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

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INTRODUCTION

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 qualitatively 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 EGFR/erbB-2 chimaera 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.

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 purified by affinity chromatography on Sepharose bound to the protein A domain of the human IgG1 molecule.

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EGF(R), epidermal growth factor (receptor); FCS, foetal calf serum; IEP, isoelectric point; NCS, newborn calf serum; TdR, thymidine; TGF-α, transforming growth factor-α.

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secreted as protein A-/factor X-growth factor fusion proteins [12] into the periplasmic space of Escherichia coli strain K874, a Δ2-protease-deficient mutant (a generous gift from Dr. K. L. Strauch and J. Beckwith, Harvard University; see also [13]). The bacteria were grown in 2YT 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 A600 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 Co-
operation, Milford, MA, USA) at a flow rate of 1 ml/min, using
a linear gradient of 0-60 % acetonitrile in 0.1 % (v/v) triflower-
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 10⁶ human EGFR/cell; or HER-14 cells,
4.0 x 10⁶ 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. Fuesig (Deutsches
KrebsforschungsZentrum, Heidelberg, Germany; see [16]). NIH-
3T3 fibroblasts transfected with a human EGFR/erbB-2 chim-
aera containing the human EGFR extracellular domain and the
transmembrane and cytoplasmic domains of erbB-2 (HER-56
cells, 1.0 x 10⁶ 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 10% (v/v)
new-born calf serum (NCS), HaCaT cells were cultured in DMEM
supplemented with 10% (w/v) foetal calf serum (FCS).

**Murine ¹²⁵I-EGF-binding competition assays**

Murine EGF (Bioproducts for Science Inc., Indianapolis, IN)
was iodinated using enzymobeads (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
plates (1.8 cm²). The medium was removed and serial dilutions
of wild-type or chimeric growth factors were added to the cells in 100 µl DMEM containing 15 mM Hepes, pH 7.7 and supple-
mented with 10% NCS (DMEM/Hepes) together with 0.1 ng/
well murine ¹²⁵I-EGF. After incubation for 2 h at room tempe-
rament, the cells were rinsed twice with ice-cold PBS supple-
mented with 0.1% (w/v) BSA and once with ice-cold PBS. Cells
were then lysed by incubation with 250 µl/well 1% (v/v) Triton
X-100 for 1 h at room temperature. Finally radioactivity present
in the cell lysate was measured by γ-counting.

The pH dependence of binding of the various chimeric
growth factors to the human EGFR was determined essentially
as described above, except that the growth factors were now
added in DMEM buffered with 50 mM BES and supplemented
with 0.1% (w/v) BSA (DMEM/BES) and the medium was
adjusted to different pHs using either NaOH or HCl. The
recombinant wild-type and mutant growth factors were tested at
a fixed concentration of 100 ng/ml.

**Mitogenic Assays**

HER-7, HER-14 or HER-56 cells were seeded in gelatinized 24-
well dishes (1.8 cm²) at a density of 6.0 x 10⁴ cells/well in 1 ml
DMEM/10% (v/v) NCS. After 24 h incubation, the medium
was replaced by 0.9 ml of DMEM/10% (v/v) Ham’s F12 medium (Gibco-
BRL) (1:1) supplemented with 30 nM Na₂SeO₃, 10 µg/ml human
transferrin and 0.5% (v/v) BSA [DFST/0.5% (v/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 [³H]thymidine (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 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 HOAc,
135 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 EGF present on the cell
surface after down-regulation by the various recombinant growth
factors, a murine ¹²⁵I-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
[γ-³²P]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 [γ-³²P]ATP
mix containing 40 mM Hepes (pH 7.4), 4 mM MnCl₂, 20 mM p-
nitrophenyl phosphate, 80 µM Na₂VO₃, 30 µM ATP, 0.4%,
(w/v) Nonidet P-40, 4 mM angiotensin I and 1 µCi [γ-³²P]ATP.
The mixture was incubated for 3 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

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The text above contains a detailed description of experimental procedures, including methods for purifying and testing recombinant growth factors, as well as assays for measuring their activity. It involves characterizing the binding of growth factors to various cell lines, assessing the internalization of these factors, and determining their tyrosine kinase activity.
RESULTS

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

Human EGF, human TGF-α and the chimeric 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 EGFRs. 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 chimaeras 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 as a result of ligand-induced down-regulation, whereas E3T, E4T and T3E4T mimic human TGF-α in their ability to induce receptor recycling. The other mutants, which behave similarly to EGF, most likely target the receptor to degradation in the lysosomes.

