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Epidermal Growth Factor-induced Protection of Tumour Cell Susceptibility to Cytolysis

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Using radiobinding, transfection and colorimetric assays, the biological significance of epidermal growth factor (EGF) and its receptor on established human tumour cell lines was investigated. The intensity of class I major histocompatibility antigen (MHC) and EGF receptor (EGFR) expression on 20 tumour cell lines was investigated and showed no direct correlation (coefficient of correlation $r = 0.43$ and $P = 0.06$). Furthermore, transfection of the β2-microglobulin gene into a class I negative bladder tumour cell line, resulting in the re-expression of fully assembled cell surface class I antigens, did not result in alteration of EGFR expression. However, there was an inverse correlation between the intensity of EGFR expression and the stimulatory response of cells to exogenously added EGF. The per cent inhibitions of cell proliferation by EGF at 100 ng/ml for A431 (highest EGFR expressor) and Scaber (lowest EGFR expressor) were 37 and −7%, respectively. The results also showed that cell lines isolated from testis tumours positive for epithelial markers (using pan keratin antibody LP34 as an epithelial marker), expressed significantly lower EGFR levels than cell lines from bladder tumours. The expression of EGFR receptor was not modulated by interferons (IFN-α and -γ and only a minor effect with IFN-β) or active supernatant containing a mixture of cytokines. Whilst the pretreatment of tumour cells with IFNs resulted in a significant increase in the susceptibility of tumour cells to interleukin-2-activated peripheral blood mononuclear cells, EGF treatment resulted in their protection. Thus, the per cent killing at an effector:target ratio of 20:1 for untreated cells and EGF (100 ng/ml), IFN-α (1000 U/ml), -β (2000 U/ml) and -γ (100 U/ml) were 53%, 33% ($P = 0.004$), 64% ($P = 0.004$), 69% ($P = 0.001$) and 66% ($P = 0.001$), respectively. These results indicate the complex interactions between EGF and EGFR and their relevance in modifying tumour cell behaviour. The hypothesis that the resistance to cytolysis of tumour cells induced by EGF stimulation may be a factor in the accelerated tumour growth seen in patients after traumatic tissue damage is discussed.

Key words: epidermal growth factor, epidermal growth factor receptor, major histocompatability complex, transfection, cytokine
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INTRODUCTION

EPIDERMAL GROWTH factor (EGF) is a small polypeptide, which was first isolated from the submaxillary gland of a mouse, is widely distributed in human tissues and large quantities are found in urine, milk and prostate secretions [1]. It is a potent mitogen for cells of epidermal origin and acts via its receptor (EGFR), a transmembrane protein with an extracellular binding domain [2].

Many malignant cells contain oncogenes whose protein products are homologous with EGFR. Oncogene activation has been shown to occur concomitantly with increased expression of EGFR and its mRNA in many tumours including brain [3], breast [4], lung [5], bladder [6] and colon [7] cancer. Neal and associates [8] reported that, in contrast to normal urothelium where EGFR is only expressed on the basal layer, 48% of 101 bladder tumour biopsies investigated expressed EGFR on all the tumour cells, and this corresponded with tumour invasiveness, poor differentiation and possibly with poor clinical prognosis.

Similar findings correlating increased expression of EGFR to poor prognosis were reported in breast cancer [9–11]. There has been controversy with regard to mechanism of increased EGFR expression by different human tumours. Many investigators have reported that cytokines such as interferons and tumour necrosis factor α could influence EGFR expression [10, 12]. The possible relevance of EGF in tumour metastasis has been reported by Shima and associates [13] showing that EGF could stimulate the production of matrix metalloproteinases which in turn are thought to be important in the destruction of matrix, and hence tumour spread.

Consideration of these and the report of Schreiber and associates [14] demonstrating the stabilising effects of major histocompatibility complex (MHC) antigens on EGFR, prompted us to investigate whether there was any correlation between the intensity of EGFR and class I antigen expression on established human tumour lines and a class I negative bladder tumour cell line before and after correction of class I antigens by gene transfection. We also examined the effects of cytokine stimulation on EGFR expression, the correlation between the intensity of EGFR and response of tumour cells to EGF, and finally the influence of EGF stimulation on tumour target susceptibility to non-MHC-restricted killing.

