Superagonistic behaviour of epidermal growth factor/transforming growth factor-α chimaeras: correlation with receptor routing after ligand-induced internalization

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Human epidermal growth factor (EGF) and human transforming growth factor alpha (TGF-α) are structurally related polypeptide growth factors that exert their mitogenic activity through interaction with a common cell-surface receptor, the epidermal growth factor receptor (EGFR). The biological effect induced by these two ligands is quantitatively similar in most cases; in some test systems, however, TGF-α functions as a more potent form of EGF. In this study, we have compared EGF, TGF-α and ten previously described chimaeras of these two ligands in terms of their ability to generate a mitogenic response in cells carrying the human EGFR, and observed that three of the mutant growth factors (E3T, E4T and T3E4T) are mitogenic at concentrations 10-fold lower than that of either wild-type EGF or TGF-α. No difference in tyrosine kinase activity of the receptor towards an external substrate was observed after binding of the various mutants. It has been established before [Ebner and Derynck (1991) Cell Regulation 2, 599–612] that EGF and TGF-α differ in the processing of the receptor–ligand complex after internalization, as a result of their different pH sensitivities of receptor binding. Similar measurements on our chimaeric mutants revealed that the above superagonists show an enhanced pH dependence of binding in comparison with EGF. Furthermore, induction of receptor recycling by these superagonists is largely comparable with that induced by TGF-α. No superagonistic behaviour was observed on a cell-line containing an EGF/erbB-2 chimera which does not show ligand-induced internalization. These data show that EGF/TGFα chimaeras can be more active than the naturally occurring ligands, and that receptor recycling after ligand-induced internalization seems to be a prerequisite for this phenomenon.

INTRODUCTION

Human epidermal growth factor (EGF) and human transforming growth factor alpha (TGF-α) are members of a large family of structurally related polypeptide growth factors that exert their biological activity by binding to the EGF receptor (EGFR) [1]. Within both growth factors, six almost identically spaced cysteines form three disulphide bridges that are known to be essential for biological activity [2]. Binding of the ligand to the extracellular domain of the EGFR causes receptor dimerization, coupled to activation of the intrinsic tyrosine kinase activity. Although EGF and TGF-α bind with a very similar affinity to their receptor [3,4], several reports have described that TGF-α appears to be more potent than EGF in a variety of biological systems [5,6]. It has been established that EGF and TGF-α induce a different intracellular routing of the receptor after ligand-induced internalization [7]. Since EGF is a more acidic molecule than TGF-α, it remains bound to the EGFR after internalization during the gradual acidification of the endocytotic vesicles [8], finally resulting in proteolytic degradation of both ligand and receptor in the lysosomes. In contrast, TGF-α rapidly dissociates from the receptor after internalization, resulting in the recycling of both receptor and ligand to the cell surface. However, in most cell systems, this difference in receptor routing does not appear to affect the mitogenic potential of these growth factors.

Lax et al. [9] have shown that although EGF and TGF-α may have similar affinity for the human EGFR, EGF has a 100-fold lower affinity than TGF-α for the chicken EGFR. Recently, we have made ten EGF/TGF-α chimaeras on the basis of exchange of domains within EGF and TGF-α that are bordered by their conserved cysteine residues. Using this approach, we concluded that the C-terminal domain of EGF is responsible for the low-affinity binding to the chicken EGFR [10]. In later studies, we have shown that replacement of Arg-45 in human EGF by the corresponding Ala of TGF-α is sufficient to induce high-affinity binding to the chicken EGFR [11], indicating that this residue may be directly involved in receptor binding.

In the present study we have measured the mitogenic capacity of the above EGF/TGF-α chimaeras towards cells expressing the human EGFR. The present data show that three mutants, designated E3T, E4T and T3E4T, induce mitogenic activity at a 10-fold lower concentration than wild-type human EGF and TGF-α, in spite of the fact that they have similar receptor-binding affinity and induce the same amount of EGFR tyrosine kinase activity. These three superagonists induce receptor recycling after ligand-induced internalization, similar to TGF-α, and have an enhanced pH dependence of binding when compared with EGF. These data are interpreted in terms of the role of EGF receptor recycling in mitogenic activation by EGF-like growth factors.

