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Original Paper

Cytokine Modulation of Epidermal Growth Factor Receptor Expression on Bladder Cancer Cells is not a Major Contributor to the Antitumour Activity of Cytokines

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Epidermal growth factor is a potential mitogen for many different human tumours. Its effect is mediated via a bispecific receptor (EGFR), the expression of which correlates well with invasive disease. We investigated the modulation of EGFR by cytokines produced following *bacillus Calmette Guerin* (BCG)-immunotherapy. Our data demonstrate the IFNγ, TNFα and IL-1α can decrease the expression of EGFR on some bladder tumour cell lines. IFNγ reduced EGFR expression on two of eight cell lines (RT4, SD). However, IL-1 and TNF did not share this activity. When cells were treated with a combination of all three cytokines, EGFR was decreased on three cell lines (RT4, RT112, SD) and furthermore, the change in the receptor expression was even more marked. Treatment with phorbol ester (thereby activating protein kinase C) resulted in rapid disappearance of the receptor from the cell surface. Interestingly, the decrease of EGFR expression did not require protein synthesis. Although the cytokines studied could down modulate EGFR, this only occurred on three out of eight cell lines; therefore, it is unlikely that the suppression of proliferative activity caused by cytokine-induced decrease of EGFR expression is central to the antitumour action of BCG therapy, but in a proportion of tumours this mechanism may be involved.

Key words: BCG, immunotherapy, epidermal growth factor receptor, cytokines, IFN, TNF, interleukin-1, bladder neoplasms, transitional cell carcinoma
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INTRODUCTION

Bacillus Calmette Guerin (BCG) immunotherapy has proved to be extremely efficient for the cure and prophylaxis of superficial bladder cancer [1, 2]. Although its mechanism has been intensively investigated and much has been learned we still lack a complete understanding of the accompanying immunological events [3]. It is thought that several arms of antitumour action are involved, namely induction of cellular-dependent cytotoxicity (LAK and BAK cells, gamma/delta T-lymphocytes subset), modulation of adhesion and co-regulatory molecules (providing physical interaction and possibly antigen presentation) and proinflammatory cytokine production which also exerts direct effects on the growth of tumour cells [3–12].

It is widely accepted that several growth factors (including epidermal growth factor (EGF)) may be involved in the malignant process [13, 14]. This ubiquitous protein has mitogenic qualities and is strongly involved in the proliferation of normal and malignant cells of different types. EGF exerts its activity through the epidermal growth factor receptor (EGFR) which also serves as a ligand for transforming growth factor alpha (TGF-α). The receptor has a molecular weight of approximately 170 kDa and structurally is similar to c-*erb*B2 and v-*erb*B1 oncogenes. Blockade of the EGFR has been successfully used to inhibit the growth of different tumours including colon, breast, lung and prostate cancers both *in vitro* and *in vivo* [15, 16].

From numerous reports we know that the pattern of the EGFR expression is different in normal and malignant urothelium and that its overexpression correlates with invasive disease, recurrence and death in bladder cancer [13, 14, 17]. Sauter and co-workers recently demonstrated that overexpression of EGFR was a stronger predictor of bladder cancer proliferation than grade or stage [18]. It has also been shown that EGF and TGFα can serve as autocrine and paracrine growth factors for transitional cell carcinoma (TCC) cells both *in vitro* and *in*

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Table 1. *The lack of correlation between the constitutive expression of the EGFR and histopathological grade or growth profile of TCC cell lines*

Cell lines	RT4	UMUC-3	RT112	MGH-U1	EJ18	J82	SD	5637
Percentage of EGFR positive cells ±S.D.	87 ± 2.2	91 ± 2.6	69 ± 1.9	72 ± 2.5	96 ± 0.9	67 ± 4.7	76 ± 3.5	98 ± 0.5
Log fluorescence intensity ±S.D.	75 ± 1.5	47 ± 2.1	54 ± 1.3	39 ± 0.5	161 ± 4	90 ± 1.8	203 ± 2.1	90 ± 1.25
Histological grade	1	1	2	3	3	3	3	3
Growth rate ($\times 10^3$/h)	1.5	4	7.5	16	4.3	0.63	5	10

The receptor expression and growth profile were determined as described in Materials and Methods. Note that the EGFR expression failed to correlate with the grade ($r_1 = 0.214$ and $r_2 = 0.417$, respectively for the percentage of positive cells and for log fluorescence intensity) and with growth profile of cancer cells (correspondingly, $r_1 = 0.043$ and $r_2 = 0.357$).

vivo [19–21]. Therefore, it is accepted that the EGF–TGFα–EGFR link may be involved in the progression of bladder cancer.

