Growth inhibition of human lung adenocarcinoma cells by antibodies against epidermal growth factor receptor and by ganglioside $G_{M3}$: involvement of receptor-directed protein tyrosine phosphatase(s)

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Summary Growth of the EGF receptor-expressing non-small-cell lung carcinoma cell line H125 seems to be at least partially driven by autocrine activation of the resident EGF receptors. Thus, the possibility of an EGF receptor-directed antiproliferative treatment was investigated in vitro using a monoclonal antibody (oEGFR ior egfr/3) against the human EGF receptor and gangliosides which are known to possess antiproliferative and anti-tyrosine kinase activity. The moderate growth-inhibitory effect of oEGFR ior egfr/3 was strongly potentiated by the addition of monosialoganglioside $G_{M3}$. Likewise, the combination of oEGFR ior egfr/3 and $G_{M3}$ inhibited EGF receptor autophosphorylation activity in H125 cells more strongly than either agent alone. A synergistic inhibition of EGF receptor autophosphorylation by oEGFR ior egfr/3 and $G_{M3}$ was also observed in the human epidermoid carcinoma cell line A431. In both cell lines, the inhibition of EGF receptor autophosphorylation by $G_{M3}$ was prevented by pretreatment of the cells with pervanadate, a potent inhibitor of protein tyrosine phosphatases (PTPases). Also, $G_{M3}$ accelerated EGF receptor dephosphorylation in isolated A431 cell membranes. These findings indicate that $G_{M3}$ has the capacity to activate EGF receptor-directed PTPase activity and suggest a novel possible mechanism for the regulation of cellular PTPases.

Keywords: epidermal growth factor receptor; monoclonal antibody; ganglioside $G_{M3}$; growth inhibition; protein tyrosine phosphatases

Epidermal growth factor (EGF) is a 6-kDa peptide (Cohen and Carpenter, 1975) that binds to a 170-kDa transmembrane receptor (EGFR) with intrinsic tyrosine kinase activity (Ullrich and Schlessinger, 1990). Ligand-induced receptor dimerization and autophosphorylation are essential subsequent steps for the initiation of intracellular events, which ultimately lead to EGF-induced cell division. The autophosphorylation of the EGFR is rapidly reverted by cellular PTPases, the identity of which remains to be established (Swarup et al., 1982; Charbonneau and Tonks, 1992; Faure et al., 1992; Pot and Dixon, 1992; Böhmer et al., 1993, 1995). Apparently, different PTPases, including those of cytosolic or transmembrane type, have the capacity to dephosphorylate autophosphorylated EGFR (Hashimoto et al., 1992; Lammers et al., 1993). Recently, the SH2-domain PTPase, PTP1C, has been shown to associate with the EGFR and to dephosphorylate it in A431 cells and in 293 cells overexpressing PTP1C and EGFR (Tomic et al., 1995). Receptor dephosphorylation is considered a major mechanism of negative regulation of receptor activity.

