequencing were internal primers in the sense direction (U amplimers) U8, U6 and U0. The sequencing reactions were performed using the Taq DyeDeoxy Terminator Cycle Sequencing kit(ABI, Foster City, CA). Purified PCR product was amplified at 95^0C for 3 minutes, then 25 cycles of 95^0C for 15s, 50^0C for 1s, 60^0C for 4 minutes, followed by cooling to 4^0C in a Perkin Elmer 9600 PCR machine. The products were then precipitated in 3M sodium acetate pH 5.2 and 100% ethanol at -70^0C overnight, centrifuged at 15,000 revs/min at 4^0C for 15 minutes, washed in 70% ethanol , centrifuged at

15,000 revs/min, and the cell pellet dried . Electrophoresis was done on a 6% denaturing polyacrylamide gel(0.4mm thick), and the bands visualised on the Applied Biosystems 373A automatic sequencer.

U8 5′- GCC CCT GTC ATC TTT TGT CCC-3′(264-284)

U6 5′- TGT CCG CGC CAT GGC CAT CTA-3′(459-479)

U3 5'- A CCT CAC TGC ATG GAC GAT CTG- 3'(109-130)

U0 5′- GAG TAT ACC ACC ATC CAC TAC-3′(673-693)

D5.1 5′- CTT GAG GGT GAA ATA CTC TCC-3′(964-984)

2.2.11. Protein Analysis

a) Preparation of total protein extracts

Subconfluent cells on 90mm diameter culture plates were infected with either wild type Ad2 or Onyx-015 at an infectivity of 100pfu/cell. At different times after infection, cells were washed twice in ice cold PBS and then lysed with 200 μL of buffer containing 0.5%NP40, 250mM NaCl, 50mM Hepes pH7.0,5mM EDTA,0.5M NaF,200mM Na_3VO_4,100mM benzamidine, 100mM PMSF,10μg/ml each of leupeptin, chymostatin, pepstatin, aprotinin . After 30 minutes on ice , the lysate was centrifuged at 15,000revs/min at 4 ^{0}C for 20 minutes to pellet insoluble debris. The protein concentrations of the supernatants was determined by the Bio-Rad (Richmond,CA) protein assay.

b) Immunoblotting

50 μg of protein was suspended in an equal volume of SDS sample buffer and separated on an SDS/10% polyacrylamide gel. After electrophoresis, the proteins were transferred to an Immobilon membrane

(Millipore) at a current of 2mA/cm^2 of gel area for 30 minutes using a semidry electroblotter. The membranes were then blocked in 5% non-fat milk in PBS/0.1% Tween 20 for 3 hours. The membranes were then incubated with the primary antibody diluted in blocking solution overnight at 4 ^{0}C and then washed with blocking solution x3 for 15 minutes each. The membranes were incubated with the appropriate secondary antibody linked to horseradish peroxidase (Santa Cruz) for 1 hour at room temperature diluted in blocking solution. After washing in PBS/0.1% Tween20 solution x3 for 15 minutes each, the membranes were visualised by enhanced chemiluminescence ECL (Amersham, UK). Autoradiograms were scanned and cropped with Adobe Photoshop and figures were prepared wih Adobe Freehand software on an Apple Macintosh computer.

The following primary antibodies were used:

Protein	Antibody	Source	Dilution
p53	rabbit polyclonal CM-5	D.Lane	1/2000
p21	rabbit polyclonal sc-397	Santa Cruz	1/500
vinculin	mouse monoclonal VIN-11-1	Sigma	1/1000
E1A	mouse monoclonal M73	Oncogene Science	1/500
E4ORF6	mouse monoclonal RSA3	T Shenk	1/5
E4ORF6/7	mouse monoclonal RSA3	T Shenk	1/5

2.3. MOUSE IN VIVO STUDIES

2.3.1. Direct intratumoural injection efficacy studies in Nude mice.

Tumour cells (10^7 cells in 200µl of PBS) were injected into the flanks of 6 week old female athymic nude mice and allowed to grow into palpable

tumours (5-8mm maximal diameter). Tumour injection was by a single puncture into the centre of the tumour and then directing the needle into four quadrants injecting 25% volume into each quadrant. Ad2 treated tumours were injected with 10^8 pfu Ad2 suspended in 100μl of PBS daily for 5 days. Control tumours were injected with vehicle (PBS) in an identical fashion. Onyx-015 treated tumours were injected with 10^8 pfu Onyx-015 in the same way. Tumour measurements were taken twice weekly by caliper measurement in 2 dimensions. The volume of each tumour was calculated using the formula $d^2l/2$, where d is the smallest diameter in mm and l is the largest diameter in mm. Tumour volumes were expressed as mm^3. The animals were humanely killed once tumours were greater than 15mm maximal diameter.

2.3.2. Tumour transplantation into C57bl mice.

PDVc57 tumour cells (10^7 cells in 200μl of PBS) were injected into the flanks of 6 week old female athymic nude mice and allowed to grow into 1cm palpable tumours. Mice were then sacrificed and the tumours dissected out. The tumours were cut into 5mm x 5mm x 5mm blocks and then transplanted into 4 week old C57bl6 mice. C57bl6 mice were anaesthetised by intraperitoneal injection with a 1:1:2 mixture of hypnorm (10mg/ml), hypnovel (5mg/ml) and H_20. Under aseptic conditions , a single cut was made on the dorsal aspect of the flank of each mouse and a small subcutaneous pocket made. The tumour block was then inserted into the pocket and skin then closed with a single metal clip which was removed on day 7. Tumours were allowed to establish for 14 days before viral injection studies were done.

2.3.3. Direct intratumoural injection efficacy studies inC57bl6 mice.

Tumours were established by tumour transplantation from PDVc57 tumours grown in nude mice. Ad2 treated tumours were injected with 10^8 pfu Ad2 suspended in 100μl of PBS daily for 5 days. Control tumours were injected with vehicle (PBS) in an identical fashion. Onyx-015 treated tumours were injected with 10^8 pfu Onyx-015 in the same way. Tumour measurements were taken twice weekly. The animals were humanely killed once tumours were greater than 15mm maximal diameter.

2.3.4. Animals and animal care.

Female athymic nu/nu mice were obtained from Harlan Olac at 4-6 weeks of age. Mice were housed 4 per cage in cages fitted with sterile filter tops and fed with sterile food and sterile water. Guidelines for animal care were strictly followed.

2.3.5. Analytical and statistical methods.

Mean tumour volumes in treated animals versus controls were compared at a given time point using the unpaired two-tailed t-test. Survival of animals in each group was analysed using the method of Kaplan and Meier, and Kaplan-Meier plots for treated and control groups were compared for statistical significance using the Log rank test.

2.3.6. In situ hybridisation for adenoviral DNA

In situ hybridisation was performed on formalin fixed paraffin embedded tumours cut into 5μm sections. Slides were deparaffinised in xylene, hydrated through ethanols 100%, 90%, 70% and then H_20. The tissue was digested with proteinase K and post fixed in 4% paraformaldehyde. Hybridisation was carried out overnight at 37^0C with 0.5μg/ml biotinylated adenovirus DNA probe (Enzo Diagnostics,Inc.Farmingdale.NY). After 3 successive washes in 1xSSC at 55^0C, an alkaline phosphatase conjugated antibiotin antibody (Vector Laboratories) was applied. NBT/BCIP was used as the chromagen and slides were counterstained with nuclear fast red (Vector Laboratories).

2.4. HUMAN IN VITRO STUDIES.

2.4.1. Human cell lines

The ovarian adenocarcinoma cell lines A2780 and its cisplatin resistent derivative A2780Cp70 were grown in RPMI medium containing 10% FCS and 10% L-Glutamine. A2780 has wild type p53 sequence and function and A2780Cp70 has wild type p53 sequence which is non-functional (Behrens et al, 1987).

All cell lines were grown at 37^0C in an incubator adjusted to 5%CO_2. Cells were routinely passaged when subconfluent by aspirating the medium, washing once with PBS, and then adding 1-2ml of trypsin solution (10%v/v trypsin,0.01%EDTA in PBS). Cells were incubated at 37^0C until they detached ,then resuspended in medium with 10% FCS to inactivate the trypsin, then seeded at the appropriate dilution.

2.4.2. Infectivity assays.

a)Ad5LacZ assay.

As described previously.

b) E1A immunofluorescence assay.

Cell monolayers on 90mm plates were grown to 70-90% confluence and then infected with Onyx-015 adenovirus at 10 and 100 pfu/cell for 90 minutes. Nonabsorbed virus was removed after 90 minutes and then medium with 2% FCS added. After 24 hours, cells were harvested by trypsinisation and then resuspended in medium. Cytospins were then made using 5 x 10^5 cells per slide and ice cold methanol fixation. Slides were washed in PBS and then with PBT(PBS plus 0.1% Tween 20) to permeabilise the cells. They were then incubated with 100μl of anti-E1A antibody (mouse monoclonal (Oncogene Science) at 1/500 dilution in PBT for 1 hour at 37^0C. The cells were washed in PBS x2 for 5 minutes each and then incubated with 100μl of FITC labelled anti-mouse IgG (Sigma) at 1/40 dilution at room temperature for 30 minutes. Cells were washed in PBS and then counterstained with DAPI solution. The slides were then examined by fluorescent microscopy. E1A positive cells stained bright green and negative cells blue with the counterstain DAPI. The number of E1A positive cells was counted in 3 high power fields and the mean % of E1A positive cells calculated.

2.4.3. Replication assays

a) Burst assay.

As described previously.

b) Hexon protein expression assay.

Cell monolayers were grown to 50% confluence on 90mm plates. One plate was infected with Ad2 at 10pfu/cell, one plate with Onyx-015 at 10pfu/cell and one plate left uninfected as a control. Nonabsorbed virus was removed after 90 minutes and then medium with 2% FCS added. After 48 hours incubation, the cells were harvested and collected into a cell pellet by centrifugation at 2,000revs/min.The cells were fixed in ice cold 70% ethanol for at least 1 hour, washed in PBS and then resuspended in 200 microlitres of anti-hexon protein antibody(Chemicon #MB805) at1/1000 dilution in PBS for 1 hour at 37^0C. The cells were washed in PBS and then resuspended in 100 μl of FITC labelled anti-mouse IgG (Sigma) at 1/40 dilution at room temperature for 30 minutes. Cells were washed in PBS and then resuspended in propidium iodide solution (10 μg/ml) for 30 minutes at room temperature. The cells were then analysed by flow cytometry. The percentage of FITC staining cells was determined for Ad2 and Onyx-015 infected cells using the control cells as a negative control for FITC. Each infection was determined 3 times and the mean percentage calculated.

2.4.4. Cell cycle analysis using BrdU and PI.

Cell monolayers were grown to 50% confluence on 90mm plates. Three plates were infected with Ad2 at 100pfu/cell, three plates with Onyx-015 at 100pfu/cell and three plates left uninfected as controls. Nonabsorbed virus was removed after 90 minutes and then medium with 10% FCS added. After 0, 24, 48 hours incubation, BrDU at a concentration of 10μM in medium was added for 1 hour. This was then aspirated, the cells washed with PBS and then

harvested and collected into a cell pellet by centrifugation at 2,000revs/min.The cells were fixed in ice cold 70% ethanol for at least 1 hour, washed in PBS x2 for 5 minutes each. The cell pellets were then resuspended in 1ml of pepsin (1mg/ml) for 30 minutes , centrifuged at 2000rpm, supernatant removed, and the cell pellet resuspended in 1ml of 2M HCL for 30 minutes. After centrifugation at 2000rpm for 5 minutes, the acid supernatant was removed and the cell pellet washed in PBS and then PBT(PBS with 0.5% BSA and 0.1% Tween 20). After centrifugation at 2000rpm for 5 minutes , the cells were resuspended in 100μl of mouse anti-BrdU(DAKO) at 1/30 dilution in PBT for 1 hour at RT. The cells were washed in PBT x2, then resuspended in 100 μl of FITC labelled anti-mouse IgG(Sigma) at 1/40 dilution at room temperature for 30 minutes.Cells were washed in PBS and then resuspended in propidium iodide solution (10 μg/ml) for 30 minutes at room temperature. The cells were then analysed by flow cytometry.

2.4.5. E2F Bandshift assay.

a) Preparation of protein extract.

Cell monolayers were grown to 50% confluence on 90mm plates. Three plates were infected with Ad2 at 100pfu/cell, three plates with Onyx-015 at 100pfu/cell and three plates left uninfected as controls. Nonabsorbed virus was removed after 90 minutes and then medium with 10% FCS added. After 0, 24, 72 hours incubation, the cells (floating and adherent) were harvested and collected into a cell pellet by centrifugation at 2,000revs/min.The cells were then washed in PBS and then 100μl of lysis buffer added. The lysis buffer was made fresh and consisted of 20mM HEPES pH7.9, 0.4M NaCl, 1mM EDTA,

20% glycerol, 1mM DTT, 1mM PMSF, 0.5μg/ml leupeptin, 0.5μg/ml aprotinin, 1μg/ml chymotrypsin. The cells were incubated on ice for 20 minutes , freeze/thawed (dry ice to ice) x2, centrifuged at 14,000rpm for 20 minutes at 4^0C and the supernatant separated into a separate eppendorf. The protein concentration was determined by the Coomassie method.

b) Labelling of E2F oligonucleotide probe.

The oligonucleotide with the consensus binding sequence for E2F is shown below:

GATCTAGT**TTTCGCGC**TTAAATTTGA

300ng of probe was end- labelled with α^{32}P labelled dGTP . The reaction mixture consisted of 300ng probe, 2μl 10x React3, 5μl α^{32}P dGTP, 2μl of Klenow enzyme (1unit/μl), and 10μl ddH_20. The mixture was incubated at RT for 30 minutes and then excess α^{32}P dGTP removed using a Qiagen nucleotide purification column.

c) Reaction E2F/probe complex formation.

To 10μg of protein extract was added 5μl of 4xReaction Buffer, 2μg of salmon sperm DNA, 300ng of double stranded competitor oligonucleotide, 0.5μl of BSA(10mg/ml) and ddH_20 to a final volume of 19μl. After incubation at RT for 10 minutes, labelled probe (equivalent to 150cps on Geiger counter) was added and then incubated for a further 20 minutes at RT. The sample was then loaded onto a 4.5% polyacrylamide gel for electrophoresis.

4x Reaction buffer consisted of 40mM HEPES pH 7.9, 400mM Kcl, 4mM EDTA, 16% Ficoll, 1mM DTT. The sequence of the competitor

oligonucleotide for E2F is shown below with nucleotide mutations shown in bold:

GATGTAGTTTTCGATATTAAATTTGA

d) Gel electrophoresis.

Samples were loaded onto a 4.5% polyacrylamide /TBE gel (Acrylamide/Bis : 29/1). Electrophoresis was carried out in 0.25x TBE running buffer at 100V for 2 hours at 4^0C. The gel was then fixed in 10% Methanol/10% Acetic acid at RT for 15 minutes with shaking, vacuum dried, and then exposed to X-Ray film.

e) Supershift assays.

To identify the E2F complexes, antibodies to E2F1, E2F4, E2F5, DP1 were added to the reaction mixture to disrupt the complexes. The antibodies used were the following:

E2F1	rabbit polyclonal	Ed Harlow
E2F5	rabbit polyclonal	Nick La Thangue
DP1	rabbit polyclonal	Nick La Thangue

2.4.6. Apoptosis detection by TUNEL staining.

Cell monolayers of A2780 on 90mm plates were grown to 50% confluence and then infected with Ad2 adenovirus or Onyx-015 at 100 pfu/cell for 90 minutes. Nonabsorbed virus was removed after 90 minutes and then medium with 10% FCS added. After 0, 24 and 72 hours cells (floating and adherent) were harvested by trypsinisation and then resuspended in medium. Cytospins were then made using 5 x 10^5 cells per slide. Cells were then fixed in

4% paraformaldehyde at RT for 30 minutes. Slides were washed in PBS and then permeabilised with 0.1% Triton X-100, 0.1% Na Citrate for 2 minutes at 4^0C. The slides were then washed in PBS and then 50µl of TUNEL reagent (Boehringer Mannheim in situ cell death detection kit fluorescein) added to the slide and incubated for 1 hour at 37^0C in an humidified chamber in the dark. The cells were washed in PBS x2 for 5 minutes each and then incubated with 100µl of anti-adenoviral hexon protein mouse monoclonal antibody (Chemicon) at 1/1000 dilution for 1 hour at 37^0C in an humidified chamber in the dark. After washing in PBS, 100µl of anti-mouse Texas Red was added at a dilution of 1/50 and the cells incubated for 1 hour at 37^0C in an humidified chamber in the dark. Slides were washed in PBS and then counterstained with DAPI/antifade solution. The slides were covered with coverslips and sealed with nail varnish. The slides were then examined by fluorescent microscopy. Apoptotic cells stained bright green, hexon positive expressing cells bright red , and uninfected cells blue with DAPI counterstain.

2.4.7. Protein Analysis

a) Preparation of total protein extracts - as described previously.

b) Immunoblotting

As described previously with the exception of gel electrophoresis for Rb protein. With Rb , electrophoresis was done on SDS/7.5% polyacrylamide gels. The polyacrylamide had a low percentage of crosslinker bis acrylamide (1:120) to obtain good separation of the different phosphorylated forms of Rb.

The following primary antibodies were used:

Protein	Antibody	Source	Dilution
p53	mouse monoclonal DO-1	Oncogene Science	1/1000
p21	rabbit polyclonal sc-397	Santa Cruz	1/500
vinculin	mouse monoclonal VIN-11-1	Sigma	1/1000
Rb	mouse monoclonal	Pharmingen	1/1000
mdm2	mouse monoclonal E10	A Levine	1/100
E1A	mouse monoclonal M73	Oncogene Science	1/500
E1B(19kDa)	rat monoclonal	Oncogene Science	1/500
E4ORF6	mouse monoclonal RSA3	T Shenk	1/5
E4ORF6/7	mouse monoclonal RSA3	T Shenk	1/5
Bcl2	mouse monoclonal	DAKO	1/1000
$Bclx_L$	mouse monoclonal	Transduction labs	1/1000
Bax	rabbit polyclonal	Pharmingen	1/1000
Bak	mouse monoclonal	Amersham	1/1000
PARP	mouse monoclonal	K Caldecott	1/1000

2.4.8.Northern Blot analysis.

a) Extraction of Total RNA from adherent cells

Total RNA was isolated by the RNAzol as described previously.

b) Northern blot transfer of RNA

As described previously.

c) Radioactive ladelling of probes by the random priming method

As described previously.

d) Northern blot hybridisation

Northern blots were prehybridised in Ambion prehyb/hybridisation solution(cat#8677) for 3 hours at the temperature of best hybridisation for each probe (42^0C for p21, mdm2, bax.). ^{32}P labelled probe was added and the membrane incubated overnight at the hybridisation temperature.The membrane was washed in low stringency wash solution(Ambion cat#8673) x2 at room temp for 10 minutes each followed by high stringency wash(Ambion cat#8674) at 42^0C x2 for 10 minutes each before being exposed to X-Ray film. Equal loading of RNA was checked by using a GAPDH probe at a hybridisation temperature of 42^0C.

2.4.9. Clonogenic assay

a) Onyx-015.

Cells were seeded at a density of 5 x 10^5 per 90mm plate and infected with Onyx-015 at an MOI of 1, 10, and 100pfu/cell with a mock uninfected control plate for 90 minutes. Excess virus was then removed and then medium with 10% FCS added. After 24 hours, cells were harvested by trypsinisation and then plated at a density of 10^3 per plate. 5 plates were used for each MOI. 10 days of incubation at 37^0C was required for colony formation to occur. After this period, medium was removed, cells washed with PBS, and then fixed with methanol for 30 minutes. After air drying the plates overnight, the colonies were stained with crystal violet and then colonies greater than 50 cells counted using the colony counter (PROTOS). The mean colony count per MOI was calculated and then expressed relative to the uninfected mock control. Graphs of percentage survival versus dose of virus (MOI) were then plotted.

b) Onyx-015 and cisplatin.

After infection with virus as described above, cells were exposed to cisplatin at either 24 hours post infection or 72 hours post infection. Cells were exposed to cisplatin for 1 hour at doses of 1, 5, 10, 20 μM for A2780 and doses 20, 40, 60, 80 μM for A2780Cp70. Cells were then trypsinised and plated out as described above.

c) Onyx-015 and radiation.

After infection with virus as described above, cells were exposed to ionising radiation from a cobalt source at either 24 hours post infection or 72 hours post infection. Cells were exposed to radiation doses of 0.25, 0.5, 1, 2Gy for A2780 and doses 0.5, 1, 2, 4Gy for A2780Cp70. Cells were then trypsinised and plated out as described above.

2.4.10. Immunoprecipitation

a)Preparation of protein lysates.

Subconfluent cells on 90mm diameter culture plates were infected with Onyx-015 at an infectivity of 100pfu/cell. At 8, 24, and 72 hours after infection, cells were washed twice in ice cold PBS and then lysed with 150 μL of RIPA buffer containing 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 150mM NaCl, 50mM Tris pH7.4, 200mM Na_3VO_4, 100mM benzamidine, 100mM PMSF,10μg/ml each of leupeptin, chymostatin, pepstatin, aprotinin . After 30 minutes on ice , the lysate was centrifuged at 15,000revs/min at 4 ^{0}C for 20 minutes to pellet insoluble debris. The protein concentrations of the supernatants was determined by the Bio-Rad (Richmond,CA) protein assay.

b)Immunoprecipitation.

The volume of 200μg of protein was estimated and the final volume adjusted to 200μl with RIPA buffer. To each sample was added 1μg of DO1 mouse monoclonal p53 antibody and the mixture incubated with agitation at 4^0C overnight. p53 protein complexes were then immunoprecipitated with 100μl of protein A sepharose (Sigma) at 4^0C for 1 hour with gentle agitation. After centrifugation at 14,000rpm for 30sec, the supernatant was removed , the cell pellet washed twice with 500μl of RIPA buffer , and then an equal volume of SDS loading buffer (50μl) added to the cell pellet (~50μl). The mixture was boiled for 3-5 minutes, centrifuged briefly at 14,000rpm for 30sec, and the supernatant removed and then loaded onto an SDS/10% polyacrylamide gel.

c)Electrophoresis and immunoblotting.

Gel electrophoresis and immunoblotting was carried out as described before. Mdm2 was detected using mouse monoclonal 2A10 supernatant at a dilution of 1/50 , followed by peroxidase conjugated goat antimouse secondary antibody and then enhanced chemiluminescence. p53 was detected using DO1 mouse monoclonal antibody, followed by peroxidase conjugated protein A as the secondary antibody (to prevent immunoglobulin heavy chains from masking the p53 signal at 55 kDa) and then enhanced chemiluminescence.

CHAPTER 3
RESULTS

3.1.IMMUNOCOMPETENT MOUSE MODEL TO TEST ONCOLYTIC ADENOVIRUSES.

3.1.1. Infectivity and replication of human adenoviruses in mouse cell lines.

a) Infectivity of rodent cell lines.

A nonreplicating E1 deleted adenovirus with a lacZ reporter construct (Kolls et al,1994) was used to determine infectivity. The percentage of beta-galactosidase positive staining cells at a multiplicity of infection (MOI) of 10 and 100 pfu/cell for several different rodent cell lines is shown in **Table 1.** Infectivity was very high for specific tissue types notably mouse epidermal cells, mouse kidney adenocarcinoma TCMK1, rat glioblastoma 9L-82, rat thyroid carcinoma VH1 VRS2(**Figure 8**), rat Morris hepatoma. Infectivity was very low in mouse Lewis lung carcinoma and rat colon carcinoma K12/TrB despite the known tropism for the respiratory and gastrointestinal tracts in humans. The infectivity of mouse 3T3 fibroblasts was very low and this was significant as much research on adenoviral replication in mouse tissue has been done on this tissue type rather than epithelial tissue (Silverstein et al,1996).When compared with the human ovarian cell line A2780Cp ,which allows efficient viral replication, the infectivity of many of the rodent cell types was surpringly higher.

Cell line	Species	Tissue	Infectivity			
			%Beta gal +ve 24hrs			
			10pfu/cell	s.e	100pfu/cell	s.e.
A2780Cp70	Human	Ovarian adenocarcinoma	14.8	0.9	40.4	1.6
B16F1	Mouse	Melanoma	0.7	0	4.8	0.8
Lewis	Mouse	Lung carcinoma	0.5	0.2	1.5	0.2
3T3	Mouse	Fibroblasts	1	0	8.5	1.3
K12/TrB	Rat	Colon carcinoma	3.5	0.6	8.3	1.1
TCMK1	Mouse	Kidney adenocarcinoma	19.5	0.8	36.6	1.6
Morris	Rat	Liver hepatoma	59.6	4.1	100	0
9L-82	Rat	Glioblastoma	36.7	2.4	100	0
VH1 VRS2	Rat	Thyroid carcinoma	31.3	3.7	46.4	3.2
A5	Mouse	Epidermal spindle Ca	22	1.8	63.5	1.9
B9	Mouse	Epidermal squamous Ca	26.7	1.9	68	3
SN161	Mouse	Epidermal sp/sq Ca	16.3	1.3	57.5	4.7
CarB	Mouse	Epidermal spindle Ca	23.8	2	49.8	4.3
P6	Mouse	Epidermal papilloma	16.9	2.2	44.5	2.8

Table 1. Infectivity of rodent cell lines

Infectivity was carried out using a nonreplicating adenovirus in which the E1 region was replaced with a lacZ reporter construct. The percentage of beta galactosidase positive cells was quantified by counting the number of blue staining cells in 5 random high power fields and expressing this as a percentage of the total number of cells in the fields. The standard error(s.e.) is shown for each infectivity result.

