Human corneal epithelial cells (HCEC) are constantly being shed from the apical surface and ultimately replaced primarily by mitosis of basal cells. Although the molecules that normally regulate turnover of epithelial cells in vivo have not been fully established, circumstantial evidence suggests that peptide growth factors and their receptors may play key roles. Epithelial cells have been shown to express receptors which specifically bind epidermal growth factor (EGF) and the structurally related protein, transforming growth factor-α (TGF-α). EGF and fibroblast growth factor also have been shown to stimulate mitosis and migration of human corneal epithelial cells in vitro.1

The source(s) of the growth factors which might regulate epithelial turnover in vivo have not been fully established. Recently, EGF was detected in tears,2 and a major source of EGF synthesis appears to be the lacrimal glands,3 suggesting that EGF may act by an exocrine mechanism to influence corneal epithelial cells. However, previous studies have shown that the tight junction complex of epithelial cells prevents penetration of hydrophilic molecules in tears, such as fluorescein, insulin, or horseradish peroxidase,4,5 unless the epithelial cell barrier has been damaged. Thus, the size and charge properties of EGF or other growth factors present in tears might limit their penetration to the basal epithelial cell layers where cell division normally occurs.

Another mechanism which has been proposed for the control of epithelial turnover in other tissues, such as the skin, is the TGF-α autocrine system. Skin epithelial cells (keratinocytes) express receptors for TGF-α and EGF, predominantly in the basal cell layers, and synthesize TGF-α messenger RNA (mRNA) and immunoreactive protein.6 To determine if human corneal epithelial cells synthesize a potential autocrine growth factor, we analyzed cultures of HCEC.
cells for the presence of TGF-α mRNA and protein, and we studied sections of human corneas for immunoreactive TGF-α protein.

Materials and Methods

Human Corneal Epithelial and Fibroblast Cell Culture

HCEC cultures were established using modifications of a previously described method. Briefly, the scleral rim was removed from a donor cornea, the endothelium was removed with a cotton-tipped applicator, and the cornea was placed epithelial side up in a 35-mm culture dish containing 3 ml of phosphate-buffered saline (PBS) and 5 U/ml of Dispase (Collaborative Research, Lexington, MA). After 30 min of incubation at 37°C, full-thickness sheets of corneal epithelium were removed by gentle dissection with a periosteal elevator, suspended in keratinocyte growth medium (Clonetics, San Diego, CA), seeded into Primaria culture flasks (Falcon, Oxnard, CA), and grown to confluence at 37°C in 5% CO₂. The purity of the epithelial cells was established by cell morphology and positive immunostaining for 64-kilodalton keratin (AE 5 monoclonal antibody; ICN, Costa Mesa, CA). All experiments with epithelial cells were performed with primary cultures.

Tenon’s capsule fibroblasts were grown from 2 mm squares of episcleral tissue obtained from human donor eyes. Tissue fragments were placed in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37°C in 5% carbon dioxide. When the outgrowths were near confluence, the cells were passaged once and then used for RNA isolation.

TGF-α Radioimmunoassay

HCEC in culture were analyzed for immunoreactive TGF-α protein using a commercially available radioimmunoassay that is highly specific for TGF-α and does not cross react with EGF at greater than 1000-fold concentration (Biotope, Seattle, WA). Briefly, a primary culture of HCEC was grown to 80% confluence in a 75-cm² culture flask (approximately 2 million cells), washed in saline and solubilized with 1 ml of 1% Triton X-100 detergent in PBS. The sample was reduced with dithiothreitol, boiled for 1 min, and assayed in duplicate at three dilutions. Displacement data were linearized by logit transformation, and the best-fit line was determined by linear-regression analysis. Slopes of lines generated by HCEC and the TGF-α standard were compared using the student t-test for slopes to determine whether the slopes were different. Concentrations of TGF-α were calculated by averaging the amounts of TGF-α interpolated from the linear regression of the standard curve and expressed as nanograms of TGF-α immunoreactivity per 10⁶ cells.