A relation between the EGF/EGFR system and malignant cell transformation has been well established in experimental systems (Downward et al., 1984; Hayman et al., 1985; Derynck et al., 1987; Di Fiore et al., 1987; Velu et al., 1987). More importantly, EGFR expression in human breast tumours has been correlated with a poor prognosis (Perez et al., 1984; Sainsbury et al., 1985; Macias et al., 1986, 1987a, b; Klijn et al., 1992), and a link between EGFR activity and the malignant process has also been suggested for a number of other epithelial tumours, including non-small-cell lung cancer (NSCLC) (Khaize et al., 1993; Modjtahedi and Dean, 1994; Fontanini et al., 1995). Therefore, the evaluation of the EGF/EGFR system as a potential target for tumour therapy is highly warranted (Baselga et al., 1994; Basalga and Mendelsohn, 1994). Different strategies have been employed to block EGFR signalling. Paradoxically, EGF itself can be inhibitory for cell growth under certain conditions (Barnes, 1982; Lombardero et al., 1986; Kamata et al., 1986). Although the exact mechanism of this effect remains elusive, this principle was used successfully in a pilot clinical trial for treatment of skin epidermoid carcinoma (Fonsecas et al., 1988). Attempts to design on a peptide basis EGF antagonists that block EGF binding to its receptor have up to now had little success (Groenen et al., 1994). Alternatively, anti-receptor antibodies have been generated in a number of laboratories and investigated with respect to a potential therapeutic application (Mueller et al., 1991; Fernandez et al., 1992; Fong et al., 1992; Modjtahedi et al., 1993; Reins et al., 1993; for a review see Baselga and Mendelsohn, 1994b). Although some of the clinical data obtained so far are encouraging, the general impression emerges from these studies that antibody treatment alone will not be sufficient to combat EGF-driven tumours, but will need combination with further antiproliferative agents (Baselga and Mendelsohn, 1994b). A further strategy to block EGFR signalling would be the specific inhibition of receptor tyrosine kinase activity (Gibbs and Oliff, 1994; Levitzki and Gazit, 1995). Recent efforts to obtain synthetic EGFR kinase inhibitors yielded quite potent and specific compounds, which might lead to useful pharmacological agents in
the future. Among various naturally occurring tyrosine kinase inhibitors, the ganglioside, $G_{M3}$, has been reported to attenuate EGFR signalling (Bremer et al., 1986; Hanai et al., 1988; Weis and Davis, 1990; Zhou et al., 1994); however, the mechanism of this effect is not well understood. Gangliosides are of low general toxicity and often have pronounced antiproliferative and differentiation-inducing properties (Hakomori, 1993; Svennerholm, 1994). In the current study, we therefore explored the possibility that a combined action of ganglioside $G_{M3}$ and an anti-EGF receptor antibody, designated αEGFR ior egfr/3 (Fernandez et al., 1992), would lead to a more pronounced inhibition of the proliferation of EGFR-expressing tumour cell lines. We demonstrate a synergistic effect of αEGFR ior egfr/3 and $G_{M3}$ on EGFR signalling activity and growth in H125 human NSCLC cells. Furthermore, our data suggest that activation of EGFR-directed PTPases by $G_{M3}$ might, at least in part, constitute the mechanism underlying the inhibition of EGFR activity by $G_{M3}$.

**MATERIALS AND METHODS**

**Cells and reagents**

Eleven different cell lines derived from human lung tumours were generously provided by Drs Gazit and Levitzki (Jerusalem, Israel), (Fry et al., 1994; Osherov and Levitzki, 1994; Levitzki and Gazit, 1995). They were dissolved in dimethyl sulfoxide (DMSO); for application in cell culture, the final DMSO concentration in the assays was ≤0.1%, only DMSO of the same concentration was included in the corresponding controls. Pervanadate was prepared from sodium orthovanadate stocks and hydrogen peroxide as described by Pumiglia et al (1992).

**EGF receptor assay**

The amount of EGF receptor in the various lung cancer cell lines was measured as previously described (Macias et al., 1986). Briefly, cells were seeded in 24-well plates (Costar) at $5 \times 10^4$ cells per well. On the next day, the culture medium was removed and the plates were washed three times with PBS containing 10 mM magnesium chloride and 10 mM calcium chloride. Then the cells were incubated with approximately 10$^6$ c.p.m. of $^{[125]}$EGF (150–200 μCi μg$^{-1}$) in the absence or presence of different concentrations of unlabelled EGF in 0.5 ml of 10 mM Tris-HCl, pH 7.4, 10 mM magnesium chloride, 0.1% bovine serum albumin (BSA) (binding buffer) for 1 h at room temperature. Thereafter, the cells were washed with binding buffer and the bound radioactivity was recovered with 100 μl of 1M sodium hydroxide and measured in a gamma counter. Non-specific binding was estimated in the presence of 1000 ng ml$^{-1}$ EGF. Data were analysed according to Scatchard’s method (Scatchard, 1949).

**Growth assay**

H125 cells were seeded in 24-well plates at $5 \times 10^4$ cells per well. One day later, the medium was replaced with RPMI medium containing 2% fetal bovine serum (PBS) and the desired effectors. This medium was changed every 2 days including renewal of effectors. At the desired time points, cell layers were washed with PBS and dissolved with 300 μl of 1.5 M sodium hydroxide for 1 h at room temperature, and the total amount of protein was determined. Alternatively, cells were labelled with $[^3]H$thymidine (1 μCi ml$^{-1}$, 2 μM thymidine) for 18–24 h. Cell protein content and $[^3]H$thymidine incorporation were linearly correlated under standard growth conditions of H125 cells.

