Journal of Cellular Biochemistry 32:293–304 (1986)
Growth Factors, Tumor Promoters, and Cancer Genes 1–12

Transforming Growth Factor-α: Structure and Biological Activities

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Two types of growth factors have been termed transforming growth factors (TGFs). One of these, TGF-α, is related to epidermal growth factor (EGF) and binds to the EGF receptor, while the other one, TGF-β, is a structurally unrelated protein with a distinct receptor. The initial observation that led to the identification of TGF-α was that some retrovirally transformed fibroblasts displayed a strongly reduced number of EGF binding sites at their surface [1]. It was subsequently shown that these cells release an EGF-like factor that is able to bind to the EGF receptors, which then become unavailable for binding of an externally added ligand. This EGF receptor binding factor was first isolated from murine-sarcoma-virus-transformed fibroblast cultures and was therefore initially called sarcoma growth factor [2]. Subsequent examination of a variety of cell sources showed that this factor was made by many more transformed cells but not by adult normal cells in culture [3–6].

Sarcoma growth factor preparations are able to induce profound morphological changes in rat fibroblasts when added to the medium. These changes result in a phenotype similar to that of virally transformed cells. Removal of these growth factor preparations results in a reversion of the cellular phenotype back toward the normal. It was also shown that these preparations enable normal anchorage-dependent rat fibroblasts to grow in soft agar. However, when these anchorage-independent soft agar colonies are selected and subsequently plated in the absence of these growth factor preparations, they grow again as normal contact-inhibited fibroblasts [2,6]. The fact that preparations of this factor were able to convert the normal rat kidney (NRK) cells into phenotypically transformed cells and the synthesis of this factor by several different transformed cells led to the name transforming growth factor.

Initially it was assumed that sarcoma or transforming growth factor was a single peptide [2]. Extensive biochemical purification and characterization showed later that the preparations consisted of the structurally unrelated peptides TGF-α and -β. While the binding to the EGF receptor is solely due to the presence of TGF-α, the profound morphological changes observed with the rat fibroblasts are due to the cooperative effect of TGF-α and -β [7]. TGF-β by itself will not induce any colony formation of

Received April 21, 1986; revised and accepted July 28, 1986.

NRK cells in soft agar. Pure TGF-α preparations will only have a minimal effect in this assay system but can elicit the formation of few relatively small colonies, which may be ascribed to the synergistic activity of low levels of active TGF-β in the bovine serum used. In contrast, the simultaneous presence of both TGF-α and -β will result in the acquisition of the transformed phenotype by normal rat kidney cells as shown by the appearance of a high number of large colonies in the soft agar assay. It should be stressed that the need for both growth factors in order to promote phenotypic transformation is dependent upon the cell system used. Both TGF-α and -β are indeed needed in the NRK system, but in many other cell systems they do not have a cooperative effect on proliferation or transformation. In some systems TGF-α (or EGF) and -β could even function as antagonists [8].

TGF-α and Its Precursor

TGFs-α have been detected in culture supernatants and extracts from several transformed rodent and human cells [2,3,9]. These TGFs-α, which all bind to the EGF receptor, display upon gel filtration a heterogeneity in apparent molecular weights ranging from a 6-kd species secreted by several tumor cell lines [9] to the 34-kd TGF-α species detected in the urine of cancer patients. The low molecular weight TGF-α species has been purified to homogeneity from several cell sources [10]. Subsequent amino acid sequencing led to the establishment of the complete amino acid sequence of the 50-amino-acid-long rat TGF-α [11]. These data on the structure of TGF-α have now been confirmed and extended by cDNA analysis. The sequence of a human TGF-α cDNA derived from a renal cell carcinoma cDNA library indicates that the 50-amino-acid TGF-α is synthesized as a larger precursor [12], as has now been confirmed by the sequence analysis of rat TGF-α cDNAs [13].