TGF-α Immunohistochemical Analysis

A pair of human corneas, which were unsuitable for transplantation, were harvested, placed in Optisol (Chiron, Irvine, CA), and then processed for immunohistochemical analysis as previously described. Briefly, the corneas were fixed in Bouin’s solution overnight, dehydrated in graded alcohol solutions, and embedded in paraffin. Sections 5-μm thick were cut and returned to water. The sections were incubated with sheep antibodies made to the mature form of human TGF-α diluted 1:1000 (East Acres Biological, Southbridge MA), followed by biotinylated goat anti-rabbit antibodies diluted 1:1000, and finally with avidin-conjugated horseradish peroxidase. 3-3’Diaminobenzidine and hydrogen peroxide were used to generate the chromogenic reaction product. Controls included sections incubated without primary anti-TGF-α antibodies or anti-TGF-α antibodies that were neutralized with 100 μg of TGF-α per milliliter of diluted antiserum.

Northern Hybridization of TGF-α mRNA

RNA isolation, agarose gel electrophoresis, and northern blotting were performed using standard techniques. Briefly, RNA was isolated using the guanidinium isothiocyanate method from three different cultures: a confluent culture of Tenon’s capsule fibroblasts, a confluent culture of HCEC, and an 80% confluent culture of HCEC. Approximately 30 μg of total RNA was isolated from each culture which contained approximately 2 million cells. Twenty micrograms of total RNA from each sample was applied to a 1% agarose-formaldehyde gel, and the RNA species were separated by electrophoresis, then blotted by diffusion in 10X SSC (1.5 mol/l sodium chloride, 0.15 mol/l sodium citrate, pH 7.0) onto a Hybond N+ membrane (Amersham, Arlington Heights, IL). The membrane was probed with 32P-labeled TGF-α complementary DNA encoding for amino acids 17-160 of the human TGF-α precursor. The TGF-α probe was generated by restriction endonuclease digestion of a clone obtained from Oncogen (Seattle, WA) and was purified by agarose gel electrophoresis and labeled with α32P-deoxycytidine triphosphate using the Prime-It random-primed labeling kit (Stratagene, La Jolla, CA). Hybridization was performed overnight at 65°C in 10 ml of 250 mmol/l sodium phosphate buffer, pH 7.4, and 1% bovine serum albumin. Then the blots were washed at 60°C in 20 mmol/l phosphate buffer, pH 7.4.
7.4, with 0.1% sodium dodecyl sulfate. The blots were then exposed to x-ray film with intensifier screens for 7 days at −80°C.

Results

TGF-α Radioimmunoassay

Logit transformation of the competitive binding data produced by dilutions of the TGF-α standard and the detergent extract of HCEC generated the lines shown in Figure 1. The slopes of the displacement lines for the TGF-α standard and HCEC sample were not significantly different (P > 0.05), indicating that the HCEC sample contained immunoreactive TGF-α protein. The concentration of immunoreactive TGF-α interpolated from the TGF-α standard curve was 3 ng/10⁶ cells. This level of TGF-α was similar to others reported for TGF-α in conditioned medium of human tumor cells, which ranged from 0.1-5 ng/10⁶ cells; it was higher than levels reported for extracts of the human tumor cells, which ranged from 6-98 pg of TGF-α/10⁶ cells.

TGF-α Immunohistochemical Analysis

Intense immunohistochemical staining was generated throughout the epithelium with the TGF-α antibody (Fig. 2, panel A). A low level of staining was present in the stroma. Very light staining of the sections was detected when the TGF-α antibody was pre-treated with TGF-α, indicating the specificity of the immunohistochemical binding (Fig. 2, panel B) or when the primary antibody was omitted (data not shown).

Northern Blot Hybridization

Autoradiography of the northern blot probed with the TGF-α complementary DNA generated a single intense band at 4.4 kilobases for both samples of HCEC (Fig. 3, panel A). The size of the TGF-α hybridization band agreed closely with its previously reported size of 4.5–4.8 kilobases for prepro-TGF-α mRNA found in human cell lines derived from different normal organs and tumors and was different from the reported size prepro-EGF mRNA of 5.2 kilobases. By contrast, no hybridization was detected for RNA isolated from Tenon’s fibroblasts. The failure to detect TGF-α mRNA in the sample of fibroblasts was not caused by degradation of the RNA or underloading of the sample because the intensities of the 28S ribosomal RNA band stained by ethidium bromide were similar and intact in all three samples (Fig. 3, panel B).

