# THE RADIOTHERAPEUTIC POTENTIAL OF THE EPIDERMAL GROWTH FACTOR RECEPTOR

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To my Mum and Dad

look - Godot's showed up

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## SUMMARY

The epidermal growth factor receptor (EGFR) is overexpressed in a wide range of tumour types. Molecules which target and sterilise EGFR-overexpressing cells, or increase their sensitivity to conventional therapies may have a beneficial effect in the clinical management of these tumours. This study describes two approaches adopted to target EGFR-overexpressing cells.

Firstly, we examined the effect of exogenous epidermal growth factor (EGF), a polypeptide growth factor which binds to and activates EGFR, on the radiation sensitivity of a panel of cell lines with a wide range of EGFR expression. This effect was assessed by colony forming assay and the results obtained were analysed using the linear quadratic model of cell survival. Our results indicated that the presence of EGF did not alter the radiosensitivity of any of the cell lines examined. This suggests that the use of EGF as a radiosensitising agent is unlikely to be of benefit in a clinical setting.

The second approach adopted was to investigate the possibility of using EGF as a means of delivering therapeutic ultra-short range radionuclides to EGFR-overexpressing cells. The short range of the particles emitted from these radionuclides (Auger electrons) requires that decay occurs in close proximity to cellular DNA for a radiobiological effect to be observed. For EGF to act as an effective delivery vehicle for these radionuclides, it must be capable of delivering the radionuclide to the nucleus of the target cell.

Studies were carried out to assess the sub-cellular distribution of EGF after receptor binding and internalisation. Using both light and elctron microscopic techniques along with cell fractionation, we established that exogenous EGF does accumulate in the nucleus of some EGFR-overexpressing cell lines under the appropriate conditions. These results suggested that EGF may be suitable as a delivery vehicle for Auger-emitting radionuclides. Finally, we examined the effect of EGF conjugated to the Auger-emitting radionuclide 123I on the clonogenic survival of a cell line panel with a wide range of EGFR expression. We observed that 123I-EGF was able to sterilise some EGFR-overexpressing cell lines, and that cell lines with a higher EGFR expression were more senitive to123I-EGF than those with a lower EGFR expression. It was also observed that incubation conditions which abolished the nuclear uptake of EGF also abolished 123I-EGF-mediated cytotoxicity, suggesting that 123I-EGF sensitivity is dependent upon the nuclear uptake of the radioconjugate.

The function of nuclear EGF remains to be established, as does the nuclear translocation mechanism. However, the possibility of EGFR-targeted therapies based upon this phenomenon, as outlined in this report, implies that further studies into the nuclear accumulation of EGF and other polypeptide growth factors are warranted.

# CHAPTER 1

# IONISING RADIATION AND CELL DEATH

## 1.1 Overview

Ionising radiation is presently one of the most widely used and efficacious cancer therapies. Generally, the range of intrinsic cellular radiosensitivity varies much less than sensitivity to cytotoxic drugs, and the emergence of clonal radioresistance occurs less frequently than does resistance to chemotherapeutic agents. The effectiveness of external beam radiotherapy, however, is limited by the amount of radiation damage sustained by non-malignant cells, primarily those of the hematopoetic system. Agents which are able to selectively sensitise tumour cells, or protect normal cells from radiation damage have the potential to improve the clinical outcome of patients undergoing standard radiotherapeutic regimes.

This thesis is concerned with the exploitation of the overexpression of the epidermal growth factor receptor (EGFR) by some tumour cell types in a radiotherapeutic context. One approach taken was to examine the effect on the radiosensitivity of tumour cells by ligand-induced activation of EGFR. Previous reports have suggested that EGFR activation results in an increase in cellular radiosensitivity in some tumour cell lines (Kwok and Sutherland, 1989, 1991b). Chapter 7 describes experiments which were performed to establish the effect of exogenous EGF on the radiosensitivity of EGFR-expressing tumour cell lines.

This thesis also describes preliminary experiments to determine the effectiveness of epidermal growth factor (EGF) as a vehicle for targeted radionuclide therapy. The specific targeting of EGFR-overexpressing tumours by EGF-radionuclide conjugates has the potential to allow tumour cell irradiation without the degree of normal tissue damage sustained by conventional external beam irradiation.

There are many radionuclides with diverse physical properties which may be suitable for targeted radiotherapy. However, one of the primary determinants of the effectiveness of any radionuclide therapy is the ability of the targeting moiety (delivery vehicle) to deliver radionuclide to the tumour site. If the radionuclide used has a very short range (less than one cell diameter), the sub-cellular localisation of the targeting vehicle becomes important. Chapter 8 describes experiments to

establish the sub-cellular localisation of exogenously added EGF, and thus its suitability as a vehicle for ultra-short range radionuclides. Chapter 9 describes experiments which demonstrate the ability of EGF-ultra-short range radionuclide conjugates to sterilise EGFR-expressing tumour cell lines.

## 1.2 Ionising radiation-induced cell death

The link between ionising radiation-induced genomic DNA damage and cell sterility is now well established. The selective irradiation of cell nucleus and membrane (by the use of 125I-IUDR and 125I-concanavalin A respectively) demonstrated that the radiation dose to the nucleus is of primary importance in determining cell survival (Warters et al, 1977). Of the various types of DNA damage sustained after irradiation, double strand breaks (dsb), and the efficiency of dsb repair have been shown to be the most important factors in radiation-induced cell death (Kemp et al, 1984; Schwartz et al, 1991). There is evidence to suggest that a single unrepaired dsb results in cell sterility, and can be regarded as a lethal lesion (Frakenberg-Schwager and Frakenberg, 1990).

## 1.3 Mechanisms of radiation-induced cell death

There are two morphologically distinct mechanisms of cell death: necrosis (mitotic cell death) and apoptosis (programmed cell death). Apoptosis is characterised by several features which are absent in necrotic cell death. These include shrunken cell size, shrunken nuclei and a characteristic DNA fragmentation 'ladder' demonstrable by electrophoresis (reviewed by Wyllie, 1993). Generally, necrosis occurs during mitosis, while apoptosis occurs during interphase. Apoptosis usually occurs rapidly after irradiation, and is thought to be a cell-regulated process (Sellins and Cohen, 1987). Necrotic cell death does not necessarily occur during the first cell division after irradiation. Introduction of dsb by either radiation or DNA-cleaving restriction enzymes results in the progressive accumulation of gross chromosome abnormalities (Bradford, 1991; Zhang and Dong, 1987). Lethally-

irradiated cells may undergo several divisions before metabolic and structural integrity is lost due to these chromosome abnormalities (Hurwitz and Tolmach, 1969).

Whilst necrotic cell death is prevalent in most cell types, radiation-induced apoptosis has been observed in an increasing range of cell types. These include lymphoid and hematopoetic cells, and undifferentiated progenitors of testicular, renal and neuronal cell lineages (reviewed by Allan, 1992).

### 1.4 Factors involved in radiation-induced apoptosis

Classical apoptosis is defined as a type of cell death that requires gene expression (Wyllie et al, 1980), and may be suppressed by inhibitors of protein or RNA synthesis. The molecular events which initiate apoptosis are not well defined. Apoptosis has been observed in some cell types after ligand-induced activation of Fas (APO-1/CD95) and tumour necrosis factor receptor (TNF-R1). However, apoptosis initiated by these mechanisms is not inhibited by protein synthesis inhibitors, suggesting that the intracellular mediators of apoptosis may already by expressed in some cells (Nagata, 1996).

Recent reports have highlighted the role of sphingomyelin metabolites in the cellular response to radiation. Sphingomyelin is an integral membrane sphingolipid which yields intracellular ceramide upon hydrolysis. Ceramide has been demonstrated to be involved in the regulation of several intracellular signal transduction pathway intermediates (including intermediates in the stress-activated kinase pathway), the phosphorylation status of the retinoblastoma gene product, and the interleukin-1β converting enzyme (ICE)- like protease family (Hannun, 1996). The result of increased intracellular ceramide concentration varies amongst cell types, but reported effects include apoptosis, cell cycle arrest, differentiation and cell senescence (Venable et al, 1995, Jayadev et al, 1995, Obeid et al, 1993)

Ionising radiation has been demonstrated to trigger the hydrolysis of sphingomyelin to produce intracellular ceramide (Haimovitz-Friedman et al, 1994 a). This effect was also observed in enucleated cells, implying that radiation has a

direct effect on radiation-sensitive intracellular targets other than genomic DNA. Subsequent investigations established that ceramide-induced apoptosis in endothelial cells could be blocked by direct activation of protein kinase C (PKC), and by indirect activation of PKC via the receptor for basic fibroblast growth factor (bFGF) (Haimovitz-Friedman et al, 1994(b); Fuks et al, 1995). Molecular events downstream of PKC in ceramide-induced apoptosis have yet to be established.

Further studies have highlighted the role of the cell membrane in the apoptotic pathway. Trolox, a vitamin E analogue which inhibits oxidative membrane damage, has been shown to protect irradiated thymocytes from radiation-induced apoptosis (Ramakrishnan et al, 1993). Also, chemical agents which cause cell membrane lipid damage are able to induce apoptosis (Langley et al, 1993).

The precise mechanism by which radiation induces sphingomyelin hydrolysis remains obscure, as does the nature of the non-DNA radiation-sensitive target.

### 1.5 The linear quadratic model of cell survival

The linear quadratic model of cell survival in response to ionising radiation was first outlined by Kellerer and Rossi (1972). This model was developed further by Chadwick and Leenhouts (1973), who arrived at the same empirical equation as Kellerer and Rossi using several different assumptions.

The equation which relates cell survival and dose is now widely used to describe the sensitivity of cells to ionising radiation, and usually takes the form:

$$\text{Survival (S)} = \exp - (\alpha d + \beta d2)$$

$\alpha$ and $\beta$ are constants, relating to different parts of a survival curve which has no shoulder nor linear portion, but is continuously bending.

The Kellerer and Rossi model makes the assumption that each cell has two 'targets', and that both must be hit to cause cell sterility. This could be achieved by a single track of radiation hitting both targets, or by two distinct ionisation events,

each hitting a distinct target. The α component is related to the probability of a single track causing lethal damage, while the β component is related to the probability of lethal damage being caused by two tracks.

The Chadwick and Leenhouts model assumes that only an unrepaired double strand break in DNA causes cell sterility, and that a single track of ionising radiation may cause either a double or single strand break. It is also assumed that if a single track causes a single strand lesion, then a second track is required to cause a further lesion in the complementary DNA strand, close enough to the primary lesion to generate what appears to be a double stranded lesion.

In relation to the survival curve produced using this model, the α component describes the initial slope of the curve, while the β component describes curvature of the line.

These models are unlikely to closely represent the actual effects of ionising radiation tracks on cellular DNA, but the linear quadratic equation does appear to fit the data obtained from cultured cell response to radiation, within the dose range normally used to examine monolayer and spheroid cultures.

### 1.6 Tumour radiobiology

Clinical management of malignant disease often utilises fractionated radiotherapy. The rationale for dose fractionation is based mainly upon the fact that tumour cells generally repair DNA damage less efficiently than normal cells. The inter-fraction time results in preferential repair and repopulation of damaged normal tissue However, several processes may affect the outcome of fractionated radiotherapy. Re-oxygenation, redistribution, and intrinsic radiosensitivity, as well as repair of DNA damage and repopulation of normal tissue, are collectively known as the five 'Rs' of radiotherapy. Re-oxygenation refers to the tumour regions which have a poor blood supply, and are thus hypoxic. Hypoxic cells are much more resistant to γ-irradiation than the same cells under oxic conditions. Under fractionated radiotherapy, hypoxic regions may undergo re-oxygenation, and thus ensure that no cell remains permanently hypoxic, and thus resistant to γ-irradiation. Redistribution

refers to cell cycle status of tumour cells, and allows cells which are initially in a relatively radiation-insensitive cell cycle phase to re-assort into more sensitive phases. Intrinsic radiosensitivity is discussed below.

### 1.6.1 *Intrinsic radiosensitivity.*

Cells from different tumour types produce different shapes of radiation survival curve. An examination of the radiation response of many cell lines and the tumours from which they were derived revealed that cell lines derived from generally radioresponsive tumours (e.g lymphomas) were more radiosensitive than cell lines derived from radioresistant tumours (e.g gliomas) (Malaise et al, 1987). The difference in radiosensitivity was only observed in the low-dose region of the survival curve (described by the $\alpha$ parameter from the linear quadratic model of cell survival).

Another parameter which allows discrimination between radiation sensitive and resistant cell lines is the surviving fraction at 2 Gy (SF2) (Steel, 1988). 2 Gy is the most common fractionated dose administered during radiotherapy. Cell lines examined by Steel (1988) showed a range of SF2 values between 0.19 and 0.52. A precise explaination of the differences in intrinsic radiosensitivity observed between different cell types remains elusive.

It is important to note that the curability of a tumour *in situ* is not wholly dependent upon intrinsic radiosensitivity. The additional influence of the remaining four 'Rs' combine to determine clinical outcome. However, induced alteration in the intrinsic radiosensitivity of a tumour cell could have important ramifications in a clinical setting.

# CHAPTER 2

# TARGETED RADIONUCLIDE THERAPY

## 2.1 Introduction

The major problem encountered in conventional radiotherapy is the unavoidable damage sustained by normal tissue. The sensitivity of normal tissue to radiation limits the total dose per fraction administered, and thus the effectiveness of the therapeutic strategy. Ideally, cancer radiotherapy involves the selective irradiation of tumour deposits without irradiation of the surrounding normal tissue.

Targeted radiotherapy involves the conjugation of radionuclides to carrier molecules which are preferentially accumulated in tumours. Systemic administration of such conjugates results in tumour cell accumulation of cytotoxic radionuclide, whilst non-tumour tissue, which does not accumulate the conjugate, is not irradiated.

The efficacy of targeted radiotherapy is dependent upon the nature of both the radionuclide and the tumour-seeking delivery vehicle.

## 2.2 Radionuclides in targeted therapy

There are many radionuclides available for conjugation to targeting vehicles, with a wide range of physical and chemical properties. Some of those of current interest are listed in table 2.1.

There are several criteria by which an appropriate radionuclide may be selected. The half-life should match as closely as possible the biological half-life of the delivery vehicle. This ensures that the majority of decay energy is deposited whilst the targeting vehicle is available for tumour association. Another important factor is the chemistry of the isotope in question. The stability of the conjugate is of primary importance, and a simple and rapid conjugation method is preferable. The most important criterion for selection of a radionuclide is the form in which the decay energy is deposited, and the range of these emissions.

### 2.2.1 *Nature and range of emitted particles*

The decay of many radionuclides includes a gamma component. These emissions, whilst of negligible therapeutic benefit, are useful in scintigraphy, allowing imaging of tumour deposits, and providing information on the distribution and tumour uptake of the conjugate. The particulate emissions of the radionuclide are responsible for radiobiological effect. The mean range of emitted particles describes the distance from the site of decay in which energy is deposited. Irradiation of the cell nucleus,

and thus DNA damage, is the primary objective of radionuclide therapy. As outlined in table 2.1, the mean range of emitted particles varies considerably. Long range β particles have a range of several cell diameters (e.g 131I), whilst Auger electrons have a mean range of less than one cell diameter (e.g 125I). The selection of an appropriate radionuclide therefore depends upon the subcellular distribution of the targeting vehicle. A vehicle which remains bound to a cell surface antigen, or is internalised into the cell cytoplasm (e.g an antibody) would require a particle emission with a range of at least one cell diameter to ensure irradiation of the cell nucleus. A targeting vehicle which localises in the cell nucleus allows the possibility of using short range Auger electron-emitting radionuclides (e.g 125I-iododeoxyuridine). Auger electron-emitting radionuclides have a greatly decreased radiobiological effect when localised in the cytoplasm or cell membrane (Warters et al, 1977). The sub-cellular localisation of a long range β-emitting radionuclide (e.g 131I) is less important, because the particle range is longer than a single cell diameter, and the nucleus of targeted cells will receive a radiation dose regardless of sub-cellular localisation.

### 2.2.2 *Linear energy transfer*

Linear energy transfer (LET) describes the energy imparted (in kiloelectron volts) to a medium by a radioactive emission travelling 1μm along its path or track (Hall, 1994). The terms low and high LET describe the relative ionising potential of different radioactive emissions. Radionuclides with low LET radiation (e.g 131I-emitted β-particles) are less effective in causing DNA damage, as energy exchanges with matter are more widely spaced, and thus less likely to interact directly with DNA. High LET radiation (e.g 125I-emitted Auger electrons) is preferable for radiobiological effect, as energy exchanges with matter occur frequently, and is more likely to result in DNA damage.

The LET nature of a radioactive emission also has implications for DNA damage repair. Ionising radiation inflicts several types of damage on DNA, including base modification and protein-DNA cross-linking as well as single and double strand breaks. The densely ionising nature of high LET radiation results in multiple lesions in a localised region of the DNA strand. The correct repair of multiple lesions

**Table 2.1** Radionuclides of interest for targeted radiotherapy (adapted from Wheldon, 1994)

| Radionuclide | Half-life | Emitted particles | Mean particle range |
|---|---|---|---|
| $^{131}I$ | 8 days | β | 5 mm |
| $^{90}Y$ | 2.7 days | β | 0.8 mm |
| $^{67}Cu$ | 2.5 days | β | 0.6 mm |
| $^{212}Bi$ | 1 hour | α | 50 μm |
| $^{211}At$ | 7 hours | α | 50 μm |
| $^{125}I$ | 60 days | Auger | > 1μm |
| $^{123}I$ | 13.1 hours | Auger | > 1μm |

occurs with a much lower frequency than single lesions, where the undamaged strand may be used as a template.

The complexity and number of DNA lesions caused by high LET radiation makes radionuclides which emit such particles especially suitable for targeted radiotherapy.

## 2.3 Targeting Vehicles

As outlined earlier, the efficacy of any targeted radionuclide therapy is dependent upon the ability to deliver radionuclide to the target cell. One of the earliest, and possibly most effective targeted radionuclide strategies is the administration of ionic 131I for the treatment of thyroid tumours. This treatment is effective due to the unique sequestration of iodine by thyroid cells. Ionic strontium-89 (89Sr) has also been used for palliative therapy of bone metastases, as it is preferentially accumulated in metabolically active bone. However, there are very few tumour types in which administration of radionuclide in ionic form would result in preferential tumour uptake. Conjugation of radionuclide to a tumour-seeking carrier molecule is usually required. Tumour cells have several biological and metabolic features which distinguish them from the surrounding normal tissue, and a variety of agents have been used to exploit these differences.

### 2.3.1 *Antibodies*

Although treatment of patients with radiolabelled antibodies raised against tumour markers was first described in 1953 (Pressman and Korngold), modern radioimmunotherapy began with the development of hybridoma technology, i.e the ability to produce large quantities of monoclonal antibody with pre-defined specificity. Monoclonal antibodies were originally envisaged as 'magic bullets', which would specifically seek out tumour-specific markers, and elicit an anti-tumour effect. However, clinical trials involving the use of naked (unconjugated) antibodies have yielded disappointing results (reviewed by Grossbard et al, 1992, Riethmuller et al, 1993), mainly due to the lack of an host antibody-dependent cytotoxic response.

Attempts to improve on these results have led to the conjugation of a wide variety of cytotoxins and radionuclides to monoclonal antibodies. However, investigators have encountered many problems with such strategies. Heterogeneity of antigen expression within the tumour mass, poor tumour vasculature, poor diffusion of antibody within the tumour mass and low levels of absolute antibody uptake in

tumours have been identified as problems affecting the efficacy of antibody-conjugate therapy (reviewed by Stold et al, 1997, Wheldon, 1994). In addition, the systemic administration of mouse monoclonal antibodies may result in a host immune response, reducing the efficacy of subsequent treatments. Attempts are currently underway to circumvent many of these problems by the use of smaller antibody fragments and 'humanised' antibodies (reviewed by von Mehren and Weiner, 1996).

In relation to immunoconjugates, radioisotopes have some advantages over cytotoxic drugs and biological toxins. Conjugation of a radionuclide with a long range β-emission results in both the target cell, and those in close proximity, being irradiated and thus sterilised. The process by which untargeted cells are killed by emissions from a neighbouring targeted cell is known as crossfire. Cytotoxic drugs or biological toxins are only capable of killing cells to which they are directly targeted, i.e. there is no crossfire effect. Heterogeneity of antigen expression within the tumour mass will have less effect on the efficacy of radioimmunoconjugates than on cytotoxic drug/toxin immunoconjugates due to the effects of crossfire. Other disadvantages of cytotoxic drug immunoconjugates are the potential denaturation of the drug after internalisation, and the sparing of tumour cells with multi-drug resistance. Biological toxins may also precipitate a host immune response. These problems are not encountered by radioimmunoconjugate strategies.

The most successful clinical application of radiolabelled antibodies is in the treatment of haematological malignancy. In particular, the treatment of B-cell lymphoma with 131I-labelled antibody has resulted in significant patient response and prolonged remission (Press et al, 1993, Kaminski et al, 1993). Haematological malignancies are good candidates for radioimmunotherapy, as the penetration problems encountered in solid tumours are greatly reduced. In addition, haematopoetic cells are generally radiosensitive, and the target antigens are not widely expressed on other tissue types.

### 2.3.2 *Nucleotide analogues*

Analogues of DNA precursor molecules (nucleotides) are incorporated into cellular DNA during the S-phase of the cell cycle, and are preferentially accumulated by proliferating cells. Deoxyuridine, an analogue of thymidine, and deoxycytidine have both been used as radioisotope delivery vehicles in cultured cell models (Makrigioros et al, 1989, Howell et al, 1991). Auger electron-emitting radionuclides are

particularly suitable for nucleotide analogue-conjugation, as the conjugate is incorporated into DNA.

Because this type of delivery vehicle exploits tumour/normal tissue proliferative differential, rather than any tumour-specific marker, systemic administration could lead to significant normal tissue toxicity. Work is currently underway to examine different types of drug administration, in an attempt to confine the biodistribution to the local tumour region, and maximise tumour uptake (Kassis et al, 1990). Tumours such as glioma are good candidates for radiolabelled nucleotide analogue therapy, as the proliferating tumour cells are surrounded by non-proliferating tissue.

### 2.3.3 *Agents which target metabolic differences of tumour cells*

Some tumour types display metabolic differences from the surrounding normal tissue. An example of this is malignant melanoma, in which the pigmented cells overproduce melanin. Compounds, such as methylene blue, which posses a high binding affinity for melanin are preferentially accumulated in melanoma cells, in proportion to the cellular melanin content. Radiolabelled methylene blue has been demonstrated to posses *in vivo* anti-tumour activity in animal models (Link and Carpenter, 1990, Link et al, 1996).

One of the most successful targeted radiotherapy strategies is the use of 131I-meta-iodobenzylguanidine (MIBG) in the treatment neuroblastoma and phaeochromocytoma. MIBG, similar in structure to noradrenalin, exploits the noradrenalin active uptake mechanism of these tumours. MIBG is currently in clinical use, both as a diagnostic tool and as a therapeutic agent.

### 2.3.4 *Growth factors and steroid hormones*

Many tumour cells overexpress, or have enhanced sensitivity to peptide growth factors. The small size and high receptor binding affinity of these peptides makes them good candidates as radionuclide delivery vehicles. Radiolabelled analogues of somatostatin have proven effective in human breast and small cell lung cancer xenograft models (Zamora et al, 1996). Ligands which bind the epidermal growth factor receptor (EGFR) have also been examined as potential delivery vehicles (Andersson et al, 1992). The use of EGFR ligands as delivery vehicles is discussed in chapter 5.

Steroid hormones are also candidates as delivery vehicles for radionuclides. Steriod hormones enter the cell by passive diffusion, and interact with specific intracellular receptors which associate with DNA in the ligand-bound state. Several tumour types, including breast and ovarian tumours, express high levels of oestrogen receptor. The DNA-association characteristic of the ligand-bound receptor allows the use of radionuclides with short-range emissions. 123I and 125I-oestrodiol derivatives have been demonstrated to selectively sterilise oestrogen receptor-positive cells (De Sombre et al, 1992, Beckman et al, 1993).

## 2.4 The therapeutic potential of Auger electron-emitting radionuclides

### 2.4.1. *Auger electron emitting radionuclides and their radioactive decay.*

Several radionuclides decay by electron capture and/or internal conversion. These terms describe the manner in which a nucleus absorbs an inner shell orbital electron (electron capture). The vacancy created in the inner shell is filled by another orbital electron from a higher energy level, generating another vacancy. The energy produced from this electronic transition may be transferred to one or more orbital electrons, causing further ionisation of the atom. This causes a cascade of electronic transitions, and a release of a large number of electrons, resulting in a highly charged atom. The atom is then charge neutralised by acquisition of electrons from the surroundings.

Emitted electrons resulting from transition from a higher to lower electron shell are termed Auger electrons, while those emitted as a result of a transition from a higher to lower energy sub-shell are termed Coster-Kronig electrons (Sastry, 1992).

The electron absorbed into the nucleus from the inner orbital shell combines with a proton to form a neutron. This process results in the emission of a γ-ray photon from the nucleus. This energy may be transferred to an orbital electron, causing further ionisation, and thus another cascade of electronic transitions as described above. This results in emission of Auger electrons. This process (internal conversion) is rarely 100 % efficient, and thus some of the energy of the photon will be released from the atom as γ-rays. The emitted γ-rays are characteristic of the radionuclide. In this report, for simplicity, all electrons emitted as a result of electron capture and/or internal conversion are termed Auger electrons.

Auger electrons generally have low energies (< 500 eV), and thus have very short ranges in biological material (< 1μm). However, the number of electrons emitted per radioactive decay can result in extremely high energy deposits within this range.

To date, the major clinical application of radionuclides which decay in either of these ways has been in radio-imaging. The technique of radio-imaging utilises the emitted γ-ray component of these radionuclides, while the Auger electrons cannot be detected due to the extremely short range of emission. Commonly encountered radionuclides in radio-imaging include 123I, 111In, 210TI and 99mTc.

### 2.4.2. *Auger electron cytotoxicity*

The decay of a radionuclide by electron capture and/or internal conversion produces Auger electrons with an extremely short range. To achieve a radiobiological effect from such electron emissions, it is necessary that the radioactive decay occurs close enough to DNA to be within the range of these electrons.

125I, incorporated into DNA in the form of the thymidine analogue 125I -5 iodo-2'-deoxyuridine (125I-IUdR) results in double-strand DNA breaks, and a decrease in cell survival and clonogenic capacity (Kirsch, 1972). The biological effects of 125I incorporation into DNA are consistent with those produced by high LET radiation, i.e. large clusters of ionisations within the range of the emitted Auger electron (Charlton, 1986). A high LET radiation track interacting with DNA will cause much greater damage than a low LET track, for example X-ray photon tracks. The relative biological effectiveness (RBE) of DNA-incorporated 125I when compared to estimated equivalent dose 250 KeV X-rays is over seven times greater (Kassis et al, 1989). However, 125I localised in the cytoplasm, cell membrane or in the extracellular medium produces a cell survival curve comparable with that achieved with low LET radiation, with an RBE similar to that observed with externally applied X-rays of estimated equivalent dose (Kassis et al, 1989), suggesting that the radionuclide must be localised within, or close to DNA for the high LET effect to be observed.

The damage caused to DNA by disintegration of incorporated 125I is very great, with each disintegration on average producing at least one double strand break (Humm and Charlton, 1989). Studies using synthetic oligonucleotides demonstrated that the majority of strand breaks occurred within 1-2 nm (approximately 5 bases) of the incorporation site, with some strand breaks occurring up to 7 nm (approximately 20 bases) from the site of incorporation (Martin and Haseltine, 1981). This study also

suggested that the decay of incorporated 125I causes multiple double strand lesions within a 10-20 base range. Typical radiation survival curves observed after treatment with high LET radiation characteristically show little curvature, and a steep 'linear' component.

The therapeutic potential of Auger electron-emitting radionuclides depends entirely upon selective nuclear delivery, as cytoplasmic localisation of these radionuclides has very little effect in either tumour or normal tissue (Kassis et al, 1989). Due to the short range of these particles, the high LET characteristic will only be observed after localisation within the targeted cell nucleus. Damage to surrounding cells should be greatly reduced compared to that induced by external beam radiotherapy, provided that an effective means of exclusive delivery to tumour cell nuclei can be developed. The photon emissions from the decay of Auger-emitting (and beta-emitting) radionuclides (either gamma or x-rays) rarely have any therapeutic benefit. However, in a clinical setting, the high concentration of radionuclide administered can result in the patient receiving a significant whole body dose from these emissions. This may be particularly important in multi-modal theapeutic strategies where the haematopoetic system is already under considerable stress.

### 2.4.3. *Delivery vehicles for Auger emitters*

The ultra-short range of Auger electrons requires a delivery vehicle which either binds, or is incorporated into DNA. Several studies have examined the potential of the use of nucleotide analogues as delivery vehicles. Deoxyuridine, a thymidine analogue, has been used to deliver 123I and 125I (in the form of iododeoxyuridine, or IdUR) and 77Br (in the form of bromodeoxyuridine, or BdUR) to DNA in cultured cell models (Chan et al, 1976, Kassis et al, 1982, Makrigiorgos et al, 1989). Similarly, deoxycytidine has been used to deliver 125I in the form of iododeoxycytidine (IdC) (Howell et al, 1991, Narra et al, 1992). All of these labelled nucleotide analogues produce high LET-type survival curves.

Molecules which bind DNA in a non-covalent manner have also been assessed as delivery vehicles for Auger emitters. Cisplatin analogues and the vitamin A analogue acetamidoiodo-proflavin, when labelled with Auger emitting radionuclide, have been shown to be highly cytotoxic (Kassis et al, 1989, Howell et al, 1994).

As outlined above (section 2.3.4), preliminary studies have also been carried out into the potential for the use of steroid hormones as delivery vehicles. These hormones enter the cell by passive diffusion, and recognise an intracellular receptor

which associates with DNA in the ligand-bound state (reviewed by Carson-Jurica et al, 1990). 123I- and 125I-labelled oestrogens have been demonstrated to be cytotoxic to oestrogen receptor-positive cells, displaying high LET-type effects (Beckman et al, 1993, DeSombre et al, 1992).

# CHAPTER 3

# THE EPIDERMAL GROWTH FACTOR RECEPTOR AND PHYSIOLOGICAL LIGANDS

## 3.1 Discovery and characterisation of EGFR and cognate ligands

### 3.1.1. *Epidermal Growth Factor*

The epidermal growth factor (EGF) was first described in 1962, when it was observed that an extract of mouse submaxillary gland was able to induce precocious tooth eruption in neonatal mice (Cohen, 1962). These effects were demonstrated to be a result of increased epidermal proliferation, hence the name epidermal growth factor. Following purification, analysis of the factor indicated that it was of low molecular weight (6 kD), and consisted of 53 amino acid residues, with three intermolecular di-sulphide bonds (Savage et al, 1972). The human equivalent of EGF, subsequently isolated from urine, was found to be identical to a peptide known as urogastrone, which acted as an inhibitor of gastric acid secretion (Gregory, 1975).

Since its discovery, EGF has been found to be the prototypic member of an extended family of peptides. These peptides all contain three di-sulphide bonds in the general sequence indicated (Carpenter and Cohen, 1990):

XnCX7CX2/3GXCX10/13CXCX3YXGXRCX9LXn

Individual members of the family are encoded by separate genes, and include transforming growth factor alpha (TGF-α), amphiregulin (AR), heparin-binding EGF-like growth factor (HB-EGF), cripto and betacellulin (BTC) (Carpenter and Cohen, 1990, Todaro et al, 1976, Shoyab et al, 1988, Higashiyama et al, 1991, Sasada et al, 1993). A viral gene frequently encoded by members of the pox virus family also produces a peptide with homology to EGF (Todaro et al, 1990). EGF-like motifs can also be found in a wide range of both secreted and cell-bound proteins. While these proteins have no EGF-like effect, they appear to have functions in development, cell adhesion and protein-protein interactions (Carpenter and Cohen 1990). It has been speculated that the growth factor/receptor system utilised by members of the EGF-like family may have evolved from an ancestral form of cell-cell communication (Prigent and Lemoine, 1992).

### 3.1.2. *The epidermal growth factor receptor*

The cellular receptor for EGF was isolated and purified to homogeneity by affinity chromatography in 1980 (Cohen et al, 1980). Both the purification and early study of the epidermal growth factor receptor (EGFR) was facilitated by the establishment of the A431 cell line derived from a human carcinoma of the vulva (Giard et al, 1973). A431 has an abnormally high EGFR content on the cell membrane, established by 125I-EGF binding as approximately 2x106 per cell (Haigler et al, 1978).

The full cDNA and amino acid sequence of the receptor was reported in 1984, which greatly enhanced the study of EGFR expression (Ullrich et al, 1984). EGFR is expressed normally in a wide range of tissues, with the notable exception of haemopoetic cells (Carpenter, 1987).

The observation that EGFR expression may be increased in human malignancies was first reported in 1984, where it was observed that both epidermal tumour biopsy material, and cell lines derived from this material, invariably has an enhanced EGFR expression (Hendler and Ozanne, 1984). More recent studies have demonstrated that EGFR overexpression is a characteristic of tumours of many diverse sites of origin. This overexpression may be involved in both the initial transformation process and in the clinical progression of the disease (Di Fiore et al, 1987, Sainsbury et al, 1987).

Subsequent investigation has established that the EGFR belongs to a family of structurally related proteins with regions of conserved sequence homology. The Type 1 (EGFR-like) growth factor receptor family includes HER 1 (also known as EGFR, erb-B1, p170 *erb-B1* ), HER 2 (erb B2, p185 *erb B2*, p185 *neu* ), HER 3 (erb B3, p160 *erb B3* ) and HER 4 (erb B4, p180 *erb B4* ) (Prigent and Lemoine, 1992, Kraus et al, 1989, Plowman et al, 1993). Whilst ligand molecules for HER 3 and 4 have been described, none has been demonstrated to be able to directly bind and activate EGFR (Carraway et al, 1994, Plowman et al, 1993). EGFR remains the best characterised member of this family.

### 3.1.3. *EGFR signalling*

Initial observations of the effect of ligand binding to the EGFR indicated that protein kinase activity was involved in the transduction of the EGF signal. The addition of EGF to partially purified extracts of A431 cell membranes resulted in a rapid increase in protein phosphorylation (Carpenter et al, 1979). Subsequent studies indicated that the EGFR itself, as well as other substrate proteins, were

phosphorylated in response to EGF stimulation (King et al, 1980). The finding that both substrate proteins and the EGFR itself were phosphorylated specifically on tyrosine residues gave rise to the hypothesis that EGF exerted its effect on receptor-expressing cells via an increase in receptor associated tyrosine kinase activity (Ushiro and Cohen, 1980). Failed attempts to dissociate the ligand binding activity from the tyrosine kinase and substrate portions of the receptor indicated that all 3 regions were contained within a single molecule (Buhrow et al, 1982).

The EGFR was the first described membrane-bound growth factor receptor with intrinsic tyrosine kinase function. Subsequent investigations have revealed many other receptor tyrosine kinases, including the receptors for insulin, insulin-like growth factor (IGF), colony stimulating factor (CSF), platelet-derived growth factor (PDGF) and the fibroblast growth factor family (reviewed by Ullrich and Schlessinger, 1990).

## 3.2 Control of normal and aberrant EGFR expression

### 3.2.1. *Regulation of EGFR expression*

The regulation of EGFR expression is a complex process, with many cellular compounds exerting regulatory influence at several stages of the transcription/translation pathway.

The EGFR gene promoter was first characterised in 1985, with the observation that the GC-rich promoter which contained an SP1 binding motif did not contain CAAT or TATA boxes (Ishii et al, 1985). Subsequent investigations have revealed a number of transcription factors which positively regulate EGFR gene transcription, including SP1, ETF1 and 2 (EGFR-specific transcription factor) (Merlino, 1990, Kageyama et al, 1988). p53 has also been demonstrated to bind and activate the EGFR promoter region (Deb et al, 1994). Additional co-operative factors, which bind to different sequences in the promoter region and enhance transcriptional activity, have been described (Maikawa et al, 1989). Transcription repressing DNA-binding proteins have also been reported, including ETR (EGFR transcriptional repressor) and GCF (GC factor) (Kageyama and Pastan, 1989, Hou et al, 1994).

The cellular expression of EGFR has been demonstrated to be regulated at the transcriptional level by a variety of physiological systems. Both progestin and glucocorticoid treatment of a human breast cancer cell line (T-47D) have been shown to result in increases in EGFR mRNA and cell surface receptor expression (Murphy

et al, 1986, 1988, Ewing et al, 1989). Growth hormone has also been demonstrated to increase EGFR expression and mRNA levels in rodent hepatic cells (Jansson et al, 1988, Ekberg et al, 1989). An interesting regulator of EGFR expression is EGF itself, which has been shown to increase EGFR mRNA transcription in two different cell lines (Clark et al, 1985, Kudlow et al, 1986). This effect is achieved as a result of an increase in the intracellular half-life of the mRNA species (Jinno et al, 1988). There is no report of any other EGFR regulation system causing this effect. Other known regulators of EGFR transcription include interferon-gamma (IFN-γ), and the negative regulators, thyroid hormone (T3) and retinoic acid (Hamburger and Pinnamaneni, 1991, Hudson et al, 1990, Grandis et al, 1996).

EGFR transcription is also upregulated by elevated intracellular concentrations of cAMP, and the protein kinase C activator, PMA (4β-phorbol 12-myristate 13-acetate) (Hudson et al, 1989), suggesting that other cellular signalling systems may affect EGFR expression.

In summary, the transcription and expression of EGFR is under precise control by a range of intracellular signalling intermediates and hormonal influences at the transcriptional and post-transcriptional level. Complex regulation of EGFR expression is essential due to the consequences of abnormal expression. These consequences are discussed in section 3.3.

### 3.2.2. *EGFR gene amplification*

The overexpression of EGFR is most commonly a consequence of increased mRNA production and/or stability. However, amplification of the EGFR gene may also be involved in receptor overexpression. EGFR gene amplification has been observed, both as double minute chromosomes and homogeneous staining regions, in tumours of several sites. Amplification is most commonly observed in gliomas, particularly glioblastoma multiforme, to a lesser degree in lung squamous cell carcinomas and head and neck squamous cell tumours (Schwechheimer et al, 1995, Gorgoulis, 1993, Saranath et al, 1993). Due to the higher incidence of gene amplification in gliomas, many of the studies addressing the characteristics of EGFR gene amplification have been carried out using material and cell lines derived from tumours of this type.

EGFR gene amplification invariably results in EGFR protein overexpression, but there appears to be no direct correlation between gene copy number and the level of protein expression (Chaffanet et al, 1992). This suggests that control of expression at

the transcriptional level remains the most important determinant of EGFR protein overexpression.

In early stage gliomas, investigators have observed other genetic alterations which usually precede EGFR gene amplification. These most commonly include loss of heterozygosity (allele loss) in regions of chromosomes 10 and 17p (Fujimoto et al, 1989, James et al, 1989). The incidence of these genetic mutations is high (LOH 10 = 60-75%, LOH 17p = 30-35%), but the influence on subsequent EGFR gene amplification is unclear (Venter et al, 1991). One study has observed that EGFR gene amplification occurs with higher frequency in patients without LOH 17p, and only occurs in patients with LOH 10 (Von Deimling et al, 1993). However, another study has indicated that EGFR gene amplification can be observed without LOH 10 (Diedrich et al, 1995). Both of these studies found that EGFR gene amplification incidence increases with age of patient.

Whilst an explaination of how chromosome 10 and 17p allele losses directly affect EGFR gene amplification remains elusive, the prognostic significance of amplification and the resultant EGFR overexpression is clear. Individuals with a glioma carrying an EGFR gene amplification have a significantly poorer prognosis (Diedrich et al, 1995).

### 3.2.3. *EGFR gene rearrangement*

As well as amplification of the EGFR gene in gliomas, there is also a high incidence of structural rearrangement of the gene, resulting in an in-frame deletion mutant EGFR protein (Sugawa et al, 1990). The most commonly observed mutant receptor is characterised by the loss of nucleotides 275-1075 in the receptor gene, encoding amino acid residues 16-273 (Wong et al, 1992). This corresponds to the deletion of exons 2 to 7, and the direct fusion of exons 1 and 8. The resultant protein, termed EGFR VIII, has almost no extracellular domain and is unable to bind EGFR ligand. This protein bears great similarity to the v-*erb* gene product, and behaves in a similar manner i.e as a constituitively active membrane-anchored tyrosine kinase (Ekstrand et al, 1994). The expression of EGFR VIII was first reported in 17% of primary glioblastoma tumours (Wong et al, 1992, Yamazaki et al, 1988). Subsequent studies have revealed that EGFR VIII expression can be observed in 16% of non-small cell lung carcinomas (Garcia de Palazzo et al, 1993), 78% of (infiltrating ductal) breast carcinomas, 75% of ovarian carcinomas, and several brain tumours, including astrocytoma (56%) and medulloblastoma (86%) (Moscatello et al, 1995).

Other EGFR deletion mutants have been reported, but occur at a much lower frequency (Garcia de Palazzo et al, 1993). These studies have also failed to detect any EGFR deletion mutant in corresponding normal tissue, suggesting that EGFR VIII expression may be exploited as a tumour-specific marker.

## 3.3. The EGFR system in human tumours

### 3.3.1. *The role of EGFR in tumour initiation and progression*

One of the earliest indications that EGFR may play a role in the development of the malignant process was the observation that the product of the v-*erb* B oncogene has many similarities to the intracellular tyrosine kinase-containing region of the human EGFR (Downward et al, 1984a). The v-*erb* B (avian erythroblastosis virus) oncogene, which induces malignancies in chickens, is derived from the avian EGFR gene and thus indicated the proto-oncogenic potential of the EGFR.

The observation that the v-*erb* B gene product contained a constituitively active tyrosine kinase domain, which appeared to be responsible for the transforming potential of the virus, gave rise to the hypothesis that the observed increase in EGFR expression in some tumour types may be responsible, or at least involved in either the malignant transformation process, or in the progression of the disease (Gilmore et al, 1985).

The role of EGFR overexpression in the transformation process has since been studied, revealing a number of interesting findings. Two independent studies determined that overexpression of normal human EGFR in NIH 3T3 fibroblasts resulted in transformation (defined by soft agar colony formation) only when the cells were exposed to an EGFR ligand (Di Fiore et al, 1987, Riedel et al, 1988). Overexpression of normal human EGFR in the absence of an activating ligand was not sufficient to confer the transformed phenotype. However, transfection of a similar cell line with an N-terminal deleted EGFR, containing no extracellular (ligand binding) domain was sufficient for transformation (Haley et al, 1989). The N-terminal mutants were shown to have a constituitively active kinase domain, similar to that observed with the v-*erb* B gene product. This study indicated that EGFR-related transformation was dependent upon the activity of the intracellular kinase domain of the receptor. It should be noted though, that overexpression of EGFR in vivo in a number of different cell types failed to produce neoplasia in transgenic animals (Merlino, 1990), suggesting that EGFR overexpression may lie downstream

of another important transformational event. Similar work using TGF-α transgenic mice demonstrated that overproduction of EGFR ligand is implicated in the development of epidermal tumours of several sites without initial EGFR overexpression (Jhappan et al, 1990, Sandgren et al, 1990, Vassar et al, 1992).

Whilst the exact role of ligand and receptor overexpression in the transformation process remains obscure, the finding that EGFR ligand is frequently detected in tumours overexpressing EGFR suggests that the formation of self-stimulating (autocrine) loops plays an important role in the pathology of these tumours (Iihara et al, 1993, Fang et al, 1996).

### 3.3.2 *EGFR overexpression in human tumours*

The EGFR is overexpressed in a wide range of sarcomas and carcinomas. Overexpression is most commonly seen in squamous cell carcinomas, and is also observed less frequently in some brain tumours, in particular malignant glioma. Common sites of EGFR overexpression are tumours of the head and neck, breast, bladder, lung (non-small cell, and lung adenocarcinomas), as well as tumours of the digestive tract and several gynaecological tumours, especially those of cervical and ovarian epithelium (reviewed by Gullick, 1991, Modjtahedi and Dean, 1994a).

The reliability of EGFR expression as a prognostic indicator remains to be fully established. Routine screening of biopsy material may help to confirm the prognostic significance of EGFR overexpression, but questions still remain over the clinical relevance of such screening. The degree of overexpression in many malignancies appears to correlate with tumour stage/grade, and thus may not have much value as a tumour independent marker (Gusterson, 1992). Also, standardisation of the screening technique is required. Many anti-EGFR antibodies do not perform reliably in paraffin-embedded sections, and data obtained using immunocytochemical techniques may be open to misinterpretation (Gusterson, 1992). Studies carried out on biopsy material using radioligand binding or radioimmunoassay can only be semi-quantitative, as the results obtained are dependent on the amount of normal tissue in the sample. The observation that production of EGFR mRNA generally correlates with expression of EGFR protein on the cell surface suggests that more accurate and informative screening of tumour samples may be possible using RT-PCR techniques (Xu et al, 1984).