MATERIALS AND METHODS

Expression and purification of recombinant growth factors

The DNA constructs for the expression of human EGF, TGF-α and EGF/TGF-α chimaeras were cloned into the pEZZ18 expression vector (Pharmacia, Uppsala, Sweden), as described [11]. Wild-type and chimaeric growth factors were expressed and
secreted as protein A-/factor X-growth factor fusion proteins [12] into the periplasmic space of Escherichia coli strain KS474, a degP-pro tease-deficient mutant (a generous gift from Dr. K. L. Strauch and J. Beckwith, Harvard University; see also [13]). The bacteria were grown in 2YTE medium [per litre: 16 g
bactotryptone/10 g yeast extract/8 g NaCl/0.4% (w/v) glucose] at 30 °C while agitating (200 rpm) until an A$_\text{oo}$ of 1.5 was reached. The fusion proteins obtained were purified using IgG-Sepharose chromatography (Pharmacia, Uppsala, Sweden) and the recovery of the chimaeras was measured by an ELISA based on competitive binding with biotin-labelled protein A [14]. The recombinant growth factors were enzymically separated from the protein A sequence by factor X digestion, after which the wild-type and mutant growth factors were purified by an additional run on an IgG-Sepharose column. Both dimeric and inactive forms with disulphide bridge mismatches were removed by reverse-phase HPLC on a Deltapak C18 column (Waters Cooperation, Milford, MA, USA) at a flow rate of 1 ml/min, using a linear gradient of 0–60% aceonitrile in 0.1% (v/v) trifluoro-acetate, as described previously [11]. Fractions (1 ml) were tested for EGFR-binding activity on HER-7 or HER-14 cells (see below), and the amount of growth factor present was deduced from the peak area at 229 nm in the HPLC chromatogram, using natural murine EGF under the same experimental conditions as the standard [11].

Cell culture

NIH-3T3 cells transfected with the wild-type human EGFR (HER-7 cells, 6.0 x 10$^5$ human EGFR/cell; or HER-14 cells, 4.0 x 10$^5$ human EGFR/cell; see [15]) were kindly provided by Dr. J. Schlessinger (New York University, NY). HaCaT, a human keratinocyte cell-line expressing the wild-type human EGFR, was a generous gift from Dr. N. E. Fusenig (Deutsches KrebsforschungsZentrum, Heidelberg, Germany; see [16]). NIH-3T3 fibroblasts transfected with a human EGFR/erbB-2 chimera containing the human EGFR extracellular domain and the transmembrane and cytoplasmic domains of erbB-2 (HER-56 cells, 1.0 x 10$^6$ receptors/cell) were used as described by Sorkin et al. [17]. All cells were cultured in gelatinized flasks: HER-7, HER-14 and HER-56 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 0.1% (w/v) newborn calf serum (NCS), HaCaT cells were cultured in DMEM supplemented with 10% (w/v) foetal calf serum (FCS).

Murine $^{125}$I-EGF-binding competition assays

Murine EGF (Bioproducts for Science Inc., Indianapolis, IN) was iodinated using enzymebeads (Bio-Rad) to a specific activity of approx. 500 Ci/mmol [18]. HER-7, HER-14, HER-56 and HaCaT cells were grown to confluency in gelatinized 24-well dishes (1.8 cm$^2$). The medium was removed and serial dilutions of approx. 500 Ci/mmol [18]. HER-7, HER-14, HER-56 and HaCaT cells were grown to confluency in gelatinized 24-well dishes (1.8 cm$^2$). The medium was then replaced by 0.9 ml of DMEM/Ham’s F12 medium (Gibco-BRL) (1:1) supplemented with 30 nM Na$_2$SeO$_3$, 10 µg/ml human transferrin and 0.5% (w/v) BSA [DFST/0.5% (w/v) BSA]. After an additional 48 h, serial dilutions of recombinant growth factors were added in 0.1 ml DMEM/Bes (pH 6.8) and after a further 8 h, 0.5 µCi [$^{32}$P]Jthymidine (TdR) was subsequently added in 0.1 ml Ham’s F12 medium. Incorporation of the tracer into the cellular DNA was determined 24 h after growth factor addition as described previously [19].