For a number malignant diseases including breast, renal and endometrial cancer, some cytokines (namely, interferons-alpha and gamma (IFNα, IFNγ) and tumour necrosis factor-α (TNFα) exert their cytotoxic effect via a decrease of EGFR expression [22–24]. In our laboratory we previously demonstrated that IFNγ, TNFα and a mixture of cytokines contained in the urine of BCG-treated patients can exert strong cytostatic and cytotoxic effects on TCC cell lines [11, 25–28]. However, it appeared that at least for IFNγ, the effect on different tumour

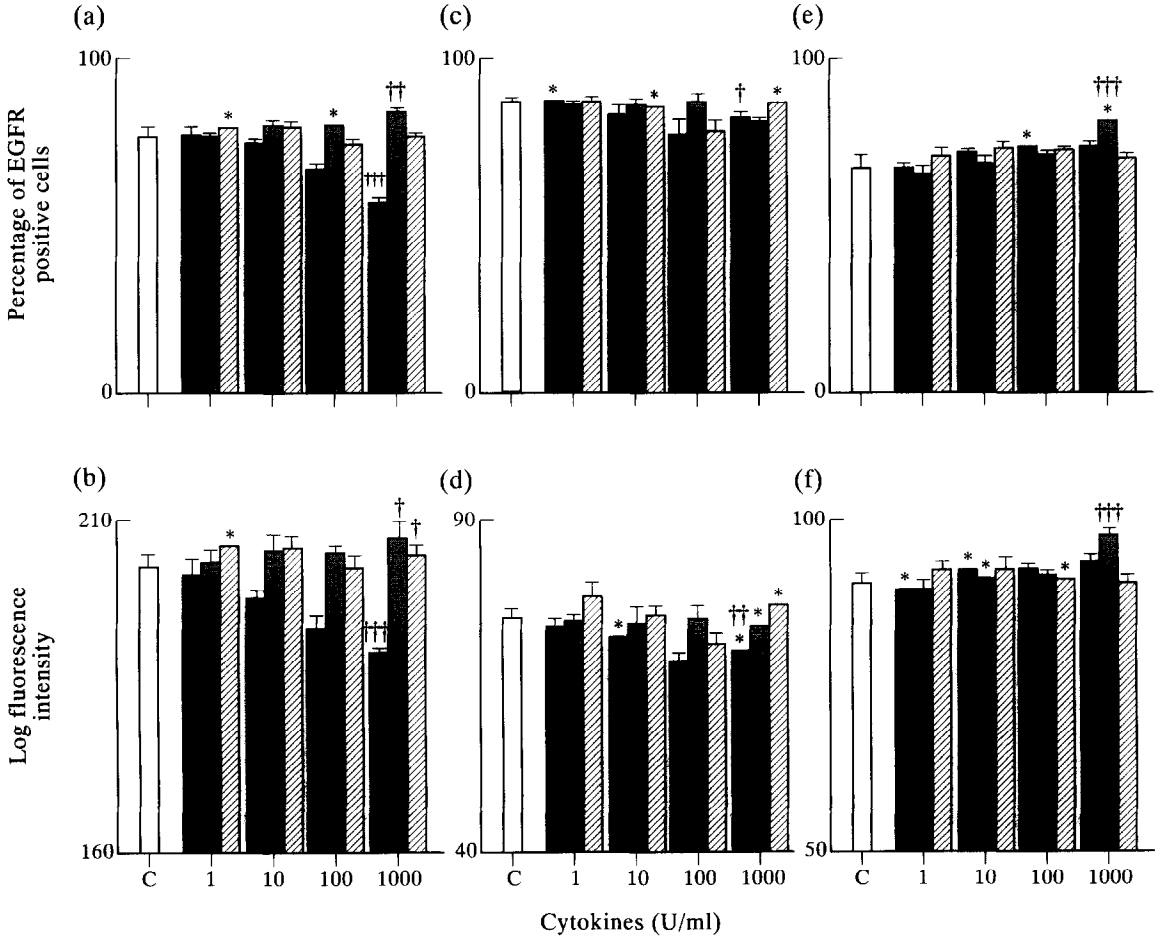

Figure 1. The effect of IFNγ, TNFα and IL-1α on EGFR expression on bladder tumour cell lines SD (a, b), RT4 (c, d), J82 (e, f) TCC cell lines. Cells were stimulated with the recombinant cytokines (IFNγ(shaded black), TNFα(shaded grey), IL-1α(hatched)) at the indicated concentrations or complete medium (C(□)) for 24 h, recovered and analysed using flow cytometry. Bars indicate the mean of at least triplicate determinations and error bars indicate the level of 1 standard deviation. * indicates a standard deviation of less than 0.5%, † shows a level of significance less than 0.05; ††, 0.005; †††, 0.0005 (versus control). Note, that interferon alone significantly down-modulated EGFR on SD and RT4 cell lines, whilst TNF enhanced EGFR expression on SD and J82 cell lines.