Whilst the prognostic implications of EGFR overexpression remain controversial, there are several reports which appear to correlate high EGFR expression with poor

prognosis. This is most apparent in superficial bladder tumours, where overexpression of EGFR is associated with tumour multiplicity, short relapse time, high recurrence rate and early death (Neal et al, 1990), while EGFR overexpression in breast tumours also correlates with poor tamoxifen response, early recurrence and death in lymph node-positive patients (Horak et al, 1991, Nicholson et al, 1990). Similarly, EGFR overexpression may predict poor survival in patients with squamous lung tumours (Hendler et al, 1989).

## 3.4. Structure of the EGFR

The EGFR is a 170 kD membrane-anchored protein, consisting of 1186 amino acids (figure 3.1). This protein is encoded by the c-erb B1 proto-oncogene, located on chromosome 7 in humans. The receptor is a single chain protein which crosses the cell membrane once, the transmembrane domain consisting of 24, mainly hydrophobic, residues.

The amino terminus of the EGFR (amino acid residues 1-621) contains the extracellular ligand binding domain, which, *in vivo*, contains a large amount of N-linked polysaccharide (Carpenter and Cohen, 1990). Also, it contains two cysteine-rich regions which can be aligned, and give the external part of the EGFR a rigid, bi-lobal structure (Hunter, 1984). Evidence suggests that the non-cysteine rich regions of the extracellular domain are involved in ligand binding (Lax et al, 1988). The cytoplasmic, carboxyl-terminus of the peptide chain (amino acid residues 645 - 1186) can be divided into 3 domains based on function:

### 3.4.1. *Juxtamembrane domain*

The juxtamembrane domain (648-668) contains several phosphorylation sites which appear to be involved in the modulation of receptor function. Thr 654, Thr 669 and Ser 671 have been implicated as substrates for Ser/Thr kinases, with Thr 654 being the major protein kinase C (PKC) substrate. PKC interaction with EGFR results in attenuation of receptor tyrosine kinase activity (Davis et al, 1985, Livneh et al, 1988) and downregulation of high affinity EGF binding (Lund et al, 1990, Countaway et al, 1990). These effects are abrogated by point mutation of the EGFR at Thr 654. Phosphorylation of Thr 669 and Ser 671 results in altered receptor internalisation kinetics (Heiserman et al, 1990). Thr 669 has been implicated as a ceramide-activated protein kinase substrate (Dressler et al, 1992, Joseph et al, 1993),

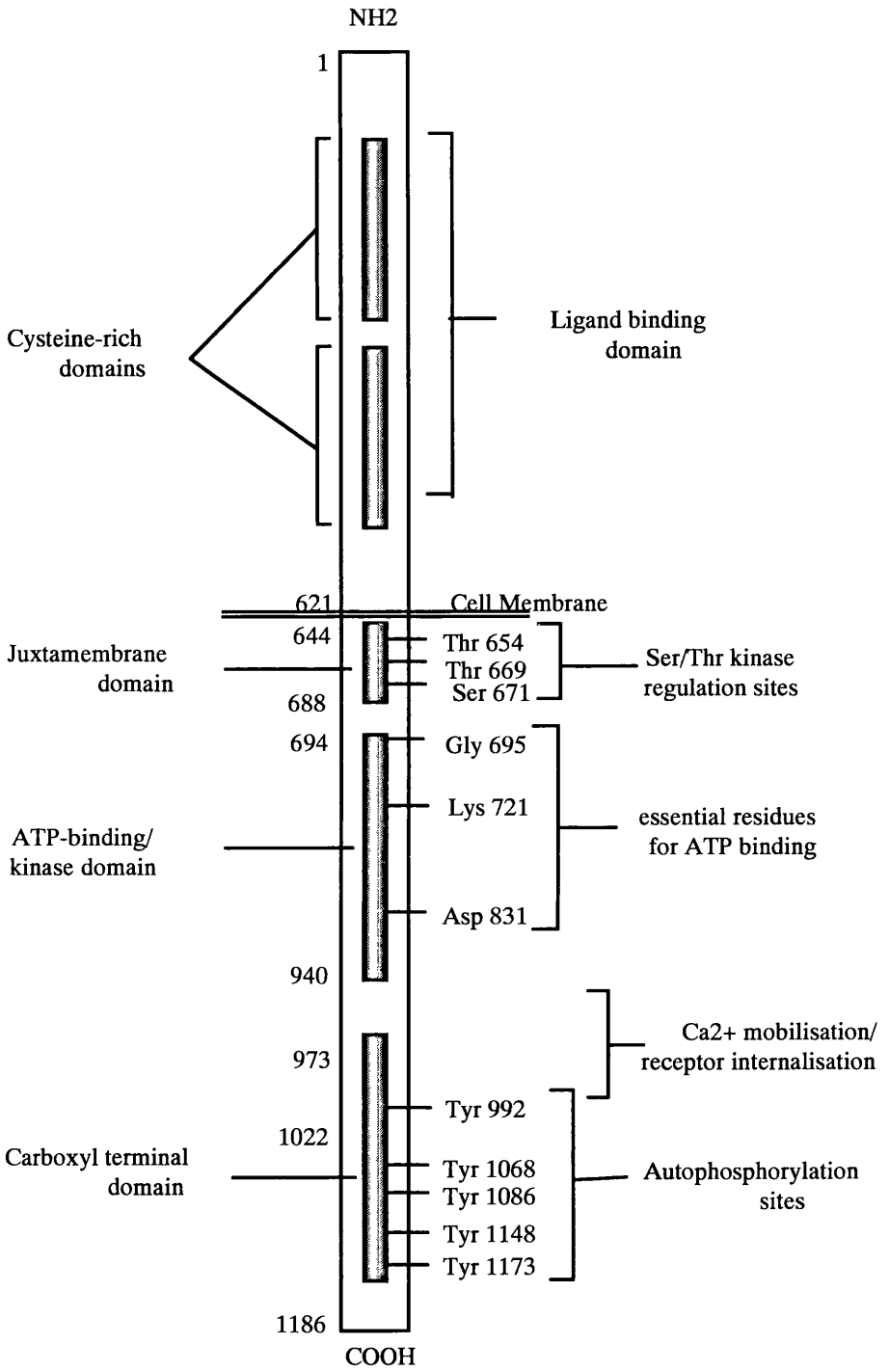



**Figure 3.1** Schematic representation of the epidermal growth factor receptor. Domains and amino acid residue numbers are indicated on the left, while individual amino acids of functional significance are indicated on the right.

and has also been found to be a phosphorylation site for MAP kinase (Takishima et al, 1991). However, more recent studies have suggested that these sites are not under direct control by PKC and MAP kinase, and that an intermediate protein may be involved in mediating the effects of these kinases on EGFR function (Griswold-Prenner et al, 1993, Morrison et al, 1996). The upregulation of PKC and MAP kinase activity by EGFR suggests the presence of auto-inhibitory loops involved in regulating EGFR tyrosine kinase activity.

The juxtamembrane domain also interacts with several proteins with signalling functions, including phosphatidylinositol-4P kinase (PIP-4 kinase), adenylyl cyclase and the cytosolic kinase, *eps* 8 (Cochet et al, 1991, Castagnino et al, 1995, Sun et al, 1995).

### 3.4.2 *Kinase domain*

The kinase domain is the most highly conserved region between members of the type 1 family of receptors. Of central importance in this domain is the ATP binding site, Lys 721. Point mutation of this site abrogates ATP binding and receptor tyrosine kinase activity (Honneger et al, 1987). This domain also contains two short sequences which are highly conserved in nucleotide binding proteins, both of which are involved in the correct orientation and binding of ATP (Wierenga and Hol, 1983, Carpenter, 1987). Within these sequences, residues Gly 695 and Asp 831 appear to be the main sites of interaction with ATP.

### 3.4.3. *Carboxyl terminal domain*

The carboxyl terminal tail domain contains two regions of functional importance. Amino acids 973 to 1022 contain a number of negatively charged residues which appear to play a role in ligand-induced EGFR internalisation. Truncation of the receptor to residue 972 results in a receptor which is unable to undergo ligand-induced downregulation (Chen et al, 1989). This region is also implicated in EGFR-mediated alterations in Ca2+ ion flux.

The carboxyl tail (residues 1022-1186) contains the main autophosphorylation sites on the receptor. Tyrosine resides 992 1068, 1086, 1148 and 1173 have all been reported as being phosphorylated in response to EGF stimulation of the receptor (Downward et al, 1984, Margolis et al, 1989, Walton et al, 1992). Phosphorylation of these sites allows the carboxyl terminus of the receptor to form binding sites for

several SH2 (src homology 2) domain proteins (Margolis et al, 1990). These proteins include the ras GTPase activating protein (GAP), phosphatidylinositol-3 kinase (PI-3 kinase), phospholipase C-gamma (PLC-γ), the src homology and collagen protein (Shc), GRB2, and the SH2 domain protein-tyrosine phosphatase (SHP-1)(Batzer et al, 1994, Tomic et al, 1995, Soler et al, 1994). Interaction with these proteins serves to link the EGFR to a variety of intracellular signalling pathways. However, point mutation of all known autophosphorylation sites, or their removal by truncation of the carboxyl tail does not prevent the phosphorylation of these proteins or the activation of downstream signalling proteins associated with EGFR stimulation (Chen et al, 1989, Li et al, 1994). The mechanisms involved in the activation of SH2 proteins by these mutant receptors have not been fully elucidated.

## 3.5. Signal transduction and signal abrogation

### 3.5.1. *Signal transduction from activated EGFR*

EGF exerts all of its cellular effects by binding the extracellular domain of the EGFR. No other mechanism of EGF-mediated alteration in cell function has been suggested. The transduction of this signal from the cell surface to nucleus has been the subject of extensive investigation, with the result that the EGFR intracellular signalling mechanism is now the best characterised of any growth factor receptor. There are several physical and biological events associated with EGFR activation and attenuation of the signal.

#### 3.5.1.1. *Ligand induced dimerisation*

First described for EGFR, and now accepted as a property of all known growth factor receptors, is the phenomenon of ligand-induced receptor dimerisation (Schlessinger and Ullrich, 1992). Receptor dimerisation is a crucial early event in the EGF response, as co-expression of extracellular-truncated and wild type EGFR results in the formation of an inactive heterodimer (Honneger et al, 1990, Kashles et al, 1991). This work was expanded upon with the demonstration that the dimeric form of the receptor was not only more catalytically active than the monomer, but that the dimer represented the high affinity component of the EGFR dual affinity ligand binding characteristic (Sorokin et al, 1994).

3.5.1.2. *Recruitment and activation of signalling intermediates*

The first reported substrate for EGFR was EGFR itself (King et al, 1980). As outlined earlier, receptor autophosphorylation increases the interaction of SH2 domain proteins (Honneger et al, 1988, 1989). These proteins assemble at the receptor, resulting in the activation of downstream signalling pathways. Many of these pathways converge at MEK kinase (MAP kinase/extracellular signal related kinase kinase), with a subsequent activation of the p42/p44 MAP kinases, a family of ser/thr kinases (Pelech and Sanghera, 1992). MAP kinases are rapidly translocated to the nucleus after activation, where they play an important role in the regulation of several transcription factors and other nuclear proteins (reviewed by L'Allemain, 1994).

3.5.1.3. *Attenuation of signalling*

The primary mechanism by which EGFR signalling is arrested is receptor endocytosis and lysosomal degradation (Chen et al, 1989). Expression of mutant receptors which do not internalise results in cell transformation *in vitro,* and enhanced tumorigenicity *in vivo* (Nishikawa et al, 1994). Receptor dimers rapidly form clusters in membrane invaginations, which are coated in clathrin, a glycoprotein which forms part of the cytoskeleton. These invaginations are then fully internalised as clathrin-coated vesicles, with the ligand-binding portion of the receptor to the inside of the vesicle (Bequinot, 1984). The vesicles are then uncoated and acidified, the fall in intravascular pH serving as a ligand-receptor uncoupling mechanism (Dunn et al, 1984, Maxfield, 1982).

Before the uncoupled ligand and receptor enter the endosome stage, a degree of receptor recycling may occur, where a proportion of the internalised receptors are returned, apparently undegraded, to the cell surface. The remaining receptor and ligand are then delivered to lysosomes where complete degradation occurs (Dunn et al, 1986).

Internalised EGFR has been demonstrated to retain signalling capacity (Vieira et al, 1996). This could allow EGFR to interact with cytoplasmic proteins which are not found at the cell membrane, resulting in EGFR regulation of other cellular systems.

# CHAPTER 4

# THE NUCLEAR UPTAKE OF POLYPEPTIDE GROWTH FACTORS

## 4.1. Transport of macromolecules into the cell nucleus

### 4.1.1. *Nucleocytoplasmic transport*

The nuclear and cytosolic compartments of a eukaryotic cell are separated by a double-membrane structure termed the nuclear envelope (Dingwall and Laskey, 1992). Since gene transcription and translation occur in different cellular compartments, macromolecules must cross the nuclear envelope in both directions to allow the cell to function normally. Transport across the nuclear envelope occurs through the nuclear pore complex (NPC), an envelope-bound multi-subunit protein complex of 108 daltons (Reichelt et al, 1990). The NPC is arranged into two ring structures, with a central pore which connects nuclear matrix (nucleoplasm) and cytoplasm (Hanover, 1992). The pore itself has a radius of 4.5 - 6.0 nM, and allows the passive diffusion of proteins up to 50 kDa, with the diffusion rate inversely proportional to protein size (reviewed by Gorlich and Mattaj, 1996). However, even small proteins have been demonstrated to enter the nucleus actively, rather than by diffusion (Breeuwer and Goldfarb, 1990).

### 4.1.2 *Nuclear localisation sequences*

Nuclear localisation sequences (NLS) are defined as "those sequences both necessary and sufficient for nuclear localisation" (Jans, 1995). Such sequences are short stretches of consecutive basic amino acids, usually 10 residues or less. A bipartite NLS comprises two such basic sequences, separated by a length of any 10 amino acids (Silver, 1991). Conjugation of these sequences to proteins which normally localise in the cytoplasm confers the ability to translocate to the nucleus. Conversely, mutation of a single residue in the NLS can severely impair nuclear localisation capacity (Kalderon et al, 1984, Lanford and Butel, 1984).

### 4.1.3 *Mechanisms involved in nucleocytoplasmic transport*

Early experiments indicated that protein translocation across the nuclear envelope is an active process. In vitro studies determined that translocation is NLS and ATP-dependent, and was inhibited at 4oC (Newmeyer et al, 1986). Subsequent studies indicated that binding of the protein to the nuclear envelope was NLS-dependent, but

was not affected by low temperature or ATP withdrawal (Newmeyer and Forbes, 1988). The translocation process could then be described in two stages; an energy-independent NPC-docking stage, and an energy-dependent translocation stage (Newmeyer and Forbes, 1988). More recently, a cytosolic NLS binding protein complex, importin αβ, has been described (Gorlich et al, 1994). The α-subunit is responsible for binding the NLS-containing protein, while the β-subunit is responsible for docking the NLS-protein-importin complex to the nuclear pore (Imamoto et al, 1995).

The active transport of the importin-NLS-protein complex through the NPC is facilitated by the hydrolysis of GTP by the Ran GTPase (TC4). (Moore and Blobel, 1993).

## 4.2. Growth factors in the cell nucleus

### 4.2.1 *Introduction*

The classical model for the action of a growth factor on its target cell consists of three individual components. The growth factor must first bind to its cognate membrane-bound receptor. The ligand-receptor complex then facilitates the activation of intracellular secondary messengers, which ultimately results in the appropriate biological response. Finally, the attenuation of this signal is achieved by internalisation and lysosomal degradation of the ligand-receptor complex. This model assumes that all of the observed cellular responses to growth factor stimulation are a result of events initiated at the cell surface. There is little doubt that this model encompasses many important features of growth factor signalling. However, the persistent and consistent reports of growth factor localisation in the target cell nucleus suggest that the model may be far from complete.

The number of growth factors which reportedly associate with the cell nucleus is growing steadily (Table 4.1). However, many of these observations remain controversial, and definitive data is awaited in several instances. The nuclear localisation of the fibroblast growth factor (FGF) family of peptides is by far the most widely investigated to date.

| Growth Factor/Hormone | Intranuclear binding/distribution | Nuclear localisation sequence | References |
|---|---|---|---|
| Acidic fibroblast growth factor (FGF-1) | Nucleus | yes | Cao et al, 1993 |
| Basic fibroblast growth factor (FGF-2) | Nucleolus | yes | Bouche et al, 1987<br>Baldin et al, 1990 |
| Epidermal growth factor (EGF) | Chromatin | no | Rackowicz-Szulcynska et al, 1986 and 1989 |
| Schwannoma derived growth factor (SDGF) | DNA | yes | Kimura et al, 1990<br>Kimura, 1993 |
| Amphiregulin | Nucleus/nucleolus | yes | Johnson et al, 1991 |
| Nerve growth factor (NGF) | Chromatin | no | Rackowicz-Szulcynska et al,1986<br>Rackowicz-Szulcynska, 1993 |
| Platelet derived growth factor (PDGF) | Chromatin | yes | Rackowicz-Szulcynska et al, 1986<br>Yeh et al, 1987 |
| Beta and gamma Interferons | Not determined | no | Kushnaryov et al, 1985 |
| Interleukin-1 alpha and beta | Not determined | no | Quarnstrom et al, 1988<br>Grenfell et al, 1989 |
| Insulin | Nuclear membrane | no | Goldfine et al, 1977 |
| Brain derived growth factor (BDGF) | Not determined | yes | Wetmore et al, 1991 |
| Heregulin β1 | Not determined | yes | Li et al, 1996 |

**Table 4.1** List of growth modulating factors with reported nuclear localisation (adapted from Burwen and Jones, 1987)

### 4.2.2. *Nuclear accumulation of the FGF peptide family*

The fibroblast growth factor (FGF) family currently consists of nine distinct members (reviewed by Baird and Klagsburn, 1991).The most detailed studies of the signalling mechanisms employed by the FGFs have concentrated on the two prototype members of the family, acidic FGF (FGF-1, aFGF) and basic FGF (FGF-2, bFGF). These growth factors bind with high affinity to distinct transmembrane receptors with intrinsic cytoplasmic tyrosine kinase activity. They also bind with low affinity to cell surface heparan-sulphate proteoglycans (HSPG), ensuring the correct orientation of the growth factor for presentation to the high affinity receptor (Basilico and Moscatelli, 1992, Yayon et al, 1991).

Both FGF-1 and FGF-2 have a wide range of biological activities in a variety of cell types. The mitogenic activities of FGF-1 and 2 are partly transduced via the intrinsic tyrosine kinase function of the FGF receptor (Friesel et al, 1989). However, another widely accepted pathway of FGF signalling is the internalisation and nuclear localisation of the growth factor.

The nuclear uptake of of FGF-2 was first observed in endothelial cells, and was demonstrated to be specific to the G1 phase of the cell cycle (Bouche et al, 1987, Baldin et al, 1990). Subsequent studies have demonstrated this to be a general effect, which can be observed in many cell types (Tessler and Neufeld, 1990, Speir et al, 1991, Hawker and Granger, 1994).

Studies into FGF-2 expression have revealed that multiple isoforms exist, due to the presence of several translation initiation codons, and that the isoforms which contain a nuclear localisation signal sequence (NLS) in the amino terminus localise in the nucleus, while those lacking a large amino terminal tail are mainly cytoplasmic (Renko et al, 1989, Quarto et al, 1991, Bugler et al, 1991).

Unlike many other growth factors, the cellular function of nuclear FGF-2 has also been investigated. Early studies indicated that the peptide may have a role in the transcriptional regulation of ribosomal RNA (Bouche et al, 1987), these findings supported by the observation that nuclear FGF-2 has opposite effects on the transcription of phosphoglycerate kinase 1 and 2 (Nakanishi et al, 1992). Similar observations have also been reported in a study on rat myoblasts (Hawker and Granger, 1994). In addition to transcriptional regulation, nuclear FGF-2 has also been implicated in the regulation of the activity of two nuclear proteins - CKII kinase and histone H1. Thus nuclear FGF-2 can indirectly affect gene expression (Amalric et al, 1994).

Investigations into the nuclear uptake of FGF-1 have also provided information on the functional significance of the NLS. Initial studies into the putative NLS in the amino terminus showed that mutation of this region reduced the mitogenic potency of FGF-1 (Imamura et al, 1990). N-terminal deletion does not affect receptor binding or receptor tyrosine kinase activation (Weidlocha et al, 1994).

The role of heparan-sulphate proteoglycans (HSPG) in the nuclear localisation mechanism of the FGF family remains controversial. The binding of FGF-2 to cell surface HSPG is essential for FGF-2 binding to high affinity receptors, and thus ligand-induced receptor mediated internalisation (Yayon et al, 1991). Nuclear populations of cell surface proteoglycans have been described by several groups (Hiscock et al, 1994, Jackson et al, 1991), although a translocation mechanism has yet to be established. Amalric and colleagues have suggested that FGF-2 and HSPG are co-transported into the cell nucleus (Amalric et al, 1994). There have also been suggestions that HSPG protects the growth factor from intracellular degradation, thereby increasing intracellular half-life and subsequent nuclear delivery (Sommer and Rifkin, 1989). The finding that FGF-dependent DNA synthesis is blocked by heparin treatment, which acts to stabilise the FGF molecular conformation, suggests that the tertiary structure of FGF may be important to nuclear localisation, and that HSPG may influence or regulate the folding of FGF (Weidlocha et al, 1992).

In summary, both FGF-1 and FGF-2 appear to exert their biological effect by a combination of two mechanisms: activation of membrane-localised, FGFR tyrosine kinase activity, and by nuclear translocation of the ligand. Nuclear uptake of exogenous FGF may occur by internalisation and nuclear translocation of FGF/FGFR complexes and/or FGF/HSPG complexes. The degree of interdependency of these postulated translocation mechanisms has yet to be established, as has a detailed translocation mechanism in each case. Nuclear FGF may act directly to regulate transcription, or indirectly via nuclear FGFR kinase, or other FGF-sensitive nuclear kinases. Both FGF-1 and 2 contain putative nuclear localisation sequences in the amino terminal of the peptide, which appear to be involved in the regulation of nuclear uptake.

### 4.2.3. *Nuclear uptake of EGFR ligands*

The EGFR binds and mediates the cellular effects of several polypeptide mitogens. There is little published work describing the nuclear accumulation of these ligands. However, investigators have noted the nuclear accumulation of one EGFR-binding

peptide, amphiregulin (AR), and its rat homologue, Schwannoma-derived growth factor (SDGF).

Immunohistochemical studies have established the nuclear localisation of AR in a range of normal and malignant tissues and cell lines (Johnson et al, 1992, Yokoyama et al, 1995, Lysiak et al, 1995). AR differs from EGF in two important respects. AR contains a nuclear localisation sequence (NLS) in the amino terminus of the peptide which is absent from EGF (Plowman et al, 1990). Secondly, AR, unlike EGF, is able to bind cell surface HSPGs (Piepkorn et al, 1994). The heparin-binding characteristics of AR appear to be essential to its mitogenic activity, as the addition of soluble heparin, heparan sulphate or hexadimethrine (a heparin antagonist) inhibit AR-induced mitogenesis (Johnson and Wong, 1994, Cook et al, 1995). None of these treatments affects EGF-induced mitogenesis.

SDGF, like amphiregulin, contains an NLS in its amino terminus and is able to bind heparin. It is readily detectable in the nucleus of cells transfected with SDGF cDNA. Deletion of the NLS results in a peptide which is able to bind and activate EGFR, but has no mitogenic capacity (Kimura, 1993). This mutation also abolishes nuclear localisation of the peptide. These observations indicate that the nuclear localisation of SDGF is essential to its mitogenic capacity. It was also observed that SDGF is able to bind DNA in a sequence-specific manner, which also suggests a nuclear role for this peptide. Similar evaluations have yet to be made of AR.

### 4.2.4. *Nuclear uptake of EGF and EGFR*

The accumulation of EGF in the cell nucleus was first reported in 1980, using cultured rat pituitary cells (Johnson et al, 1980). Since then, work has centred mainly on the physiological nature of this accumulation, and the identification of the nuclear EGF binding site. As yet there is very little work published on the function of nuclear EGF and its receptor, or the part that this may play in the response of the cell to EGF stimulation.

Immunoflourescent staining of the A431 squamous carcinoma cell line using polyclonal antibodies raised to purified EGFR demonstrated that internalised EGFR localised mainly in the perinuclear region. This accumulation appeared to be EGF-dependent, and was enhanced in the presence of the lysosomal protease inhibitor, chloroquine (Murthy et al, 1986).

Another study examining 125I-EGF distribution in fractionated nuclei demonstrated that EGF was not associated with the perinuclear membrane but was

tightly bound to chromatin. The accumulation of chromatin-bound EGF was maximal after 48 hrs exposure to EGF. This study also demonstrated that an antibody raised to the EGFR ligand binding domain was able to prevent EGF binding to chromatin, and thus that the chromatin binding site was "EGFR-like" (Rakowicz-Szulczynska et al, 1986).

A subsequent study into the EGF binding characteristics of a hepatic nuclear fraction showed that the nuclear EGF binding site displayed dual affinity binding, with binding constants similar to the membrane bound EGFR (Ichii et al, 1988). It was also shown that EGF stimulation of this fraction was unable to enhance ligand-induced autophosphorylation, suggesting that the putative nuclear EGFR was already maximally autophosphorylated.

Most of the experiments up until this point had been performed in the presence of chloroquine, a lysosomal protease inhibitor, which prevents metabolism of EGF resulting in enhanced nuclear delivery. A study in 1989 investigated nuclear delivery under physiological conditions without the use of chloroquine (Rakowicz-Szulczynska, 1989). It was shown that EGF binding to chromatin was highly specific and that an anti-EGFR antibody raised to the EGF binding site bound to the same chromatin 'receptor' as EGF. EGF remained bound to chromatin in the presence of 2M NaCl, which discounted the possibility of non-specific association. The same study also demonstrated that EGF bound to specific regions in the chromatin structure. EcoR1 digestion of 125I-EGF-labelled chromatin produced a distinct band upon electrophoresis, rather than a smearing pattern which would be seen if EGF association was at random sites.

The role of the cell surface receptor in nuclear EGF delivery was examined using the technique of physical reconstitution of EGFR from donor hepatic membranes to receptor-deficient fibroblasts (Jiang and Schindler, 1990). This technique ensured that there was no endogenous nuclear EGFR, and that any EGFR found subsequently in the nucleus was translocated, cell surface receptor. This study demonstrated that nuclear accumulation of EGF occurred at a rate similar to those previously reported, and that translocated EGFR could be detected in the cell nucleus. These experiments were carried out at 4oC, at which internalisation of EGFR is inhibited (Carpenter and Cohen, 1976). At this temperature, no nuclear delivery of EGF was observed, further implicating the role of normal membrane receptor function in the delivery of growth factor to the nucleus.

A revealing study into the physiology of nuclear delivery of EGF noted that hepatic nuclei from partially hepatectomised rats appeared to bind less EGF than control

nuclei (Marti et al, 1991). It was also established by ELISA that hepatectomised rats have higher circulating EGF levels than control rats. The authors hypothesised that the decrease in EGF binding sites in the nucleus was due to prior occupation of these sites by endogenous EGF.

Further studies have produced interesting observations in proliferating tissue. An investigation into anti-EGFR antibody binding in normal skin cells showed that in 20% of keratinocyte samples, there was strong immunolabelling of the cell nucleus with a stronger superimposed labelling of what was assumed to be chromatin (Pierard-Franchimont et al, 1991). In the population of cells showing nuclear immunoreactivity, there was a marked decrease in membrane and cytoplasmic EGFR. A similar pattern been observed in hepatocytes of bile duct-ligated rats (Oguey et al, 1992). Three days after ligation, rat livers displayed decreased cytoplasmic and increased nuclear EGFR. Both of these studies suggest that increased nuclear EGFR may be linked to DNA synthesis.

More recent studies into nuclear EGFR have attempted to establish whether the receptor has a signalling function within the nucleus. Using anti-phosphotyrosine antibodies together with anti-EGFR antibodies, it was shown that after incubation of HN5 (squamous cell carcinoma) cells with EGF, there was a 4-fold increase in nuclear EGFR, along with a doubling in the amount of EGFR-dependent phosphotyrosine, suggesting that the nuclear receptor may retain its tyrosine kinase activity (Holt et al, 1994). This study also reported that nuclear EGFR associated with chromatin. In a subsequent report the same group demonstrated using immunoelectron microscopy, that the ligand-mediated nuclear translocation of EGFR and the concomitant EGFR-dependent increase in nuclear phosphotyrosine was serum dependent (Holt et al, 1995). Previous studies had demonstrated that EGF and EGFR are delivered to the nucleus in an undegraded form, (Rakowicz-Sulczynska, 1989, Jiang et al, 1990) but these were the first reports describing functional, nuclear EGFR. The nature of the tyrosine kinase substrates and the consequences of their phosphorylation are yet to be established.

Another revealing study has suggested a direct consequence of EGF in the cell nucleus. Examination of nuclei isolated from a human choriocarcinoma cell line and from normal human placenta revealed the presence of functional EGFR (Cao et al, 1995). This group also found that addition of EGF to the same isolated nuclei caused a reduction in the transcription rate of the chorionic gonadotropin receptor gene (CGR).

In addition to the cell lines and tissues outlined above, nuclear accumulation of EGF or nuclear EGFR populations have also been observed in tumour sections of bladder and cervical epithelium, as well as malignant and non-malignant mesothelioma, hepatocytes, fallopian tube, placental trophoblast, bovine corneal endothelium, rat epithelium and murine chondrocytes (Lipponen and Eskelinen, 1994, Groppinger et al, 1989, Tervahauta et al, 1993, Ramael et al, 1991, Raper et al, 1987, Lei and Rao, 1992, Arnholdt et al, 1991, Savion et al, 1981, Green et al, 1987, Tajima et al, 1993). However, many of these studies have employed positive nuclear immunoreactivity as the determinant of nuclear EGFR, which falls short of conclusive proof of nuclear EGFR populations. This technique only indicates the presence of "EGFR-like" epitopes in the cell nucleus.

In summary, it has been demonstrated that both EGF and EGFR can be detected in the cell nucleus in a variety of tissue types and cultured cell lines. EGF, EGFR protein and tyrosine kinase activity have been detected in the nuclei of cells with and without exogenous EGF treatment. Much of the evidence suggests that translocation of ligand-bound receptor from the cell surface is involved in the nuclear accumulation mechanism. The nature of the apparent chromatin association has yet to be fully elucidated, as has the detail of the receptor translocation mechanism. The correlation between increased nuclear delivery of EGF and DNA synthesis/transcription also requires further examination before any definitive conclusions can be drawn regarding the respective roles of EGF/EGFR chromatin binding and nuclear tyrosine kinase activity in the cellular response to EGF.

### 4.2.5. *Methods used in the detection of nuclear EGF and EGFR*

Methods used to assay the nuclear uptake of EGF and EGFR fall into two broad categories - those which involve the microscopic detection of EGF/EGFR in intact cells, and studies involving intact nuclei isolated from cells treated with labelled EGF (usually 125I-EGF). However, there are inherent problems with both techniques which must be taken into account before definitive conclusions about EGF/EGFR nuclear localisation can be drawn.

Microscopic examination of intact cells can provide qualitative data about the intracellular localisation of labelled EGF. Semi-quantitative analysis may also be possible, but is dependent upon the sensitivity of the microscopic technique employed. The amount of EGF which localises in the nuclei of cells treated in the absence of endosomal protease inhibitors (e.g. chloroquine) is usually very low (<

1% of cell-associated EGF) (Johnson et al, 1980, Savion et al, 1981), and the use of classical EM immunogold techniques results in a low number of grains per nucleus (Holt et al, 1994, 1995). It would be preferable to use a technique which allows similar unequivocal nuclear localisation, but which has a higher sensitivity.

The isolation of intact nuclei is most commonly performed by washing cells in a detergent-containing buffer. Washing with detergent disrupts the lipid cell membrane and membranous cytosolic vesicles such as endosomes, without disruption of the protienatious inner nuclear membrane. However, it is not possible to simply equate isolated nuclei with a detergent-insoluble fraction. It is important to control for the uptake of labelled EGF by nuclei during the isolation procedure, to confirm that EGF released from cytosolic vesicles is not taken up non-specifically. Assessment of the contamination of the isolated nuclear pellet by cell membrane and cytosolic organelles must also be performed. Membrane contamination can be assessed by membrane-specific enzyme activity. The most common is 5'-nucleotidase, although other membrane-specific marker enzymes have been used (Burwen and Jones, 1987). EGF-containing lysosomes are another potential source of contamination. Some investigators have used the activity of the lysosome-specific acid phosphatase enzyme to assess lysosomal contamination (Savion et al, 1981, Holt et al, 1994). However, this does not control for contamination by other EGF-associated cytosolic organelles, such as detergent-insoluble actin filaments, which have been shown to bind EGFR (Rijken et al, 1995, den Hartigh et al, 1997).

It is also important to note that neither technique is able to exclude the possibility that the positive nuclear signal is derived from a degradation product of EGF, which is rapidly metabolised after internalisation. This possibility and its implications are discussed in chapter 8.

# CHAPTER 5

# EGFR-TARGETED THERAPEUTIC STRATEGIES

## 5.1. Introduction

The rationale for EGFR-directed, anti-cancer therapy is now well established. The role of EGFR overexpression in tumour initiation and disease progression, along with the observation of autocrine and paracrine stimulation via increased ligand production has led many investigators to attempt to interrupt EGFR signalling function by the use of monoclonal antibodies (mAb) raised against either the receptor or ligand. In addition, many compounds have now been developed which inhibit the intrinsic tyrosine kinase activity of the receptor, and thus prevent EGFR signalling.

The overexpression of EGFR in many tumour types, compared to surrounding normal tissue, confers a degree of tumour-specificity which may be exploited therapeutically. Many of these strategies involve the conjugation of a toxin, anti-proliferative drug or radionuclide to an EGFR-seeking molecule, usually a monoclonal antibody or receptor ligand, to achieve a tumour/normal tissue dose differential.

## 5.2. Monoclonal antibody-based strategies

### 5.2.1 *Anti-EGFR monoclonal antibody therapy*

There are several anti-EGFR mAbs with anti-proliferative activity both *in vitro* and *in vivo* . Some of these have been evaluated clinically.

Initial studies in cell lines with a mouse anti-human EGFR IgG1 (mAb 225) and IgG2a (mAb 528) found that these antibodies were effective in blocking EGF/TGF-α binding, EGFR kinase activity, and EGF-dependent proliferation in cell lines and xenograft models (Mendelssohn, 1990). Neither of these antibodies attached directly to the ligand binding portion of the receptor, but to a region close enough to interfere with ligand/receptor binding. Neither was able to activate the EGFR kinase function, but the rate of receptor internalisation after mAb binding was identical to that of ligand-bound receptor (Sunada et al, 1986).

A phase 1 clinical trial suggested that patients were able to tolerate the presence of saturating doses of mAb (300 mg total dose) for over 3 days without toxic effect, and that tumour uptake of mAb was significant (Mendelssohn, 1990). However, the effectiveness of anti-EGFR mAb as a single-mode therapy is affected by two major

limitations. Firstly, xenograft models indicate that while these antibodies inhibit tumour growth, the growth inhibitory effect is reversible, and no cytotoxicity is observed. Secondly, administration of these antibodies elicits a host immune response which can affect the efficacy of subsequent antibody administrations (Divgi et al, 1991).

Another antibody to reach clinical trial is mAb 425 (also known as EMD 55 900) (Murthy et al, 1987). Again, this mAb is a mouse anti-human EGFR IgG2a, with similar receptor binding properties to mAb 225 and 528. In common with these antibodies, mAb 425 was effective in inhibiting the growth of tumours in xenograft models, and exhibited low toxicity at phase I trial. However, this mAb had limited therapeutic benefit in preliminary phase II trial (Schnurch et al, 1994; Stragliotto et al, 1996).

Studies have also been carried out with an anti-EGFR IgG2b (ICR62) (Modjtahedi et al, 1993). Treatment of squamous cell tumour xenografts with this antibody resulted in the appearance of markers of terminal differentiation (Modjtahedi et al, 1994). The authors concluded that differentiation of tumour cells may play an important role in the therapeutic effect of this treatment. A phase I trial indicated that administration of this mAb was well tolerated, but as yet there is no data indicating therapeutic benefit (Modjtahedi et al, 1996).

### 5.2.2 *Anti-EGFR mAb/cytotoxic combination therapy*

Combined administration of mAbs 225 or 528 with conventional chemotherapeutic drugs has been evaluated in xenograft models. Combination treatment of mAb and doxorubicin, or mAb and a cisplatin analogue have both resulted in tumour eradication in a proportion of tumour-bearing athymic mice (Baselga et al, 1993, Fan et al, 1993). The anti-tumour effect of these combined therapies appears to be at least additive. Clinical trials based on these observations are currently underway.

### 5.2.3 *Anti-EGFR mAb-toxin conjugates*

Many EGFR-targeting conjugates have employed either EGF or TGF-α as the targeting vehicle. However, some studies with anti-EGFR mAb conjugates have been undertaken. One experimental therapy involved conjugation of ricin, a plant-derived toxin which acts by inactivation of ribosomal subunits, to mAb 225 (Masui et al, 1989). A similar study involved an anti-EGFR mAb-gelonin conjugate. Gelonin also

acts as a protein synthesis inhibitor by inactivation of ribosomal subunits (Hirota et al, 1989). While these immunoconjugates were cytotoxic to EGFR-bearing cell lines and xenograft models, the extreme toxicity of the plant-derived toxins requires that the degree of tumour-specific targeting, and the stability of the immunoconjugate are accurately established. The widespread distribution of normal EGFR-bearing cells may preclude such immunotherapies, and few are currently being studied.

Anti-EGFR antibodies have also been used to target conventional cytotoxic drugs to EGFR-bearing tumours. Doxorubicin, conjugated via a dextran bridge to another anti-EGFR IgG2a (mAb 108) showed increased efficacy when compared to equivalent free doxorubicin doses in cell line and xenograft models (Aboud-Pirak et al, 1989). However, the immunoconjugate had reduced antigen binding capacity, and thus reduced tumour/normal tissue dose differential. A similar study involving a doxorubicin-mAb 425 immunoconjugate demonstrated that mAb-doxorubicin conjugate was more cytotoxic than doxorubicin alone in a metastatic melanoma model (Sivam et al, 1995).

Another interesting approach to antibody therapy led to the generation of an anti-EGFR (mAb 225)/interleukin 2(Il2) chimera (Naramura et al, 1993a). Il2 acts via its receptor on T-cells to amplify the host immune response, and thus the chimera may enhance the host anti-tumour immune response. In xenograft models, the chimera was able to stimulate cellular cytotoxicity mediated through activated mononuclear effector cells at the equivalent free IL2 dose, and displayed the same antigen-binding capacity as intact mAb 225 (Naramura et al,1993b).

### 5.2.4 *Radio-immunoconjugates*

Anti-EGFR mAbs have also been conjugated to radioisotopes. Whilst initial studies have focused on the tumour imaging potential of these radioimmunoconjugates, some studies have indicated a possible therapeutic response. Epenetos and colleagues have reported a beneficial response in glioma patients using an 131I-labelled anti-EGFR mAb which binds to the receptor at a region distinct from the growth factor binding site (Epenetos et al, 1985, Kalofonos et al, 1989). This mAb does not induce receptor internalisation upon antigen recognition.

Two other groups have investigated the potential of mAb 425 conjugated to 125I and 131I. 125I-mAb 425 has been demonstrated to significantly increase the survival of patients with malignant glioma (Brady et al, 1990a and b). Further studies in xenograft models suggested that 125I-mAb 425 has a greater anti-glioma effect than

either 131I-mAb 425 or unlabelled antibody (Bender et al, 1992). These findings imply that elements of the decay of 125I have an important cytotoxic effect when conjugated to anti-EGFR antibody.
125I-mAb 425 has successfully undergone phase II clinical trial, and is currently the most clinically promising anti-EGFR mAb conjugate (Brady et al, 1992, Snelling et al, 1995). Studies have indicated that mAb 425 localises in the nuclei of cells derived from high-grade glioma. The intranuclear concentration of antibody was enhanced by chloroquine, in a similar manner to chloroquine-enhanced nuclear EGF uptake (Emrich et al, 1994). However, chloroquine did not enhance tumour uptake of 125I-mAb 425 in xenograft models using the same cell lines (Emrich et al, 1996).

## 5.3 Ligand based targeting strategies

All of the ligand-based EGFR targeting strategies currently under investigation employ either EGF or TGF-α. It is not yet clear whether the other EGFR ligands are suitable targeting vehicles in the context of these strategies, or whether their individual characteristics may enhance the potency of a particular conjugate.

### 5.3.1 *Ligand-cytotoxin fusion proteins/conjugates*

The rationale behind the use of ligand-exotoxin conjugates is identical to that of antibody-exotoxin conjugates, i.e receptor- mediated, tumour cell-specific delivery of cytotoxin. A major difference lies in the methods employed in their synthesis. Fusion of cDNA encoding both ligand and exotoxin, and expression in a suitable expression system (e.g. E. coli.) allows intact ligand-exotoxin fusion protein to be purified from whole cell lysates or inclusion bodies. The fusion peptide approach ensures that the ligand and toxin are fused at the correct amino acid residues, so that the fusion peptide retains maximal EGFR binding capability and minimal non-specific (i.e. toxin receptor-mediated) toxicity. This approach results in a significant improvement in product yield over even the most efficient chemical conjugation methods.

The most extensively studied fusion toxin was first described by Pastan and colleagues in 1987. TP40 was generated by the fusion of TGF-α and the Pseudomonas exotoxin PE40 (Chaudhary et al, 1987). This fusion peptide has affinity for EGFR comparable to native TGF-α, and is toxic to cells in an EGFR-dependent manner (Pastan and Fitzgerald, 1991). Modifications to the toxin region have resulted in several fusion peptides (TGF-α-PE38, PE35/TGF-α-KDEL) with

several hundred-fold increases in *in vitro* cytotoxicy over TP40 (Phillips et al, 1994, Theuer et al, 1994). TP40 cytotoxicity correlates directly with EGFR expression in a variety of cell lines and xenograft models, without bone marrow cell damage (Baldwin et al, 1996, Kirk et al, 1994, Siegall et al, 1990, Berger et al, 1995).

A similar approach has led to the generation of DAB389EGF. This molecule, a fusion of portions of diphtheria toxin with EGF, also inhibits cellular protein synthesis in an EGFR expression-dependent manner (Shaw et al, 1991). More recent studies confirmed that DAB389EGF-mediated cytotoxicity correlated with EGFR expression in a panel of breast cancer cell lines (Osbourne, 1996). One advantage of DAB389EGF over TP40 is that it irreversibly binds EGFR (Shaw et al, 1991). This suggests that the serum concentration required for efficacy may have to be maintained for a shorter period for therapy using than TP40. Clinical trials are currently underway.

### 5.3.2 *Ligand-Based EGFR targeted radiotherapy*

The potential of EGF-radionuclide conjugates for use as tumour-imaging agents has already been established, using 123I-, 99mTc-, 111In- and 76Br- EGF (Schatten et al, 1991, Capala et al, 1997, Remy et al, 1995, Scott-Robson et al, 1991). The potential of therapeutic ligand-radioconjugates has been examined by Carlsson and colleagues. Initial studies in glioma cell line models demonstrated that 131I-EGF preferentially killed EGFR-overexpressing cells (Capala and Carlsson, 1991). Subsequent work has addressed the problem of intracellular EGF degradation which could result in loss of intracellular radionuclide. Conjugation of large (20kD), biologically inert, dextran chains to 131I-EGF significantly increased the intracellular radionuclide retention time, and was demonstrated to have a greater radiobiological effect than 131I-EGF (Andersson et al, 1992, Carlsson et al, 1994). These observations have provided the basis for a novel approach to boron neutron capture therapy. 10B is a non-radioactive, non-toxic boron isotope which, upon exposure to a thermal neutron field, decays into highly ionising 4He2+ (alpha) and 7Li3+ ions, both with a mm range (Barth et al, 1990). 10B-EGF conjugates may facilitate tumour uptake of 10B. Subsequent exposure of the tumour region to a neutron field should result in tumour-associated 10B activation and cell sterilisation without irradiation of regions of non-tumour 10B uptake (Carlsson et al, 1994). The conjugation of boron to EGF is more complicated than halogenation methods and the requirement of a large number of 10B atoms to sterilise cells (109 per cell) are potential problems with

this therapeutic mode. However, a recent report described an EGF-boron conjugate incorporating a large number of boron atoms which retained EGFR binding and internalisation capability (Capala et al, 1996). Such developments hold promise for clinical implementation of boron neutron capture therapy.

## 5.4 Aims of this study

The purpose of this study was to investigate novel radiotherapeutic strategies based upon the overexpression and subcellular distribution of EGFR by tumour cells.

