Detection of Transforming Growth Factor-α mRNA and Protein in Rat Lacrimal Glands and Characterization of Transforming Growth Factor-α in Human Tears

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Purpose. To assess whether the lacrimal gland is a possible site of synthesis of transforming growth factor-α (TGF-α) and to characterize TGF-α biochemically in human tears.

Methods. Reverse transcription–polymerase chain reaction (RT–PCR) amplification was used to analyze rat lacrimal glands for the presence of TGF-α mRNA. Specific monoclonal antibodies were used to localize TGF-α immunohistochemically in lacrimal gland tissue of rats. Human tears were analyzed for immunoreactive TGF-α protein using a specific radioimmunoassay, and the molecular weight of TGF-α in tears was characterized by Western blot analysis.

Results. RT–PCR amplification of rat lacrimal gland RNA generated a band of the predicted 492 base pairs for TGF-α mRNA. Immunohistochemical staining of rat lacrimal gland localized TGF-α protein to lacrymocytes constituting acini but not to interacinar and intraacinar ducts of lacrimal glands. Western blot analysis of human tears detected a single band at MWt 16,000. Logit transformation of radioimmunoassay data for tears and TGF-α standard generated parallel displacement lines, indicating the presence of immunoreactive TGF-α levels in human tears with an average concentration of 100 ± 20 pg/ml (mean ± SEM).

Conclusions. Rat lacrymocytes synthesize TGF-α mRNA and protein, and human tears contain immunoreactive TGF-α, suggesting that the lacrimal gland may be an exocrine source for TGF-α in tears. The single MWt 16,000 form of TGF-α in human tears appears to be generated by an unusual proteolytically processing of the pro-TGF-α transmembrane precursor protein.


Tears provide substances, including bacteriostatic proteins such as immunoglobulins and lysozyme, lipids, mucins, and electrolytes, that are critical for normal corneal physiology.¹ The discovery of epidermal growth factor (EGF) in human tears and the expression of EGF receptor by corneal cells enlarged the concept for the role of tears in corneal physiology to include providing an important mitogen for corneal cells.²,³ Although the physiological roles for EGF in tears have not been established definitively, it has been suggested that EGF may act to stimulate the healing of epithelial injury by promoting migration and mitosis of epithelial cells.³ This concept is strengthened by reports⁵,⁶ of increased rates of epithelial wound healing in animals and patients with the topical application of EGF in eye drops. The effects of tear-borne EGF on corneal wound healing may not be limited to epithelial cells but may include effects on stromal fibroblasts and endothelial cells as well. Cultures of human stromal fibroblasts and endothelial cells were reported to express EGF receptors and to increase DNA synthesis during culturing with EGF.⁶,⁷ Topical application of EGF eye drops increased tensile strength of corneal incisions.⁴,⁵ Thus, EGF in tears may play important roles in promoting corneal wound healing after superficial and penetrating injury.

We recently reported⁸ the detection of transforming growth factor alpha (TGF-α) in human tears.
Structurally, TGF-α is related to EGF and binds to the EGF receptor. The effects of TGF-α on corneal cells have not been established, but the effects of EGF and TGF-α have been examined in other systems. In general, the biologic effects of TGF-α and EGF on most cells are similar, but there are several reports of qualitative and quantitative differences. For example, TGF-α was a more potent stimulator of angiogenesis and skin wound healing than EGF, and TGF-α induced more rapid release of previously incorporated calcium from bone cultures than EGF, and TGF-α induced the formation of large colonies of keratinocytes compared to the size of colonies induced by EGF. Also, TGF-α stimulated and EGF inhibited the proliferation of lung carcinoma cells, and EGF, but not TGF-α, induced persistent desensitization of contraction with indomethacin-treated stomach smooth muscle cell preparations.

The sources of TGF-α in tears have not been investigated fully. We recently reported that human corneal epithelial cells contain TGF-α mRNA and protein. However, because of the limited diffusion through the epithelium of charged proteins, we speculated that TGF-α produced by corneal epithelial cells would not contribute significantly to the levels of TGF-α present in tears. Instead, we speculated that TGF-α produced by epithelial cells would act predominantly by an autocrine or juxtacrine mechanism to influence basal epithelial cells. This prompted us to examine other sources for TGF-α in tears. The lacrimal gland is a major source of tear fluid proteins, and its role in the secretion of EGF has been emphasized. The purpose of this study was to investigate the potential of the lacrimal gland to synthesize and secrete TGF-α into tears and to further biochemically characterize TGF-α in tears.