Table 2. Modulation of EGFR expression (percentage of positive cells ± standard deviation) on TCC cell lines by various cytokines

Treatment	Percentage of cells expressing EGFR for different TCC cell lines							
	RT4	UMUC3	RT112	MGH-U1	EJ18	J82	SD	5637
Control	87 ± 2.2	91 ± 2.6	69 ± 1.9	72 ± 2.5	96 ± 0.9	67 ± 4.7	76 ± 3.5	98 ± 0.5
IFNγ, 1000 U/ml	82 ± 2.1	93 ± 4.5	80 ± 1.6	75 ± 0.8	98 ± 0.5	74 ± 2.5	57 ± 1.8	98 ± 0
TNFα, 1000 U/ml	81 ± 1.8	96 ± 1.4	72 ± 1.4	77 ± 0.9	98 ± 0	82 ± 1.1	84 ± 1.7	98 ± 0.5
IL-1α, 1000 U/ml	86 ± 1.2	93 ± 1.3	66 ± 0.9	69 ± 0	97 ± 0.5	71 ± 2.1	77 ± 0.8	98 ± 0.5
IFNγ and TNFα, 1000 U/ml	66 ± 1.7	97 ± 0.5	60 ± 4.1	77 ± 2.4	98 ± 0	79 ± 2.5	54 ± 1.9	98 ± 0.5
IFNγ, TNFα and IL-1α, 1000 U/ml	51 ± 4.5	97 ± 0.9	65 ± 0.9	77 ± 3.3	97 ± 0.5	69 ± 3.6	59 ± 3	98 ± 0.5

Maximum responsiveness is shown (upon treatment with cytokines at a concentration of 1000 U/ml). The receptor expression was determined as described in Materials and Methods. Experiments performed at least in triplicate.

Table 3. Modulation of EGFR expression on TCC cell lines by various cytokines

Treatment	Log fluorescence intensity for EGFR for different TCC cell lines							
	RT4	UMUC3	RT112	MGH-U1	EJ18	J82	SD	5637
Control	75 ± 1.5	47 ± 2.1	54 ± 1.3	39 ± 0.5	161 ± 4.0	90 ± 1.8	203 ± 2.1	90 ± 1.3
IFNγ, 1000 U/ml	70 ± 0.7	61 ± 0.7	58 ± 0.5	38 ± 0.5	164 ± 0.8	94 ± 1.1	190 ± 0.8	102 ± 0.5
TNFα, 1000 U/ml	74 ± 0.4	81 ± 6.3	55 ± 0.5	36 ± 0.8	163 ± 1.5	98 ± 0.8	207 ± 2.4	104 ± 0.7
IL-1α, 1000 U/ml	77 ± 0.7	56 ± 3.7	53 ± 0	36 ± 0	160 ± 1.3	91 ± 1.1	205 ± 1.5	100 ± 1.2
IFNγ and TNFα, 1000 U/ml	71 ± 0.5	74 ± 0.5	50 ± 0	36 ± 1.3	169 ± 1.3	98 ± 1.3	189 ± 0.4	98 ± 2.7
IFNγ, TNFα and IL-1α, 1000 U/ml	65 ± 0.7	67 ± 13.2	50 ± 0.8	37 ± 0.8	165 ± 1.4	95 ± 0.8	192 ± 1.9	96 ± 1.1

Results presented as a change in the log fluorescence intensity ± standard deviation. Demonstrated maximum responsiveness (upon treatment with cytokines at a concentration of 1000 U/ml). The receptor expression was determined as described in Materials and Methods. Experiments performed at least in triplicate.

cell lines was not dependent on the expression of cytokine receptors or their affinity for this ligand [29] but rather correlated with the modulation of EGFR expression [25]. This conclusion remains to be verified in an extended study.

Recently it has been shown that TCC cells can produce a number of cytokines (including interleukin-1 alpha (IL-1α) TNFα and some as yet unidentified proteins) [30–32]. Hayashi and co-workers produced evidence that at least one of them, namely IL-1α, serves as an autocrine growth factor for TCC cancer cells [32]. Following immunotherapy the production of a "cocktail" of cytokines and their receptors is observed (IL-1, IL-2, IL-5, IL-6, IL-8, IL-10, IL-12, IL2-R, IL-6R, TNFR, IFNγ, TNFα, sCD14 etc), which can synergise with or antagonise each other [33–36] and unpublished data).

The aim of this study was to investigate any possible correlation between the cytostatic effect of cytokines and the modulation of EGFR expression and also to determine whether cytokines can synergise in this respect.

