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Epidermal Growth Factor and Transforming Growth Factor α Bind Differently to the Epidermal Growth Factor Receptor

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ABSTRACT: Epidermal growth factor (EGF) and transforming growth factor α (TGFα) compete with each other for binding to the EGF receptor. These two growth factors have similar actions, but there are distinguishable differences in their biological activities. It has never been clear how this one receptor can mediate different responses. A monoclonal antibody to the EGF receptor (13A9) has been identified which has only small effects on the binding of EGF to the EGF receptor, but which has very large effects on the binding of TGFα to the EGF receptor; 5 μg/mL antibody has been shown to totally block 0.87 μM TGFα from binding to purified EGF receptor and to lower both the high- and low-affinity binding constants of TGFα binding to EGF receptor on A431 cells by about 10-fold. The 13A9 antibody causes a 2.5-fold stimulation of the tyrosine kinase activity of partially purified EGF receptor, compared to a 4.0-fold stimulation of the tyrosine kinase activity by EGF under the same conditions. The data suggest either that the antibody stabilizes a conformation of the EGF receptor which is not favorable for TGFα binding or that it blocks a part of the surface of the receptor which is necessary for TGFα binding but not EGF binding.

Transforming growth factor α (TGFα) is a 50 amino acid polypeptide found in the supernatants of a wide variety of tumor cell lines and virally transformed cells (Todaro et al., 1980, 1985; Ozanne et al., 1980; Roberts et al., 1980). The amino acid sequence of TGFα has 35% identity with the epidermal growth factor (EGF) sequence, which includes the conservation of all six cysteine residues (Derynck et al., 1984; Marquardt et al., 1984). Two-dimensional NMR studies provide evidence that in solution the two proteins have very similar polypeptide chain folds (Montelione et al., 1989). TGFα has been shown to compete with EGF for binding to

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the EGF receptor (Todaro et al., 1980; Carpenter et al., 1983; Massague, 1983b).

In a wide variety of in vitro and in vivo assays, TGFα and EGF have similar modes of action. However, there are distinguishable differences in the biological activities of these two proteins. When tested for their ability to release $^{45}Ca^{2+}$ from labeled murine calveria and rat fetal long bones, TGFα and EGF show very different potencies. In the murine calveria system, TGFα is 3–10 times more potent than EGF, and in the rat long bone system, TGFα induces a pronounced release of $^{45}Ca^{2+}$ in a dose-dependent manner, whereas EGF shows no significant effect (Stern et al., 1985; Ibbotson et al., 1986). In a study of the effect of TGFα and EGF on angiogenesis, it was shown that although both TGFα and EGF induce neovascularization, TGFα induces the effect at a concentration at which EGF is totally ineffective (Schreiber et al., 1986). When the effects of EGF and TGFα on arterial blood flow in vascular beds were compared, the two proteins showed equal potency, but the maximal response obtained for TGFα was much larger than for EGF. In addition, upon repeated exposure of the vascular beds to TGFα or EGF, the system became desensitized to EGF, but was still fully responsive to TGFα (Gan et al., 1987).

How do the cells in these assays mediate a different response to TGFα than to EGF? In 1982, Massague et al. reported that TGFα interacts with a 60-kDa protein as well as the EGF receptor. He suggested that this 60-kDa protein mediated the induction of a transformed phenotype by TGFα (Massague et al., 1982). Later, however, it was shown by Carpenter et al. (1983) that induction of anchorage-independent growth by TGFα could be blocked by antibodies raised to the EGF receptor. Since that report, there has been no evidence presented which supports the presence of a second receptor for TGFα.

Different biological responses to TGFα and EGF could be mediated by the EGF receptor if the two growth factors bound to different sites in the receptor or if the two proteins caused different conformational changes in the receptor. In theory, either of these models could affect the signal transduction system. We have now found an antibody which blocks the binding of TGFα to the human EGF receptor but has no affect on the affinity of EGF for binding to the EGF receptor. The results suggest that one of the above models may indeed be responsible for the differences in biological activities of these two proteins.

Materials and Methods

Receptor-grade EGF from mouse submaxillary glands was purchased from Sigma Chemical Co. ^{125}I-labeled EGF and ^{125}I-labeled TGFα were prepared using chloramine-T (Hunter & Greenwood, 1962). Wheat germ agglutinin–agarose was purchased from Vector Laboratories, Burlingame, CA. A431 cells were obtained from the University of California at San Francisco tissue culture facility and maintained on 50:50 Dulbecco's-modified Eagle's medium and F12 medium supplemented with 5% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Membranes from the A431 cells were prepared according to the method of Thom et al. (1977).