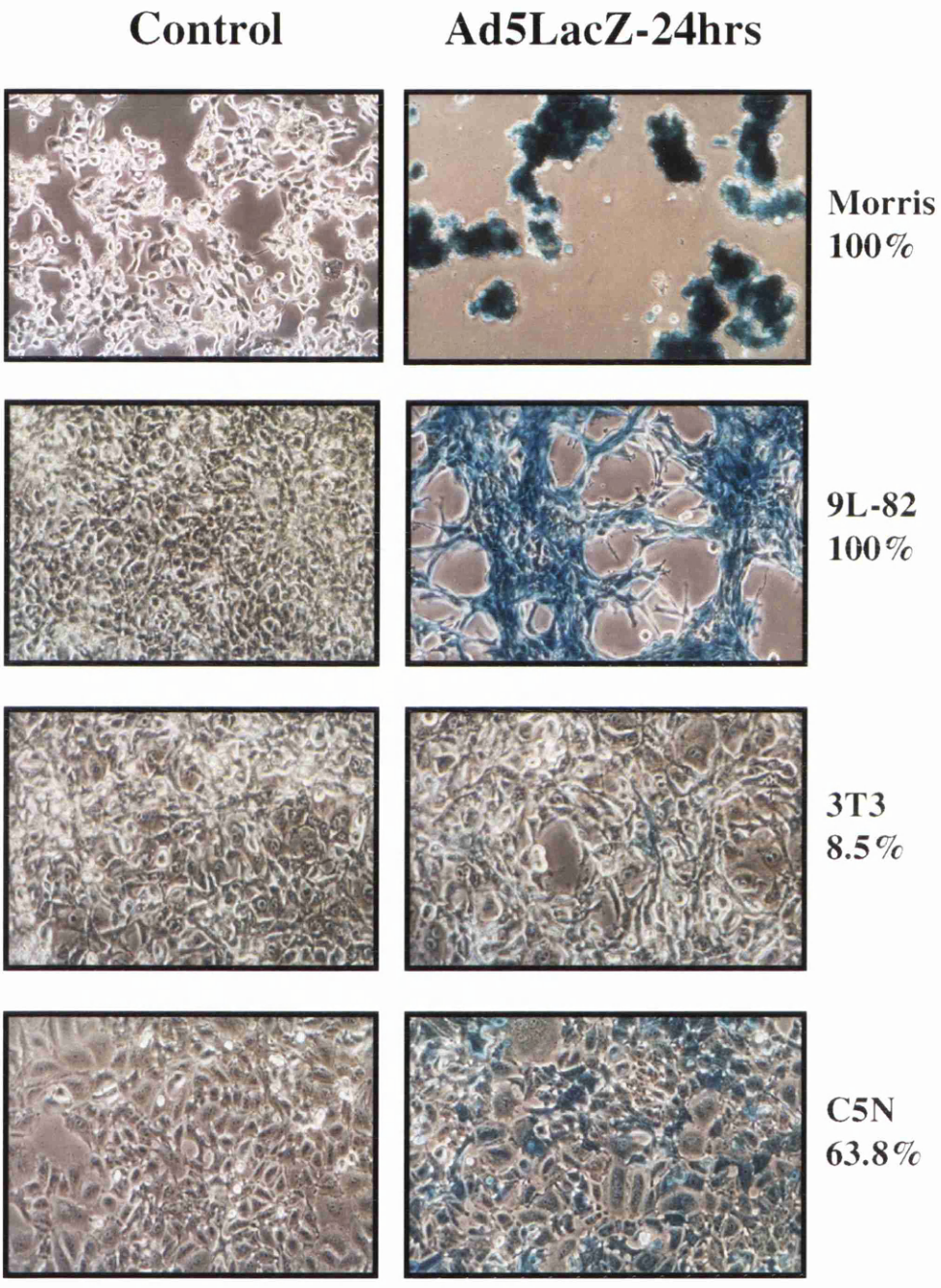


Figure 8. Infectivity of rodent cell lines.

Infectivity was determined using an E1 deleted adenovirus with a lacZ reporter gene. Beta galactosidase staining was carried out at 24 hours post infection and the number of blue staining cells calculated. Figure 8shows examples of staining in different cell lines at an MOI of 100pfu/cell.

b) Replication and cytopathic effect of rodent cells infected with wild type Ad2.

Cytopathic effect assays using wild type adenovirus Ad2 were done at a MOI of 10pfu/cell. **Table 2** shows that cytolysis occurred in all the mouse epidermal cells, and to a lesser extent in thyroid carcinoma VH1 VRS2 cell lines. Some cytolysis was also present in rat colon carcinoma K12/TrB despite the low infectivity in this cell line.The presence of the late phase hexon protein was detected by direct immunofluorescence. All cell lines which showed a positive CPE were positive by immunofluorescence for hexon protein. It was interesting to note that the cell lines 9L-82 and TCMK1 which were highly infectable with the CMVlacZ virus showed no CPE or positive immunofluorescence which indicated that there was a block to replication in these cell lines as no late phase proteins were expressed. **Figure 9** shows a typical example of hexon protein staining and CPE in the epidermal cell line B9.

c) Productive adenoviral infection in mouse epidermal cells.

To determine if a productive infection could be produced in mouse cells, burst assays were done with wild type Ad2. The burst ratio was expressed as the concentration of virus at 72 hrs divided by the concentration of virus at 4 hrs post infection. **Figure 10a** shows the results of burst assays in cell lines B9, SN161 compared to the human ovarian cell line A2780Cp.The burst ratio for A2780Cp is x50 greater than B9, x25 greater than SN161. **Figure 10b** shows that the infectivities of each cell line are similar at 10pfu/cell as

Cell line	Species	Tissue	CPE	Hexon staining
B16F1	Mouse	Melanoma	-	-
Lewis	Mouse	Lung carcinoma	-	-
3T3	Mouse	Fibroblasts	-	-
K12/TrB	Rat	Colon carcinoma	+	+
TCMK1	Mouse	Kidney adenocarcinoma	-	-
Morris	Rat	Liver hepatoma	-	-
9L-82	Rat	Glioblastoma	-	-
VH1 VRS2	Rat	Thyroid carcinoma	+	+
A5	Mouse	Epidermal spindle Ca	+	+
B9	Mouse	Epidermal squamous Ca	+	+
SN161	Mouse	Epidermal sp/sq Ca	+	+
CarB	Mouse	Epidermal spindle Ca	+	+
P6	Mouse	Epidermal papilloma	+	+
C5N	Mouse	Immortalised keratinocyte	+	+

Table 2. - Cytopathic effect(CPE), and immunofluorescence(IF)for hexon protein of wild type adenovirus infected rodent cell lines.

Cytopathic effect and immunofluorescence were done at a MOI of 10pfu/cell. For CPE, plus and minus symbols refer to the presence or absence of cytolysis. For hexon protein staining, plus and minus symbols refer to the presence or absence of green fluorescent hexon protein staining cells.

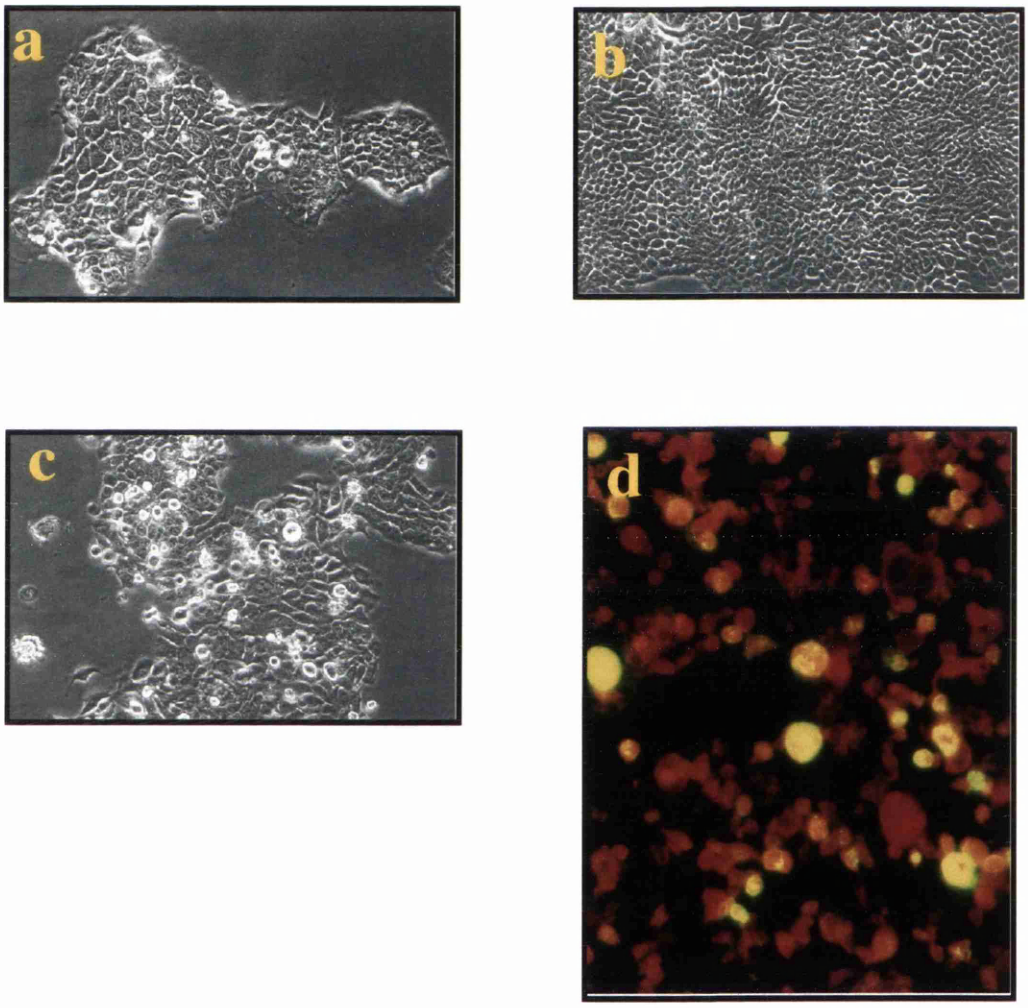


Figure 9. Cytopathic effect and hexon protein staining in cell line B9.

Figure 9a and 9b show uninfected cells at 0 hours and 72 hours respectively. Figure 9c shows cells undergoing cytolysis at 72hours post infection with Ad2 at 10pfu/cell.Figure 9d shows B9 cells stained for adenovirus hexon protein at 72 hours post infection. Green staining cells are hexon positive cells. Uninfected cells stain red with the counterstain Evans blue.

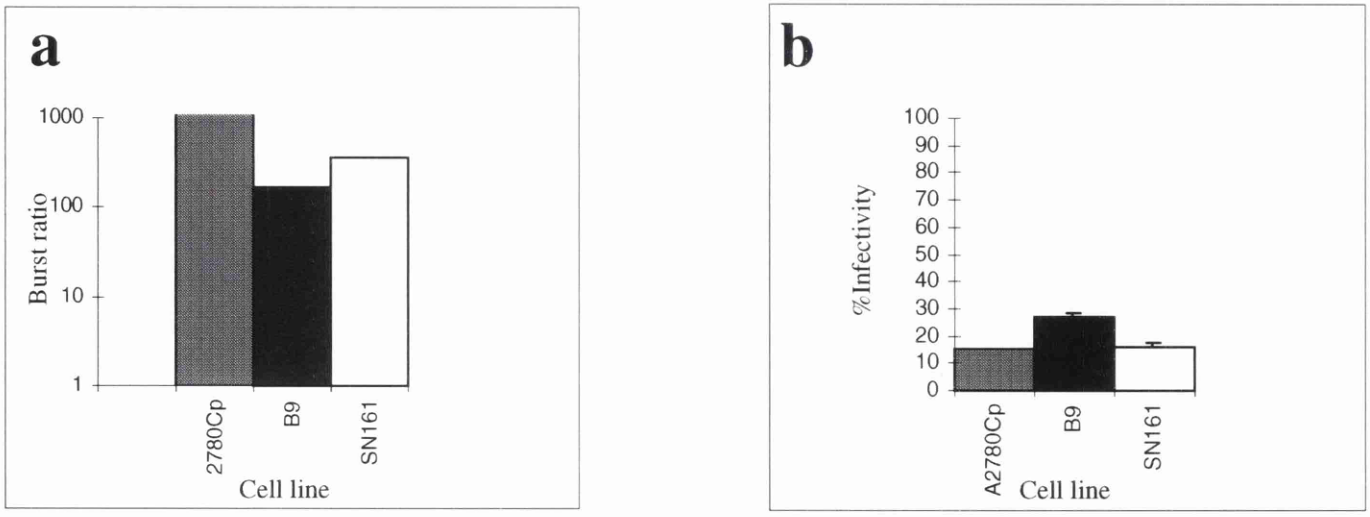


Figure 10.

Burst assay (a) and infectivity assay (b) on mouse epidermal cell lines compared to human ovarian cell line A2780Cp70.

determined using the LacZ adenovirus. These results show that mouse epidermal cell lines can produce a productive viral yield but that the efficiency is x25-50 less than human cells.

d) Early adenoviral gene expression of infected rodent cells.

Previous results on rodent fibroblasts have suggested that the expression of adenovirus E1A is repressed following viral uptake by the presence of a trans-acting transcriptional repressor protein ϕAP3 (Fognani and Babiss ,1993). In order to determine whether this mechanism is operative in a wide range of rodent cell types, protein lysates were made from cells 24hours after infection with Ad2 at a MOI of 100pfu/cell. Western immunoblotting was carried out using the mouse monoclonal antibody M73 which detects the E1A proteins. A vinculin mouse monoclonal antibody was used as a loading control. **Figures 11a and 11b** show that all rodent cells were able to express the early gene E1A proteins after infection. E1A expression was expressed relative to vinculin expression for each cell line using densitometry **(Table 3)** and then expressed as a percentage relative to the cell line with maximum E1A expression which was Morris liver hepatoma cell line .

Within the limits of this experiment (vinculin levels may also vary between different cell lines) there was no obvious correlation between E1A expression and infectivity e.g. the Morris hepatoma cell line which is highly infectible has the highest E1A expression, but cell line 9L-82 which is equally infectible has only 20% E1A expression compared to Morris. Similarly, TCMK1 is relatively highly infectible but has low E1A expression. Within the

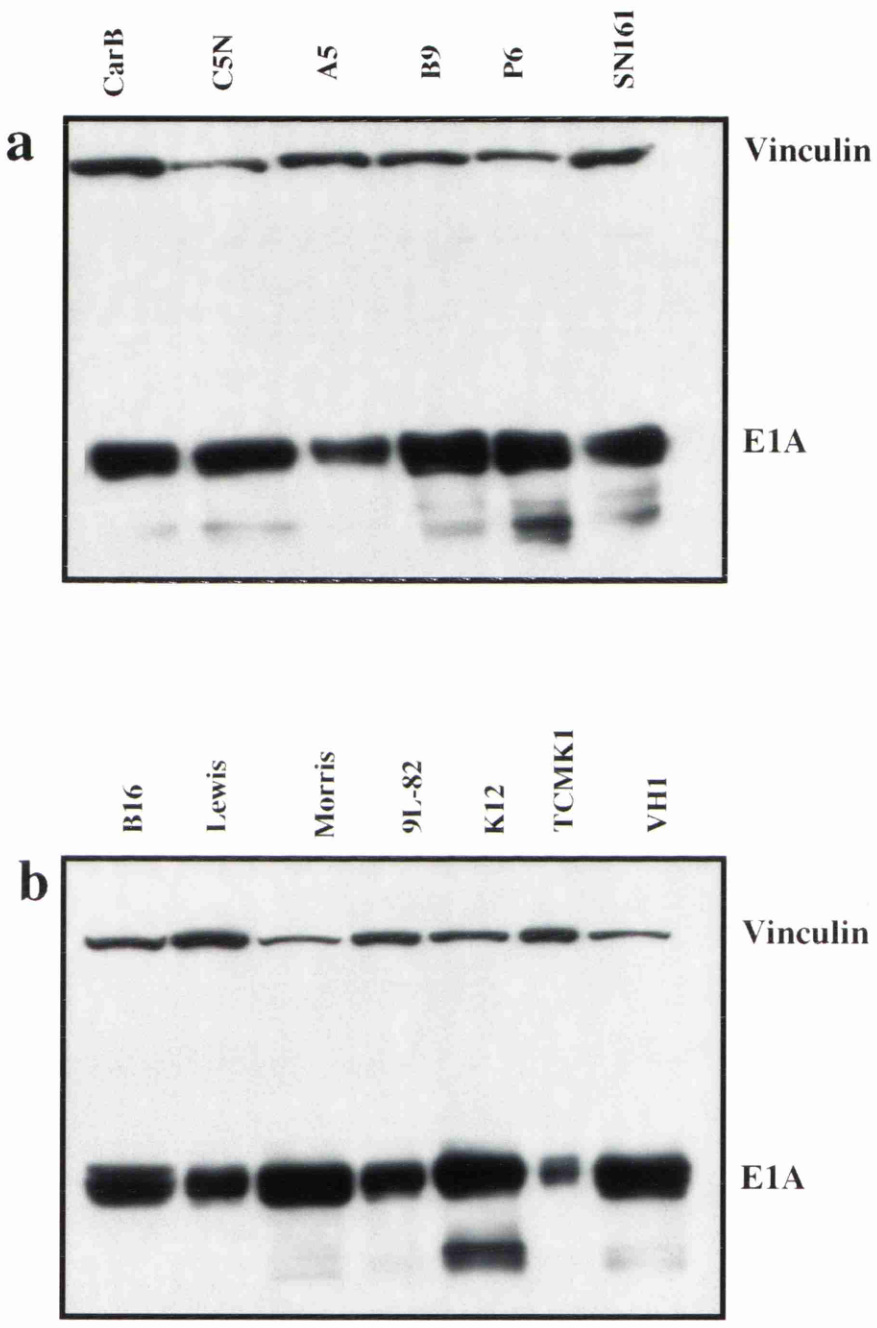


Figure 11. E1A expression of infected rodent cells.

Figure 11a shows E1A expression in mouse epidermal cell lines. E1A expression is lower in the spindle cell lines CarB and A5 compared the other cell lines which are squamous. Figure 11b shows E1A expression in different cell types.

Cell line	Species	Tissue	Relative E1A exp^{n}
			%
B16F1	Mouse	Melanoma	30.8
Lewis	Mouse	Lung carcinoma	12.3
3T3	Mouse	Fibroblasts	ND
K12/TrB	Rat	Colon carcinoma	48.9
TCMK1	Mouse	Kidney adenocarcinoma	6.3
Morris	Rat	Liver hepatoma	100
9L-82	Rat	Glioblastoma	19.2
VH1 VRS2	Rat	Thyroid carcinoma	85.2
A5	Mouse	Epidermal spindle Ca	11.3
B9	Mouse	Epidermal squamous Ca	36.2
SN161	Mouse	Epidermal sp/sq Ca	20.8
CarB	Mouse	Epidermal spindle Ca	14.8
P6	Mouse	Epidermal papilloma	56.8
C5N	Mouse	Immortalised keratinocyte	61.8

Table 3. Relative E1A expression of wild type adenovirus infected rodent cell lines. E1A expression was expressed relative to vinculin expression for each cell line using densitometry and then expressed as a percentage relative to the cell line with maximum E1A expression which was Morris liver hepatoma cell line.

epidermal cell lines tested, all of which have comparable infectivities, the E1A expression also varied .

There was also no obvious correlation between E1A expression levels in the different tissue types and ability to support replication, e.g. Morris hepatoma showed high infectivity, high E1A expression, but no evidence of replication as determined by hexon protein staining. In fact, hexon protein expression was only present in the colon cell line K12/TrB and the thyroid cell line VH1 VRS2 and in both of these cases was much less than squamous epidermal cell lines tested. This indicated that there was a block to late phase protein expression in most tissue types with the exception of mouse epidermal cells. However, when we compared E1A expression with replication in the epidermal cell lines we found a relatively good correlation. We compared E1A expression levels in two independent pairs of well differentiated squamous cell lines (B9 and E4) with their undifferentiated spindle counterparts (A5 and H11 respectively). In each of these pairs of cells the spindle variant was derived from the same primary tumour as the squamous cell (Stoler et al,1993; Burns et al ,1991). **Figure 12** shows that E1A expression correlated with the degree of cellular differentiation, with poorly differentiated cells i.e. spindle cell lines showing reduced expression. The more differentiated cells also were more permissive for replication, as shown by the relatively increased staining with anti-hexon protein antibodies. Preferential replication of Ad2 for well differentiated cells has also been reported for human keratinocytes (Laporta and Taichman ,1981).

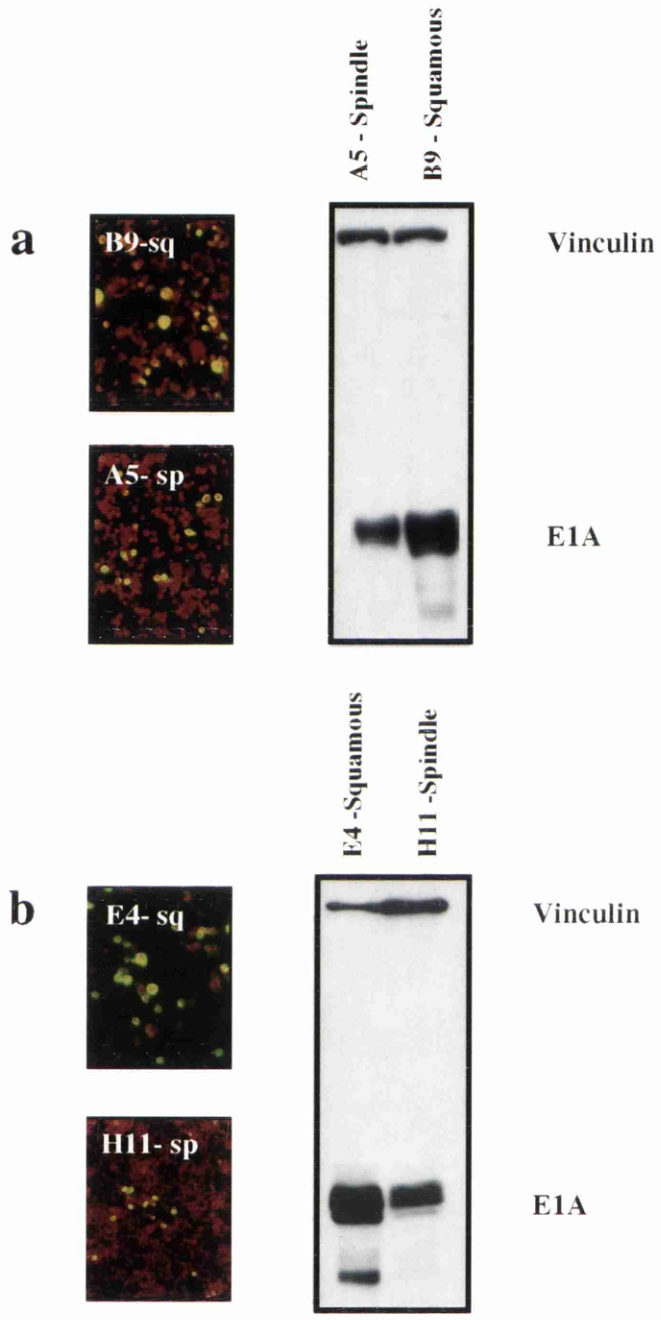


Figure 12. E1A expression and hexon protein staining in squamous/spindle paired cell lines.

Figure 12a shows that E1A expression and hexon protein expression is greater in the squamous phenotype B9. Similarly, in Figure 12b, the squamous cell line E4 also expresses E1A and hexon protein to a greater extent than the spindle cell line H11.

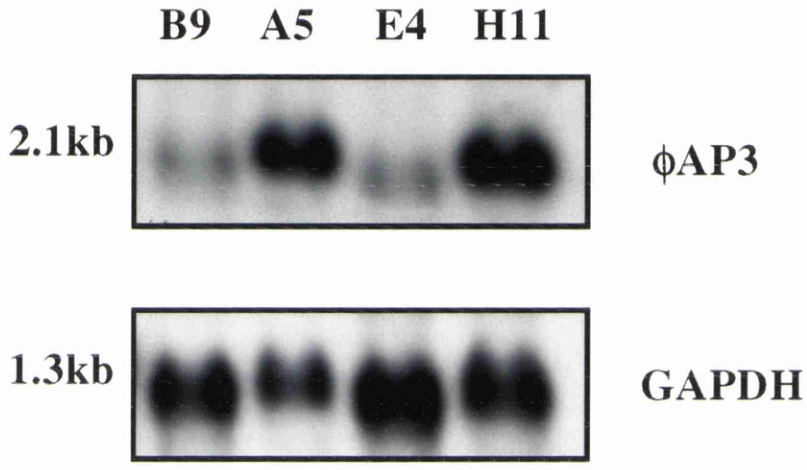


Figure13. mRNA expression of ϕAP3 in squamous/spindle paired epidermal mouse cell lines.

The expression of ϕAP3 is much lower in the spindle phenotype for both pairs of cell lines.This suggests that reduced expression of E1A in the spindle cell lines is due to high expression of the ϕAP3 protein.

e) Expression of φAP3 determines E1A expression in mouse epidermal cell.

We hypothesised that the reduced E1A expression in the spindle epidermal cells may be due to repression of the E1A promoter by the nuclear factor ϕAP3 , as described in mouse fibroblasts by Fognani and Babiss ,1993. We therefore examined the expression of ϕAP3 mRNA in the paired cell lines A5(spindle) vs B9(squamous) and H11(spindle) vs E4(squamous). We made a 651bp probe to detect the ϕAP3 mRNA which was of length 2.1kb using GAPDH as a loading control. **Figure 13** shows that in both cell pairs ϕAP3 mRNA expression was 20 fold higher in the spindle cell phenotype (A5 and H11) compared to the squamous cell phenotype (B9 and E4 respectively) This suggested that reduced expression of E1A and therefore reduced replication in the poorly differentiated cell type (i.e. spindle) was due to high expression of the ϕAP3 gene.

3.1.2. Selective replication of an E1B deficient adenovirus in mouse epidermal cell lines.

a) p53 sequence and protein expression of mouse epidermal cell lines.

The p53 sequence of the mouse epidermal tumour cell lines A5, B9, PDVc57 and SN161 have all been previously published (Burns et al,1991) and are mutant. The p53 sequence spanning the highly conserved domains II-V in the cell lines CarB(carcinoma), P1(papilloma) , P6(papilloma) and C5N(immortalised keratinocyte) were amplified by polymerase chain reaction (PCR)

Cell line	Phenotype	Treatment	Codon	Mutation	amino acid change	Reference
A5	Spindle carcinoma	DMBA	236	TGC>TTC	Cys>Phe	Burns et al,1991
			241	AAC>ATC	Asn>Ile	
			246	CTT>CTA	No change	
B9	Squamous carcinoma	DMBA	236	TGC>TTC	Cys>Phe	Burns et al,1991
			241	AAC>ATC	Asn>Ile	
			246	CTT>CTA	No change	
SN161	Sq/Sp carcinoma			8bp deletion	Frameshift	
PDVc57	Squamous carcinoma	DMBA	231	ATG>GTG	Met>Val	Burns et al,1991
P1	Papilloma	DMBA/TPA	wt	wt	none	Haddow et al,1991
P6	Papilloma	DMBA/TPA	wt	wt	none	Haddow et al,1992
CarB	Spindle carcinoma	DMBA/TPA	wt	wt	none	Diaz-Guerra et al,1992
1^0 mouse ker	Keratinocyte	none	wt	wt	none	
C5N	Immortalised keratinocyte	none	wt	wt	none	Kulesz-Martin et al,1983

Table 4. p53 gene sequence for mouse epidermal cell lines.

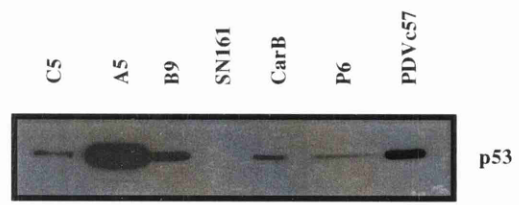


Figure 14. Western immunoblot for p53 protein expression in mouse epidermal cell lines.

Cell lines C5N,CarB and P6 have wild type p53 and low protein expression. Cell lines A5,B9 and PDVc57have mutant p53 and high protein expression. Cell line SN161 has a deletion in p53 gene and does not expressthe protein.

, fragments purified and directly sequenced using internal primers. All 4 cell lines were shown to have wild type sequence.**Table4**

Protein lysates were made from exponentially growing cells and Western immunoblotting carried out to detect p53 protein expression using the rabbit polyclonal antibody CM5 which is specific for mouse p53. **Figure 14** shows that the 4 cell lines with wild type p53 also have very low protein expression, whereas the mutant p53 cell lines A5, B9, PDVc57 express very high levels of protein.The cell line SN161, which has an 8 base pair deletion in the p53 sequence expressed no detectable p53 protein. Thus the protein expression results are consistent with the mutational analysis.

b) p53 functional status by radiation induced G1 arrest.