### 5.4.1 *Characterisation of a panel of EGFR-expressing cell lines*

Experiments described in this thesis were performed using a panel of eleven cell lines (table 6.1). Chapter 6 describes the determination of the growth parameters, EGFR expression and EGFR signalling capacity of these cell lines.

### 5.4.2 *EGF-mediated enhancement of cellular radiosensitivity*

Chapter 7 describes experiments performed to establish the effect of EGF on the radiosensitivity of a panel of cell lines with widely varying EGFR expression.

### 5.4.3 *Nuclear accumulation of EGF*

Chapter 8 describes experiments performed to assess the suitability of EGF as a delivery vehicle for Auger-emitting radionuclides. Nuclear uptake of EGF by EGFR expressing cell lines is examined using both previously characterised and novel EGF detection methods.

### 5.4.4 *Effect on clonogenicity of 123I-EGF*

Chapter 9 describes preliminary experiments to determine the effect of 123I-EGF treatment on a panel of cell lines with widely varying EGFR expression.

# CHAPTER 6

# CHARACTERISATION OF A PANEL OF EGFR-EXPRESSING CELL LINES

## 6.1 Introduction

### 6.1.1 *Derivation of cell lines*

A panel of cell lines was selected for use in the experiments described in this report (table 6.1). Seven of these cell lines are derived from human squamous cell carcinomas of various sites including head and neck (B2A4, HN5, SCC 12), vulva (A431), cervix (CaSki, SiHa) and bladder (EJ). Two cell lines derived from breast adenocarcinomas were also included (MCF-7 and MDA-MB 231). In addition, two murine fibroblast cell lines were examined (HER 14 and K721A) (Honegger et al, 1987). These cell lines are stable transfections of NIH 3T3 fibroblasts devoid of endogenous EGFR. HER 14 is transfected with cDNA encoding wild type human EGFR, whilst K721A is transfected with cDNA encoding a mutant human EGFR. The mutation introduced results in the substitution of the lysine residue at position 721 of the receptor for an alanine residue. Lys721 in the wild type EGFR has been identified as the intracellular ATP binding site of the receptor. Substitution of this residue renders the receptor incapable of binding ATP, and thus ligand-induced autophosphorylation.

The breast adenocarcinoma-derived cell line MDA-MB 231, whilst expressing functional EGFR, is not responsive to stimulation by EGF (Davidson et al, 1987). The particular downstream signalling event responsible for the lack of response to ligand-induced EGFR activation have not been fully elucidated.

### *6.1.2 Aims of this study*

Experiments described in this chapter were designed to characterise a panel of tumour cell lines with respect to doubling time, EGFR expression and EGFR signalling capacity. The purpose of this investigation was to establish a panel of cell lines with a wide range of EGFR expression, suitable for the the study of EGFR-mediated cellular events.

**Table 6.1** Panel of Cell Lines

| Cell Line | Tissue origin | Cell bank Number. | Reference |
|---|---|---|---|
| A431 | Human squamous epidermal carcinoma (Vulva) | ATCC - CRL 1555 | Giard et al, 1973 |
| B2A4 | Human squamous epidermal carcinoma (Head and Neck) | N/A | Cowley et al, 1984 |
| HN5 | Human squamous epidermal carcinoma (Head and Neck) | N/A | Easty et al, 1981 |
| CaSki | Human squamous epidermal carcinoma (Cervix) | ATCC - CRL 1550 | Patillo et al, 1977 |
| HER 14 | Murine fibroblast (NIH 3T3) transfected with wild-type EGFR cDNA | N/A | Honneger et al, 1987 |
| K721A | Murine fibroblast (NIH 3T3) transfected with Δ721 EGFR cDNA | N/A | Honneger et al, 1987 |
| MCF -7 | Human adenocarcinoma (Breast) | ATCC- HTB 22 | Soule et al, 1973 |
| SiHa | Human squamous epidermal carcinoma (Cervix) | ATCC- HTB 35 | Friedl et al, 1970 |
| SCC 12 | Human squamous epidermal carcinoma (Head and Neck) | N/A | Rheinwald and Beckett, 1981 |
| MDA-MB 231 | Human adenocarcinoma (Breast) | ATCC- HTB 26 | Cailleau et al, 1974 |
| EJ | Human squamous epidermal carcinoma (Bladder) | ECACC - 85061108 | Hall et al, 1983 |

## 6.2 Materials and Methods

### *6.2.1 Cell Culture*

A panel of eleven cell lines was selected for this study (table 6.1). Cell lines were maintained in the cell culture medium and serum concentration described in table 6.2, supplemented with L-glutamine (2mM), penicillin(100 I.U./ml), streptomycin (100μg/ml), amphotericin-B (2.5μg/ml) and sodium bicarbonate to a pH of 7.4. All cell lines were grown in a 2% CO2 atmosphere, at 37oC. The cells were screened every 8 weeks for mycoplasma infection, and shown to be consistently free from infection.

### *6.2.2 Growth characteristics and doubling times*

Cells were cultured in 24 well plates (Nunclon, Roskilde, Denmark) at 1x104 cells/well, and left for 24 h in an incubator at 37oC in a 2% CO2 atmosphere. Cells were then washed in PBS, followed by the addition of fresh medium.
Cells were removed from the appropriate wells by trypsinisation, and counted in a Coulter counter (Coulter Electronics, Luton, U.K.).
Cell number per well was determined every 24 h. Medium was replaced every 48 h to ensure consistent conditions throughout the course of the experiment. Each data point was determined in triplicate.

### *6.2.3 EGFR ligand binding analysis*

Cells (between 5x104 and 5x105, depending upon cell line) were grown overnight in 24-well plates. The original medium was then removed, and cells were washed twice with 1 ml binding buffer (PBS containing 70 μg/L bacitracin, 0.1% (w/v) bovine serum albumin, pH 7.4). Cells were then incubated with human 125I-EGF (Amersham International, Buckinghamshire, U.K., specific activity 1440 Ci/mmol) at a concentration range 0.05 to 5.5 nM, total volume 140 μl, for 1 h at 20oC.
The radioactive solution was then aspirated, and the cells washed three times with ice-cold binding buffer. The cells were then lysed in 1 ml of 1M NaOH containing 0.1% SDS (w/v), the lysate transferred to Eppendorf tubes, and radioactivity measured in a gamma counter (Canberra Packard, Berkshire, UK.).

**Table 6.2** . Cell culture conditions

| Cell line | Cell culture medium | Serum content |
|---|---|---|
| A431 | Ham's F-10/D-MEM (50:50 v/v) | 10% Foetal Calf Serum (FCS) |
| B2A4 | Ham's F-10/D-MEM (50:50 v/v) | 2% FCS |
| HN5 | Ham's F-10/D-MEM (50:50 v/v) | 10% FCS |
| CaSki | Ham's F-10/D-MEM (50:50 v/v) | 10% FCS |
| HER 14 | Ham's F-10/D-MEM (50:50 v/v) | 10% Donor Calf Serum (DCS) |
| K721A | Ham's F-10/D-MEM (50:50 v/v) | 10% DCS |
| MCF -7 | RPMI 1640 | 10% FCS |
| SiHa | Ham's F-10/D-MEM (50:50 v/v) | 10% FCS |
| SCC 12 | Ham's F-10/D-MEM (50:50 v/v) | 10% FCS |
| MDA-MB 231 | RPMI 1640 | 10% FCS |
| EJ | Ham's F-10/D-MEM (50:50 v/v) | 10% FCS |

Non-specific binding was assessed in the presence of a 100-fold excess of unlabelled human EGF.

Receptor affinity and abundance were determined by Scatchard analysis (Scatchard, 1949), using the binding data analysis program LIGAND (Munson and Rodbard, 1979). Each data point was determined in triplicate.

### *6.2.4 Preparation of whole cell lysate*

When the cell cultures attained 70% confluence, medium was removed, and replaced with medium containing the desired concentration of EGF was added, and the cells were placed in an incubator at 37oC for 15 min. The medium containing EGF was then aspirated and the cells washed twice with PBS. The cells were then lysed in sample buffer (0.0625 M Tris-HCl, 10% (w/v) sucrose, 5% (v/v) β-mercaptoethanol, 2% (w/v) SDS, 5mM sodium fluoride, 1mM sodium orthovanadate, pH 6.8) on ice for 1 hour.

The lysate was then centrifuged at 20 000 x g for 5 min. The supernatant was removed and stored at -70oC until required. A small aliquot was removed for protein estimation (Bio-Rad, Beaconsfield, U.K.).

### *6.2.5 Preparation of membrane-rich cell fraction*

Cells were grown to 70% confluence, the medium aspirated, and the cells washed twice in ice-cold PBS. The cells were then harvested, resuspended in 10 ml ice-cold PBS containing 0.007% (w/v) bacitracin (PBS-B), and homogenised in a tight-fitting glass-glass homogeniser. The cell homogenate was then centrifuged at 20 000 x g at 4oC for 10 min, and the supernatant was removed and kept on ice.

The pellet was resuspended in 1ml PBS-B and sonicated (MSE, Essex, U.K) for 3 x 10 seconds at the highest setting. Again, the homogenate was centrifuged at 20 000 x g and the supernatant kept on ice.

The supernatant was then centrifuged in a Beckman Optima TLX ultracentrifuge (Beckman, Fullerton, U.S.A.) at 100 000 x g for 30 mins. The pellet was then resuspended in PBS-B, and stored at -70oC.

*6.2.6 SDS/polyacrylamide gel electrophoresis (SDS/PAGE)*

The methodology is essentially the same as the Tris/glycine buffered electrophoresis system described by Laemmli (1970).

Protein samples, prepared as described in sections 6.2.4 and 6.2.5 were diluted in an equal volume of sample buffer (section 6.2.4), and incubated at 100oC for 4 mins. 20 μl loading buffer (33% (v/v) glycerol, 20% (w/v) SDS, 0.05% (w/v) bromophenol blue) was then added to each sample.

Samples were applied to a polyacrylamide gel, which was cast using the Protean II vertical gel casting apparatus (Bio-Rad).

Electrophoresis was carried out at a constant current of 50 mA (4-6 hours), or 10mA (18-24 hours), until the bromophenol blue dye marker was 0.5 cm from the bottom of the plate.

*6.2.7 EGFR detection by western blotting*

Proteins separated by SDS/PAGE were transferred to Immobilon P blotting membrane (Millipore, Bedford, U.S.A.) by means of an Immobilon semi-dry electroblotter. Transfer was made at constant current of 0.8mA/cm2 for 60 mins. When protein transfer was complete, the blockade of non-specific binding to the membrane was achieved by immersion of the membrane in blocking solution (5% (w/v) milk powder prepared in Tris-buffered saline with 0.05% (v/v) Tween 20) overnight at 4oC.

Mouse monoclonal antibody, (mAb F4 - 1μg/ml in blocking solution), specific for an epitope on the EGFR, was then applied to the membrane and allowed to bind for 2 h at room temperature. The membrane was then washed in TBS-T (Tris-buffered saline with 0.05% (v/v) Tween 20) for 15 mins.

Anti-mouse IgG-horseradish peroxidase (Amersham) at a dilution of 1:5000 was then added, and allowed to bind for 15 min. The membrane was then washed for 2 hours at room temperature in TBS-T, dried, and coated in a hydrogen peroxide-luminol mixture (ECL solution)(Amersham).

The membrane was dried, exposed to Kodak X-OMAT film for 2 min and then developed.

### *6.2.8 32P-labelling of the EGFR*

An aliquot of cell membrane preparation (section 6.2.5) was added to an equal volume of NP-40 solution (50 mM NaCl, 10 mM Tris-HCl, 0.5% (v/v) NP-40). EGF was added to the desired final concentration and allowed to stand on ice for 30 min.

γ-32P dATP (Amersham, specific activity 3000Ci/mmol) was added to a final concentration of 2 μM, vortexed gently, and allowed to stand on ice for 1 h.

### *6.2.9 Immunoprecipitation*

3ml of 10% (w/v) immobilised Staphylococcus aureus (immuno-precipitin) was washed twice by centrifugation with NP-40 solution (section 6.2.8). Centrifugations were carried out at 10 000 x g for 5 min at 4oC. After the final wash, the pellet was resuspended in 3 ml NP-40 solution containing 100 μl of 10% (w/v) BSA.

4 μg mAb F4 was pre-absorbed to 900 μl washed immuno-precipitin, for 1 hour at room temperature. 100 μl aliquots of this mixture were then added to protein samples prepared as described in section 6.2.5, and placed on ice for 1 hour. To establish that mAb F4 binding to EGFR was specific, a competitor peptide (peptide 2E) which contains the same amino acid sequence as the epitopic recognition site on EGFR, was added to control samples.

After centrifugation at 20 000 x g for 5 min at 4oC, the supernatants were discarded. The pellets were washed 3 times in NP-40 solution and resuspended in 100 μl sample buffer (section 6.2.4).

20 μl loading buffer (section 6.2.6) was then added to each sample before incubation at 100oC for 4 min. The samples were then centrifuged at 20 000 x g for 5 min at 4oC, the pellet discarded, and the supernatant was electrophoresed on an 8% polyacrylamide gel.

Finally, the gel was dried, covered in Saran wrap, and exposed to Kodak X-OMAT film for 4 to 6 hours at -70oC.

## 6.3 Results

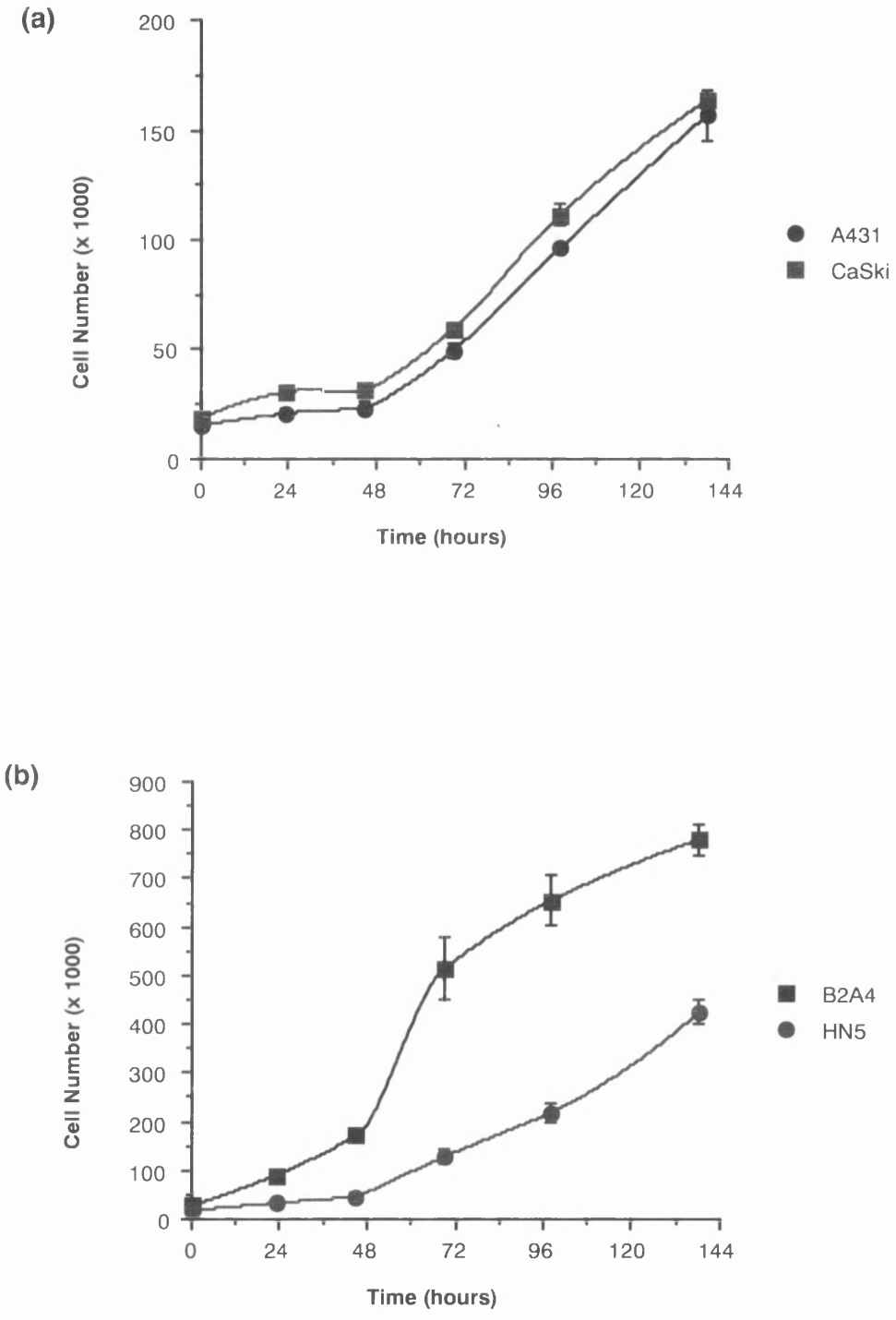
### *6.3.1 Growth characteristics and doubling times*

The growth characteristics of the cell line panel are depicted in figure 6.1 (a-f). The population doubling times, calculated from the linear portion of the growth curve, were as follows :
A431 -33 hours, B2A4 -20 hours, CaSki -28 hours, HN5 -35 hours, K721A - 24 hours, HER 14 -20 hours, SCC12 -36 hours, MDA-MB 231 - 35 hours, SiHa -38 hours, EJ - 28 hours, MCF-7 -26 hours.

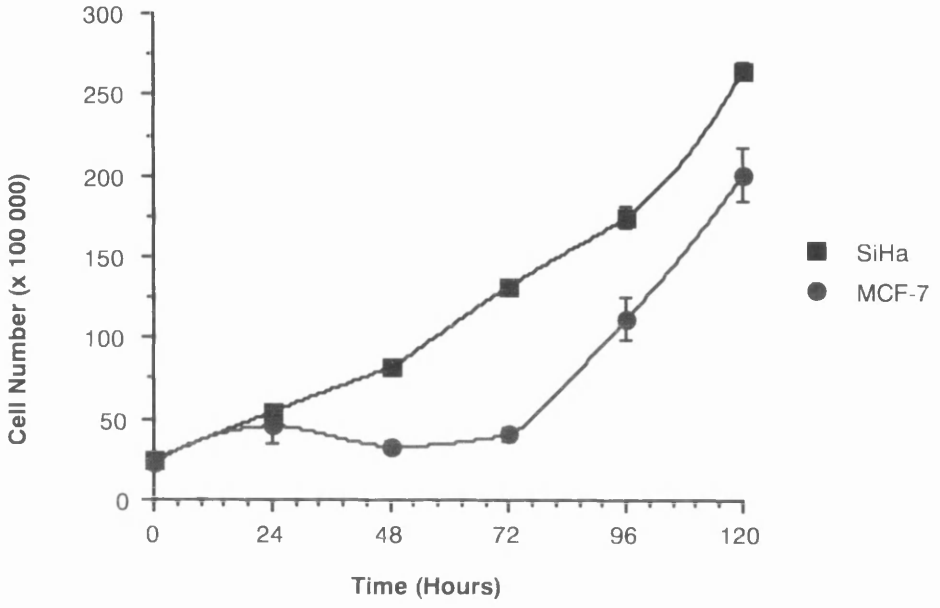
### *6.3.2 EGFR ligand binding analysis*

Scatchard plots were prepared from the data obtained by ligand binding analysis (figure 6.2. (a-f)). Receptor concentrations and dissociation constants (kDs) are tabulated (table 6.3.). Cell lines were ranked according to EGFR expression, and tabulated (table 6.4)

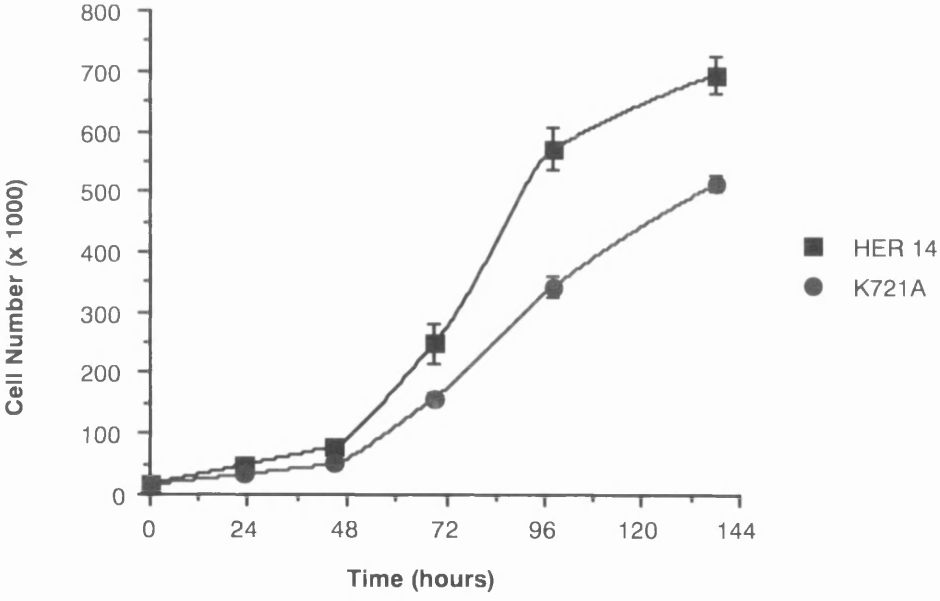
**Fig 6.1** Growth characteristics of cell lines



(c)


300
250
200
150
100
50
0
Cell Number (x 100 000)
0
24
48
72
96
120
Time (Hours)
SiHa
MCF-7

(d)


800
700
600
500
400
300
200
100
0
Cell Number (x 1000)
0
24
48
72
96
120
144
Time (hours)
HER 14
K721A

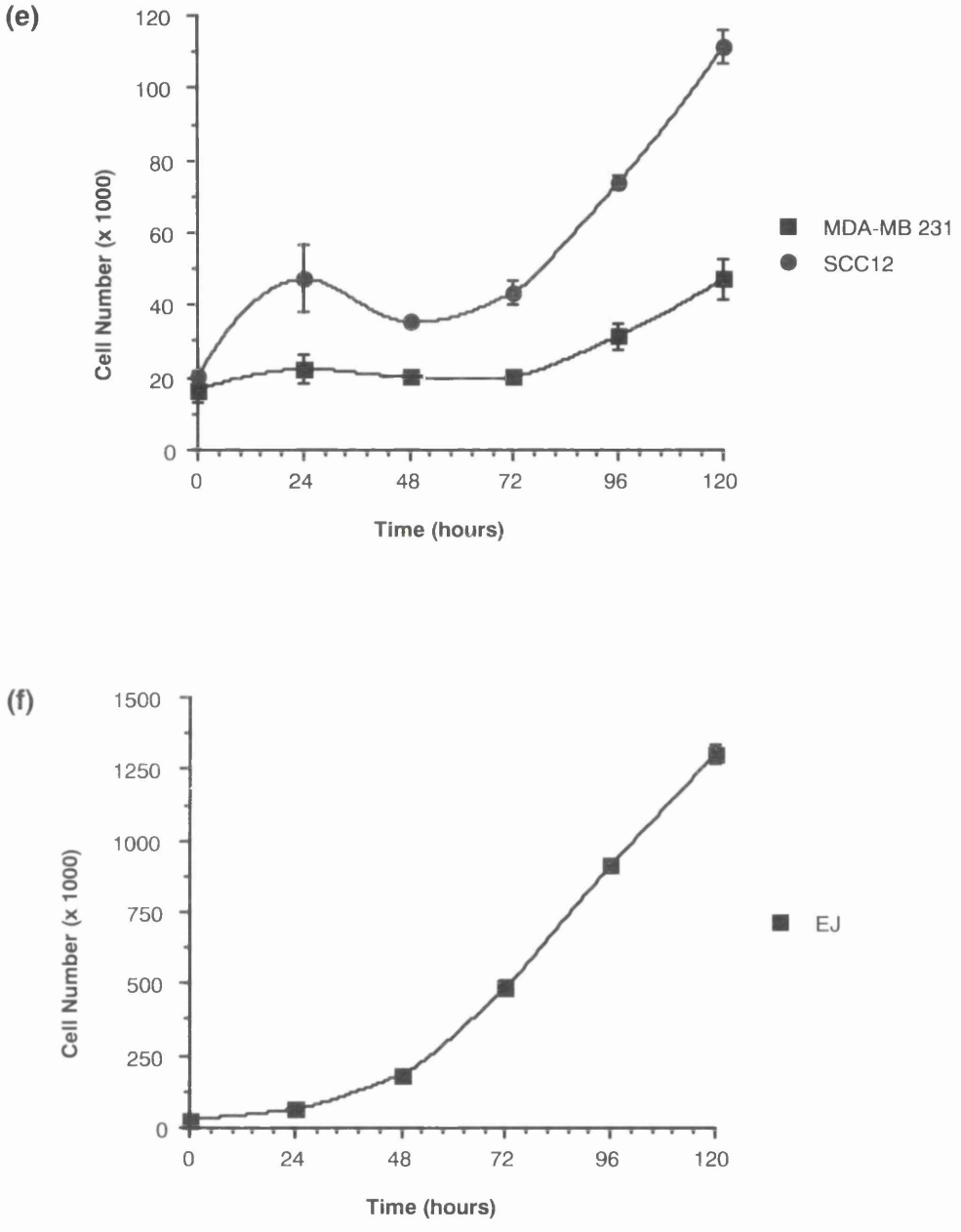
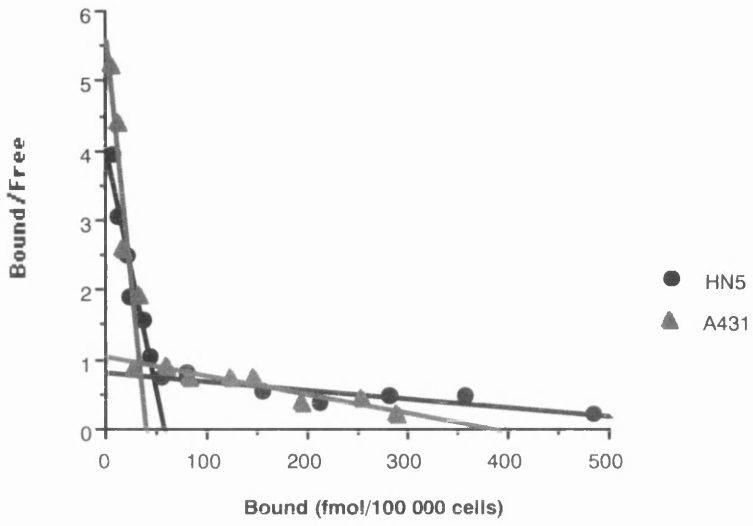


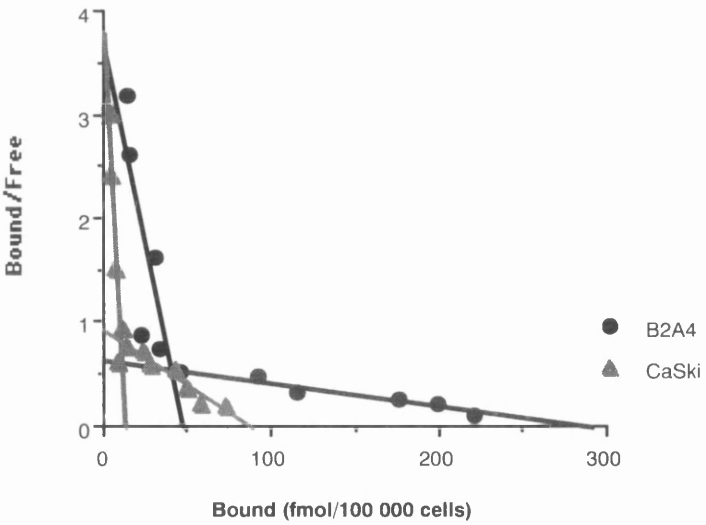

**Fig 6.1** . Points are means and standard deviations of triplicate determinations. Variations were smaller than the symbols where no error bars are shown.

**Fig 6.2** Scatchard plots of EGFR ligand binding data obtained from a panel of cell lines

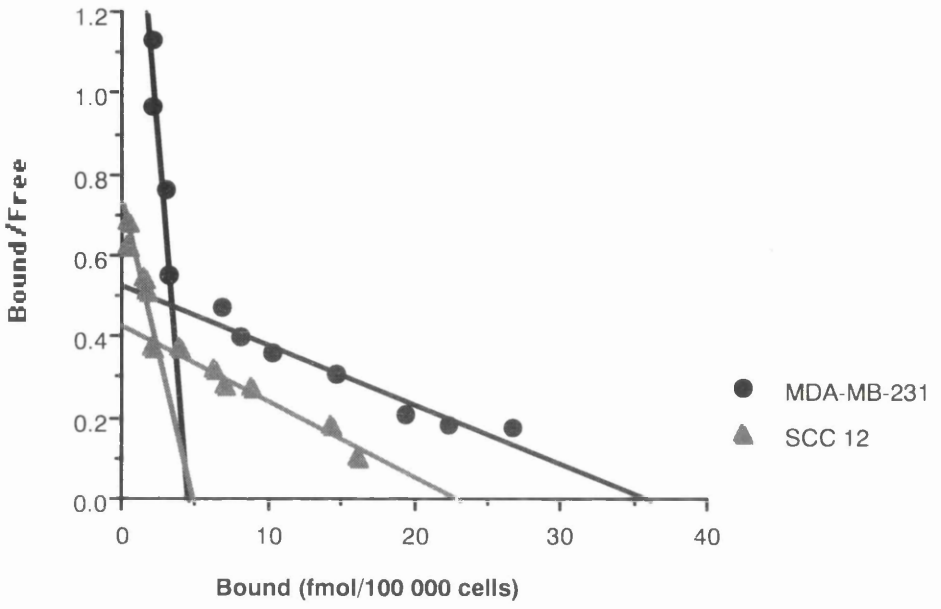
(a)



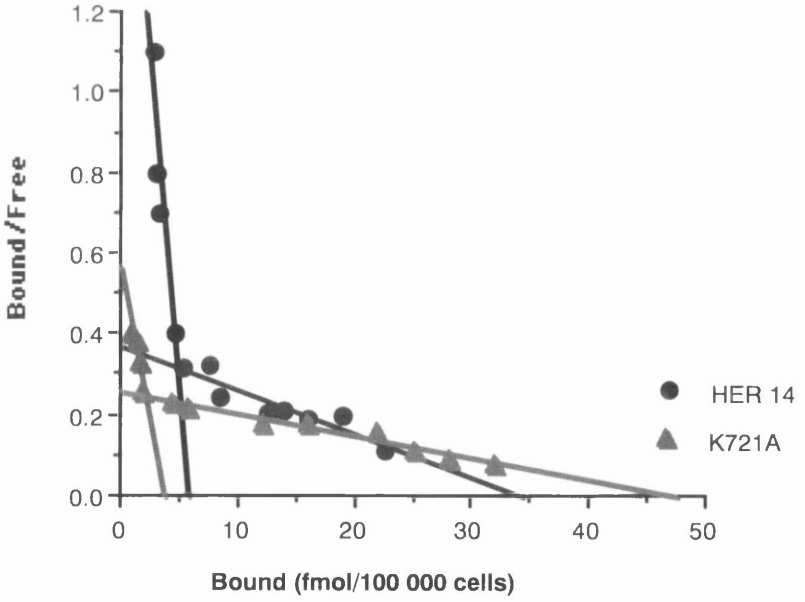

(b)



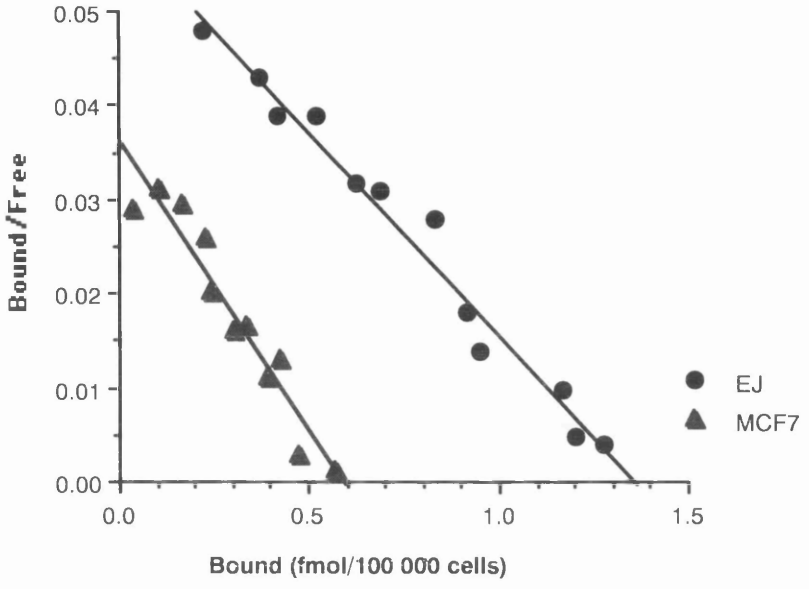
(c)


1.2
1.0
0.8
0.6
0.4
0.2
0.0
Bound/Free
0
10
20
30
40
Bound (fmol/100 000 cells)
MDA-MB-231
SCC 12

(d)


1.2
1.0
0.8
0.6
0.4
0.2
0.0
Bound/Free
0
10
20
30
40
50
Bound (fmol/100 000 cells)
HER 14
K721A

(e)




(f)

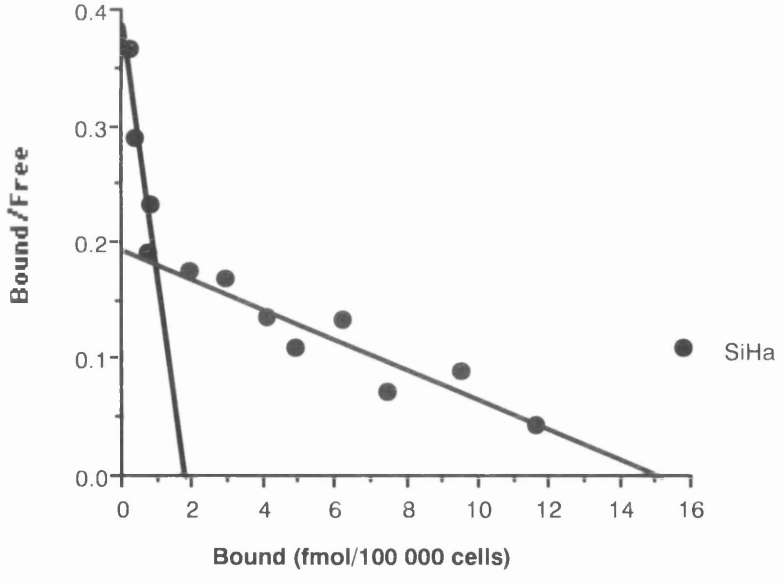



**Fig 6.2** Scatchard plots of ligand binding data obtained from a panel of cell lines. Each plot is a representative example of a single triplicate determination. Error bars are omitted for clarity.

**Table 6.3** EGFR concentration and dissociation constants of cell lines

| | High Affinity | | Low Affinity | | Total (x 100 000) |
|---|---|---|---|---|---|
| Cell line | Kd (nM) | Number (x 100 000) | Kd (nM) | Number (x 100 000) | |
| A431 | 0.12 +/- 0.02 | 2.0 +/- 0.4 | 4.37 +/- 0.23 | 23.7 +/- 1.3 | 25.7 +/- 0.9 |
| B2A4 | 0.16 +/- 0.04 | 2.6 +/- 0.1 | 8.77 +/- 1.81 | 15.7 +/- 0.6 | 18.3 +/- 0.7 |
| HN5 | 0.25 +/- 0.03 | 3.6 +/- 0.5 | 12.12 +/- 0.24 | 40.5 +/- 1.4 | 44.2 +/-1.2 |
| CaSki | 0.09 +/- 0.03 | 0.7 +/- 0.1 | 2.33 +/- 0.55 | 5.1 +/- 0.4 | 5.8 +/- 0.6 |
| HER 14 | 0.08 +/- 0.01 | 0.3 +/- 0.08 | 1.97 +/- 0.69 | 1.85 +/- 0.6 | 1.85 +/- 0.7 |
| K721A | 0.23 +/- 0.09 | 0.2 +/- 0.04 | 2.72 +/- 0.61 | 2.7 +/- 0.3 | 2.90 +/- 0.4 |
| SCC 12 | 0.33 +/- 0.08 | 0.3 +/- 0.11 | 3.92 +/- 0.88 | 1.41 +/- 0.6 | 1.71 +/- 0.8 |
| SiHa | 0.18 +/- 0.10 | 0.1 +/-0.06 | 6.58 +/- 3.39 | 0.9 +/- 0.2 | 1.0 +/- 0.3 |
| MDA-MB 231 | 0.12 +/- 0.06 | 0.3 +/- 0.14 | 5.30 +/- 1.24 | 2.2 +/- 0.9 | 3.1 +/- 0.9 |
| MCF-7 | | | 3.79 +/- 0.86 | 0.04 +/- 0.01 | 0.04 +/- 0.01 |
| EJ | | | 3.07 +/- 0.40 | 0.08 +/- 0.01 | 0.08 +/- 0.01 |

**Table 6.3** Data obtained from ligand binding analysis was analysed using LIGAND software. Results are means and standard deviations of two triplicate determinations.

*6.3.3 Immunodetection of EGFR*

Figure 6.3 (a) Immuno-detection of EGFR by western blotting. The relative density of each band is expressed as a percentage of the most dense band (lane 4). Cell lines were ranked according to the relative density, and tabulated (table 6.4). The highest ranked cell line was HN5, whilst the lowest ranked cell line was MCF-7. Of the two fibroblast cell lines, HER 14 was ranked higher than K721A.

(b) Detection of EGFR by 32P-labelling followed by immunoprecipitation. 32P-labelled EGFR appears as a band at 170 kDa. The membrane-rich-fraction prepared from HN5 cells provided the strongest signal (lane 4). 32P-labelled EGFR could not be detected in membrane preparations extracted from K721A cells (lane 8).

## Fig 6.3 Detection of EGFR by $^{32}$P labelling/immunoprecipitation and western blotting

Fig 6.3(a)

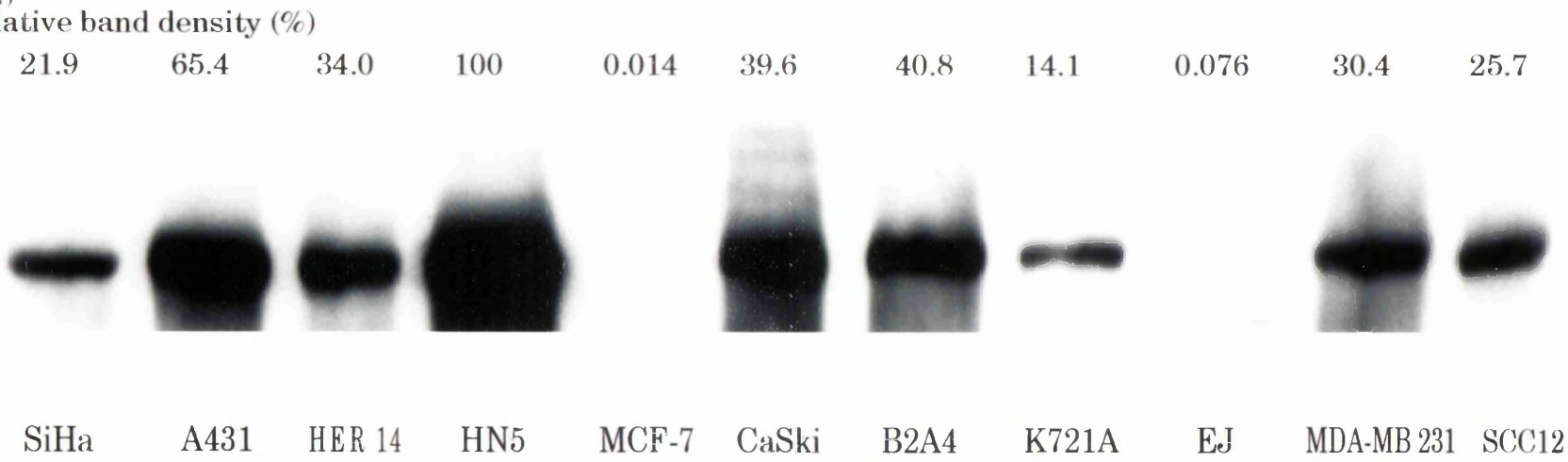



Fig 6.3(b)

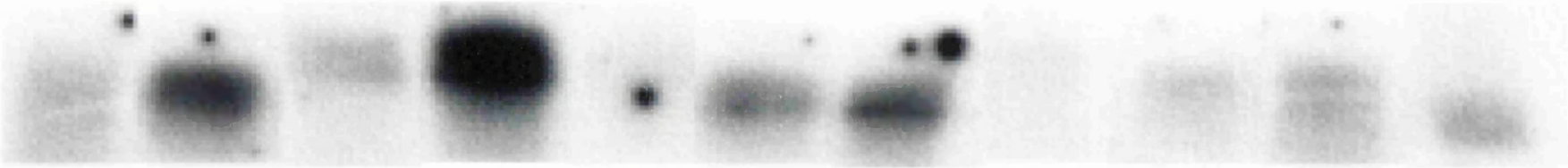


Fig 6.3(a) Detection of EGFR in a panel of cell lines by western blotting. 50 μg of whole cell lysate from each cell line was loaded per lane. EGFR appears as a band at 170 kDa. The density of the 170 kDa band was assessed by densitometry. The relative density of each band is indicated as a percentage of the densest band (lane 4).

Fig 6.3(b) Detection of EGFR by 32p-labelling followed by immunoprecipitation. 25μg of a membrane-rich fraction was loaded per lane. The presence of a band at 170 kDa indicates that the EGFR is phosphorylated in response to EGF stimulation.

### *6.3.4 Effect of EGF pre-stimulation on immunodetection of EGFR in A431 cells*

Figure 6.4.(a) demonstrates the effect of EGF pre-stimulation of A431 cells prior to western blotting. Each lane was loaded with 50 μg protein of a whole cell lysate of A431 cells which were pre-stimulated with a range of concentrations of EGF, as described in section 6.2.4

Lane 1. 50 μg A431 whole cell lysate, prestimulated with 25ng/ml hEGF
Lane 2. 50 μg A431 whole cell lysate, prestimulated with 10 ng/ml hEGF
Lane 3. 50 μg A431 whole cell lysate, pre-stimulated with 5 ng/ml hEGF
Lane 4. 50 μg A431 whole cell lysate, pre-stimulated with 1 ng/ml hEGF
Lane 5. 50 μg A431 whole cell lysate

Figure 6.4.(b) demonstrates the effect of EGF and peptide 2E on the detection of EGFR by 32P-labelling and immunoprecipitation.

Lane 1. 25 μg A431 membrane, with 1μg/ml hEGF
Lane 2. 25 μg A431 membrane, with 5 μg/ml hEGF
Lane 3. 25 μg A431 membrane, with 10 μg/ml hEGF
Lane 4. 25 μg A431 membrane, with 10 μg/ml hEGF, precipitated with normal rabbit serum
Lane 5. 25 μg A431 membrane, with 10 μg/ml hEGF, and 20 μg/ml peptide 2E
Lane 6. 25 μg A431 membrane, with 10 μg/ml hEGF, and 10 μg/ml peptide 2E
Lane 7. 25 μg A431 membrane, with 10 μg/ml hEGF, and 1 μg/ml peptide 2E

**Fig 6.4.** Effect of EGF on detection of EGFR in A431 cells by $^{32}P$ labelling/immunoprecipitation and western blotting



Fig 6.4.(a)

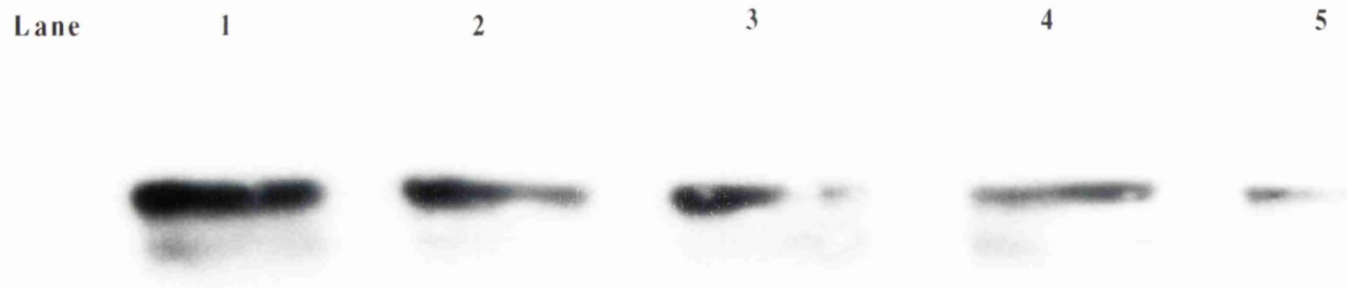



Fig 6.4.(b)

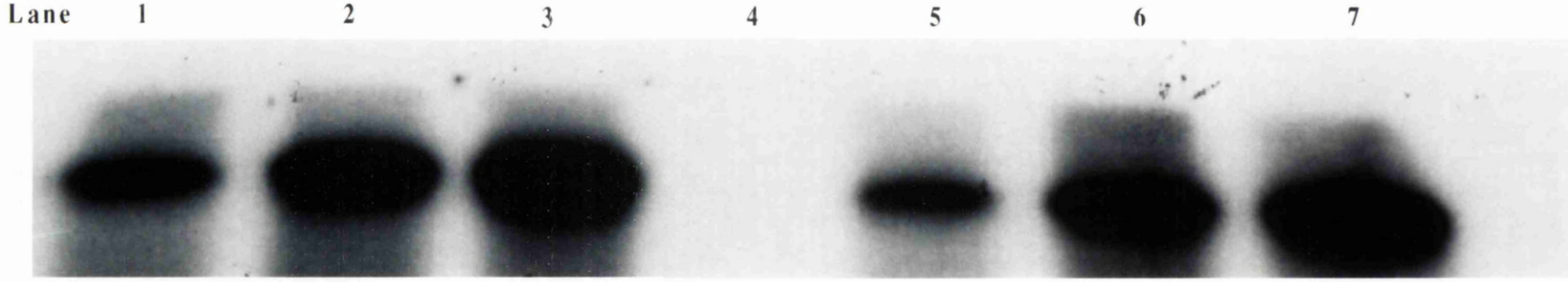


Figure 6.4 (a) demonstrates that the antibody mAb clone F4 showed greater affinity for EGFR which had been exposed to EGF prior to separation by SDS-PAGE (lanes 1-4), than to EGFR which had not been pre-stimulated (lane 5).