MATERIALS AND METHODS

Interferon (IFN) and monoclonal antibodies

IFN-α, -β and -γ were obtained from Wellcome, ASTA Pharma (Bioferon) and Biogen, respectively. The monoclonal antibody W6/32 was used for the detection of all β2-microglobulin (β2-m)-associated HLA-A,B,C, while unassociated β2-m molecules were detected using BBM.1 [15]. Intact HLA-DR molecules were detected using the HB55 antibody [16]. The EGFR antibody was obtained from the Imperial Cancer Research Fund (London, U.K.).

Development of cell lines and lymphocyte preparation

The list of established cell lines used in this study (Table 1) has been described previously [17]. Attempts were also made to develop new cell lines and led to the development of Lanc, Ha, Wil and Fen lines. Briefly, surgically removed tumour biopsies were used for developing cell lines, along with peripheral blood lymphocytes (MNCs) which were prepared as described previously [18]. Tissues were finely cut before being passed through a sieve with the aid of a sterile syringe plunger. After washing, tissue cells were cultured overnight at 37°C in RPMI, containing 10% fetal calf serum (FCS), before non-adherent cells and cell debris were removed and replaced by fresh medium. The adherent cells were fed until confluence (1–2 × 10^6/25 cm^2 flask) and were expanded by trypsinisation and subculturing at lower seed (0.5 × 10^6/25 cm^2 flask) in new culture flasks.

Table 1. Expression of epidermal growth factor receptor, keratins and MHC class I antigens on established human cell lines

Cell line	Class I	EGFR	Keratin
Testis			
Ep2102	660 ± 108	96 ± 5	++++
Lanc	274 ± 10	109 ± 1	++
Tera II	240 ± 49	174 ± 44	5%++
Ha	84 ± 14	202 ± 16	++++
Tera I	63 ± 12	139 ± 15	++++
Bladder			
J82	2065 ± 408	697 ± 15	++
T24	1786 ± 91	749 ± 149	−
TccDes	1736 ± 427	705 ± 25	nd
TccSup	1680 ± 67	304 ± 50	−
Wil	1208 ± 67	338 ± 21	+++
RT112	1020 ± 57	1125 ± 139	++++
Scaber	920 ± 109	390 ± 50	++++
RT4	566 ± 161	142 ± 22	++++
Fen	132 ± 20	132 ± 14	+++
Fen 3	1412 ± 71	158 ± 28	+++
Others			
SKV14	1624 ± 287	1053 ± 44	+++
A431	1121 ± 67	2275 ± 315	++++
T47D	1105 ± 77	137 ± 18	−
JEG3	747 ± 107	290 ± 38	++++
MCF7	1187 ± 42	109 ± 18	+++
Overall mean ± S.D.	981 ± 622	466 ± 532	

Results of three replicates are expressed as mean ± S.D. (cpm).
Intensity of keratin (LP34 monoclonal antibody) staining of the cells is shown as ++++ for strong positive and − negative expression. The $r = 0.43$ and $P = 0.06$ for EGFR and class I comparison.
Fen 3 is the Fen line after β2-microglobulin gene transfection.
nd, not detectable.

Immunohistochemistry

Exponentially growing cells were trypsinised, placed (1 × 10^4 cells/well) on to sterile multichamber microscope slides (Gibco), and incubated for 48 h at 37°C, after which the cells were air dried, fixed in acetone for 5 min and stored at −40°C until use. The expression of test antigens was determined using an immunoperoxidase technique as described previously [19].

Radiobinding assay

Tumour cells (1 × 10^4/well) were prepared as described in the immunocytochemistry section, and aliquotted into flat-bottomed microtitre plates. IFN-γ was added, and after 48 h of incubation, appropriate concentrations of specific monoclonal antibodies (MAbs) (50 μl/well, in three replicates) containing 0.02% sodium azide were added and incubated for a further

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45 min at room temperature. After three washes, 50 μl of diluted (in RPMI containing 10% FCS and 0.02% azide) iodinated rabbit anti-mouse antibody (50 000 cpm/well, Amersham, U.K.) was added and incubated for 45 min. Following three washes, the cells were lysed with 100 μl/well of 2% (v/v) Triton X-100 in water, and the degree of radioactivity in the supernatants was measured using a gamma counter.