MATERIALS AND METHODS

Cell lines

Eight human TCC lines: RT4 (histopathological tumour grade 1), RT112 (grade 2), MGH-U1 (grade 3, supplied by Dr J. Masters, Institute of Urology, London, U.K.), UMUC-3 (grade 1), EJ18, 5637, J82 and SD (grade 3, Dr P. Perlmann, Sweden) were used in the present study [37]. These cell lines have proved to be stable, free from mycoplasma infection and their cytological appearance and growth characteristics have remained representative of the parent tumours.

The cells were routinely grown in RPMI-1640 medium (Gibco, Uxbridge, U.K.) supplemented with 5% foetal calf serum (Sera-Lab, Crawley Down, U.K.), sodium pyruvate (5 mM) and L-glutamine (2 mM). For routine purposes and analysis, cells were recovered by trypsinisation (trypsin/EDTA, 0.5 g/l trypsin and 0.2 g/l disodium EDTA).

Cytokines, antibodies, micro-organisms and other reagents

Highly purified recombinant IFNγ was purchased from Boehringer Mannheim (Lewes, U.K) and contained 2.5×10^7 U/mg protein and less than 2 U/mg of endotoxin. Highly purified recombinant TNFα and IL-1α were the kind gift of Behringwerke (Marburg/Lahn, Germany).

Monoclonal mouse antibody to human epidermal growth factor receptor (EGFR) was obtained from Genzyme Diagnostics (West Malling, U.K.). This antibody recognises the extracellular domain of the human EGFR and competes with EGF for the binding site. Sheep anti-(mouse-IgG) $F(ab')_2$ fragment conjugated to fluorescein isothiocyanate (FITC) was obtained from Sigma (Poole, U.K.).

BCG micro-organisms were obtained from Allergen (Russian strain, derivative from Pasteur strain, Stavropol, Russia). This strain was effective in the induction of ICAM-1 on TCC cells (unpublished data). Phorbol myristate acetate (PMA) was purchased from Sigma (Poole, U.K.).

Experimental strategy and quantification of surface antigen expression

The TCC cells in mid log phase in 24 well tissue culture plates were stimulated with recombinant cytokines. The medium with