HaCaT cells were seeded at a density of 3.0 x 10$^4$ cells/well in 1 ml DMEM/10% (v/v) FCS and grown for 3 days to 50% confluency. The medium was then replaced by 0.9 ml DFST/0.5% BSA and 24 h later serial dilutions of recombinant growth factors were added in 0.1 ml DMEM/Bes (pH 6.8) supplemented with 0.1% BSA. [$^{3}H$]TdR incorporation was determined 24 h after growth factor addition as described above.

Ligand-induced internalization of the human EGFR

HER-7 or HER-56 cells were grown to confluency in gelatinized 24-well dishes. Subsequently the medium was replaced by 400 µl DFST medium and the recombinant chimaeric proteins were added to the cells in 100 µl DMEM/Bes (pH 6.8) to a final concentration of 100 ng/ml, with or without 0.3 mM monensin (Sigma, St. Louis, MO) and incubated for 1 h at 4 °C. Then the cells were incubated at 37 °C for the indicated time periods (see Results section), after which all non-internalized receptor-bound ligand was removed by an ice-cold acid wash (5 mM HCl, 0.35 mM NaCl, 2.5 mM KCl, pH 2.5). The cell medium was neutralized by rinsing twice with DMEM/10% (v/v) NCS and incubation was continued for 1 h at 37 °C with 1 ml of the same medium, to allow any internalized intact receptors to recycle to the cell surface. To quantify the number of human EGFR present on the cell surface after down-regulation by the various recombinant growth factors, a murine $^{125}$I-EGF binding experiment was performed for 1 h at 4 °C.

Stimulation of receptor tyrosine kinase

The growth-factor-dependent tyrosine kinase activity of the human EGFR was determined by measuring the incorporation of [$^{32}$P]ATP (ICN Biomedicals Inc., Costa Mesa, CA) into the synthetic polypeptide substrate angiotensin I (Sigma, St. Louis, MO). Membranes from HER-7 cells were isolated according to the method of Thom et al. [20] and preincubated for 60 min at room temperature with increasing concentrations of growth factors in 20 µl PBS/0.1% (w/v) BSA. The tyrosine kinase reaction was initiated by the addition of 20 µl of ‘2x’$^{32}$P-assay mix’ containing 40 mM Hepes (pH 7.4), 4 mM MnCl$_2$, 20 mM p-nitrophenyl phosphate, 80 µM Na$_2$VO$_3$, 30 µM ATP, 0.4% (w/v) Nonidet P-40, 4 mM angiotensin I and 1 µCi [$^{32}$P]ATP. The mixture was incubated for 5 min at 30 °C, after which the reaction was terminated by the addition of 50 µl 5% (v/v) trichloroacetic acid and 20 µl of 2.5% (w/v) BSA. Aliquots of 20 µl of acid-soluble material were spotted onto phosphocellulose paper (Whatman P81). The filters were washed six times with...
7.5 mM phosphoric acid and subsequently dried. The radioactivity incorporated into the peptide substrate was measured by liquid scintillation counting.

RESULTS

Mitogenic response of EGF/TGF-α chimaeras towards human EGFR

Human EGF, human TGF-α and the chimaeric proteins were expressed in E. coli and purified by reverse-phase HPLC, as described [11]. The absorbance at 229 nm of the active peak was taken as a measure of the amount of growth factor present. All mutants tested showed, to within a factor of 2, a similar receptor-binding affinity to the human EGF as murine EGF from a natural source, in agreement with data of van de Poll et al. [11]. Nomenclature used for the EGF/TGF-α chimaeras was similar to that used before [10]; e.g. E3T stands for a growth factor with EGF sequences N-terminal of and TGF-α sequences C-terminal of the third cysteine.