**METHODS**

**Detection of TGF-α mRNA in Rat Lacrimal Glands by Reverse Transcription Polymerase Chain Reaction**

Procedures involving experimental animals were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research under a protocol approved by the Animal Care and Use Committee of the University of Florida. Analysis of rat lacrimal gland for TGF-α mRNA was performed using RT-PCR amplification of total RNA isolated as described previously. Briefly, lacrimal glands were excised from adult (300 gm) male Sprague-Dawley rats, immediately homogenized in 4 M guanidinium isothiocyanate containing 25 mM sodium citrate, pH 7, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol, and then extracted with phenol, chloroform, isooamy alcohol (1:0:0:5:0:2, vol/vol/vol) at pH 4 for the preparation of total RNA. For a positive control, total RNA was isolated from cultures of SW48 cells, a human colon cancer line, which is known to synthesize TGF-α mRNA and protein. A negative control containing no RNA was used to assure no cross-contamination of samples. Two micrograms of total RNA was reverse transcribed with a human TGF-α specific downstream primer 5'-CCCTGGGTCTCTTCA-GAGGAC-3' as described previously. The RT reactions were performed for 10 minutes at an annealing temperature of 25°C, 60 minutes at an extension temperature of 42°C, and 5 minutes at a final temperature of 95°C to inactivate the reverse transcriptase and RNasin. With the addition of the upstream primer 5'-ATGGTCCCCTCGGCTGGACAG-3', the TGF-α cDNA was amplified beginning with the addition of Taq polymerase after a 5-minute hot start at 95°C. This was followed by 36 cycles of amplification of 95°C for 1.5 minutes, at 57°C for 2 minutes, and at 72°C for 3 minutes. The amplified products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. Using these primers, the predicted sizes of the authentic TGF-α amplicons is 495-bp product for human and 492-bp for rat. These primers corresponded to amino acids 1 to 160 of the TGF-α precursor protein and include the entire sequence of the mature TGF-α 50-mer. Also, the amplified TGF-α cDNA spanned 5 introns; thus, amplification of contaminating genomic DNA would generate products substantially larger than the predicted 495-bp product.

**Immunohistochemical Analysis of TGF-α in Rat Lacrimal Glands**

Immunohistochemical detection of TGF-α was performed as described previously. Briefly, lacrimal glands from five adult male Sprague-Dawley rats were fixed in Bouin's solution and embedded in paraffin. The specimens were cut into 6-μm sections, mounted on coated slides, deparaffinized, and rehydrated. After inactivation of endogenous peroxidase activity by incubation of the tissues in 3% hydrogen peroxide, nonspecific binding of immunoglobulin G was blocked by preincubation with specific serum using the ABC Kit (Vector, Burlingame, CA). The sections were then incubated for 2 hours with primary rabbit antibodies against human TGF-α. The slides were washed with phosphate-buffered saline and incubated for 2 hours at room temperature with goat anti-rabbit immunoglobulin G (ABC Kit) coupled to horse radish peroxidase. The sections were washed and incubated with diaminobenzidine substrate until colored bands were visible. The reaction was stopped with water washing. Sections were photographed with Normarski interference contrast optics.
TGF-α Radioimmunoassay of Tear Fluid

In the first experiment, a pool of human tears was evaluated for the presence of immunoreactive TGF-α protein using a specific radioimmunoassay. The specimens were collected in compliance with the tenets of the Declaration of Helsinki, informed consent was obtained, and the protocol was approved by the Institutional Human Experimentation Committee. Briefly, tear fluid samples were collected using capillaries after stimulation of reflex tearing with onion vapor. A pool of tears (10 ml) was collected from five non-contact lens wearing individuals without ocular disease, and the pool was assayed in triplicate at four dilutions. Displacement data for tears and authentic TGF-α were linearized by logit transformation, and the best-fit lines were determined by linear regression analysis. Slopes of lines generated by the tear sample and by the TGF-α standard were compared by t-test for slopes to determine if the lines were significantly nonparallel.

In the second experiment, TGF-α levels were measured in individual samples of reflex tears collected from five men and five women (age range, 30 to 45 years) without apparent ocular disease. Samples were assayed in duplicate without dilution. Concentrations of TGF-α were calculated by averaging the amounts of TGF-α interpolated from the linear regression of the standard curve and were expressed as picograms of TGF-α per milliliter of tears. The detection limit of the assay was approximately 50 pg of TGF-α in the assay. Epidermal growth factor does not cause measurable displacement in the TGF-α radioimmunoassay at concentrations less than 10,000 pg/ml.