4 cell lines were analysed for radiation induced G1 arrest- CarB, C5N, B9,SN161. Both CarB and C5N which have wild type p53 showed a G1 arrest at 10-12 hours post radiation as shown in **Figure 15**. For C5N the S phase fraction decreases from 42.7% to 19.5% and the G1 fraction increases from 36.1% to 55% from 4 hours to 10 hours post radiation. The S and G1 phase fractions in the control samples remain unchanged. For CarB, we also see a decrease in the S phase fraction (for both diploid and tetraploid populations) at 10 hours post radiation and this is associated with an increase in the G1 phase fraction. Western immunoblotting for p53 and p21 protein also show induction of both p53 and p21 with radiation.(Debbie Stuart- unpublished results) . In contrast, the cell lines B9(point mutation in p53) and SN161 (8bp deletion in p53) showed no G1 arrest although both showed a very strong G2 arrest as shown in **Figure 16.**

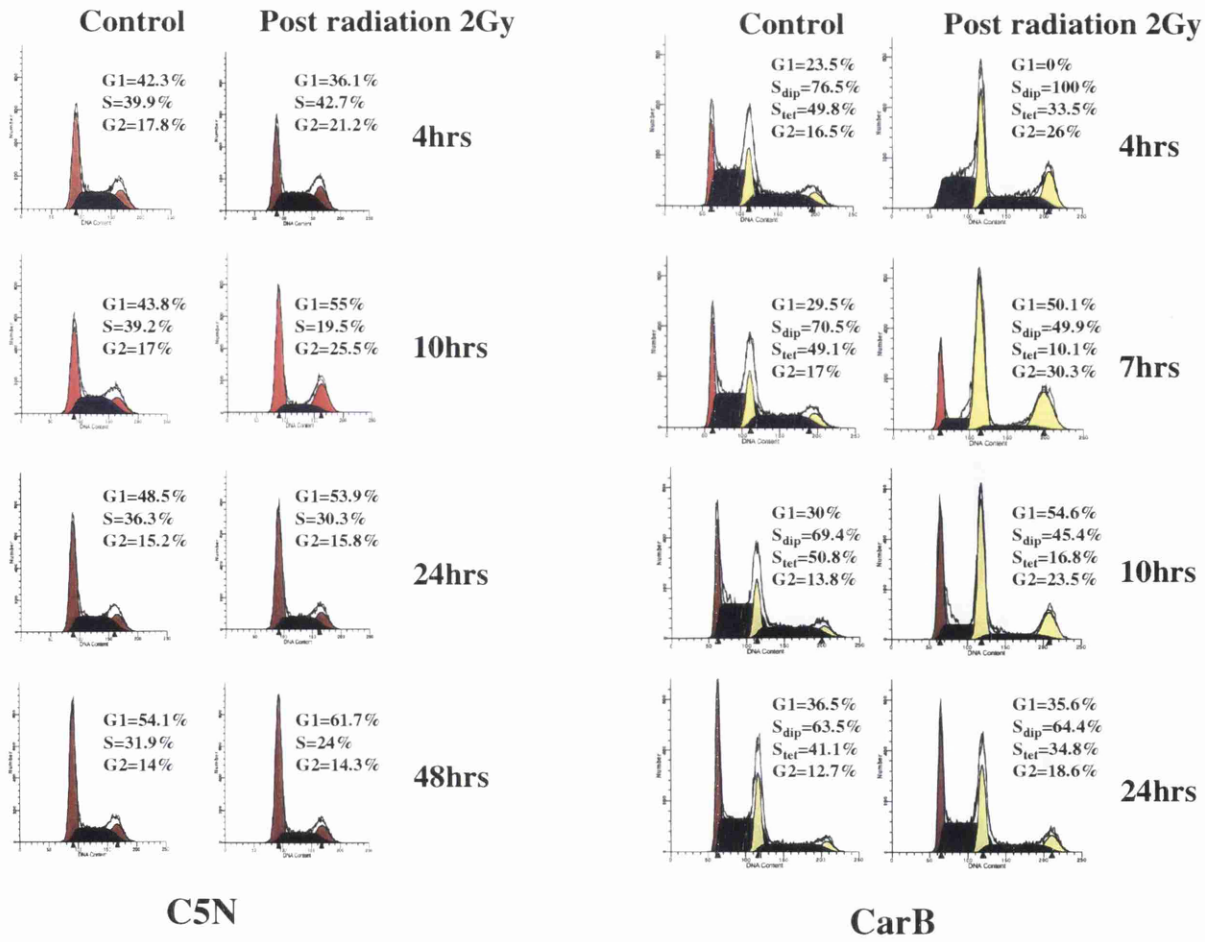


Figure15. p53 functional status of C5N and CarB by radiation induced G1 arrest.

Both cell lines show a G1 arrest at 10 hours post radiation. Cell line CarB also shows a G2 arrest at 4 hours post radiation.For CarB, G1 refers to the diploid population, G2 refers to the tetraploid population, "dip" refers to diploid and "tet" refers to tetraploid.

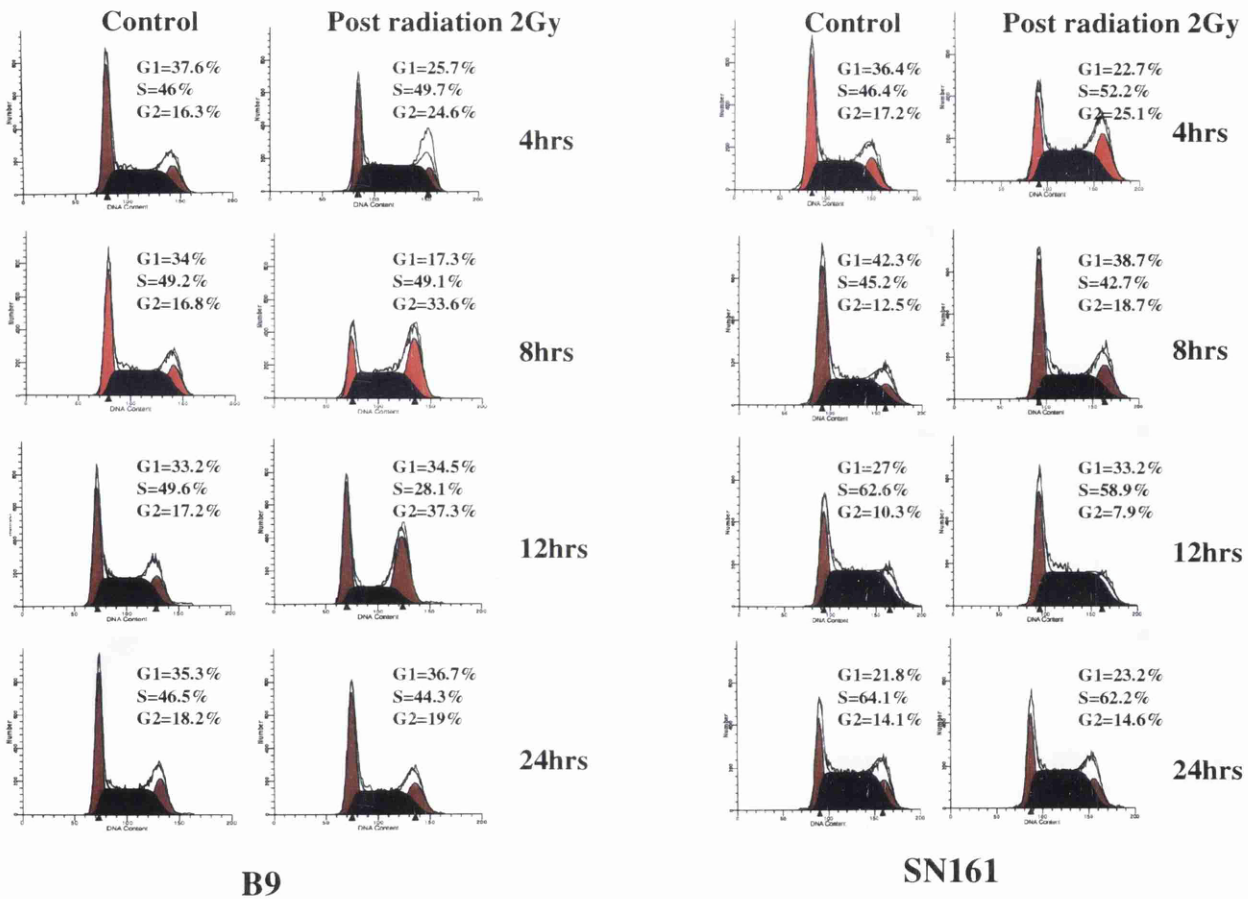


Figure 16. p53 functional status of B9 and SN161 by radiation induced G1 arrest.

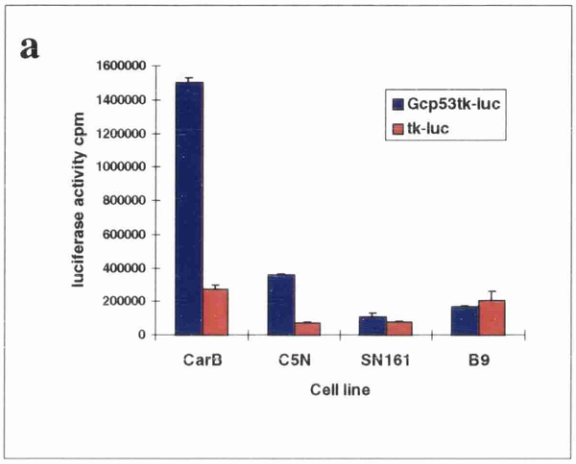
Neither cell line shows a G1 arrest post radiation indicating no p53 function. Both cell lines undergo a G2 arrest- B9 at 8-12 hours and SN161 at 4-8 hours post radiation.

c) p53 functional status by luciferase reporter assay.

Figure 17a shows the results of transfecting a luciferase reporter with the p53 consensus binding sequence (p53tkluc) compared to a control without the p53 sequence (tkluc). Both CarB and C5N showed increased expression compared to controls indicating functional p53 in both of these cell lines. The cell lines with mutant p53-B9 and SN161 showed no increase in luciferase expression which indicated no functional p53 and this correlated with the mutational analysis of these cell lines. As a control to check the p53 functionality of this reporter construct, wild type p53 was cotransfected with the reporter construct in the cell line SN161 which has a deletion in p53 **Figure 17b**. As expected luciferase activity increased enormously in the presence of wild type p53.

d) Selective replication of Onyx-015 in p53(-) mouse epidermal cell lines.

Table 5 shows the range of mouse epidermal cells tested. CPE assays and hexon protein staining were done on each cell line after infection with wild type Ad2 or Onyx-015 at a MOI of 10pfu/cell. Ad2 showed no selectivity and was able to replicate in all these cell lines. However, Onyx-015 could only replicate in those cell lines with mutant p53 demonstrating the selectivity of this virus. **Figure 18a** shows positive cytolysis after Onyx-015 infection for the mutant cell line SN161 compared to cell line P6 which has wild type p53. **Figure 18b** shows positive hexon protein staining in the mutant p53 cell line SN161 but no staining in P6.



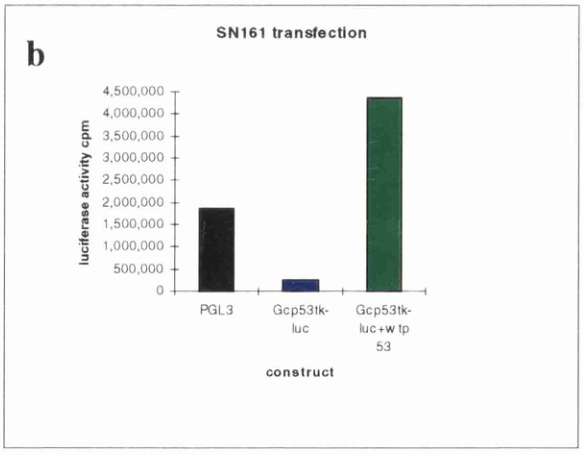


Figure 17.

p53 luciferase reporter assays on cell lines CarB,C5N,B9,SN161.

In Figure17a ,both CarB and C5N show a large increase in luciferase activity after transfection with the p53 reporter construct (Gcp53tk-luc). SN161 and B9 show similar activities for the vector alone(tk-luc) and p53 construct. In Figure17b, we have cotransfected the p53 reporter construct with wt p53 in SN161 to show that the reporter construct functions in the presence of wt p53. PGL3 is a luciferase construct used to show the efficiency of transfection control.

Cell line	p53	CPE/IF	CPE/IF
		Onyx-015	Ad2
A5	-	+	+
B9	-	+	+
H11	-	+	+
E4	-	+	+
SN161	-	+	+
PDVC57	-	+	+
CarB	+	-	+
P1	+	-	+
P6	+	-	+
1^0 mouse ker	+	+	+
C5N	+	+	+

Table 5. Cytopathic effect(CPE) and immunofluorescence (IF)for hexon protein in Ad2 and Onyx-015 infected p53(+) and p53(-) mouse epidermal cell lines.

CPE and immunofluorescence were carried out at a MOIof 10pfu/cell. Plus or minus symbols refer to the presence or absence of cytolysis (for CPE) or green fluorescent hexon protein staining cells (for IF).

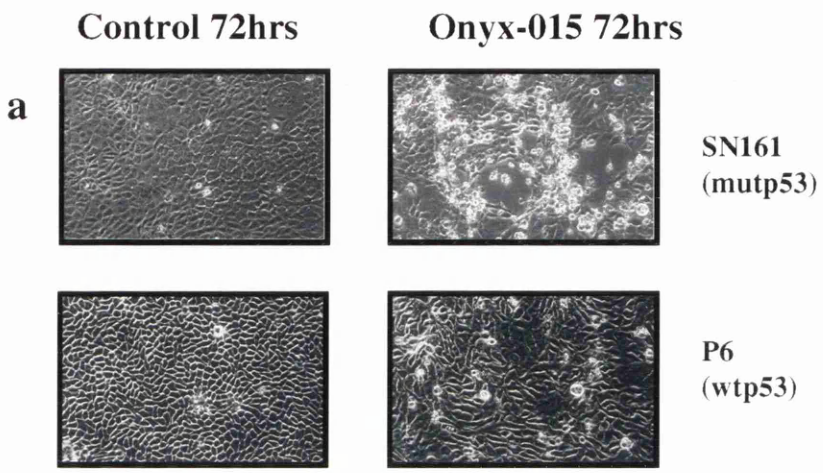


Figure 18a.

After infection with Onyx-015, the cell line SN161(mutant p53) shows cytolysis, whereas the cell line P6(wild type p53) shows markedly reduced cytolysis.

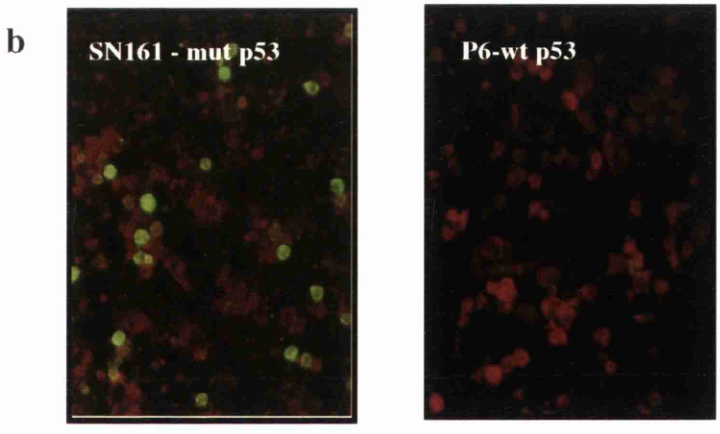


Figure 18b.

The cell line SN161(mutant p53) shows positive hexon protein staining after infection with Onyx-015 at MOI of 10pfu/cell. The cell line P6(wild type p53) shows no hexon protein staining.

e) Productive adenoviral infection in SN161.

We have previously shown that human adenovirus can produce a productive infection in mouse epidermal cell lines. To determine if Onyx-015 could also produce a productive infection, the cell line SN161 was infected with wild type Ad2 or Onyx-015 and serial plaque assays carried out over a time course of 72 hours. **Figure 19** shows that both viruses produced a productive infection and the burst increase were very similar.

f) Replication of Onyx-015 in primary mouse keratinocytes.

Although we have shown that Onyx-015 will selectively replicate in mouse epidermal cell lines with mutant p53, we also found that primary mouse keratinocytes allow replication and lysis. This result would suggest that although primary keratinocytes have wild type p53 sequence, the function may be repressed. Transgenic mice with a lacZ reporter adjacent to the p53 consensus binding site have been made recently to examine p53 function in mouse tissues in response to DNA damage (Hall et al,1996) In response to ionising radiation , mouse keratinocytes in these mice fail to show p53 transcriptional activity(Debbie Stuart unpublished results). This suggests that in primary mouse keratinocytes, the p53 gene is functionally inactive. Weinberget al,1995 showed that p53 mediated transcriptional activity increased with differentiation in epidermal keratinocytes. Since primary keratinocytes are largely derived from the basal layer (undifferentiated) of the epidermis , then this may also suggest that p53 transcriptional activity is less active.

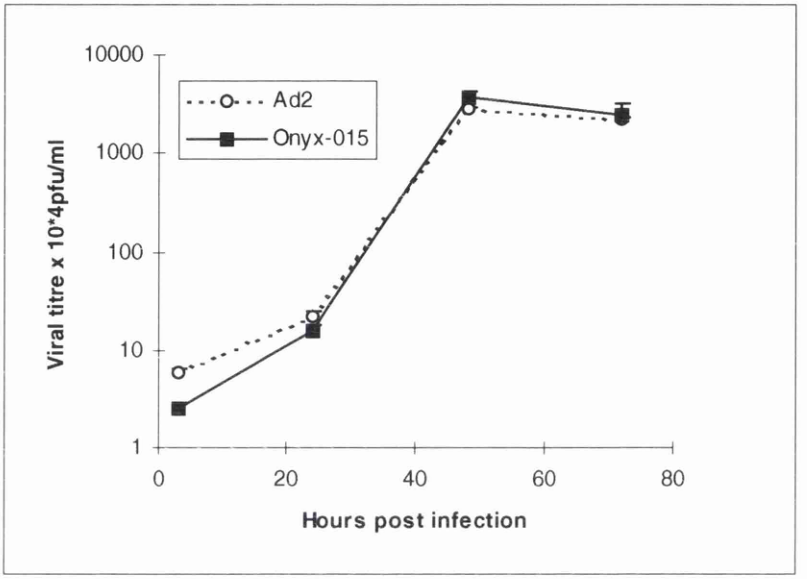


Figure19.

Serial plaque assay on mouse epidermal cell line SN161 infected with Ad2 or Onyx-015 at an MOI of 10pfu/cell.

Both viruses produce a x1000 fold increase in virus particles over a 48 hour period. This shows not only that mouse epidermal cells can produce an adenoviral burst but also that the E1B deleted adenovirus,Onyx-015, will produce a comparable viral yield to the wild type virus Ad2.

g) Replication of Onyx-015 is dependent on p21 level in wild type p53 cell lines.

The cell line C5N, which we have shown to have p53 wild type sequence and function, also allows replication to occur. To further examine the mechanism behind this we carried out Western immunoblotting on the cell lines with wild type p53 following infection with Onyx-015. Cell lines CarB, P6 and C5N were tested. **Figure 20** . In all 3 cell lines, there was a large induction of p53 protein levels. This effect of E1B deleted adenoviruses in human cell lines with wild type p53 has been shown before (Grand et al,1994). p53 increases in response to adenoviral infection and remains high due to the absence of the E1B gene since the 55kDa protein product is required for p53 degradation. In all cell lines, the p21 level also rises indicating that p53 remains functional. However in the cell line C5N the level of p21 drops rapidly at 72hours post infection despite high levels of p53, whereas in the cell line CarB and P6 the levels of p21 remain high. This may suggest that loss of p21 is required for Onyx-015 to replicate in cell lines with wild type p53.

h) Loss of p21 is due to post-transcriptional degradation and also transcriptional repression.

The loss of p21 may be due to loss of p53 transcriptional activity since Onyx-015 still expresses the E4ORF6 protein which has been shown by Dobner and Shenk, 1996 to inhibit directly the transcriptional activity of p53. Alternatively, loss of p21 may be due to post-transcriptional degradation. Recently, it has been shown that overexpression of the cellular factor $p120^{E4F}$

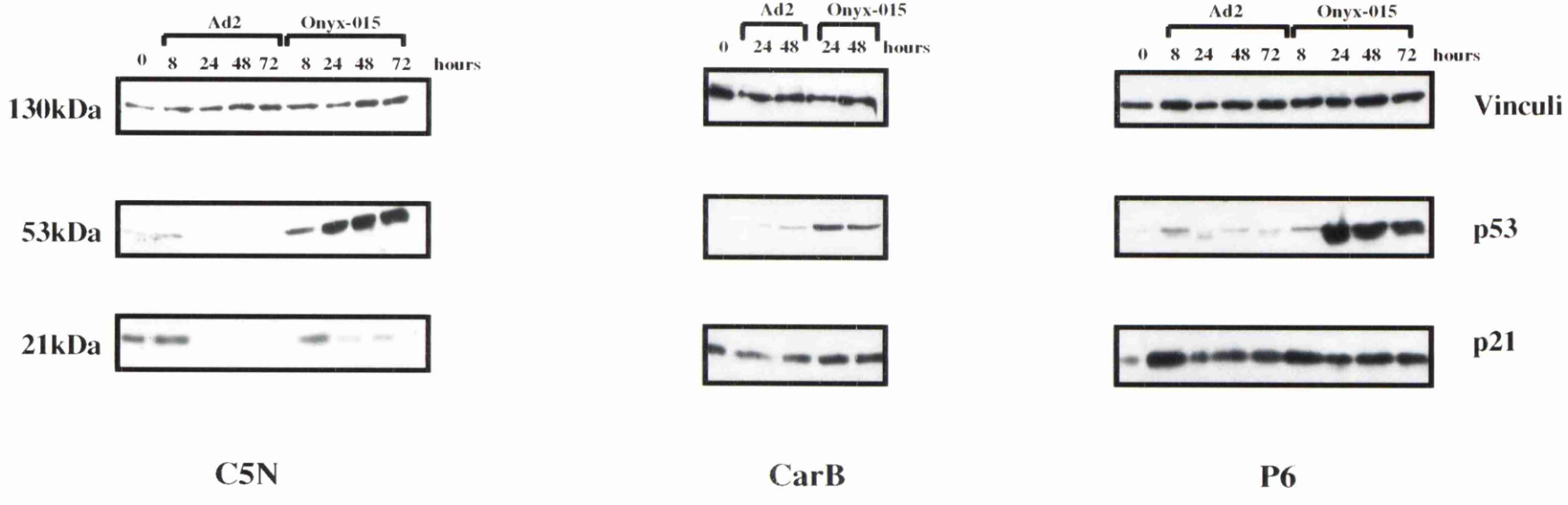


Figure 20. Western immunoblots of p53, p21 expression in cell lines C5N, CarB and P6 post infection with Ad2 and Onyx-015 at an MOI of 100pfu/cell.

All 3 cell lines have wild type p53 sequence and function. After infection with Onyx-015, no replication occurs in cell lines CarB and P6 but replication does occur in C5N. p53 protein levels are induced following Onyx infection in all 3 cell lines . However in C5N, p21 levels are reduced , whereas they are increased in CarB and P6. Thus loss of p21 protein may be a requirement for efficient replication to occur with Onyx-015.

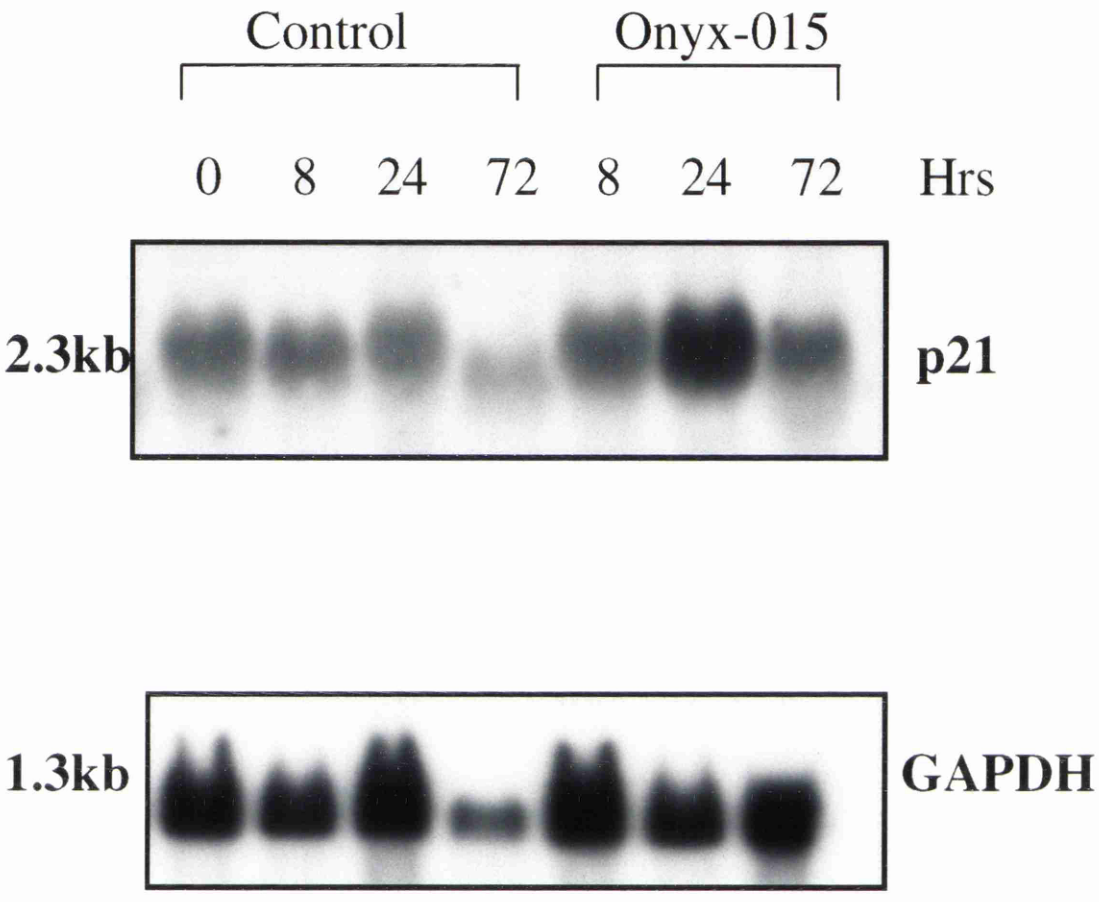


Figure 21. mRNA expressin of p21 in cell line C5N afterinfection with Onyx-015 at an MOI of 100pfu/cell.