Figure 1(A) shows that EGF, TGF-α and most of the chimaeras tested have similar mitogenic activity towards HER-14 cells. However, three chimaeras, designated as E3T, E4T and T3E4T, already show a mitogenic response at at least 10-fold lower concentrations, in spite of the fact that their receptor-binding affinity is similar to that of the other growth factors tested. Similar results were obtained on HER-7 cells, although the extent of growth stimulation was less than on the HER-14 cells, due to the partly transformed character of these cells.

To be certain that this superagonistic response is not an artefact related to the particular cell lines used in these studies, a similar experiment was performed on a non-transformed human keratinocyte cell line (HaCaT) carrying endogenous human EGFs. Figure 1(B) shows that in this cell-line also dose-response curves for mitogenic activity by the above superagonists are shifted to significantly lower concentrations. For reasons currently not well understood, however, the maximum levels of growth stimulation induced by some chimaerae appear to be significantly higher than those observed for EGF and these superagonists.

EGF receptor-induced tyrosine phosphorylation

The observation that E3T, E4T and T3E4T are capable of inducing a superagonistic response prompted us to evaluate the possibility that these ligands are able to convert the EGFR into a more active conformation. Therefore some of the ligands were tested for their ability to induce dose-dependent tyrosine phosphorylation of angiotensin I, an external substrate molecule for the activated EGFR. Figure 2 shows the incorporation of radiolabelled phosphate into angiotensin I, mediated by membrane preparations of HER-7 cells. No significant differences were observed in receptor tyrosine kinase activity when comparing the dose-response curves of the superagonists with those of the wild-type growth factors (Figure 2A) and non-superagonists (Figure 2B).

Receptor routing induced by EGF/TGF-α chimaeras

It has been shown by Ebner and Derynck [7] that human EGF and human TGF-α induce a different routing of the ligand–receptor complex after internalization. Figure 3(A) shows that incubation of HER-7 cells with wild-type human EGF and various chimaeras causes a strong decrease of cell-surface receptors in spite of the fact that their receptor-binding affinity is similar to that of the other growth factors tested.

To evaluate whether the differences observed for TGF-α and the superagonists are due to alterations in receptor recycling and not to differences in their ability to induce receptor internalization, the same experiment was repeated in the presence of monensin, which is known to inhibit the recycling of transmembrane receptors [21], including the EGFR [23]. Figure 3(B) shows that in the presence of monensin, the extent of ligand-
induced down-regulation of the receptors is very similar for all ligands, indicating that under the experimental conditions tested, the rate of ligand-induced receptor internalization does not differ for superagonists and non-superagonists.

**Mitogenic response of HER-56 cells expressing an internalization-impaired hEGFR/erbB-2 chimera**

To investigate whether differences in receptor routing induced by the various EGF/TGFα chimaeras may account for the difference in mitogenic potency, superagonists and non-superagonists were tested for their ability to stimulate the proliferation of quiescent HER-56 cells expressing a human EGF/FerbB-2 chimera. This chimaeric receptor, which contains the human EGFR extra-cellular domain and the transmembrane and intracellular domain of erbB-2, can be activated upon human EGF binding and is able to generate a potent mitogenic signal [23]. However, the ligand-induced internalization of this receptor is much slower and hardly any down-regulation of the receptor takes place [17]. The EGF/TGFα chimaeras bound to this chimaeric receptor with an affinity similar to that of the wild-type growth factors (results not shown). Furthermore, Figure 4(A) shows that upon ligand binding the receptor remains located at the cell surface, irrespective of whether incubation was with wild-type or mutant growth factors, and that no significant ligand-induced receptor internalization occurs. When tested for their ability to induce a mitogenic response in HER-56 cells, chimaeric and wild-type growth factors were now almost equally potent (Figure 4B). This
Superagonistic behaviour of growth factor chimaeras