Western Blot Analysis of Tears for TGF-α Protein

The size of immunoreactive TGF-α species in human tears was investigated by Western blot analysis using a modification of a previously described procedure. Briefly, 10 ml of tears was collected by reflex tearing from one normal individual. The tear samples were centrifuged at 10,000 g for 5 minutes, and the supernatant solution was removed and lyophilized. The tear sample was dissolved in 100 μl of sodium dodecyl sulfate (SDS) sample buffer containing β-mercaptoethanol and boiled for 5 minutes. Fifty microliters of the tear sample was loaded onto a 15% SDS-polyacrylamide gel, and 5 μg of recombinant human TGF-α similarly denatured in SDS sample buffer containing β-mercaptoethanol was loaded as a positive control. Low molecular weight standards were run in adjacent wells (BioRad Laboratories, Richmond, CA). After separation by electrophoresis, the samples were transferred to polyvinylidifluoride membrane at 70 V for 2 hours. The membrane was blocked in a solution of Tris-buffered saline containing 3% gelatin and incubated at 23°C in the same buffer containing a 500-fold dilution of the rabbit anti-human TGF-α serum for 12 hours at 22°C. The blot was washed three times, incubated with goat anti-rabbit antiserum coupled to alkaline phosphatase for 2 hours at room temperature, washed again, and substrate was added and color generated. Controls that tested the specificity of the autoradiographic bands were performed by incubating membranes with anti-TGF-α antiserum preabsorbed with recombinant human TGF-α or incubating membranes with only secondary antibody. Molecular weights of immunoreactive bands were calculated from the graph of the ratio of migration distance of prestained molecular weight standards divided by migration distance of bromophenol blue dye versus log of molecular weight of standard proteins.

RESULTS

Analysis of Rat Lacrimal Gland for TGF-α mRNA

Reverse transcription-polymerase chain reaction amplification of total RNA isolated from the SW48 human colon cancer cell line, which has been reported to synthesize TGF-α mRNA and protein, generated a single band at 495 bp, which is the predicted size of the PCR product for human TGF-α (Fig. 1). RT-PCR amplification of total RNA from rat lacre-
mal gland generated a single band of 492 bp, indicating the presence of TGF-α mRNA in lacrimal gland tissue. We reported previously that RT-PCR amplification of TGF-α from rat parotid and submandibular glands generated a 492-bp product that hybridized with a TGF-α-specific cDNA probe. Definitive proof that the 492-bp product generated from the rat lacrimal gland was amplified from TGF-α mRNA would require Southern blot analysis with a TGF-α-specific cDNA probe or digestion of the 492-bp product with an endonuclease that would yield two fragments of unique size. No amplification product was detected in the negative control lane containing no RNA.

**Localization of TGF-α Protein in Rat Lacrimal Glands**

The immunohistochemical staining for TGF-α in the lacrimal glands revealed specific staining for TGF-α protein predominantly in the parenchymal portion of the gland, whereas staining was absent in the capsule or connective tissue (Fig. 2a). In acinar cells, staining was concentrated close to the lumen opposite the basally located nuclei. Specific staining was not observed in the interacinar and intraacinar ducts of the lacrimal glands. Control sections stained with antiserum preabsorbed with TGF-α were devoid of staining (Fig. 2b).

**TGF-α Radioimmunoassay of Human Tears**

Shown in the inset of Figure 3, serial dilutions of the sample of human tears produced proportional displacement of 125I-TGF-α binding in the radioimmunoassay. Logit transformation of the displacement data for tears and authentic TGF-α generated lines with equations of $y = 4.96 - 1.59\log X$ ($r^2 = 0.97$) for the TGF-α standard and $y = 4.33 - 1.79\log X$ ($r^2 = 0.88$) for tears. The slopes of the logit lines generated by the TGF-α standard and the pooled sample of tears were compared by $t$-test for slopes and were found not to be significantly different ($P > 0.05$). This result indicates the presence of authentic immunoreactive TGF-α protein in human tears. In the second experiment, the individual samples of tears collected from five male and five female volunteers were measured for TGF-α. The average concentration of TGF-α for the five men was $95 \pm 10$ pg/ml (mean ± SE), and for the five women it was $107 \pm 23$ pg/ml, which was not significantly different. The overall average concentration in the 10 individual tear samples was $100 \pm 10$ pg/ml.