Transfection

Transfection was carried out using the lipofectin technique as described previously [20]. Briefly, 0.5×10^6 of exponentially growing adherent cells (25 cm^2 flask) were washed with sterile phosphate-buffered saline (PBS) and 5 ml of optimem was added. The cells were then incubated for 4 h at 37°C. Genomic DNAs containing 2 μg/ml of β2-m gene and 2 μg/ml of pSV2neo marker gene, diluted with 50 μl of PBS and 2.5 ml of optimem in a bijou tube, were mixed with the contents of a second bijou tube consisting of 150 μl lipofectin and 2.5 ml of optimem. This mixture was then added to the culture flask treated with the optimem.

Cells were incubated overnight at 37°C before replacement of the medium with fresh RPMI, containing 10% FCS, for a further 10 h of incubation. The supernatant was then substituted by fresh medium containing genetacine (500 mg/ml found to be sufficient to kill 100% of cells during the first weeks of culture). After 2 weeks of culture, the surviving cells were cloned and positive cells were selected using W6/32 as a marker.

Cell lysis and total protein determination

Washed cell pellets were immersed for 30 min in NP40 lysis buffer containing 0.5% NP40, 10 mM Tris–HCl pH 7.4, 150 mM NaCl and 2 mM PMSF at 4°C. Insolubilised material was subsequently removed by centrifugation at 10 000*g* for 10 min. Protein concentration measurement of the resulting lysate was performed using the bicinchoninic acid protein assay (BCA) commercially adapted for use in microtitre plates (Pierce Ltd).

Colorimetric (MTT) assay

This was carried out using the MTT (3-[4,5-dimethylthiazol-2yl]-2,5 diphenyl tetrazolium bromide) assay described by Hussain and associates [21]. Exponentially growing cells were treated with trypsin and EDTA (0.05% + 0.02%) for 5 min, washed, resuspended in RPMI containing 10% FCS (Gibco), and plated at 10×10^3 per well (in 100 μl volume) in flat-bottomed microtitre plates (Nunc). After an overnight incubation at 37°C under 5% CO_2, the cells were loaded with 10 μl of freshly prepared and millipore-filtered MTT (5 mg/ml in PBS), and incubated for a further 4 h. The medium was then replaced with 100 ml acidified (0.04 M HCl) β-isopropanol (Sigma,), and left for 30 min at room temperature for colour development, before being read by an ELISA reader (570-nm filter).

RESULTS

EGFR and class I antigen expression on cell lines

Using a radiolabelled binding technique, the levels of EGFR expression on human cell lines were investigated. As can be seen from Table 1, the mean ± S.D. for EGFR and class I antigens on 20 cell lines were 981 ± 622 and 466 ± 532 cpm (correlation coefficient $r = 0.43$, $P = 0.06$, respectively). In addition, the bladder lines expressed higher intensities of EGFR than the testis lines (seven of ten verses one of five with mean values greater than 200 cpm, respectively; $0.05 < P > 0.01$). These results indicated no correlation between the intensity of EGFR and class I antigen expression, and that there was a tendency for bladder cell lines to express more EGFR than testis cell lines, despite their epithelial nature (as shown with LP34 antibody recognising pan keratins).

Effects of class I correction by gene transfection on EGFR expression

A well-established bladder tumour cell line, defective for class I antigens and shown to be corrected following β-2m gene transfection [22], was used to assess the influence of class I antigen on EGFR expression. As can be seen from Table 2, the values for class I antigens (detected by W6/32 monoclonal antibody, which only reacts with fully assembled cell surface class I antigens) before and after gene transfection were 132 ± 20 and 1412 ± 71 cpm ($P = 0.0001$), respectively. The values for EGFR were 132 ± 14 and 158 ± 28 cpm ($P \geqslant 0.05$), respectively. Similar values for class II antigens on cells before and after IFN-γ (100 U/ml) stimulation were, respectively, 160 ± 30 and 1811 ± 91 before transfection and 108 ± 11 and 2184 ± 113 cpm following transfection. The results also indicated that while both cell types responded to IFN-γ but not IFN-α or -β for induction of class II antigens, they showed no change in the intensity of EGFR expression. Thus, the mean ± S.D. for class II antigens on Fen cells before and after IFN-γ stimulation on untransfected and transfected cells were 160 ± 30 and 1811 ± 91 cpm ($P = 0.0001$) and 108 ± 11 and 2184 ± 113 cpm ($P = 0.0001$), respectively. The corresponding values for EGFR were 132 ± 14 and 167 ± 3 cpm ($P > 0.05$) and 158 ± 28 and 160 ± 52 cpm ($P > 0.05$). These results indicated that under conditions where cells were stimulated by a cytokine like IFN-γ, the levels of EGFR remained unchanged.