Figure 6.4 (b) demonstrates the effect of EGF on EGFR 32P-incorporation. Lanes 1-3 contain 25 μg protein of A431 membrane-rich fraction stimulated with increasing concentrations of EGF. The increasing density of the band at 170 kDa indicated that 32P-incorporation was EGF-dependent.

Lanes 4-7 indicate that the immunoprecipitation technique was antibody dependent. Lane 4 contains 25 μg protein labelled with 32P, which has been precipitated with whole rabbit serum, rather than anti-EGFR antibody. The absence of a band at 170 kDa demonstrated the dependence of this detection upon the presence of mAb clone F4. Lanes 5-7 demonstrate that peptide 2E, which contains the same amino acid sequence as the epitopic recognition site on EGFR, was able to compete with EGFR for the binding of mAb clone F4.

Table 6.4 indicates the rank order of EGFR expression, determined by ligand binding analysis and western blotting, of the cell line panel. Most cell lines were ranked similarly by both methods. The EGFR expression of the K721A cell line was ranked lower by western blotting than by ligand binding analysis. Statistical analysis was performed using the Wilcoxton signed rank test. The p value obtained (0.433) was greater that 0.05, indicating that there was no significant difference in the rank order of EGFR expression determined by these two methods.

**Table 6.4** . Comparison of the rank order of EGFR expression determined by ligand binding analysis and western blotting in a panel of cell lines.

| | Ligand binding analysis | Western blotting |
|---|---|---|
| Cell line | Rank order (1= highest) | Rank order (1= highest) |
| HN5 | 1 | 1 |
| A431 | 2 | 2 |
| B2A4 | 3 | 3 |
| CaSki | 4 | 4 |
| MDA-MB 231 | 5 | 6 |
| K721A | 6 | 9 |
| HER 14 | 7 | 5 |
| SCC12 | 8 | 7 |
| SiHa | 9 | 8 |
| EJ | 10 | 11 |
| MCF-7 | 11 | 10 |

**Table 6.4** Values represent the EGFR expression rank order of the cell line in the panel (1=highest). Rank orders were determined from ligand binding data (table 6.3.) and the relative density of the 170 kDa band observed by western blotting (figure 6.4 a). Wilcoxton signed rank significance > 0.05.

## 6.4 Discussion

The purpose of the experiments described in this section was to characterise a panel of cell lines with respect to EGFR expression and signalling capacity, to enable the investigation of EGFR-mediated cellular events.

EGFR expression was determined by western blotting using a monoclonal antibody which recognises a sequence on the intracellular portion of the receptor (residues 985-996). This antibody was chosen in preference to those which recognise the extracellular portion of the receptor, because the latter antibodies tend to bind more strongly to the receptor used in the primary inoculation. For example, EGFR1 binds with stronger affinity to receptors expressed on A431 cells than other cell lines (Ozanne, personal communication). This is possibly due to differences in the glycosylation of the extracellular region between cell lines.

Figure 6.3 (a) demonstrates EGFR detection in all of the cell lines examined by western blotting. Densitometric assessment of the intensities of the 170 kDa band indicated a wide range of receptor expression, and hence suitability of the panel of cell lines for the study of EGFR-mediated cellular events.

The number of EGFR was determined by ligand binding analysis. This technique assays the binding of a labelled EGFR ligand (125I-EGF) to a known number of cells (or a known protein content of homogenised tissue) at different ligand concentrations. From this data, receptor concentration and affinity can be estimated.

Figure 6.2 (a-f) and table 6.3. show the results obtained using ligand binding analysis. In nine cell lines, a bi-phasic binding curve was obtained. Two cell lines (MCF-7 and EJ) showed linear binding characteristics. Dissociation constants (Kd) ranged from 0.08 - 0.33 nM (high affinity), and 1.97 - 12.12 nM (low affinity). Previous studies have demonstrated that, in intact cells, the extracellular region of the EGFR is insensitive to trypsin, and that tyrpsinisation of intact cells does not result in a decrease of in 125I-EGF binding capacity (Carpenter, 1987).

EGFR signalling capacity was assessed by *in vitro* kinase 32P-labelling, followed by immunoprecipitation using the same monoclonal antibody used for western blotting. The phosphorylation of EGFR after stimulation with EGF is now well documented (King et al, 1980). Using γ-32P-ATP as substrate, the degree of 32P incorporation is a function of both the receptor tyrosine kinase activity and receptor concentration. Figure 1.7.3 (b)

indicates that all of the cell lines examined, with the exception of K721A, expressed EGFR which was capable of autophosphorylation after stimulation with EGF.

It was observed (figure 6.4.(a)) that the antibody used for the detection of EGFR (mAb clone F4) bound with greater affinity to ligand-bound, or active receptors than to inactive receptors. There are two possible explanations for this phenomenon. EGFR ligand binding to the receptor may cause a change in the three-dimensional conformation of the receptor, which is more easily recognised by the antibody. Another possibility is that Tyr 992, a residue within the epitopic site recognised by mAb F4, is a receptor autophosphorylation site (Walton et al, 1990). It is possible that the antibody has a greater affinity for the epitope when this site is phosphorylated.

Figure 6.4 (b) shows the effect of EGF concentration on the amount of 32P-labelled EGFR detected by immunoprecipitation and autoradiography. Increasing EGF concentration resulted in an increase in the density of the 170 kDa band. The dependence of the immunoprecipitation technique on the specific binding of the antibody to the receptor is also demonstrated. The addition of increasing concentrations of a peptide which competes with EGFR for antibody binding (peptide 2E) resulted in a decrease in the amount of 32P-labelled receptor precipitated.

Table 6.4 shows a comparison of the concentration of EGFR detected by both western blotting and ligand binding analysis. An strong correlation was observed between the results obtained using these methods. One exception was the K721A cell line, in which proportionately less EGFR was detected by western blotting.

The quantification of EGFR concentration using western blotting is problematic, as several factors may influence the degree of specific antibody binding. Figure 6.4 (a) demonstrates that mAb clone F4 bound to active (ligand-bound) receptor with greater affinity than inactive receptor. Alterations in external EGFR-ligand concentration will therefore affect the result obtained. Whilst no additional EGF was included in the cell lysate incubation mixtures prior to western blotting (figure 6.3 (a)), the external EGFR ligand concentration may vary for two reasons. Firstly, the extracellular concentration of EGFR-ligands in culture fluid depends on the concentration and type of serum used. Serum concentration and type were not constant (table 6.2.) Another source of EGFR ligand is the cells themselves. Many EGFR-expressing cell lines secrete EGFR-ligands, mainly TGF-$\alpha$, into the extracellular medium, resulting in autocrine and paracrine

stimulation (e.g. Hirsch et al, 1996). Thus the results obtained using western blotting with mAb clone F4 are a function of both receptor number and external EGFR-ligand concentration.

A further complicating factor is the unique expression of kinase-deficient EGFR by K721A cells. This receptor is incapable of activation by EGF, unlike the wild-type EGFR expressed by the other members of the cell line panel (figure 6.3 (b)). Therefore the increase in affinity of mAb clone F4 for active EGFR may be dependent upon activation of the intrinsic receptor kinase, and may not be due solely to the receptor binding to an appropriate ligand. This may explain why EGFR detection by this method does not exactly mirror the results obtained by ligand binding analysis (table 6.3). For these reasons, western blotting should be regarded as a semi-quantitiative method of EGFR detection.

The analysis of ligand binding data by Scatchard plot is a common method of estimation of EGFR number and affinity in cell lines or tissue samples (Scatchard, 1949). Parameters are obtained by plotting bound ligand against the bound/free ligand ratio and extrapolating the best fit line to the x axis. The dissociation constant (Kd) is the negative reciprocal of the slope of the best fit line. This system is susceptible to many possible inaccuracies, which may explain the divergence in reported results between groups of investigators.

The experimentally derived data makes the assumption that both of the ligand species (EGF and 125I EGF) are identical with respect to receptor binding, and that they are able to compete equally for EGFR. The Chloramine T method of iodination (Hunter and Greenwood, 1962), produces a small proportion of 125I-EGF product which is able to form covalent cross-links with the EGFR. This is also true of 125I EGF synthesised by the Iodo-Gen method (Fraker and Speck, 1978). Binding by this cross-linking species cannot be overcome by excess unlabelled EGF and this affects the derived Kd (Comens et al, 1982).

It is also assumed that 125I EGF is a single iodinated molecular species, in which all iodinated molecules behave identically. Since it is known that one compound is produced in the labelling reaction which affects the derived Kd, it is possible that additional sub-species are present which may also cause an alteration in the apparent Kd. Another source of inter-laboratory variation in data derived by Scatchard analysis may be the type of unlabelled EGF used. Little account is usually taken of the different binding

characteristics displayed between recombinant and purified EGF, or in the differences between human and murine EGF. While the differences are probably small, the additive effects of errors such as these can give rise to large variations in derived parameters, especially when these parameters are obtained by extrapolation of a best-fit line and not directly from the data itself.

The technique of Scatchard analysis is also open to misinterpretation in a number of respects. Firstly, mathematical models have demonstrated that at low receptor concentrations, the Kd becomes a linear function of the receptor concentration (Carpenter, 1987). This may explain the dual affinity binding characteristics displayed by EGFR. Recently, a stable active dimeric form of the EGFR has been synthesised which displays binding characteristics similar to the high affinity component of the bi-phasic Scatchard curve. (Sorokin et al, 1994). This confirms that observed dual binding affinity is not simply an artifact of the detection method. Also, binding studies carried out at room temperature are prone to error due to internalisation of ligand. It has been demonstrated that EGFR internalisation is reduced, but not abolished at 20oC, compared with 37oC (Carpenter and Cohen, 1976). Unlabelled ligand can not compete for internalised EGFR-125I-EGF complexes, and will lead to an increase in the estimation of receptor number.

Despite these qualifications, the data presented in table 6.3 characterising the cell lines used in this investigation provides Kd values and receptor numbers per cell which are similar to previously published values for these cell lines (Reiss et al, 1991, Kwok and Sutherland, 1991, Cowley et al, 1984, Honegger et al, 1987). It can be concluded from this data that the cell lines used in this study are as previously reported with regard to receptor number and binding affinity.

# CHAPTER 7

# THE EFFECT OF EPIDERMAL GROWTH FACTOR ON CELLULAR RADIOSENSITIVITY

## 7.1 Introduction

There is a growing interest in the use of cytokines and growth factors to modify the cellular response to ionising radiation. The identification of chemical agents which may increase the sensitivity of tumours, or decrease the sensitivity of non-malignant tissue to radiation are potentially of considerable significance, given the widespread use of radiotherapy in cancer treatment. In particular, several studies have focused on the radioprotective effect of cytokines in heamatopoietic tissue. Erythropoetin, GM-CSF, interleukin-1 (IL-1), interleukin-11 (IL-11) and basic fibroblast growth factor (bFGF) have all been demonstrated to increase radioresistance both *in vitro* and in animal and human studies (Santucci et al, 1994, Neta et al, 1989, Uckun et al, 1989, Gallicchio et al, 1991, Goff et al, 1997). In addition, bFGF has also been shown to protect endothelial cells against radiation-induced apoptosis (Fuks et al, 1994). There are several possible mechanisms underlying these observations. IL-1 has been demonstrated to induce mitochondrial superoxide dismutase, resulting in a radioprotective effect in bone marrow stem cells (White and Tsan, 1994, Eastgate et al, 1993), while bFGF-induced radioresistance appears to be mediated through an FGF receptor/protein kinase C-dependent pathway (Haimovitz-Friedman et al, 1994). Subsequent studies have indicated that activated PKC may act to inhibit apoptotic signals generated by the radiation-induced hydrolysis of membrane sphingomyelin (Fuks et al, 1995). Interleukin-11 has been shown to reduce cellular production of tumour necrosis factor (TNF), whose effects are partly mediated through the sphingomyelin pathway (Redlich et al, 1996).

The ability of EGF to modify the cellular radiosensitivity of epithelial cells was first examined by Kwok and Sutherland (1989). They demonstrated that EGF added to cell culture medium enhanced the sensitivity of a human carcinoma cell line to ionising radiation. Further investigation showed that this radiosensitisation could be observed in a panel of human squamous cell lines which expressed different levels of EGFR. This effect appeared to be dependent upon the expression of high affinity EGF binding sites, and was maximal in all cell lines at 10 ng/ml EGF. (Kwok and Sutherland, 1991(a)). Examination of damage repair kinetics showed that EGF-dependent sensitisation was not related to alterations in potentially lethal damage repair, or sub-lethal damage repair (Kwok and Sutherland, 1991(b)). A further report from this group suggested that cell cycle distribution may be an important factor in the sensitisation effect (Kwok and Sutherland, 1992). An examination of the

combined effects of radiation and EGF showed that EGF increased the length of G2-M arrest caused by γ-irradiation (Laderoute et al, 1994). From these observations, the authors formed the hypothesis that the increase in G2-M arrest may be cytotoxic to a sub-population of cells, which undergoes apoptosis. This hypothesis, however, remains to be proven.

Other investigators have also studied the effect of EGF on the radiosensitivity of a variety of cell lines. The addition of EGF to hormone-deprived MCF-7 cells increased radioresistance when compared to hormone-deprived controls (Wollman et al, 1994). Another study reported that pre-incubation of two different squamous cell carcinoma cell lines with exogenous EGF reduced the radioresistance without alteration of cell cycle distribution (Bonner et al, 1994). There has, as yet, been no report of EGF-induced alteration of radiosensitivity *in vivo*.

### 7.1.1 *Aim of this study*

The aim of this study was to characterise the effect of exogenous EGF on the radiosensitivity of the panel of EGFR-expressing cell lines described earlier (table 6.1). Experiments were also designed to establish the effect of EGF on the proliferation and cloning efficiency of the cell line panel.

## 7.2 Materials and methods

### 7.2.1 *Analysis of growth response to EGF*

1x104 cells per well were grown in 24 well plates under the conditions described in table 6.2. After 24 hours, the FCS content of the medium was changed from 10% to 0.5% (v/v), and the cells were incubated for a further 24 hours. This medium was then replaced with medium containing 0.5% (v/v) FCS plus a range of concentrations of murine EGF.

Cells were then counted every 24 hours using a Coulter counter (Coulter Electronics). In an attempt to maintain a constant EGF concentration, EGF-containing medium was renewed every 48 hours. All determinations were performed in triplicate. Statistical analysis of the results was carried out using the paired Student's t-test.

7.2.2 *Radiation survival assay*

A feeder layer of cells was prepared by irradiating a single cell suspension using a 60Co source at a dose rate of approximately 1.75 Gy/min, until a dose of 60 Gy had been given. Feeder cells were either used immediately or stored for a maximum of 48 hours at 4oC.

Viable cells were prepared by harvesting at 70% confluence by trypsinisation. The cells were then transferred to 25 cm2 tissue culture flasks (between 500 and 20 000 cells per flask), containing the stated medium and serum conditions (table 6.2.). Feeder cells were then added, so that each flask contained a total of 5 x104 cells. Three flasks containing 5x104 viable cells were also prepared to allow the measurement of multiplicity. The flasks were then placed in a 2% CO2 atmosphere for 24 hours.

The multiplicity factor (n) for each assay was established by scoring the number of single, double and treble divisions which occurred in the flasks containing only viable cells after 24 hours, and determining the mean number of divisions per cell.

Cells to be irradiated were then placed under a 60Co source and given a dose from 2 to 8 Gy, at a dose rate of approximately 1.75 Gy/min.

Immediately after irradiation, the original culture medium was removed and replaced with medium containing normal serum plus the appropriate concentration of human EGF. Flasks were then incubated at 37oC for 10 to 14 days to allow colony formation.

Colonies were stained with Carbol-fuschin. Those colonies which contained 50 or more cells were scored as viable.

The actual surviving fraction (f) was calculated from the observed surviving fraction (F) using the equation :

$$f= 1 - (1-F)1/n$$

as previously described (Elkind and Whitmore, 1967).

## 7.3 Results

### 7.3.1 *Effect of EGF on cellular proliferation*

The effect of EGF on the proliferation of a panel of 11 cell lines was examined. The proliferative effect of EGF at two concentrations (10 and 25 ng/ml) was established at 24 hour intervals over a five day period.

EGF displayed a bi-functonal growth modulatory effect on this cell line panel. The proliferation of HN5, B2A4, CaSki, ($p < 0.01$) EJ and A431 ($p < 0.05$) was inhibited significantly at both 10 and 25 ng/ml EGF (figures 7.1 a-k and figure 7.2). A positive proliferative effect was observed in HER14 ($p < 0.01$), MCF 7 and SiHa ($p < 0.05$) at the same concentrations of EGF. EGF treatment failed to modify the proliferative capacity of K721A, MDA-MB 231 and SCC 12.

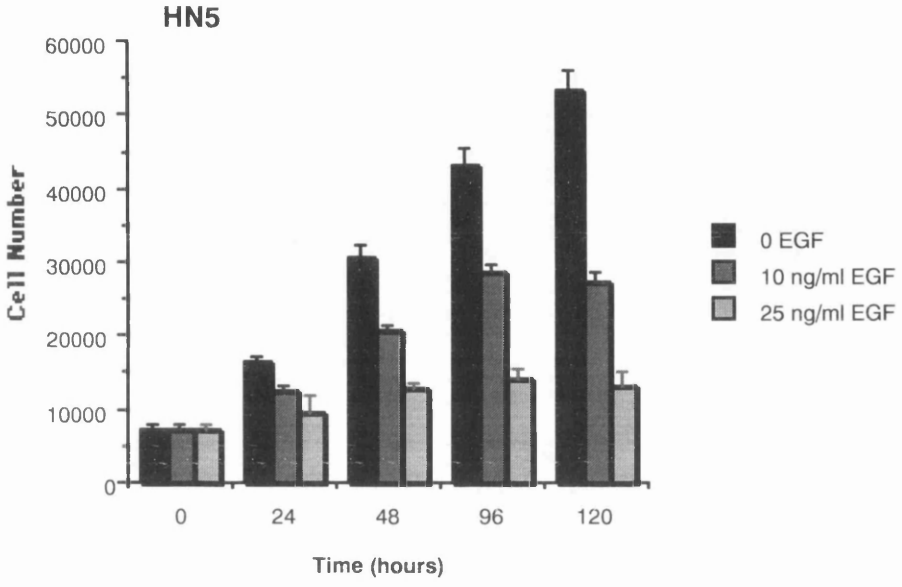
### 7.3.2 *Effect of EGF on cloning efficiency*

The effect of EGF on cloning efficiency was established. Cloning efficiency is calculated from the number of colonies (of 50 or more cells) which are eventually formed following the seeding of a known number of single cells, expressed as a percentage.

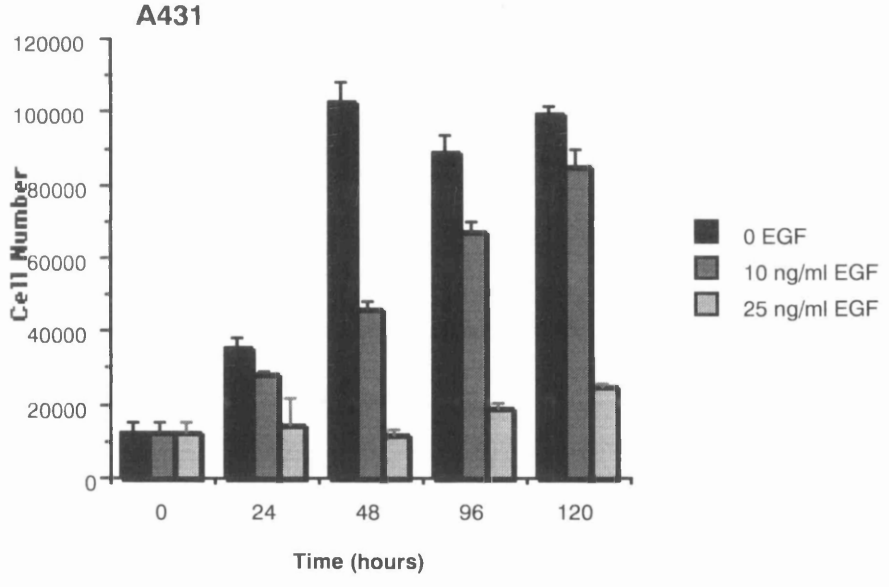
The presence of EGF in the tissue culture medium during the colony-forming period reduced the cloning efficiency of A431, B2A4, CaSki and HN5 cell lines (table 7.1). Of these four cell lines, only A431 was able to form colonies in the presence of 10 ng/ml EGF. The maximal EGF concentration which permitted colony formation with B2A4 and CaSki cell lines was 3 ng/ml. Colony formation with HN5 cells was only possible in an EGF concentration of 1 ng/ml. No significant alteration in the cloning efficiency of the remaining cell lines (HER14, K721A, MCF-7, SiHa, MDA-MB 231, SCC12 and EJ) was observed at 10 ng/ml EGF (table 7.1.).

**Fig 7.1** Effect of EGF on the proliferation of a panel of cell lines

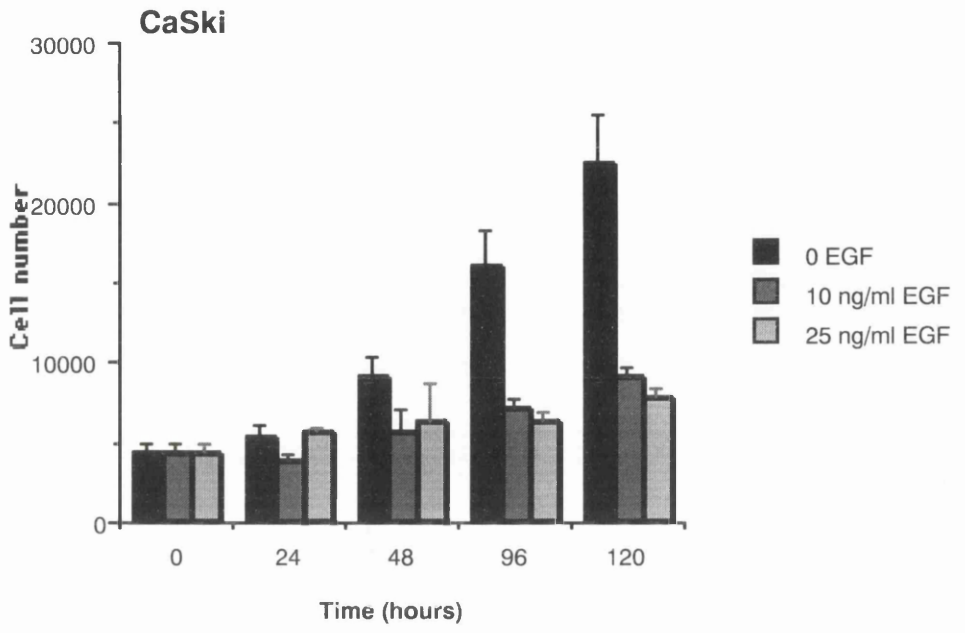
(a)



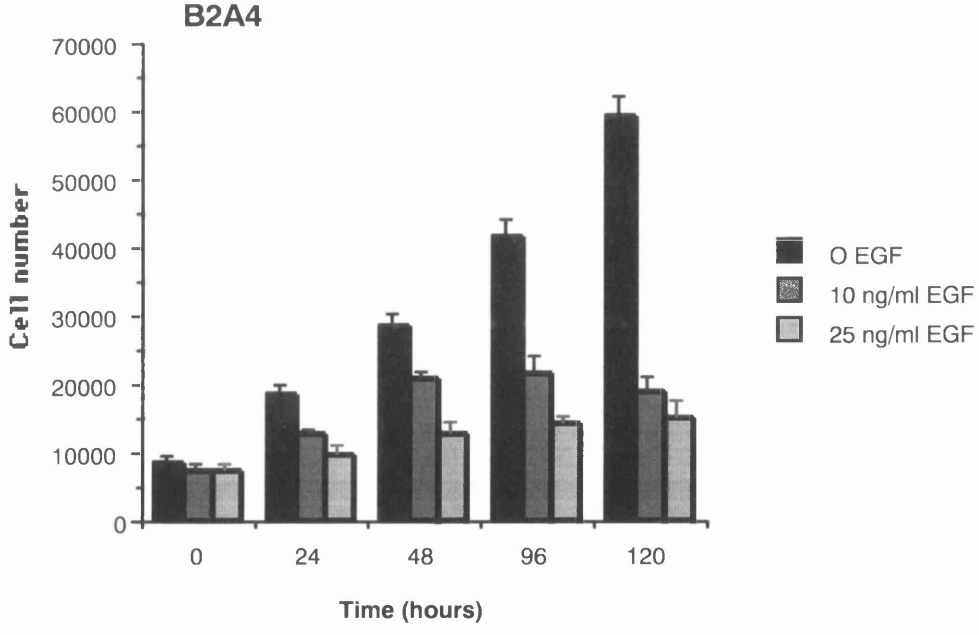

(b)



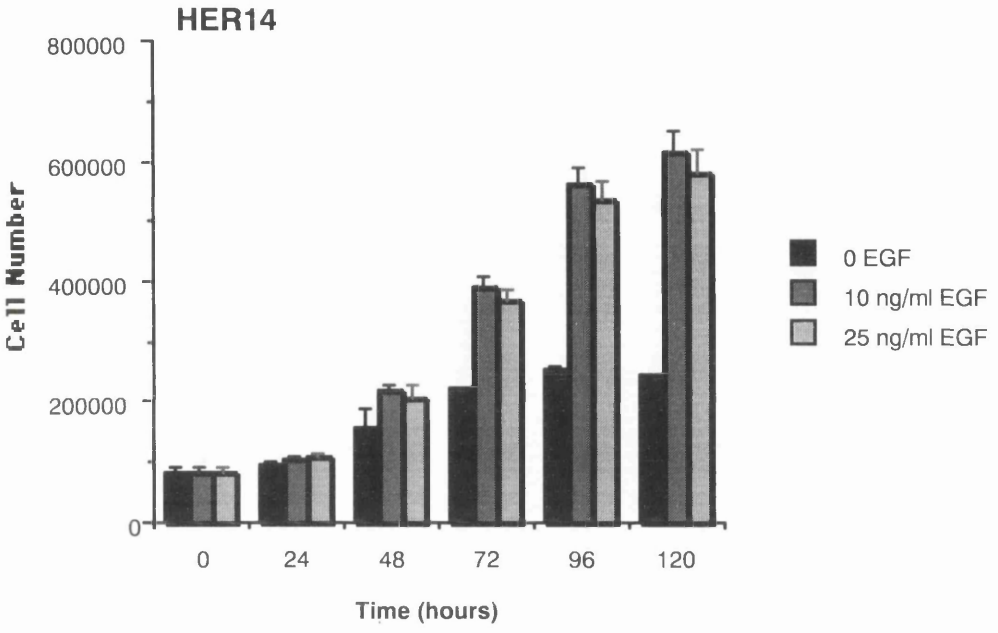
(c)


CaSki
30000
20000
10000
0
Cell number
0
24
48
96
120
Time (hours)
0 EGF
10 ng/ml EGF
25 ng/ml EGF

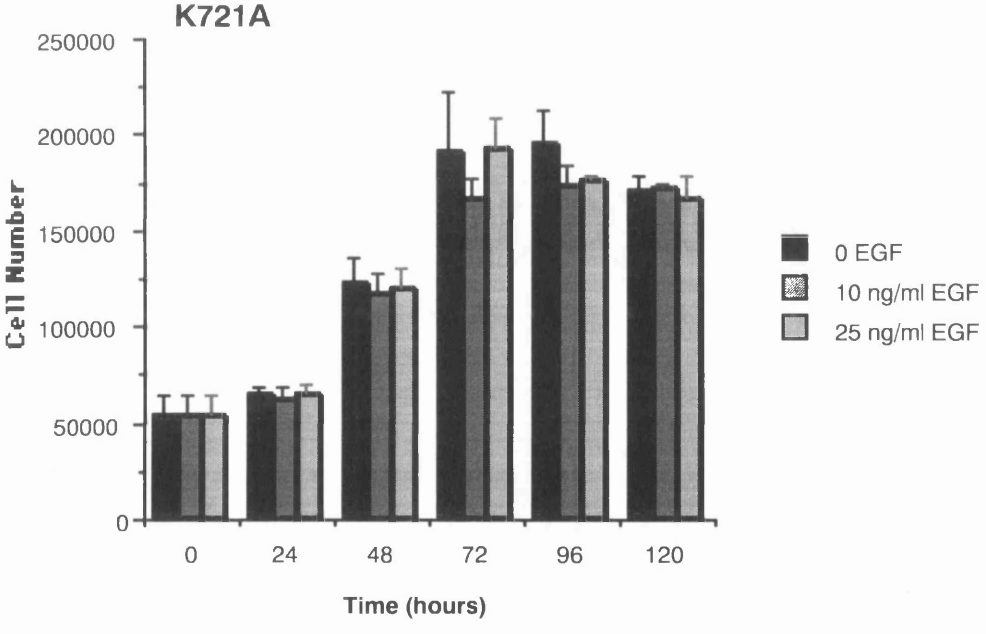
(d)


B2A4
70000
60000
50000
40000
30000
20000
10000
0
Cell number
0
24
48
96
120
Time (hours)
O EGF
10 ng/ml EGF
25 ng/ml EGF

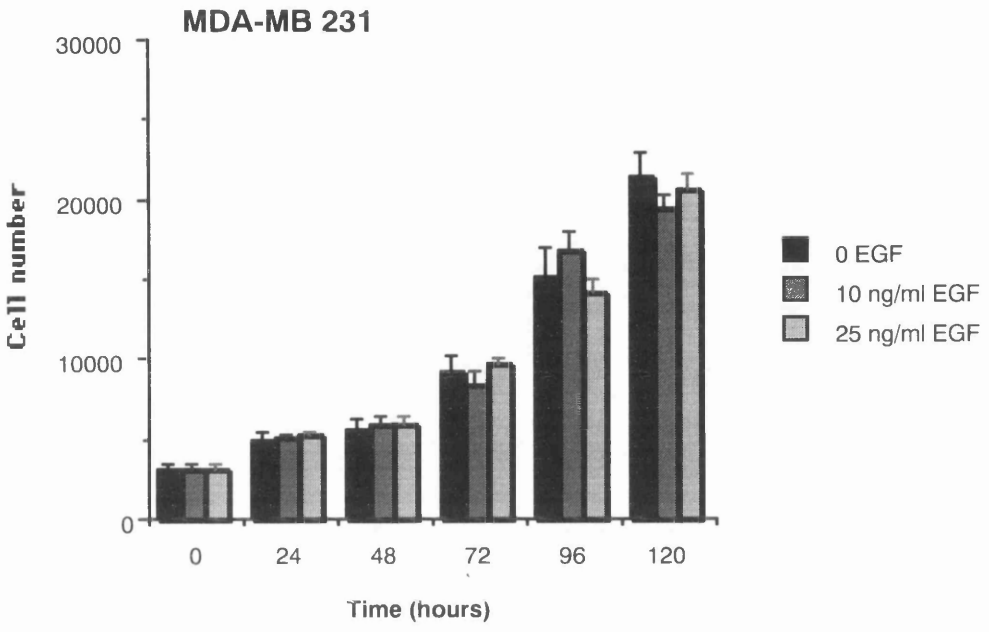
(e)


HER14
800000
600000
400000
200000
0
Cell Number
0
24
48
72
96
120
Time (hours)
0 EGF
10 ng/ml EGF
25 ng/ml EGF

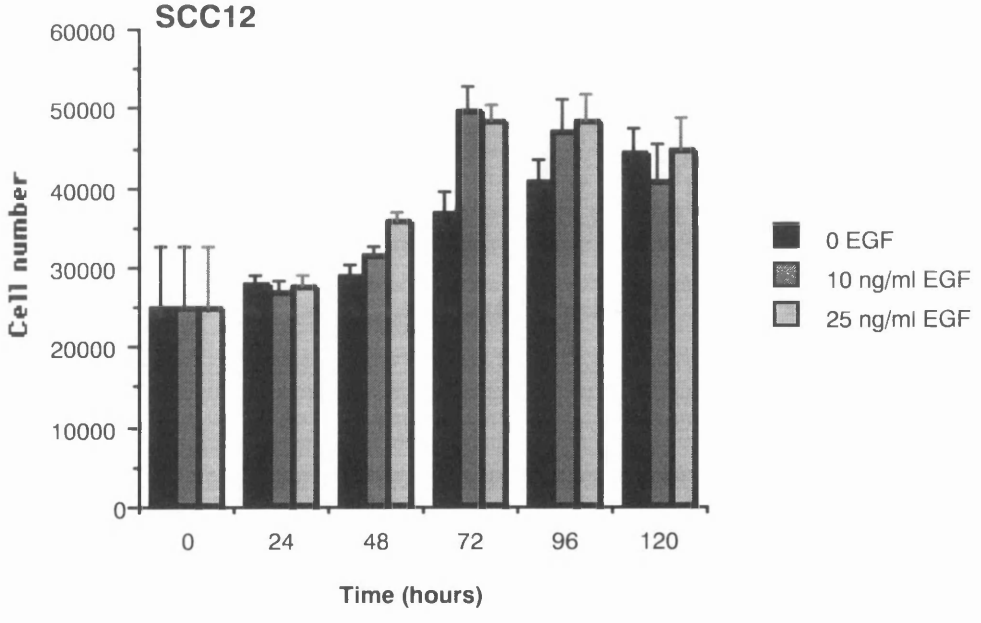
(f)


K721A
250000
200000
150000
100000
50000
0
Cell Number
0
24
48
72
96
120
Time (hours)
0 EGF
10 ng/ml EGF
25 ng/ml EGF

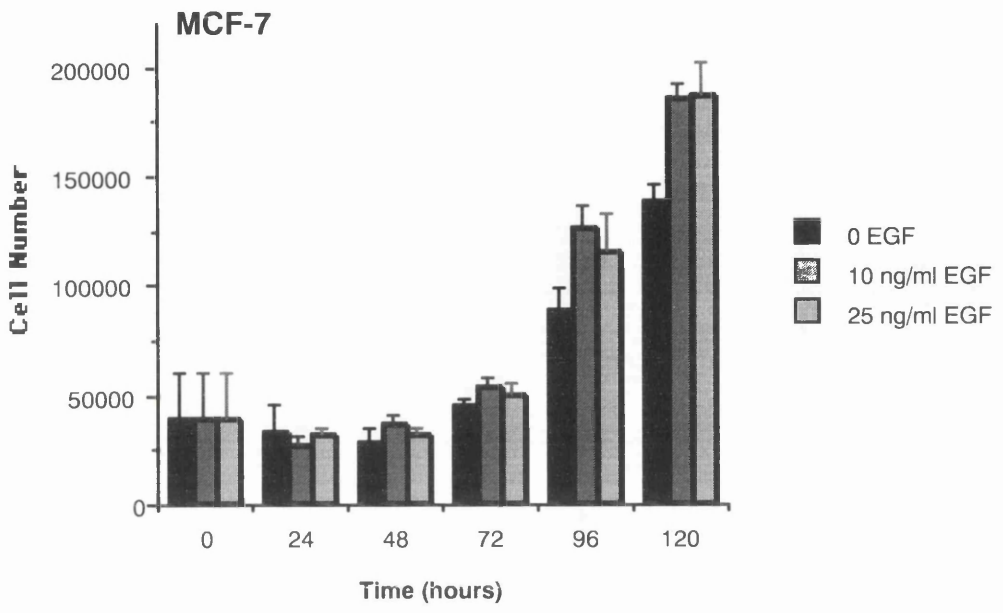
(g)


MDA-MB 231
30000
20000
10000
0
Cell number
0
24
48
72
96
120
Time (hours)
0 EGF
10 ng/ml EGF
25 ng/ml EGF

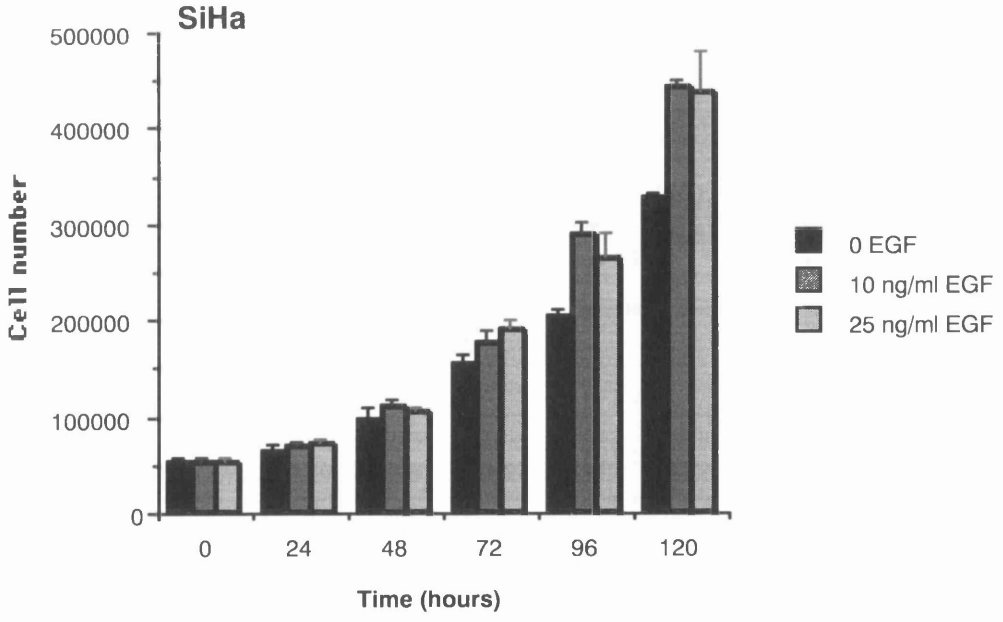
(h)


SCC12
60000
50000
40000
30000
20000
10000
0
Cell number
0
24
48
72
96
120
Time (hours)
0 EGF
10 ng/ml EGF
25 ng/ml EGF

(i)


MCF-7
Cell Number
200000
150000
100000
50000
0
0
24
48
72
96
120
Time (hours)
0 EGF
10 ng/ml EGF
25 ng/ml EGF

(j)


SiHa
Cell number
500000
400000
300000
200000
100000
0
0
24
48
72
96
120
Time (hours)
0 EGF
10 ng/ml EGF
25 ng/ml EGF

(k)

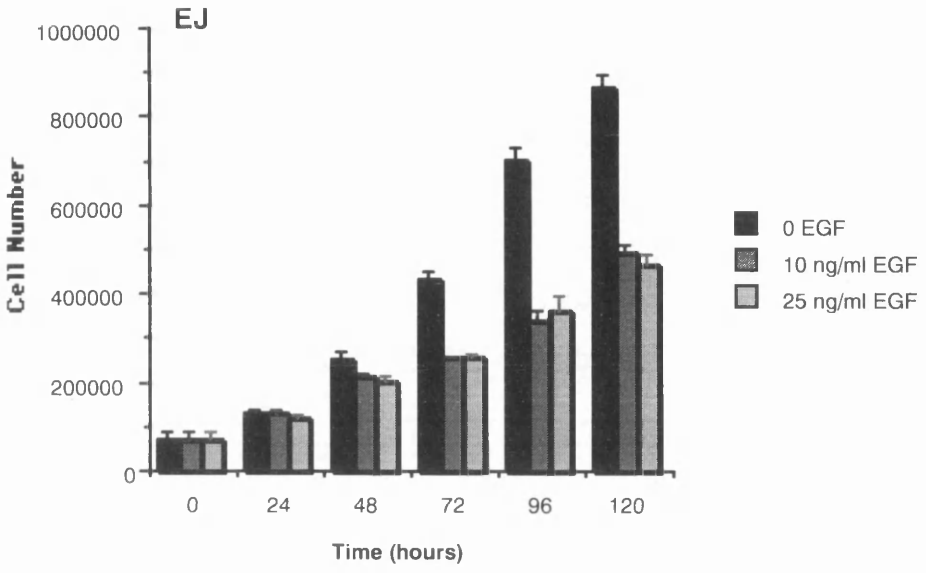



**Fig 7.1** Effect of EGF on the proliferation of 11 EGFR-expressing cell lines. Columns represent triplicatE determinations +/- S.D.

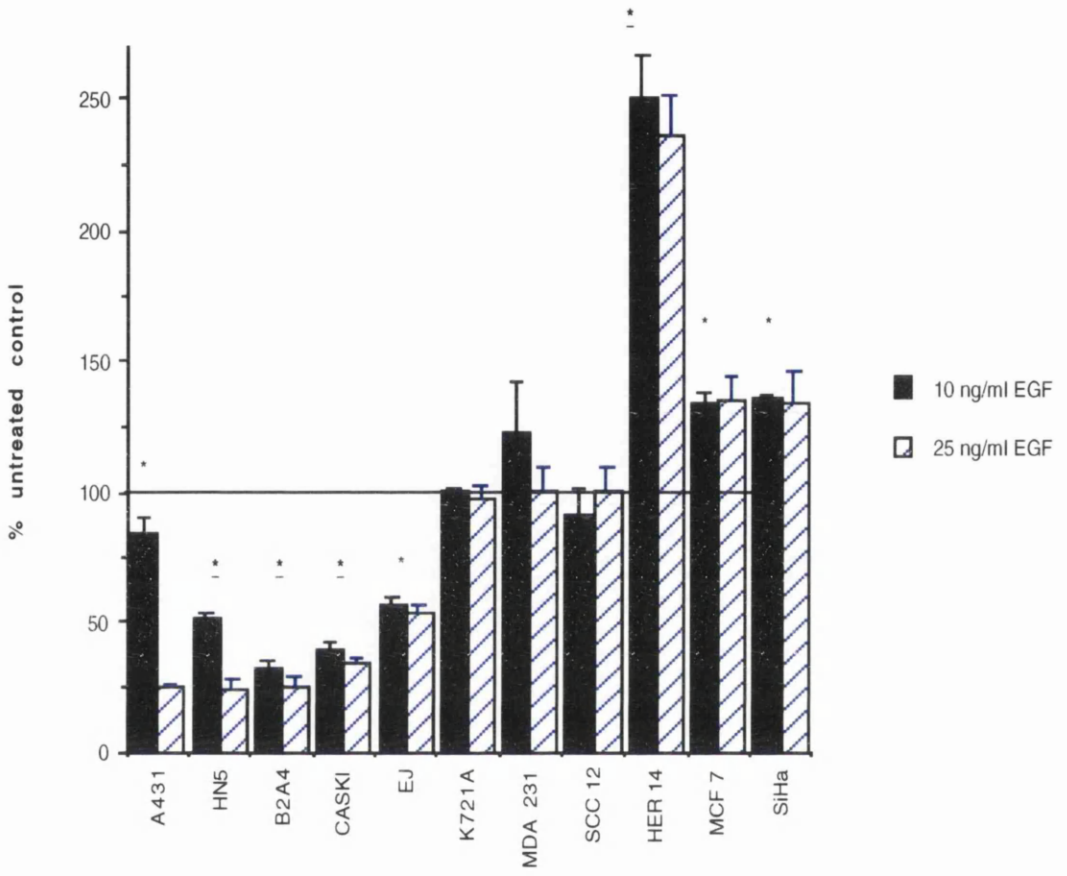



**Fig 7.2** Effect of EGF on cell number after 120 hours incubation. Columns represent the effect of 10 or 25 ng/ml EGF on cell number when compared to untreated controls (100%). Data was analysed using the paired Student's t-test

* represents both columns $p < 0.05$

$\underline{*}$ represents both columns $p < 0.01$

| | cloning efficiency (%) | | |
|---|---|---|---|
| Cell line | no EGF | with EGF | EGF Concentration(ng/ml) |
| A431 | 34 +/- 9 | 14 +/- 3 | 10 |
| B2A4 | 29 +/- 4 | 14 +/- 2 | 3 |
| HN5 | 31 +/- 7 | 21 +/- 4 | 1 |
| CaSki | 23 +/- 4 | 14 +/- 4 | 3 |
| HER 14 | 25 +/- 6 | 21 +/- 7 | 10 |
| K721A | 26 +/- 7 | 21 +/- 3 | 10 |
| MCF7 | 47 +/- 13 | 45 +/-8 | 10 |
| EJ | 26 +/-3 | 29+/-6 | 10 |
| SiHa | 23 +/- 2 | 18 +/- 4 | 10 |
| MDA-MB 231 | 37 +/- 6 | 41 +/- 3 | 10 |
| SCC 12 | 16 +/- 6 | 17 +/- 5 | 10 |

**Table 7.1.** Effect of EGF on the cloning efficiency of eleven cell lines. Results from at least three experiments performed in triplicate. Figures are means +/- S.E.M.