Figure 4  Down-regulation of the EGFR/erbB-2 chimaera in HER-56 cells (A) and mitogenic stimulation of HER-56 cells (B)

Down-regulation of the chimaeric receptor in HER-56 cells was measured as described in the legend of Figure 3 still in the absence (solid lines) or presence (hatched lines) of 0.3 mM monensin. Mitogenic stimulation of HER-56 cells was assessed by measuring [3H]thymidine incorporation 24 h after growth factor addition. Values are given as percentages of the stimulation obtained with 100% binding at variable pH (100% competition at pH 7.7). Data in (A) and of (B) represent the means of four independent experiments in triplicate. S.E.M. is within 5% for all data points.

Figure 5  pH-dependent inhibition of murine [125I]-EGF binding to HER-7 cells

Fibroblasts were grown to confluence, after which a binding competition experiment was performed at room temperature in the presence of 1 ng/ml murine [125I]-EGF and 100 ng/ml of human EGF (+), TGF-α (bold +), E3T (●), T3E (○), E4T (▼), T4E (◆), E3T4E (□), T3E4T ( ■ ), T6E (○), EOT (●). Data represent the means of three independent experiments in triplicate.

suggests that E3T, E4T and T3E4T are not superagonistic when compared with wild-type ligands in cells where the receptor is not internalized after activation.

pH-dependent binding of EGF/TGF-α chimaeras to the human EGFR

The ability of EGF to induce degradation of the human EGFR, and of TGF-α to induce receptor recycling, has been related to their difference in pH dependence of binding [7].

Figure 5 shows the ability of the various mutants, added at a fixed concentration of 100 ng/ml, to compete with murine [125I]-

EGF for binding to the human EGFR at different pH values. The data are presented relative to the binding ability at pH 7.7, which is similar for all growth factors tested [10]. These results show that every ligand has its own characteristic pH dependence of binding, in which TGF-α and T3E4T rapidly lose their affinity for the receptor upon acidification, E3T and E4T show an intermediate binding property, whereas binding of EGF and all other ligands to the EGFR is much less pH-sensitive. These results show that the ability of the superagonists to induce receptor recycling, similar to TGF-α, is paralleled by an enhanced pH dependence of binding.

DISCUSSION

In the present study we have investigated the mitogenic potential of ten previously characterized EGF/TGF-α chimaeras, and shown that three of them, E3T, E4T and T3E4T, behave as superagonists for the human EGFR receptor. The observation that these superagonists can induce mitogenic activation at much lower levels of receptor occupation suggests that these ligands are either able to induce the EGFR to form a more active conformation, or that they may be able to activate individual receptor molecules for a longer period of time. The observation that tyrosine kinase activity of the activated receptor towards an external substrate molecule, such as angiotensin, is not different for the superagonists suggests that the first option is not likely, although more quantitative data will be required to exclude this possibility. The observation that no superagonistic activity is seen on the internalization-deficient human EGFR/erbB2 chimera suggests that receptor internalization may be important for the observed enhanced mitogenicity. It has been established that, in the case of EGF, internalized receptor–ligand complexes are stable during gradual acidification in the endocytic vesicles, resulting in degradation of both receptor and ligand in the lysosomes. In the case of TGF-α, the ligand dissociates from the internalized receptor–ligand complex as soon as the pH is lowered below 6.0, resulting in receptor recycling [8]. Ebner and Derynck [7] have suggested that this difference in routing of the EGFR after ligand-induced internalization could account for the more potent activity of TGF-α in some systems, although it is not clear
why enhanced activity of TGF-α is then not a more general phenomenon.

