EGF, Basic FGF, and TGF Beta-1 Messenger RNA Production in Rabbit Corneal Epithelial Cells

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The polymerase chain reaction (PCR) was used to demonstrate that rabbit corneal epithelial cells produce messenger RNAs coding for epidermal growth factor (EGF), basic fibroblast growth factor (FGFb), and transforming growth factor beta-1 (TGFβ1) ex vivo and in primary culture. EGF, FGFb, and TGFβ1 mRNAs were detected in central and peripheral ex vivo epithelial tissue in wounded and unwounded rabbit corneas. Southern blots of the PCR products were probed with oligonucleotides to demonstrate that the appropriately sized amplification products were specific. These results suggest that corneal epithelial cells produce growth factors that may have autocrine or paracrine effects on epithelial cells, and possibly other cells of the cornea. The functions, if any, performed by these growth factors in corneal epithelial wound healing are yet to be elucidated. Invest Ophthalmol Vis Sci 33:1987–1995, 1992

Although contrasting results have been reported, studies have demonstrated that exogenous epidermal growth factor (EGF), basic fibroblast growth factor (FGFb), and transforming growth factor beta (TGFβ) can modulate corneal epithelial cell proliferation in wound healing models in vitro and in vivo. For example, Mishima et al. reported that TGFβ had an antagonistic effect on the EGF-stimulated proliferation of rabbit corneal epithelial cells in culture. Petroustoso et al. demonstrated that EGF and FGFb increased the rate of rabbit corneal epithelial wound healing in vivo. In addition, receptors for EGF have been identified on the surface of corneal epithelial cells. In that study, an increase in EGF receptor protein levels on rabbit corneal epithelial cells was noted after anterior keratectomy. To our knowledge, there have been no reports of the endogenous production of growth factors by corneal epithelial cells.

Autocrine and paracrine production of growth factors have been shown to have a role in the physiology of many types of cells. Autocrine and paracrine regulatory systems provide mechanisms for cells to modulate their own viability and growth, and the viability and growth of adjacent cells, through the production of specific growth factors and the corresponding receptors. For example, we have recently demonstrated that EGF, EGF receptor, TGFβ1, FGFb, and interleukin-1 alpha (IL-1α) mRNAs are produced in cultured human corneal endothelial cells.

In the present study, the polymerase chain reaction has been used to demonstrate that rabbit corneal epithelial cells produce messenger RNAs coding for EGF, FGFb, and TGFβ1 ex vivo and in vitro. The growth factors specified by these mRNAs could modulate the growth and viability of the cells of the epithelial surface. Similarly, they could have important functions in regulating the wound healing response of cornea.

Materials and Methods

Corneal epithelial cells for culture were removed from rabbit eyes obtained commercially (Pel Freez Biologicals, Rogers, AK). Eyes were maintained at 4°C after enucleation and used within 24 hr of the rabbits' deaths. Epithelial cells were cultured in Costar (Cambridge, MA) T-25 flasks according to a previously described method. Fetal calf serum and other reagents used for tissue culture were obtained from Hazelton Biologics, Inc. (Lenexa, KS). Cells were fed twice a week with complete medium.

Ex vivo wounding experiments were performed with male New Zealand white rabbits (approximately 3 kg). Animals were anesthetized by intramuscular injection of a mixture of 30 mg/kg ketamine and 3 mg/kg xylazine and topical application of one drop of proparacaine HCl 0.5% prior to wounding or collection of epithelial specimens. Central epithelial defects
were produced in one eye of six rabbits by initially marking the cornea with a penetrating central wound of the epithelium using a 4 mm punch biopsy trephine (Acuderm, Inc, Ft. Lauderdale, FL). Full thickness epithelium within this mark was abraded with a sterile spatula. Two rabbits served as controls and were not wounded. Gentamicin drops (0.3%) were applied four times a day to wounded and control eyes. Animals were divided into four groups for collection of epithelium from the corneas (two control, two 6 hr, two 24 hr, and two 72 hr after the initial epithelial defect).

Fluorescein was placed into the eye with an applicator strip, and the size of the corneal epithelial defect was recorded prior to harvesting the epithelium. Each cornea was irrigated with balanced salt solution, and a 6 mm trephine was used to make a central perforating epithelial mark. The latter mark was concentric with the original defect in the wounded corneas. A sterile spatula was used to remove all epithelium (full thickness) within the 6 mm zone without penetrating the underlying stroma. Epithelium was immediately transferred into 0.75 ml of guanidinium thiocyanate (GTC) solution in a 1.5 ml conical tube for RNA extraction. A separate spatula was used to remove all the remaining peripheral epithelium (full thickness) in a single quadrant (Fig. 1). Epithelium was removed to within 0.5 mm of the limbus under high magnification. Limbal blood vessels were peripheral to the area of removal of epithelium and no bleeding was produced by the scrapping. The peripheral epithelium was transferred to a separate 1.5 ml conical tube containing 0.75 ml of GTC solution for RNA extraction. Another sample was prepared similarly by pooling all of the corneal epithelium from four unwounded rabbit corneas. This investigation conformed to the ARVO Resolution on the Use of Animals in Research.