TGFα. Recombinant human TGFα was purified as a fusion protein from *Escherichia coli* containing the plasmid pTE5 (Derynck et al., 1984). The TGFα was released from the fusion protein by the action of cyanogen bromide and purified to homogeneity by reverse-phase HPLC (Winkler et al., 1986).

TGFα–Agarose. TGFα–agarose was made by coupling 3.2 mg of TGFα to 5 mL of Reacti-Gel (Pierce Chemical Co.) in 0.1 M sodium bicarbonate at pH 9.0 for 96 h. The resin was blocked for 4 h with 1 M ethanolamine, washed with 1 M sodium chloride, and equilibrated with 40 mM Hepes, pH 7.5, containing 0.2% Triton X-100, 10% glycerol, and 2 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) (HTGE buffer) prior to use.

EGF Receptor. EGF receptor was purified from A431 cells after solubilization of the cells in 10 mM ethanolamine, pH 10.2, containing 10% glycerol, 1% Triton X-100, and 10 mM ethylenediaminetetraacetic acid (EDTA). The extract was applied to a wheat germ agglutinin–agarose column, and the column was washed with 40 mM Hepes buffer, pH 7.5, containing 10% glycerol, 0.2% Triton X-100, 2 mM EGTA, and 150 mM sodium chloride. EGF receptor was eluted from the column with wash buffer containing 0.3 M *N*-acetylglucosamine. The EGF receptor was dialyzed into HTGE buffer and loaded onto a TGFα–agarose column. The column was washed with HTGE buffer and eluted with the same buffer, containing 1 M sodium chloride. Purified EGF receptor was stored at −70 °C.

Monoclonal Antibody Production. Monoclonal antibodies to the EGF receptor were produced by immunizing BALB/c mice initially with intact A431 cells followed by wheat germ agglutinin–agarose-purified EGF receptor. Splenocytes were fused with the mouse myeloma line X63-Ag8.653 (Kearney et al., 1979) using previously described methods (Oi & Herzenberg, 1980). Hybridoma supernatants were screened initially for immunoglobulin production followed by an antigen-capture RIA with immobilized monoclonal antibody binding [^{32}P]ATP, autophosphorylated EGF receptor. EGF receptor binding was verified by 7.5% SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) and autoradiography. The 13A9 antibody, at 40 μg/mL, did not detect either EGF or TGFα coated on plastic ELISA plates and also did not show any reactivity with the HER-2 receptor in an immunoprecipitation assay (Fendly et al., unpublished results). At 10 μg/mL, the antibody did not show any interference in our TGFα ELISA assay, which utilizes both a monoclonal and a polyclonal antibody. In addition, 13A9 reacted with a lower molecular weight form of the EGF receptor produced by cells treated with tunicamycin, suggesting that the antibody is directed toward an epitope in the polypeptide chain of the EGF receptor rather than an epitope formed by carbohydrate residues (Fendly et al., unpublished results). The monoclonal antibody was purified from murine ascites fluid by chromatography on protein A–Sepharose and was quantitated by the absorbance at 280 nm, assuming an $\epsilon^{1\%}$ of 13.6.

^{125}I-EGF and ^{125}I-TGFα Binding and Displacement from Purified EGF Receptor. The displacement of ^{125}I-EGF or ^{125}I-TGFα from purified EGF receptor by EGF or TGFα was measured in 250 μL of 20 mM Hepes, pH 7.5, containing 10% glycerol and 0.1% Triton X-100 (HGT buffer). A total of 50 000 cpm of ^{125}I-EGF or ^{125}I-TGFα (125–200 μCi/μg) and 0–1.2 μg of unlabeled EGF or TGFα were incubated with the receptor in the presence or absence of 5 μg/mL 13A9 for 1 h at room temperature. The receptor was collected by incubating the reaction mixture with 50 μL of a 50% slurry of concanavalin A–Sepharose (Pharmacia) for 1 h. Four milliliters of 20 mM Hepes, pH 7.5, was then added, and the samples were centrifuged for 5 min at 1000*g*. The supernatant was aspirated and the pellet counted in an IsoData γ counter. Data were fit to a four-parameter logistic model to obtain values for 50% displacement of ^{125}I-EGF or ^{125}I-TGFα.