**EGFR autophosphorylation and dephosphorylation assays**

Subconfluent cultures of H125 or A431 cells in six-well plates (Falcon) were treated overnight with serum-free medium. The medium was changed and the desired agents were added as described in the figure legends. Then the cells were stimulated with 1 μg ml$^{-1}$ EGF for 1 min at room temperature or left unstimulated. Cell extracts were prepared as described (Tomic et al., 1995), using 200 μl of lysis buffer per well. About 20 μg or 5 μg of protein per lane of H125 or A431 cell extract, respectively, was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with anti-phosphotyrosine antibodies (RC20-peroxidase conjugate, Transduction Laboratories) and ECL (Amersham) detection. Quantification was performed by densitometric scanning of the films and analysis using the program NIH image 1.52. To evaluate the amount of EGFR analysed, the blots were stripped and reprobed with the
Table 1  EGF receptor expression in human lung cancer cell lines as revealed by a radio receptor assay

| Cell line     | Amount of receptor* (sites per cell) | Dissociation constant* (Kd, nM) |
|---------------|-------------------------------------|---------------------------------|
| H125 (ADC)    | 2.1±0.20 x 10^4                     | 1.7±0.33                        |
| H23 (ADC)     | 2.9±1.90 x 10^4                     | 6.0±1.40                        |
| H661 (LCC)    | 2.4±0.45 x 10^4                     | 1.2±0.09                        |
| H157 (LCC)    | 7.0±2.80 x 10^4                     | 1.4±0.56                        |
| U1810 (LCC)   | 1.3±0.26 x 10^4                     | 1.7±0.26                        |
| U1906 (SQC)   | 2.5±1.44 x 10^4                     | 2.5±1.30                        |
| U1690         | 2.5±1.44 x 10^4                     | 2.5±1.30                        |
| U2020         | 2.5±1.44 x 10^4                     | 2.5±1.30                        |

*[^125I]EGF binding was measured by a standard technique as described under Materials and methods, and the data were analysed according to Scatchard (1949). NSCLC, non-small cell lung carcinoma; SCLC, small-cell lung carcinoma; ADC, lung adenocarcinoma; LLC, lung large-cell carcinoma; SQC, squamous cell carcinoma.

Figure 1  Effect of EGF on the growth of H125 NSCLC cells. H125 cells were cultured for 7 days in the absence or presence of different concentrations of EGF as indicated. Thereafter, the total amount of cells was measured by protein determination as described in Materials and methods. Data points are the means ± s.d. of triplicates calculated as a percentage of control.

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RESULTS

EGF receptor expression in lung cancer cell lines

In order to choose a suitable in vitro system for evaluation of EGFR-directed antiproliferative treatments, different lung cancer cell lines were compared for expression of EGFR using a binding assay with [^125I] EGF. The results are summarized in Table 1. Except H661 cells, all other NSCLC cell lines tested exhibited moderate to high levels of EGFR expression. Among the SCLC cell lines tested, only U1690 revealed EGFR expression as detectable with this assay. Therefore, most of the further investigation was performed with H125 cells, which express 2.1 x 10^4 EGFR sites per cell.

Growth modulation of H125 cells by EGF and EGFR-specific tyrosine kinase inhibitors

When H125 cells were treated with EGF at 10, 100 and 1000 ng ml^{-1} in the presence of 1% fetal calf serum (FCS), the growth rate increased in a dose-dependent manner (Figure 1), albeit to a moderate extent. The relatively low potency of exogenously added EGF might be due to the constitutive presence of endogenous TGFs, which is known to be expressed in H125 cells (Söderblad et al., 1988). In order to test whether an autocrine pathway involving the EGFR contributes to the growth of these cells, the effect of a recently discovered highly specific EGFR tyrosine kinase inhibitor, designated AG1478 (Osherov and Levitzki, 1994; Levitzki and Gazit, 1995), was tested. H125 cells were cultured in the absence or presence of AG1478. AG1478 treatment effectively inhibited stimulation of EGFR autophosphorylation by exogenously added EGF (Figure 2A). Culturing H125 cells in the presence of AG1478 and in the absence of exogenously added EGF reduced the growth rate of the cells to about 69% in a concentration as low as 0.1 μM and to 3% at 1 μM (Figure 2B). In contrast, H661 NSCLC cells, which have undetectable EGFR levels (Table 1), are resistant to treatment with AG1468, indicating that the effect of H125 is specific and non-toxic. Identical results were obtained with another EGFR tyrosine kinase inhibitor, AG1517 [PD 153035 (Fry et al., 1994)] (Figure 2B). These findings suggest a partial dependence of H125 cell growth on EGFR signalling even in the absence of exogenously added EGF.