Human TGF-α is encoded by a 4.5–4.8-kb mRNA. cDNA clones derived from either a renal cell carcinoma or a fibrosarcoma cell line reveal that the 50-amino-acid TGF-α is initially translated as an internal part of a 160-amino-acid precursor from which it is derived after proteolytic cleavage (Figs. 1 and 2.) The initiator ATG is followed by a short hydrophobic sequence between positions 8 and 18, suggesting the presence of an amino-terminal signal sequence. Comparison with other signal sequences suggests that the cleavage by the signal peptidase could occur following the Ala at position 19, the Cys at position 20, or the Ala at position 22, although no experimental proof for this is available. The Asn-Ser-Thr triplet at positions 25–27 could possibly be a site for N-glycosylation.

Comparison of the precursor sequence with the available direct amino acid sequencing data of rat, mouse, and human TGF-α [9,11] starts with the N-terminus Val-Val at positions 40–41 and ends at the Leu-Ala dipeptide (amino acids 88–89; Fig. 1). In order to generate the 50-amino-acid TGF-α, proteolytic cleavage of the precursor must occur at both the amino and carboxy terminus between an alanine residue and valine dipeptide. This Ala-Val-Val trimer is located within the sequence Val-Ala-*Ala-Val-Val* at the amino terminus of the 50-amino-acid TGF-α and within the similar sequence *Ala-Val-val*-Ala-Ala at its carboxyl end. Proteolytic processing of a precursor protein by a protease with such specificity has not been described for any other polypeptide. In contrast, cleavage of precursors for polypeptide hormones often takes place at dibasic residues. An extremely hydrophobic domain begins nine residues downstream of the carboxy terminus of the 50-amino-acid TGF-α. This region, which consists almost exclusively of isoleucines, leucines, and valines, is 23

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                                                                      20
Human:  Met Val Pro Ser Ala Gly Gln Leu Ala Leu Phe Ala Leu Gly Ile Val Leu Ala Ala Cys
Rat:                Ala                         Leu                 Leu Val     Val
                                                                                  ____
                                                                      40
Human:  Gln Ala Leu Glu Asn Ser Thr Ser Pro Leu Ser Ala Asp Pro Pro Val Ala Ala Ala VAL
Rat:                              Pro             ---     Ser
        ____________________________________________________________________________
                                                                      60
Human:  VAL SER HIS PHE ASN ASP CYS PRO ASP SER HIS THR GLN PHE CYS PHE HIS GLY THR CYS
Rat:                        LYS                         TYR
        ____________________________________________________________________________
                                                                      80
Human:  ARG PHE LEU VAL GLN GLU ASP LYS PRO ALA CYS VAL CYS HIS SER GLY TYR VAL GLY ALA
Rat:                            GLU                                               VAL
        ____________________________
                                                                     100
Human:  ARG CYS GLU HIS ALA ASP LEU LEU ALA Val Val Ala Ala Ser Gln Lys Lys Gln Ala Ile
Rat:                                                                          ----------
                                                                     120
Human:  Thr Ala Leu Val Val Val Ser Ile Val Ala Leu Ala Val Leu Ile Ile Thr Cys Val Leu
Rat:    ----------------------------------------------------------------------------------
                                                                     140
Human:  Ile His Cys Cys Gln Val Arg Lys His Cys Glu Trp Cys Arg Ala Leu Ile Cys Arg His
Rat:    -----                                                             Val
                                                                     160
Human:  Glu Lys Pro Ser Ala Leu Leu Lys Gly Arg Thr Ala Cys Cys His Ser Glu Thr Val Val
Rat:
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Fig. 1. Comparison of the amino acid sequences for the human and rat TGF-α precursors. Only the residues different from the human sequences are indicated for the rat precursor. The sequence of the 50-amino-acid fully processed TGF-α is shown in capitals and is overlined. The 23-amino-acid hydrophobic sequence that may function as a transmembrane region is underlined with a dashed line.

amino acids long (residues 99–121; Fig. 1) and is flanked by paired basic amino acids at positions 96–97 and 127–128. The length of the hydrophobic domain and the basic character of the flanking amino acids is characteristic of transmembrane domains of membrane proteins. This would thus suggest that the TGF-α precursor, following the removal of the NH_2-terminal signal peptide, would be inserted into the membrane (Fig. 2). Subsequently, the Ala-Val-Val-specific protease, which is likely located at the external side of the membrane, would then cleave the external segment of the precursor and in this way release the 50-amino-acid TGF-α. It may be conceivable that not all cells have this protease. This could then result in the continuous anchorage of the TGF-α precursor in the membrane and the absence of TGF-α in the medium. The sequence downstream of the 23-amino-acid hydrophobic domain is very rich in cysteines. Indeed, seven cysteines are present in the 37 amino acids at the C-terminus of the precursor. If the TGF-α precursor constitutes a transmembrane protein, then this C-terminal portion would remain at the cytoplasmic side of the membrane and probably not undergo any disulfide bond formation in this protein domain.