The effect of EGF (100 ng/ml) on the metabolic activity (as measured by optical density, OD) of the cells before (Fen) and after gene transfection [Fen 2 (cells which survived G418 treatment but were negative for class I antigens) and Fen 3 (cells which survived G418 and were positive for class I antigens)] remained unchanged (Table 3), and was similar to the effect of IFN-γ (10 U/ml). These results indicated that the correction of class I antigens did not influence the intensity of EGFR expression or the response of the cells to EGF.

Relationship between the intensity of EGFR expression and the response to EGF

The effects of EGF on the A431 cell line was investigated to establish optimum conditions. The results showed that the per cent inhibition of the cells metabolic activity at 1, 10 and 100 ng/ml EGF on cells (10×10^3 cells/well) cultured in flat-bottomed microtitre plates for 24 h were 4.6, 32.2 and 40.4%, respectively, and these conditions were used for the subsequent experiments. EGF was tested on six cell lines, A431, RT112, T24, TccDes, J82 and Scaber, expressing varying intensities of EGFR expression and the results are presented in Table 4. As can be seen, there was an inverse correlation between the intensity of EGFR expression and the response of cells to EGF ($r = 0.95$, $P = 0.003$). The values of EGFR expression for A431 and Scaber lines were, respectively, 2275 ± 315 and 390 ± 50 cpm, and the corresponding values for cell proliferation inhibition in response to 10 and 100 ng/ml of EGF were 32 and 37%, and −8 and −7%, respectively.

Table 2. Effects of interferons (IFN) on major histocompatability complex (MHC) antigens and epidermal growth factor receptor (EGFR) expression of a class I negative cell line before and after gene transfection and correction of class I antigens

	Non-transfected			Transfected		
Cell line	Class I	Class II	EGFR	Class I	Class II	EGFR
NT	132 ± 20	160 ± 30	132 ± 14	1412 ± 71	108 ± 11	158 ± 28
γ-100	149 ± 14	1811 ± 91	167 ± 3	1914 ± 358	2184 ± 113	160 ± 52
α-1000	136 ± 15	197 ± 3	143 ± 35	1855 ± 22	106 ± 15	122 ± 3
β-4000	133 ± 29	128 ± 39	170 ± 6	2056 ± 48	114 ± 18	238 ± 31

Results are expressed as mean ± S.D. (cpm) of three replicates using a binding assay. IFNs were used at U/ml and the cells were cultured for 48-h duration. NT denotes untreated cells.

Table 3. Effect of epidermal growth factor (EGF) on stimulation of a class I negative bladder tumour cell line before and after β2-microglobulin gene transfection and correction of class I antigens

Cell line	Fen	Fen 2	Fen 3
NT	0.42 ± 0.01	0.48 ± 0.01	0.47 ± 0.02
EGF 100 ng/ml	0.39 ± 0.01	0.45 ± 0.01	0.44 ± 0.02
IFN-γ 10 U/ml	0.42 ± 0.01	0.46 ± 0.02	0.45 ± 0.02

Results are expressed in optical density of mean ± S.D. of three replicates. Cells were incubated with cytokines for 24 h before colorimetric assay. Fen, Fen 2 and Fen 3 are bladder cell lines before transfection and after transfection (surviving G418 selection treatment), but class I negative and class I positive, respectively. NT denotes untreated cells.

Table 4. Effects of epidermal growth factor (EGF) on cell lines expressing different intensities of EGF receptor

	EGF (ng/ml)			
Cell line	NT	10	100	EGFR (cpm)
A431	0.93 ± 0.07	32%†	37%†	2275 ± 315
RT112	0.76 ± 0.02	0.4%	3.3%	1125 ± 139
T24	0.54 ± 0.02	0.5%	−6%	749 ± 149
TccDes	0.75 ± 0.02	−12%*	−4%	705 ± 25
J82	0.73 ± 0.01	−16%*	−16%*	697 ± 15
Scaber	0.64 ± 0.02	−8%*	−7%	390 ± 50

Results of NT (untreated cells) are expressed as mean ± S.D. of optical densities of three replicates in the MTT assay. The values for EGF are expressed as per cent inhibition compared with NT cells cultured with EGF for 24 h, and EGFR values are expressed in cpm using a binding assay. * and † denote the significance levels of $=0.05$ and <0.05, respectively. Coefficient of correlation between EGFR expression and response to EGF (100 ng/ml) was $r = 0.95, P = 0.003$.