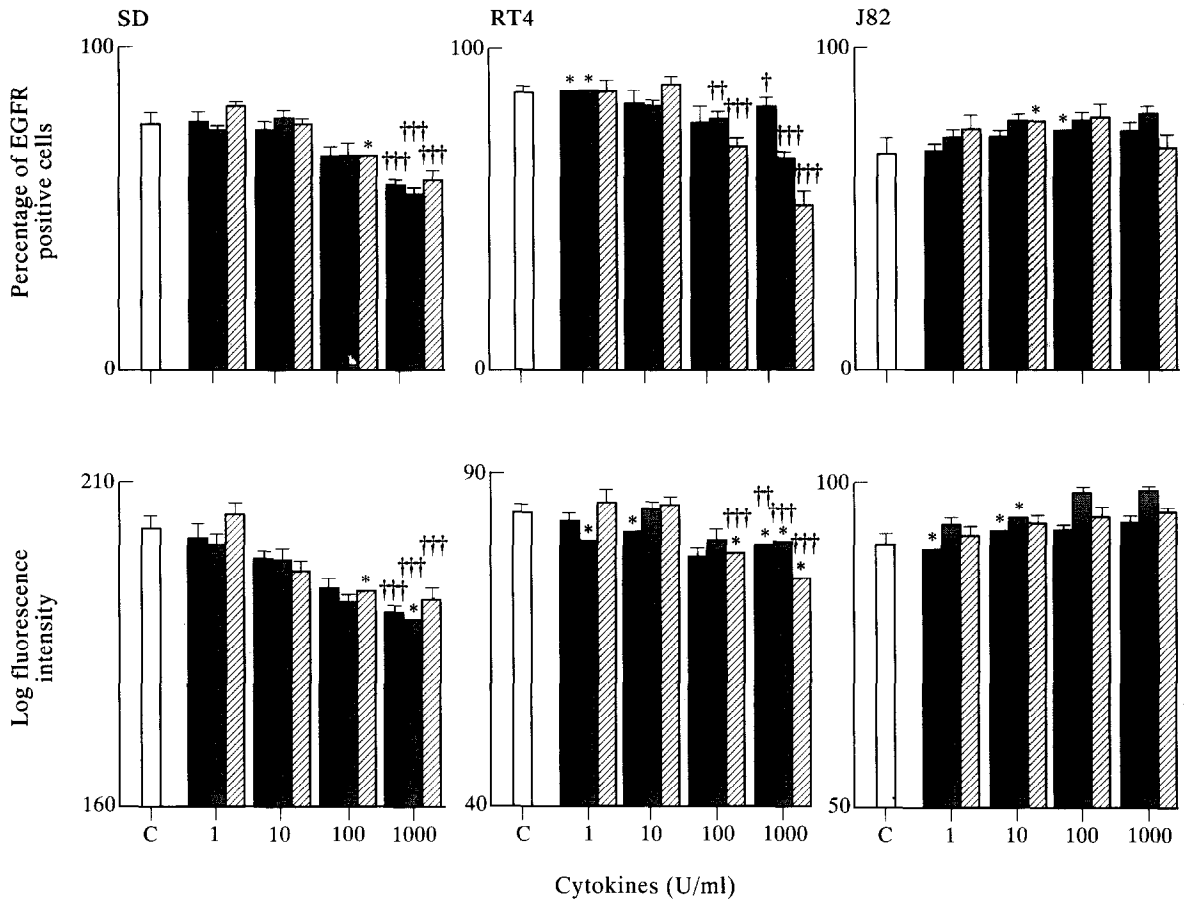

Figure 2. The effect of the combinations of cytokines on EGFR expression on bladder tumour cell lines SD (a, b), RT4 (c, d), J82 (e, f) TCC cell lines. Cells were incubated with cytokines (IFNγ(shaded black), IFNγ and TNFα(shaded grey), IFNγ, TNFα and IL-1α(hatched)) at the indicated concentrations or complete medium (C(□)) for 24 h and analysed using flow cytometry. Bars indicate the mean of at the least triplicate determinations and error bars indicate the level of 1 standard deviation. * indicates a standard deviation of less than 0.5%. † shows a level of significance less than 0.05; ††, 0.005; †††, 0.0005 (versus control). Notably, the combination of cytokines markedly down-regulated EGFR on the RT4 cell line.

or without cytokines was replaced every 48 h. After the time indicated, cells were recovered using trypsin/EDTA. All experiments for responding cell lines (RT4, SD) were performed at least three times in triplicate.

Single colour immunofluorescence was performed on bladder cancer cell lines stained indirectly with monoclonal antibodies as described previously [30]. Non-viable cells were gated out of the window and at least 5000 events were accumulated using linear amplification of fluorescence on Coulter EPICS-C. The growth rate was assessed by counting cells following recovery from tissue culture plates using trypsin/EDTA.

Statistical analysis

Statistical analysis was performed using the unpaired *t*-test and other statistical functions provided in Statview 512 and Cricket Graph III software, using a Macintosh computer.

RESULTS

Constitutive expression of EGFR

We have investigated whether cytokine modulation of EGFR plays any role in the antitumour actions of BCG immunotherapy. We studied the effect of IFNγ, TNFα and IL-1α alone and together on EGFR expression by TCC cell lines. All eight cell lines constitutively expressed high levels of EGFR (Table 1). However, the level of its expression failed to correlate with the histopathological grade of cells or with the growth rate of cells.

Modulation of EGFR on TCC cells by IFNγ, TNFα, IL-1α

In two out of eight cell lines IFNγ induced a dose-dependent reduction in EGFR expression (Figure 1, Tables 2 and 3). The maximal effect was detected with 1000 U/ml of IFNγ. For SD cell lines the marked decrease of EGFR expression was observed as a decrease in both the percentage of positive cells and the fluorescence intensity. However, modulation of EGFR on RT4 cells was mainly in the change of fluorescence intensity. In contrast, TNFα moderately but significantly enhanced EGFR expression on SD and J82 cell lines ($P_2 < 0.05$ and $P_2 < 0.0004$, respectively), whilst IL-1α failed to modulate EGFR expression on any cell line.

Taking into account that during immunotherapy a "cocktail" of cytokines is observed we investigated whether a combination of cytokines was effective in modulating EGFR expression. The combination of TNFα and IFNγ significantly decreased EGFR expression on the RT4 cell line (Figure 2). A further reduction in the number of positive cells and the intensity of fluorescence was noted on the simultaneous addition of all three cytokines. This effect was apparent with a cytokine concentration of 100 U/ml. Modulation of EGFR expression on RT112 by IFNγ and TNFα also was significant ($P < 0.03$; data not shown). Combined stimulation with all three cytokines did not affect EGFR levels on the remaining five cell lines.