The rat TGF-α precursor, deduced from cDNA cloning [13], is 159 amino acids long, ie, one residue shorter than the human counterpart, and has all the features described above for the human precursor (Fig. 1). Comparison of the deduced rat and human precursor sequences indicates a very strong homology. Only four differences are observed in the 50-amino-acid-long TGF-α sequence. The precursor sequence downstream of the TGF-α peptide is even more conserved and displays only conservative differences between both species. This striking conservation of the sequence, which encompasses the putative transmembrane region and the potentially cytoplasmic segment, suggests a potentially important biological function for the

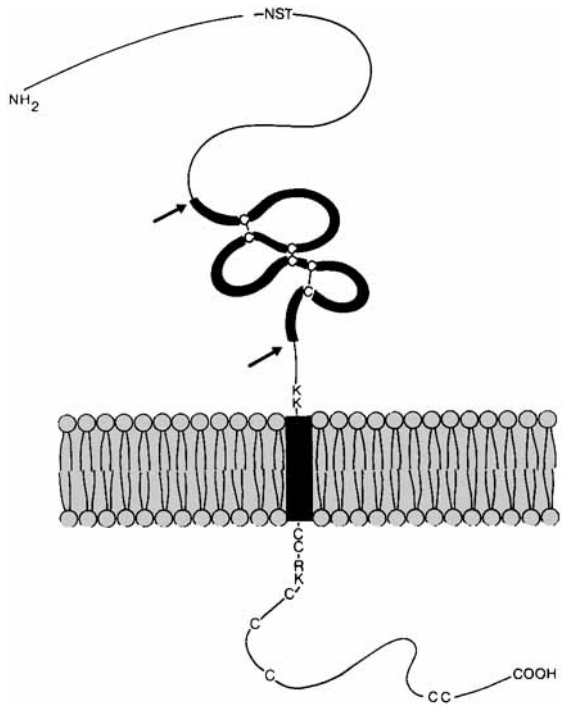

Fig. 2. Depiction of a hypothetical model of the TGF-α precursor as a transmembrane protein. The NH_2-terminal signal sequence is shown as already cleaved from the precursor. The 50-amino-acid TGF-α with its three proposed cysteine (C)-disulfide bridges is shown as a heavy line, flanked by the proteolytic cleavage sites (arrows). The boxed transmembrane region is flanked at each side by two basic amino acids (KK and RK). The carboxy-terminal cytoplasmic domain shown below the membrane is rich in cysteines (C).

C-terminus. However, it is as yet unclear what the physiological role of this peptide would be. It could be proposed that the intracellular segment is cleaved from the transmembrane region and thus exerts a biological function as a separate entity. However, cleavage at the Arg-Lys residues that flank the putative transmembrane region at the cytoplasmic side is unlikely, since the processing protease that cleaves at the dibastic peptide is presumably located at the external side of the membrane. Alternatively, the cytoplasmic peptide would remain covalently attached to the transmembrane region, and it could then be suggested that it plays a role in some type of signal transduction, possibly in a way similar to a receptor. The high number of Cys-residues would then probably have biological significance. However, it is not known if the TGF-α precursor, perhaps in its unprocessed form, can function as a receptor molecule as has been proposed for the much larger EGF precursor. Whatever the function of the C-terminal segment of the precursor may be, it is important to recognize that the synthesis and secretion of TGF-α goes together with the synthesis of the C-terminal precursor segment, which may have separate activities in the physiology of the cell.