Figure 1 Mitogenic stimulation of HER-14 and HaCaT cells

The mitogenic stimulation of HER-14 (A) and HaCaT (B) cells was investigated. The relative mitogenic activity of hEGF (+), hTGF-α (bold +), E3T (●), T3E (▲), T4E (▼), T3E4T (■), T4E6T (♦), E3T4E (□), T4E4T (▲) and E4T6E (△) was assessed by measuring [3H]Tdr incorporation 24 h after growth factor addition. The stimulation is shown as a percentage of the control value (1.55 ± 0.16) x 10⁶ c.p.m. and in control cells, without growth factor addition, (35.8 ± 3.6)% of this value. Data on HER-14 cells were based on six independent experiments in triplicate, with the following mean and S.E.M. values for the EC₅₀ (in ng/ml): EGF, 0.782 ± 0.024; TGF-α, 0.715 ± 0.042; E3T, 0.142 ± 0.003; E4T, 0.097 ± 0.017; T3E4T, 0.100 ± 0.012. Data on HaCaT cells represent the mean and S.E.M. of three independent experiments in triplicate, as follows (in ng/ml): EGF, 0.196 ± 0.012; TGF-α, 0.172 ± 0.012; E3T, 0.011 ± 0.002; E4T, 0.003 ± 0.001; T3E4T, 0.015 ± 0.001.
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 EGFR/erbB-2 chimera. This chimaeric receptor, which contains the human EGFR extracellular 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

**Figure 2** Phosphorylation of angiotensin I by the human EGFR present in membrane preparations of HER-7 cells

Receptors were incubated for 60 min at room temperature with various concentrations of human EGF (+), human TGF-α (bold +), E3T (●), T3E (○), E4T (▼) and T4E (▲). Angiotensin I and [γ-32P]ATP were added and the mixture was incubated for 3 min at 30 °C. Incorporation of 32P into angiotensin I was subsequently determined as described in the Materials and methods section. In (A) the superagonists E3T and E4T are compared with wild-type EGF and TGF-α, whereas in (B) the non-superagonists T3E and T4E are compared with the superagonist E4T and wild-type EGF. Data represent the means of duplicate experiments.

**Figure 3** Internalization of the cell-surface EGF-receptor in HER-7 fibroblasts

Confluent monolayers of both cells were treated with an excess of unlabelled hEGF (+), human TGF-α (bold +), E3T (●), T3E (○), E4T (▼), T4E (▲), T6E (●), E6T (♦), E3T4E (□), T3E4T (■), T4E6T (▲) and E4T6E (▲) for 1 h at 4 °C. Internalization was induced by transferring the cells to 37 °C for the indicated time periods, in the absence (A) or presence (B) of 0.3 mM monensin. Cell-surface EGF-binding capacity of the cells was subsequently assayed by an incubation with 1 ng/ml murine 125I-EGF for 1 h at 4 °C, after acid-stripping of receptor-bound ligand followed by a neutralization step with DMEM/Hepes, pH 7.7. Data represent the means of three independent experiments in triplicate.
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 EGF 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 endocytotic 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 chimera of 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 chimeras 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 chimeras 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 pKα 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, a protein complex containing the receptor and a second messenger protein can be formed under physiological conditions and that tyrosine kinase activity can be induced in the absence of ligand. However, the specific type of receptor activation is still a matter of debate [28].

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 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 EGF/R 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-3-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 EGF/R 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 low-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 EGF/R can still have mitogenic activity very similar to that of wild-type EGF [35–39]. 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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