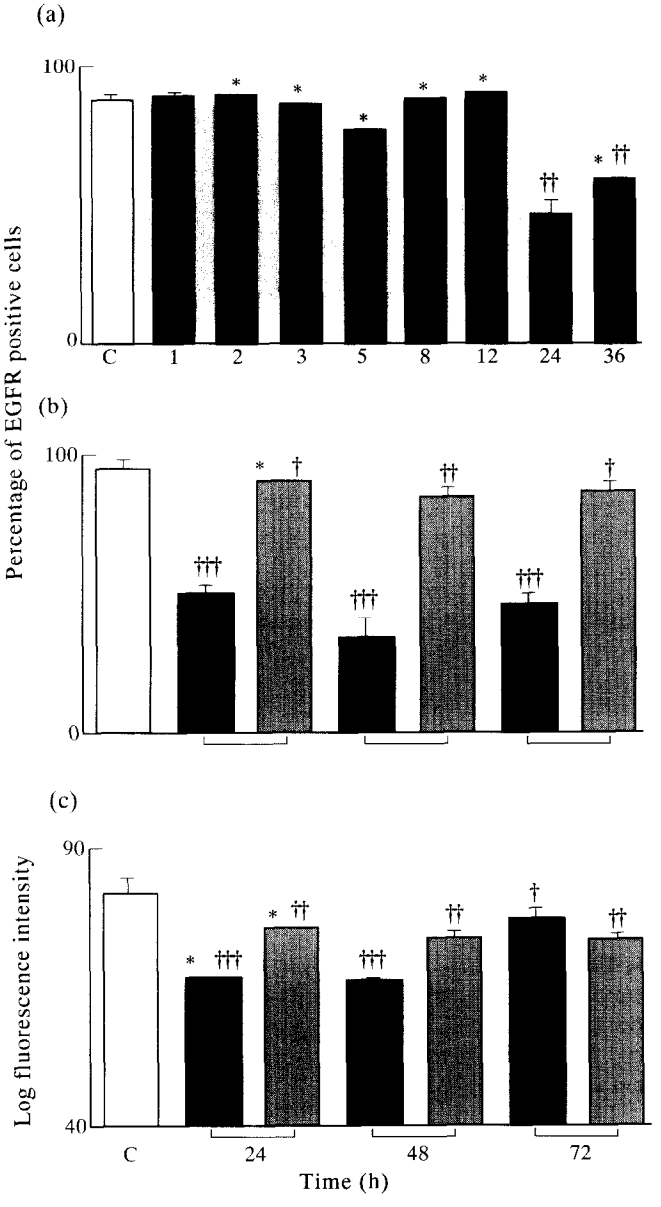

Figure 3. The kinetics of the modulation of EGFR expression by cytokines on the RT4 TCC cell line following 1–36 h (a) and 24–72 h (b, c) incubation with cytokines. Cells were incubated with complete medium (C(□)) or cytokines (a mixture of IFNγ, TNFα and IL-1α(shaded black)), or IFNγ alone (shaded grey) (concentration 1000 U/ml) for the indicated period of time, recovered and analysed using flow cytometry. Bars indicate the mean of at least triplicate determinations and error bars indicate the level of 1 standard deviation. * indicates a standard deviation of less than 0.5%. † shows a level of significance less than 0.05; ††, 0.005; †††, 0.0005 (versus control). Note, the maximal decrease of EGFR expression occurs after 24 h of stimulation.

The kinetics of EGFR expression modulation

The kinetics of EGFR modulation on RT4 and SD cells was studied. Cells were stimulated with a mixture of IFNγ, TNFα, IL-1α (1000 U/ml each) for the period of time indicated. The effect was maximal after 24 h of continuous incubation ($P < 0.005$; Figure 3a). The change in fluorescence intensity mirrored the change in the percentage of cells which expressed EGFR ($P < 0.0001$; data not shown). We demonstrated elsewhere that the cytostatic effect of IFNγ and BCG-micro-organisms occurred within first 48 h of incubation [11]; therefore, we investigated whether cytokines modulated receptor expression with prolonged incubation. No further decrease of EGFR expression was observed with prolonged incubation (up to 72 h) with either the mixture of cytokines or IFNγ alone (Figure 3b,c). These were similar for RT4 and SD cell lines (data not presented).

Effect of activation of PKC and inhibition of protein synthesis on EGFR expression

To further evaluate the possible mechanism of reduced expression of EGFR, we performed studies using PMA which activates protein kinase C (PKC). We previously showed that BCG microorganisms and PMA (a potent activator of PKC-dependent signalling pathways) induce ICAM-1 on a number of TCC cell lines (MGH-U1, EJ18, SD, J82 [30] and unpublished data). Surprisingly, all cell lines which were susceptible to cytokines (RT4, RT112, SD), but not the unresponsive cell lines, responded to PMA with the percentage of EGFR-positive cells and fluorescence intensity being reduced (data not shown). Moreover, for two cell lines (RT4, RT112) the change of EGFR expression by PMA was greater than with cytokines. Notably, high grade cell lines (SD and RT112) responded to a lesser extent than low grade cells (RT4). The effect of PMA on EGFR expression was dose and time-dependent becoming significant at the concentration 0.1 ng/ml and maximal at 100 ng/ml (Figures 4 and 5). Interestingly, the kinetics of the modulation of EGFR expression by PMA were different from that by cytokines, being faster and evident after 1 h.