Growth modulation of H125 cells by anti-EGF receptor antibody αEGFR ior egfr3 and ganglioside G_{13}

The previously described anti-EGFR receptor antibody, αEGFR ior egfr3 (Fernandez et al., 1992), is known to inhibit EGF binding and EGFR signalling, and we wished to explore its potential usefulness for growth inhibition of EGFR-expressing NSCLC. As shown in Figure 3A, αEGFR ior egfr3 inhibited the growth of H125 cells. Whereas 10 μg ml^{-1} αEGFR ior egfr3 used in this assay only had a small inhibitory effect, the cell growth rate was reduced to 50% and 41% at 30 and 100 μg ml^{-1} respectively. The addition of an unrelated murine monoclonal antibody (αCD3 ior i-3) with identical isotype had no effect on proliferation of H125.
concentrations were antibodies. (Figure 3) Effect of the monoclonal anti-EGF receptor antibody αEGFR ior egfr3 on growth and EGFR autophosphorylation of H125 cells. (A) H125 cells were cultured for 7 days in the absence or presence of different concentrations of the monoclonal antibodies as indicated. Thereafter, the total amount of cells was measured by protein determination. Data points are means ± s.d. of triplicates calculated as a percentage of control. (B) Serum-deprived subconfluent H125 cells were treated with different concentrations of AG1478 or AG1517 as indicated and then [3H]thymidine incorporation was measured. Data points are the means ± s.d. of triplicates calculated as a percentage of control.

Figure 2 Effect of the specific EGF receptor tyrosine kinase blockers on EGFR autophosphorylation and growth of H125 cells. (A) Serum-deprived subconfluent cultures of H125 cells were treated with different concentrations of AG1478 for 2 h. Thereafter, the cells were stimulated with EGF (or not, as indicated), cell extracts were prepared and the EGFR phosphorylation was analysed by SDS-PAGE and immunoblotting with antiphosphotyrosine antibodies. (B) H125 cells or the EGFR receptor-negative NSCLC H661 cells were cultured for 3 days in the absence or presence of different concentrations of AG1478 or AG1517 as indicated and then [3H]thymidine incorporation was measured. Data points are the means ± s.d. of triplicates calculated as a percentage of control.

cells. Similar results were obtained with other EGFR-positive cell lines, such as H157 and U1752 (data not shown).

We then tested the possibility of enhancing the antiproliferative effect of αEGFR ior egfr3 by simultaneous addition of ganglioside G3M, G3M at 0.5 and 5 μM had little effect on H125 cell growth (Figure 4A) and inhibited growth partially at 20 μg ml⁻¹. However, when combined with αEGFR ior egfr3, 5 μM ganglioside, which alone was ineffective, potentiated the inhibitory effect produced by αEGFR ior egfr3 and reduced growth to about 12% of control. G3M (20 μM) plus αEGFR ior egfr3 almost completely inhibited cell growth and partially resulted in cell death. This cytotoxic effect was strictly mediated by EGFR, because the growth of the EGFR-negative cell line H661 was not significantly affected by the combination of αEGFR ior egfr3 and 5 μM G3M (Figure 4B). Also, the synergistic growth inhibition was specific for G3M, since De-N-acetylganglioside at 20 μM alone had only a slight effect on H125 cell growth and did not further enhance the growth inhibition exerted by αEGFR ior egfr3 (Figure 4A). A very similar synergistic growth inhibition of H125 cells was also observed with the combination of another anti-EGFR antibody, αEGFR mab425, and G3M. Also, αEGFR mab425 and G3M synergistically inhibited growth of the EGFR-expressing NSCLC cell line, U1752 (data not shown).

Effect of the anti-EGF receptor antibody, αEGFR ior egfr3, and ganglioside G3M on EGFR signalling activity