As mentioned above, several larger forms of TGF-α besides the 50-amino-acid species can be detected in the medium of transformed cells. Genomic hybridizations have not revealed the existence of more than one TGF-α gene [12], which suggests that the larger forms may be derived from the same gene and thus from the same

precursor. The fact that several larger TGF-α species isolated from retrovirally transformed cells react with a polyclonal antiserum to the C-terminus of the 50-amino-acid TGF-α supports this view [14]. Proteolytic processing at different sites of the precursor, eg, at the dibasic residues, or the lack of processing could then result in the generation of various other forms. The larger forms could possibly be due to some form of aggregation to other proteins or to a type of dimerization or oligomerization.

Structural Homology With EGF

TGF-α and EGF have an unambiguous sequence homology [11,12]. Twenty-one of the 50 residues of human TGF-α, including all six cysteines, are found in corresponding positions in the human EGF sequence, while rat TGF-α and human EGF have 17 amino acids in common. However, there is a major difference in isoelectric point, ie, 6.8 for rat TGF-α and 4.5 for mouse EGF. The positions of the three disulfide bonds in murine EGF have been determined [15]. It is likely that the same disulfide bridges exist in TGF-α, since both peptides bind to the same receptor. This would imply the presence of disulfide bridges between the first and third, second and fourth, and fifth and sixth cysteines (Fig. 2). The homology between TGF-α and EGF is most concentrated in the third disulfide-bounded loop of the peptides, suggesting that this region could be most important in the binding to the receptor. This suggestion may be strengthened by the fact that synthetic peptides corresponding to only this loop are able to bind to the receptor, albeit with a much lower affinity [16].

A 140-amino-acid-long polypeptide encoded by vaccinia virus [17] contains a sequence that appears to be closely related to EGF and TGF-α. The conservation of all six cysteines in this polypeptide segment suggest that post-translational processing could result in the release of a peptide, which, owing to a disulfide bond configuration similar to that of EGF and TGF-α, could bind to the EGF/TGF-α receptor. Experimental evidence shows that vaccinia-virus-infected cells do indeed release an EGF receptor binding protein, which has been named vaccinia virus growth factor (VVGF). Biochemical characterization indicates that VVGF is 77 amino acids long and is glycosylated, in contrast to EGF or TGF-α. N-terminal sequencing of VVGF shows that the first cysteine is preceded by a 25-residue-long segment, which is much longer than the corresponding sequence in TGF-α and EGF [18].

All three peptides that bind to the same receptor and presumably have a homologous disulfide bridge formation, EGF, TGF-α, and VVGF, are initially synthesized as larger precursors. The human TGF-α [12] and VVGF [17,18] precursors are 160 and 140 amino acids long, while the murine EGF precursor is 1,217 residues long [19,20]. The corresponding EGF/TGF-α-like sequences in all three precursors are flanked by sequences that also show an unambiguous sequence homology. In all three precursors, the EGF/TGF-α-like sequence is closely followed at a similar distance by a hydrophobic domain, which could act as a transmembrane region. However, the sites of proteolytic cleavage, which results in the release of both peptides, are certainly dissimilar in sequence, thus indicating a different processing mechanism. No clear sequence homology is present in the C-terminal regions, which supposedly are located at the cytoplasmic side of the membrane.

Cellular Sources of TGF-α Synthesis

TGF-α activity was initially detected in culture supernatants of rodent fibroblasts transformed with Moloney or Kirsten murine sarcoma viruses [3–6]. An initial

survey of various transformed cell lines indicated that EGF receptor binding activity could be found predominantly in the medium of retrovirally transformed cells, but to a much lesser extent in cells transformed with DNA viruses or chemical carcinogens [6]. However, it has been reported that transformation by SV40 [21] or polyoma [22] will induce TGF-α secretion. In the latter case, transfection of rat cells with the DNA segment coding for middle T is sufficient to induce both the transformed phenotype and TGF-α production. The close correlation between TGF-α synthesis and transformation is also illustrated by experiments with rat cells transformed with a Kirsten murine sarcoma virus, which secretes TGF-α only when phenotypic transformation occurs at the permissive temperature [4].