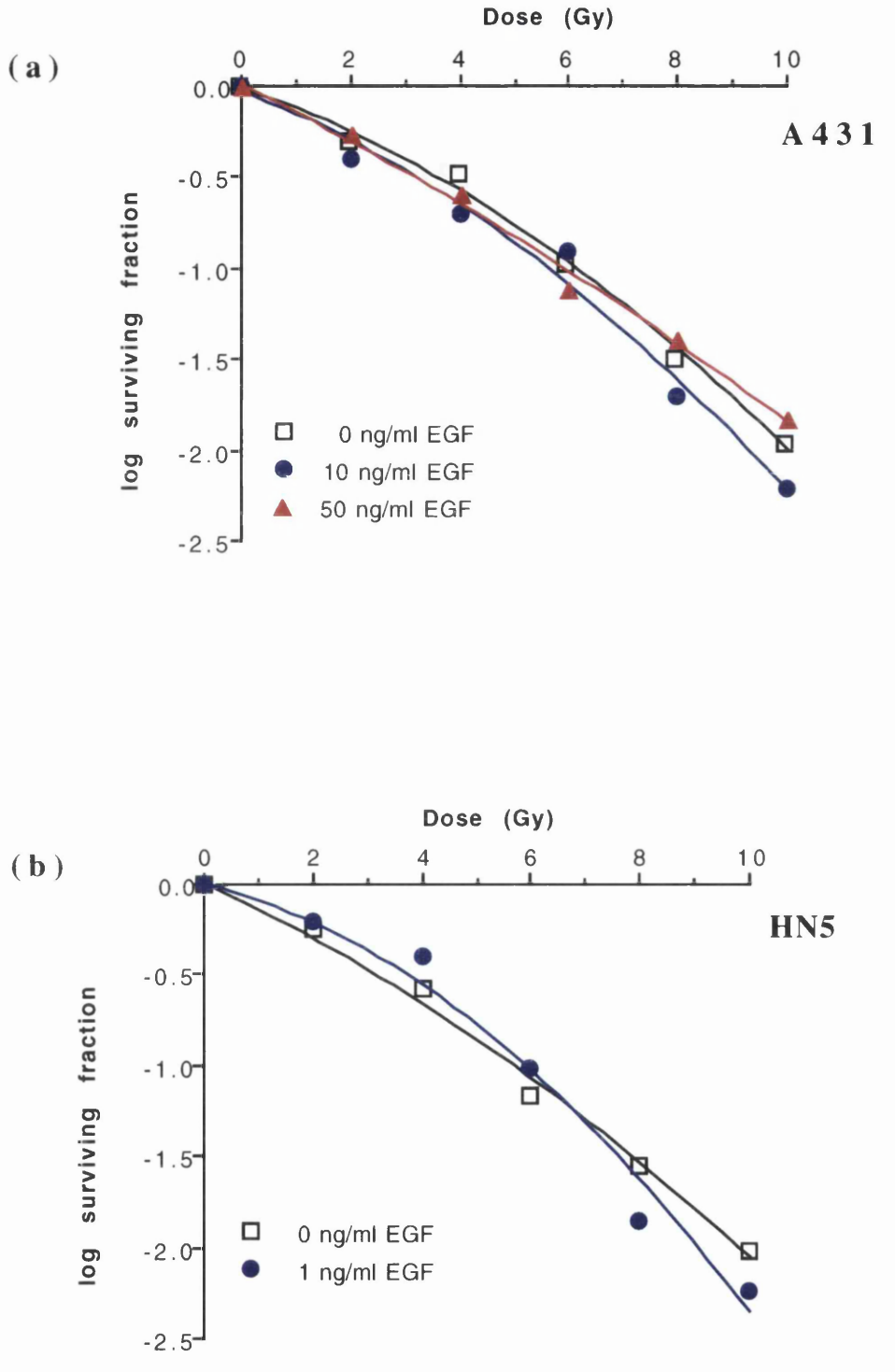
### 7.3.3 *Effect of EGF on radiation sensitivity*

The effect of post-irradiation EGF administration on radiation sensitivity was established by colony forming assay. The data from the colony forming assay was fitted to the linear quadratic model of cell survival, described in secton 6.1. Radiobiological parameters obtained from the model were tabulated (table 7.2).

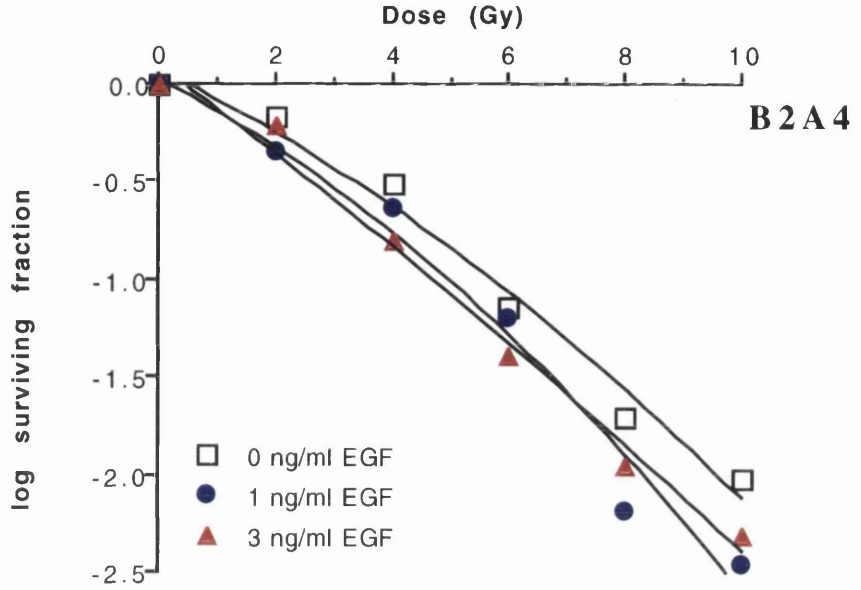
The data from the radiation survival assay was fitted to the linear quadratic model of cell survival (figure 7.3). The parameters $\alpha$ and $\beta$, as well as the surviving fractions at 2 and 10 Gy (SF2 and SF10 respectively) were obtained using this model (table 7.2). Statistical analysis was carried out using the paired Student's t-test. The p value was used to indicate statistical significance of differences in radiosensitivity and was derived by comparison of the SF2 and SF10 values of the EGF treated cells with the untreated controls.

Of the cell lines examined, none showed a statistically significant alteration in the SF2 value at any EGF concentration (table 7.2). Likewise, the SF10 values showed no significant EGF-modulation, with the exception of CaSki cell line, which showed an increased radioresistance when treated with EGF at 1 and 3 ng/ml.

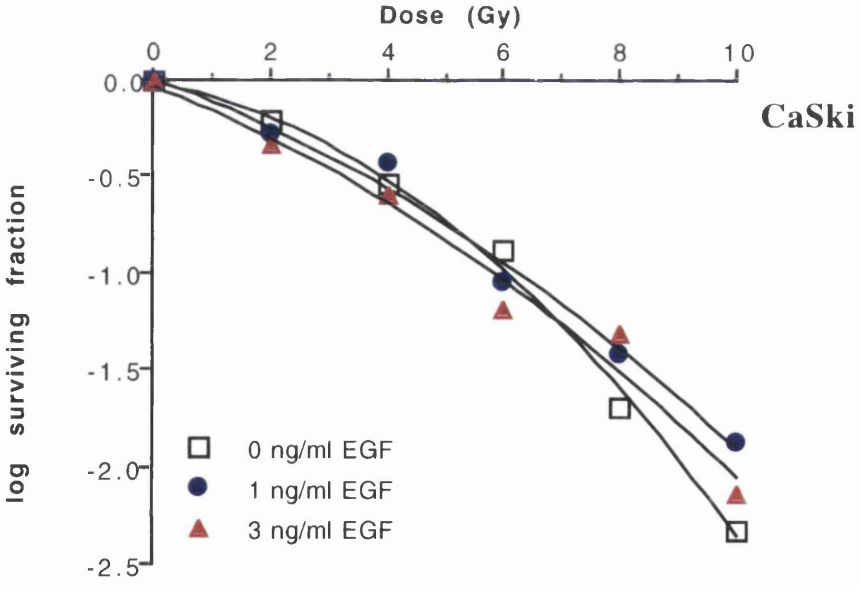
**Fig 7.3** Effect of EGF on the radiation dose/response plots of a panel of cell lines



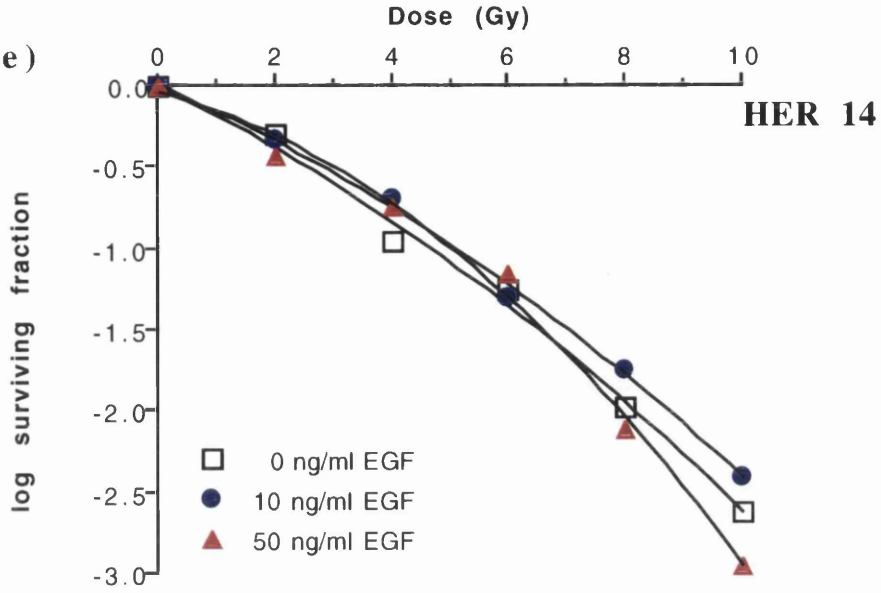
(c)


Dose (Gy)
0
2
4
6
8
10
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
log surviving fraction
B2A4
0 ng/ml EGF
1 ng/ml EGF
3 ng/ml EGF

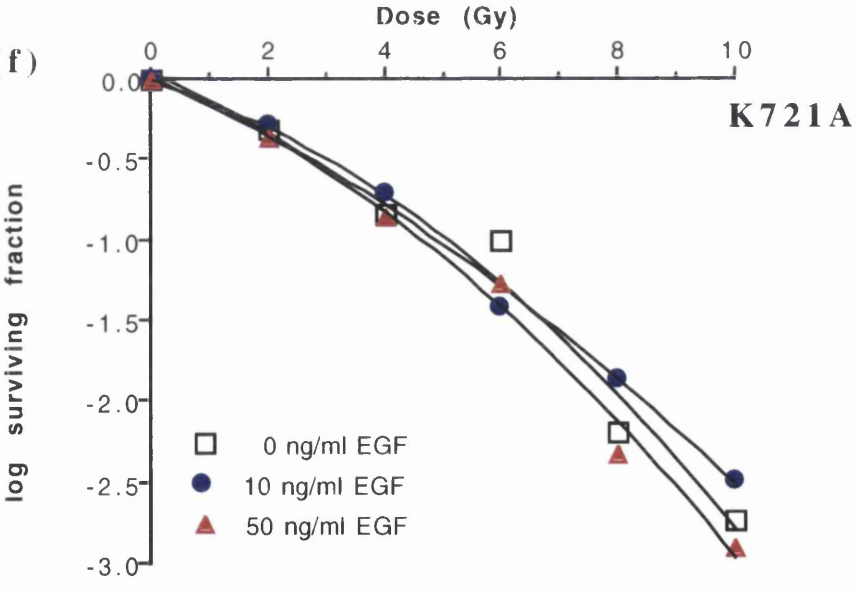
(d)

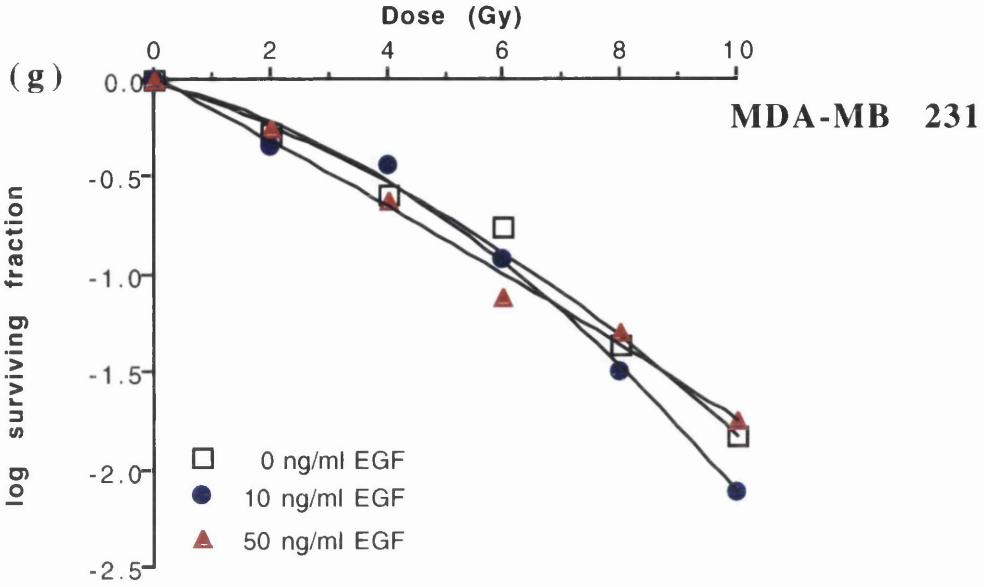

Dose (Gy)
0
2
4
6
8
10
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
log surviving fraction
CaSki
0 ng/ml EGF
1 ng/ml EGF
3 ng/ml EGF

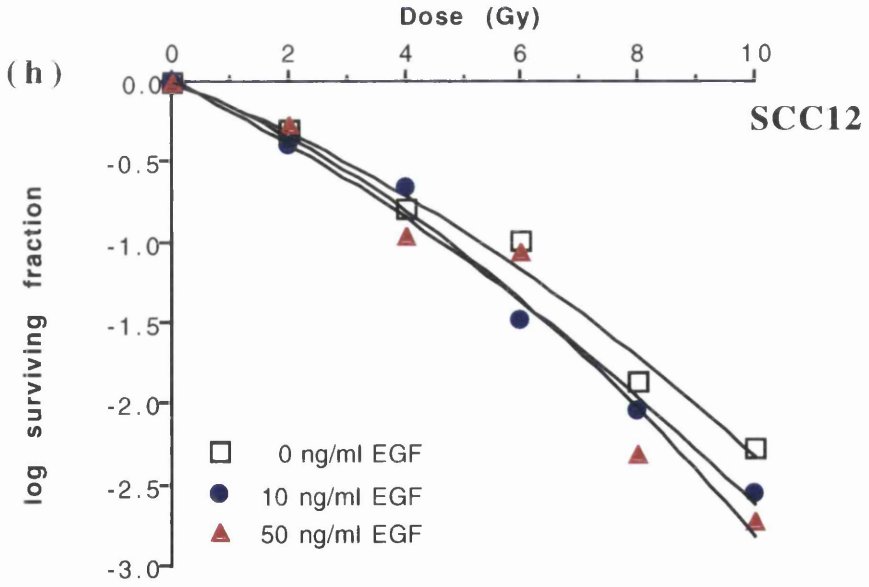
(e)


Dose (Gy)
0
2
4
6
8
10
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
log surviving fraction
HER 14
0 ng/ml EGF
10 ng/ml EGF
50 ng/ml EGF

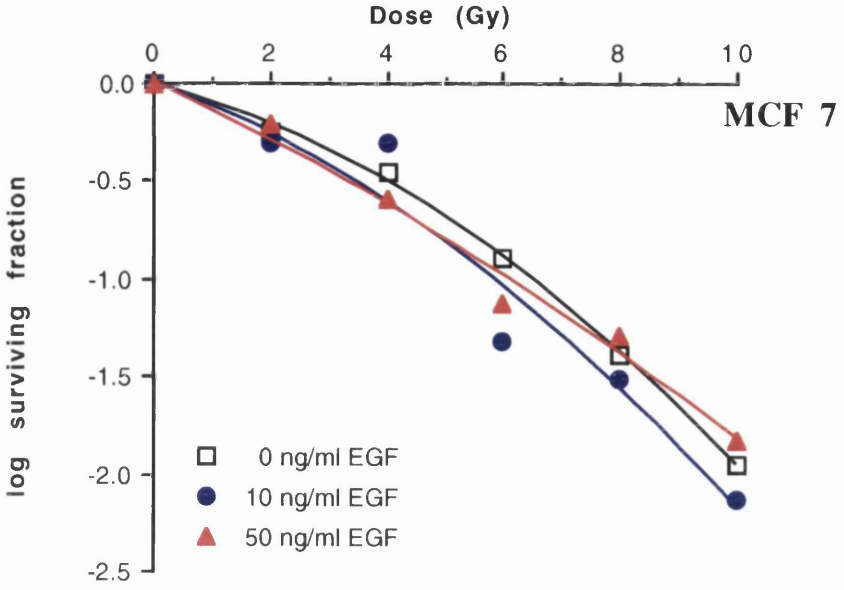
(f)


Dose (Gy)
0
2
4
6
8
10
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
log surviving fraction
K721A
0 ng/ml EGF
10 ng/ml EGF
50 ng/ml EGF

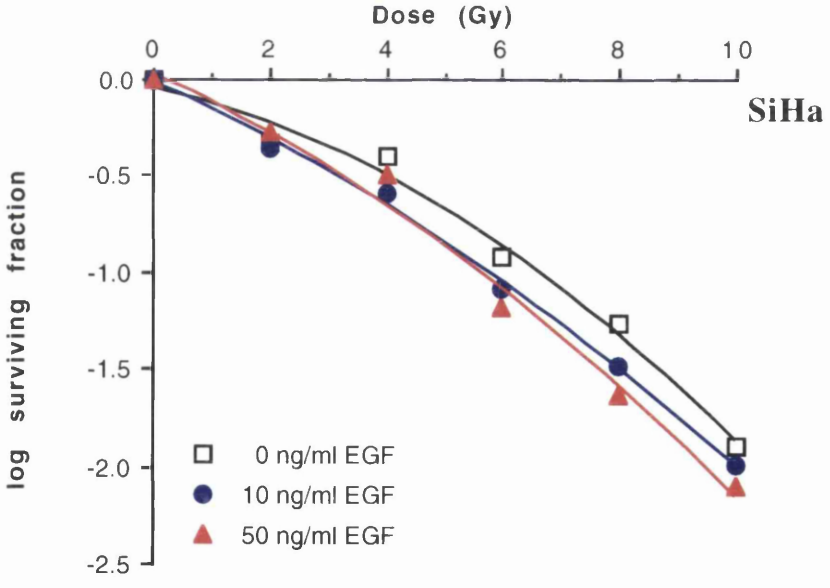

(g)
Dose (Gy)
0
2
4
6
8
10
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
log surviving fraction
MDA-MB 231
0 ng/ml EGF
10 ng/ml EGF
50 ng/ml EGF


(h)
Dose (Gy)
0
2
4
6
8
10
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
log surviving fraction
SCC12
0 ng/ml EGF
10 ng/ml EGF
50 ng/ml EGF

(i)


Dose (Gy)
0
2
4
6
8
10
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
log surviving fraction
MCF 7
0 ng/ml EGF
10 ng/ml EGF
50 ng/ml EGF

(j)


Dose (Gy)
0
2
4
6
8
10
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
log surviving fraction
SiHa
0 ng/ml EGF
10 ng/ml EGF
50 ng/ml EGF

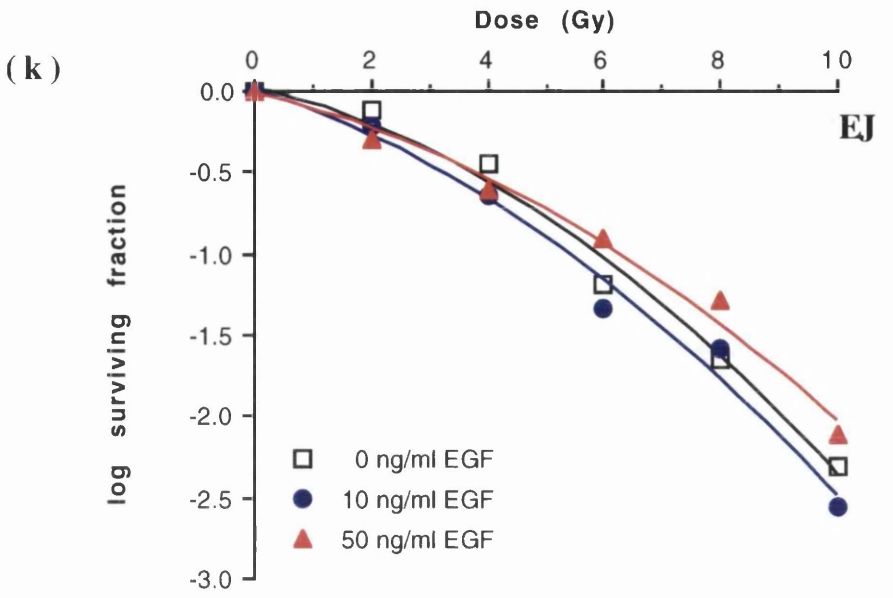



**Fig 7.3** Effect of EGF on the radiation dose/response of eleven cell lines. Curves were fitted to the data using the linear quadratic model of cell survival. Results are from three separate determinations in triplicate. Error bars removed for clarity.

| Cell line | EGF conc. (ng/ml) | α ($Gy^{-1}$) | β ($Gy^{-2}$) | SF2 | SF10 (x $10^{-3}$) |
|---|---|---|---|---|---|
| A431 | 0 | 0.238 +/- 0.037 | 0.0221 +/- 0.0044 | 0.569 +/- 0.144 | 10.15 +/- 2.81 |
| | 10 | 0.279 +/- 0.078 | 0.0231 +/- 0.0092 | 0.522 +/- 0.108 | 6.10 +/- 2.79 |
| | 50 | 0.333 +/- 0.041 | 0.0091 +/- 0.0048 | 0.495 +/- 0.079 | 14.41 +/- 3.62 |
| HN5 | 0 | 0.307 +/- 0.046 | 0.0169 +/- 0.0054 | 0.506 +/- 0.186 | 8.57 +/- 2.24 |
| | 1 | 0.163 +/- 0.095 | 0.0380 +/- 0.0113 | 0.620 +/- 0.118 | 4.38 +/- 2.95 |
| B2A4 | 0 | 0.226 +/- 0.061 | 0.0297 +/- 0.0072 | 0.565 +/- 0.234 | 5.35 +/- 1.34 |
| | 1 | 0.327 +/- 0.114 | 0.0272 +/- 0.0136 | 0.466 +/- 0.102 | 2.50 +/- 1.77 |
| | 3 | 0.425 +/- 0.081 | 0.0127 +/- 0.0096 | 0.406 +/- 0.098 | 4.01 +/- 1.17 |
| CaSki | 0 | 0.130 +/- 0.044 | 0.0411 +/- 0.0053 | 0.654 +/- 0.073 | 4.47 +/- 0.93 |
| | 1 | 0.245 +/- 0.056 | 0.0193 +/- 0.0067 | 0.567 +/- 0.149 | ¶ 12.52 +/- 4.84 |
| | 3 | 0.296 +/- 0.085 | 0.0174 +/- 0.0101 | 0.516 +/- 0.088 | ¶ 9.09 +/- 2.28 |
| HER 14 | 0 | 0.394 +/- 0.058 | 0.0208 +/- 0.0069 | 0.418 +/- 0.094 | 2.43 +/- 1.06 |
| | 10 | 0.345 +/- 0.031 | 0.0209 +/- 0.0036 | 0.461 +/- 0.133 | 3.92 +/- 0.49 |
| | 50 | 0.294 +/- 0.071 | 0.0432 +/- 0.0084 | 0.516 +/- 0.105 | 1.16 +/- 1.72 |
| K721A | 0 | 0.262 +/- 0.118 | 0.0381 +/- 0.0140 | 0.508 +/- 0.100 | 1.61 +/- 1.47 |
| | 10 | 0.364 +/- 0.054 | 0.0216 +/- 0.0064 | 0.443 +/- 0.048 | 3.03 +/- 0.85 |
| | 50 | 0.337 +/- 0.081 | 0.0346 +/- 0.0095 | 0.444 +/- 0.071 | 1.08 +/- 0.61 |
| MCF 7 | 0 | 0.177 +/- 0.019 | 0.0272 +/- 0.0023 | 0.629 +/- 0.128 | 11.22 +/- 5.19 |
| | 10 | 0.241 +/- 0.130 | 0.0258 +/- 0.0155 | 0.557 +/- 0.061 | 6.81 +/- 3.87 |
| | 50 | 0.310 +/- 0.061 | 0.0108 +/- 0.0073 | 0.515 +/- 0.139 | 15.30 +/- 6.22 |
| MDA 231 | 0 | 0.239 +/- 0.052 | 0.0172 +/- 0.0062 | 0.579 +/- 0.063 | 16.41 +/- 6.81 |
| | 10 | 0.172 +/- 0.052 | 0.0316 +/- 0.0061 | 0.625 +/- 0.054 | 7.60 +/- 3.44 |
| | 50 | 0.353 +/- 0.048 | 0.0048 +/- 0.0057 | 0.484 +/- 0.123 | 18.13 +/- 7.06 |
| SCC 12 | 0 | 0.321 +/- 0.081 | 0.0215 +/- 0.0096 | 0.483 +/- 0.086 | 4.70 +/- 1.21 |
| | 10 | 0.406 +/- 0.077 | 0.0198 +/- 0.0091 | 0.410 +/- 0.111 | 2.38 +/- 2.07 |
| | 50 | 0.331 +/- 0.147 | 0.0318 +/- 0.0173 | 0.454 +/- 0.058 | 1.52 +/- 1.97 |
| SiHa | 0 | 0.192 +/- 0.057 | 0.0237 +/- 0.0068 | 0.619 +/- 0.144 | 18.32 +/- 7.93 |
| | 10 | 0.316 +/- 0.028 | 0.0142 +/- 0.0033 | 0.502 +/- 0.158 | 10.25 +/- 4.28 |
| | 50 | 0.287 +/- 0.062 | 0.0210 +/- 0.0074 | 0.518 +/- 0.101 | 6.94 +/- 3.73 |
| EJ | 0 | 0.173 +/- 0.070 | 0.0370 +/- 0.0082 | 0.611 +/- 0.067 | 4.43 +/- 1.55 |
| | 10 | 0.253 +/- 0.088 | 0.0319 +/- 0.0103 | 0.531 +/- 0.122 | 3.28 +/- 0.89 |
| | 50 | 0.201 +/- 0.068 | 0.0265 +/- 0.0081 | 0.602 +/- 0.089 | 9.47 +/- 4.70 |

**Table 7.2** Radiobiological parameters obtained from clonogenic assay of cell lines. α and β values are means of three experiments, +/- S.D. SF2 and SF10 values were obtained from the model, and represent three separate determinations +/- S.E. ¶ denotes $p < 0.05$

## 7..4 Discussion

The experiments described were designed to establish the effect of exogenous EGF on the sensitivity of a panel of cell lines to ionising radiation. The effect of EGF on the growth and cloning efficiency of each cell line was established using standard techniques (Freshney, 1987). The effect of EGF on the radiation sensitivity of each cell line was then established by colony forming assay.

The colony forming assay was performed by seeding the viable cells in flasks prior to irradiation. The cells were then allowed to attach overnight, and an estimate of the average number of doublings made immediately prior to irradiation. Immediately after irradiation, EGF was added to the culture medium, and the cells were incubated until colonies formed. This method was used in preference to seeding the cells after irradiation, as it resulted in a higher cloning efficiency. The effect of the overnight incubation on the viable cell number was taken into account in the final calculation of the surviving fraction. This method did not result in errors greater than those observed when cells were seeded post-irradiation.The multiplicity value obtained using this method was typically small ( 2-5% +/- SEM) The SEM of triplicate determinations was typically 10% (Between 2-5 cells per 1000). The effect of this error on the final SF2 value was negligible, and was therefore not included in the results presented in table 7.2.

The data obtained from the colony forming assay was fitted using the linear quadratic model of cell survival. This model differs from the multi-targel model in that all of the parameters derived from this model are obtained directly from the equation of the curve, rather than by extrapolation. The n value in the multi-target model is obtained by extrapolation of the linear component of the line to the y-axis. As with any extrapolated value, small differences in observed data may be amplified erroneously by the extrapolation, leading to a misinterpretation of the data. The $\alpha$ and $\beta$ coefficients, SF2 and SF10 values derived from the linear quadratic model are much more stable, and thus less prone to misinterpretation.

The effect of EGF on the growth of the different cell lines was established (figures 7.1(a-k) and 7.2). The growth of three cell lines (HER14, MCF-7 and SiHa) was stimulated by EGF at 10 and 25 ng/ml. Five cell lines (A431, B2A4, HN5, CaSki and EJ) were inhibited by EGF at the same concentrations, while the growth of three cell lines (K721A, MDA-MB 231 and SCC12) was unaffected by EGF.

The effect of EGF on the cloning efficiency of the cell lines in the panel was also established. Seven cell lines (HER14, K721A, MCF-7, EJ, SiHa, MDA-MB 231 and SCC12) did not show a significant alteration in cloning efficiency in the presence of 10 ng/ml EGF (table 7.1.). Four cell lines (A431, B2A4, HN5 and CaSki) showed a reduction in cloning efficiency in the presence of EGF. Colony formation in three cell lines (B2A4, HN5 and CaSki) was completely abolished in the presence of EGF at 10 ng/ml. Colony formation by these cell lines was observed at 3, 1 and 3 ng/ml EGF respectively (table 7.1.). In each case, a significant reduction in cloning efficiency was observed when compared to untreated controls. A431 cells showed a significant decrease in cloning efficiency at 10 ng/ml EGF.

The effect of EGF on the radiosensitivity of the cell line panel was then determined. In the cell lines examined, there was no statistically significant radiosensitisation effect at any concentration of EGF (figure 7.3 and table 7.2.). We observed a significant increase in the SF10 value for the CaSki cell line at both 1 and 3 ng/ml EGF ($p < 0.05$). However, an increase was not apparent in the SF2 value, and the lack of alteration of either the $\alpha$ or $\beta$ coefficients suggests that the radiation response of CaSki is not significantly altered by EGF.

The data shown in figure 7.1 indicate the growth rates of the panel of cell lines examined in the presence of different concentrations of EGF. Our observation of a growth inhibitory effect of EGF on some tumour-derived squamous cell lines has also been documented by others (Gill and Lazar, 1981, Kamata et al, 1986, Hirari, 1988). The mechanisms underlying this inhibition are unclear. However, it has been suggested that an EGFR-mediated increase in the cyclin-dependent kinase inhibitor p21 may be responsible (Fan et al, 1995).

Our observation of the effect of EGF upon cell growth on the CaSki cell line (figure 7.1 (c)) is at variance with a previously published report (Kwok and Sutherland, 1991a), who reported that EGF has little or no inhibitory effect on this cell line. Interestingly, our observation of the growth of HN5 cells in the presence of EGF (figure 7.1.(a)) is similar to the results reported in the above publication. That is, after five days in medium containing 25 ng/ml EGF, the cell population underwent between one and two doublings. However, as shown in figure 7.1 (a), after an initial increase in cell number between 0 and 48 hours, the population size of HN5 cells remained constant, suggesting almost complete inhibition of growth. The possibility of this type of growth curve having been produced by the rate of increase in number of a putative cellular sub-population resistant to the effects of EGF being paralleled

by the rate of death of the normal cell population is discounted by the apparent failure of colony formation over a 14 day assay period. The data published by Kwok and Sutherland (1991a), however, does not allow this possibility to be discounted.

The present data indicates that EGF had no significant effect on the radiation sensitivity of the cell lines examined. There are several previously published reports of studies of EGF-mediated alteration in radiation sensitivity. One study involving human colon cancer cells reported radiosensitisation by TGF-α (Leith and Michelson, 1991). The cell line examined (HCT-8) had an EGFR expression level of 104 per cell, with maximal radiosensitisation observed at concentrations of TGF-α in excess of 200 ng/ml. This concentration is well in excess of physiological levels. Therefore, the effect observed is probably independent of the cell surface EGFR expression. Other investigators observed EGF-mediated radiosensitisation in two cell lines derived from squamous cell tumours of the head and neck (Bonner et al, 1994). This effect was only apparent when the cells were pre-incubated in EGF at 10 ng/ml for 24 hours prior to irradiation. Kwok and Sutherland (1991a) reported an EGF-mediated radiosensitisation effect, which was maximal at 10 ng/ml EGF, in all of the cell lines which they studied, but found that EGF administration either before or during irradiation had no effect upon the radiosensitivity of the cell. Only the post-irradiation administration of EGF resulted in alteration in radiosensitivity. The divergence of these reports is significant because they suggest that the radiosensitisation observed is effected by different mechanisms.

EGF has also been reported to have a radioprotective effect in some cell lines. Wollman and colleagues (1994) have reported that EGF reduces radiosensitivity in MCF-7 cells. Unfortunately, it is not possible to directly compare this observation with the data presented in this report, as the experiments were performed using low serum concentrations, and EGF was added prior to irradiation. Another study using prostate epithelial cells observed that growth factor deprivation after irradiation resulted in increased radiosensitivity (Howard et al, 1995). Replacement of growth factors, including EGF, abrogated this effect.

The present data is at variance with the results previously published by Kwok and Sutherland (1991a), who reported EGF-mediated radiosensitisation in three of the cell lines used in our study (A431, HN5 and CaSki). We were unable to observe similar responses in these cell lines, or any other from the panel of cell lines used in this study. There are several possible explanations for these differences:

(a) The cloning efficiencies of the cell lines examined in this study (table 7.1.) are significantly lower than those previously reported (Kwok and Sutherland, 1991a). This may be due to differences in age and passage number resulting in the evolution of altered growth characteristics. However, ligand binding analysis of the cell lines used in the present study (table 6.3.) indicated that the EGFR expression and binding affinities were similar to those of Kwok and Sutherland.

(b) A further methodological difference between this investigation and that of Kwok and Sutherland (1991a) is the absence of an irradiated feeder layer in the latter report. The authors stated, however, that cell seeding density had no effect on the radiosensitisation effect.

(c) There may be variations in the EGF concentration in the culture medium during the colony forming period. Cells were incubated in medium containing FCS or DCS (table 6.2) for the duration of the colony forming period. The concentration of serum-derived EGF, or any other members of the EGF family, is unknown and varies between serum batches. Also, no account was taken of the cellular uptake and degradation of the exogenous EGF, or the cellular production of EGF or TGF-α. Consequently, the EGFR ligand concentration is not constant throughout the colony forming period.

A more recent report has suggested a mechanism by which an observed EGF-mediated radiosensitisation of A431 cells may occur (Laderoute et al, 1994). This study indicated that EGF increased the length of radiation-induced cell cycle arrest in the G2-M phase. The authors hypothesised that the observed radiosensitisation effect was a result of an increase in apoptotic cell death as a consequence of arrest in the G2-M phase. The initiation of apoptosis in the G2-M phase, however, has only been observed in the presence of toxic concentrations of cytotoxic drugs, particularly cisplatin (Sorensen et al, 1990), and there is no evidence to suggest that the onset of apoptosis in this phase of the cell cycle is directly linked to the DNA damaging action of this drug. Indeed, there is experimental evidence which indirectly contradicts the above hypothesis. One study observed an inverse relationship between the length of radiation-induced G2-M delay and apoptosis in HeLa cells (Bernhard et al, 1996). Other investigators have demonstrated that an FGF-4-mediated enhancement of the radiation-induced G2 arrest in adrenal carcinoma cells resulted in increased cell survival (Jung et al, 1994). Both of these observations imply that an increase in the G2-M population does not correlate with an increase in apoptosis.

The published literature examining EGF-mediated alteration in cellular radiosensitivity contains many inconsistencies. The various effects of incubation of cells with EGF pre-and post-irradiation require clarification, and standardisation of the methodologies used would be of benefit. Another obstacle to resolving this issue is establishing cultures in media whose EGFR-ligand content is accurately quantified. This would greatly simplify comparison of results from different studies. The results presented in this report indicate that EGF-mediated increase in radiation sensitivity of squamous carcinoma cells is unlikely to be of benefit in a clinical context.

# CHAPTER 8

# ACCUMULATION OF EGF IN THE NUCLEI OF EGFR-EXPRESSING CELL LINES

## 8.1 Introduction

As outlined in section 2.2.1, one of the criteria used in the selection of an appropriate radionuclide for conjugation to a delivery vehicle is the sub-cellular distribution of the vehicle in the target cell. The experiments described in this and the subsequent chapters were designed to evaluate the suitability of EGF as a vehicle for the delivery of Auger-emitting radionuclides to EGFR-expresing cells. Due to the short range of Auger electrons, high LET-type DNA damage is only observed after radionuclide decay occurs in close proximity to cellular DNA. Therefore, the suitabililty of EGF as a delivery vehicle for Auger-emitting radionuclides is dependent upon the nuclear uptake of EGF by EGFR-expressing cells. This chapter is concerned with the detection and characterisation of the nuclear accumulation of EGF by EGFR-expressing cell lines.

### 8.1.1 *Aims of this study*

The experiments described in this chaper were designed to determine whether exogenous EGF accumulated in the nuclei of the EGFR-expressing cell line A431. Two microscopic techniques, not previously used for nuclear EGF detection, were employed. In addition, experiments were designed to establish the kinetics of nuclear accumulation in the same cell line. Experiments were also performed to evaluate nuclear EGF uptake in cell lines with various levels of EGFR expression.

## 8.2 Materials and methods

### 8.2.1 *Detection of nuclear EGF by laser scanning confocal microscopy*

Glass microscope coverslips (13mm diameter) were immersed in PBS containing 13.3 μg/ml poly-L-lysine for 30 mins at 37oC. Coverslips were then washed 3 times by immersion in PBS, and placed individually in the wells of 24 well tissue culture plates. 5x104 A431 cells were then added to the wells in a volume of 0.5 ml, and incubated overnight at 37oC. Medium was then removed and replaced with medium containing 50 ng/ml of an EGF-biotin conjugate (Boehringer Mannheim) and incubated at the required temperature for 24 hours. Coverslips with attached cells

were then removed from the wells, and washed 3 times by immersion in staining solution (DMEM containing 20mM HEPES, 20mM HEPES (Na+ salt), 0.05% (w/v) NaN3, pH 7.4). Cells were then fixed by immersion in paraformaldehyde solution (PBS containing 4%(w/v) paraformaldehyde, 1mM CaCl2, 0.5 mM MgCl2, pH 7.4) for 15 minutes at 20oC.

Fixed cells were washed three times by immersion in staining solution, and permeablised by immersion in PBS containing 0.1% (v/v) Tween 20 for 10 seconds, followed immediately by three washes in staining solution.

To detect the presence of EGF-biotin in the cell, 50 µl avidin-FITC conjugate (25µg/ml in staining solution) was applied to the coverslips, and incubated for 30 min in a sealed container at 20oC, followed by three washes in staining solution.

Coverslips were then placed, with cells down, in a drop of Vectashield mounting medium containing 0.3 µg/ml propidium iodide and sealed with clear nail varnish.

Slides were viewed on a Nikon Diaphot - TMD microscope attached to a Bio-Rad MRC 600 confocal laser scanner. The krypton/argon laser excited at 488 and 568 nm, with red and green fluorescent emissions collected at 588 and 552 nm respectively.

Non-specific binding of avidin-FITC was assessed by viewing cells treated with avidin-FITC only. Fluorescence observed using these control cells was subtracted from that observed in the treated cells.

### 8.2.2 *Detection of nuclear EGF using Electron Spectroscopic Imaging (ESI)*

(This work was carried out in collaboration with Dr Max Huxham, electron microscopy unit, University of Glasgow)

A431 cells were incubated with 50 ng/ml EGF-biotin in medium with or without serum for 24 hours at 37oC. Cells were washed with PBS and removed from the culture surface by trypsinisation. Cells were harvested in serum-free medium and pelleted by centrifugation. The cell pellets were chemically fixed in 4% (w/v) paraformaldehyde, 0.5% (v/v) glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) for 1 hour at 4oC. The pellets were then washed in buffer and dehydrated in an ethanol series using a progressive lowering of temperature technique (from 4oC to -35oC) and then finally embedded in Lowicryl HM20 methacrylate resin. The resin was polymerised at -35oC using UV light for 48 hours. Sections were cut to a thickness of 70-80 nm, and picked up on to 400 mesh gold grids and then immediately incubated

on droplets in a moist chamber with streptavidin-10nm gold diluted 1/80 (v/v) with PBS containing 0.1% (w/v) fatty-acid-free BSA for 36 hours at 4oC. The sections were then washed three times in PBS/BSA followed by a single wash in water prior to examination without further staining. To maintain control of the staining protocol, sections from the plus-serum and minus-serum cell pellets were incubated simultaneously.

Sections were examined at 20000 x magnification at 80 kV using a Zeiss TEM 902 energy filtering transmission electron microscope in order to enhance visualisation of both the gold probe and cellular material without the use of conventional heavy metal stains. This allowed both unequivocal identification of gold particles by ESI and the determination of local macromolecular mass by measurement of the relative elemental nitrogen content of defined nuclear and cytoplasmic areas included in the analysis using serial electron energy loss spectroscopy (SEELS) (Huxham et al, 1992). This approach allowed the assessment of the relationship between the number of gold particles and the relative density of the intracellular domain with which they were associated, rather than simply the cross-sectional area.

For ESI, cell sections were visualised at E=265 eV using a 15eV spectrometer exit slit and a focused beam. The number of gold particles per square micron of nuclear and cytoplasmic domains for each cell pellet was counted. To control for the non-specific association of streptavidin-gold, streptavidin-gold-only treated cells were also assessed for nuclear and cytoplasmic gold particles. The values obtained were then subtracted from the values obtained from the EGF-biotin-treated cells.

For SEELS analysis, an intermediate aperture was used to define pairs of nuclear and cytoplasmic domains of 1.5 μm2 (n=15 for each domain). Relative intensity ratios for nitrogen were calculated using in-house software.

### 8.2.3 *Radio-iodination of EGF*

Radio-iodination of murine epidermal growth factor (mEGF) was carried out using Iodo-beads (Pierce, Rockford, U.S.A.) at room temperature. Carrier-free Na125I (Amersham), diluted in 100mM phosphate buffer, pH 6.5, was allowed to react with one washed Iodo-bead for 5 minutes. mEGF, also diluted in 100mM phosphate buffer, was then added (approximately 1μg mEGF per 10 MBq of Na125I) and the reaction allowed to proceed for 12 minutes. The optimal reaction time was established by instant thin layer chromatography (ITLC). After completion of the reaction, 125I-EGF was separated from unincorporated 125I using a 5 ml pre-packed

disposable desalting column, equilibrated with PBS (Pierce). The specific activity of 125I-EGF was 3.4 - 4.5 MBq/μg.

### 8.2.4 *Isolation of intact cell nuclei*

Isolation of intact nuclei was performed using a modification of the method of Murawaki et al (1990), which is a modification of the method described by Rakowicz-Szulczynska (1986).

Cells were harvested by trypsinisation, washed in PBS, and the pellet suspended in 1ml of nuclear isolation buffer (0.35M sucrose, 1% (v/v) Triton X-100, 12mM β-mercaptoethanol, 1.5mM MgCl2, 10mM KCl, 10mM Tris-HCL, pH 7.6) and transferred to 1.5 ml Eppendorf tubes. The suspension was then centrifuged at 800 x g for 10 mins at 4oC.