**Western Blot Analysis of Human Tears**

The immunoreactive TGF-α protein in human tears was characterized further by Western blot analysis. As shown in Figure 4, the pool of human tears contained a single band at approximate MWt 16,000 Da. No immunoreactive band was detected at MWt 6,000 Da, which is the size of the TGF-α monomer released from the transmembrane TGF-α precursor by proteolytic processing at two sites. The TGF-α standard generated a single intense autoradiographic band at approximate MWt 6,000 Da. Control membranes incubated with rabbit anti-TGF-α serum preabsorbed with TGF-α protein or incubated with only the second antibody did not produce detectable autoradiographic bands, demonstrating that the bands detected with the anti-

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FIGURE 2. Localization of TGF-α in rat lacrimal glands. Immunohistochemical staining for TGF-α in acinar cells (A), whereas control sections incubated with antiserum preabsorbed with TGF-α remained negative (B). (arrows) Lumen of acini. Bars = 100 μm.
TGF-α serum specifically recognized immunoreactive TGF-α proteins.

DISCUSSION

Because the lacrimal gland is a major source of tear proteins, we investigated the possibility that the lacrimal gland is an exocrine source of TGF-α in tears. RT-PCR amplification of total RNA isolated from rat lacrimal glands using primers unique for TGF-α nucleotide sequence generated a band of predicted size, suggesting that rat lacrimal gland contains TGF-α mRNA and, therefore, has the potential to synthesize TGF-α. The detection of immunoreactive TGF-α protein within the acinar cells of rat lacrimal glands strongly suggests that the TGF-α mRNA is translated into TGF-α protein. It is interesting to note that there was substantial heterogeneity in the staining for TGF-α in acinar cells, both within and between acinar complexes. It is possible that this pattern of staining reflects significant variation in the extent of TGF-α synthesis or secretion among acinar cells. In contrast, immunohistochemical localization of EGF in the lacrimal gland and the submandibular gland of rats revealed that the highest concentration of EGF was located primarily within ductal cells rather than the acinar cells. This suggests that the cellular sites of synthesis of EGF and TGF-α in rat lacrimal glands are different. TGF-α appears to be synthesized primarily by acinar cells, whereas EGF is synthesized primarily by ductal cells. The detection of TGF-α mRNA and protein within the acinar cells of the rat lacrimal gland suggests that TGF-α protein is synthesized by the acinar cells into tear fluid.

To assess further whether the TGF-α protein is secreted into the lumen of the acini and ultimately into tear fluid, we analyzed human tears for immunoreactive TGF-α protein. Analysis of a pool of human tears generated a displacement line in a radioimmunoassay specific for TGF-α, which was parallel to authentic recombinant human TGF-α, indicating the presence of TGF-α protein in human tears. The concentration of TGF-α was similar in a small collection of tears from five male and five female volunteers. We had detected a significantly higher level of TGF-α in tears collected from male (n = 16) volunteers than in female volunteers (n = 30). The small number of samples in this study and the variation in concentrations may have prevented determination of whether there is a difference in concentration of TGF-α in tears of men and women.
TGF-α Production by Lacrimal Gland

TGF-α in tears could be contributed by other sources, including plasma and cells of the ocular surface. A vascular origin for TGF-α in tears seems unlikely because TGF-α in plasma (average, 28 pg/ml) is approximately threefold lower than the concentration of TGF-α in tears.27 TGF-α in tears could be derived, in part or totally, from the corneal epithelial cells that we previously reported18 express TGF-α mRNA and protein. However, release of TGF-α into tears probably would be restricted to the superficial layer of epithelial cells because of the difficulty charged protein molecules have in penetrating the stratified corneal epithelial layer. If concentrations of TGF-α remain constant in reflex tears, it is strong circumstantial evidence for regulated secretion of TGF-α from the lacrimal gland. We recently reported that the tear fluid concentration of TGF-α decreased significantly (P < 0.002) with increasing total time of tear collection. This suggests that TGF-α constitutively may be secreted from the lacrimal gland or that the reserves of TGF-α stored in the lacrimal gland are small. More definitive proof that human lacrimal glands synthesize and secrete TGF-α into tears will require analysis of normal human lacrimal gland tissue for TGF-α mRNA and protein and of collecting lacrimal duct fluid for TGF-α analysis.