The reported secretion of TGF-α by retrovirally transformed fibroblasts and the apparent lack of it by normal fibroblasts in cultures led us to examine a large variety of human tumor cell lines and surgically removed tumors for the presence of TGF-α mRNA [23]. This study showed that TGF-α mRNA could not be detected in any of ten tumor cell lines of hematopoietic origin. On the other hand, it was detectable in many solid tumors or cell lines derived from such origin. While TGF-α mRNA is present in several sarcomas or sarcoma cell lines, its occurrence is biased toward carcinomas and tumors of neuroectodermal origin. TGF-α mRNA is most consistently synthesized in renal carcinomas and in squamous carcinomas, irrespective of their location, but can also be frequently found in many mammary carcinomas and in tumors of neuronal origin. It is possible that TGF-α is also consistently synthesized by some other tumor types, but the low number of samples of a particular tumor type precludes generalizations. The occurrence of TGF-α mRNA in such a large variety of solid tumors suggests that the synthesis of TGFα may play a biological role in malignant transformation and tumor development in vivo. The synthesis of TGF-α by these tumors could then explain the presence of a high molecular weight EGF receptor binding factor that reacts with specific anti-TGF-α antibodies in the urine of some cancer patients and not in the urine of normal controls [24,25].

The initial observations suggested that secretion of TGF-α is tightly linked with malignant transformation. Accordingly, TGF-α could not be detected in medium from normal cells in vitro and is not known to be made in normal fully developed tissues. However, it should be stressed that only a very limited number of observations have been reported, and that as yet it cannot be excluded that TGF-α may play a role in the normal physiology of the adult organism. Recent evidence from specific antibody-based detection and Northern hybridizations have indicated that TGF-α is synthesized during early fetal development [25,13]. The TGF-α expression in the murine fetus appears to peak around day 9 and quickly levels off, so that there is no detectable TGF-α birth (day 21). This indicates that TGF-α may function as a normal embryonic version of a family of EGF-related growth factors. The expression of the gene may be reinitiated during the process of malignant transformation and tumor development, indicating that TGF-α is an oncodevelopmental antigen.

Role of TGF-α in Malignant Transformation

It has now been convincingly illustrated that the induction of anchorage independence by the original TGF preparations in normal rat fibroblasts was due to the cooperative activity of TGF-α and TGF-β [7]. As these soft agar assays are usually performed in the presence of serum that contains a variety of growth factors, it cannot be excluded that still other factors may cooperate. TGF-α by itself can exert mitogenic

activities but may not be very effective in inducing anchorage independence. It is, however, important to recognize that both in vitro and in vivo the cells are continuously exposed to an environment of growth factors. Therefore, a change in expression levels of one particular factor may in a cooperative fashion trigger major changes in the behavior and the phenotype of particular cells. It is thus conceivable that initiation of TGF-α synthesis may trigger or contribute to phenotypic transformation owing to cooperativity with TGF-β or other factors. It has been postulated that during the transformation process, TGFs exert their action via an autocrine mechanism, whereby they help sustain the transformed character of the same cells from which they are secreted [26,27]. In the case of TGF-α, this would be due to an interaction of the growth factor with the EGF receptor, which would then induce a down-regulation of the ligand-receptor complex and induce subsequent physiological changes. Such an autocrine mechanism could explain the initial observation that retrovirally transformed cells have a lower number of EGF binding sites at their surface as a result of endogenous TGF-α secretion [1]. While TGF-α synthesis could contribute to malignant transformation through an autocrine mechanism, it could also exert some activities on other cell populations via a paracrine mechanism.