We also investigated whether protein synthesis was involved in the modulation of EGFR expression by cytokines or PMA. On two cell lines, RT4 and SD, pretreatment of cells with cycloheximide prior to stimulation with the cytokines or PMA (in the concentration previously demonstrated to stop protein synthesis [6, 8]) failed to prevent modulation of EGFR expression (data not shown). Finally, as we have recently shown that BCG micro-organisms directly exert a potent cytostatic effect on the panel of TCC cell lines used [11] we decided to examine whether they modulate EGFR expression. Stimulation with the optimal concentration of viable BCG micro-organisms for 24 h failed to decrease EGFR expression on RT4 to a significant extent (data not shown).

DISCUSSION

Although BCG immunotherapy for superficial bladder cancer is highly effective, the precise mechanisms involved remain to be elucidated. Overexpression of EGFR is a strong negative prognostic factor associated with high proliferation of tumour cells and invasive disease, recurrence and death [13, 14]. Previously, we have shown that IFNγ and TNFα may exert their potent cytostatic effect on bladder cancer cells by decreasing EGFR expression [25]. In the present study we confirm these findings and demonstrate that (1) the combination of IFNγ, TNFα and IL-1α cytokines may have a greater effect; and (2) that cytokines may exert their effect through PKC-dependent pathways, yet independently of protein synthesis. Although we have shown elsewhere that IFNγ had a strong cytostatic effect on all eight bladder cancer cell lines [11], in the present study it decreased receptor expression on only two out of eight cell lines.

In patients receiving intravesical BCG therapy for TCC we observed the production of a mixture of cytokines (IFN-γ, TNF-α, IF-1, IL-2, IL-5, IL-6, IL-10, IL-12, etc [33–35] and

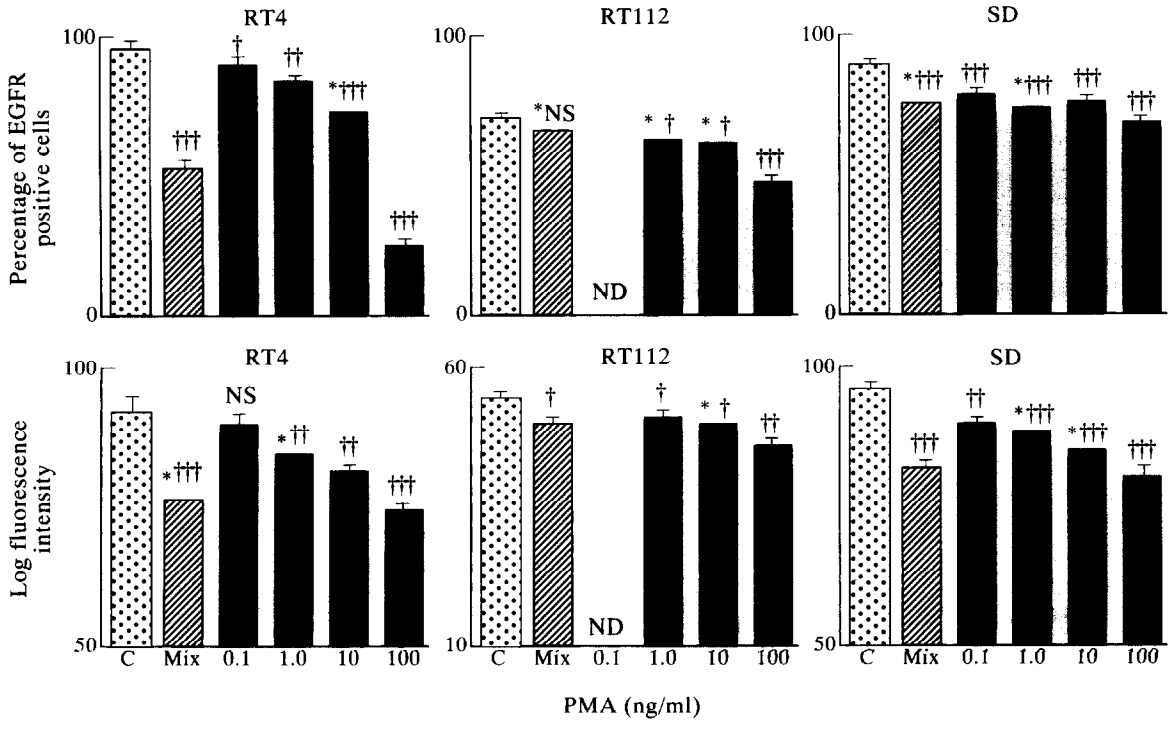

Figure 4. The dose-dependent decrease of EGFR expression by PMA on RT4, RT112 and SD bladder cancer cell lines. Cells were continuously incubated with complete medium (C(dotted)), a mixture of IFNγ, TNFα and IL-1α (1000 U/ml)(Mix, hatched), or PMA at the concentrations indicated (shaded black) for 24 h, recovered and analysed with the aid of flow cytometry. Bars indicate the mean of at least triplicate determinations and error bars indicate the level of 1 standard deviation. * indicates a standard deviation of less than 0.5%; ND, no data obtained; † shows a level of significance less than 0.05; ††, 0.005; †††, 0.0005 (versus control); NS, the change failed to reach a significant level.