^{125}I-EGF and ^{125}I-TGFα Binding and Displacement from A431 Cells. A431 cells were seeded at 1×10^4 cells/well in 96-well "Removawell" tissue culture plates (Dynatech) and

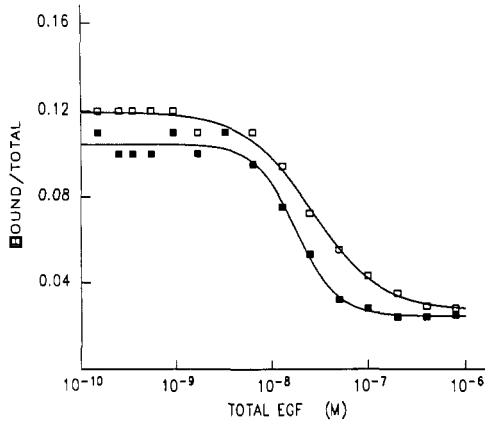

FIGURE 1: Displacement curve for the competition of 51 000 cpm of ^{125}I-EGF (126 μCi/μg) with (□) EGF and (■) EGF in the presence of 5 μg/mL 13A9 for binding to purified human EGF receptors.

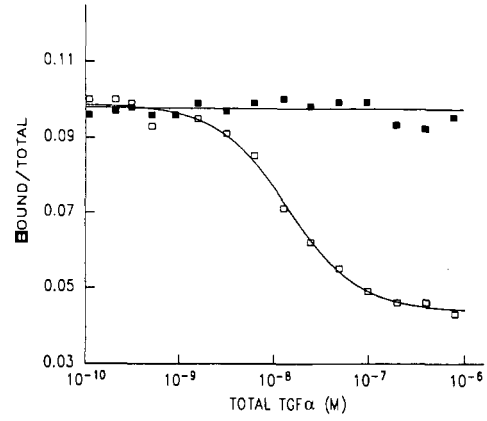

FIGURE 2: Displacement curve for the competition of 53 000 cpm of ^{125}I-EGF (169 μCi/μg) with (□) TGFα and (■) TGFα in the presence of 5 μg/mL 13A9 for binding to purified human EGF receptors.

allowed to grow for 24–48 h. The cells were washed 3 times with 100 μL of ice-cold 50:50 Dulbecco's-modified Eagle's medium and F12 medium containing 0.1% bovine serum albumin and 20 mM Hepes buffer, pH 7.2 (binding media). A total of 20 000 cpm of ^{125}I-EGF or ^{125}I-TGFα (150–200 μCi/μg) and 0–10 pmol of unlabeled EGF or TGFα were incubated with the cells in 200 μL of binding media at 4 °C for 90 min in the presence or absence of 5 μg/mL 13A9 antibody. The cells were then washed 4 times with 200 μL of cold binding media, and the wells were removed and counted in an IsoData γ counter. Data were analyzed by using a version of the program LIGAND (Munson & Robard, 1980), adapted for UNIX by R. Vandlen (Genentech).

TGFα Competition with Monoclonal Antibody for Binding to the EGF Receptor. The competition of unlabeled TGFα and the monoclonal antibody 13A9 for binding to membrane-bound EGF receptors was determined by measuring the amount of TGFα which remained unbound to A431 cell membranes in the presence of varying amounts of antibody; 12.5 ng of TGFα was incubated with A431 membranes in the presence of 0.3 ng–25 μg of purified antibody in 0.5 mL of PBS containing 1% BES and 0.1% BSA for 1 h at room temperature. The membranes were removed from the solution by centrifugation in an Eppendorf centrifuge for 30 min. The TGFα left in the supernatant was measured in a sandwich-type ELISA assay which is sensitive to 0.1 ng of TGFα/mL. The presence of 13A9 in the solutions was shown to have no effect on this ELISA assay.

Autophosphorylation of the EGF Receptor. Stimulation of the tyrosine kinase activity of the EGF receptor by EGF, TGFα, and the monoclonal antibody, 13A9, was tested by measuring the rate of autophosphorylation of the receptor after exposure to these reagents. Wheat germ agglutinin–agarose-purified EGF receptor was incubated with 0.6 μM EGF or TGFα in the presence or absence of 20 μg/mL monoclonal antibody 13A9 in HTG buffer for 1 h at room temperature. The samples were then incubated with [γ-^{32}P]ATP (Amersham Corp.) in the presence of 5 mM $MgCl_2$ and 1 mM $MnCl_2$ for 3 min on ice. The reaction was stopped by the addition of hot Laemmli SDS sample buffer with 1 mg/mL dithiothreitol, and the samples were run on a 7.5% SDS Laemmli gel (Laemmli, 1970). Phosphorylated EGF receptor bands were cut from the gel and counted in a Beckman scintillation counter after visualization on X-ray film.