Much attention has been focused in the last few years on whether the secretion of a growth factor can induce malignant transformation via an autocrine mechanism. One of the best studied systems to date is the woolly monkey simian sarcoma virus (SSV), which contains the v-*sis* gene, which is highly homologous to the c-*sis* gene coding for the B chain of platelet-derived growth factor [28,29]. Experimental evidence indicates that the binding of the v-*sis* gene product to the receptor for platelet-derived growth factor is responsible for the SSV transformation via an autocrine mechanism [30,31]. This is in agreement with the experimentally induced phenotypic transformation that is due to the overexpression of the human cellular homologue, the c-*sis* gene [32]. An autocrine mechanism has also been invoked for the phenotypic changes triggered by superinfection of v-*myb*-transformed chicken myeloblasts with retroviruses carrying src-related oncogenes. This superinfection induced growth factor independence in these cells, which are otherwise dependent for their growth on the presence of a specific myelomonocytic growth factor [33]. In another hematopoietic system, transfection of recombinant retroviral vectors that express granulocyte-macrophage colony stimulating factor (GM-CSF) into a hematopoietic precursor cell line resulted in the acquisition of malignant characteristics by these cells. This was confirmed by the ability of these GM-CSF producing cells to develop leukemias in mice [34]. As illustrated by these three examples, cellular endogenous production of a growth factor can induce malignant transformation. Overproduction of TGF-α could thus possibly be involved in transformation via a similar autocrine mechanism. The fact that TGF-α mRNA is most consistently produced in squamous carcinomas, which all contain relatively high levels of EGF receptor mRNA, would be in agreement with this hypothesis. However, it has not yet been reported whether constitutive expression of TGF-α is sufficient to induce malignant transformation via an autocrine mechanism. Also, while TGF-α expression is quite common in solid tumors and tumor cell lines, it is still debatable if the autocrine mechanism of transformation by TGF-α or by any growth factors will induce or contribute to the development of human malignancies in vivo.

Biological Activities of TGF-α: Comparison With EGF

Studies of the biological activities of TGF-α have been very much hampered by the low availability of sufficient pure TGF-α from transformed fibroblasts. The need

to carry out the biological experiments with TGF-α devoid of any other biologically active peptides is exemplified by the fact that the initial TGF preparations contained both TGF-α and -β, which resulted in the induction of anchorage independence, in contrast to results with homogeneously pure TGF-α and EGF. Most experiments with natural TGF-α have used rat TGF-α from Snyder-Theilen feline-sarcoma-virus-transformed rat fibroblasts [10,11]. The determination of the complete sequence of the 50-amino-acid rat TGF-α [11] has enabled the direct synthesis of larger amounts of rat TGF-α by solid phase techniques [35]. The isolation of a human TGF-α cDNA has also led to the synthesis and purification of relatively large amounts of human TGF-α from properly engineered *Escherichia coli* [12].

It is generally accepted that the biological actions of TGF-α, like the actions of other polypeptide hormones, are mediated through the binding to specific cell surface receptors. Earlier studies have indicated that TGF-α can interact not only with the EGF receptor but also with a 60-kd membrane component that does not bind EGF. It was proposed that this 60-kd protein was a putative TGF-α receptor species, which may mediate TGF-α effects that are directly involved in the induction of the transformed phenotype [36]. However, the induction of anchorage independence can be neutralized by blocking antibodies raised against the EGF receptor [37], which make the identity of the 60-kd protein as a specific TGF-α receptor unlikely. The binding of TGF-α to the EGF receptor makes it possible to quantify TGF-α on the basis of a generally used radioreceptor assay in which TGF-α competes with ^{125}I-EGF for receptor binding. Comparison between murine EGF and the 50-amino-acid TGF-α secreted by transformed rat fibroblasts has indicated that both ligands exhibit a remarkably similar mode of interaction with the EGF receptor and that both peptides compete for receptor binding with the same potency and to the same extent [38]. However, the 50-amino-acid recombinant human TGF-α purified from *E. coli* cultures and subsequently refolded appears to be only about half as potent in EGF receptor binding as murine EGF (M. Winkler, personal communication). Comparison of binding characteristics has revealed that natural rat TGF-α requires a stringent pH optimum for receptor binding in contrast to EGF. Exposure of placental membranes, which contain EGF receptors, to several lectins will modify the binding of EGF or TGF-α in a parallel way. Continued exposure of A431 cells to either TGF-α or EGF induces down-regulation of the receptors according to similar kinetics [38]. Interaction of TGF-α to the EGF receptor will also mimic the action of EGF to activate a receptor-associated kinase [39]. While our current knowledge thus indicates that TGF-α may exert its activities through the EGF receptor, it is not known how EGF receptors with different stages of affinity will behave in vivo with respect to their binding properties for TGF-α or EGF.