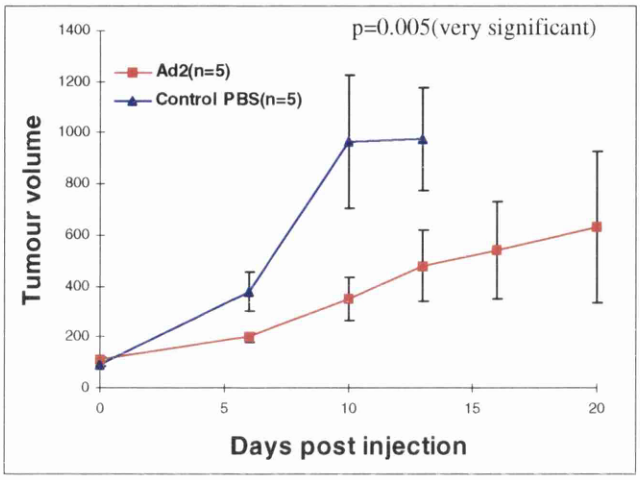
p21 mRNA expression is increased at 24 hours post Onyx infection. However in figure 20, p21 protein levels are reduced compared to 8 hours post infection despite high levels of p53 protein. This indicates that the reduction in p21 protein at 24 hours is by post-translational degradation. At 72 hours post Onyx infection, the mRNA p21 levels are reduced compared to 24 hours yet figure 20 shows high levels of p53 protein. This would suggest that there is also p53 transcriptional repression at 72 hours, possibly by the adenoviral protein E4ORF6.

leads to stabilisation of p21 protein by a p53 independent post-transcriptional mechanism (Fernandes et al,1998). It has also been shown that adenoviral E1A can downregulate expression of $p120^{E4F}$ (Fernandes et al,1997) and therefore reduce the half-life of p21 protein. To determine whether the loss of p21 was transcriptional or post-transcriptional, mRNA levels of p21 in Onyx-015 infected and mock infected C5N were quantified by Northern blotting. **Figure 21.** From this figure we can see that the level of p21 mRNA increases 24 hours post infection. However in figure 20, p21 protein levels are reduced compared to 8 hours post infection despite high levels of p53 protein. This indicates that the reduction in p21 protein at 24 hours is by post-transcriptional degradation. At 72 hours post Onyx infection, the mRNA p21 levels are reduced compared to 24 hours yet figure 20 shows high levels of p53 protein. This would suggest that there is also p53 transcriptional repression at 72 hours, possibly by the adenoviral protein E4ORF6.(Dobner and Shenk ,1996).

3.1.3. In Vivo Studies.

a) Anti-tumour activity of Onyx-015 in B9 subcutaneous xenografts in nude mice.

The mutant p53 mouse squamous epidermal cell line B9 was selected for in vivo testing. Subcutaneous xenografts were formed in nude mice and once tumours were 5-10mm in maximum diameter, the tumours were injected at 10^8pfu with either wild type adenovirus or Onyx-015 daily for 5 days. Control tumours were injected with diluent of PBS. Tumour diameters were then measured twice weekly and growth curves plotted. **Figure 22a** shows the growth curve for Ad2 and **Figure 22b** shows the growth curve for Onyx-015.



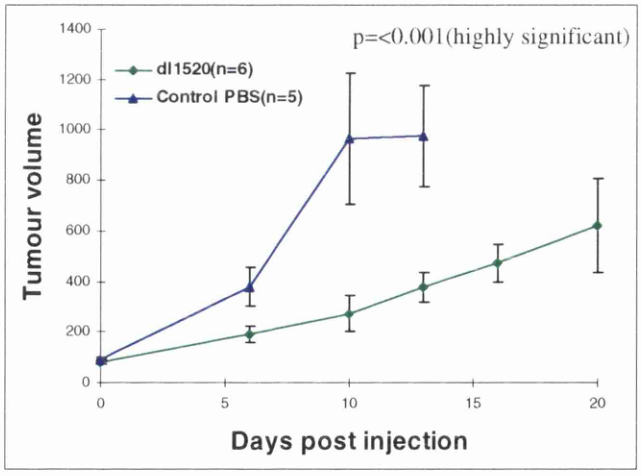


Figure 22. Growth curves for B9 nude mouse xenografts injectedwith Ad2 or Onyx-015.

Injection with either Ad2 or Onyx-015 at 10^8pfu for 5 daily injections produced a reduction in growth compared to control mice. Both curves were statistically significant with a p value of 0.005 for Ad2 (very significant)and <0.001 for Onyx-015(highly significant).

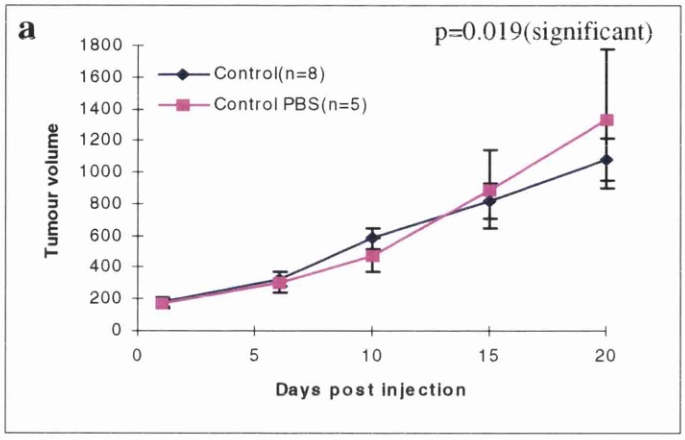
As can be seen, control tumours continued to grow exponentially whereas Ad2 and Onyx-015 injected tumours underwent tumour regression. Both curves were statistically analysed using unpaired two-tailed t-test and found to be statistically significant. The p-value for Ad2 versus Control was 0.005 and the p-value for Onyx-015 versus Control was <0.001.

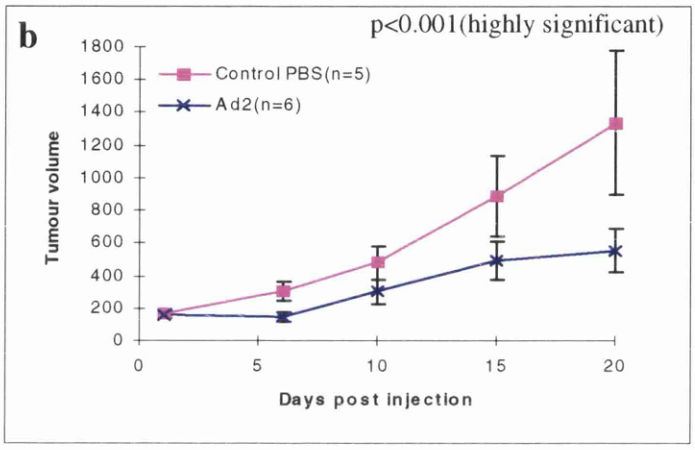
b) Anti-tumour activity of Onyx-015 in PDVc57 subcutaneous xenografts in nude mice.

The mouse cell line PDVc57 has mutant p53 and is a squamous epidermal cell line. This cell line grows in the syngeneic host C57bl6 mice and therefore is a good model to compare efficacy of virus treatment in both an immunoincompetent host (nude mouse) and an immunocompetent host (C57bl mouse).

i) Efficacy.

Subcutaneous xenografts were formed in nude mice and once tumours were 5-10mm in maximum diameter, the tumours were injected at 10^8pfu with either wild type adenovirus or Onyx-015 daily for 5 days. Control tumours were injected with diluent of PBS. In addition, an extra control arm was included to compare the effect of PBS injection with no injection of diluent. Tumour diameters were then measured twice weekly and growth curves plotted. **Figure 23a** shows the growth curve for PBS versus no injection, **Figure 23b** shows the growth curve for Ad2 versus PBS and **Figure 23c** shows the growth curve for Onyx-015 versus PBS. As can be seen, PBS control tumours continued to grow exponentially and at a slightly faster rate than uninjected control tumours (p=0.019). This showed that there was no





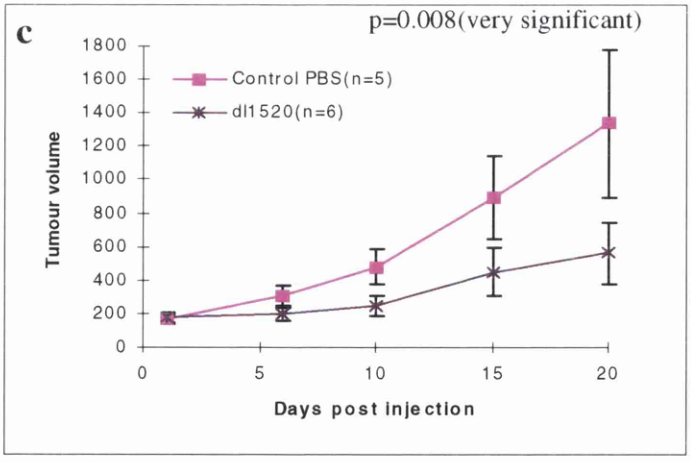


Figure 23. Growth curves for PDVc57 xenografts in nude mice injected with Ad2 or Onyx-015(dl1520) or control solution PBS. Tumour volumes are in mm^3.

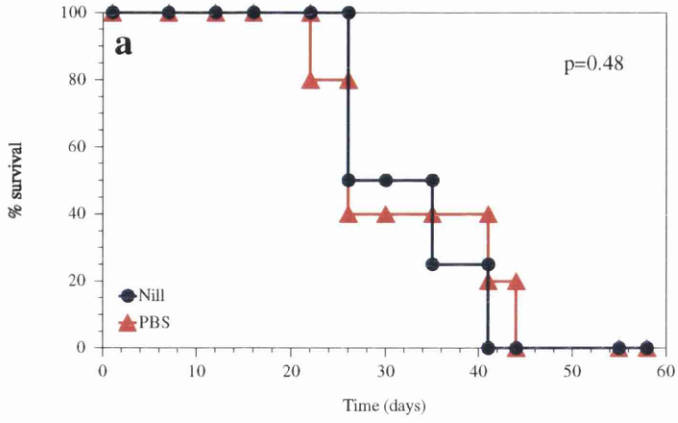
antitumour effect with the injection procedure alone. Ad2 and Onyx-015 injected tumours had much slower growth rates compared to PBS control tumours. Both curves were statistically analysed using unpaired two-tailed t-test and found to be statistically significant. The p-value for Ad2 versus PBS was <0.001 and the p-value for Onyx-015 versus PBS was 0.008.

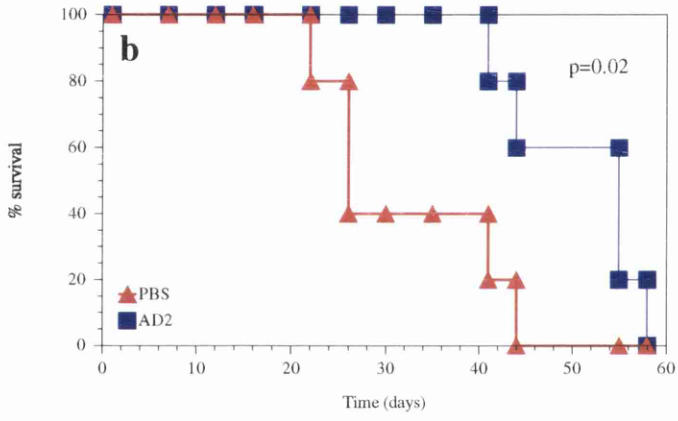
ii)Survival.

Survival of animals in each group was analysed using the method of Kaplan and Meier, and Kaplan-Meier plots for treated and control groups were compared for statistical significance using the Log rank test. **Figure 24a** shows the survival plot for PBS versus no injection, **Figure 24b** shows the survival plot for Ad2 versus PBS and **Figure 24c** shows the survival plot for Onyx-015 versus PBS. As can be seen, the survival plots for PBS versus no injection were similar and not statistically significant (p=0.48). The survival plot for Ad2 versus PBS was significant with a p value of 0.02. The survival plot for Onyx-015 versus PBS is shown on **Figure 24c.** Although the plots show a greater survival for Onyx-015 injected tumours, the plots were not statistically different (p=0.18).

iii)Viral replication in tumours.

To determine whether or not the anti-tumour effect of Ad2 and Onyx-015 was due to viral replication, it was necessary to show the presence of viral replication in the injected tumours. Tumours were stained by in situ hybridisation using a probe against adenoviral DNA. **Figures 25** shows examples of typical staining seen. The cells staining positive were always located at the border between necrosis and viable tumour. All viral injected tumours stained positive by in situ hybridisation. These results showed that the





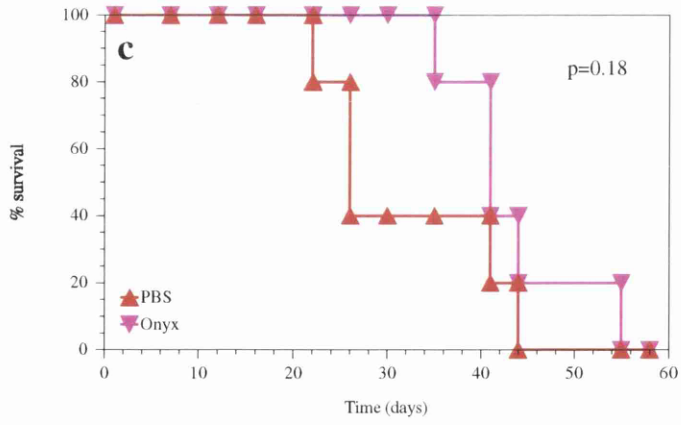


Figure 24.
Survival curves for nude mice with PDVc57 xenografts injected with Ad2 or Onyx-015.

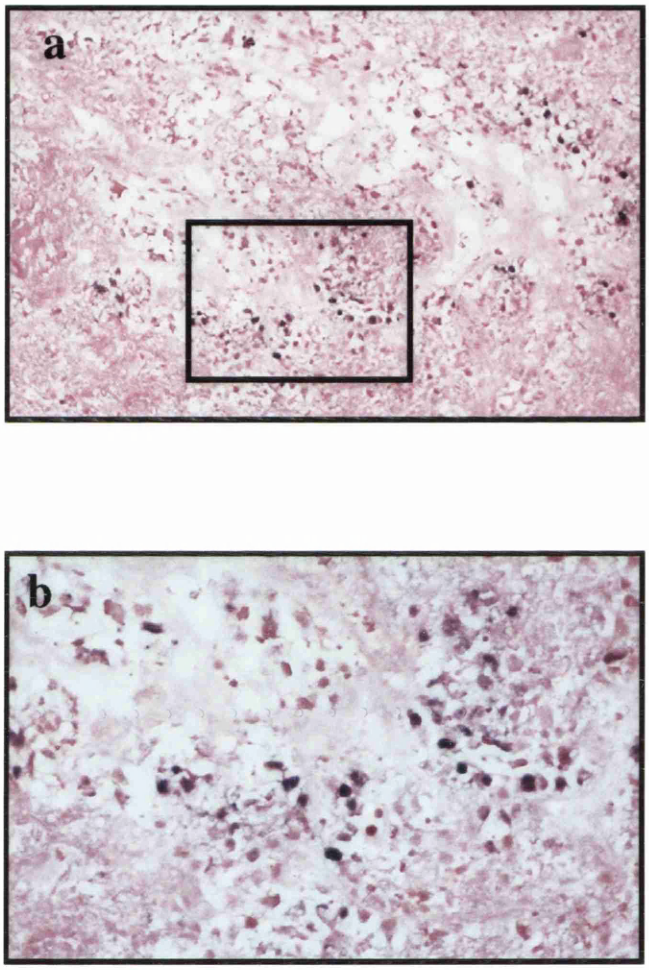


Figure25. Detection of adenoviral DNA in PDVC57 nude mouse xenografts by in situ hybridisation.

Cells expressing adenoviral DNA stain dark blue and uninfected cells stain red with the counterstain nuclear fast red.Figure 25a is at low power and figure 25b at high power. The cells expressing adenovirus were always located at the area between necrotic tissue and viable tumour.

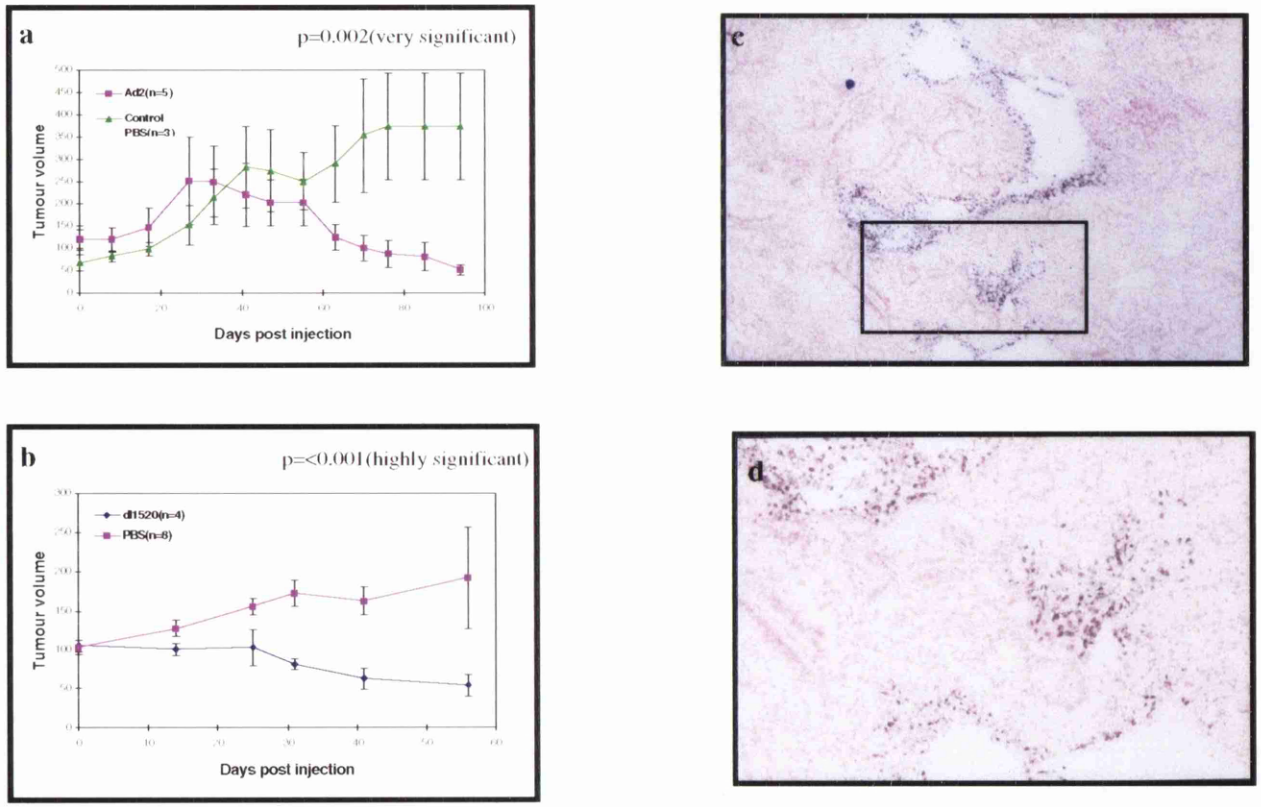


Figure 26. Growth curves for human nude mouse xenografts BICR16 and detection of adenoviral DNA in tumours by ISH.

With the human HNSCC cell line BICR16 , nude mouse xenografts underwent regression (figure a/b) after injection with 5 daily injections with either Ad2 or Onyx-015 at 10^8pfu per injection. This is in contrast to the mouse cell lines B9 and PDVc57 which only showed a reduced growth rate of tumours. Figure c/d show adenoviral DNA detected by in situ hybridisation at low and high power.This shows that there is more adenoviral positive staining cells in comparison to the PDVc57 nude mouse tumours. This indicates that there is reduced replication in the PDVc57 model and this accounts for the fact we only see a reduction in growth rate of tumours rather than tumour regression.

anti-tumour effect observed in the mouse model was due to viral replication. However , if we compare the degree of replication in the PDVc57 mouse xenograft with a xenograft derived from a squamous cell cancer cell line from humans (BICR16) we can see that the extent of replication is lower (**Figure 26c/d**). This is in keeping with our *in-vitro* results in which we showed that replication in the mouse cell line is ~x50 less efficient than the human cell line. Also if we compare the growth curves of PDVc57 xenografts with BICR16 xenografts we can see that in the human xenografts we observe regression of the tumours but in the mouse xenografts we only see delayed growth and not regression (**Figure 26a/b**). Therefore in the mouse model, the faster rate of growth of the cell line combined with the reduced viral replication of virus in the mouse cell line results in delayed growth as opposed to regression of tumours.

c) Anti-tumour activity of Onyx-015 in PDVc57 subcutaneous xenografts in syngeneic C57bl6 mice.

i) Efficacy.

Subcutaneous xenografts were formed in syngeneic mice by tumour transplantation and allowed to grow to 5-10mm in maximum diameter. The tumours were then injected at 10^8pfu with either wild type adenovirus or Onyx-015 daily for 5 days. Control tumours were injected with diluent of PBS. In addition an extra control arm was included to compare the effect of PBS injection with no injection of diluent. Tumour diameters were then measured twice weekly and growth curves plotted. **Figure 27a** shows the growth curve for PBS versus no injection, **Figure 27b** shows the growth curve for Ad2

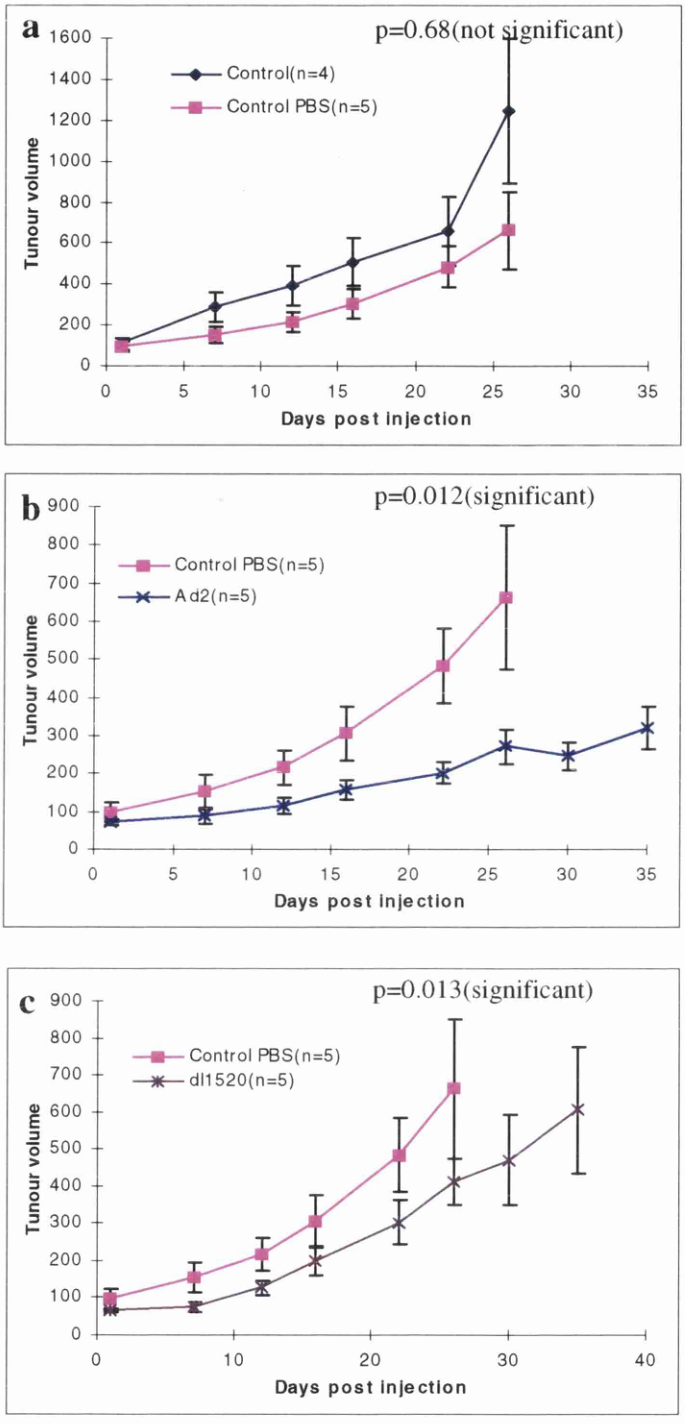


Figure 27. Growth curves for PDVc57 xenografts in syngeneic mice injected with Ad2 or Onyx-015(dl1520) or control solution PBS. Tumour volumes are in mm^3.

versus PBS and **Figure 27c** shows the growth curve for Onyx-015 versus PBS. As can be seen, PBS control tumours continued to grow exponentially and there was no difference to the growth curve of the uninjected group (p=0.68). Ad2 and Onyx-015 injected tumours had much slower growth rates compared to PBS control tumours. Both curves were statistically analysed using unpaired two-tailed t-test and found to be statistically significant. The p-value for Ad2 versus PBS was 0.012 and the p-value for Onyx-015 versus PBS was 0.013. Thus, even though these mice had an intact immune system virus injection was still able to reduce the growth of the tumours.

ii)Survival.

Figure 28a shows the survival plot for PBS versus no injection, **Figure 28b** shows the survival plot for Ad2 versus PBS and **Figure 28c** shows the survival plot for Onyx-015 versus PBS. As can be seen, the survival plots for PBS versus no injection were similar and not statistically significant (p=0.41). The survival plot for Onyx-015 versus PBS was significant with a p value of 0.04. The survival plot for Ad2 versus PBS is shown on **Figure 28b.** Although the plots show a greater survival for Ad2 injected tumours, the plots were not statistically different (p=0.15).

iii)Viral replication in tumours.