RESULTS

Figure 1 shows the competition between ^{125}I-EGF and unlabeled EGF for binding to purified EGF receptors. In the absence of the monoclonal antibody, 13A9, 50% displacement of the labeled EGF is seen at an EGF concentration of 21 nM. In the presence of the antibody, there are small differences in the displacement curve. The decrease in the maximum fraction of bound EGF in the presence of 13A9 suggests that there is a small decrease in the number of EGF binding sites, although this decrease does not occur with every preparation of EGF receptor. A small but reproducible shift in the displacement curve to lower EGF concentrations is also observed, suggesting that the antibody has caused the remaining EGF binding sites to have a slightly higher affinity for EGF (50% displacement is calculated to be 11 nM).

In Figure 2, the competition of ^{125}I-EGF and unlabeled recombinant human TGFα for binding to purified EGF receptors is shown. Fifty percent displacement of the labeled EGF is seen at 12 nM TGFα. In the presence of the monoclonal antibody 13A9, there is no displacement of ^{125}I-EGF by TGFα, even at 0.87 μM TGFα. This suggests that in the presence of antibody 13A9, TGFα is prevented from binding to the EGF receptor. The same result was seen when EGF receptor related protein, a truncated form of EGF receptor secreted from A431 cells (Weber et al., 1987), was used (data not shown). This form of the receptor contains only the extracellular portion of the EGF receptor, fused to 17 amino acids of unknown origin (Merlino et al., 1985). The data indicate that the effect of the 13A9 antibody is exerted solely through its interaction with the extracellular domain of the receptor. When the same experiment is done with A431 cell membranes, where the receptor is separated from the reaction mixture by filtration rather than by the use of concanavalin A–Sepharose, there again was no competition of TGFα with ^{125}I-EGF in the presence of the monoclonal antibody (data not shown). The results suggest that the effect of the antibody on TGFα competition with EGF is not due to the method used to separate the EGF receptor from the unbound growth factors.

The effect of 13A9 on competition between ^{125}I-TGFα and unlabeled TGFα for binding to purified EGF receptor is shown in Figure 3. In the absence of 13A9, the receptor shows 50% displacement of labeled TGFα at a total TGFα concentration of 45 nM. Again, however, in the presence of 13A9, there is no observable binding of TGFα to the receptor.

Since purified EGF receptor preparations have a much lower affinity for EGF or TGFα compared to receptors on the cell surface, displacement experiments were repeated on A431 cells in the presence and absence of the 13A9 antibody. The results, shown in Figures 4 and 5, show two classes of binding sites for both EGF and TGFα. The dissociation constants calculated for EGF binding were 0.06 and 3.8 nM, and those for

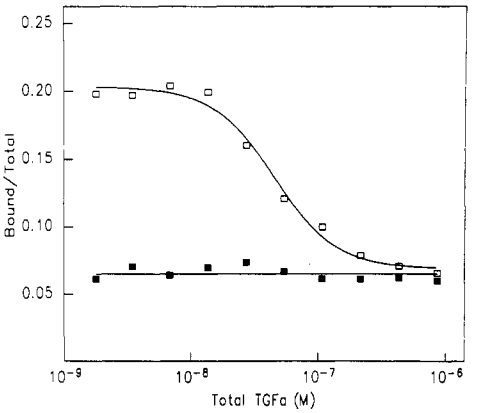

FIGURE 3: Displacement curve for the competition of 47 000 cpm of ^{125}I-TGFα (159 μCi/μg) with (□) TGFα and (■) TGFα in the presence of 5 μg/mL 13A9 for binding to purified human EGF receptors.