The present data show that the currently characterized EGF/TGF-α chimaeras with superagonistic activity induce receptor recycling instead of receptor degradation, as observed similarly for TGF-α. In line with the model postulated by Ebner and Derynck [7] there seems to be a correlation between receptor recycling and the pH dependence of binding of the mutants, since all three superagonists show clearly reduced binding at acidic pH when compared with EGF, although, in particular, binding of E3T and E4T seems less pH-sensitive than that of TGF-α. Furthermore, in the case of binding of insulin and insulin-like growth factors to their receptors, a direct correlation has been observed between pH dependence of binding and receptor recycling after ligand-induced internalization [24]. In contrast with the model of Ebner and Derynck [7], however, we observed no direct correlation between the overall isoelectric point (IEP) of the various EGF/TGF-α chimaeras and their pH dependence of binding. This is illustrated by a comparison of Table 1 and Figure 3 which show that the superagonists E3T and E4T have IEP values similar to those of EGF, but which induce receptor recycling in a similar manner to TGF-α. Moreover, the chimaeras E6T and T6E differ substantially in their IEPs, but show almost identical pH dependence of binding (see Figure 5). Table 1 also shows that TGF-α and the superagonists have a relatively high histidine content, which is the amino acid with the pKₐ value of its side chain closest to the pH range used in Figure 5. Further research will be required to establish a causal relationship between pH dependence of binding and the presence of specific histidine residues [25].

There is increasing evidence that tyrosine kinase receptors can still be active in generating second messengers after ligand-induced internalization [26,27]. This suggests that, in the case of EGF, receptors can have a long signalling period, both initially at the cell surface and during the course of internalization [28], but because of lysosomal degradation, each receptor can be used only once [29,30]. In the case of TGF-α, signalling is restricted to the cell surface but receptors can be used more than once as a result of recycling. Under most experimental conditions these two signalling mechanisms seem to converge to induce similar mitogenic responses. If the hypothesis that the intensity of mitogenic stimulation through the EGF-R is limited by ligand-induced receptor internalization is correct, this would encourage a detailed comparison of the receptor internalization and routing kinetics induced by wild-type ligands and superagonists to be made.

It is well known that the EGFR belongs to a multigene family, which also includes erbB2, 3 and 4. Recent studies have indicated that EGF-like growth factors can induce receptor dimerization, and that heterodimers, in particular with erbB2, are mitogenically very potent [31–33]. Preliminary studies using interleukin-dependent myeloid cells transfected with only erbB1 suggest that the superagonistic activity of E3T, E4T and T3E4T is intrinsic to the human EGF receptor, and does not require receptor heterodimerization (A. E. G. Lenferink and Y. Yarden, unpublished work). Moreover, it is well known that the human EGFR can occur in both a high- and a low-affinity state [9], and it has been suggested that EGF-induced mitogenesis is primarily mediated by the so-called high-affinity receptors [34,35]. On cells extracted with Triton X-100, which are devoid of so-called high-affinity receptors, we observed however similar binding affinities of the wild-type ligands and the superagonists, indicating that superagonists do not preferentially interact with such high-affinity sites.

Many studies have been performed to elucidate the structure-function relationship of EGF and TGF-α with the final aim of developing a receptor antagonist [1,25,36]. The present observations indicate that mutants with similar binding activities can evoke different mitogenic responses, and from that point of view it can be concluded that it is possible to uncouple the process of ligand binding from the subsequent process of ligand-induced receptor activation, which is a prerequisite for the design of such an antagonist. In agreement with this observation, recent studies indicate that EGF-like growth factors with low affinity for the EGFR can still have mitogenic activity very similar to that of wild-type EGF [35,37–39]. The current study, however, is the first to show that growth factors with a binding affinity similar to that of EGF can have enhanced mitogenic activity.

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REFERENCES


Table 1 Charge and histidine content of EGF/TGF-α chimaeras

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<tr>
<th>Growth factor</th>
<th>IEP*</th>
<th>No. of histidine residues†</th>
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<tr>
<td>Human EGF</td>
<td>4.50</td>
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<tr>
<td>Human TGF-α</td>
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</tr>
<tr>
<td>T4E6T</td>
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</table>

* Isoelectric point (IEP) calculated on the basis of the known pKₐ values of individual amino acid side chains (also see [40]).
† Total histidine content of EGF/TGF-α chimaeras.
Super agonistic behaviour of growth factor chimaeras


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