To determine whether or not the anti-tumour effect of Ad2 and Onyx-015 was due to viral replication, it was necessary to show the presence of viral replication in the injected tumours. Tumours were stained by in situ hybridisation using a probe against adenoviral DNA. None of the mice injected with Onyx-015 showed any evidence of viral DNA in the tumours. Only 2/6 of the Ad2 injected tumours showed viral DNA. This is in comparison to the nude

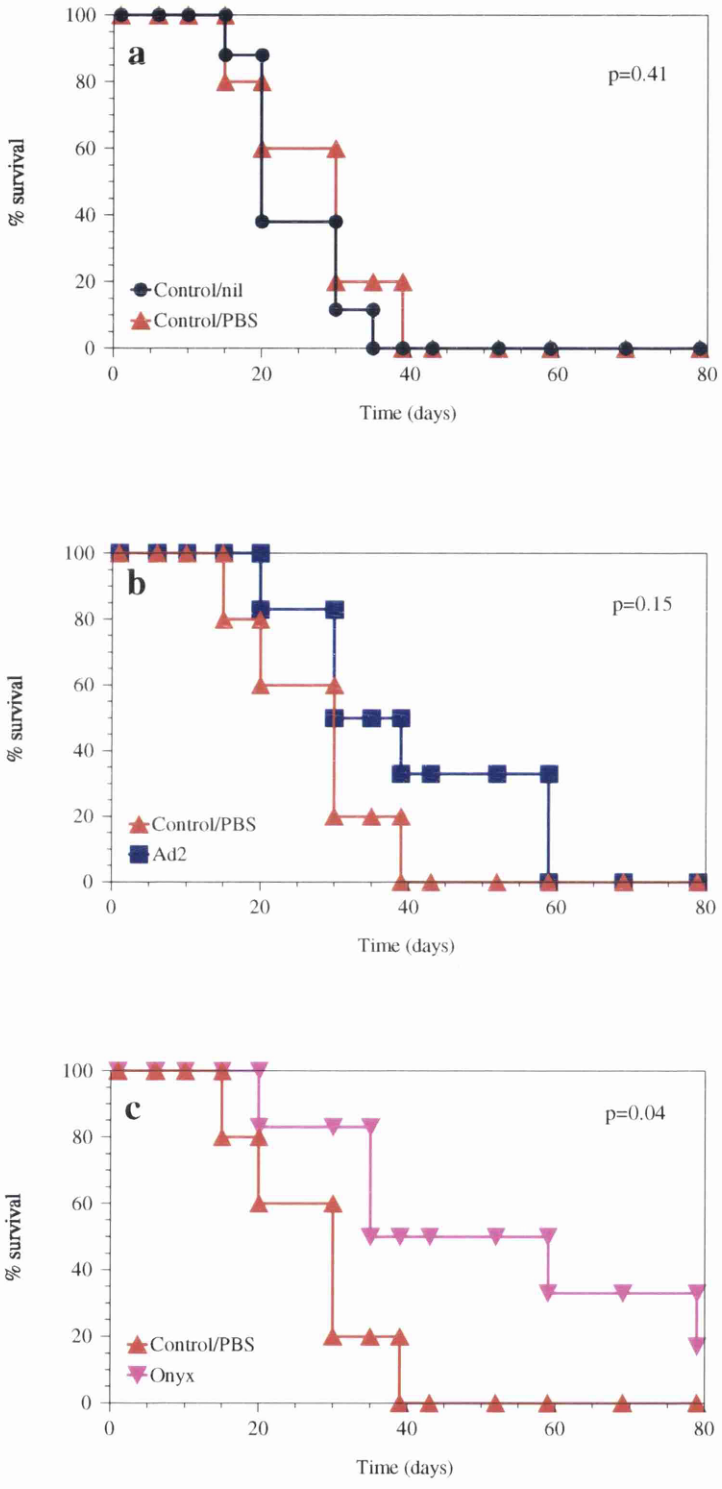


Figure 28.
Survival curves for syngeneic mice with PDVc57 xenografts injected with Ad2 or Onyx-015.

mouse model where all tumours showed evidence of viral DNA in both Onyx-015 and Ad2 injected groups. In addition, the degree of replication seen in the syngeneic mouse tumours was also much less than the nude mouse model.

These results suggested that the immune system may be preventing efficient replication of virus, either by a rise in neutralising antibody or cytotoxic T lymphocyte infiltration. Blood samples from the mice were not taken for anti-adenovirus neutralising antibody. However, Smith et al,1996 have reported a rise in antibody titre in C57bl mice when injected with a nonreplicating adenoviral vector. Tumour samples were not stained for CD4 or CD8 lymphocytes since no antibodies are commercially available. The fact that we see both an antitumour effect and survival advantage to virus in the syngeneic host could be due to either the effect of immune effector cell infiltration, the initial effect of viral replication before virus is eliminated, or a combination of these two.

3.1.4. Conclusions

In conclusion, in vitro experiments in mouse cell lines have shown that human adenoviruses will infect rodent cell lines with variable infectivity. In mouse epidermal cells, a productive viral infection can be produced whereas there is a block to replication in other cell types. In mouse epidermal cells, the restriction point to replication is at the expression of the early gene E1A. Replication is greatest in squamous epidermal cell lines in which there is a high expression of E1A. We have shown that there is a correlation between the level of E1A and the nuclear factor φAP3 , a transcriptional repressor of the E1A promoter.

Using mouse epidermal cell lines of known p53 status and function, the selective replication of Onyx-015 for p53(-) cells is in general agreement with that reported in human cell lines with a few exceptions. Some cell lines with wild type p53 do allow replication of Onyx-015 and this may be related to the ability for Onyx-015 to repress p21 levels as shown for the cell line C5N.

Using the , squamous epidermal cell line PDVc57 , in vivo studies have shown efficacy in both nude mouse and syngeneic mouse models. However, replication is markedly reduced in the syngeneic host suggesting limitation of viral replication by the immune system.

3.2. MECHANISTIC STUDIES ON THE SELECTIVE REPLICATION OF ONYX-015 IN P53(-) CELL LINES.

Several publications have disputed the p53 selectivity of the Onyx-015 virus. Goodrum and Ornelles,1997 showed that replication only occurred in cells which were in the S phase of the cell cycle and that this was independent of the p53 status of the cell lines studies. However although the p53 sequence of the cell lines used in this study was known, the functional status was not known. Hall et al,1998 recently suggested that adenovirus induced cell death was greater in cell lines which had wild type p53. However this paper only looked at cell death in the cell lines studies but not the mechanism of cell death. i.e. viral replication or apoptosis. Therefore, another aim of this thesis was to further examine the mechanism of selectivity of Onyx-015 for p53(-) cells. To study this we utilised the human ovarian adenocarcinoma cell lines

A2780 (functional p53) and A2780Cp70 (non-functional p53 cisplatin resistant variant). To do this it was necessary to

1. Determine whether the infectivities of A2780 and Cp70 to adenoviruses were comparable.

2. Determine whether replication of adenovirus was greater in Cp70 compared to A2780.

3. Explain mechanistically the differences in replication by cell cycle analysis, p53 protein changes, and by examining the mode of cell death i.e. virus induced cytolysis or apoptosis.

3.2.1. Infectivity and replication of Onyx-015 in the human ovarian adenocarcinoma cell lines A2780 and A2780Cp70.

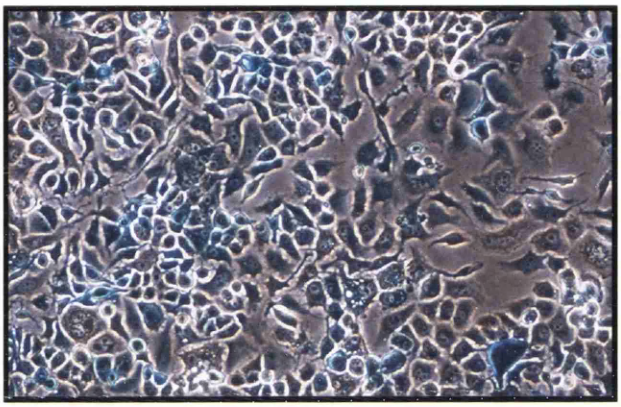
a) Infectivity of A2780 and A2780Cp70 to adenoviruses.

i) Ad5lacZ infectivity assay.

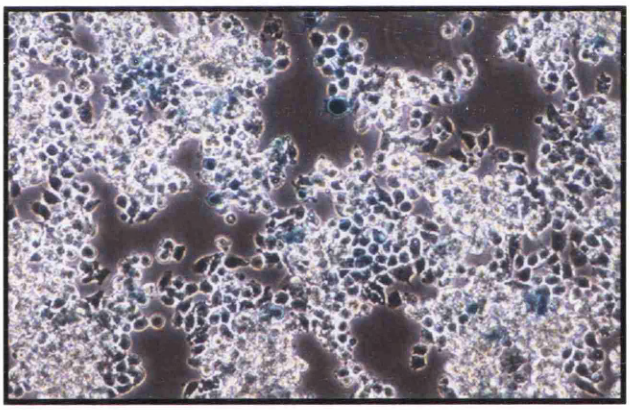
The nonreplicating E1 deleted adenovirus with a lacZ reporter construct was used to determine infectivity. The percentage of beta-galactosidase positive staining cells was determined at MOI of 10 and 100 pfu/cell as described before. The percentage of positive staining cells was used as an indicator of infectivity. This is shown in **Table 6.** From this table we can conclude that the infectivity was similar for both A2780 and Cp70 at both an MOI of 10 and 100pfu/cell. Examples of positive staining for cell lines Cp70 and A2780 at an MOI of 10pfu/cell is shown in **Figure 29.**

Cell line	Infectivity assay	Infectivity			
		10pfu/cell	s.e.	100pfu/cell	s.e.
A2780	% beta gal +ve at 24 hrs.	12	1.2	56.3	3.4
	%E1A +ve at 24 hrs.	8.4	0.7	51.3	4.5
Cp70	% beta gal +ve at 24 hrs.	14.7	2.3	57.1	3.6
	%E1A +ve at 24 hrs.	10.8	0.8	52.1	4.7

Table 6. Infectivity of A2780 and Cp70 to adenovirus by Ad5lacZ infectivity assay and by E1A immunofluorescence .



A2780Cp70
24hours



A2780
24hours

Figure 29. Beta galactosidase staining in A2780 and Cp70 infected with Ad5lacZ adenovirus at MOI of 10pfu/cell.

Cells infected with adenovirus Ad5lacZ stain blue. The percentage of infected(blue) cells to uninfected (nonblue) cells was calculated and was ~12-15% for both cell lines.

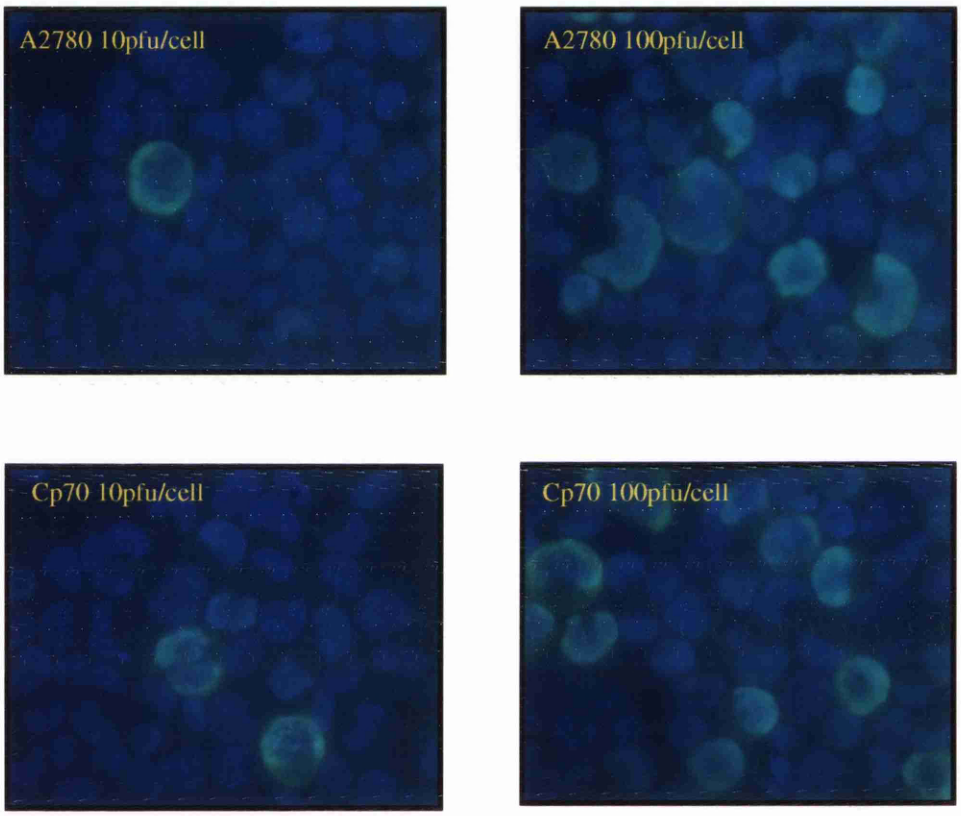


Figure 30. E1A immunofluorescence of Onyx-015 infected cells.

Cell lines A2780 and A2780Cp70 were infected with Onyx-015 at an MOI of 10 and 100pfu/cell for 24 hours. The percentage of cells infected was determined using immunofluorescent detection of cells expressing the adenoviral E1A protein. Green stained cells are labelled with an FITC labelled mouse monoclonal antibody to adenoviral E1A. Uninfected cells stain blue with the counterstain DAPI. This shows similar infectivities for both cell lines at MOI of 10(~8-10%) and 100pfu/cell(~50%).

ii) E1A infectivity assay.

Infectivity was also determined by an E1A immunofluorescence assay. Cells were infected with Onyx-015 at MOI of 10 and 100pfu/cell and the mean percentage of cells expressing E1A protein at 24 hours determined. The results are shown in **Table 6** and **Figure 30.** This showed that similar numbers of cells expressed E1A after infection suggesting equivalent infectivity for A2780 and Cp70.

b) Replication of Onyx-015 in A2780 and A2780Cp70.

i) Burst assay.

To determine if a productive infection could be produced, burst assays were carried out with wild type Ad2 and Onyx-015 at an MOI of 10pfu/cell. The burst ratio was expressed as the concentration of virus at 72 hrs divided by the concentration of virus at 4 hrs post infection. **Figure 31a** shows the results of burst assays. From this we can conclude that a productive infection occurs in the cell line Cp70 but not A2780 and this would agree with the p53 selectivity of Onyx-015. To further examine this burst assays were also carried on mutant p53 transfectants of A2780. We can see from the figure that A2780 transfected with the mutant p53 (valine 153) allows a productive infection to occur compared to vector alone or A2780.This again confirmed the p53 selectivity of Onyx-015.

ii) Hexon protein expression assay.

Replication was also determined by quantifying the percentage of cells expressing the late phase hexon protein by FACS analysis. These results were

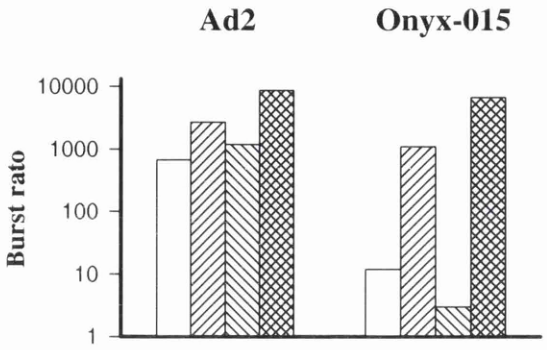


Figure 31a

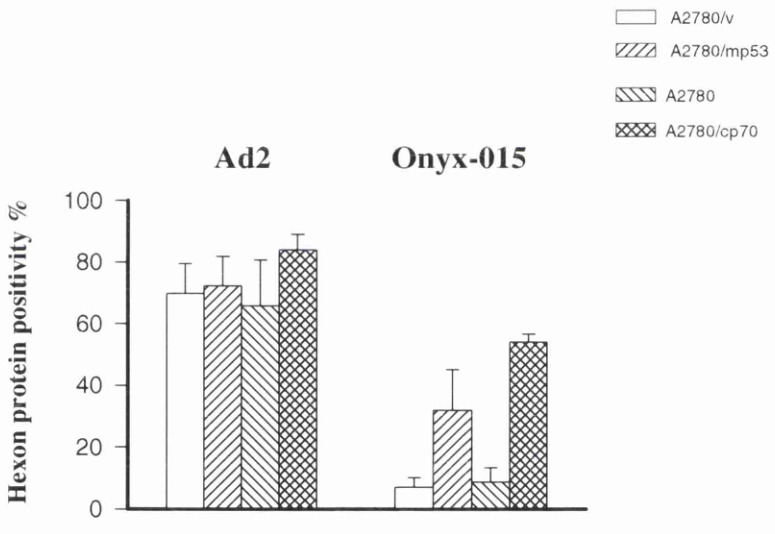


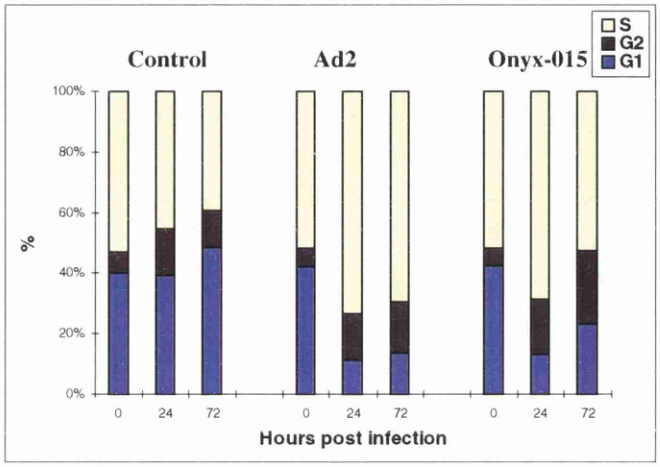
Figure 31b

Figure 31a/b. Burst assays(a) and hexon protein replication assays(b) for A2780, Cp70 , p53 mutant transfectant cell line A2780(mut) and vector alone transfectant cell line A2780(v).

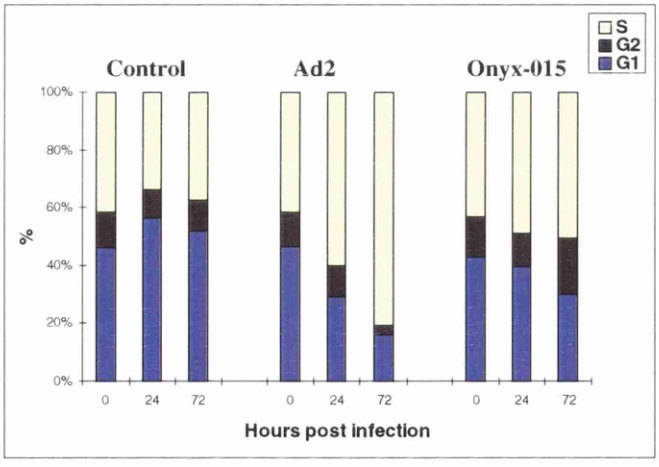
obtained by Dr Young Tae Kim and are shown in **Figure 31b**. These also showed that the percentage of hexon positive cells was greater in Cp70 compared to A2780.

c) Replication in Cp70 is due to an increase in S phase entry.

For replication to occur, adenovirus requires the host cell to be in S phase since the adenoviral replication machinery is dependent on the host cell's DNA polymerase for replication. We therefore predicted that the percentage of cells in S phase would increase in Cp70 cells infected with Onyx-015 but that this would not occur in the cell line A2780 which does not allow replication. With wild type Ad2, the percentage of cells in S phase should increase in both Cp70 and A2780. To test this hypothesis we infected cells at an MOI of 100 pfu/cell and then determined the percentage of cells in S phase by pulse BrdU labelling. Cells were pulsed with BrdU for 1 hour at time points 0, 24 and 72 hours post viral infection and the percentage staining positively detected using an FITC labelled anti-BrdU antibody by flow cytometry. **Figure 32** shows the results obtained by 2 repeat experiments. As can be seen , Ad2 induces S phase entry in both cell lines (no selectivity) at 24 hours post infection compared to uninfected controls in which there is a reduction in S phase cells. **Figure 33** shows an example of BrdU cell cycle analysis in cell line Cp70. With Onyx-015 , there is an increase in S phase cells in Cp70 over control but only a very small increase in S phase cells in A2780.



A2780Cp70



A2780

Figure 32. Cell cycle analysis of A2780 and Cp70 infected with Ad2 or Onyx at MOI 100pfu/cell.

Infected cells were harvested at 0,24 and 72 hours post infection . S phase cells were labelled with BrdU and detected with antiBrdU antibody linked to FITC. $G_1$and G_2 phase cells were detected by propidium iodide staining. Quantification of cells in S,$G_1$and G_2 phases of the cell cycle was done by flow cytometry and presented as a bar chart as shown above. In Cp70, there is an increase in the % cells in S phase at 24 hours post infection with both Ad2 and Onyxinfection. However in A2780, the increase in S phase cells only occurs with Ad2 infected cells with only a very slight increase in Onyx infected cells.

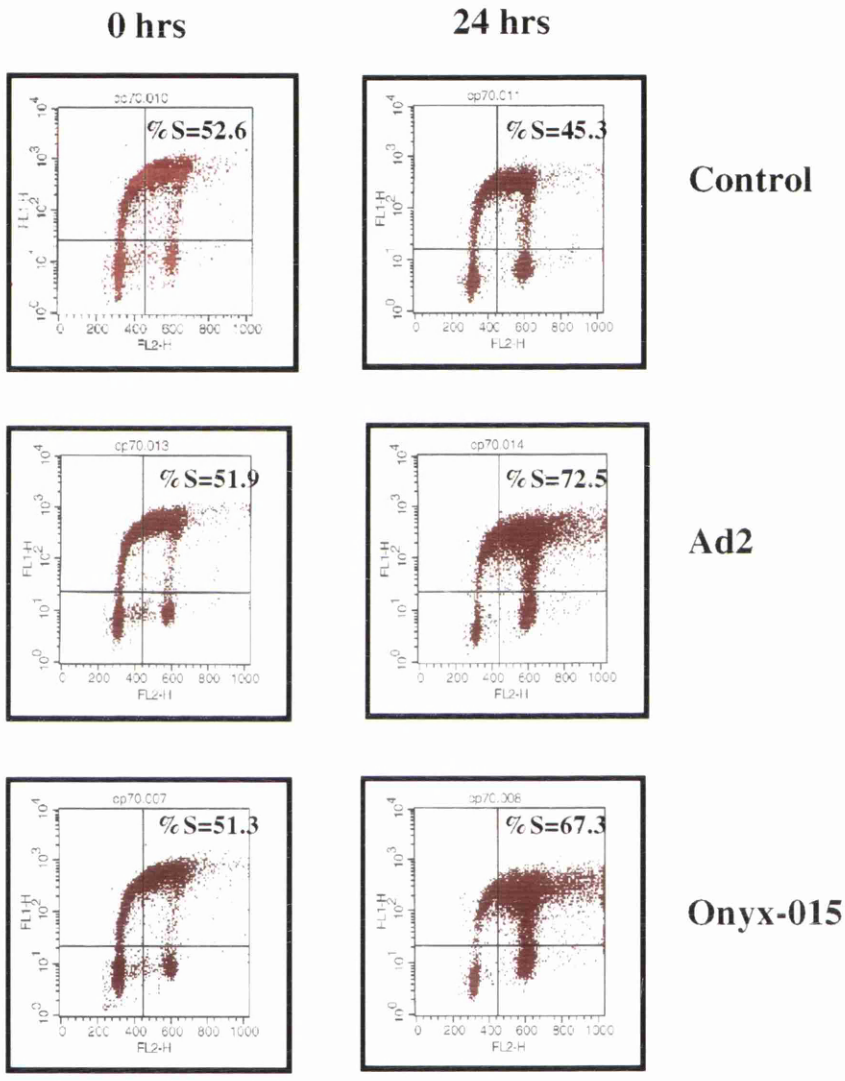


Figure 33. Cell cycle analysis by BrDU labelling of Cp70 infected with Ad2 or Onyx-015 at MOI of 100pfu/cell.

The percentage of cells in S phase was determined by BrDU labelling and flow cytometry. Cells in S phase are those in the upper quadrants(upper left quadrant represents cells in early S and upper right quadrant represents cells in late S phase.) In Cp70, there is an increase in the % cells in S phase for both Ad2 and Onyx-015 infected cells.

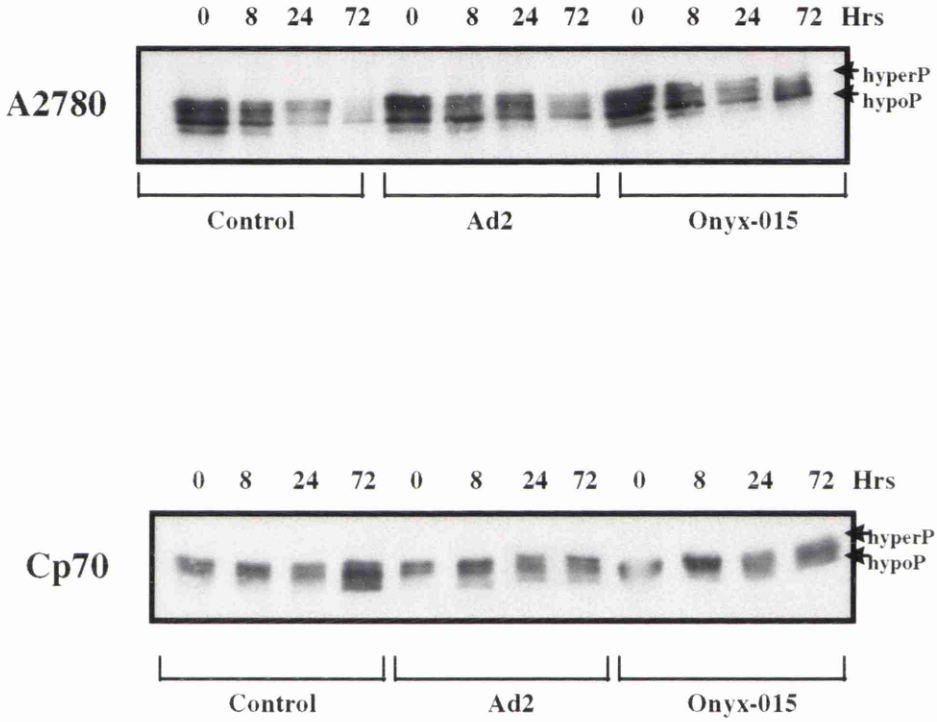


Figure34. Western immunoblot of Rb phosphorylation in A2780 and Cp70 after infection with Ad2 or Onyx-015 at MOI of 100pfu/cell.

There is no significant change in Rb phosphorylation status in either cell line indicating that Rb phosphorylation is not required for S phase entry after adenoviral infection.

d) S phase entry is not mediated by Rb phosphorylation.

The retinoblastoma protein plays a key role in S phase entry at the G1/S checkpoint (Weinberg,1995 for review). In its active form it exists as a hypophosphorylated form bound to E2F. Cyclin dependent kinases phosphorylate Rb into the hyperphosphorylated form (inactive) and this causes the release of E2F . E2F then activates genes essential for DNA replication such as dihydrofolate reductase (DHFR) , DNA polymerase α, thymidine kinase, thymidylate synthase by binding to E2F sites in the promoters of these genes. This therefore allows S phase entry to occur. We determined the Rb phosphorylation status by Western blotting over a time course in both Cp70 and A2780 infected with Ad2 or Onyx-015. **Figure 34** shows the Western immunoblots obtained. From these we can conclude that there was no significant change in phosphorylation state in either cell line indicating that phosphorylation of Rb was not the determinant for S phase entry.

e) S phase entry is mediated by E2F induction.