As sufficient quantities of TGF-α are becoming available, EGF and TGF-α are being compared for their biological activities. It is now well established that the full induction of anchorage independence of normal rat kidney fibroblasts by the earlier sarcoma growth factor or transforming growth factor preparations is due to the cooperative effect of both TGF-α and -β [7]. Pure TGF-α and EGF are equally potent in these soft agar colony formation assays in the presence of TGF-α, and both growth factors appear therefore to be interchangeable. The absence of exogenously added TGF-α results in a highly depressed efficiency of induction of anchorage independence, but again the concentration-dependent response curves for EGF and TGF-α are superimposable [12]. It is assumed that the low but still significant responses seen

in the soft agar assay with either of these peptides is due to the presence of TGF-β in the serum.

One of the first biological activities established for EGF was that it is able to induce precocious eyelid opening in newborn mice. Injection of EGF into newborn mice accelerates eyelid opening in a concentration-dependent way, from 12 days in the absence of exogenous growth factor to 8 days at the maximal EGF concentration. Comparison of human TGF-α with murine or human EGF indicates that also in this assay both growth factors induce similar responses [40]. In addition to the effects on eyelid opening, both EGF and TGF-α also induce other changes in the somatic development of the mouse. They induce accelerated tooth eruption, retard the growth rate, and inhibit hair growth. While it has been reported that TGF-α and EGF do not differ significantly in these activities [41], it is important to evaluate these data with caution, since the group treated with EGF consisted only of two or four animals, depending upon the experimental parameter examined. Also, the control animals were injected with EGF only at a single concentration that presumably triggers maximal responses. Because of these limitations, it is impossible to derive relevant conclusions concerning the relative responses and especially the relative potencies of both growth factors. In addition, the photographic evidence of the effects on hair growth and morphology would suggest that TGF-α exerts more pronounced effects on the hair morphology and on hair follicle development [41].

Cell ruffling is a very early response of cells in culture to administration of various growth factors. Both TGF-α and EGF are able to induce rapid and transient ruffling responses in sparsely cultured cells. At lower doses, the magnitude and duration of the responses to either factor is similar, but at high doses the maximal responses for both parameters are higher with TGF-α than with EGF. Pretreatment of the cells with TGF-β greatly enhances the ruffling response to TGF-α but will antagonize the EGF-induced ruffling [42]. TGF-α and EGF have also been compared for their ability to induce proliferation of human epidermal cells. Also, in this cell culture system differences in activity between EGF and TGF-α are apparent. TGF-α elicits a greater effect in inducing the formation of epidermal cell colonies than does EGF (Barrandon and Green, personal communication).

The biological activities of EGF and TGF-α have been compared in several other systems, which also revealed differential responses to both factors. The ability of a factor to induce release of calcium ions in a bone organ culture is often studied in two established systems. Either murine calvaria or fetal rat long bones prelabeled with $^{45}Ca^{2+}$ are incubated in the presence of the factor. An increase of Ca^{2+} release owing to the presence of the growth factor is a measure of bone resorption and could bear relevance to hypercalcemia in vivo. Both EGF and TGF-α are able to induce Ca^{2+} release in the calvaria system, but TGF-α is about three- to ten-fold more potent than EGF and induces a response at concentrations as low as 0.5 ng/ml. The difference is more striking in the fetal rat long bone system, since TGF-α induces a pronounced Ca^{2+} release in a dose-dependent manner, while EGF does not trigger any statistically significant effect [43,44]. Studies on cultured cells indicate that this induction of bone resorption by TGF-α may be due to an inhibition of osteoblast activity as measured by the effects on collagen synthesis and by an activation of the osteoclast population [44]. In a recently developed in vitro system, TGF-α was about 10- to 100-fold more potent than EGF in stimulating the proliferation of osteoclast-precursor cells [45]. Hypercalcemia in vivo is often observed in conjunction with

advanced stages of malignancies. It can therefore be speculated that the strong potency of TGF-α to induce bone resorption in vitro may have significance in vivo. This hypothesis may be enforced since TGF-α mRNA is produced most consistently in squamous, renal, and mammary carcinomas and in melanomas [23], which often induce malignancy-associated hypercalcemia [46]. It should be emphasized, however, that other factors have been implicated in hypercalcemia and that parathyroid hormone-like polypeptides released by several tumor cells can also induce bone resorption [46]. It is therefore likely that a single factor may not be responsible for all cases of malignancy-associated hypercalcemia, and it is also possible that several factors in conjunction with each other may trigger hypercalcemia in vivo. If TGF-α released by the tumor cells indeed induces hypercalcemia in vivo, then these activities are exerted via a paracrine or even an endocrine mode.