To evaluate whether the enhanced growth inhibition of H125 cells exerted by αEGFR ior egfr3 in the presence of the ganglioside G3M is mediated by an enhanced inhibition of EGFR signalling activity, the effect of both agents on EGFR autophosphorylation was investigated. Intact H125 cells were pretreated with αEGFR
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ior egf/r3, G₄₃ or both. Thereafter, the cells were stimulated with EGF, lysed and the extent of EGFR autophosphorylation was measured by immunoblotting with anti-phosphotyrosine antibodies. EGFR autophosphorylation was inhibited by αEGFR ior egf/r3 in a dose-dependent manner (Figure 3B). Treatment of the cells with 50 μg ml⁻¹ αEGFR ior egf/r3 resulted in a reduction of EGFR autophosphorylation to 43% (Figure 4C, upper panel). G₄₃ treatment of the cells alone had little effect at 20–50 μM G₄₃ and required as much as 200 μM G₄₃ to obtain a reduction in receptor autophosphorylation to 47%. This finding is in accordance with earlier observations, indicating that rather high concentrations of G₄₃ are required to inhibit EGFR signalling activity in A431 cells (Zhou et al., 1994). Combined treatment of the cells with αEGFR ior egf/r3 and G₄₃ drastically reduced EGFR autophosphorylation, already at 20 μM G₄₃ to 28% and down to 17% at 200 μM. The level of EGFR protein was essentially unaffected by the different treatments (Figure 4C, lower panel). Thus, the combined action of αEGFR ior egf/r3 and G₄₃ leads to an inhibition of EGFR autophosphorylation, which is qualitatively matching the observed synergistic inhibition of H125 cell growth.

For comparison, the effects of G₄₃ and αEGFR ior egf/r3 on EGFR autophosphorylation in A431 human epidermoid carcinoma cells were investigated. As shown in Figure 5A, similarly to H125 cells, combined treatment of A431 cells with G₄₃ and αEGFR ior

Figure 4 Effect of the combination of ganglioside G₄₃ and the monoclonal anti-EGF receptor antibody αEGFR ior egf/r3 on EGFR autophosphorylation and growth of H125 cells. (A) H125 cells were treated without ganglioside (control) [], or with 0.5 μM G₄₃ [▲], 5 μM G₄₃ [○], 20 μM G₄₃ [●], 20 μM De-N-acetyl G₄₃ [■], in the presence or absence of anti-EGF receptor antibody αEGFR ior egf/r3 as indicated for 7 days. Thereafter, the total amount of cells was measured as described in Figure 1. Data points are the means ± s.d. of triplicates calculated as a percentage of control. (B) the same assay as in A with the EGFR receptor-negative NSCLC cell line H661. (C) Serum-deprived subconfluent cultures of H125 cells were treated with ganglioside G₄₃ or the anti-EGF receptor antibody αEGFR ior egf/r3 or the combination of both as indicated for 2 h. Thereafter, the cells were stimulated with EGF or not as indicated, extracted and the EGFR phosphorylation was analysed by SDS-PAGE and immunoblotting with antiphosphotyrosine antibodies (upper panel). The numbers underneath the lanes represent the percentage of the autophosphorylation signal compared with the control in the absence of G₄₃ or αEGFR ior egf/r3, as revealed by densitometric scanning. The blot was stripped and reprobed with anti-EGFR-CT antibodies to verify that similar amounts of receptor are present in the individual lanes (lower panel). (D) H125 cells were treated for 30 min with pervanadate or not (as indicated) and subsequently with G₄₃ at different concentrations. Then, EGFR receptors were immunoprecipitated from cell extracts and the tyrosine phosphorylation state was evaluated by immunoblotting as in C.

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were with ganglioside cells. Ganglioside described Figure and B; stimulated EGF-stimulation dephosphorylation autoradiography band. Itg GM3 (jiM) for addition of Materials and methods. The initiatied EGF-11, serum-deprived as Fig 6B. Also, GM3-mediated inhibition of EGF autophosphorylation in H125 cells is prevented by pervanadate treatment (Figure 4D). These findings indicate that activation of EGF-directed PTPases rather than direct tyrosine kinase inhibition by GM3, might at least contribute to the observed attenuation of EGF autophosphorylation. To test directly whether GM3 has the capacity to activate EGF-directed PTPase activity, in vitro PTPase assays employing A431 cell membranes were carried out. Membranes were pretreated with GM3 or vehicle, the EGF was stimulated and autophosphorylation was allowed to occur in the presence of [γ32P]ATP. The autophosphorylation was quenched by addition of EDTA, and EGF dephosphorylation by the endogenous PTPases was monitored by analysis of the receptor phosphorylation state at different time points thereafter. As shown in Figure 6, EGF dephosphorylation is clearly accelerated in the presence of 60 μM GM3.