It is known that the adenoviral E1A protein can interact directly with Rb and cause the release of E2F(Fattaey et al,1993; Ikeda et al,1993). To determine whether or not E2F increased after adenoviral infection we utilised an E2F band shift assay using the human E2F consensus binding sequence. E2F supershift assays were done using antibodies to E2F1, E2F4, E2F5 and DP1 to determine the E2F subtype which was induced. **Figures 35 and 36** show that with Ad2 infection there is a very large increase in E2F4 and E2F5 in both Cp70 and A2780 at 24 hours which then drops rapidly at 48-72 hours. With Onyx-015 infection, we also saw a similar increase in E2F4/5 in both cell

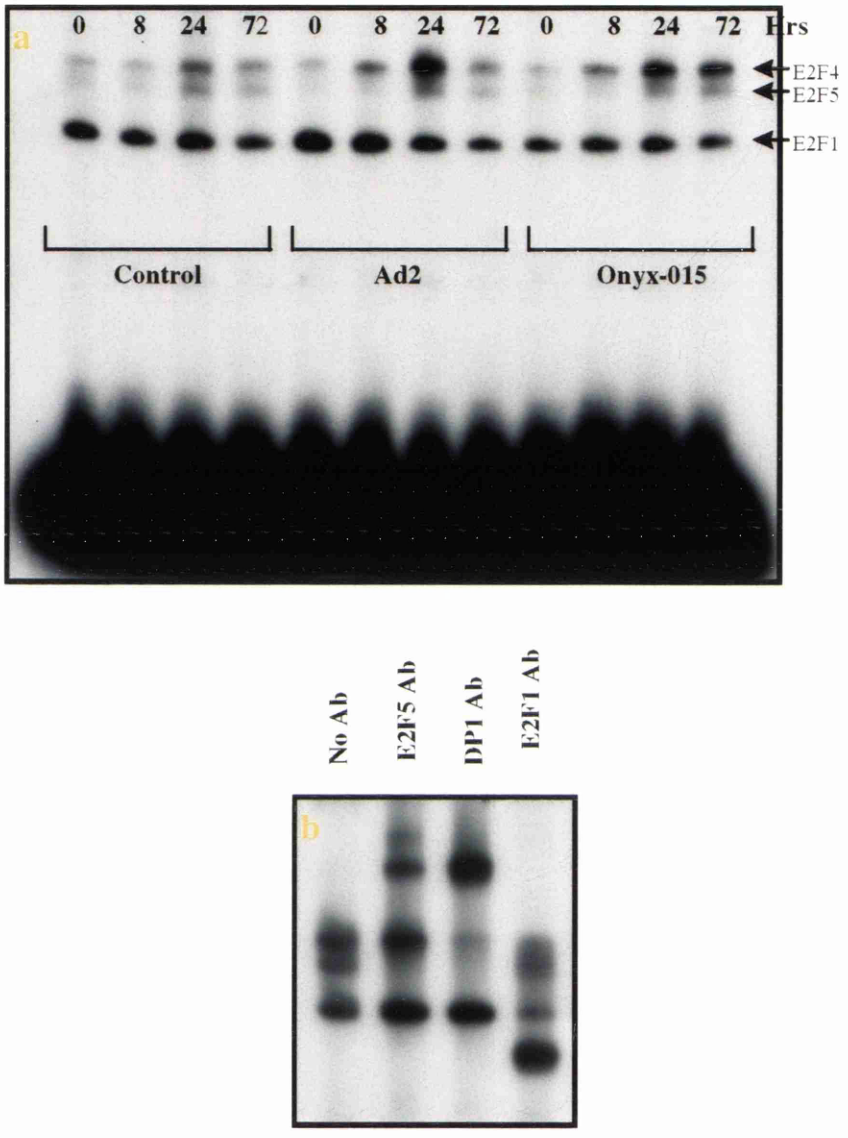


Figure 35. A2780Cp70 E2F Bandshift assay.

Figure 35a shows the effect of Ad2 and Onyx-015 infection at an MOI of 100pfu/cell on Cp70. In both cases E2F induction occurs at 24 hours post infection.The predominant E2F subtype which is induced is E2F4. Figure 35b shows a supershift assay using antibodies to E2F5,DP1,E2F1 to disrupt the E2F complexes. The supershift assay identifies which band represents each E2F subtype.

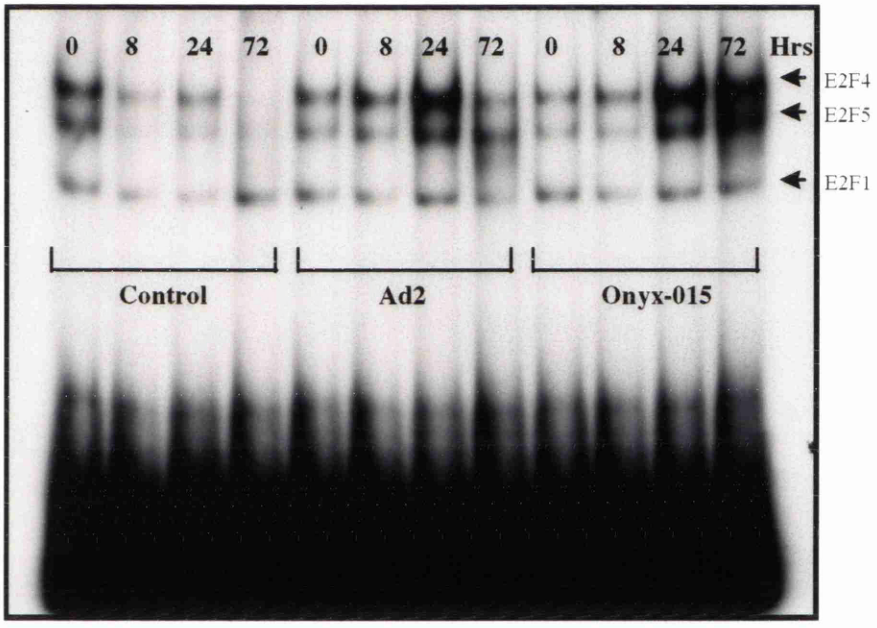


Figure 36. A2780 E2F Bandshift assay.

Figure 36 shows the effect of Ad2 and Onyx-015 infection at an MOI of 100pfu/cell on A2780. In both cases E2F induction occurs at 24 hours post infection.The predominant E2F subtype which is induced is E2F4 and also E2F5

lines. Although the induction of E2F would explain the increase in S phase cells with Ad2 infection in both cell lines and also Onyx infection in Cp70, it does not explain why E2F induction in A2780 infected with Onyx-015 does not cause S phase entry. In fact, the induction in A2780 with Onyx-015 is equivalent to that found with Ad2 infection yet Ad2 produces a much larger increase in S phase cells. One possible explanation for this could be that high levels of E2F in the presence of wild type p53 induces apoptosis as reported by Qin et al,1994 and Wu et al,1994. Therefore replication would not occur because the cells are forced into apoptosis. We therefore decided to determine whether or not there was an increase in apoptosis in A2780 after Onyx-015 infection and also if p53 increased after Onyx-015 infection.

3.2.2. Onyx-015 induces p53 dependent apoptosis in A2780.

a) Clonogenic survival of A2780 infected with Onyx-015.

A2780 cells were infected with Onyx-015 at a MOI of 1,10 and 100pfu/cell for 24 hours , cells harvested and then 10^3 or 10^4 cells plated out and clonogenic survival determined at 10 days. **Figure 37** shows the survival obtained. As can be seen at 100pfu/cell only 10-15% cells survive infection.We have already shown that viral replication does not occur in A2780 and therefore the clonogenic survival curve can not be explained by viral replication. We therefore examined for evidence of apoptosis in A2780.

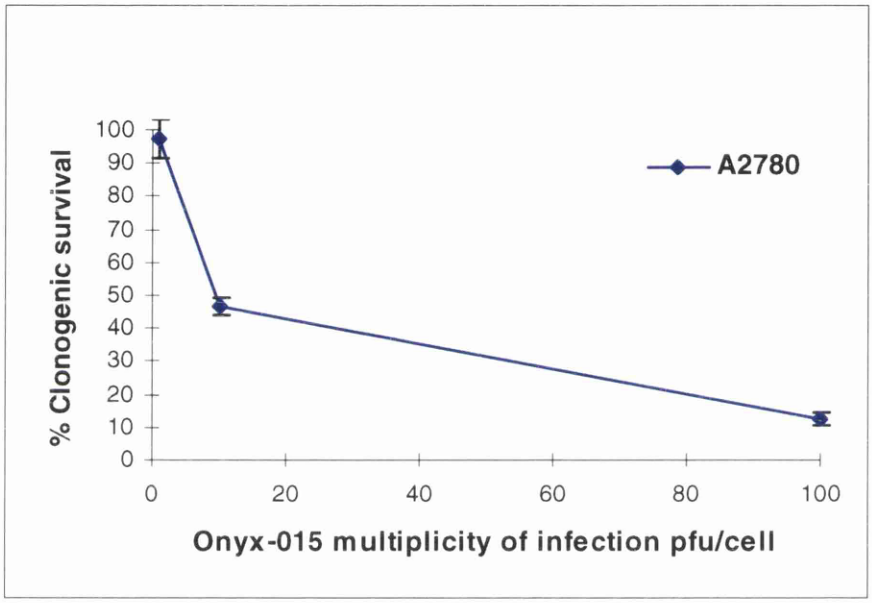


Figure 37. Clonogenic survival of A2780 after 24 hours infection with Onyx-015.
Cell monolayers were infected at 1,10 and 100pfu/cell for 24 hours. 10^3 or 10^4 cells were then plated out and clones allowed to grow for 10 days. Clones greater than 50 cells were then counted.

b) Onyx-015 induces apoptosis in A2780.

i) Evidence for apoptosis by cell morphology.

Cells infected with Onyx-015 at an MOI of 100pfu/cell and then harvested by trypsinisation at 0, 24, 72 hours and cytospins made. The cells were stained with haematoxylin and then examined for apoptotic bodies. **Figure 38** shows that at 72hours there is clear evidence for apoptosis with cellular fearures of apoptosis appearing in the infected cells compared to control uninfected cells.

ii) Evidence for apoptosis by PARP cleavage.

In apoptosis , activation of caspases causes cleavage of many proteins including PARP (Poly ADP-ribose polymerase). PARP is cleaved from a 117kDa protein into an 85kDa and 32kDa protein and this can be detected by Western blotting using an anti-PARP antibody. This antibody will detect both the 117 and 85kDa proteins. As can be seen from **Figure 39** , A2780 infected cells showed evidence of PARP cleavage with Onyx-015 and also Ad2 at the 72 hour time point . This was further evidence of apoptosis occuring in A2780.

iii) Evidence for apoptosis by TUNEL staining.

Cytospins were also stained for apoptosis using the terminal deoxynucleotidyl-transferase-mediated dUTP nick end-labelling (TUNEL) to detect cleaved DNA in apoptotic cells. **Figure 40** shows Onyx-015 infected cells undergoing apoptosis as indicated by the bright green fluorescent bodies. Non apoptotic cells stain blue with the counterstain DAPI. Cells which express the late phase adenovirus hexon protein are stained red with an antihexon antibody linked to Texas Red. As can be seen Ad2 infected cells stain

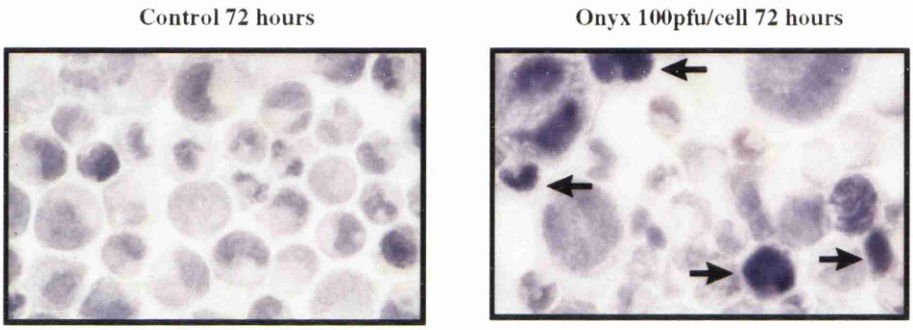


Figure 38. Haematoxyllin staining of A2780 cells infected with Onyx-015 at MOI 100pfu/cell

After infection with Onyx-015, cells undergo apoptosis at 72 hours post infection. Typical densely staining shrunken cells undergoing apoptosis are arrowed.

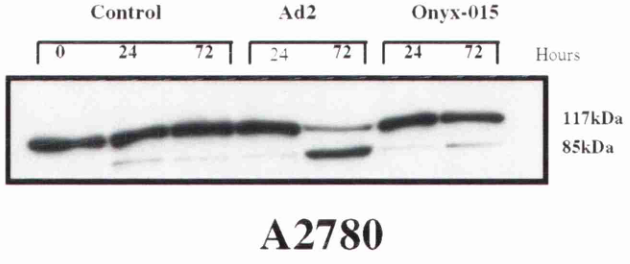


Figure 39. Western immunoblot of PARP protein cleavage in A2780 infected with Ad2 or Onyx-015.

After infection with Ad2 or Onyx-015, PARP protein cleavage occurs with a reduction in the 117kDa protein and an increase in the 85kDa protein.

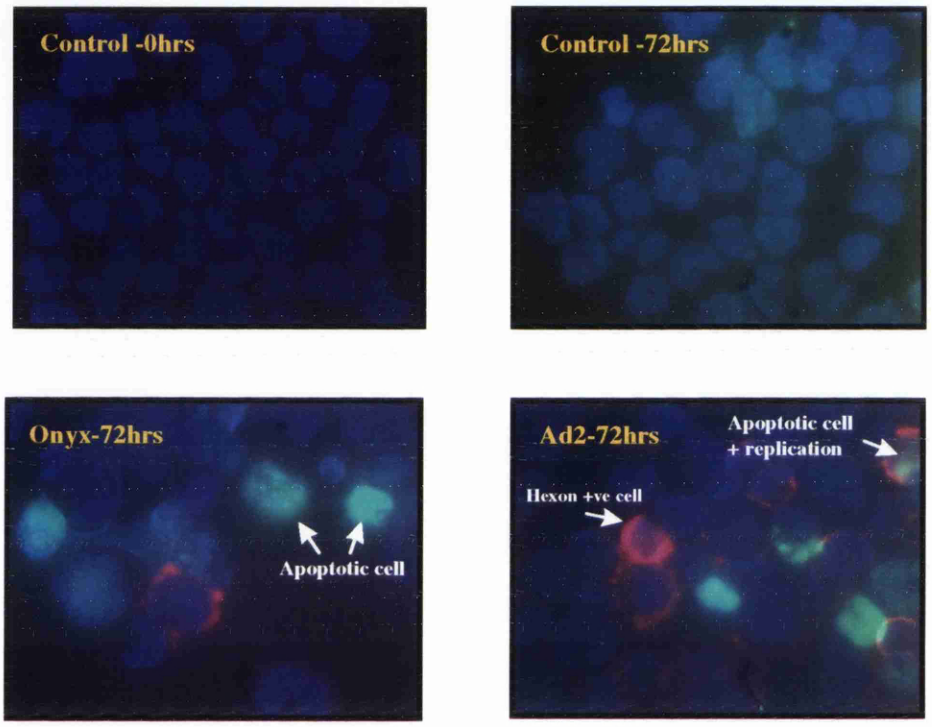


Figure 40. Apoptosis in A2780 by TUNEL staining.

After infection with Onyx-015 at 100pfu/cell, many apoptotic cells are seen at 72 hours. These stain green with the FITC labelled TUNEL reagent. Cells were also stained for hexon protein expression using an antibody linked to Texas Red. In Onyx-015 infected cells very few cells stain for hexon protein indicating no viral replication is occurring. In Ad2 infected cells, replication occurs as indicated by the many red staining cells. In addition apoptotic cells are seen in Ad2 infected cells . Some cells stain for both hexon protein and also for apoptosis indicating that before apoptosis occurs a degree of viral replication can still occur.

positively for hexon protein but very few Onyx-015 infected cells since replication occurs with Ad2 but not Onyx-015. Interestingly, although replication occurs in Ad2 infected cells we also see evidence of apoptosis occuring.

iv) Apoptosis involves an increase in bax and a reduction in bclxl protein

To determine what proteins might be involved in apoptosis, we looked at the Bcl2 family of proteins. These included the survival factors Bcl2 and $bclx_L$ and the proapoptotic factors bax and bak. **Figure 41** shows the results obtained. As can be seen levels of bak and Bcl2 remain unchanged. However we see a reduction in $bclx_L$ and an increase in bax at the 72 hour time point. This suggests that these 2 proteins are involved in viral induced apoptosis in this cell line.

c) Apoptosis is p53 dependent and is induced by adenoviral E1A and repression of adenoviral E1B19kDa protein.

i) p53 induction by Onyx-015 is by adenoviral E1A..

Adenoviral E1A is known to induce p53 (Debbas and White,1993; Lowe and Ruley,1993). With wild type adenovirus the large E1B 55kDa protein binds to the N terminal end of p53 and represses transcriptional activity of p53 (Debbas and White,1993). In addition, E1B 55kDA, along with the E4ORF6 protein ,complex to p53 mediating the degradation of p53 via the ubiquitin degradation pathway (Querido et al,1997; Steeganga et al,1997). Thus in cells infected with Ad2 we would expect to see a reduction in p53 protein expression. However with adenoviruses deleted in the E1B 55kDA region, no p53 degradation would occur (Grand et al,1994). We therefore wanted to

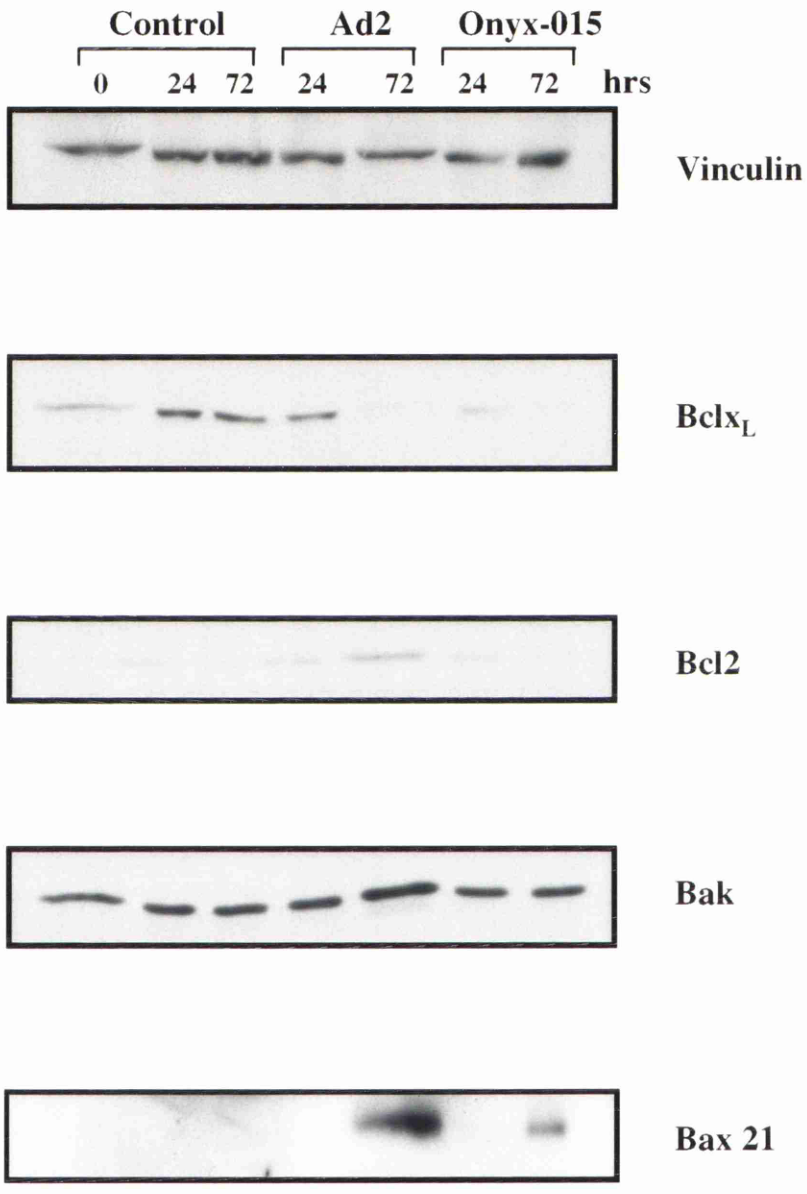


Figure 41. Western immunoblotting for the Bcl2 family of proteins in Onyx-015 and Ad2 infected A2780 cells.

In both Ad2 and Onyx-015 infected cells there is a reduction in Bclx$_L$ and an increase in Bax protein at 72 hours post infection. Thus apoptosis is being mediated by these 2 proteins.

examine p53 expression in cells infected with Onyx-015 and also Ad2. **Figure 42** shows Western blotting for E1A, E1B19kDa, p53. As can be seen, with Ad2 infected cells p53 is initially induced and then drops rapidly to undetectable levels by 72 hours. With Onyx-015 infected cells however we see an increase in p53 protein expression which continues to high levels at 72 hours. **Figure 43** shows p53 staining of A2780 cytospin preparations infected with Onyx-015. This shows very clear accumulation of nuclear p53 over 72 hours.

ii) Apoptosis is not inhibited by adenoviral E1B19kDa.

Interestingly the antiapoptotic protein E1B19kDa is expressed very weakly in A2780 (compared to Cp70- see figure 48) and is therefore not able to inhibit apoptosis. The expression of E1B19kDa is however higher in the Ad2 infected cells. This could prevent apoptosis occuring and allow replication to occur in Ad2 infected cells. This could explain why replication is seen with Ad2 infected cells but not Onyx-015 infected cells. We do not know why there is this difference in E1B 19kDa levels . One possibility could be that E1B19kDa is repressed by p53 and since p53 is degraded in Ad2 infected cells and not Onyx-015 infected cells repression of E1B19kDa would cease to occur at 24-72hours post infection. If this indeed was the case we expect to see high levels of E1B19kDa in cells which had non-functional p53 such as in Cp70. This is indeed what we do see **(Figure 48).**

iii) E4ORF6 does not repress p53 transcriptional activity

Dobner and Shenk ,1996 reported that E4ORF6 is able to repress p53 transcriptional activity alone. Western blotting for E4ORF6, bax , p21 was carried out to examine this **(Figure 44)** . We initially see an induction of p21 but this drops rapidly at 72 hours and this might suggest that either

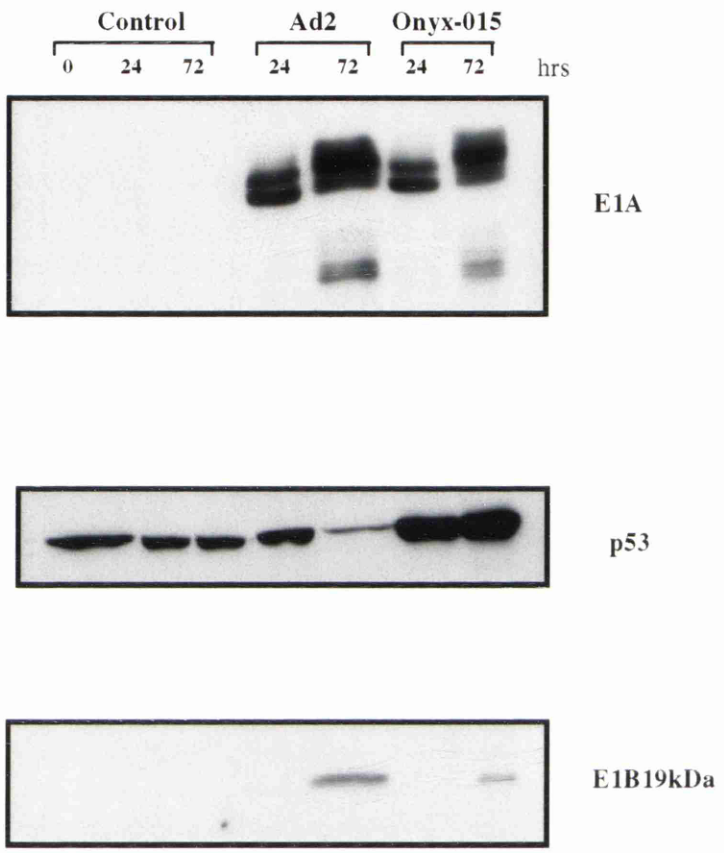


Figure 42. Western immunoblot of E1A, p53 and E1B19kDa protein expression in A2780 after infection with Ad2 or Onyx-015.

After infection with either Ad2 or Onyx-015, E1A is expressed and this causes an induction in p53 as shown. In Ad2 infected cells, the level of p53 drops at 72 hours due to degradation by the E1B55kDa protein. After infection with the E1B55kDa deleted adenovirus Onyx-015, the p53 level remains high. The antiapoptotic protein E1B19K is very weakly expressed and therefore unable to inhibit p53 mediated apoptosis. The level of E1B19K is higher in the Ad2 infected cells which could suggest that it may be controlled by the expression of p53 in the cells.

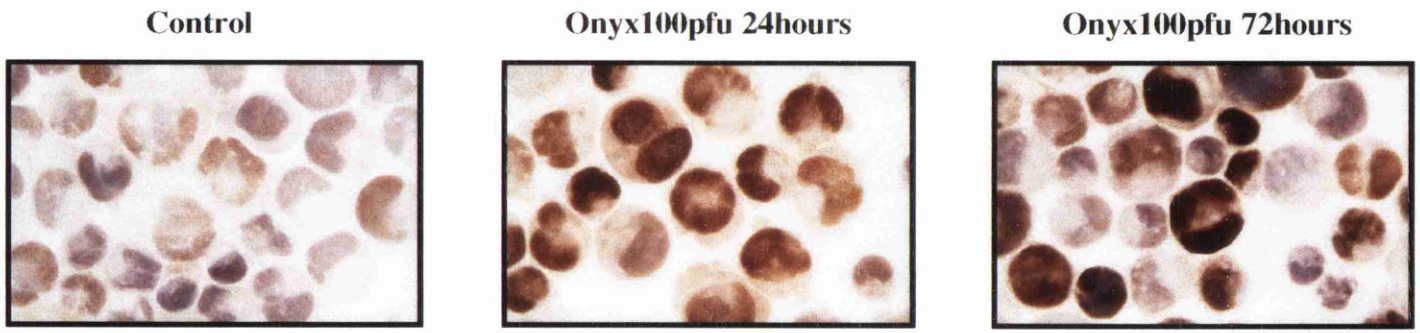


Figure 43. p53 staining of A2780 cells infected with Onyx-015 at MOI of 100pfu/cell.
After infection with Onyx-015 at MOI of 100pfu/cell, p53 induction occurs as indicated by the intense nuclear staining of the p53 protein at 24 hours. At 72 hours both nuclear and cytoplasmic p53 staining is present.

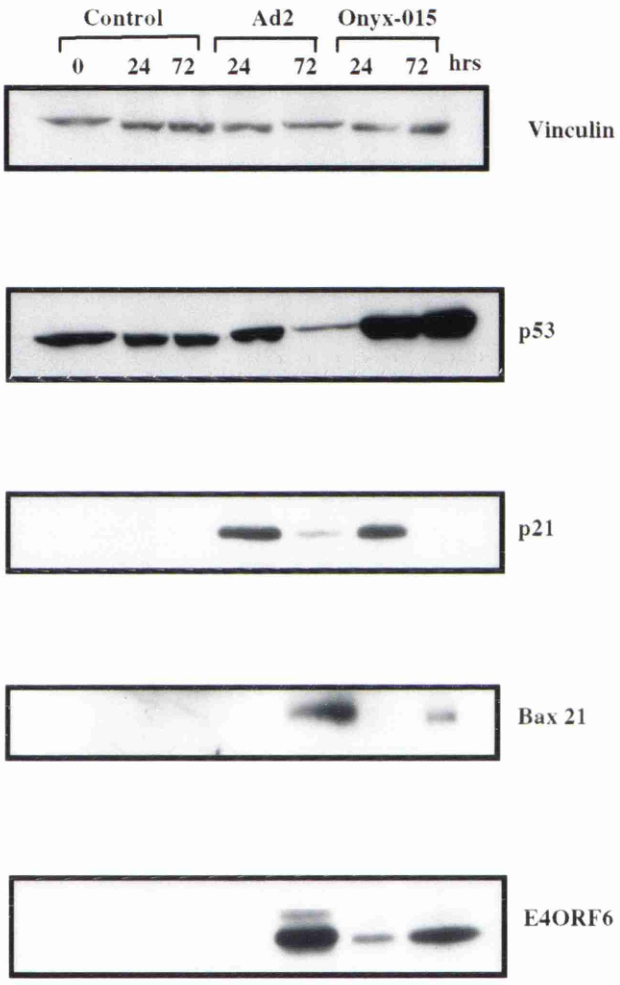


Figure 44. Western immunoblots for p53,p21,bax and E4ORF6 in A2780 infected with Ad2 or Onyx-015.