TGF-α has also been tested for its effect on angiogenesis. Different quantities of TGF-α or EGF were absorbed to blue Sepharose and subcutaneously implanted in the hamster cheek pouch. This in vivo system allows the monitoring of the extent of neovascularization that is due to the formation of capillaries that migrate toward the site of implantation. EGF is a relatively poor inducer of angiogenesis, but TGF-α is able to induce neovascularization at low concentrations that are without any effect in the case of EGF. However, both factors were equally efficient in inducing their mitogenic effects on cultured endothelial cells and on several other cells in vitro [47]. Thus, the differential angiogenic response cannot be explained by simple differences in mitogenicity on the capillary endothelial cells. It is well recognized that tumor cells secrete angiogenic factors and that tumor-derived angiogenesis is crucial to tumor development. It is possible that TGF-α, which is apparently synthesized by many tumors, could play a role in the induction of neovascularization in the tumor, could play a role in the induction of neovascularization in the tumor. This would be in agreement with the lack of TGF-α synthesis by cell lines derived from hematopoietic tumors, which do not require neovascularization. It is important, however, to recognize that several tumors have been shown to synthesize other potent angiogenic polypeptides, which could either alone or in combination with TGF-α induce neovascularization in the tumor. It is not yet known to what extent TGF-α or these other factors will induce angiogenesis in the different tumor types in vivo. The production of TGF-α by the tumor may thus be important to tumor development in at least two ways. It could act as a stimulator of tumor cell proliferation in an autocrine way, but it could also contribute to the induction of tumor-derived angiogenesis using a paracrine mechanism. The latter activity of TGF-α could also be physiologically important during early fetal development [48,49].

EGF exerts a potent activity on vascular tissue, which results in an increase in regional arterial blood flow in a variety of vascular beds. TGF-α and EGF displayed an equal potency in this system, but the maximal response obtained for TGF-α was much higher than with EGF. In addition, prior exposure of the vascular tissue to TGF-α markedly desensitized the arterial system to EGF but not to TGF-α. The synthesis of TGF-α by tumor cells and the fact that TGF-α does not cause desensitization to its own action may suggest that it could play a persistent role in the local vascular hyperdynamic state associated with malignancy (M.D. Hollenberg, personal communication).

The results briefly discussed above indicate that TGF-α and EGF behave differently in several biological systems. In many cases, TGF-α is much more potent

than EGF and seems to behave as a superagonist. In other systems, the responses elicited by both factors are very similar, if not identical. In any case, it is important that TGF-α and EGF should not be considered as mere analogues because they bind to the same receptor and cannot be discriminated in their biological effects in some assays. It could be argued that some quantitative differences in response by TGF-α or EGF may be due to differential stability in the assay systems or to differential aggregation with binding proteins. However, such an explanation does not agree with the data obtained in cell culture or in the in vivo eyelid opening assay, nor could it account for differences in maximal effects or for the qualitatively different responses observed for both growth factors. It is unclear how both TGF-α and EGF, which bind to the same receptor, can trigger differential responses. It has been proposed that there are EGF receptors with high and low affinity for EGF, and it may be possible that TGF-α and EGF will exhibit differences in their binding to these. One could also postulate that there are differences in behavior of the ligand-receptor complex during internalization. It is also possible that receptor binding of EGF or TGF-α will trigger different effects in some specialized cell types such as osteoblasts or endothelial cells in contrast to, for example, fibroblasts. Detailed studies on receptor-ligand interactions and subsequently triggered physiological events will therefore be needed to explain the differential activities of TGF-α and EGF.

ACKNOWLEDGMENTS

I acknowledge all colleagues at Genentech who contributed to the determination of the human TGF-α cDNA sequence and its expression in bacteria: E. Chen, D. Eaton, D. Goeddel, and M. Winkler. I thank M.D. Hollenberg (University of Calgary) and H. Green (Harvard University) for allowing the communication of data prior to submission of a manuscript. J. Arch is acknowledged for her skillful assistance in the preparation of this manuscript.

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