Possible influence of cytokines on the expression of EGFR

In order to investigate whether the expression of EGFR was under cytokine control, cell lines including Fen (before and after β2-m gene transfection) were exposed to IFNs (α, β and γ) or active supernatant (prepared from mitogen-activated peripheral blood mononuclear cells of a normal individual for 48 h at 37°C) for 48 h in medium containing 10% FCS. As can be seen from Table 5, the results demonstrated that at optimum concentrations (as assessed for HLA antigen induction, established from previous studies [18]) only IFN-β resulted in a small but significant increase in EGFR expression. Thus, the values for untreated and IFN-β-treated Fen cell line were 132 ± 14 and 170 ± 6 cpm ($P = 0.04$), respectively. The respective values for Fen3 cell line were 158 + 28 and 238 + 13 cpm ($P = 0.03$).

The active supernatant, which is known to contain a variety of cytokines [23], also failed to have any significant effects on the levels of EGFR expression. The lack of response was not due to the absence of cytokine receptors on the tumour cells since under the same conditions, IFN-γ stimulation resulted in the induction of MHC class II antigens (Table 2).

Effects of EGF on susceptibility of tumour target to non-specific killing

Non-MHC restricted killing (NK) of tumour cells by interleukin-2 (IL-2)-activated cells was investigated using the Fen cell line. Tumour cells were treated with IFNs or epidermal growth factor (EGF) and cultured for 48 h at 37°C before cytotoxicity assay. As can be seen from Figure 1, all three IFNs increased the susceptibility of the target cells at all the effector:target ratios. In contrast, EGF-treated cells were found to be protected from this killing. Thus, at an effector:target ratio of 20:1 the per cent killing for untreated cells, EGF (100 ng/ml), IFN-α (1000 U/ml), IFN-β (2000 U/ml) and IFN-γ (100 U/ml)-treated cells were 53%, 33% ($P = 0.004$), 64% ($P = 0.004$), 69% ($P = 0.001$) and 66% ($P = 0.001$), respectively. A similar degree of significance was observed for the lower effector:target ratios. These results indicated that at a non-toxic concentration

Table 5. Effects of cytokines on induction of epidermal growth factor receptor (EGFR) on different tumour cell lines

Cell line	Fen	Fen 3	A431	T24
NT	132 ± 14	158 ± 28	1965 ± 395	711 ± 117
γ-100	167 ± 43	130 ± 6	1399 ± 99	755 ± 143
α-1000	143 ± 35	122 ± 3	1611 ± 229	749 ± 108
β-4000	170 ± 6*	238 ± 13*	ND	ND
A.Sup 2.5%	ND	ND	1784 ± 27	761 ± 37

Results are expressed as mean ± S.D. cpm of three replicates using a binding assay. ND and A.Sup denote not done and active supernatant, respectively. Interferons were used at U/ml. * P values of <0.05 using paired t-test. NT denotes not tested.

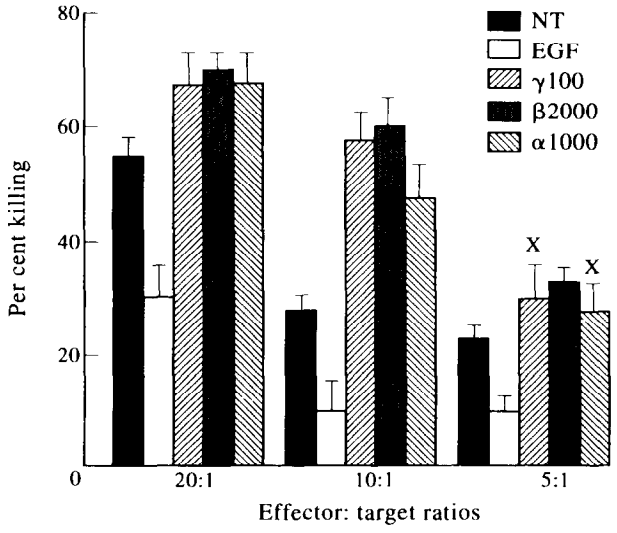

Figure 1. Effects of pretreatment of Fen cells with cytokines before cytotoxicity assay. *P* values comparing treated with untreated cells at 20:1 (effector:target ratio). Epidermal growth factor = 0.004, interferon (IFN)-α = 0.004, IFN-β = 0.001 and IFN-γ = 0.001. Apart from columns indicated with X, all the other results gave *P* values between $P = < 0.05$ and $P = < 0.001$ using paired *t*-test.

of cytokines, EGF was protective, while IFNs increased the susceptibility of the tumour target to non-MHC killing by IL-2-activated cells.