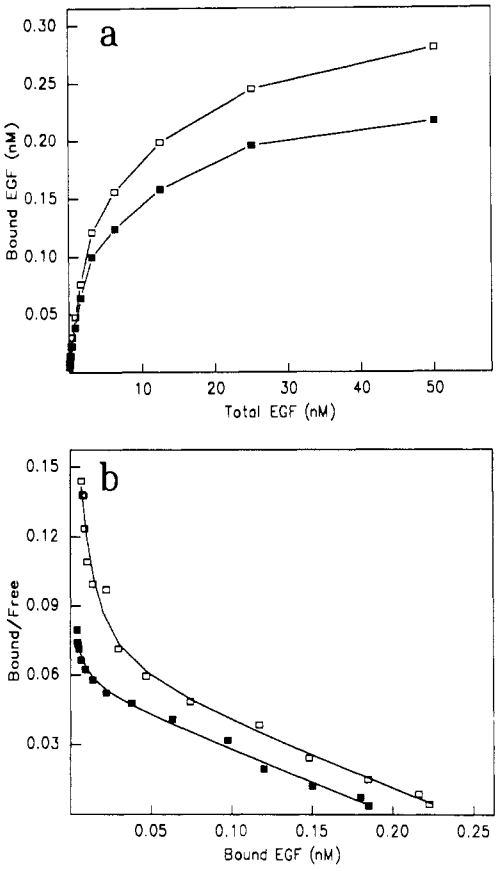

FIGURE 4: (a) EGF binding curves carried out on A431 cells at 4 °C and (b) Scatchard analyses of the binding data. Binding was carried out with 0–10 pmol of unlabeled EGF in the presence of 24 000 cpm of ^{125}I-EGF (196 μCi/μg) and in the absence (□) or presence (■) of 5 μg/mL 13A9.

TGFα binding were 0.02 and 9.6 nM. In the presence of 5 μg/mL 13A9, there were reproducible small changes in the EGF binding curve. Scatchard analyses using the LIGAND program (Munson & Robard, 1980) suggest that there is no significant change in the affinity of the EGF for the receptor but rather there is a significant decrease (to about 0.3 of the value obtained with no antibody) in the number of high-affinity binding sites. In the case of TGFα binding to A431 cells in the presence of 13A9, there are much larger changes. The Scatchard analyses showed the high-affinity dissociation constant to have increased from 0.02 to 0.25 nM and the low-affinity dissociation constant to have increased from 9.6

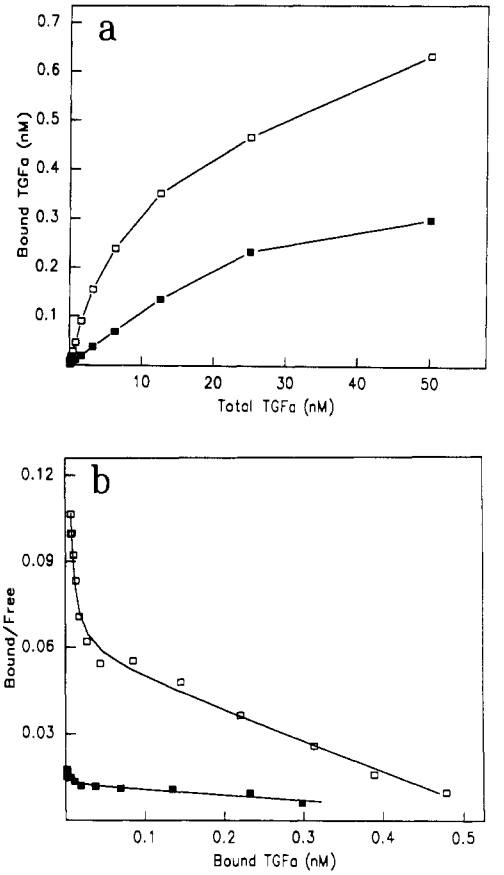

FIGURE 5: (a) TGFα binding curves carried out on A431 cells at 4 °C and (b) Scatchard analyses of the binding data. Binding was carried out with 0–10 pmol of unlabeled TGFα in the presence of 22 000 cpm of ^{125}I-TGFα (159 μCi/μg) and in the absence (□) or presence (■) of 5 μg/mL 13A9.