**DISCUSSION**

Lung cancer cells, in particular NSCLC, frequently express EGFR. Thus, although the prognostic significance of EGFR expression in lung carcinoma is currently controversial (Modjtabadi and Dean, 1994), the EGFR might present a target for antiproliferative treatment. We compared a limited number of lung carcinoma cells for expression of EGFR by a ligand-binding assay. In accordance with previous observations, five out of six NSCLC cell lines contained measurable levels of EGFR sites per cell, while only one out of five SCLC cell lines was EGFR positive. H125, an NSCLC line with about 2 x 10^5 EGFR receptors per cell, was chosen to investigate the antiproliferative potency of an EGFR-blocking monoclonal antibody (αEGFR 1046) towards NSCLC. Apparently, the growth of this cell line is at least partly dependent on autocrine activation of the EGFR, since: (1) the cells are known to express TGFα (Söderdahl et al., 1988) in addition to EGFR; (2) the cells respond only moderately to exogenous EGF; and (3) most importantly, the cells are growth inhibited by very low doses of highly specific EGFR tyrosine kinase inhibitors, whereas NSCLC cells not expressing EGFR were completely refractory to the drugs. The anti-EGF receptor antibody, αEGFR 1046, reduced the growth of H125 cells under the same culture conditions to 41%, without cytotoxic effect. These results match well with those from ongoing in vivo studies with αEGFR 1046. No relevant toxic effects have been found; however, the cytostatic effects on the tumours observed so far are only moderate (unpublished data). Similar results have been reported for other studies employing EGFR-blocking antibodies (Baselga and Mendelsohn, 1994b), suggesting the need to combine this treatment with another antiproliferative principle. We therefore

egr/f3 inhibits EGFR autophosphorylation. In comparison with H125 cells, αEGFR 1046 alone was less effective, and GM3 was more effective in inhibiting EGFR activity in A431 cells. The combined inhibition of receptor autophosphorylation by both agents was more pronounced in A431 than in H125 cells (Figures 4B and 5A).

**GM3 activates EGFR-directed PTPase activity**

Interestingly, when the A431 cell treatment was performed in the presence of pervanadate, a potent inhibitor of PTPases (Pumiglia et al, 1992), the inhibition of EGFR autophosphorylation by GM3, as well as the synergistic inhibition of EGFR autophosphorylation by GM3 and αEGFR 1046, was abolished (Figure 5C). Also, GM3-mediated inhibition of EGFR autophosphorylation in H125 cells is prevented by pervanadate treatment (Figure 4D). These findings indicate that activation of EGF-directed PTPases rather than direct tyrosine kinase inhibition by GM3, might at least contribute to the observed attenuation of EGFR autophosphorylation. To test directly whether GM3 has the capacity to activate EGF-directed PTPase activity, in vitro PTPase assays employing A431 cell membranes were carried out. Membranes were pretreated with GM3 or vehicle, the EGF was stimulated and autophosphorylation was allowed to occur in the presence of [γ32P]ATP. The autophosphorylation was quenched by addition of EDTA, and EGF dephosphorylation by the endogenous PTPases was monitored by analysis of the receptor phosphorylation state at different time points thereafter. As shown in Figure 6, EGF dephosphorylation is clearly accelerated in the presence of 60 μM GM3.

**Figure 5** Synergistic inhibition of EGF receptor autophosphorylation by ganglioside GM3 and anti-EGF receptor antibody αEGFR 1046 in A431 cells. (A) Serum-deprived subconfluent cultures of A431 cells were treated with ganglioside GM3 and 1046 as indicated for 2 h. Thereafter, the cells were stimulated with EGF as indicated and cell extracts were analysed by SDS-PAGE and immunoblotting with antiphosphotyrosine antibodies. (B) The membrane blot was stripped and reprobed with αEGFR-CT antibodies as described in Materials and methods. (C and D) The same experiment as in A and B; however the cells were incubated with pervanadate for 30 min before EGF stimulation.