DISCUSSION

The findings of this investigation showed that there was no association between the intensity of class I antigen and EGFR expression, and the correction of class I antigens on a class I negative cell line did not influence the intensity of EGFR expression or the response of cells to EGF. There was an inverse correlation between the intensity of EGFR expression and the response of cells to exogenously added EGF and EGFR expression was not modulated by IFN-α and -γ. Treatment of tumour cells with EGF prior to cytotoxic testing resulted in protection from killing.

These results indicated the complex interactions between EGF and its receptor in determining the proliferative response of epithelial cells and the protective effect of EGF on tumour targets to cytolytic cells. These phenomena may have important implications for tumours of epithelial origin, not only in terms of the tumour response to exogenous EGF, but also for the induction of tumour resistance to cytolysis.

During the past few years, there has been an increasing number of reports demonstrating the increased expression of EGFR in a variety of human tumours, including brain [3], breast [4], lung [5], colon [7] and bladder [6] cancers. It has also been shown that this increased expression was associated with poor prognosis. Schreiber and associates [14] reported the critical role of class I antigens for stabilisation of EGFR. This would imply that high EGFR-expressing tumours ought to express high or at least normal levels of class I antigens. This has clearly been shown not to be the case, and indeed loss of or reduced MHC class I antigens have found to be frequent occurrences in human tumours including breast [24], bladder [19] and colorectal [25] carcinoma.

Our findings that there was no correlation between the intensity of class I antigens and EGFR expression on tumour cell lines is consistent with the lack of association. Furthermore, if the cell surface class I antigens was an important factor for stabilising EGFR, then one would have expected that the insertion of β2-m into a β2-m-defective cell line (which resulted in correction of cell surface class I antigens) would have altered the intensity of EGFR expression and/or the response of the cells to exogenously added EGF, which was clearly not the case (Tables 2 and 3).

Transformed cells, unlike normal cells, grow *in vitro* with only simple nutrients. This has led to the conclusion that tumour cell lines produce and respond to their own growth factors in a autocrine manner [26]. Iihara and associates [27] argued that this might be the mechanism by which human oesophageal carcinoma escapes normal growth control mechanism(s). In this study, we compared the intensity of EGFR expression on tumour lines and the response of the cells to exogenously added EGF (Table 4). The results showed that there was an inverse correlation between the two parameters, indicating that there might be an optimum intensity of EGFR and the concentration of EGF for maximum tumour proliferation. The implication of this hypothesis is that it is only possible to establish the true autocrine environment of epithelial tumour by determining not only the levels of EGFR expression on tumour cells, but also the availability of EGF at the tumour site. Currently, we are examining EGF production by tumour cell lines *in vitro* to address this question.

In recent years, the use of cytokines, such as IFNs (α, β and γ), as therapeutic agents in human cancers has stimulated a great deal of interest into their exact mode of action [28]. We hypothesised [18] that the capacity of IFNs to induce MHC antigens and hence increase tumour visibility to cytolytic T-cells might be part of IFN's therapeutic efficacy. Scambia and colleagues [29] reported yet another interesting concept, arguing

that following *in vivo* treatment of endometrial adenocarcinoma patients with IFN-α, there was a significant increase in the expression of oestrogen receptors with a concomitant decreased in EGFR expression. This led to the conclusion that IFN-α treatment pushes the tumour cells from undifferentiated, aggressive to more differentiated and more benign phenotypes. This observation, however, could not be confirmed by Budillon and associates [30], where it was shown that instead of a decrease, IFN-α increased the expression of EGFR on their tumour model. Our findings, measuring cell surface expression of EGFR by a radiolabelled binding technique showed that IFN-α and -γ or the active supernatant (containing a whole battery of cytokines) could not influence the expression of EGFR on a variety of cell lines tested. These discrepancies may in part be due to different tumour models and methods of detection of EGF and more studies are required for further clarification.