After infection with Ad2, p53 degradation occurs by 72 hours. This is associated with a reduction in p21 protein levels but an increase in bax. After infection with Onyx-015, p53 stabilisation occurs. However we still see a reduction in p21 levels suggesting either transcriptional repression of p53 by E4ORF6 or protein degradation of p21. Bax protein levels are increased at 72 hours. Northern blotting was carried out to determine whether loss of p21 transcriptional or post translational.

transcription of p53 is being repressed or post-transcriptional degradation of p21 occurs as is seen in the cell line C5N. However, with bax we see an increase in levels at 72 hours. We therefore carried out Northern blots to examine the mRNA levels of p21,mdm2, bax post infection **Figure 45a.** As can be seen , p21,mdm2 and bax RNA levels do not decrease at 72 hours . This indicates that transcriptional repression of p53 by E4ORF6 does not occur in this cell line. We did observe this however in the mouse cell line C5N. The loss of p21 protein expression in A2780 is therefore post transcriptional . This may be due to caspase degradation p21 in the process of apoptosis as reported by Levkau et al,1998.

iv) mdm2 mediated degradation of p53 is inhibited post Onyx-015 infection.

Figure 42 shows that p53 protein levels increase markedly post Onyx infection . Northern analysis as shown above would suggest that p53 remains transcriptionally active. This would suggest that mdm2 mediated degradation of p53 is inhibited, possibly by inhibition of mdm2/p53 complex formation. To examine this, p53 complexes were immunoprecipitated with DO1 antibody and then mdm2 detected with 2A10 mouse monoclonal antibody **(Figure 45b).** From this figure, mdm2 levels initially increase in parallel with the p53 protein levels. However, at 72 hours post infection the level of mdm2 drops markedly while that of p53 increases indicating that complex formation at 72 hours post viral infection is inhibited. de Stanchina ,1998 recently reported that adenoviral E1A induces p53 by induction of p19 . p19 has been reported to inhibit mdm2 mediated degradation of p53 by Zhang et al,1998 and Pomerantz et al,1998 . We can therefore conclude that Onyx-015 may mediate p53 induction by the same mechanism.

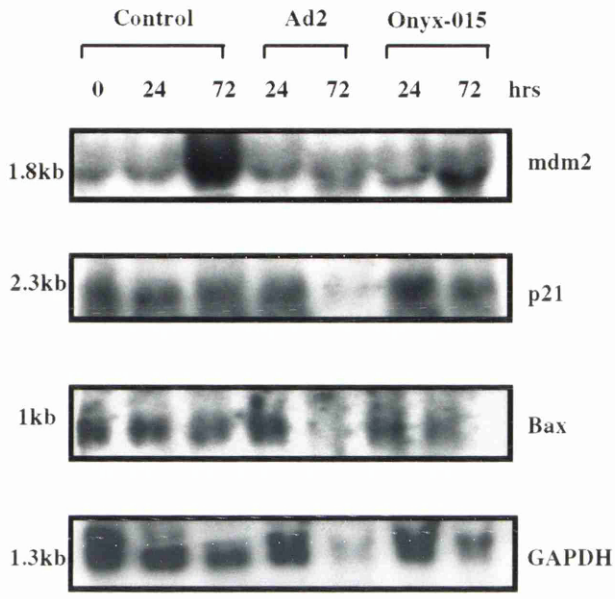


Figure 45a. mRNA expression of p21 ,mdm2 and bax in A2780 infected with Ad2 and Onyx-015 at MOI of 100pfu/cell.

GAPDH is used as a control for loading. All lanes are equally loaded except for lane 5. There is no evidence for a reduction in mRNA levels in p21.mdm2 or bax at 72 hours post Onyx-015 infection.

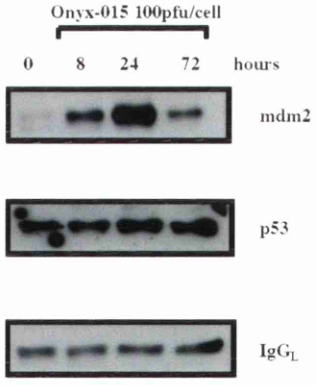


Figure 45b. p53 and mdm2 protein expression on immunoprecipitation of p53 in cell line A2780 infected with Onyx-015 at MOI of 100pfu/cell.

p53 was immunoprecipitated using the mouse monoclonal antibody DO-1. Mdm2 protein complexed to p53 was determined using the mouse monoclonal antibody 2A10 . p53 protein levels were determined using the same antibody used for immunoprecipitation and using proteinA-hrp as the secondary antibody. From this figure, after viral infection p53 levels increase over 72 hours as expected. mdm2 levels also increase for the first 24 hours indicating transcriptional activation by p53. However the level drops rapidly at 72 hours. This may be due to E1A induction of p19 , which prevents mdm2 interacting with p53.

3.2.3. Onyx-015 kills A2780Cp70 by cytolysis and not by apoptosis.

a) Onyx-015 does not induce PARP cleavage.

We have already shown that Onyx-015 can replicate efficiently in the cell line Cp70. Apoptosis does not occur in this cell line as shown by a lack of PARP cleavage after infection (**Figure 46**).

b) Effect of Onyx-015 on Bcl2 family of proteins.

Further evidence for no apoptosis is shown in **Figure 47** which shows Western blots for the Bcl2 group of proteins. As can be seen, there is no change in any of the survival or apoptotic factors. We do see an increase in bak expression in both Ad2 and Onyx-015 infected cells but this is not significantly different from the increase seen in the control samples at the same time points.

c) High expression of E1B 19kDa inhibits apoptosis.

Figure 48 shows the expression of the adenoviral E1B19kDa protein . In Cp70 this protein is expressed to much higher levels than A2780 and this would inhibit apoptosis. This also suggests that E1B19kDa protein may be repressed by p53.

3.2.4. Conclusions

In conclusion, we have shown that selective replication for Onyx-015 occurs in the cell line A2780Cp70 (non-functional p53) compared to the parental cell line A2780 (functional p53). Replication is dependent on S phase entry of the host infected cell and this is mediated by E2F induction by

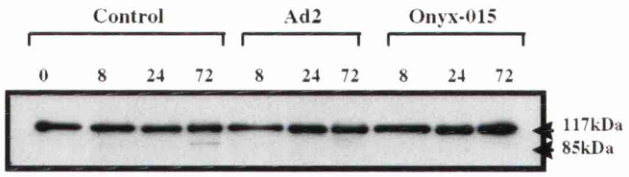


Figure 46. Western immunoblot for PARP protein cleavage in A2780Cp70.

A2780Cp70 shows no evidence of apoptosis as indicated by lack of PARP cleavage on infection with either Ad2 or Onyx-015. Only the uncleaved 117kDa protein is detected on Western blotting.

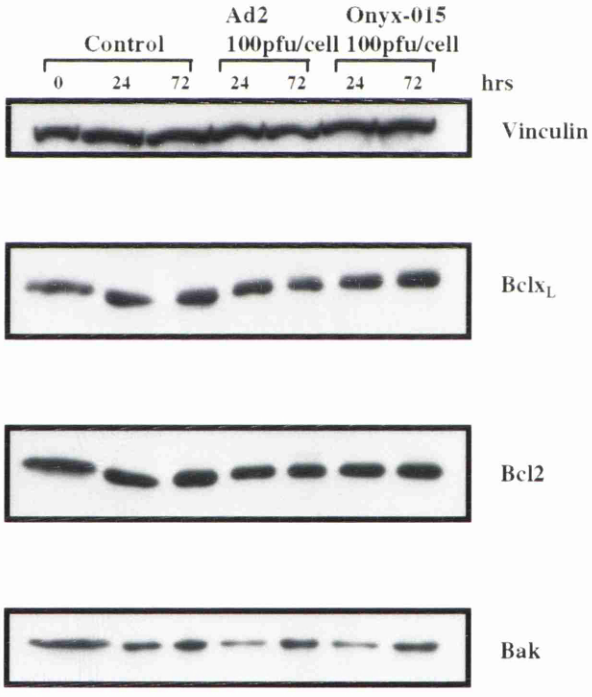


Figure 47. Western immunoblots for Bclx$_L$, Bcl2, Bak in A2780Cp70.

Cp70 has very high levels of the survival factors Bclx$_L$ and Bcl2 which do not decrease on infection with either Ad2 or Onyx-015. Although the apoptotic factor Bak increases on infection, the level is not significantly different from the uninfected protein level. In addition, the apoptotic protein Bax is not detectable in uninfected or infected cells.Therefore overall no is no evidence for apoptosis in Cp70.

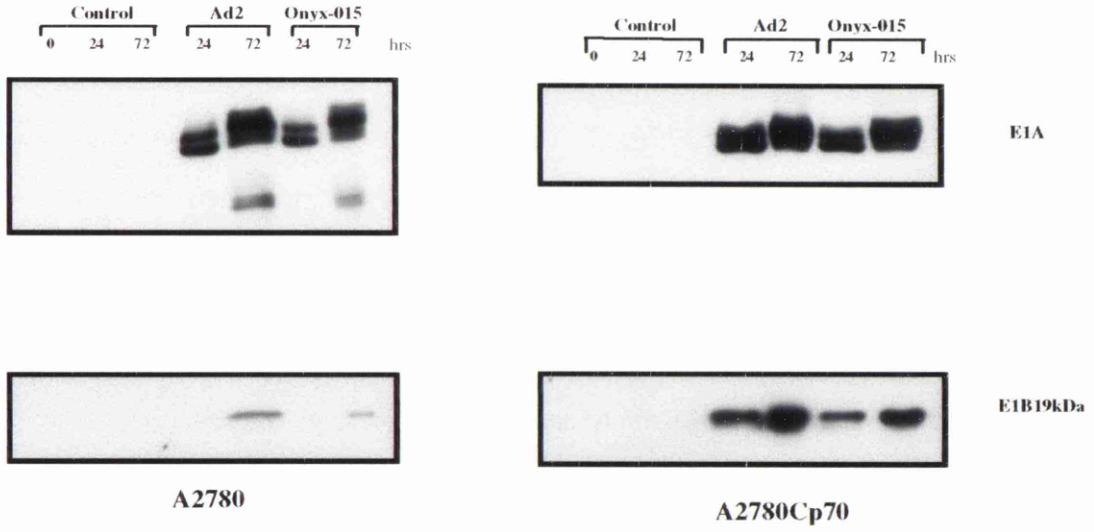


Figure 48.Western immunoblots for E1A and E1B19KDa proteins in A2780 and Cp70 cell lines.

After infection with either Ad2 or Onyx-015, both cell lines express high levels of E1A protein. However Cp70 expresses very high levels of the E1B19kDa protein compared to A2780. This protein is an inhibitor of apoptosis and the high level in Cp70 may account for the lack of apoptosis seen in Cp70. The low level seen in A2780 may account for the high level of apoptosis seen in this cell line. This may suggest that p53 in A2780 represses E1B19kDa expression.

adenoviral E1A. In cell line A2780, E2F induction occurs but S phase entry is limited by p53 mediated apoptosis. Thus Onyx-015 cannot produce a productive viral yield in A2780 due to apoptosis of the host cell. It is likely that the p53 induction seen is due to inhibition of mdm2 mediated p53 degradation due to the induction of p19 by adenoviral E1A. In contrast, A2780Cp70 does not undergo apoptosis in response to virus infection and this allows Onyx-015 to produce a viral yield. The lack of apoptosis seen in Cp70 is due to absence of functional p53. High expression of the anti-apoptotic factor E1B19kDa also prevents apoptosis in Cp70.

3.3. COMBINATIONAL THERAPY OF ONYX-015 WITH DNA DAMAGING AGENTS CISPLATIN AND RADIATION.

Studies have shown that transfecting in the E1A gene into tumour cells which are either wild type or mutant p53, induces apoptosis (Sanchez-Prieto et al,1998). Moreover this apoptosis is enhanced when combined with DNA damaging agents (Sanchez-Prieto et al,1996). Rather than transfecting E1A into tumour cells , it would be simpler to infect with an adenovirus which retains the E1A region such as with Onyx-015. Our objective was therefore to determine whether or not Onyx-015 could increase cytotoxicity to DNA damaging agents in non-functional p53 and also functional p53 cell lines. To study this we utilised the human ovarian adenocarcinoma cell lines A2780 (functional p53) and A2780Cp70 (non-functional p53 cisplatin resistant variant).

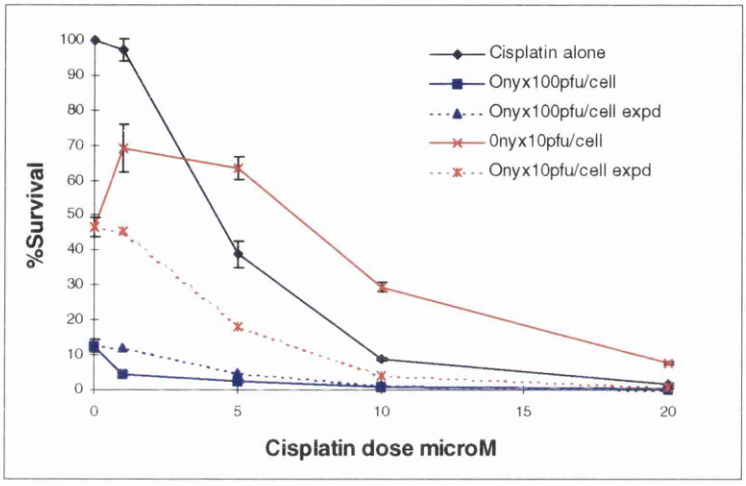
3.3.1. Effect of Onyx-015 on cytotoxicity induced by cisplatin and radiation in A2780.

a) Infection with Onyx-015 for 24 hours followed by cisplatin is antagonistic.

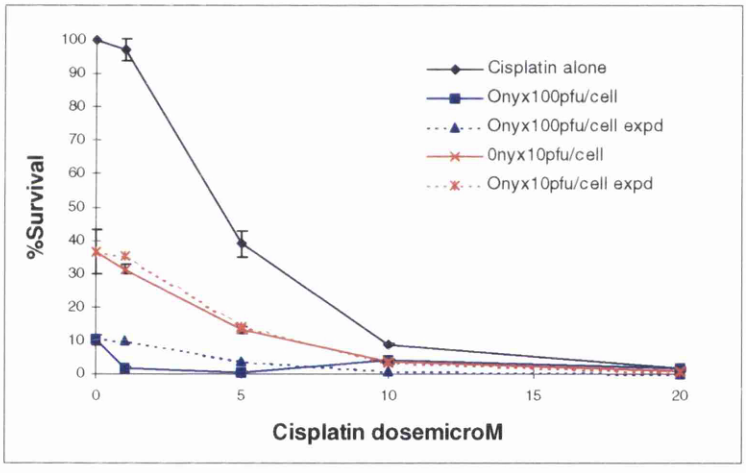
Cytotoxicity to Onyx-015 and to DNA damaging agents was determined by clonogenic assay. Cell monolayers were initially infected with ONYX-015 at MOI of 10 or 100pfu/cell followed by exposure to cisplatin at a range of concentations for 1 hour. Cells were then plated out for clonogenic assay. As can be seen from **Figure 49,** infection for 24 hours followed by cisplatin produces a survival curve which suggests antagonism since the actual curve obtained at 10pfu/cell shows a better clonogenic survival than the predicted survival (dotted curves). This could be explained in terms of p53 functionality. We have already shown that p21 is induced at 24 hours post infection and this may reduce the number of cells in S phase at this time point. Since cisplatin is an S phase specific drug it would be less effective at this time point and there would be an antagonistic interaction between virus and cisplatin.

b) Infection with Onyx-015 for 72 hours followed by cisplatin is additive.

After exposure to virus for 72 hours followed by cisplatin the survival curves show additive cytotoxicity as shown in **Figure 49.** p21 is no longer detectable at 72 hours and therefore we may expect more cells to be in S phase which would allow cisplatin to form adducts and induce cytotoxicity. Thus the antagonistic interaction observed at 24 hours would not be present at 72 hours.



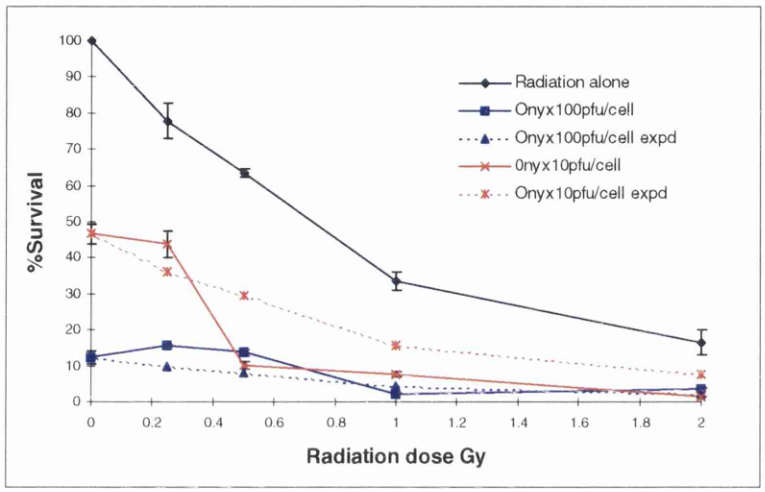
Onyx 24hours A2780



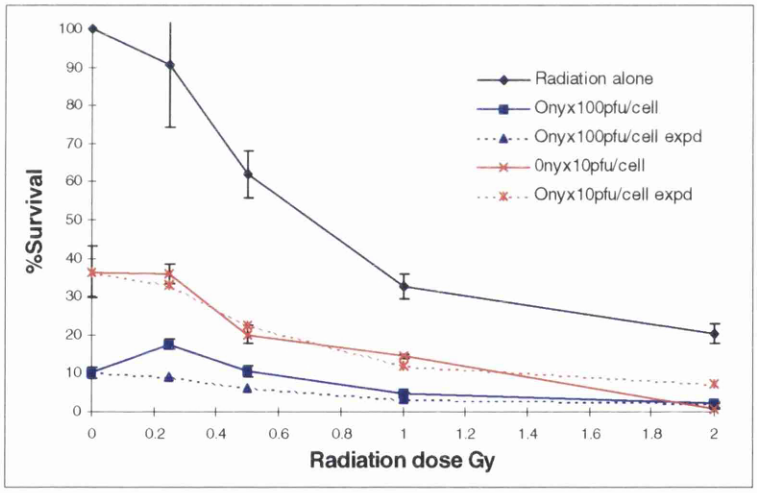
Onyx 72hours A2780

Figure 49. Clonogenic survival of A2780 after infection with Onyx-015 followed by cisplatin exposure.

Cells were infected with Onyx-015 at MOI of 10 and 100pfu/cell for either 24 hours (Figure 49a) or 72 hours (Figure 49b) and then exposed to cisplatin at 1, 5, 10 and 20 microM for 1 hour. The clonogenic survival plot for 24 hour exposure to Onyx-015 shows that the observed survival at MOI 10pfu/cell was greater than the predicted survival(calculated by multiplying the values for cisplatin alone by values for virus alone). This suggested an antagonistic interaction between Onyx infection and cisplatin. The clonogenic survival plot for 72 hour exposure to Onyx-015 shows observed survival equivalent to the predicted.This suggested an additive effect.



Onyx 24hours A2780



Onyx 72hours A2780

Figure 50. Clonogenic survival of A2780 after infection with Onyx-015 followed by radiation exposure.

Cells were infected with Onyx-015 at MOI of 10 and 100pfu/cell for either 24 hours (Figure 50a) or 72 hours (Figure50b) and then exposed to ionising radiation at 0.25 ,0.5 , 1and 2Gy. The clonogenic survival plot for 24 hour and 72 hour exposure to Onyx-015 shows that the observed survival at both MOI's is equivalent to the predicted.This suggested an additive effect.

c) Infection with Onyx-015 for 24 hours followed by radiation is additive.

After exposure to Onyx-015 for 24 hours followed by radiation **Figure 50** the survival curve for 10pfu/cell MOI shows additive cytotoxicity. Antagonism is not seen as with cisplatin possibly because radiation is not S phase specific in its mechanism of cell kill but will induce DNA strand breaks at any phase of the cell cycle. Since radiation and also Onyx-015 can both induce a p53 dependent apoptosis , both treatments combined would produce enhanced toxicity.

d) Infection with Onyx-015 for 72 hours followed by radiation is additive.

After exposure to Onyx-015 for 72 hours followed by radiation **Figure 50** the survival curves show additive cytotoxicity.

These results with either cisplatin or radiation suggest that combination therapy with Onyx-015 and DNA damaging agents enhances cytotoxicity in cells with p53. This would suggest that if both agents are given systemically, there may be an increase in toxicity in normal (functional p53) tissue and clinically this would be undesirable. In local delivery of virus, as with intratumoural therapy, this is less of a problem. In this case, Onyx-015 would replicate in tumours with mutant p53 and replication would then stop on reaching the normal surrounding tissue. This is because the normal (non tumour) cells would undergo apoptosis preventing further spread of virus into normal tissue. This normal tissue toxicity may be increased if virus is combined with DNA damaging agents but this would not be as serious as systemic toxicity.

With heterogeneous tumours which have both wild type and mutant p53 cells, Onyx-015 would kill mutant p53 cells by replication and wild type p53 cells by apoptosis. Combining therapy with DNA damaging agents would enhance this effect.

Radiotherapy is frequently used to treat primary HNSCC which have a lower incidence of p53 mutations. The mechanism of cell death is largely p53 dependent apoptosis. Theoretically it is possible that a preinjection of tumour with Onyx-015 24 hours before radiotherapy may sensitise these tumours to radiation .

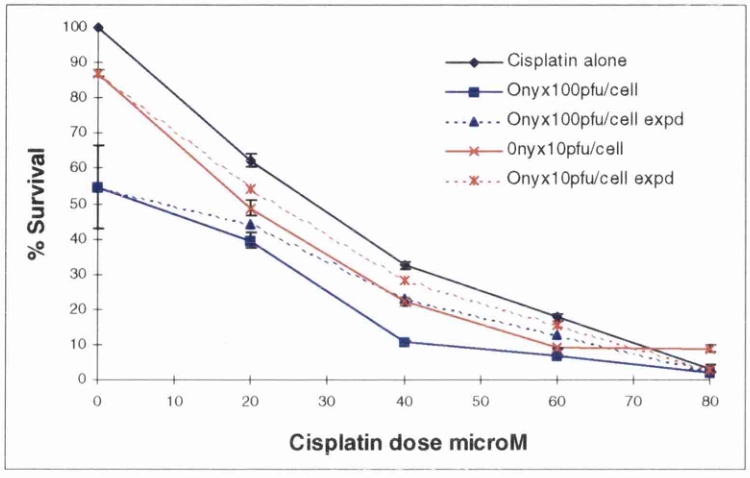
3.3.2. Effect of Onyx-015 on cytotoxicity induced by cisplatin and radiation inCp70.

a) Infection with Onyx-015 for 24 hours or 72 hours followed by cisplatin is synergistic.

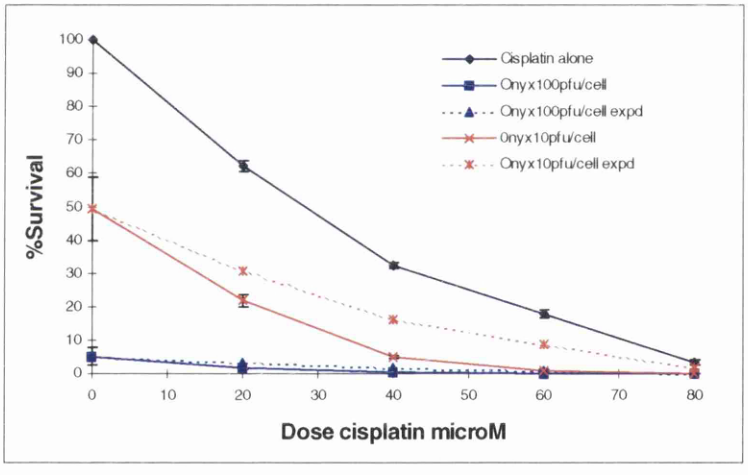
Figure 51 shows the survival curves for combination with cisplatin. Exposure to Onyx-015 for either 24 hours or 72 hours followed by cisplatin is synergistic in both cases. The cytotoxicity is greatest after 72 hours since adenovirus has had greater time to replicate and cause cytolysis.

b) Infection with Onyx-015 for 24 hours or 72 hours followed by radiation is synergistic.

Figure 52 shows the survival curves for combination with radiation. Exposure to Onyx-015 for either 24 hours or 72hours followed by radiation is synergistic in both cases. The cytotoxicity is again greatest after 72 hours since adenovirus has had greater time to replicate and cause cytolysis.



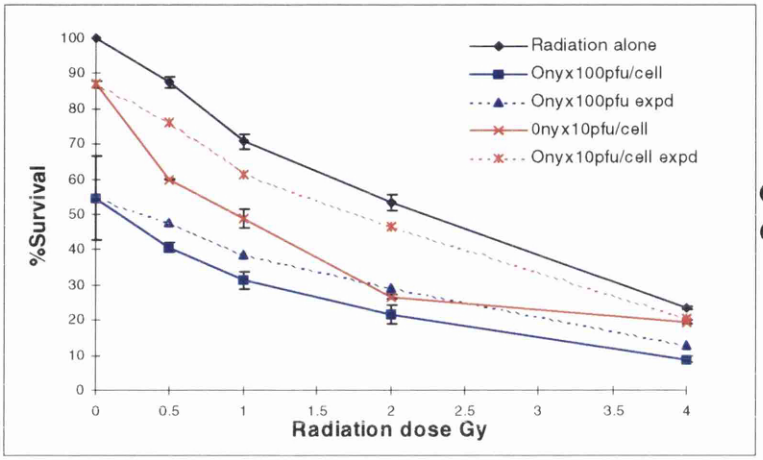
Onyx24hours Cp70



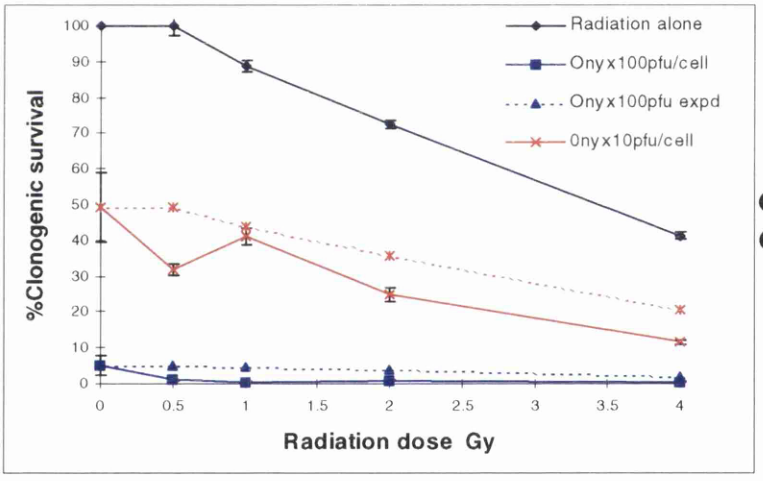
Onyx72hours Cp70

Figure 51. Clonogenic survival of A2780Cp70 after infection with Onyx-015 followed by cisplatin exposure.