Results of these experiments and of the previous reports of EGF in human tears indicate that both growth factors are present in human tears. The concentration of EGF in reflex tears is approximately 950 ng/ml, which is substantially higher than the concentration of TGF-α (100 to 250 ng/ml).9 Nevertheless, this concentration of TGF-α (20 pM) is within a range that could be expected to stimulate physiological responses of corneal cells, such as chemotaxis.28 The presence of both EGF and TGF-α in biologic fluids is not uncommon and has been reported for milk,29 saliva,30 and urine.31 However, this makes us question why the lacrimal gland synthesizes two growth factors of the same growth factor family. It is possible that EGF and TGF-α have similar, but not identical, effects on corneal cell physiology and wound healing. At present, the effects of EGF and TGF-α on corneal cells have not been compared directly, but differences between the effects of the two factors have been detected in other epithelial cell systems.13,15,16 These different biologic effects of EGF and TGF-α on epithelial cells may be caused by differences in the interaction of EGF and TGF-α with their common receptor, as was observed with A431 human epidermoid carcinoma cells.31 A second advantage of expressing two similar growth factors is that the regulation of their transcription, translation, and activation may be different. This would provide greater flexibility of the lacrimal gland to respond to changes of the ocular surface. For example, levels of TGF-α mRNA increased ninefold during liver regeneration, whereas EGF mRNA was not synthesized by hepatocytes.32

Further analysis of a sample of human tears for immunoreactive TGF-α protein by Western blot analysis produced an unexpected result. Human tears contained a single band of immunoreactivity with MWT 6,000 Da. Interestingly, no band was detected in the range of MWT 6,000 Da, which is the size predicted for TGF-α monomer when it is cleaved from the transmembrane precursor at the two previously described processing sites.10,11 One possibility for this result is that pro-TGF-α was processed at an alternative site(s) during maturation in the lacrimal gland, which generated a MWT 16,000 Da monomer. Pro-TGF-α is synthesized as a 160-amino acid glycoprotein with a hydrophobic transmembrane sequence of 23 amino acids that begin at residue 99.10,11 The mature 50 amino acid soluble form of TGF-α, isolated from conditioned medium of human cells in culture, presumably is generated by proteolytic cleavage with an unidentified elastase-like enzyme at two sites of pro-TGF-α: at the N-terminal sequence, Ala-Ala-Val-Val, and at the C-terminal sequence, Leu-Leu-Val-Val-Val. However, if proteolytic cleavage of pro-TGF-α occurs at different sites, soluble forms of TGF-α with greater molecular weights would be generated. For example, a single cleavage at the normal C-terminal site between amino acids Ala-Val would produce a soluble form of TGF-α consisting of 88 amino acids and approximate MWT 9,000. Also, if cleavage occurred between the double lysines Lys-Lys of pro-TGF-α by a trypsin-like enzyme, a soluble form of TGF-α consisting of 96 amino acids and approximate MWT 10,000 would be generated. The presence of carbohydrate side chains would add additional mass. Certain tumor-derived cells and retrovirally transformed cells release soluble forms of TGF-α, called “meso-TGF-α,” that are not processed at the two sites Ala-Val sites and that contain the N-terminal glycosylated region of the precursor still linked to the mature 50-amino acid TGF-α sequence.11 In addition, the majority of immunoreactive TGF-α in human milk elutes from column chromatography with a size substantially larger than MWT 6,000.28 This suggests that the cleavage of the N-terminus of pro-TGF-α may be relatively inefficient in some cell types. Furthermore, pro-TGF-α normally contains both N-linked and O-linked glycan chains attached to the N-terminal portion that precedes the mature TGF-α 50-mer sequence. The extent of N-linked glycosylation varies in several cell types and could alter processing sites and molecular weights of soluble forms. To distinguish between these possibilities, human TGF-α would have to be purified from tears and the amino acid sequence would have to be determined to establish the N- and C-terminal processing sites.
that the lacrimal gland synthesizes and secretes growth factors, including TGF-α, that have the potential to influence corneal epithelial cell physiology, including enhancing healing of corneal wounds. Additional investigation is needed to show definitively that TGF-α is secreted by the lacrimal gland into human tears and to determine (a) the pattern of regulation of TGF-α synthesis and levels in tears after corneal injury, (b) how pro-TGF-α is processed by the lacrimal gland, and (c) whether there are important differences in the biologic effects of TGF-α and EGF on corneal cells.

**Key Words**
epidermal growth factor (EGF), lacrimal gland, tear fluid, transforming growth factor-α (TGF-α), wound healing

**References**

TGF-α Production by Lacrimal Gland