The supernatant was discarded, and the pellet washed 5 times by centrifugation in nuclear isolation buffer. The nuclear pellet was then washed twice in PBS.

A sample was then analysed using phase-contrast light microscopy to confirm that the nuclear pellet was free from cell debris.

### 8.2.5 *Determination of membrane contamination of nuclear pellet (i)*

To determine the level of membrane contamination in an isolated nuclear pellet, 1x106 A431 cells were plated out in a 60mm Petri dish, and allowed to attach. Medium was then removed, and replaced with 3 ml fresh medium containing 3μCi 3H thymidine (S.A. 120 Ci/mmol, Amersham,), and cells incubated overnight at 37oC. Medium was then removed, the cells washed twice in PBS, and incubated in fresh medium for 4 hours at 37oC, to ensure complete incorporation of all intracellular 3H-thymidine. Medium was then replaced with 2ml medium containing 25 ng/ml 125I-EGF (section 8.2.3) and incubated at 4oC with gentle agitation for 4 hours. Nuclei were extracted as described above (section 8.2.4), but the entire procedure was carried out at 4oC. Twenty μl of the suspension was removed after each wash, counted for 125I content using a gamma counter (Canberra Packard) and 3H content using an LS 5000 LE liquid scintillation counter (Beckman).

Whilst 3H cannot be detected by a gamma counter, 125I can be detected by the 3H channel on the liquid scintillation counter. The relationship between 125I cpm obtained by gamma detection, and cpm obtained by liquid scintillation was linear

(figure 8.6). 3H counts were calculated by initial determination of 125I cpm by gamma detection, followed by liquid scintillation counting. The cpm obtained by liquid scintillation contained two components - 3H and 125I-derived counts. 125I-derived counts were calculated from the standard curve (figure 8.5), and subtracted from the total.

*Determination of membrane contamination of nuclear pellet (ii)*

The level of membrane contamination in an isolated nuclear preparation was also determined by assay of the activity of the plasma membrane-marker enzyme 5'-nucleotidase. The method is similar to that previously described (Newby et al, 1975).

1x106 A431 cells were cultured in 60mm Petri dishes, and allowed to attach. Cells were harvested by trypsinisation, and washed twice in PBS. Nuclei were then isolated as described above (section 8.4.2). Each sample was washed up to six times by centrifugation in nuclear isolation buffer (figure 8.7). 5'-nucleotidase activity was determined by measuring the release of 3H-adenosine from 3H-AMP. The nuclear pellets were washed twice in PBS and sonicated (as described in section 6.2.5) and then added to 500µl of assay buffer (100 µM AMP, 2-3 x105 c.p.m. of 3H-AMP (Amersham, S.A. 22 Ci/mmol), 50 mM Tris-HCl, pH 8.0.) A small aliquot of the homogenate was removed for protein determination.

Samples were then incubated at 37oC for 2 hr with agitation. The reaction was terminated by addition of 100µl 0.15M ZnSO4. AMP was then precipitated from the mixture by the addition of 100µl Ba(OH)2. The supernatant, containing adenosine only, was then added to 5ml liquid scintillant, and radioactivity was measured in an LS 5000 LE scintillation counter (Beckman).

8.3.6 *Determination of nuclear uptake of EGF*

To determine the nuclear uptake of EGF, 1x106 cells were cultured in 60mm Petri dishes, and allowed to attach overnight. Medium was then removed, and replaced with fresh medium containing 3µCi 3H-thymidine. The cells were then incubated overnight at 37oC. After incubation, the medium was removed, and the cells washed twice in PBS. Finally, cells were incubated in fresh medium for 4 hours to ensure complete incorporation of the remaining intracellular 3H-thymidine.

125I-EGF was then added at the required concentration and incubated for various lengths of time.

The number of nuclei in each assay was determined by incubating control flasks, identical to those above, with unlabelled EGF instead of 125I-EGF. After the incubation period, cells were trypsinised, and counted using a Coulter counter. The remaining cells were then assayed for 3H by liquid scintillation.

Intact nuclei were then extracted as described (section 8.4.2), and the nuclear pellets assayed for 125I and 3H. The 3H cpm were adjusted for 125I-specific counts as previously described (section 8.2.5).

## 8.3 RESULTS

### 8.3.1 *Detection of nuclear uptake of EGF by laser scanning confocal microscopy*

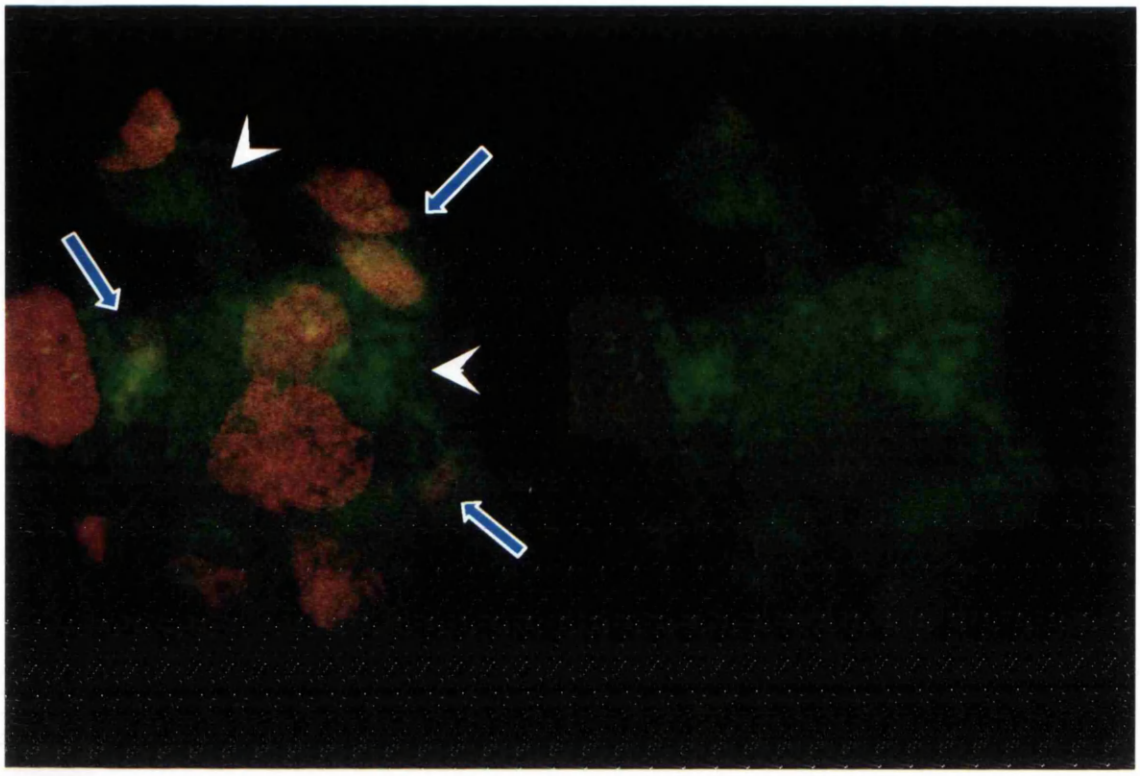
Figure 8.1 shows A431 cells which were incubated with 50 ng/ml EGF-biotin conjugate as described in section 8.2.1. The effects of serum deprivation and low incubation temperature on nuclear uptake of EGF-biotin were also examined (figures 8.2 and 8.3).

Figure 8.1 (a-c) show sequential optical sections of A431 cells. The optical sections (Z-steps) are 2 μm apart. The depth of field in each image was 1 μm. Green fluorescence indicates EGF-biotin-avidin-FITC, while red fluorescence indicated localisation of DNA-incorporated propidium iodide. Yellow fluorescence indicated co-localisation of both red and green fluorochromes. The optical sectioning technique allowed discrimination between cytoplasmic staining which overlies the nucleus, (equivalent to a false positive result), and genuine nuclear localisation. Genuine nuclear localisation was retained throughout the depth of the nucleus (indicated by blue arrows), whilst the cytoplasmic EGF-biotin distribution altered (arrowheads). Figure 8.1 demonstrates that exogenously added EGF could be detected in the nucleus of A431 cells after incubation under the conditions described. The majority of EGF-biotin localisation was intracellular, with little binding to the cell surface, indicating both receptor-ligand complex internalisation and cell surface receptor downregulation.

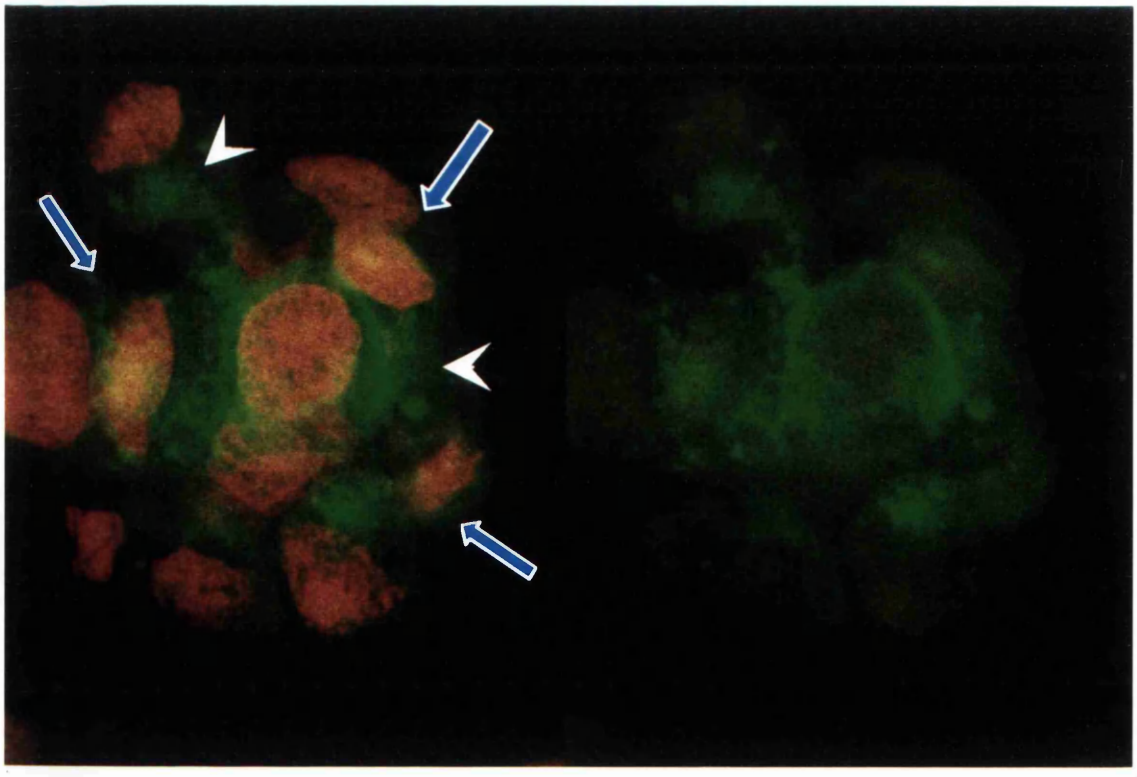
Figure 8.2 demonstrates the effect of incubation of A431 cells with 50 ng/ml EGF-biotin conjugate at 4oC for 24 hours. EGF binding to the cell surface was prominent, indicating an inhibition of receptor internalisation and downregulation. Less

**Fig 8.1** (a-c) Detection of EGF-biotin in the nuclei of A431 cells by LSCM

(a)



(b)



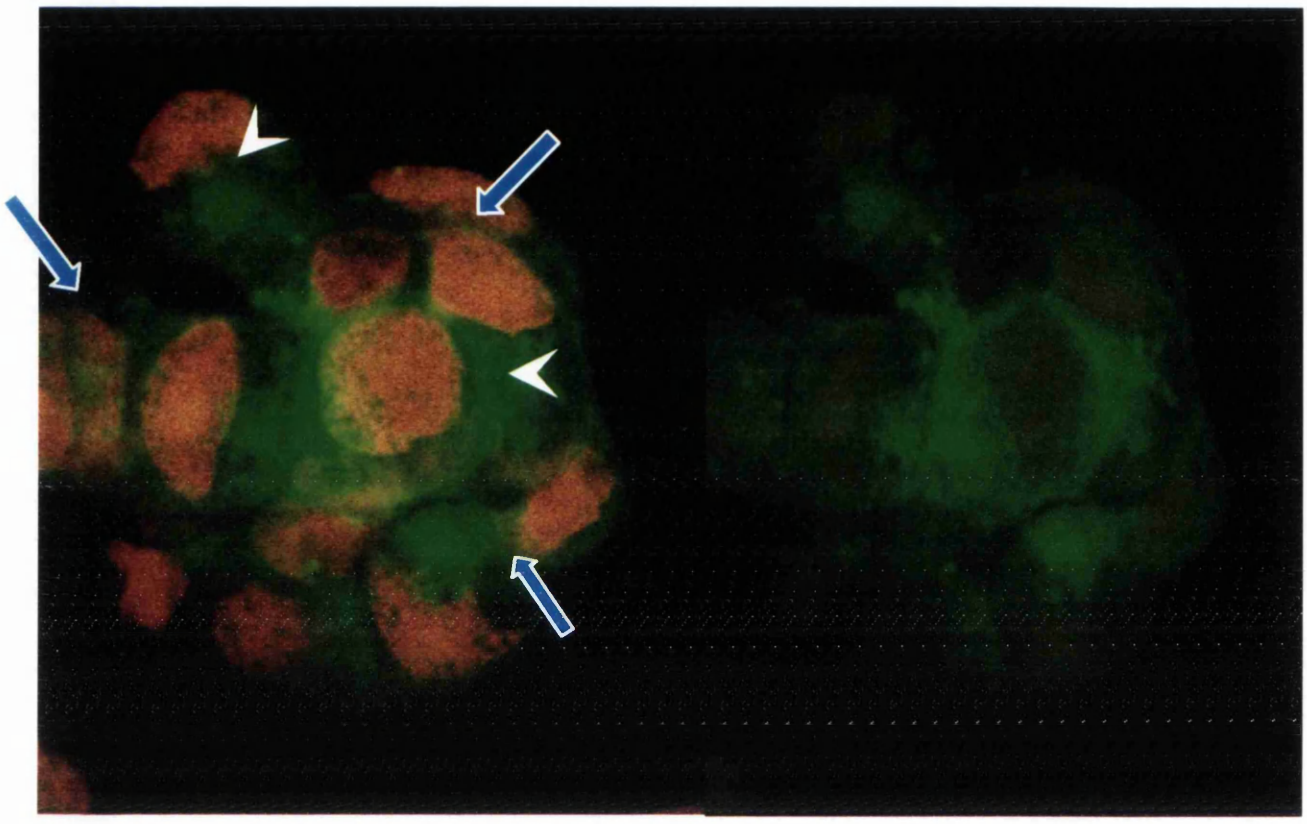


**Fig 8.1** (a-c) Detection of EGF-biotin in the nuclei of A431 cells. Cells were treated with EGF-biotin as described in section 8.2.1.. Images are sequential optical sections with a depth of field of 1μm. Each optical section is 2μm apart. Images on the left show nucleus (red fluorescence) and EGF-biotin (green fluorescence).Co-localisation of both fluorochromes appears as yellow. Images on the right indicate the distribution of EGF-biotin only (green flourescence). EGF-biotin is retained throughout the depth of the nucleus, as indicated by blue arrows .The distribution of non-nuclear EGF-biotin alters with each optical section (white arrowheads). Magnification x 600.

**Fig 8.2** Effect of low temperature on the nuclear uptake of EGF-biotin

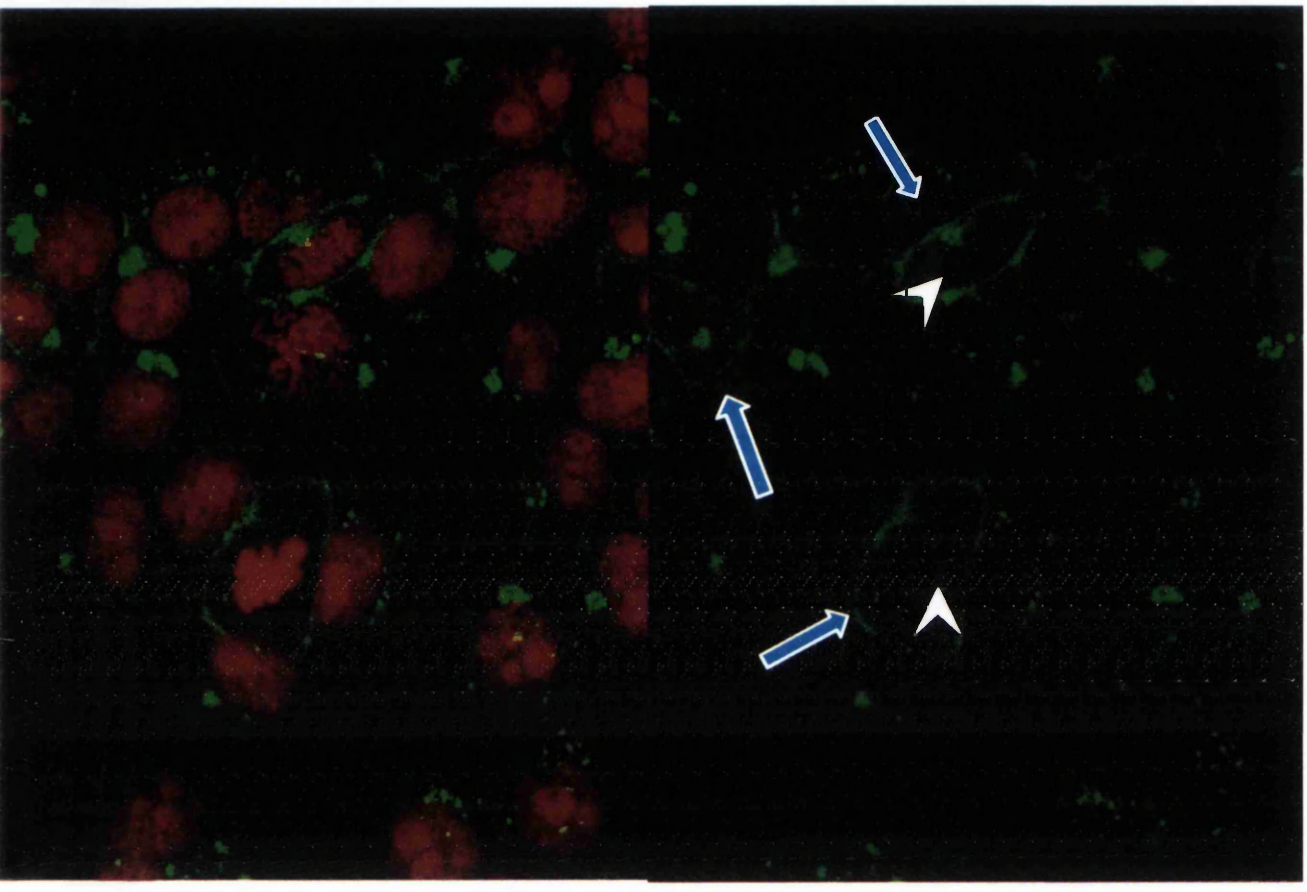


**Fig 8.2** Effect of low temperature on the nuclear uptake of EGF-biotin.. Cells were treated with EGF-biotin as described in section 8.2.1. Images are optical sections with a depth of field of 1μm.The image on the left shows nucleus (red fluorescence) and EGF-biotin (green fluorescence). The image on the right indicates the distribution of EGF-biotin only (green flourescence). EGF-biotin is found associated with the cell surface (blue arrows). No nuclear uptake of EGF-biotin can be observed (arrow heads). Magnification x 400.

**Fig 8.3** Effect of serum-deprivation on the nuclear uptake of EGF-biotin

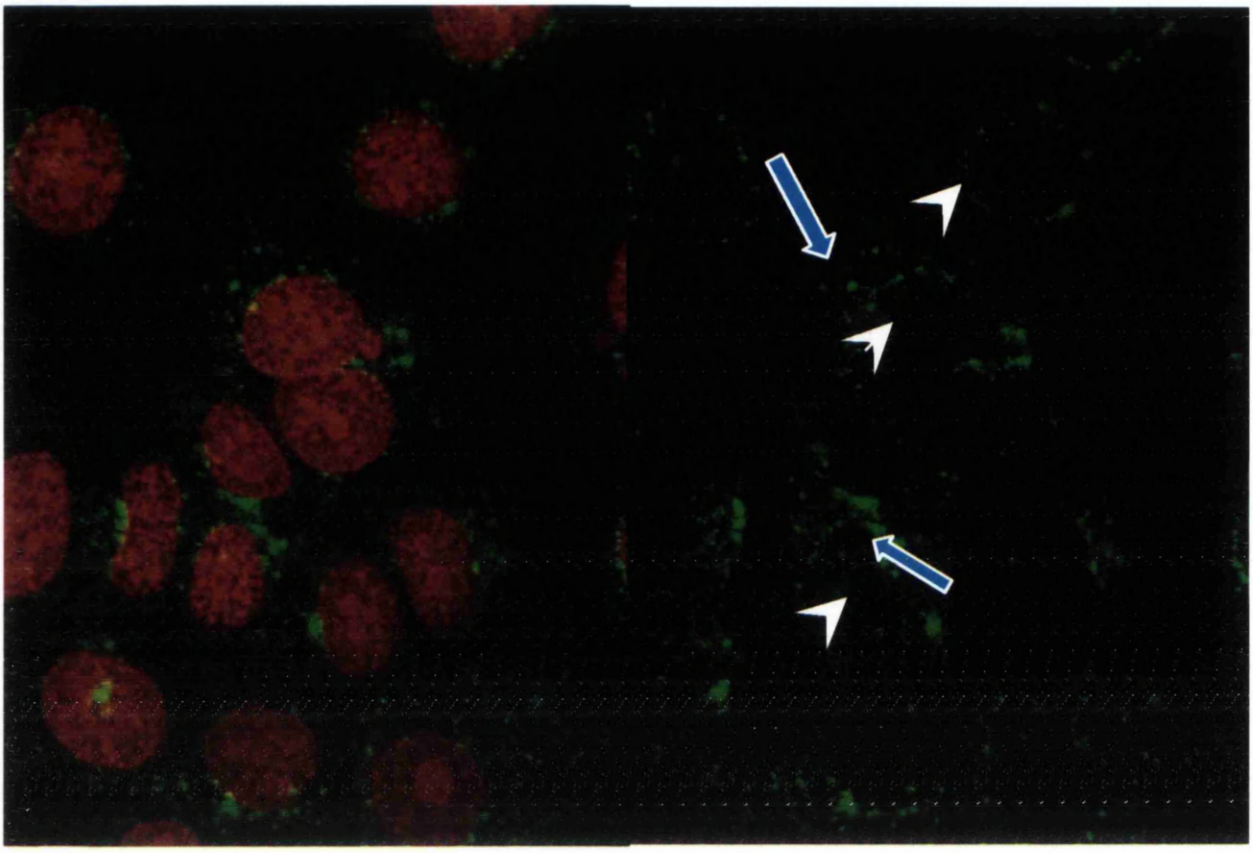


**Fig 8.3** Effect of seum-deprivation on the nuclear uptake of EGF-biotin. Cells were treated with EGF-biotin as described in section 8.2.1. Images are optical sections with a depth of field of 1μm. Images on the left show nucleus (red fluorescence) and EGF-biotin (green fluorescence).. The image on the right indicates the distribution of EGF-biotin only (green flourescence). EGF-biotin is observed in the perinuclear region (blue arrows). Very little EGF-biotin is associated with the cell membrane. EGF-biotin does not associate with the cell nucleus (white arrowheads) Magnification x 400.

intracellular EGF was observed, with an absence of localisation in the cell nucleus. The effect of serum starvation on nuclear accumulation is also demonstrated (figure 8.3). Serum-starved A431 cells were incubated in 50 ng/ml EGF-biotin for 24 hours at 37oC in serum-free medium. Cell surface staining was less intense than observed in cells incubated at 4oC, suggesting that receptor downregulation was not affected by serum-free conditions. Discreet regions of perinuclear staining (blue arrows) also indicated that the normal receptor internalisation mechanism was not impaired under conditions of serum-starvation. However, no localisation of EGF-biotin in the cell nucleus could be observed (arrowheads), indicating that the entry of EGF into the cell nucleus was serum-dependent.

### 8.3.2 *Detection of nuclear uptake of EGF by electron spectroscopic imaging (ESI)*

The relative distribution of EGF-biotin-streptavidin-gold complex between nucleus and cytoplasm was assessed using ESI in intact A431 cells incubated either with or without serum.

Figure 8.4 shows the average number of gold particles per square μm (n=30) in both nucleus and cytoplasm. Cytoplasmic EGF predominantly associated with vesicles. Regions of vesicle-associated EGF in the cytoplasm were excluded from the analysis, and the data presented represents 'free' cytoplasmic EGF.

Nuclei of cells incubated with serum contained a mean of 62.5 gold particles per μm2, while nuclei of cells incubated without serum contained a mean of 13.5 gold particles per μm2. This corresponds to a 4.3 fold increase in nuclear-associated EGF in the serum-containing sample over the serum-free sample.

SEELS analysis indicated a nuclear/cytoplasmic nitrogen intensity ratio of 2.75 (data not shown). This suggests that this concentration of EGF in the nucleus is not simply attributable to increased nuclear mass density.

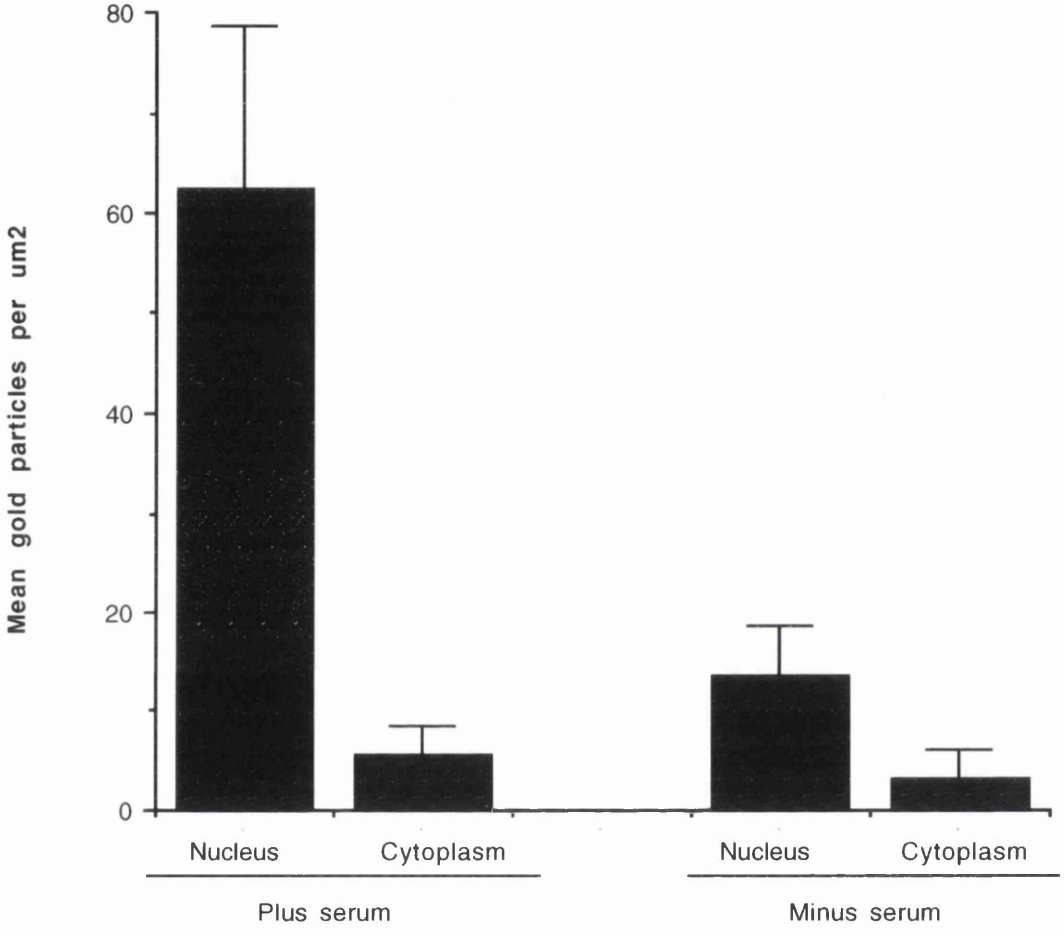



**Fig 8.4** ESI determination of nuclear and cytoplasmic EGF-biotin-streptavidin-gold in A431 cells. A431 cells were treated with 50 ng/ml EGF-biotin as described in section 8.3.2. 1.5 $mm^2$ areas of cytoplasm and nucleus were examined for the presence of EGF-biotin-streptavidin-gold. Columns represent means and standard deviations (n=30)

### 8.3.3 *Determination of the level of membrane contamination in isolated nuclear preparations*

Figure 8.5 shows the relationship between 125I-dependent counts detected on a gamma counter, and 125I-dependent counts detected on the 3H channel by liquid scintillation. This standard curve was used to calculate the number of 125I-dependent and 3H-dependent counts in dual-labelled (3H and 125I) isolated nuclei samples.

Figure 8.6 demonstrates the level of contamination of cell membrane in a nuclear pellet extracted as described in section 8.3.4. Performing the 125I-EGF labelling and nuclear extraction procedures at 4oC ensured that all 125I-EGF association with the cell was membrane-bound, as no internalisation of EGF occurs below 10oC (Carpenter and Cohen, 1976). 125I counts in the nuclear pellet represented membrane contamination. 3H-thymidine counts were used as a measure of the number of nuclei in each sample assayed, as the mean concentration of 3H-thymidine incorporation per nucleus was constant. Results are expressed as the proportion of 125I counts to 3H counts, as a percentage of control (no wash) levels. The results shown in figure 8.7 demonstrate that 125I contamination was 1.15% +/- 0.15% of the intact cell control after 5 washes in nuclear isolation buffer. This value represents the percentage of total cell membrane which remains associated with the nuclear pellet after 5 washes. Subsequent washes were unable to remove any further contamination.

Membrane contamination of isolated nuclear preparations was also determined by assay of the activity of the plasma membrane-marker enzyme 5'-nucleotidase (figure 8.7). Results represent the formation of 3H-adenosine from 3H-AMP, expressed as a percentage of the value obtained from the intact cell sample. These indicate that 5'-nucleotidase activity was reduced to 2.1% +/- 1.6% of the intact cell control control after 6 washes in nuclear isolation buffer. Subsequent washes were unable to remove any further contamination. It is important to note that both of these experiments were performed to assess the degree of contamination of the isolated nuclear pellet from cell membrane only. The results shown in figures 8.7 and 8.8 do not take account of the degree of contamination of the nuclear pellet from cytosolic, particulate components.

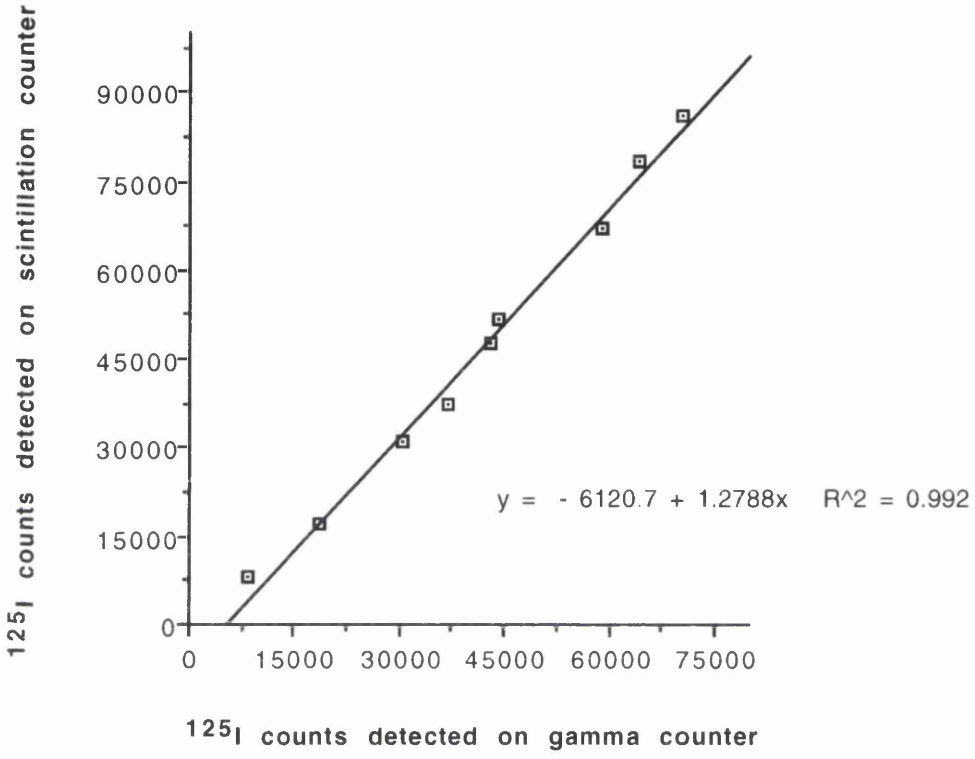



**Fig. 8.5** Standard curve indicating the relationship between $^{125}$I- counts obtained by gamma detection and liquid scintillation. Results shown are combined from two separate determinations.

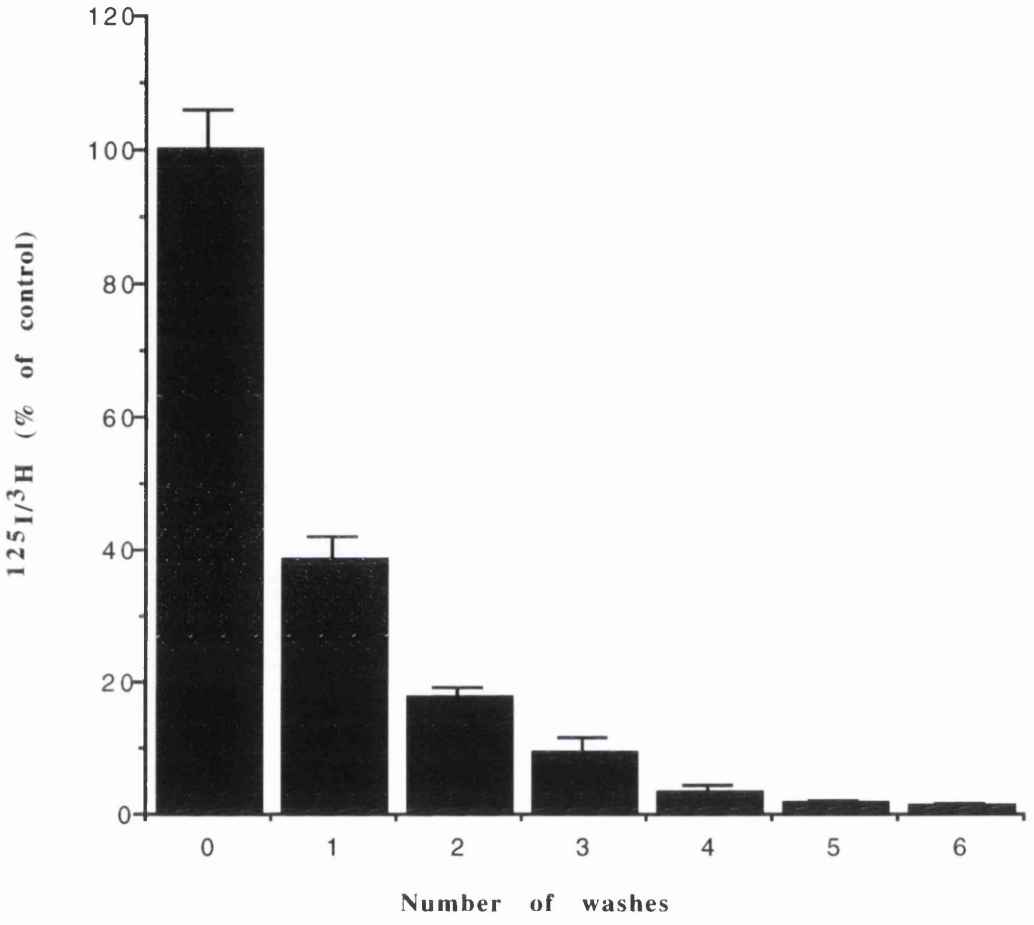



**Fig 8.6** Determination of the contamination of extracted nuclei by membrane-bound $^{125}$I-EGF. A431 cells incubated with $^{125}$I-EGF at 4$^{o}$C were washed successively in nuclear isolation buffer (section 8.3.4). Columns represent the amount of $^{125}$I-EGF, as a function of incorporated $^{3}$H-thymidine, detected after each wash. The results are expressed as a percentage of $^{125}$I-EGF contained in unwashed (intact cell) preparations. Columns represent means and standard deviations of triplicate determinations.

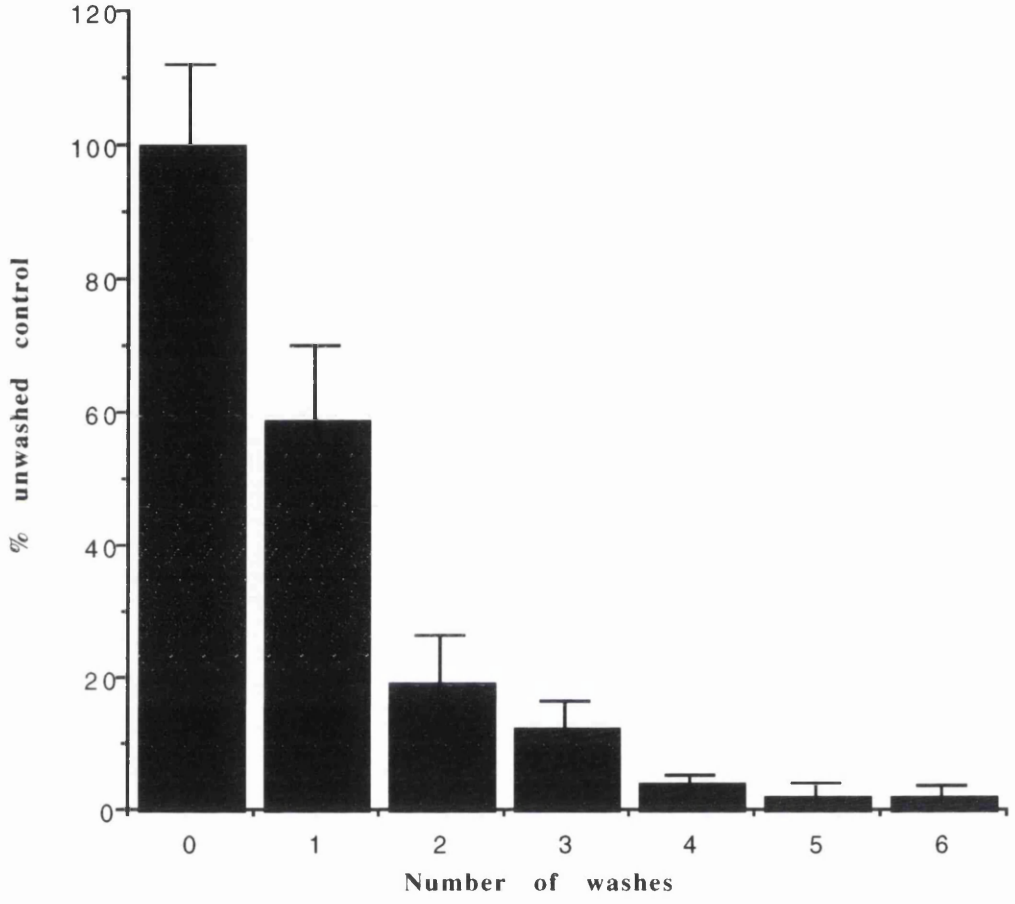



**Fig 8.7** Removal of 5'-nucleotidase activity from isolated nuclei. A431 cells were washed successively in nuclear isolation buffer (section 8.3.4). Contamination of nuclei by plasma membrane was assessed by assay of the membrane marker enzyme 5'-nucleotidase. Columns represent the relative 5'-nucleotidase activity detected after each wash. 25 µg of protein was assayed in each sample. Columns represent means and standard deviations of triplicate determinations.

*8.3.4 Assay of nuclear EGF uptake using isolated intact nuclei*

Having established that contamination of isolated nuclei by cell membrane was minimal, the nuclear uptake of 125I-EGF was assesed using nuclei isolated from 125I-EGF-treated A431 cells. The results obtained are presented in figure 8.8. This demonstrates the accumulation of EGF in the nuclei of A431 cells after incubation with 50 ng/ml 125I-EGF for 24 hours at 37oC (column 1). The accumulation was significantly reduced by lowering the incubation temperature to 4oC (column 2), or by pre-incubation of the cells for 24 hours in serum-free medium (column 4), indicating that nuclear uptake of EGF was both temperature- and serum-dependent. Incubation of nuclei extracted from untreated A431 cells with 50 ng/ml 125I-EGF (column 3) did not result in uptake. This excludes the possibility that 125I-EGF released by disruption of cytosolic EGF-accumulating structures (e.g endocytic vesicles) was taken up during the nuclear isolation procedure. In addition, the data shown in column 4 represents an important control. Serum starvation does not effect the normal internalisation and lysosomal targeting of the EGF/EGFR complex (Carpentier et al, 1980, also figure 8.3). The data shown in column 4 can therefore be interpreted as an indication of the amount of contamination obtained from cytosolic and cytoskeletal structures, as well as from cell membrane. The significant difference observed between serum-starved and serum-containing samples indicates that the observed nuclear 125I-EGF uptake in the serum-containing sample does not result from contamination of the isolated nuclear pellet by other cellular components.

The 125I-EGF used had a specific activity of 4 MBq/μg.

*8.3.5 Effect of EGF concentration and incubation time on nuclear EGF uptake.*

Figure 8.9 shows the effect of incubation time on the nuclear accumulation of exogenously added 125I-EGF. A431 cells were incubated in 50 ng/ml 125I-EGF, with or without a 100-fold excess (5 μg/ml) of unlabelled EGF, at 37oC for a range of incubation times. Statistical analysis was carried out using the paired Student's T-test. A statistically significant increase ($p < 0.05$) in nuclear accumulation of 125I-EGF was observed after 8 hours. Nuclear uptake of 125I-EGF had not reached saturation by 24 hours incubation: the longest incubation period examined. A non-

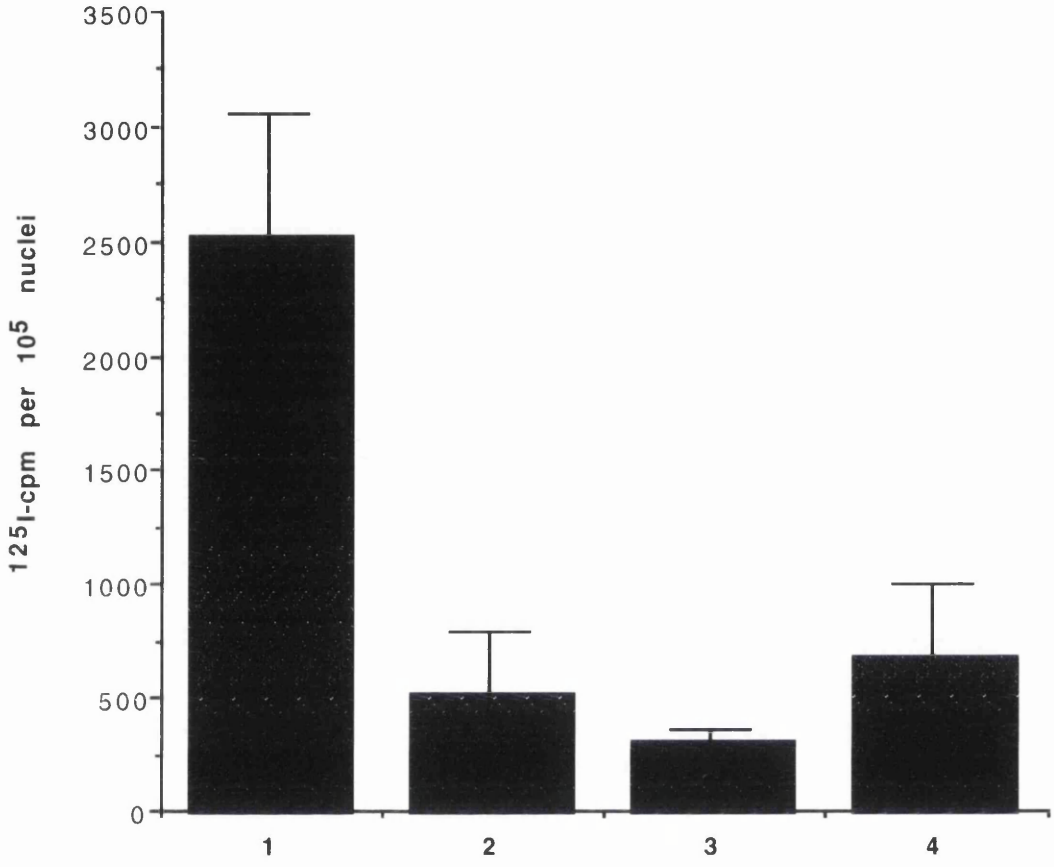



**Fig 8.8.** Effect of serum deprivation and low temperature on $^{125}$I-EGF uptake in A431 cell nuclei Intact nuclei isolated from A431 cells incubated with 50 ng/ml $^{125}$I-EGF for 24 hours at 37$^{o}$C (column 1) and 4$^{o}$C (column 2). Column 3 indicates nuclei isolated from untreated A431 cells, which were subsequently incubated for 4 hours with 50 ng/ml $^{125}$I-EGF to establish the uptake of EGF by isolated nuclei. The effect of serum starvation on nuclear EGF uptake is also demonstrated (column 4). A431 cells were grown in serum-free medium for 24 hours prior to incubation with 50 ng/ml $^{125}$I-EGF, also in serum-free conditions. Results are means of two triplicate determinations +/- S.E.M. $^{125}$I-EGF specific activity was 4 MBq/μg.