**Figure 6** Effect of ganglioside GM3 on EGFR receptor dephosphorylation in A431 cell membranes. A431 cell membranes were incubated with or without 60 μg ml⁻¹ GM3 as indicated and EGF. EGFR autophosphorylation was initiated by addition of [γ32P]ATP and allowed to proceed for 10 min on ice. Thereafter, the reaction was quenched by addition of EDTA and receptor dephosphorylation was monitored by analysing aliquots corresponding to 10 μg of membrane protein at the indicated time points by SDS-PAGE and autoradiography for the EGF receptor phosphate content. The relative radioactivity in the receptor bands was quantified with a Phosphorimager. (A) Autoradiograph; (B) time course of receptor dephosphorylation as obtained by Phosphorimager analysis of the relative radioactivity in the EGFR receptor band. The depicted experiment is one of three with identical results.
investigated the effect of a combined antiproliferative cell treatment with the anti-EGF receptor antibody, αEGFRiod egf/r3, and gangliosides, again using the NSCLC line H125 as a target. Indeed, ganglioside G_{4α} (but not De-N-acetylganglioside) was found greatly to potentiate the effect of αEGFR iod egf/r3 on the growth of these EGFR-expressing cells leading to almost complete growth arrest and cytotoxicity. This effect was dependent on the presence of EGFR, since growth of an NSCLC line lacking EGFR expression was completely unaffected by the combined treatment. Synergistic growth inhibition was also observed using another NSCLC cell line (U1752) as target or using the combination of another anti-EGFR antibody (αEGFR mab425) and G_{4α} for the treatment, suggesting that the observed synergism is general. Experiments are underway to analyse further this new cytostatic concept in experimental tumours in vivo. A similar synergistic growth inhibition by a combination of an anti-EGFR antibody and a synthetic tyrosine kinase inhibitor has been observed for a squamous cell carcinoma by Yoneda et al (1991).

When we compared the effect of various treatments of H125 cells on cell growth with that on EGFR autophosphorylation activity, we observed an overall correlation of the inhibitory effects. Cell growth was blocked by specific EGFR tyrosine kinase inhibitors, the anti-EGF receptor antibody αEGFR iod egf/r3 inhibited receptor autophosphorylation and the combination of αEGFR iod egf/r3 and G_{4α} had a stronger effect on EGFR autophosphorylation than either agent alone. Taken together, these correlations suggest that the observed EGFR tyrosine kinase inhibition is causally related to growth inhibition. Some differences in the dose - response characteristics for growth inhibition and kinase attenuation were, however, observed. Most notably, the combined inhibition by αEGFR iod egf/r3 and G_{4α} was less pronounced on the level of H125 cell EGFR autophosphorylation than on the level of cell growth. These quantitative differences might be a result of the somewhat different treatment and assay schedules for the two parameters. It is, however, currently not possible to exclude additional effects of the agents used on other cellular systems contributing to the observed growth inhibition.

Growth factor receptor signalling activity at the level of receptor autophosphorylation is the net result of the action of receptor PTK and opposing PTPases. We therefore investigated whether the synergistic inhibitory effect of αEGFR iod egf/r3 and G_{4α} on the EGFR autophosphorylation involved merely tyrosine kinase inhibition or possibly also effects on the EGFR-directed PTPase(s). Interestingly, the attenuation of EGFR autophosphorylation by G_{4α} in H125 cells and in A431 cells is abrogated by pretreatment of the cells with the PTPase inhibitor, pervanadate. Furthermore, G_{4α} has the capacity to activate EGFR dephosphorylation in A431 cell membranes in vitro. Thus, activation of EGFR-directed PTPases by G_{4α} seems to be involved in the attenuation of EGFR signalling activity, possibly in addition to a direct tyrosine kinase inhibition (Zhou et al, 1994). It seems tempting to speculate that PTPase activation is also likely to be involved in the enhancement of anti-EGFR antibody-mediated growth inhibition by G_{4α} in H125 NSCLC cells. The identity of the activated PTPase(s) is currently unknown. The SH2-domain PTPase 1C has been shown to attenuate EGFR signalling in A431 cells; however, it is unlikely that this cytosolic PTPase is affected by exogenously added G_{4α}. Rather transmembrane PTPases (Charbonneau and Tonks, 1992; Pot and Dixon, 1992) are candidate targets for G_{4α} action. One could envisage G_{4α} effects on such PTPases either via the membrane spanning or via the extracellular protein domains. The observed activation by G_{4α} might help to identify further PTPases involved in EGFR dephosphorylation and might present a new regulatory principle for PTPases. Furthermore, our findings lend support to the concept that activation of growth factor receptor-directed PTPases could be employed as a mechanism for novel antiproliferative agents.

**ABBREVIATIONS**

EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EDTA, ethylenediamine tetraacetic acid; FCS, fetal calf serum; DMSO, dimethyl sulfoxide; SCLC, small-cell lung carcinoma; NSCLC, non-small-cell lung carcinoma; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; PTPase, protein tyrosine phosphatase.

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