Discussion

Our results demonstrated that primary cultures of HCEC synthesized TGF-α mRNA in vitro and contained a substantial amount of TGF-α immunoreactive protein. In addition, the TGF-α immunostaining pattern of human corneas indicated that TGF-α was present throughout the corneal epithelium in vivo. The detection of immunoreactive TGF-α protein in sections of human corneas suggests that the synthesis of TGF-α also occurs in vivo and implies that the synthesis of TGF-α mRNA and protein detected in vitro were not entirely the result of an artifact induced by culturing the epithelial cells. The immunostaining pattern observed for TGF-α in the cornea was similar to the immunostaining pattern reported for TGF-α in the skin; in the latter, immunoreactive TGF-α was detected throughout the epidermis of neonatal foreskin. Previous reports have shown that expression of the EGF receptor in the skin was heavily concentrated in the basal cell layers of the epidermis, suggesting that regulation of mitosis is controlled in part by EGF receptor expression rather than by different patterns of TGF-α expression. Because it is well established that HCEC express the EGF receptor and that TGF-α binds to the EGF receptor, these data serve as a basis for the proposal that TGF-α acts through an autocrine mechanism to influence HCEC mitosis and normal turnover. Our finding that human corneal epithelial cells synthesized TGF-α mRNA and contained TGF-α immunoreactive protein raises the question of what regu-
Fig. 2. Immunohistochemistry analysis of human corneal epithelial cells for TGF-α. Sections of human cornea were incubated with anti-human TGF-α serum (A) or with anti-human TGF-α serum preincubated with recombinant human TGF-α (B) then incubated with biotinylated second antibody followed by avidin-conjugated horseradish peroxidase. TGF-α immunoreactive protein was localized by diamobenzidene chromogenic reaction product.

Fig. 3. Northern hybridization analysis for TGF-α mRNA. Total RNA was isolated by guanidinium isothiocyanate method from pure cultures of human Tenon’s fibroblasts (lane 1), or subconfluent cultures (lane 2) or confluent cultures (lane 3) of human corneal epithelial cells. Twenty micrograms of RNA was chromatographed by agarose gel electrophoresis, blotted onto Hybond membrane, and hybridized under high-stringency conditions with 32P-labeled cDNA probe encoding for TGF-α (A). A single hybridization band of 4.4 kilobases (kb) was detected by autoradiography for both cultures of epithelial cells but not for fibroblasts. Ethidium bromide staining of the membrane (B) shows equal loading of nondegraded RNA for each lane. Migration of RNA size standards are shown on right.

Previous studies with skin epidermal cells indicated that EGF and TGF-α both stimulated synthesis of TGF-α in vitro. It would be of interest to determine if (1) factors such as age influence TGF-α synthesis; (2) agents that stimulate epithelial regeneration in vivo, such as EGF, increase epithelial cell synthesis of TGF-α; or (3) drugs that slow epithelial healing, such as antiinflammatory corticosteroids, reduce epithelial cell synthesis of TGF-α. In addition, it would be important to determine if other corneal cells such as stromal fibroblasts and endothelial cells synthesize growth factors such as TGF-α which might act by paracrine mechanisms to stimulate epithelial cell turnover or wound healing.

Our results and those of others led us to propose the following hypothesis integrating the role of exocrine and autocrine growth factors in the regulation of epithelial cell mitosis in normal turnover and epithelial wound healing. Normal turnover of epithelial cells may be stimulated primarily by the autocrine action of peptide growth factors such as TGF-α. Growth factors that are synthesized by the lacrimal glands and secreted into the tears may play a more minor role.
in the normal turnover of basal epithelial cells as a result of the potential difficulty which the large hydrophilic proteins may have in penetrating through the overlying layers of stratified epithelium. However, growth factors present in tears may act as a reservoir of mitogens which are immediately available to increase migration and mitosis of epithelial cells after an injury has disrupted the epithelial barrier. Clinical support for this hypothesis is provided by the observation that patients with congenital alacrima, who must lack exocrine secretion of growth factors, have a corneal epithelium indicating that exocrine growth factors secreted by the lacrimal gland are not required to maintain epithelial cell turnover. However, persons with congenital alacrima often have recurring epithelial defects. Thus, exocrine secretions of EGF or other growth factors from the lacrimal gland do not appear to be required for maintaining a corneal epithelium, but they do appear to be important for healing epithelial defects. Although further research is needed to establish definitively the relative importance of the autocrine and exocrine systems in regulation of corneal epithelial cell turnover and wound healing, the synthesis of TGF-α mRNA and protein by the epithelium adds another dimension to the molecular regulation of epithelial cell mitosis.

Key words: corneal epithelium, TGF-α, radioimmunoassay, immunohistochemistry, mRNA

References