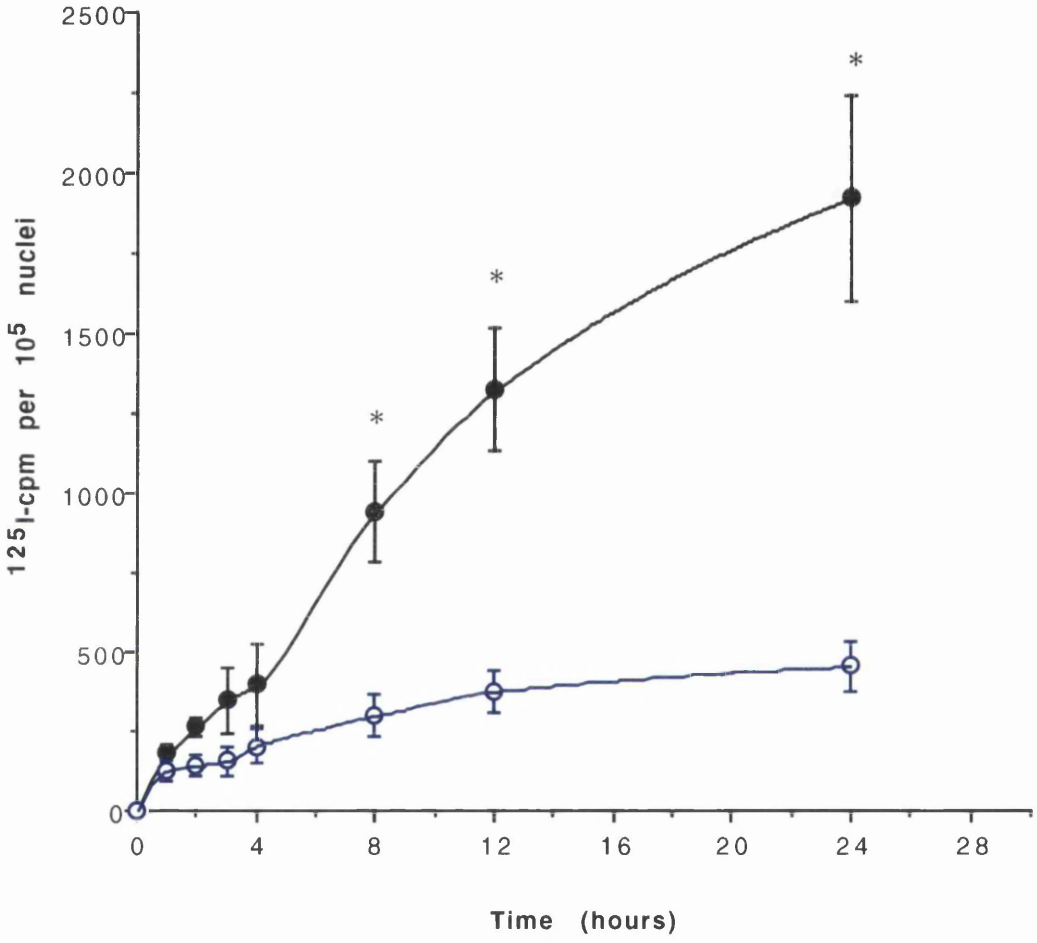



**Fig 8.9** Effect of incubation time on the nuclear uptake of $^{125}$I-EGF in A431 cells Cells were incubated as described in section 8.3.6 with 50 ng/ml $^{125}$I-EGF in the presence (open circles) or absence (full circles) of 5 µg/ml unlabelled EGF for the times indicated. Each point represents the mean of at least two triplicate determinations +/- S.E.M. * denotes $p < 0.05$.

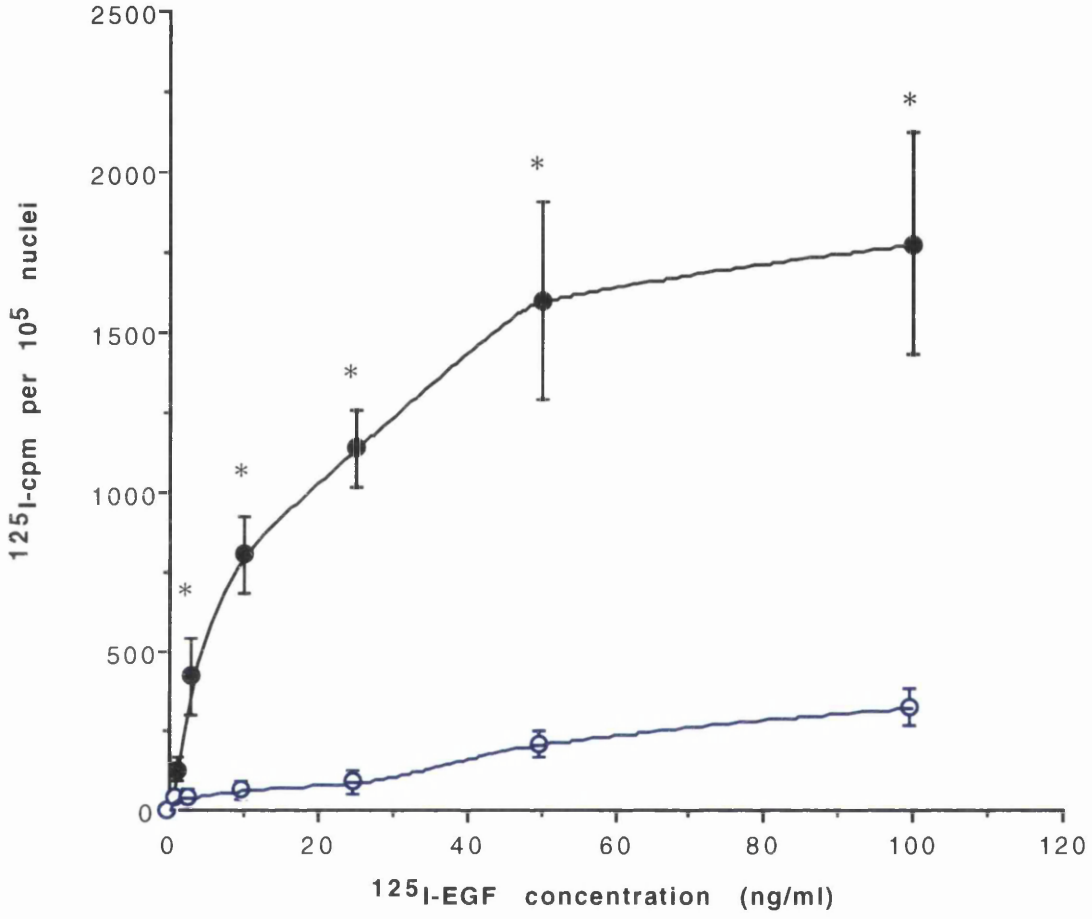



**Fig 8.10** Effect of $^{125}$I-EGF concentration on the nuclear uptake of $^{125}$I-EGF in A431 cells. Cells were incubated as described in section 3.3.4. with the $^{125}$I-EGF concentration indicated in the presence (open circles) or absence (full circles) of a 100-fold excess of unlabelled EGF for 24 hours. Each point represents the mean of at least two triplicate determinations +/- S.E.M. * denotes $p < 0.05$.

statistically significant increase in nuclear accumulation of 125I-EGF was observed between 0-4 hours incubation.

The effect on nuclear accumulation of increasing extracellular 125I-EGF concentration is shown in figure 8.10. A431 cells were incubated at the indicated 125I-EGF concentrations, with or without a 100-fold excess of unlabelled EGF, for 24 hours at 37oC. Statistical analysis was carried out using the paired Student's T-test. A statistically significant increase ($p < 0.05$) in nuclear 125I-EGF was observed at 5 ng/ml. Nuclear 125I-EGF uptake was maximal at 50 ng/ml over the 24 hour incubation period.

Having established the 125I-EGF concentration and incubation time which results in maximal nuclear 125I-EGF uptake, experiments were performed to establish the degree of nuclear 125I-EGF uptake in different EGFR-expressing cell lines under these conditions. The results obtained are presented in figure 8.11. This demonstrates the nuclear uptake of 125I-EGF by four EGFR-expressing cell lines. The cell lines (A431, HN5, B2A4 and CaSki) were incubated with 50 ng/ml 125I-EGF for 24 hours at either 4oC or 37oC. Intact nuclei were extracted and assayed for 125I and 3H uptake as described in sections 8.3.4 and 8.3.5.

All four cell lines showed a statistically significant increase in nuclear 125I-EGF uptake when incubated at 37oC, compared to 4oC. The increases in each cell line were : A431 - 5.7 fold, B2A4 - 5.6 fold, HN5 - 6.8 fold and CaSki - 4.5 fold.

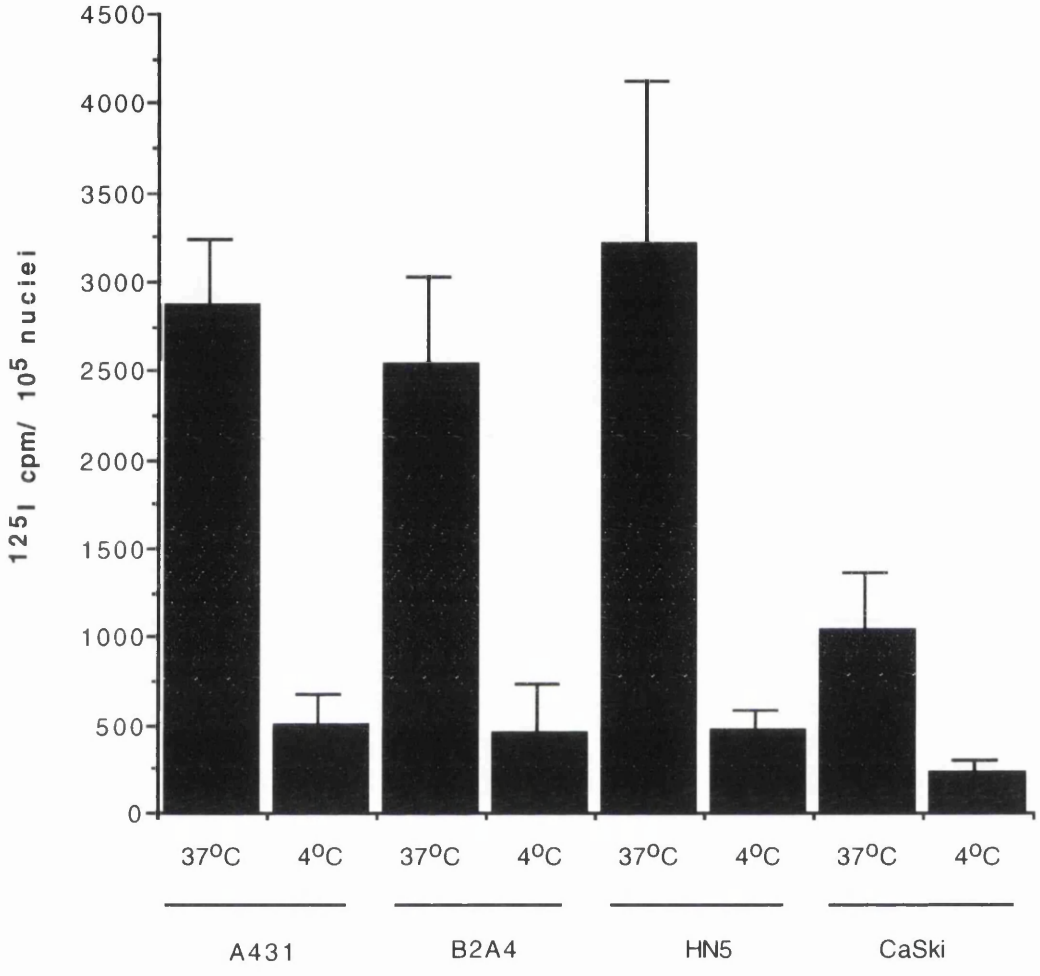



**Fig. 8.11** Nuclear accumulation of $^{125}$I-EGF in a panel of squamous carcinoma cell lines. Four squamous carcinoma cell lines were incubated with 50 ng/ml $^{125}$I-EGF for 24 hours at either 4°C or 37°C as described in section 8.3.6 Each column represents the means of at least two separate triplicate determinations +/- S.E.M. In each instance, a statistically significant increase was observed when cells were incubated at 37°C, compared to 4°C ($p < 0.05$).

## 8.4 Discussion

### 8.4.1 *Microscopic examination of intact cells*

The microscopic examinations described in this chapter were designed to investigate whether exogenously applied EGF, conjugated to biotin, accumulated in the nucleus of EGFR-expressing cell lines. Using several different techniques, we were able to detect the presence of label in the nucleus of cell lines with a high EGFR expression. Also presented is preliminary characterisation of the conditions required for this nuclear accumulation, as well as a comparison of nuclear uptake in a panel of squamous carcinoma cell lines. It is important to note that no experiment was performed to establish the exact nature of the species which eventually accumulates in the nucleus, and the data presented cannot rule out the possibility that some form of intracellular metabolism of labelled EGF has occured before nuclear uptake. This possibility is discussed later in this chapter. However, for the purposes of this report, the term "nuclear EGF" describes the species derived from biotin-labelled or $^{125}$I-labelled EGF which was detected in the cell nucleus.

Accumulation of nuclear EGF was assessed using two separate microscopic techniques; laser scanning confocal microscopy (LSCM) and electron spectroscopic imaging (ESI). These had not previously been used to examine EGF nuclear localisation. There are reports of nuclear localisation of EGF and EGFR using conventional fluorescence and electron microscope techniques (Jiang and Schindler, 1990, Holt et al, 1994 and 1995), but the reliability of these observations has been questioned (Hopkins, 1994).

Using optical sectioning on LSCM, it was possible to discriminate between genuine EGF nuclear localisation and cytoplasmic EGF which overlay the nucleus. This was not possible using conventional fluorescence microscopy. LSCM also allows visualisation of smaller quantities of fluorochrome than conventional fluorescence techniques.

ESI allows identification of labelled EGF, and some discrimination of cellular structures without the need for the heavy metal stains used in conventional electron microscopy, again reducing the possibility of artifacts. ESI also allowed rapid and accurate screening of designated intracellular regions for the presence of gold label.

The results obtained from LSCM indicated the presence of nuclear EGF in A431 cells after incubation with 50 ng/ml EGF-biotin for 24 hours at 37oC in the presence

of serum. Removal of serum or reduction of incubation temperature from 37oC to 4oC markedly reduced nuclear localisation (figures 8.1 , 8.2 and 8.3).

This technique, whilst producing clear images, can only be used to produce data of a qualitative nature. Quantification of nuclear EGF using LSCM could be open to misinterpretation, and has not been attempted.

Results obtained using ESI can be semi-quantitiative. While absolute quantitation is not possible, it is possible to compare the relative numbers of particles detected in different cellular compartments. Comparison of 1.5 μm2 regions of nucleus and non-vesicle containing cytoplasm was carried out. A431 cells treated in the presence of serum showed a 4.3-fold increase in nuclear gold particles when compared to the nuclei of cells treated in serum-free medium (figure 8.4). The amount of non-vesicular EGF detected in the cytoplasm was small. Similar results were obtained using 125I-EGF isolated nuclei, where a 5.7 fold increase in nuclear EGF was observed following treatment under identical conditions (figure 8.11.)

One drawback using this technique is the poor quality of photographic images obtained. The low intensity of heavy metal staining results in images of low resolution. Therefore, in terms of resolution of intracellular structure, ESI images are inferior to classical EM immunogold representations. However, the accurate and unequivocal detection of gold particles, combined with the semi-quantitative nature of the data obtained results in a useful novel technique for the detection of nuclear EGF and other polypeptide growth factors.

An important consideration when using microscopic techniques such as those employed in this study is the low absolute quantity of label detected in the nucleus, compared to the total cellular uptake of labelled EGF. Whilst quantitation of the total cellular uptake of EGF-biotin has not been attempted in this study, previous reports have indicated that nuclear EGF constitutes approximately 1% of the total cell-associated EGF, after incubation with EGF for 24 hours in the the absence of chloroquine (Johnson et al, 1980). Such low quantities of nuclear EGF allow the possibility that the observed nuclear accumulation is simply due to a leakage of a degradation product from the lysosome, or an artifact of the cell fixation procedure, rather than a specific uptake of nuclear EGF. These qualifications have previously been applied to microscopic examinations of nuclear EGF accumulation (Hopkin, 1994).

We have attempted to address this question by the use of control populations of serum-starved cells. Serum-starvation does not prevent EGF/EGFR complex internalisation, or subsequent lysosomal targeting (Carpentier et al, 1980). The total

cellular EGF uptake in both serum-treated and serum-starved populations should therefore be equivalent. However, the effects of serum-starvation on the intracellular metabolism of EGF has not yet been fully established, and the possibility remains that metabolism of EGF is altered under different serum conditions, giving rise to particular degradation products observed only in the serum-containing cell population. For these reasons, it is not possible to conclude that the observed nuclear accumulation is that of intact EGF. Possible resolutions of this problem are discussed later in this chapter.

### *8.4.2 Evaluation of intact isolated nuclei as a model system for the study of nuclear EGF uptake*

The use of isolated intact nuclei to study the nuclear uptake of peptides is prone to artifacts. A standard method of obtaining isolated nuclei involves washing whole cells in a detergent-containing buffer. This removes the plasma membrane, and releases the cytosolic contents, including detergent-insoluble intact nuclei.

Of fundamental importance is the level of contamination of the nuclei with plasma membrane and other cytoskeletal structures, which may bind the peptide of interest. This can be determined using a variety of methods, including microscopic morphological assessment and the assay of membrane-bound marker enzymes.

In this study, two methods of membrane contamination assessment were used. 5'-nucleotidase activity is a commonly used marker of plasma membrane contamination in isolated nuclear samples (Burwen and Jones, 1987). 5'-nucleotidase activity (assayed by the formation of 3H-adenosine from the substrate 3H-AMP) is only present on the cell membrane, and can be used as a marker of membrane contamination (Newby ey al, 1975). After 6 washes in detergent-containing buffer, 5'-nucleotidase activity was reduced by 97.9% of that observed in intact cells (figure 8.7), indicating minimal plasma membrane contamination of the isolated nuclear cell pellet.

The second method utilised to determine membrane contamination was designed to follow closely the method used to determine specific EGF nuclear localisation. Incubation of intact A431 cells at 4oC with 125I-EGF results in binding of 125I-EGF only to the cell surface, as internalisation of EGF is inhibited at this temperature (Carpenter and Cohen, 1976). 125I counts are therefore an indication of membrane contamination in the subsequently isolated nuclear pellet. After 6 washes in detergent-containing buffer, membrane-bound 125I-EGF counts were reduced by

98.9% of that observed in intact cells (figure 8.6), indicating minimal plasma membrane contamination of the isolated nuclear cell pellet.

It is more difficult to assess the degree of contamination from cytoplasmic components in the isolated nuclear pellet. Many studies have employed morphological assessment, using both light and electron microscopy, to demonstrate that cytoplasmic contamination is minimal (Jiang and Schindler, 1990, Raper et al, 1987). In addition, assay of lysosome-specific enzymes, such as acid phosphatase, have been used to determine the degree of lysosomal contamination of the nuclear pellet (Johnson et al, 1980, Holt et al, 1995). However, the observation that EGFR is able to bind to actin filaments at the cell membrane after ligand-binding demonstrates that the lysosome may not be the only potential source of cytoplasmic contamination (Rijken et al, 1995, den Hartigh et al, 1997).

The observation that serum-starvation inhibits the nuclear translocation of EGF and EGFR allows a more reliable control for cytoplasmic contamination (Holt et al, 1996). Serum-starvation does not prevent EGF/EGFR complex internalisation, or subsequent lysosomal targeting (Carpentier et al, 1980). Taken together, these observations indicate that nuclei isolated from serum-starved, 125I-EGF-treated cells controls for the amount of contamination obtained from cytosolic 125I-EGF-binding components. The data presented in figure 8.8 indicate that nuclei isolated from 125I-EGF-treated, serum-starved cells contain significantly lower amounts of 125I than nuclei isolated from serum-containing cells. This demonstrates that the observed nuclear uptake of 125I-EGF is not due to contamination from cytosolic components

The determination of the number of nuclei in a given sample can also present problems. Because nuclei have a tendency to aggregate, quantitation using a Coulter counter or haemocytometer is unreliable. Determination of DNA content using a fluorescent stain such as Hoesht 33258 (*bis* benzimide trihydrochloride) is commonly used as a method of quantifying the nuclear pellet. In this study, it was observed that a simple 3H-thymidine pulse- labelling procedure provided consistent results, and allowed quantitiation of nuclei to be performed in preparations of both intact cells and isolated nuclei.

### *8.4.3 Accumulation of nuclear 125I-EGF in A431 cells*

Intact A431 cells were treated with 125I-EGF under various conditions, and the nuclear uptake determined by isolation of the nuclei followed by assaying for 125I and 3H uptake. For each experimental treatment, a separate 3H-thymidine-only

control experiment was performed, as alterations in temperature, serum concentration and EGF concentration can influence 3H-thymidine incorporation.

Nuclear accumulation of 125I was observed after incubation of A431 cells with 50 ng/ml 125I-EGF for 24 h. This was abolished when cells were treated at 4oC, or in serum-free conditions (figure 8.8). Nuclei isolated from untreated cells and incubated in 125I-EGF failed to take up significant amounts of the labelled ligand, indicating that the observed nuclear accumulation occured prior to cell disruption, and was not an artifact of the isolation procedure.

### *8.4.4 Effect of ligand concentration and incubation time on nuclear uptake of 125I-EGF*

Ligand concentration- and time-dependent increases in nuclear EGF accumulation were observed (figures 8.9 and 8.10). Statistically significant ($p < 0.05$) quantities of nuclear 125I were observed after exposure of cells to 5 ng/ml 125I-EGF for 24 hours, with maximal uptake observed at 50 ng/ml 125I-EGF over the same incubation period. Incubation of cells with a higher concentration of 125I-EGF did not increase specific nuclear uptake, suggesting that the uptake mechanism is saturable.

Using a single 125I-EGF concentration (50 ng/ml) for varying incubation periods, statistically significant nuclear uptake was observed after 8 h incubation at 37oC ($p < 0.05$). Nuclear uptake did not appear to be maximal after 24 h, suggesting that a longer incubation period may result in higher nuclear 125I uptake.

### *8.4.5 Comparison of nuclear 125I-EGF uptake in a cell line panel*

Nuclear 125I-EGF accumulation was assesed in a panel of EGFR-expressing cell lines (Table 6.1). Cells were incubated in 50 ng/ml 125I-EGF for 24 h at 37oC. Significant nuclear 125I-EGF accumulation was observed in four cell lines (A431, HN5, B2A4 and CaSki). Nuclear uptake in these cells was 4.5 - 6.8 fold higher than control cells incubated at 4oC (figure 8.11). No significant nuclear 125I-EGF uptake was observed in the remaining cell lines.

### 8.4.6 *General discussion*

Several attempts have been made to establish the nuclear localisation of EGF and EGFR after receptor-mediated internalisation. The earliest studies (Johnson et al,

1980, Savion et al, 1981) used cells which were treated in the presence of chloroquine, which prevents endosomal acidification by inhibition of the membrane-bound Na+/H+ exchange pump (Carpenter and Cohen, 1976). This results in inhibition of endosomal proteases, and also prevents the pH-dependent uncoupling of the ligand-receptor complex. The overall effect observed is a large increase in intracellular EGF concentration, and consequently a large increase in nuclear localised EGF. The reason for the use of chloroquine to study nuclear EGF localisation was that significant amounts of EGF could not be detected in the cell nuclei without treatment (Johnson et al, 1980).

Our data was obtained using cell lines with a very high EGFR expression (table 6.3) which allowed assessment of nuclear localisation without the use of chloroquine or other protease inhibitors.

Several observations made in the present studies are supported by the results of previously published work. Firstly, nuclear localisation of EGF appears to dependent on internalisation, presumably along with the EGFR. This is indicated by the lack of nuclear EGF uptake when the incubation was carried out at 4oC. Receptor-mediated internalisation of EGF is inhibited at this temperature, compared to 37oC. Other investigators have reported an inhibition of nuclear EGF accumulation (Johnson et al, 1980, Rakowicz-Szulczynska et al, 1989), and of nuclear accumulation of EGFR (Murthy et al, 1986, Holt et al, 1994) at 4oC.

We have also demonstrated that serum is required to facilitate nuclear EGF accumulation (figures 8.3, 8.4 and 8.8). While there is no published evidence which directly supports this, indirect confirmation is provided by the observation of serum-dependent nuclear localisation of EGFR in an EGFR-transfected fibroblast cell line (Holt et al, 1995). Two separate reports examining intracellular EGFR distribution failed to observe nuclear EGFR translocation (Carpentier et al, 1987, Miller et al, 1986). However, both of these investigations were performed in serum-free conditions.

The incubation of untreated, detergent-extracted, intact nuclei with 125I-EGF does not result in binding or nuclear uptake (figure 8.8). Similar findings in nuclei isolated from other cell line cultures have also been reported (Johnson et al, 1980, Savion et al, 1981).

The effects of incubation time and external EGF concentration on the maximal uptake and rate of uptake of EGF vary widely amongst published reports. This may be in part due to the different cell lines used in the different studies, and also partly

due to the use in some studies of chloroquine and other lysosomal protease inhibitors. This makes direct comparison of the data presented here difficult.

Two reports of EGF nuclear localisation describe a lag-phase in the nuclear uptake of EGF (Johnson et al, 1980, Savion et al, 1981). No significant nuclear uptake of EGF was observed bt these workers for the first 6-12 hours of incubation. Nuclear EGF concentration then rose, and a plateau is observed at 24-30 hours. Our results confirm this finding. Figure 8.9 indicates a rise in nuclear EGF concentration between 4-8 hours, and sub-maximal accumulation after 24 hours. Nuclei assayed 0-4 hours after exposure of cells to 125I-EGF showed a small, non-statistical increase in EGF concentration. In contrast to these findings, a study using a fibroblast cell line observed half-maximal nuclear accumulation of EGF between 2-5 hours incubation (Jiang and Schindler, 1990). However, the authors state that nuclear EGF (in the form 125I-EGF) binding was low, making this observation difficult to assess.

There is only one report of the effect of external EGF concentration on the nuclear accumulation of EGF (Savion et al, 1981). Using corneal endothelial cells, the authors observed maximal nuclear uptake of EGF using 20 ng/ml EGF for an incubation period of 30 hours. In our study, maximal nuclear uptake of EGF in A431 cells was observed at 50 ng/ml EGF for a 24 hour incubation period (figure 8.10). It is difficult to draw a direct comparison between these two observations, as the presence of chloroquine in the former study would have affected the nuclear uptake kinetics. However, the observation that the nuclear uptake of EGF was saturable is common to both reports.

It is probable that intracellular EGF concentration is an important determinant of nuclear EGF concentration. In previous reports, chloroquine has been used to increase intracellular EGF concentration (Jiang and Schindler,1990, Savion et al, 1981, Johnson et al, 1980). All of these reports indicate that chloroquine-induced increases in intracellular EGF concentration were paralleled by similar increases in nuclear EGF concentration. Since EGF internalisation is dependent upon EGFR, it is likely that, in the absence of protease inhibitors, cell surface EGFR expression is an important determinant of the intracellular EGF concentration. Figure 8.11 shows the maximal nuclear accumulation of EGF in a panel of cell lines which were all derived from squamous cell carcinomas. The cell surface EGFR expression of each of these cell lines was established experimentally (table 6.3). The results presented (figure 8.11) indicate that three of the cell lines (A431, B2A4 and HN5) accumulated approximately equal amounts of nuclear EGF when incubated under identical conditions. While the EGFR expression differs widely amongst these cell lines, they

all have uncommonly high EGFR expressions (1.8-4.4 x 106/cell) when compared to the numbers of receptors observed in primary cultures from tumours. CaSki express a lower number of receptors per cell than the other three cell lines (5.8 x 105/cell). The accumulation of EGF in CaSki nuclei was found to be lower than the other cell lines examined. Cell lines with even lower EGFR expressions were examined in this study, but no significant nuclear EGF uptake could be detected. This suggests that the nuclear uptake of EGF may be related to the cell surface expression of EGFR.

A question which was not addressed in this study is the nature of the EGF-derived compound which accumulates in the cell nucleus. EGF is rapidly degraded after internalisation, with an intracellular half-life of 20 mins (Carpenter and Cohen, 1976). It is possible that the species which localises in the cell nucleus is not intact EGF, but a degradation product. However, there is some published evidence which suggests that nuclear 'EGF' may not be significantly degraded. Early studies of the nuclear uptake of EGF were performed in cells treated with endodomal and lysosomal protease inhibitors such as chloroquine and leupeptin (Johnson et al, 1980, Savion et al, 1981). This treatment of cells resulted in a large increase in both cytoplasmic and nuclear 125I-EGF concentration. The observation that inhibition of degradation of internalised EGF results in increased nuclear accumulation suggests that the species taken up by the nucleus is not a degradation product of EGF. However, these observations are not conclusive evidence that nuclear EGF is undegraded

Some studies which have addressed this question have concluded that precipitation with anti-EGFR antibodies indicates that nuclear EGF is undegraded (Savion et al, 1981, Raper et al, 1987). This interpretation should be treated with caution, as antibody binding merely indicates the presence of intact epitope, and not intact EGF.

Data presented in this chapter also suggests that nuclear EGF may not be significantly degraded. Similar patterns of nuclear uptake were observed using both EGF-biotin and 125I-EGF (figures 8.1-3 and figure 8.8). Conjugation of biotin to EGF occurs at lysine residues, whilst iodination typically occurs at tyrosine residues. Again, this suggests that the eventual nuclear EGF species is not a product of the complete degradation of EGF. However, this cannot be interpreted as conclusive evidence that nuclear EGF is not a metabolite of the native EGF species.

Given the uncertainty over the physical nature of nuclear EGF, there is another possible interpretation of the data presented in this chapter. As outlined earlier in this chapter, the significant difference in the nuclear EGF uptake observed between serum-starved and serum-competent cells may result from differences in the

intracellular metabolism of EGF in the two cell populations. If the species which is detected in the nucleus is a product of the cytoplasmic degradation of EGF, then the uptake of nuclear EGF would then be a function of the total cellular EGF uptake, and/or of the rate of metabolism of cytoplasmic EGF. It would also cast doubt upon the specificity of uptake of nuclear EGF, as data similar to that presented in this report could be explained by the the non-specific nuclear accumulation of such a degradation product. In keeping with this possible interpretation, the results presented in this chapter indicate that the highest absolute nuclear EGF concentrations were observed in cell lines with highest EGFR expression, and thus the highest total cellular EGF uptake.

To resolve this uncertainty, an investigation into the physical nature of nuclear EGF should be carried out. It may be possible to determine the molecular weight of nuclear EGF by carrying out SDS-PAGE analysis of nuclear fractions from $^{125}$I-EGF-labelled cells. However, it may prove difficult to resolve small alterations in molecular weight between 125I-EGF and nuclear 125I-EGF. Chromatographic techniques could also be used to determine some of the physical properties of nuclear EGF. Gel filtration chromatography, using a matrix with an appropriate molecular weight sieving range, should provide a more accurate molecular weight determination than SDS-PAGE. Reverse phase fractionation of nuclear extracts from 125I-EGF-treated cells could also resolve the question of the degradation of nuclear EGF. Comprison of the elution profiles of nuclear and 125I-EGF would indicate differences in the structures of these molecules. In addition, peptide sequence analysis following reverse phase purification of nuclear EGF should provide exact details of the amino acid sequence of the nuclear species.

### 8.4.4 *Conclusions*

The data presented in this chapter demonstrated that, after application of exogenous labelled EGF, the presence of the label could be detected in the nucleus of EGFR-expressing cell lines. The highest amounts of nuclear label could be detected in cell lines with high EGFR expression. This accumulation also appeared to be dependent upon incubation temperature and serum concentration. The amount of nuclear uptake varied with incubation time and exogenous ligand concentration. Whilst an investigation into the nature of the nuclear EGF-derived species requires to be carried out to establish the specific nature of this uptake, these observations imply that EGF

may be suitable for the delivery of short range Auger-emitting radionuclides to the nuclei cells which overexpress EGFR.

Chapter 9 describes experiments designed to assess the radiobiological effect of EGF labelled with an Auger-emitting radionuclide (123I) on the same panel of EGFR-expressing cells.

# CHAPTER 9

# THE EFFECT OF 123I-EGF ON THE CLONOGENICITY OF EGFR-EXPRESSING CELL LINES

## 9.1 Introduction

As outlined earlier (section 2.3.1), the use of radionuclide conjugates in targeted therapy have several advantages over both cytotoxic drug and biological toxin conjugates as targeted therapeutic agents. The development of novel tumour-targeting radiopharmaceuticals may be of clinical benefit. This chapter describes preliminary studies into the radiobiological effect of an Auger-emitting radionuclide (123I) conjugated to EGF, on a panel of EGFR-expressing cell lines.

### 9.1.1 *Particle range and tumour curability*

The range of particles emitted by radionuclides has important dosimetric implications for tumour curability. Mathematical models have indicated, assuming homogeneous distribution of radionuclide, that tumour deposits which are smaller than the mean particle range will not recieve the maximum achievable radiation dose. This is because a proportion of the decay energy is deposited outside of the tumour mass (Humm, 1986). Subsequent mathematical modelling studies have indicated that for each radionuclide, there is an optimal tumour size for cure (Wheldon et al, 1991, O'Donoghue et al, 1995). The clinical implication of these calculations is that very small tumour deposits may be more resistant to some types of radionuclide therapy, and could be sites of disease recurrence.

Studies employing EGFR-seeking radio-conjugates have mainly focused on the use of the β-emitting radionuclide 131I (Epenetos et al, 1985, Kalofonos et al, 1989, Capala and Carlsson, 1991). This radionuclide is most effective in sterilising tumour deposits of 2.6 - 5 mm diameter, due to the mean emitted β-particle range of 800 μm (Wheldon et al, 1991, O'Donoghue et al, 1995). Tumour deposits with smaller dimensions may be more resistant to therapy. EGFR-targeting using radionuclide conjugates with ultra-short particle ranges may facilitate the sterilisation of very small tumour deposits or single cells.

### 9.1.2 *Short-range particle-emitting radionuclide conjugates*

Two strategies for short-range radionuclide targeting of EGFR-expressing tumour cells have been described. The first involves the tumour targeting of the non-radioactive isotope 10B (Carlsson et al, 1994). The interaction of 10B with a thermal neutron field causes nuclear fission of the isotope, yielding high LET radiation in the

form of short-range α-particles and Li3+ ions (Barth et al, 1990). The ability to deliver 10B to EGFR-expressing cells by the use of EGF-10B conjugates has been demonstrated (Capala et al, 1996). However, the clinical use of this conjugate may be limited by the availability of the means of generating an activating thermal neutron beam.

The second involves the use of a monoclonal antibody (mAb 425) conjugated to 125I (Brady et al, 1990 a and b). Whilst this agent demonstrated therapeutic potential, limitations to its efficiency may result from incomplete tumour penetration (Mairs et al, 1990). In addition, 125I may not be the most appropriate radionuclide conjugate. As outlined in section 2.2, the radionuclide half-life should closely match the biological half-life of the delivery vehicle. The biological half-life of most monoclonal antibodies is much shorter than the half-life of 125I (half-life = 60 days). Therefore, the use of alternative Auger electron-emitting radionuclides such as 123I (half-life = 13.1 hours) may result in increased energy deposition to tumour and enhanced tumour cell sterilisation.

### 9.1.3 *Aims of this study*

The aim of this study was to determine the effect of 123I, conjugated to EGF, on the clonogenic capacity of a panel of cell lines with widely varying EGFR expression.

## 9.2 Materials and methods

### 9.2.1 *Iodination of EGF*

Carrier-free Na123I was obtained from Cygne, Eindhoven, The Netherlands and murine EGF was obtained from Boehringer Mannheim. Iodination was carried out using the Iodogen method as described in section 8.2.3. For 123I labelling, 1μg mEGF was incubated in buffered conditions with 74 MBq Na123I. The resulting specific activity of 123I-EGF was 51-59 MBq/μg. Unlabelled EGF was then added to achieve a specific activity of 50 MBq/μg. As a control, 123I was also conjugated to fatty-acid-free BSA (Sigma, U.K.) in an identical manner.

### 9.2.2 *Colony forming assay*

5x104 cells were seeded in the wells of a 24-well plate and allowed to attach. Medium was removed, and replaced with fresh medium containing either 123I-EGF or unlabelled EGF to a final concentration of 50 ng/ml. Cells were then incubated for a further 24 hours using the conditions described in section 6.2.2. After incubation, cells were washed three times in PBS and removed from the wells by trypsinisation. Cell numbers were established by counting control, unlabelled, EGF-treated cells using a haemocytometer

A feeder layer of sterilised cells was prepared for each cell line, as described in section 7.2.2. Treated cells were then seeded in 10 cm Petri dishes (1000 - 3000 cells, depending upon the individual growth characteristics of each cell line), and feeder cells added to a total cell number of 5 x104 per Petri dish. Cells were then incubated for 10-14 days to allow colony formation. Colonies were stained with Carbol-fuschin. Those colonies which contained 50 or more cells were scored as viable.

## 9.3 Results

### 9.3.1 *Effect of 123I-EGF on clonogenicity of A431 cell line*

The results shown in figure 9.1 are expressed as surviving fraction relative to untreated control cells. All incubations were carried out at 37oC, in a 2% CO2 atmosphere. Results are means and standard deviations from at least two separate triplicate determinations. Statistical analysis was performed using the paired Student's T-test. * denotes $p < 0.05$ compared to untreated control (column 1). Figure 9.1 demonstrates the effect of EGF, 123I-EGF and Na123I on the clonogenic capacity of A431 squamous cell line. Cells were incubated under the conditions indicated for 24 hours. Clonogenic capacity was assessed by colony forming assay.

50 ng/ml unlabelled EGF reduced the clonogenic capacity of A431 cells by 52%, indicating that EGF is cytotoxic to a sub-population of A431 cells. 123I-EGF (50 ng/ml, 2.5 MBq/ml) reduced the clonogenic capacity of A431 cells by 81.3% when compared to unlabelled EGF-treated A431 cells, indicating that 123I-EGF is more toxic to A431 cells than unlabelled EGF. Incubation of cells with unconjugated Na123I (2.5 MBq/ml) had only a minimal effect on clonogenicity (clonogenic capacity reduced by 11% +/- 14%), indicating that 123I is not toxic to A431 cells unless it is conjugated to EGF. Similarly, 123I-BSA (2.5 MBq/ml) had a minimal

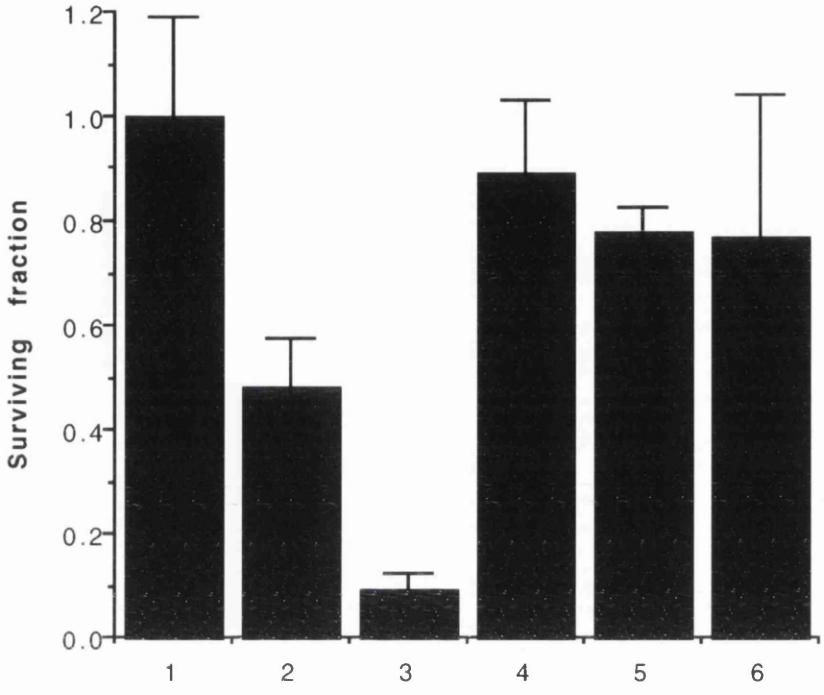



**Fig 9.1** Effect of $^{123}$I-EGF on the clonogenic capacity of A431 cell line.

Column 1 - Untreated control A431 cells

Column 2 - Control A431 cells incubated for 24 hours with 50 ng/ml unlabelled EGF

Column 3 - A431 cells incubated for 24 hours with 50 ng/ml $^{123}$I-EGF (2.5 MBq/ml)

Column 4 - A431 cells incubated with 2.5 MBq/ml Na $^{123}$I

Column 5 - A431 cells incubated with $^{123}$I-BSA (2.5 MBq/ml)

Column 6 - A431 cells incubated with 50 ng/ml $^{123}$I-EGF (2.5 MBq/ml) in the presence of 5µg/ml unlabelled EGF

effect on clonogenicity, indicating the dependence of conjugation to EGF for 123I-mediated cytotoxicity. The presence of 5μg/ml unconjugated EGF greatly reduced 123I-EGF-mediated cytotoxicity, suggesting that 123I-EGF-mediated cytotoxicity is dependent upon binding to EGFR.

### 9.3.2 *Effect of low temperature and seum starvation on 123I-EGF-mediated cytotoxicity*

The results shown in figure 9.2 are means and standard deviations from at least two separate triplicate determinations. Serum-starved cells were incubated in serum-free medium for 24 hours before addition of EGF. Statistical analysis was performed using the paired Student's T-test. * denotes $p < 0.05$.

Incubation of A431 cells with 123I-EGF for 24 hours at 4oC dramatically reduced 123I-EGF-mediated reduction in clonogenicity. However, incubation of serum-starved A431 cells for 24 hours with 123I-EGF in serum-free conditions did not fully abolish 123I-EGF-mediated cytotoxicity (clonogenic capacity reduced by 62% +/- 8%).

### 9.3.3 *Assesement of the 123I-EGF-mediated cytotoxicity in a panel of EGFR-expressing cell lines*

Figure 9.3 demonstrates the effect on clonogenicity of 123I-EGF on a panel of cell lines with differing EGFR expressions, and differing responses to EGF (figure 7.2 and table 6.3). Figure 9.3(a) indicates clearly that the three cell lines examined (A431, B2A4, HN5) all display a reduced clonogenic capacity after treatment with 123I-EGF, when compared to cells treated with unlabelled EGF. The reductions in clonogenic capacity observed were : A431 - 90.7% +/- 5%, HN5 - 91.3% +/- 4%, B2A4 - 78.5% +/- 9%.

Figure 9.3 (b) shows the clonogenicity of three cell lines (CaSki, HER14, K721A) whose response to 123I-EGF treatment was less dramatic. The reductions in clonogenic capacity observed were: CaSki - 21% +/- 11%, HER14 - 16% +/- 15%, K721A - 45.5% +/- 10%. Of these three cell lines, only K721A showed a response to 123I-EGF treatment which was significantly different from the unlabelled EGF control. CaSki and HER14 showed a small, statistically non-significant reduction in clonogenicity. Figure 9.3 (c) indicates that the three cell lines MCF-7, SiHa and EJ showed no reduction in clonogenic capacity after treatment with 123I-EGF.