Cells were infected with Onyx-015 at MOI of 10 and 100pfu/cell for either 24 hours (Figure51a) or 72 hours (Figure51b) and then exposed to cisplatin at 20, 40, 60 and 80 microM for 1 hour. The clonogenic survival plots show that the observed survival at each MOI was less than the predicted survival(calculated by multiplying the values for cisplatin alone by values for virus alone). This suggested a synergistic interaction between Onyx infection and cisplatin.



Onyx24hours Cp70



Onyx72hours Cp70

Figure 52. Clonogenic survival of A2780Cp70 after infection with Onyx-015 followed by radiation exposure.

Cells were infected with Onyx-015 at MOI of 10 and 100pfu/cell for either 24 hours (Figure 52a) or 72 hours (Figure 52b) and then exposed to ionising radiation at 0.5, 1, 2, 4Gy. The clonogenic survival plots show that the observed survival at each MOI was less than the predicted survival(calculated by multiplying the values for radiation alone by values for virus alone). This suggested a synergistic interaction between Onyx infection and radiation.

CHAPTER 4
DISCUSSION

4.1. IMMUNOCOMPETENT MOUSE MODEL TO TEST ONCOLYTIC ADENOVIRUSES.

4.1.1. Infectivity and replication of human adenoviruses in mouse cell lines.

Replication of human adenoviruses in mouse cells is thought to be severely limited due to low infectivity, repression of the transcription of E1A, or lack of synthesis of late structural proteins. We have shown in this report that the infectivity of rodent cells is extremely variable, ranging from less than 1% to about 60% after infection with 10pfu/cell. Highly infectible cell lines included several which were derived from mouse epidermis, and individual lines from mouse glioblastoma 9L-82, mouse kidney adenocarcinoma, rat thyroid carcinoma, and rat Morris hepatoma. Poor infectivity was seen in 3T3 fibroblasts, colon carcinoma, lung carcinoma and melanoma cells.

In tissues which were highly infectible, replication as determined by CPE assays and hexon protein staining was highest in mouse epidermal cells which have not to our knowledge been studied before for adenoviral infection. Using burst assays we have shown that productive infections occur in these cell lines, although the process is less efficient than in the control human cells tested (ovarian carcinoma A2780Cp70 cells). Replication in all other tissue types tested was undetectable, with the exception of the colon cell line K12/TrB and the rat thyroid cell line VH1 VRS, both of which showed poor replication in comparison to the epidermal cell lines. In agreement with the work of others (Silverstein et al, 1986), we have shown that 3T3 fibroblasts are poorly infectible, and do not allow virus replication. This has been attributed to

repression of E1A synthesis (Babiss et al,1993) and a failure to synthesise late structural proteins due to the instability of newly synthesised viral DNA (Eggerding et al, 1986) .

Although all of the cells tested showed expression of the adenovirus early gene product E1A, there was no overall correlation with the level of infectivity or ability to support replication of the virus. Thus, although E1A is clearly essential for replication, it is not in itself sufficient, and the host cell can block replication at a level downstream from E1A expression. The cellular factor(s) responsible for this block appear to be absent from epidermal cells, which was the only cell type tested which gave rise to a burst of infectious virus. The mouse epidermal cell lines tested were of comparable infectivity, but varied in the expression of E1A and hexon protein. E1A expression correlated with replication (as assessed by late protein expression) such that cells which had high E1A expression also expressed high hexon protein. The most striking observation was that squamous epidermal cells (well differentiated) gave high E1A expression and replication, while clonally related spindle epidermal cells (poorly differentiated) derived from the same tumours showed low expression of E1A and hexon protein. This suggested that the expression of early and late adenoviral genes was dependent on the state of differentiation of the cell. Laporta et al, 1981 have reported that in human keratinocytes , Ad2 replication, as is also the case for human papilloma viruses, is most efficient in the suprabasal cells (differentiated) compared to the basal cells (nondifferentiated). Our results on squamous and spindle carcinoma cells would also indicate that replication is better in the more differentiated cell phenotype, although the mechanistic basis for these observations is unclear.

Aneskievich et al (1990) reported that the block to replication in the human basal cells was not in early gene expression but was rather in late adenoviral gene expression. In contrast, our results show that the level of E1A correlates quite positively with the extent of replication and late protein expression, but negatively with the degree of cell differentiation. One possible explanation for this may be that during the transition from a squamous to a spindle phenotype, the epidermal cells assume a more fibroblastic character, generally switching off expression of the epidermal keratins and E-cadherin, and switching on fibroblast markers such as vimentin (Stoler et al,1993). Previous work from others (Fognani and Babiss ,1993) has shown that fibroblasts produce a trans-acting transcriptional repressor which suppresses the activity of the E1A promoter. Such a factor, if produced at elevated levels after the squamous-spindle transition, could account for the observed decrease in replication capacity of the spindle cells.

To examine this hypothesis in more detail, we analysed the expression of the nuclear factor ϕAP3 , which has been shown to repress the E1A promoter in mouse fibroblasts as described by Fognani and Babiss, 1993. By testing related pairs of cells of different phenotype (i.e. squamous vs spindle from the same tumour) we have shown that the expression of ϕAP3 is approximately 20 fold higher in the spindle phenotype . We can conclude that in mouse epidermal cells, the restriction to replication is at the level of early gene expression and that this is probably determined by the cellular factor ϕAP3.

In conclusion, we have shown that a series of restriction points exist which control the ability of human adenoviruses to replicate in rodent cells. Similar restrictions probably exist also in human cells, which show wide

variations in their ability to be infected by and support replication of adenovirus (Steegenga et al, submitted). Our observations are therefore unlikely to be restricted to rodent cells, although further investigations in this area are necessary. The restriction points we have seen are at the level of virus entry, expression of the early gene product E1A, and subsequent initiation of viral replication and late protein synthesis. These studies demonstrated that most or all of these restrictions are surprisingly absent in mouse epidermal cells, which showed the highest ability to support productive replication, leading to a burst of infectious virus. Replication is most robust in well differentiated epidermal tumour cells (squamous phenotype) compared to poorly differentiated cells (spindle phenotype), probably due to the relative levels of E1A and its transcriptional repressor ϕAP3 . These observations are not attributable to more relaxed control of DNA replication in epidermal tumours, since additional studies using primary epidermal keratinocytes have demonstrated a similar level of replication and hexon protein staining to that seen in the tumour lines (data not shown).

These studies therefore establish the basis for the further development of epidermal tumour models for testing replication competent adenoviral therapy for cancer. Using mouse squamous epidermal tumour cell lines, or primary tumours induced by chemical carcinogens, it may be possible to test the efficacy of oncolytic adenoviruses in immunocompetent mice and examine the role of both neutralising antibody and cytotoxic T lymphocytes responses in viral replication and cytolysis.

4.1.2. Selective replication of an E1B deficient adenovirus in mouse epidermal cell lines.

The use of replicating oncolytic adenoviruses in cancer therapy may be potentially advantageous if we can selectively target tumour cells leaving normal tissue unaffected. The E1B deleted adenovirus, Onyx-015, may be therapeutically useful in the treatment of a wide range of tumours since p53 abnormalities are very common in human cancer. However, little is known of the effect of the immune system on this virus. To develop an immunocompetent mouse model to test this virus we tested a wide range of mouse epidermal cells of known p53 sequence and function to determine if we could demonstrate the same selectivity for p53(-) mouse cells. The cell lines B9, A5, PDVc57 which have point mutations in p53 and the cell line SN161 which has an 8bp deletion in p53 all show positive CPE's and hexon protein staining. In contrast, the cell lines P1, P6, CarB with wild type p53 did not. This was very encouraging as it showed that the same selectivity was demonstrable in mouse tissue. Interestingly the cell line C5N which has normal p53 sequence and function as determined by G1 arrest in response to radiation, also showed good replication. We have proposed that the mechanism for replication of Onyx-015 in cell lines with wild type p53 is dependent upon 2 restriction points- these are the E1A expression in infected cells and secondly the ability for virus to induce S phase cells for virus to replicate. In CarB, there is low E1A expression due to high ϕAP3 (data not shown). In addition, p21 is induced which would decrease the number of S phase cells. Therefore, CarB has 2 restriction points which will prevent replication. In C5N, we have high E1A expression and also loss of p21 expression at 72 hours post infection.

Both high E1A and low p21 would favour efficient viral replication and this would account for replication of Onyx-015 in this cell line. In P6, high E1A expression occurs as it has a squamous phenotype. This would favour replication. However, p21 is induced by Onyx-015 and remains high throughout infection. This would reduce S phase cells and therefore restrict viral replication.

Northern blot analysis of p21 mRNA expression in C5N , shows that the loss of p21 is due to a combination of p53 transcriptional repression and also p21 protein degradation. Repression of p53 activity can occur via the large E1B 55k protein which binds directly to the N terminal end of p53. This protein is absent in Onyx-015 and therefore is not responsible for the repression observed. The viral protein E4ORF6 is expressed however. This has been reported to repress p53 transcriptional activity directly by binding to the C terminal end of p53 and causing a conformational change in p53 (Dobner and Shenk ,1996). This is the most likely mechanism for p53 transcriptional repression and the reduction in p21 mRNA levels seen at 72 hours post infection. However, p21 protein degradation also occurs and is probably the main mechanism for loss of p21 expression. It has recently been reported that p21 is cleaved by caspase activation in apoptosis causing loss of the C terminal end of p21 and therefore loss of the nuclear localisation signal (Levkau et al,1998). This is not the cause of p21 loss in C5N since there was no evidence for apoptosis in this cell line after Onyx-015 infection.(data not shown). Recently, it has been shown that overexpression of the cellular factor $p120^{E4F}$ leads to stabilisation of p21 protein by a p53 independent post-transcriptional mechanism (Fernandes et al,1998). It has also been shown that adenoviral E1A

can downregulate expression of $p120^{E4F}$ (Fernandes et al,1997) and therefore reduce the half-life of p21 protein. Since E1A is highly expressed in C5N , $p120^{E4F}$ may be downregulated in C5N leading to reduced stability of p21 protein. This could also explain why CarB has high p21 expression since E1A expression in this cell line is low and we would therefore predict high $p120^{E4F}$ expression. However, this hypothesis would not explain why P6 has high p21 expression since E1A in this cell line is high and is comparable to that of C5N. We are currently looking at the expression of $p120^{E4F}$ in these cell lines.

In conclusion, we have shown that the E1B deleted adenovirus, Onyx-015, will selectively replicate in mouse cell lines with mutant p53 as described for human tumour cell lines by Bischoff et al, 1996. In addition however we have also shown that some cell lines with wild type p53 (C5N) will also support replication and this was dependent upon 2 factors- the E1A expression after infection and also the p21 level after infection.

4.1.3. In Vivo Studies.

Based on the in vitro data on mouse epidermal cell lines, 2 squamous epidermal cell lines were chosen for in vivo testing- B9 and PDVc57. Subcutaneous tumours were formed and then injected with either Ad2 or Onyx-015 virus. In both cases, virus injection was able to cause delayed growth of tumours and the results were statistically significant. Survival of mice injected with virus was also improved. Evidence for viral replication was detected by in situ hybridisation in all Ad2 and Onyx injected tumours but was found to be less than in human tumour xenografts(BICR16). The reduced replication in the mouse epidermal xenografts is compatible with that seen in vitro where we

showed a x25-x50 fold reduction in viral replication. This would account for the fact that we do not see tumour regression as with human tumour xenografts, only delayed growth. In addition, the doubling time of mouse epidermal cells is 12 hours compared to BICR16 which is 24 hours. Thus, absence of tumour regression is due to more rapidly growing tumours and reduced efficiency of replication in mouse epidermal xenografts. Nevertheless, we are able to show a therapeutic effect which enables us to examine the role of the immune system in a syngeneic host.

To examine the role of the immune system and viral replication, PDVc57 xenografts were formed in their syngeneic host C57bl/6 mice. Again virus injected tumours grew more slowly than control(PBS) injected tumours for both Ad2 and Onyx-015 indicating that virus was having a therapeutic effect. However, when in situ hybridisation was carried out , none of the Onyx injected tumours showed evidence of virus and only 2 of the Ad2 injected tumours showed evidence of virus. This was evidence that virus replication was repressed in the syngeneic host and suggested that virus elimination by the immune system occurred. The reason for efficacy may be due to the initial viral replication before the immune system cleared the virus, or may be due to an immune effector cell infiltration into the tumour. At present there is no commercially available antibody to detect mouse CD4 or CD8 lymphocytes and therefore the question of increased immune infiltration remains unanswered. However, using this model we can now examine the effect of immunosuppressants e.g. cyclophosphamide, dexamethasone on viral replication and tumour growth. Smith et al,1996 showed that cyclophosphamide and deoxyspergualin(DSG) given at time of first adenovirus

injection in C57bl mice prevented the formation of neutralising antibody and permitted an effective second administration of vector. We would therefore expect improved efficacy in the syngeneic host on giving immunosuppresants. Humoral immunity could also be suppressed by using γ interferon and IL-12 (Yang et al,1995) or by using viruses with non crossreactive serotypes (Mastrangeli et al,1996). Alternatively the virus could be encapsulated with poly(lactic-glycolic) acid copolymer (PLGA) as reported by Pekarck et al,1994. This has been reported to reduce immunogeneicity to second adenoviral inoculation in vivo (Beer et al,1998). The cytotoxic T cell response has also been suppressed in mice following CTLA Ig administration(Kay et al,1995). All of these experiments are currently planned for use in the immunocompetent mouse model.

4.2. MECHANISTIC STUDIES ON THE SELECTIVE REPLICATION OF ONYX-015 IN P53(-) CELL LINES.

We have shown that Onyx-015 virus infects the ovarian carcinoma cell lines A2780 and Cp70 with comparable infectivities using a lacZ reporter virus and also E1A immunofluorescent assay. When infected with the E1B deleted adenovirus, Onyx-015, burst assays and hexon protein assay show that replication is more productive in the cell line Cp70 which has non-functional p53. Very poor replication is found in A2780 which has wt p53 sequence and function. Studies by Kim et al (unpublished results) on p53 mutant transfectants of A2780 showed increased replication when A2780 was stably

transfected with a dominant negative p53 gene. This confirmed the selectivity for virus replication for cells with non-functional p53.

The cell line Cp70 is a cisplatin resistant derivative of A2780 and has non-functional p53. Several other cisplatin resistant variants of A2780 have been established in the laboratory (MCP1-MCP9) . As with Cp70, these cells show increased replication with Onyx-015 in comparison to A2780 by a factor of x5-x10 (Kim et al ,unpublished results).

Recent papers by Ridgeway et al,1997 and by Goodrum and Ornelles,1997 and by Hall et al,1998 have suggested that replication of E1B deleted adenoviruses showed no correlation to p53 status, although in all three papers the p53 function of the cell lines used was not tested. Goodrum suggested that replication was cell cycle dependent and that replication was more productive if cells were in S phase of the cell cycle and that this was independent of p53 status. Hall showed that viral induced cell death was more efficient in wild type p53 cell lines but failed to show the mechanism of cell death,i.e. apoptosis or viral replication. To further examine the mechanism of replication of Onyx-015 in Cp70 (non-functional p53) versus A2780 (functional p53) , we hypothesised replication was dependent on the host infected cells being in S phase. This effect may be mediated by the E1A gene by interacting with the Rb/E2F complex causing release of free active E2F and G1-S phase progression as described previously. For cells with non-functional p53 (Cp70) , this would allow viral DNA replication to occur resulting in cytolysis. For cells with functional p53 (A2780) , it is known that E1A can induce p53 and that this can lead to apoptosis (Lowe and Ruley,1993) . The mechanism as to how E1A induces p53 has only recently been shown to

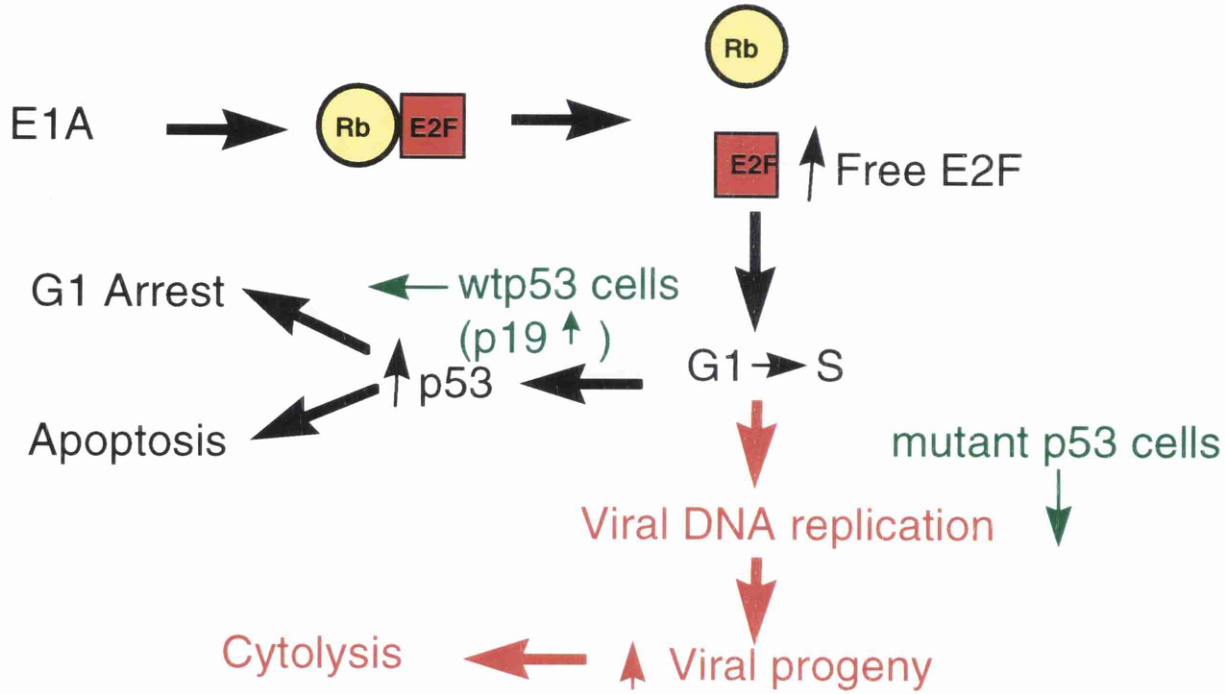


Figure 53. Proposed mechanism for selective replication of Onyx-015 in p53(+) and p53(-) cells.

involve the tumour suppressor $p19^{ARF}$ (de Stanchina et al,1998) which is able to prevent mdm2 degradation of p53. This induction of p53 by p19 may result in cell cycle arrest or apoptosis and this would prevent S phase cells increasing on viral infection. Since Onyx-015 retains the E1A gene we may expect p53 mediated arrest or apoptosis to occur. However, the situation is complicated by the fact that Onyx-015 retains the E1B19K gene which produces a protein which is anti-apoptotic (Debbas and White,1993). **Figure 53** illustrates the model suggested.

To test this hypothesis, cell cycle analysis was done at a MOI of 100pfu/cell using pulse BrdU labelling to measure cells in S phase. Both A2780 and Cp70 were infected with Ad2 or Onyx-015 over a 72 hour period and the percentage S phase , G1 and G2 phase cells estimated at 0, 24 or 72 hours post infection. For Cp70 , there is an increase in S phase cells with both Ad2 and Onyx-015. For A2780, Ad2 infection causes the percentage of S phase cells to increase greatly at 24 hours and then reduce whereas with Onyx-015 the percentage of cells in S phase changes very little on infection. This picture would fit exactly with our hypothesis. Western blotting showed that the increase in S phase cells was not dependent upon a change in Rb phosphorylation from the hypophosphorylated form to the hyperphosphorylated form. However, using bandshift assays for E2F, we have shown that both Ad2 and Onyx-015 cause E2F induction to occur. E2F exists in 5 different subtypes, E2F1-5 (Moberg et al,1996). The most abundant subtype in cells tends to be E2F4 and this is the most potent transcriptional activator after E2F-1. With Cp70, E2F4/5 both increase with Ad2 or Onyx-015 infection. This would cause S phase progression allowing viral replication and cytolysis to occur. With

A2780, E2F4/5 induction also occurs with Ad2. However, it was unexpected to see an even greater increase with Onyx-015 which was inconsistent with our hypothesis and also did not agree with the cell cycle data. It has recently been reported that overexpression of E2F-1 can cause apoptosis alone (Hung et al,1997) , but particularly in the presence of high p53 levels (Wu et al,1994). Therefore high E2F and p53 levels seen in A2780 may cause apoptosis and this would explain our cell cycle data and also the poor replication seen in A2780.

We have shown by cell morphology, PARP cleavage and TUNEL staining that Onyx-015 causes apoptosis in A2780. Indeed if a clonogenic survival curve is carried out on A2780 over a range of MOI, we see a decrease in cell survival with increasing MOI, an effect due to apoptosis not viral replication. Western blotting for p53 protein shows a large increase with Onyx-015 . This effect of E1B deleted adenoviruses in cells with wt p53 has already been reported by Grand et al,1994. We have shown that p53 induced by Onyx-015 is not transcriptionally repressed by E4ORF6 as reported by Dobner and Shenk,1996 since mRNA levels of p21, bax and mdm2 levels do not decrease post infection. We have also shown by immunoprecipitation studies that p53 levels remain high due to inhibition of mdm2 mediated degradation and we have suggested that this effect is most likely mediated by adenoviral E1A induction of p19 as reported by de Stanchina,1998. Thus high levels of functional p53 are induced in the cell line A2780. This , together with the high E2F induction would cause apoptosis. Analysis of the Bcl-2 group of proteins has shown that apoptosis involves a reduction in $bclx_L$ and an increase in bax protein. In comparison, cell line Cp70 showed no evidence of apoptosis and

there was no change in the Bcl2 family of proteins. This would fit very well with the pathway we have hypothesised.

We then asked the question why apoptosis should occur in A2780 since Onyx-015 still retains the E1B19K gene which has been reported to be a potent inhibitor of bax. When we examined the levels of E1B19kDa protein expressed in Cp70 and A2780 at the same MOI, we were surprised to see high levels in Cp70 and very low levels in A2780. Thus apoptosis in Cp70 would be inhibited by the E1B19kDa protein allowing cells to remain in S phase and allowing viral replication and cytolysis to occur. In contrast, E1B19kDa protein is weakly expressed in A2780 and therefore p53/E2F induced apoptosis is allowed to proceed. This difference in E1B19kDa protein levels suggested that E1B19K gene may be downregulated in A2780 , possibly by p53 itself. To examine this further we are currently screening wild type p53 and mutant p53 tumour cell lines to determine whether or not mutant p53 cells have high E1B19kDa protein expression and wild type p53 cells have low E1B19kDa protein expression.

4.3. COMBINATIONAL THERAPY OF ONYX-015 WITH DNA DAMAGING AGENTS CISPLATIN AND RADIATION.

It has already been reported by Sanchez-Prieto et al,1998 that E1A has an antitumour effect in vivo and that this is dependent on E1A binding to p60,p105,p107.Similarly Shisler et al,1996 has reported that the induction of susceptibility to TNF by E1A is also dependent on binding of E1A to either p105(Rb) or p300 to induce DNA synthesis. E1A transfected into tumour cells

also increases the cytotoxicity to DNA damaging agents. Lowe and Ruley,1993 reported that fibroblasts with wtp53 transfected with E1A were sensitised to both radiation and cytotoxics. This was a p53 dependent mechanism since null p53 fibroblasts were not sensitised. The mechanism of signaling of E1A to p53 has only recently been described and involves induction of p19 as described above (de Stanchina et al,1998). p19 inhibits mdm2 degradation of p53 thus leading to p53 stabilisation. This mechanism is distinct from the pathway of p53 activation and stabilisation by DNA damaging agents which involves the phosphorylation of serine 15 of p53. This prevents mdm2 interacting with p53. Because these mechanisms of p53 stabilisation are different ,synergy should exist between E1A and DNA damaging agents. However, Sanchez-Prieto et al,1995 have also showed increased sensitivity to DNA damaging agents irrespective of p53 status. In addition, Brader et al,1997 showed increased sensitivity to various cytotoxics in the cell line SKOV3(mutant p53) transfected with E1A. They hypothesised that this may be due to a decrease in p185 levels though Frisch et al,1991 showed that increased sensitivity also occurred in cells which did not express p185.

Since Onyx-015 retains the E1A gene we hypothesised that this virus may also increase the sensitivity to cytotoxics and to radiation in both wt and mutant p53 cell lines. We again used the paired cell lines A2780 and Cp70 to determine this. In A2780, we found increased cytotoxicity to radiation but the effects were additive in nature, not synergistic as one might have predicted. With cisplatin, antagonism occurred at 24 hours but additivity at 72 hours exposure to virus. This could be explained in terms of the p21 levels in A2780 in which we found induction of p21 at 24 hours with a reduction at 72 hours.

The induction of p21 at 24 hours is p53 mediated and would result in G1 arrest of cells. Since cisplatin is S phase specific agent, it would have less effect at 24 hours postinfection. As can be seen, p21 levels drop rapidly at 72 hours and this would allow more cells to be in S phase and for cisplatin to be more effective. The mechanism for p21 reduction at 72 hours may in part be due to p53 transcriptional repression by E4orf6 (Dobner and Shenk ,1996). However , Northern blot analysis showed no reduction in p21 mRNA levels as was seen in C5N mouse epidermal cell line. Therefore in A2780 the main mechanism of p21 loss is by post-transcriptional degradation. p21 cleavage has recently been reported to occur by caspase activation in apoptosis and this may be the reason for the reduction in p21 seen (Levkau et al,1998). In addition, adenoviral E1A may be downregulating $p120^{E4F}$ leading to reduced p21 stability. It is also known that E1A can activate the ubiquitin degradation pathway causing proteolysis of topoisomerase II(Nakajima et al,1996).

In the cell line Cp70, the cytotoxicity to both cisplatin and radiation is increased following infection with Onyx-015 both for 24 hours and 72 hours virus exposure. The effects are synergistic which suggests there is an interaction between virus and cisplatin or radiation. This interaction may be between cisplatin/radiation and the E1A gene of Onyx-015 . It is possible that the E1A protein induces a reduction in p185 and therefore increases sensitivity to cytotoxics (Brader et al,1997) in infected cells . It would therefore be interesting to look at p185 expression following Onyx-015 infection.

Clinically the combination of virus with DNA damaging agents is important. The combination should have greater efficacy than either agent alone in tumours with non-functional p53. In addition in tumours which are

heterogeneous with both wtp53 and mutant p53 cells, Onyx-015 would kill mutant p53 cells while chemotherapy/radiation would kill wtp53 tumour cells. However, toxicity may also be increased since we have shown that in cells with wild type p53, Onyx-015 although not replicating, will induce p53 mediated apoptosis and this effect is enhanced if combined with chemotherapy or radiation. At present use of this virus is confined to intratumoural imjection of HNSCC , pancreas, liver metastases and therefore the local tissue toxicity may not be too problematic. However, if systemic administration of Onyx-015 is given in combination with chemotherapy then there is potential for more serious toxicity to occur. Therefore, in vivo toxicity experiments in mice using systemic virus delivery and chemotherapy will have to be carried out before this therapy enters clinical trials.

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