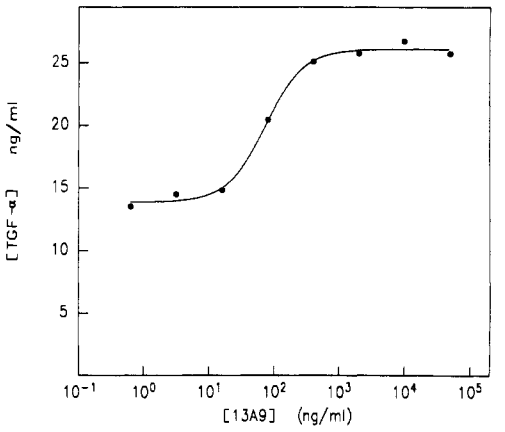

FIGURE 6: Amount of TGFα, determined by ELISA, which remains unbound to A431 cell membranes in the presence of varying amounts of 13A9 monoclonal antibody. The concentration of TGFα added to each tube was 25 ng/mL.

to 54 nM. As with EGF, 13A9 seems to have caused the loss of about two-thirds of the high-affinity binding sites with little change in the concentration of low-affinity binding sites.

To investigate whether the effect of the monoclonal antibody on TGFα binding is an artifact of the iodination of TGFα, we measured the levels of unbound TGFα in the presence of varying amounts of 13A9 in suspensions of A431 cell membranes. The results, shown in Figure 6, show that, again, the monoclonal antibody 13A9 competes with unlabeled TGFα for binding to EGF receptors. We observe 50% displacement of TGFα occurring at about 100 ng/mL 13A9. The binding

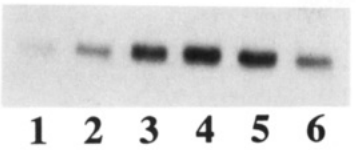

FIGURE 7: Radiogram of phosphorylated EGF receptor, resolved on a 7.5% SDS Laemmli gel. [γ-^{32}P]ATP was incorporated into partially purified EGF receptor from A431 cells after the receptor was incubated for 1 h with the following: Lane 1, no added growth factor; lane 2, 20 μg/mL 13A9; lane 3, 0.6 μM EGF; lane 4, 0.6 μM EGF and 20 μg/mL 13A9; lane 5, 0.6 μM TGFα; lane 6, 0.6 μM TGFα and 20 μg/mL 13A9.

Table I: Autophosphorylation of the EGF Receptor

conditions	amount of [γ-^{32}P]ATP incorporated (pmol)	x-fold stimulation
basal level	0.21	
20 μg/mL 13A9	0.52	2.5
0.6 μM EGF	0.85	4.0
0.6 μM TGFα	1.03	4.9
0.6 μM EGF, 20 μg/mL 13A9	1.06	5.0
0.6 μM TGFα, 20 μg/mL 13A9	0.61	2.9

of 13A9 to the EGF receptor on A431 cells may be a little weaker, showing 50% displacement of ^{125}I-TGFα at about 500 ng/mL (data not shown).

Since the 13A9 monoclonal antibody causes large changes in the binding of TGFα to the EGF receptor, we wondered whether the antibody would cause a stimulation of the EGF receptor tyrosine kinase activity, as has been shown many times for EGF and TGFα. The results, shown in Figure 7, and summarized in Table I, show that 13A9 causes some stimulation of the EGF receptor, although the stimulation is not as high as the stimulation caused by either EGF or TGFα alone. Surprisingly, 13A9 also caused a small increase in the level of autophosphorylation caused by EGF when it was added to the EGF receptor in addition to EGF. This would suggest that the mechanism of tyrosine kinase stimulation by 13A9 is different than the mechanism by which EGF causes tyrosine kinase stimulation, and therefore the effects are somewhat additive. As expected, when 13A9 is added to TGFα during the incubation with EGF receptor, there is only a 2.9-fold stimulation of the EGF receptor tyrosine kinase, about the same level as that caused by 13A9 alone. This is consistent with the TGFα not binding to the EGF receptor in the presence of 13A9.

Discussion

Although murine EGF and recombinant human TGFα have only a 35% amino acid sequence identity, they have been shown by two-dimensional NMR studies to have almost identical chain folds (Montelione et al., 1989). They have also been shown to have similar affinities for binding to the human EGF receptor, and they show competition for binding to the receptor (Massague, 1983a). Despite these similarities, EGF and TGFα have distinguishable biological activities.

We have now shown that a monoclonal antibody raised against the EGF receptor, 13A9, has quite different effects on the binding of TGFα to the human EGF receptor than it has on the binding of EGF to the human EGF receptor. The presence of 13A9 has only small effects on EGF binding to the EGF receptor but totally blocks 0.87 μM TGFα from competing with EGF for binding to the purified receptor. Furthermore, the antibody appears to displace TGFα from the EGF receptor in A431 cells by causing a 10-fold increase in the dissociation constant for TGFα binding. This evidence suggests that these ligands bind differently to the human EGF receptor, even though the difference is not reflected in the affinities of the two growth factors for the cellular receptor. The difference in their interaction with the EGF receptor may account for the differences in the biological activities of EGF and TGFα.