Although the increased expression of EGFR in human tumours has been well established, the possible implications of such an abnormality for therapeutic application has not been studied in detail. Gillies and colleagues [31] reported an interesting approach in which they made a genetic construct of EGF and CD3 genes and used it for the production of a bifunctional antibody half anti-CD3 (pan T cell marker) and half anti-EGFR. The use of the hetero-conjugate antibody was found to improve the killing capacity of the cytolytic tumour infiltrating lymphocytes (TILs) against a specific tumour target expressing EGFR. The findings of our investigation may provide another approach for improving tumour target killing. As described, pretreatment of tumour cells with exogenously added EGF resulted in tumour protection from lysis by cytolytic cells. This implied that it might be possible to investigate the significance of this *in vitro* observation by using an animal model of bladder tumours. Animals with a uroethelium showing increased expression of EGFR could be pretreated with anti-EGFR antibody prior to treatment with immunostimulatory cytokines such as IL-2. The aim would be to mask EGFR on tumour cells and prevent them from interacting with the surrounding EGF. Although indirect, this approach may prove to be useful for rendering tumour cells less resistant to cytolytic activity of IL-2-activated killer cells. This could have clinical applications, and experiments are in progress for further investigations.

Since the observation of Joynes [32], confirmed more recently by Alexander and colleagues [33], it has been well established that traumatic injury accelerates tumour cell growth. Recent experiments by Skipper and associates [34] have suggested that release of injury-induced cytokines such as EGF may be a factor in this effect. The observation in this report that EGF induced resistance in tumour cells to cytolysis provides an additional mechanism as to why tumour cells may grow after tissue injury, and justifies increased interest in the use of neoadjuvant treatments which suppress tumour and reduce tumour cell spill before minimum surgery.

In summary, the findings of this investigation have identified possible mechanisms to explain why excessive production of EGF may lead to increased malignancy and puts forward a possible new approach which might be clinically significant for treating patients with epithelial tumours with increased expression of EGFR.

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Growth Inhibition by 8-Chloro Cyclic AMP of Human HT29 Colorectal and ZR-75-1 Breast Carcinoma Xenografts is Associated with Selective Modulation of Protein Kinase A Isoenzymes

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Significant dose-related inhibition of growth of HT29 human colorectal cancer xenografts and ZR-75-1 breast cancer xenografts in immune-suppressed mice was induced by the cyclic AMP analogue, 8-chloroadenosine 3′,5′-cyclic monophosphate (8-Cl-cyclic AMP) when given by alzet mini-pumps over a 7-day period at doses of either 50 or 100 mg/kg/day. Levels and types of cyclic AMP binding proteins were measured by ligand binding and photoaffinity labelling, respectively, in tumours harvested at the end of the treatment period. Compared with levels in tumours from control animals, values of tumour cyclic AMP binding proteins from treated animals were significantly reduced. These effects were associated with an apparent modulation of the types of cyclic AMP binding proteins, 8-Cl-cyclic AMP-treated xenografts displaying a reduced ratio of RI/RII isoforms compared with untreated control tumours.

Key words: 8-Cl-cyclic AMP, growth inhibitors, breast/colorectal xenografts, protein kinase A
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INTRODUCTION

CYCLIC AMP is a ubiquitous regulatory molecule whose major actions appear to be modulated through cyclic AMP-dependent protein kinases protein kinase A [1]. Two major subtypes of the cyclic AMP-dependent protein kinases have been identified, differing in their regulatory subunits and referred to as type I (RI) and type II (RII) cyclic AMP-dependent regulatory subunits or cyclic AMP binding proteins [2, 3]. It has been suggested that the relative proportion of RI to RII expressed in cells may influence states of proliferation and differentiation [4]. Evidence has also been presented that malignant cells tend to overexpress the RI subunit at the expense of RII [5–8] and that strategies based upon redressing the balance of isoforms might reduce cellular proliferation and tumour progression [5, 9, 10]. Amongst such approaches has been the use of the cyclic AMP analogue 8-chloroadenosine 3′,5′ cyclic monophosphate (8-Cl-cyclic AMP) [11, 12]. This drug has been shown to cause growth inhibition in a range of cancer cell lines maintained in culture [8, 9, 11, 13].

The aim of the present study was to determine whether 8-Cl-cyclic AMP given *in vivo* to mice bearing xenografts of cancer cells was capable of exerting anti-tumour effects and whether these were associated with change in levels and types of cyclic AMP binding proteins.

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