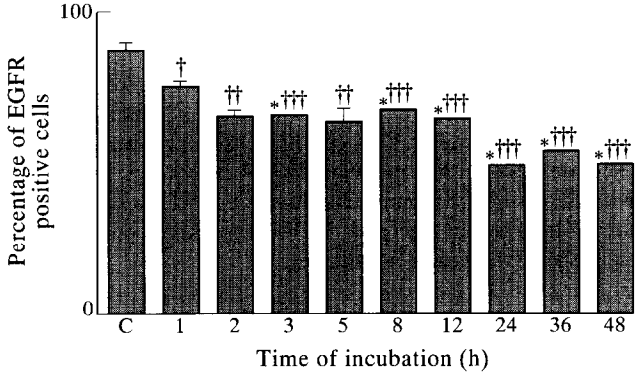

Figure 5. The kinetics of the modulation of EGFR expression by PMA on the grade 1 (RT4) TCC cell line. Cells were incubated with complete medium (C) or PMA (100 ng/ml) for the indicated period of time, recovered and analysed using flow cytometry. Bars indicate the mean of at least triplicate determinations and error bars indicate the level of 1 standard deviation. * indicates a standard deviation of less than 0.5%, † shows a level of significance less than 0.05; ††, 0.005; †††, 0.0005 (versus control).

unpublished data). The combination of cytokines used in this study also was more effective than IFNγ alone, however, the mixture of cytokines showed a marked decrease of the EGFR again only on three out of eight cell lines. BCG micro-organisms recently shown to have a potent cytostatic effect [11] also failed to modulate EGFR expression on cancer cells. Collectively, these observations suggest that cytokines produced during immunotherapy may exert their direct cytostatic/cytotoxic effects on tumour cells through mechanisms independent of decrease of EGFR expression. Our data agree with the recent findings of Flam and colleagues who using an immunohistochemical method reported no difference in the EGFR expression between responders and non-responders during immunotherapy [38], although their data possibly can be re-evaluated using more quantitative approaches.

The way in which cytokines decrease EGFR expression remains unknown. Currently we are expanding our study to see whether the effect of cytokines involves biosynthesis, internalisation or shedding of EGFR. Recently apoptotic death has been demonstrated in bladder tumours ([39], S. Paulie, personal communication). It should be noted that the observed kinetics of cytotoxicity of TCC cells mediated by immunocompetent cells and cytokines were different [9, 26]. It is possible that TCC cells may undergo apoptosis following cytokine treatment. In this case apoptotic cells may be gated out of the fluorescent studies that subsequently could affect the results. We are currently investigating this theory.

Taking into account that TNFα is constitutively produced by three cell lines (SD, EJ18, MGH-U1) and that a few cytokines (e.g. GM-CSF and G-CSF) have been shown to promote the growth of some bladder cancer cells, it is of potential interest that TNFα can upregulate EGFR expression, suggesting its function as a growth factor for some bladder neoplasms ([27], unpublished data). Ourselves and other groups have recently demonstrated spontaneous production of other cytokines (IL-1, IL-6, IL-8 etc.) by TCC cells and, consequently, it was proposed that some may act as autocrine growth factors ([30–32, 40] and unpublished data). Our further studies revaled that IL-1, IL-6 and IL-8 did not promote the growth of TCC cells and that neutralising antibodies to IL-1α and Il-1β also failed to modulate

the growth pattern of tumour cells ([11] and unpublished data). The contradictory results in respect to IL-1α action may represent a difference of cell lines in different laboratories [11, 37]. However, if these pro-inflammatory cytokines do not serve as growth factors, it is tempting to speculate that they may benefit tumours without directly modulating growth. This may occur through enhancement of metastasis (possibly associated with upregulation/activation of adhesion molecules on endothelium and/or cancer cells [41–43]), increase in resistance of antineoplastic antibiotics [44] or through the elimination of gamma/delta T lymphocytes (due to up-regulation of ICAM-1 [5, 30, 45]).

Finally, one of the "shortfalls" of the present study is a lack of data on the possible functional effect of decreased EGFR expression, i.e., response of cell lines to EGF and TGFα following cytokine treatment. However, the authors found it difficult to separate cytokine effects which are dependent and independent of EGFR modulation.

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