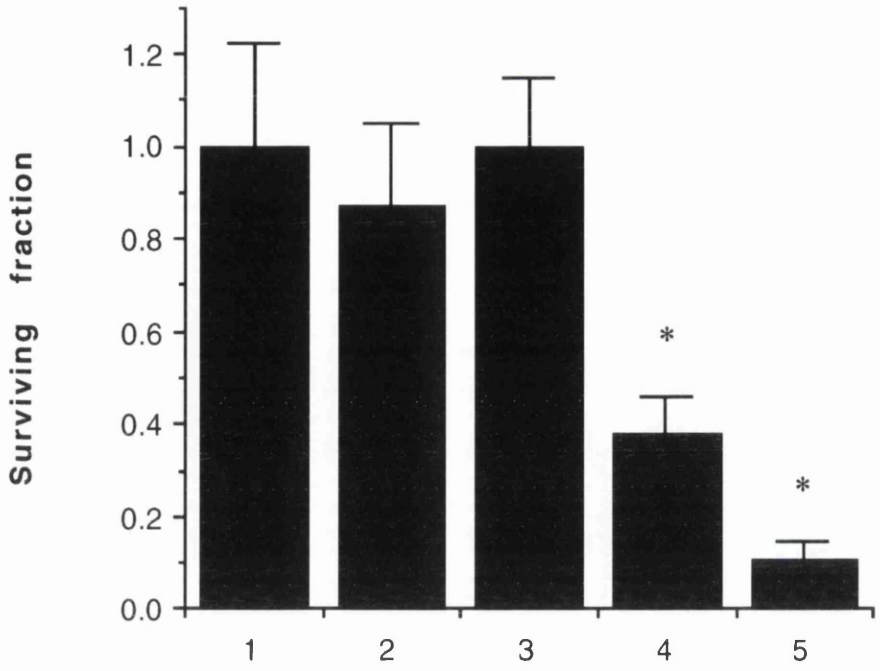



**Fig 9.2** Effect of incubation temperature and serum conditions on $^{123}$I-EGF-mediated alteration in A431 clonogenic capacity

Column 1 - Control A431 cells incubated with 50 ng/ml unlabelled EGF for 24 hours at 4$^{o}$C

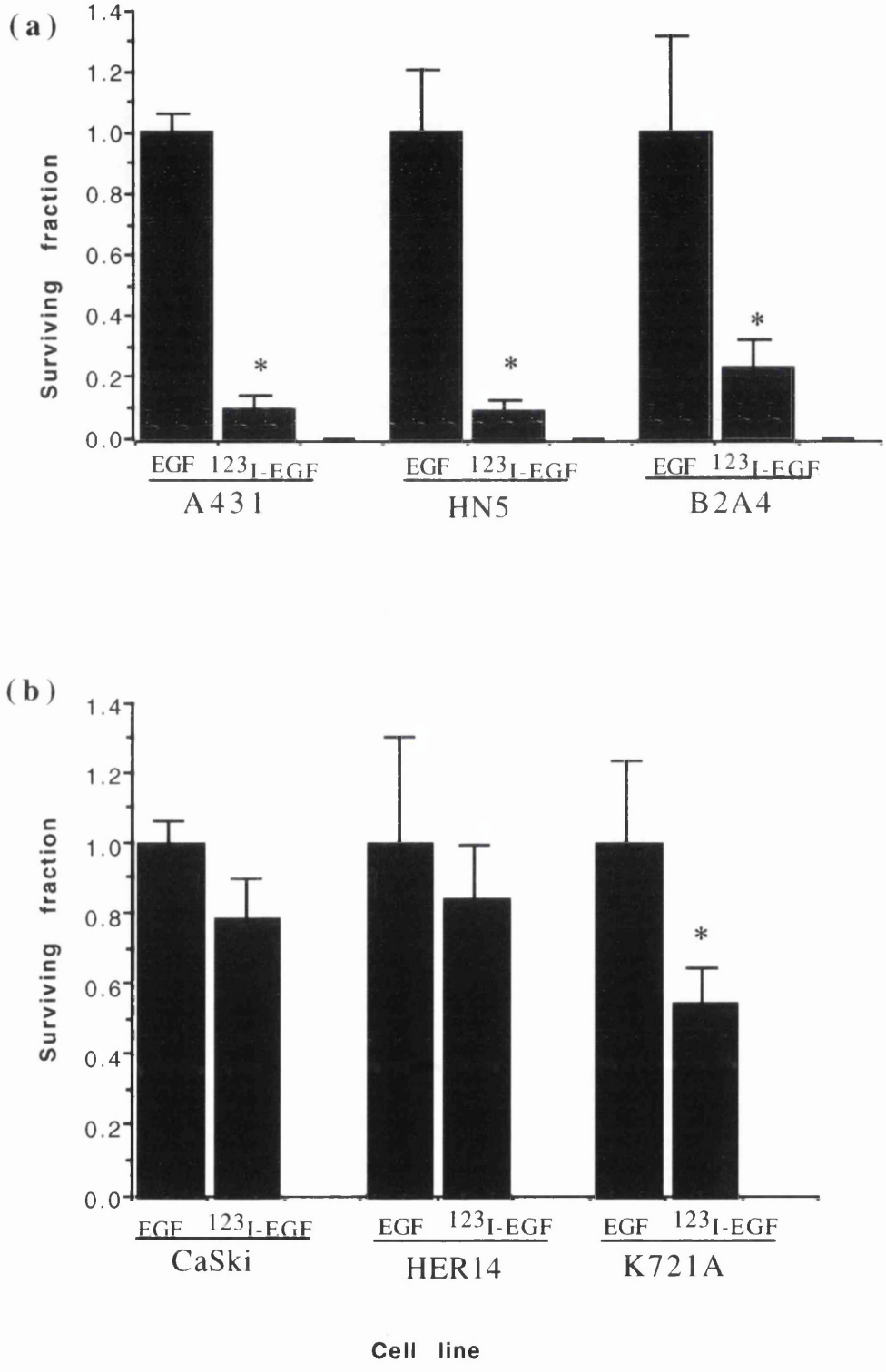
Column 2- A431 cells incubated with 50 ng/ml $^{123}$I-EGF (2.5 MBq/ml) for 24 hours at 4$^{o}$C

Column 3 - Serum-starved control A431 cells incubated with 50 ng/ml unlabelled EGF for 24 hours at 37$^{o}$C in serum-free medium

Column 4 - Serum-starved A431 cells incubated with 50 ng/ml $^{123}$I-EGF (2.5 MBq/ml) for 24 hours at 37$^{o}$C in serum-free medium

Column 5 - Positive control. A431 cells incubated with 50 ng/ml $^{123}$I-EGF (2.5 MBq/ml) for 24 hours at 37oC in serum-containing medium

**Fig 9.3** Effect of $^{123}$I-EGF on the clonogenic capacity of a panel of cell lines with varying EGFR expression



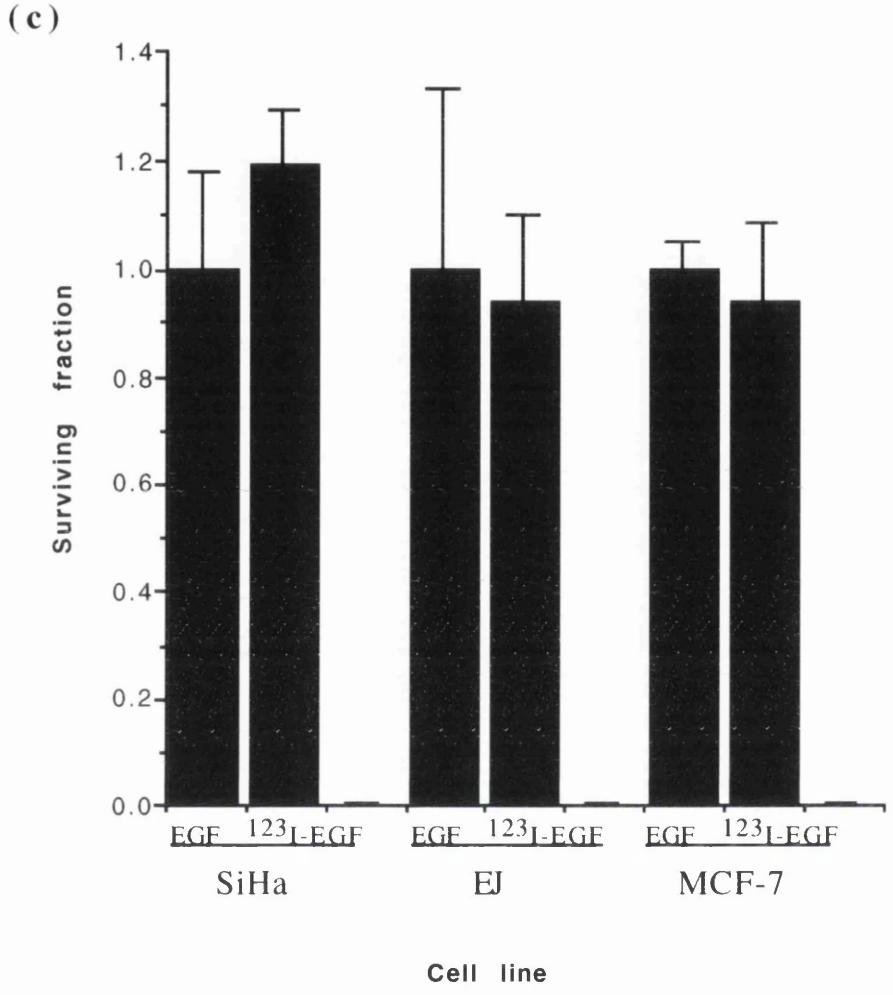



**Fig 9.3** Effect of $^{123}$I-EGF on the clonogenic capacity of a panel of EGFR-expressing cell lines. Cells incubated with 50 ng/ml unlabelled EGF or 50 ng/ml $^{123}$I-EGF (2.5 MBq/ml) for 24 hours at 37$^{o}$C. Results are means and standard deviations from at least two separate triplicate determinations. Statistical analysis performed using the paired Student's T-test. * denotes $p < 0.05$.

## 9.4 Discussion

The experiments described in this section were designed to establish whether EGF conjugated to an Auger-emitting radionuclide had therapeutic potential against cells which express EGFR. Auger emitting radionuclides require to be localised in the cell nucleus, preferably in close proximity to DNA, to cause DNA strand breaks and cell sterility (Bloomer et al, 1981). The results described in chapter 8 indicated that exogenously added EGF, or one of its metabolites, can accumulate in the cell nucleus under certain conditions, indicating that EGF has potential as a delivery vehicle for therapeutic Auger emitting radionuclides.

The radionuclide used in this series of experiments was 123I-iodine. This was selected for several reasons. Firstly, iodination of EGF is a simple procedure, and the optimal conditions for EGF iodination had previously been established (section 8.2.3). Another important consideration was the half-life of the radionuclide. 123I has a much shorter half-life (13.1 hours) than other Auger emitting iodine isotopes, such as 125I (60 days). The practical significance of the difference in half-life is that 123I will release a much larger proportion of its decay energy in a given time period than 125I. The assessment of the effect of 125I on clonogenic capacity usually requires that treated cells be stored at -170oC to allow 125I-decay-mediated, high LET damage to accumulate before seeding (Hofer et al, 1992). The use of 123I allows this procedure to be omitted, as approximately 75% of the total decay energy will be released in the 24 hour (two half-life) incubation period.

We have established that incubation of some of the cell lines with EGF caused a reduction in plating efficiency (table 7.1). To control for this effect, the clonogenicity of 123I-EGF treated cells was compared to cells treated with an identical concentration of unlabelled EGF.

Figure 9.1 indicates that treatment of A431 cells with 123I-EGF caused a reduction in clonogenic capacity when compared to cells treated with unlabelled EGF. This effect was not apparent when the cells were incubated with the equivalent radioactivity (2.5 MBq/ml) in the form Na123I. This indicates that nuclear uptake of 123I- is negligible, and that the effect on clonogenicity of the long-range gamma component of the 123I decay over 24 h is minimal. As a further control, A431 cells were incubated with 123I-BSA. This conjugate had only a minimal effect on clonogenicity, demonstrating that 123I-mediated cytotoxicity is EGF-dependent. Futher evidence for the role of EGFR in 123I-EGF-mediated cytotoxicity comes from the observation that incubation of cells with an excess of unconjugated EGF resulted

in abolition of the effect (column 6). This suggests that EGFR binding was essential for 123I-EGF-mediated cytotoxicity.

The effects of low incubation temperature and serum-starvation on 123I-EGF-mediated reduction in clonogenicity were also examined. Incubation of A431 cells with 123I-EGF at 4oC abolished 123I-EGF mediated cytotoxicity. Serum-starvation did not result in a complete loss of 123I-EGF dependent cytotoxicity, but a substantial reduction was observed (figure 9.2.).

The effect of 123I-EGF treatment on the clonogenicity of a panel of cell lines was examined to determine the effect of EGFR expression. 123I-EGF caused a significant reduction in clonogenicity in four cell lines (A431, HN5, B2A4, K721A), a non-significant trend towards reduction in two cell lines (CaSki, HER 14), and had no effect on three cell lines (MCF-7, SiHa, EJ) (figure 9.3).

The results presented in this chapter support the hypothesis that EGF has the potential to deliver radionuclides to the nucleus of cells expressing EGFR. The finding that neither unconjugated 123I nor membrane-localised 123I-EGF (as a result of incubation at low temperature) had no significant effect on clonogenicity indicate that the gamma photons emitted by 123I decay are not responsible for the toxicity of 123I-EGF. It follows that the Auger component of 123I decay is probably responsible. The average range of Auger electrons is very short (less than 1μm), implying that 123I-EGF must be localised close to DNA.

This is the first report of an EGFR ligand conjugated to an Auger emitting radionuclide causing cell sterility. However, similar observations have been made with an anti-EGFR monoclonal antibody labelled with another Auger emitter, 125I (Bender et al, 1992). 125I-mAb 425 has been shown to be more effective than 131I-mAb 425 at sterilising glioma cells both in vivo and in vitro. 131I has a much higher energy emission than 125I, and the emitted β-particle has a range of several cell diameters (Humm, 1986). The observation that 125I-labelled antibody is more effective in sterilising cells than 131I-labelled antibody could be explained by the greater radiobiological effectiveness of Auger electrons. Such a radiobiological effect could only occur if the 125I-labelled antibody comes into close proximity with DNA. The nuclear localisation of this antibody has been demonstrated in cell line models (Emrich et al, 1994). Interestingly, mAb 425 internalises after binding EGFR. There is no report of a similar finding with antibodies which do not internalise after EGFR binding.

The data presented in this chapter, and the observations made by others outlined above, do not constitute definitive proof of nuclear uptake of EGF or antibody. Whilst the mean range of Auger electrons is <1μm, mathematical models indicate that 123I and 125I may emit electrons with a significantly longer range (Sastry, 1992). Under normal conditions, internalised EGFR accumulates in vesicles in the perinuclear region (Murthy et al, 1987). It is possible that the release of Auger electrons from this site could cause DNA damage. However, this would constitute only a small fraction of the total energy released per decay, as over 70% of DNA strand breaks occur within 1.5 nm of a DNA-incorporated 125I- decay site (Martin and Haseltine, 1981). DNA damage resulting from the decay of 123I in the cytoplasm is unlikely to explain the radiobiological effects observed in this report. Other investigators have reported that cytoplasmic localisation of 125I, in the form 125I-iododihydrorhodamine, does not result in significant radiotoxicity (Kassis et al, 1989). Also, this data does not confirm that the species responsible for the sterilisation effect is native 123I-EGF. As discussed in chapter 8, it is possible that the 123I-EGF may have been metabolised before nuclear uptake.

The finding that serum-starvation, whilst apparently abolishing EGF nuclear localisation in A431 cells (figures 8.3 and 8.4) yet not completely inhibiting 123I-EGF-mediated cell sterilisation (figure 9.2) may be a reflection of the relative sensitivity of the methods used to detect nuclear EGF. In the cell line HN5, after EGF treatment, found that serum starvation resulted only in partial inhibition of nuclear uptake of EGFR (Holt et al, 1995). The authors suggested that autocrine stimulation of EGFR was able to maintain the functional permeability of the nuclear pore under serum-free conditions.

The effect of 123I-EGF on a panel of cell lines provided insight into the effect of EGFR expression. Of the four cell lines which showed a reduction in clonogenicity after 123I-EGF treatment, three (A431, HN5 and B2A4) had the highest EGFR expression in the panel examined (table 6.3). Several studies have indicated that the intracellular concentration of a range of EGF-conjugates correlates with EGFR expression (Arteaga et al, 1994, Jinno et al, 1996, Cristiano and Roth, 1996). Therefore it is possible that nuclear uptake of 123I-EGF is dependent upon the cell surface EGFR expression, and consequent intracellular concentration. Evidence to support this hypothesis comes from the use of lysosomal protease inhibitors in early studies of EGF nuclear localisation (Johnson et al, 1980). Prevention of proteolytic degradation of intracellular EGF resulted in an increase in intracellular EGF concentration, and a parallel increase in nuclear EGF uptake. The finding that K721A

cells are sensitive to 123I-EGF treatment may also support this hypothesis. K721A cells are non-EGFR expressing NIH-3T3 fibroblasts transfected with an EGFR deficient in the kinase domain. These receptors internalise normally when bound to EGFR ligand, but do not undergo ligand-induced downregulation (Honneger et al, 1987). HER 14 cells are identical in every respect to K721A except that they are transfected with wild-type EGFR. Therefore ligand binding in this cell line induces downregulation of EGFR expression (Honneger et al, 1987). Treatment of both cell lines with EGF results in K721A cells expressing a much larger number of cell surface receptors than HER 14 (Chen et al, 1989). This should lead to a higher intracellular EGF concentration, and may also explain the difference in sensitivity to 123I-EGF between these two cell lines. However, as outlined in chapter 8, it is possible to interpret this data in another way. If the eventual nuclear species is a product of the cytoplasmic degradation of 123I-EGF, then the amount of nuclear uptake becomes a function of the total cellular EGF concentration and/or the rate of EGF metabolism. Without a detailed examination of the nuclear 123I-carrying species, it is not possible to conclude that the amount of nuclear 123I-EGF is soley dependent upon the cellular EGFR expression.

It is more difficult to explain why CaSki cells, which express a high number of EGFR (table 6.3), and take up detectable amounts of nuclear 125I-EGF (figure 8.11) do not show significant sensitivity to 123I-EGF (figure 9.3(b)). CaSki nuclei took up significantly less 125I-EGF than A431, B2A4 or HN5 cells, and it is probable that 123I-EGF nuclear uptake mirrors this. It may be that the amount of nuclear 123I-EGF taken up by CaSki cells represents a threshold concentration for sensitivity in this cell line. This hypothesis could be tested by treating CaSki cells with 123I-EGF of higher specific activity.

### 9.4.1 Conclusions

The results presented in this chapter indicate that incubation of some EGFR-expressing cell lines with exogenous $^{123}$I-EGF results in a reduction in clonogenicity. The ultra-short range of Auger electrons emitted following the decay of this radionuclide implies that the radioactive decay occurs in the region of cellular DNA. It is therefore possible to conclude that $^{123}$I, conjugated to EGF, localises in or close to, the nucleus of some EGFR-expressing cell lines. However, as outlined above and in chapter 8, it is possible that $^{123}$I-EGF is metabolised in the cell cytoplasm before nuclear uptake, and thus it is not possible to conclude that the eventual nuclear

species is intact $^{123}$I-EGF. However, these results suggest that some EGFR-expressing cell lines are sensitive to $^{123}$I-EGF, possibly in a EGFR-dependent manner, and that $^{123}$I-EGF has potential as a therapeutic agent against EGFR-positive cells.

# CHAPTER 10

# CONCLUSIONS AND FUTURE STUDIES

## 10.1 Findings of this study

This report contains in several interesting findings which may have implications in the treatment of EGFR-expressing tumours. Firstly, we observed that EGF treatment does not affect the sensitivity of EGFR-expressing cell lines to gamma irradiation, contrary to previous reports. This finding implies that administration of EGF is unlikely to improve upon the efficacy of radiotherapy regiemes. Secondly, we have demonstrated that treatment of EGFR-expressing cell lines with exogenous EGF results in nuclear uptake of EGF. This observation suggests that EGF may have a specific role in the regulation of nuclear function, and may have implications in the design of novel pharaceuticals aimed at inhibition of EGFR-mediated signalling cascades. Thirdly, we have demonstrated that EGF conjugated to an Auger-emitting radionuclide is cytotoxic to cells in an EGFR-dependent manner. Our data also shows that nuclear localisation of the conjugate is essential for cytotoxicity to be observed. This suggests that high LET radiation in the form of short-range Auger electrons may be responsible for the cytotoxic effect. The design of novel Auger electron-emitting radiopharmaceuticals could improve the efficiency of sterilisation of micrometasasis, resulting in improved patient response to therapy.

The principle aims of this study were to investigate methods by which tumour-cell overexpression of EGFR could be exploited in a radiotherapeutic context. The results obtained in this study raise several questions of both academic and practical relevance which need to be addressed by further studies.

## 10.2 Growth factor-mediated alteration in radiation sensitivity

### 10.2.1 *Administration of EGF before and after irradiation*

We attempted to establish the effect of EGF administration after irradiation on the radiosensitivity of EGFR-positive cell lines. The results presented show that EGF did not modify the radiosensitivity of eleven EGFR-expressing cell lines, including A431, HN5 and CaSki. These findings were opposite to previously published results, where EGF administration after irradiation resulted in the radiosensitisation of the cell lines listed above (Kwok and Sutherland 1991 a). Possible explanations for the differences observed are discussed in section 7.4.

There is confusion in the published literature regarding the effect on radiosensitivity of EGF administration before and after irradiation. One study found that EGF-mediated radiosensitisation could only be observed if cells were pre-incubated with EGF before irradiation (Bonner et al, 1994), whilst another found that only post-irradiation EGF administration resulted in radiosensitisation (Kwok and Sutherland, 1991 a). It is difficult to draw conclusions from these observations, since both studies involved the use of cell lines cultured in serum, which contains EGFR ligands. In addition the degree of endogenous EGFR ligand production by the cells was not taken into account in either study. In both studies, therefore, activation of EGFR occurs both pre- and post-irradiation, regardless of the timing of exogenous EGF administration. The control of the signalling capacity of the EGFR, by the use of antibodies which bind to ligand, and prevent ligand-receptor association (neutralising antibodies), or non-activating anti-EGFR antibodies which compete for ligand binding, could ensure that cells are not inappropriately stimulated by EGFR-ligand derived from serum, or the cells themselves.

### 10.2.2. *Mechanisms involved in altered radiation sensitivity*

Future studies are required to understand why our observations of the effect of EGF on radiation sensitivity differ from those previously reported. As discussed in section 7.4, the responses of A431, HN5 and CaSki cell lines to EGF, and the degree of EGFR expression in each cell line were similar to those reported by Kwok and Sutherland (1991 a). This implies that the divergence in the post-irradiation EGF response is not due to alterations in EGFR expression or the EGFR signal transduction pathway.

It is possible that clonal evolution of the cell lines has resulted in an altered radiation response. It may be possible, using subtractive hybridisation, to identify specific genes, differentially expressed in the two cell populations, (radiosensitising and non-radiosensitising), which are involved in the cellular response to radiation damage. The use of subtractive hybridisation usually results in the identification a large number of genes which are differentially expressed between two cell populations. However, a comparison of two cell populations derived from a single clone should result in a smaller number of differentially expressed genes, facilitating the identification of important genes.

The scope of these investigations could be widened to include radiation-resistant sub-clones of cell lines derived from other tissue types. Radiation-resistant clones of

cell lines derived from neuroblastoma and Burkitt's lymphoma have previously been described (Russell et al, 1995, Michael et al, 1997). The identification of common genetic indicators of radiation resistance could have important implications in the understanding of the mechanisms involved in cellular radiation sensitivity.

Laderoute et al, (1994) observed alterations in the cell cycle distribution of irradiated, EGF-treated, A431 cells when compared to untreated irradiated cells. The authors hypothesised that these cell cycle alterations resulted in an increase in the proportion of cells undergoing apoptosis. Similarly, Russell et al (1995) observed that a radiation-resistant sub-clone, derived from a neuroblastoma cell line, was less prone to radiation-induced apoptosis. A radiation-resistant sub-clone derived from Burkitt's lymphoma was found to be defective in the ceramide signalling pathway (Michael et al, 1997). The role of ceramide in the apoptotic pathway (discussed in section 1.4) suggests that differences in the degree of radiation-induced apoptosis may also account for the radiation-resistant phenotype observed in these cells.

Analysis of genes, differentially expressed between parent and radiation-resistant cell populations, or sensitising and non-sensitising populations, which are involved in apoptotic signalling, or regulation of cell cycle, could provide genetic indicators predictive of radiation response and, eventually, cellular targets for pharmacological intervention.

It is important to note that subtractive hybridisation will only identify differentially expressed genes. Genetic mutations resulting in altered protein function could also be responsible for the observed differences in EGF-mediated radiosensitisation. Such mutations could not be identified by subtractive techniques.

## 10.3 The nuclear accumulation of EGF

### 10.3.1 *Regulation of nuclear EGF uptake*

As described in sections 8 and 9, cell lines with high EGFR expression accumulate more 125I-EGF in the cell nucleus, and are more sensitive to 123I-EGF treatment than cell lines with low EGFR expression. The possibility that nuclear uptake of EGF is dependent upon the level of EGFR expression has already been discussed (section 9.4). However, there are other possibilities. The three cell lines with the greatest sensitivity to 123I-EGF treatment (A431, HN5 and B2A4) are all growth-inhibited by high concentrations of EGF (figure 7.2.). Growth inhibition by EGF results in cell cycle arrest in the G1 phase of the cell cycle (McLeod et al, 1986), possibly by

induction of the p21*waf1* cyclin dependent kinase inhibitor (Fan et al, 1995). There are several proteins whose intracellular localisation is known to be cell-cycle dependent (Jans et al, 1995 and references therein). The nuclear localisation or nuclear exclusion of these proteins may be regulated by phosphorylation by cyclin-dependent kinases. Significantly, the nuclear uptake of bFGF is observed mainly in the G1 phase of the cell cycle (Bouche et al, 1987, Baldin et al, 1990), although there is no data to suggest a role for cyclin dependent kinases in this process.

It is possible that the levels of nuclear EGF detected in the cell lines indicated above are due not only to the high EGFR expression, but also due to the EGF-mediated increase in the length of the G1 phase. This may also partially explain the difference in sensitivity to 123I-EGF observed between transfected fibroblast cell lines K721A and HER 14. EGF stimulates growth of HER 14, but has no effect on the growth of K721A, as this cell line expresses a non-signalling EGFR (figure 6.3 b). Mitogenic stimulation by EGF results in a shortening of the G1 phase of the cell cycle (e.g Xiong et al, 1996). HER 14 cells may be less sensitive to 123I-EGF because EGF stimulation causes a decrease in the length of time spent in G1 phase, compared to K721A.

Future studies into the nuclear uptake of EGF should include an investigation into the cell cycle-dependence of nuclear translocation. An understanding of the factors which govern the nuclear uptake of EGF may also help to elucidate the specific role of EGF in the cell nucleus. Previous studies into the cell-cycle dependence of nuclear uptake of aFGF have been performed on cells which were serum-starved, resulting in their entering Go phase (Imamura et al, 1994). This ensured a cell cycle- synchronous population prior to FGF stimulation. This study demonstrated that serum-starvation inhibited nuclear EGF accumulation (figures 8.3, 8.4 and 8.8). Whilst examination of the cell cycle-dependence of EGF uptake would require a synchronised cell population, the use of cells which were synchronised by other methods, e.g. mitotic shake-off, would be preferable.

In addition, our data suggests that an investigation into the effect of tyrosine kinase inhibitors on nuclear EGF uptake should be performed. We have shown that K721A cells were more sensitive to 123I-EGF-mediated sterilisation than HER 14 cells (figure 9.3 b). Possible explanations for this observation (dependence of 123I-EGF toxicity upon ligand-induced EGFR downregulation and cell cycle status) are discussed in section 9.4. Pharmacological inhibition of EGFR kinase activity could result in an increase in nuclear EGF uptake due to inhibition of ligand-induced receptor downregulation, and also by abolishing the EGF-mediated shortening of the

G1 cell cycle phase. Experiments designed to investigate the effects of EGFR-specific- and broad spectrum tyrosine kinase inhibitors on nuclear EGF uptake could provide information about the role of intracellular signalling pathways in the regulation of his system.

### 10.3.2 *The nature of nuclear EGF*

As discussed in chapters 8 and 9, the data presented in this report cannot exclude the possibility that the eventual nuclear EGF species is a metabolite of native EGF, despite some evidence to suggest that nuclear EGF may not be significantly degraded. This question may have more than simply academic significance. If the species taken up by the nucleus is a large metabolite of EGF, it may be possible to design vehicles, based upon specific EGF truncations, which are taken up with greater efficiency by the nucleus. In addition, resolution of this question may also provide important information regarding the nuclear translocation mechanism.

Experiments could be performed to establish the nature of nuclear EGF. Immunoprecipitation followed by SDS-PAGE would indicate the molecular weight of the nuclear species. In addition, chromatographic analysis of nuclear EGF could establish whether there are differences between nuclear and native EGF. Purification followed by peptide sequence analysis would establish the precise nature of nuclear EGF, although obtaining the required quantities of nuclear EGF for this procedure represents a significant undertaking.

### 10.3.3 *The intranuclear localisation of EGF*

Data obtained using ESI (section 8.2.2) indicated that nuclear EGF was not exclusively associated with either nuclear membrane or nucleolus. This finding was confirmed by the observations using LSCM, which indicated that EGF was evenly distributed throughout the cell nucleus. However, the sensitivity of cells to 123I-EGF suggests, given the ultra-short range of Auger electrons emitted by this radionuclide, that nuclear EGF may be associated with DNA. Other investigators have indicated that EGF and 'EGFR-like' epitopes associate specifically with chromatin (Pierard-Franchimont et al, 1991, Rakowicz-Szulczynska et al, 1986, 1989, Holt et al, 1994). In particular, one study demonstrated that EGF binds tightly to chromatin at specific sites (Rakowicz-Szulcynska, 1989). However, EGF lacks a DNA-binding consensus

sequence, implying that it may interact with DNA as part of a complex with other nuclear proteins.

There is no published evidence to indicate a specific function for nuclear EGF. However, the interaction of EGF with DNA suggests that it may have a role in the regulation of gene transcription. An investigation into the DNA-binding site of EGF may provide more information of the biological systems involved. In addition, it is important to establish the nuclear proteins with which EGF interacts. Co-immunoprecipitation of nuclear EGF, using isolated intact nuclei, could provide information about these proteins, and also the systems affected by nuclear EGF.

### 10.3.4 *Nuclear translocation mechanism*

Reports of the nuclear accumulation of some growth factors remain controversial. Part of this controversy concerns the lack of identification of a translocation mechanism. However, the unequivocal and specific nuclear uptake of AR, SDGF and the FGFs indicates a physiological mechanism of translocation of growth factors from the cell membrane to the nucleus. Evidence from this study and others (Jiang and Schindler, 1990) appears to indicate that nucleocytoplasmic transport occurs at the nuclear pore. Serum starvation and low temperature reduce nuclear uptake of both EGF and EGFR (figures 8.2, 8.3 and Holt, 1995). Similar conditions have also been shown to reduce the nuclear uptake of other proteins (Jiang and Schindler, 1990).

It is unclear how EGF (or any other peptide growth factor), which is contained entirely within endocytic vesicles after internalisation, is able to interact with the nuclear pore. The perinuclear localisation of the endocytic vesicle may provide the opportunity for interaction with the translocation apparatus, but there is no evidence to indicate that this occurs.

It is also unclear how EGF, which lacks a nuclear localisation sequence, is targeted to the nuclear pore. The EGF molecule is small enough to pass through the nuclear pore by diffusion, but this is an unlikely mechanism of translocation as cytoplasmic EGF is exclusively found in endocytic vesicles. The lack of free cytoplasmic EGF implies that the endocytic vesicle must be the source of eventual nuclear EGF.

One possibility is that EGF is translocated together with EGFR. EGFR has a putative nuclear localisation sequence in the cytoplasmic domain (residues 645-658, sequence **RRR**HIV**RKR**TL**RR,** where **R**= arginine and **K**=lysine). This sequence remains cytoplasmic after internalisation, and is available to interact with the

importin αβ complex, which is essential for docking with the nuclear pore (Imamato et al, 1995). Experiments to determine whether EGFR co-immunoprecipitates, or co-localises with the importin αβ complex would provide evidence to support this hypothesis.

Acidification of the early endosome acts to dissociate ligand from receptor, and only a proportion of the endosomal EGFR will be in a ligand-bound state (Maxfield, 1982, Dunn et al, 1986). This may explain the differences observed between the kinetics of nuclear EGF uptake (figure 8.9), and EGFR uptake (Holt et al, 1994 and 1995).

Two other EGFR ligands, AR and SDGF, localise in the cell nucleus (Johnson et al, 1991, Kimura, 1993). In common with the FGF family, they both contain an NLS. The nuclear uptake of these peptides is dependent upon the cell surface expression of EGFR, providing more evidence of the importance of the receptor in the nuclear translocation process.

Mutation of the SDGF NLS results in a peptide which binds and activates EGFR, but does not localise in the nucleus (Kimura et al, 1993). This appears to contradict the hypothesis that the nuclear translocation of EGFR-ligand may occur by the NLS-dependent co-transport of the EGFR. However, the observations made may be interpreted differently. The acidified endosome contains free ligand and receptor, along with actively signalling ligand-receptor complexes (Vieira et al, 1996). Free SDGF, bearing an NLS will be able to bind directly to the importin αβ complex, resulting in transport through the nuclear pore. SDGF without a functional NLS will only be able to associate with importin when bound to EGFR, with nuclear uptake dependent upon the EGFR NLS. Since the endosome contains both free ligand and receptor, as well as ligand-receptor complexes, only a proportion of the total endosomal NLS-mutated-SDGF will be translocated across the nuclear membrane.

In this study, we were only able to detect nuclear 125I-EGF in cell lines with a very high EGFR expression which were incubated for several hours in a high concentration of 125I-EGF. Since NLS-mutant-SDGF and wild-type EGF have similar affinities for the EGFR (Kimura, 1993), it would be expected that, if the same translocation mechanism was involved in each case, that the nuclear uptake kinetics of EGF and NLS-mutant-SDGF would also be similar. It is unlikely, therefore, that nuclear 125I-NLS-mutant SDGF could be detected, given the ligand concentration and EGFR expression of the cell lines used in the study, explaining the observed loss of nuclear uptake. Future studies should test this hypothesis. Such experiments could involve the use of 123I-labelled SDGF mutants in a similar manner to the experiments detailed in this report using 123I-EGF. The generation of a cell line

which expresses an EGFR with a mutated NLS would also provide a useful tool in the elucidation of the nuclear translocation mechanism.

## 10.4 Targeted sterilisation of tumour cells using Auger-emitting radionuclides

### 10.4.1 *125I-EGF nuclear uptake and 123I-EGF-mediated cell killing*

We have demonstrated that exogenously applied EGF (in the form 125I-EGF) can be detected in the nuclei of several cell lines. The same cell lines were sensitive to treatment with 123I-EGF. Three cell lines (A431, HN5 and B2A4), which accumulate most nuclear 125I-EGF also show the greatest sensitivity to 123I-EGF-mediated sterilisation. Sensitivity to 123I-EGF is reduced by low temperature and serum starvation. These conditions also reduce the nuclear uptake of 125I-EGF (figures 8.8 and 9.2). These findings suggest that cellular sensitivity to 123I-EGF is dependent upon the amount of 123I-EGF in the cell nucleus. The experiments performed in this report were designed to determine whether EGF is a suitable vehicle for the delivery of therapeutic short-range radionuclides to EGFR expressing cells. They were not intended to determine whether sensitivity to 123I-EGF is a marker of EGF nuclear localisation. This could be determined by performing a dose/survival experiment. Survival curves produced by exposure to high LET radiation have distinctive profiles. A dose/survival curve specific for high LET radiation would indicate that 123I-EGF-mediated cell sterility was caused by Auger electron emission, and consequently that EGF must be localised in the cell nucleus. Another distinctive feature of Auger electron-mediated DNA damage is the lack of an oxygen-enhancement effect (Hall, 1994). Experiments to determine 123I-EGF mediated cell sterilisation under hypoxic conditions could also provide evidence of an Auger electron effect.

Whilst the data presented in this report does not prove that Auger electrons are responsible for 123I-EGF mediated cell sterility, there is evidence to indicate that this is a reasonable assumption. Localisation of 123I-EGF at the cell membrane, as a result of incubation at 4oC has no effect on clonogenic capacity (figure 9.2). The same applies to cells incubated in medium containing unconjugated 123I (figure 9.1). Both of these findings indicate that the photon component of the decay emission of 123I is not responsible for the observed reduction in clonogenic capacity.

Taken together with traditional approaches, this assay provides yet more evidence of the nuclear uptake of EGF.

### 10.4.2 *EGFR-ligands as delivery vehicles*

Both EGF and TGF-α have been used to deliver therapeutic toxins or radionuclides to EGFR-overexpressing cells (Chaudhary et al, 1987, Shaw et al, 1991, Capala and Carlsson, 1991). The toxins conjugated to EGF and TGF-α act in the cytosol to effect cell kill. The sub-cellular localisation of 131I-EGF or 10B-EGF conjugates will not greatly alter their radiotoxic effects, due to the relatively long range of the decay emissions from these radionuclides. The cytotoxicity of these conjugates correlates with the intracellular concentration of toxin or radionuclide (Andersson et al, 1992, Carlsson et al, 1994), whilst sub-cellular localisation of the internalised conjugate is of minor importance.

None of the more recently discovered EGFR binding ligands (AR and HB-EGF) have been used in this manner. These ligands bind EGFR with similar affinity to EGF, suggesting that they would not offer any advantage, as delivery vehicles of toxins or radionuclides, over EGF or TGF-α.

However, the observation of high LET effects from Auger-emitting radionuclides is entirely dependent upon the nuclear localisation of the radionuclide. We have demonstrated that EGF, which localises in the cell nucleus, has potential as a delivery vehicle for Auger-emitting radionuclides.

Amphiregulin has also been observed to localise in the cell nucleus (Shoyab et al, 1988). Unfortunately, there is no published study of an investigation of the comparative rates or maximal nuclear uptake of AR and EGF. Given the unequivocal nature of the reports of AR nuclear uptake, and the relative difficulty in observing EGF nuclear uptake, AR may be superior to EGF as a nuclear delivery vehicle for 123I.

Future studies should assess the sensitivity of EGFR-expressing cells to 123I-AR, compared with 123I-EGF.

HB-EGF has a structure which is similar to AR. In common with AR, it binds heparin and contains a putative NLS (Higashiyama et al, 1991). There has been no study carried out to determine the sub-cellular localisation of HB-EGF. However, the similarity in structure to AR, coupled with a putative NLS suggests that HB-EGF may localise in the cell nucleus. It is possible that HB-EGF may have potential as a

delivery vehicle for Auger-emitting radionuclides. Experimental evidence is required to confirm this hypothesis.

### 10.4.3 *Sphingomyelin hydrolysis and apoptosis*

The mechanisms involved in radiation-induced cell death, and the role of sphingomyelin hydrolysis in radiation-induced apoptosis are discussed in chapter 1.

Our observations of 123I-EGF mediated cell killing have been interpreted as a result of nuclear accumulation of EGF, and Auger-electron mediated DNA damage. From our data it is not possible to discount the possibility that the cell kill resulted from irradiation of the cell membrane by 123I-EGF, inducing the intracellular accumulation of ceramide, leading to apoptosis. Irradiation of cell membrane preparations has previously been demonstrated to result in ceramide production (Haimovitz-Friedman et al, 1994) However, some of our observations suggest that this mechanism of cell kill is unlikely. Incubation of A431 cells with 123I-EGF at 4oC does not result in a significant reduction in clonogenic capacity (figure 9.2). At this temperature, ligand-induced internalisation and downregulation of the EGFR is inhibited (Carpenter and Cohen, 1976). The amount of receptor-bound, and thus membrane-associated, 123I-EGF is significantly higher after incubation at 4oC than at 37oC, a temperature at which ligand-induced receptor internalisation and downregulation is not inhibited. If membrane irradiation and sphingomyelin hydrolysis was responsible for the cell killing observed, the effect would be expected to increase after incubation with 123I-EGF at 4oC. Such an increase was not observed.

It is possible, though, that low temperature may affect the ceramide-activated signal transduction pathway, resulting in abrogation of the apoptotic signal, which could also explain our results.

Future studies must determine the mechanism of 123I-EGF-mediated cell killing by assay of apoptotic markers, or by morphological assessment of cells. In addition, a 123I-EGF dose/survival curve typical of high LET radiation would also indicate direct DNA damage by 123I-EGF.

### 10.4.4 *123I-EGF as a therapeutic agent*

We have shown that 123I-EGF is able to sterilise some EGFR-expressing cell lines. Agents which sterilise cells in an EGFR-dependent manner may be useful in the treatment of tumours which overexpress EGFR. There are several reasons why the development of Auger-emitting radionuclide therapies may improve on current therapeutic strategies.

Current ligand-based targeted radionuclide strategies (discussed in section 5.3) centre mainly on the use of β-emitting radionuclides. β-particles have a range of several cell diameters, and are thus capable of sterilising the targeted cell as well as those in close proximity which are not directly targeted. This effect is useful because it reduces the effect of heterogeneous uptake of targeted radionuclide within the tumour mass. However, as discussed in section 9.1.1, small tumour masses may be disproportionately resistant to β-particle therapy.

Auger-emitting radionuclides cause cell sterilisation only when the radionuclide decays in close proximity to DNA, due to the ultra-short range of Auger electrons. Only cells which are directly targeted are sterilised, and no cross-fire effect is observed. Heterogeneous uptake of Auger-emitting radionuclides results in the sparing of non-targeted tumour cells. Therefore, 123I-EGF may not be suitable as a single agent therapeutic modality. However, combinations of EGFR-targeted β- and Auger-emitting radionuclides could result in improved clinical outcome, as Auger-emitting radionuclides are particularly suitable for the sterilisation of small tumour masses, which are spared by β-particle therapy.

Another potential clinical application of 123I-EGF is in the purging of bone marrow, or peripheral blood stem cell collections used for autologous bone marrow rescue. It is essential that haematopoetic stem cells to be reinfused are free from tumour cells, as these may be responsible for disease recurrence. As discussed above, Auger-emitting radionuclides are particularly suitable for the sterilisation of micrometastes. In addition, because EGFR is not expressed by hematopoetic cells, it would represent a tumour cell-specific target in this system. Sterilisation of non-tumour cells would be greatly reduced using 123I-EGF, compared to a β-emitting radionuclide-EGF conjugate, as only EGFR-expressing cells would be sterilised.

EGFR-targeted radionuclide strategies take advantage of the overexpression of EGFR, and preferential accumulation of radionuclide by tumour cells, as opposed to non-tumour cells. However, systemic administration of EGF-radionuclide conjugates

results in uptake by a wide variety of cell types which express EGFR. One of the major sites of non-tumour EGF uptake is the liver, which concentrates EGFR ligands from the circulation. Liver damage is a potential limitation to EGFR ligand-based therapies.

There is evidence to suggest that systemic administration of EGF-Auger-emitting, radionuclide conjugates may result in less damage to the liver than β-emitting radionuclide conjugates. Several studies of EGF nuclear localisation have been performed on cells isolated from liver regenerating after partial hepatectomy (Marti et al, 1991, Ichii et al, 1988). The authors observed that hepatocytes isolated from these animals showed increased nuclear EGF, when compared to cells isolated from non-regenerating liver. Nuclear accumulation of EGF by hepatocytes isolated from normal liver, or fully regenerated liver, was much less, even though the cells retained the capacity to bind EGF.

As discussed above, the cytotoxicity of Auger-emitting radionuclides is dependent upon nuclear localisation, whilst the sub-cellular localisation of β-emitting radionuclides is of lesser importance. The non-nuclear localisation of EGF in normal hepatocytes should render them insensitive to 123I-EGF-mediated cytotoxicity. However, similar uptake of β-emitting radionuclides would result in cell killing, due to the longer range of these particles. Thus systemic administration of Auger-emitting radionuclide-EGF conjugates may result in less liver damage than would be expected from similar administration of β-emitting radionuclide conjugates. This hypothesis, however, requires experimental investigation.

## 10.5 Conclusions

We have observed the accumulation of nuclear EGF by cell lines which overexpress EGFR, and demonstrated that this phenomenon can be exploited to deliver therapeutic short range radionuclides to the nuclei of these cells. Many of the factors which control the nuclear uptake of EGF remain to be elucidated, as does the function of nuclear EGF, and the nuclear translocation mechanism. Investigation of these mechanisms may lead to a greater understanding of the ways in which peptide growth factors exert their influence on normal and malignant cells.

The development of novel therapeutic strategies based on these observations depends on our understanding of the factors which govern nuclear EGF uptake. A significant amount of work is required before appropriate targeting strategies can be designed and optimised. However, preliminary results presented here indicate that the

nuclear localisation of peptide growth factors may be exploited therapeutically, and in a manner which may complement and improve on therapies both in clinical use, and those being considered for clinical use.

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