The differences in the effect of 13A9 on TGFα binding to the EGF receptor as compared to its effect on EGF binding to the same receptor may be explained by using the following models. The EGF receptor could contain two distinct binding sites, one for EGF and the other for TGFα. These sites could be very far apart from each other in the receptor. Competition between the two ligands would occur because of a conformational change in the receptor upon the binding of one ligand and which would cause a displacement of the other ligand when bound to the receptor. In this case, the 13A9 could affect TGFα binding and have very little affect on EGF binding either by sterically blocking the TGFα binding site or by causing a conformational change which precludes TGFα binding. A conformational change caused by the antibody in this model may be similar to the conformational change caused by EGF.

Another model which would explain the observed effects of the 13A9 antibody would be that both EGF and TGFα bind to the same pocket of the receptor, thereby competing with each other for binding, but each ligand induces a different conformation of the receptor, which allows for its distinguishable biological activity. In this case, the monoclonal antibody would not be acting as a steric inhibitor of ligand binding but may be causing a conformational change of the receptor which only allows for EGF binding.

13A9, which binds to the extracellular, amino-terminal peptide portion of the EGF receptor, causes stimulation of the tyrosine kinase activity of the receptor. The tyrosine kinase domain is located on the intracellular, carboxy-terminal portion of the EGF receptor. Stimulation of the tyrosine kinase is most probably transmitted through the receptor either by a conformational change in the receptor or through antibody-mediated dimerization of the receptor. If dimerization of the EGF receptor occurs in the presence of 13A9, it could happen by virtue of the bivalent nature of the antibody or through a mechanism similar to EGF-induced aggregation of EGF receptors (Yarden & Schlessinger, 1987). The mechanism of autophosphorylation stimulation by 13A9 cannot be exactly the same as that caused by EGF binding because the antibody does not stimulate the tyrosine kinase activity to the same extent as EGF and seems to cause a small increase in the level of EGF stimulation. There is not enough evidence to determine whether the change which causes 13A9 to stimulate the tyrosine kinase is the same change which causes the inhibition of TGFα binding to the EGF receptor.

The fact that 13A9 causes a decrease in the number of both EGF and TGFα high-affinity EGF receptor sites in A431 cells suggests that large amounts of 13A9 may prevent the aggregation of EGF receptors on the cell surface. It has been suggested that the dimer form of the receptor has a higher ligand binding affinity than the monomer form of the EGF receptor (Yarden & Schlessinger, 1987). Inhibition of dimerization could occur when two antibody molecules bind to neighboring receptor molecules, sterically preventing dimerization. Small amounts of the antibody, however, may still cause dimerization of the receptor, stimulating autophosphorylation. A titration of 13A9 on A431 cells in the presence of 10.8 nM TGFα shows a 14% increase in binding

of TGFα at 6.8 ng/mL 13A9, whereas higher concentrations of 13A9 show a marked inhibition in the binding of TGFα (greater than 75% inhibition at 15 μg/mL 13A9, data not shown).

A study which suggests that different factors affect the binding of EGF than affect the binding of TGFα was reported by Lax et al. (1988b). In this study, EGF and TGFα were shown to have very different affinities for binding to the chicken EGF receptor expressed in mouse cells. It was reported to require 200 times more EGF than TGFα to achieve 50% displacement of ^{125}I-TGFα. This difference is mainly due to a 50-fold decrease in the affinity of EGF for the chicken receptor as compared to the human EGF receptor. The differential binding occurs despite the fact that the chicken and human EGF receptors share a 75% identity in amino acid sequence in the domain between the two cysteine-rich regions as well as throughout the entire receptor.

Identification of the peptide isolated after cross-linking of ^{125}I-EGF to the human EGF receptor, followed by cyanogen bromide cleavage, has suggested that the region between the two cysteine-rich domains of the EGF receptor (residues 314–474) is responsible for most of the interactions that define the ligand specificity for binding of EGF to the EGF receptor (Lax et al., 1988a). It has not been shown whether TGFα also binds to this domain.

Registry No. EGF, 62229-50-9; EGF growth factor receptor tyrosine kinase, 79079-06-4.

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