Total cellular RNA also was isolated from two confluent primary rabbit corneal epithelial cell cultures. The cells were rinsed twice with Hank’s balanced salt solution, and 1.5 ml of GTC solution was transferred onto the monolayer of cells. Each ex vivo epithelial specimen, including the corneal epithelial sample that was pooled from four unwounded corneas, was homogenized with a pestle in the 0.75 ml of GTC solution containing 0.75 ml of GTC solution for RNA extraction. Another sample was prepared similarly by pooling all of the corneal epithelium from four unwounded rabbit corneas. This investigation conformed to the ARVO Resolution on the Use of Animals in Research.

First strand complementary DNA (cDNA) was prepared from total cellular RNA. For each in vitro sample, the reverse transcriptase reaction was performed in a single 100 µl reaction with all of the RNA isolated from an individual culture flask. For each ex vivo epithelial specimen, all of the isolated RNA was included in a 50 µl reaction. The specific reaction conditions have been described previously. The polymerase chain reaction (PCR) primers used for EGF, FGFb, TGF/β1, and beta actin also have been described. The expected size for each growth factor or beta actin PCR product amplified from cDNA is provided in Table 1. All PCR primers and probes were synthesized by Midland Certified Reagent Co. (Midland, TX) and purified by high-pressure liquid chromatography. All four primer pairs were designed so that amplification of contaminating genomic DNA sequences would produce PCR products substantially larger than PCR products amplified from cDNA (beta actin, 790 base pairs; EGF, 18 panes were designed so that amplification of contaminating genomic DNA sequences would produce PCR products substantially larger than PCR products amplified from cDNA (beta actin, 790 base pairs; EGF, 18
Table 1.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Size*</th>
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<tbody>
<tr>
<td>Beta actin</td>
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<tr>
<td>EGF</td>
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<td>422</td>
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<td>TGFβ1</td>
<td>266</td>
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* Size is the expected length of the amplified sequence, including the 5' clamps and restriction sites using the PCR primers described in a previous study.13

11,900 base pairs; FGFb,19 33,900 base pairs; and TGFβ1,20 minimum of 366 base pairs, but far longer because only 100 nucleotides of the long intron within the relevant genomic interval have been determined), because intron sequences that were excised during RNA processing would be included in genomic DNA targets. PCR amplification of each sequence was performed with 5 µl of in vitro or 10 µl of ex vivo cDNA sample in a total volume of 100 µl using 2.5 units of Taq polymerase (Perkin Elmer Cetus Corporation, Norwalk, CT) and 1.5 mmol/l magnesium, as previously described.21,22 Control reactions without template were included in each PCR amplification performed with each primer pair to monitor for sequence contamination. Programmable temperature cycling (Ericomp, Inc., La Jolla, CA) was performed with the following cycle profile: denaturation, 4 min at 94°C; followed by 40 cycles of annealing, 2 min at 55°C; extension, 3 min at 72°C; and denaturation 90 sec at 94°C. Unless otherwise specified, all reagents were obtained from Sigma (St. Louis, MO).

Horizontal 1.5% agarose (US Biochemical Corp., Cleveland, OH) gel electrophoresis was performed using 27 µl of each PCR product and 3 µl of 10× loading buffer per lane with a 120 ml gel run in a wide Mini-Sub cell electrophoresis apparatus (Bio Rad, Richmond, CA). Phi × 174 RF DNA/Hae III fragments (Bethesda Research Laboratories, Gaithersburg, MD) were used as molecular size standards. Ethidium bromide (Sigma) at a final concentration of 0.0001 mg/ml was added to the running buffer, and a voltage of 80 V was applied until the loading dye had traveled two-thirds the distance to the end of the gel.

Southern hybridization was performed using previously described 30-mer oligonucleotide probes that hybridized to internal regions within the coding strand of the amplified sequences to confirm that the PCR amplified products were derived from messenger RNAs coding for EGF, FGFb, and TGFβ1. The expected sizes of the amplified cDNA sequences are provided in Table 1. Five microliters of each PCR product were resolved by 1.5% agarose gel electrophoresis. Phi × 174 RF DNA/Hae III fragments were used as molecular size standards. Separated PCR amplification products were deposited on a supported 0.45 µm nitrocellulose filter (Nitroplus 2000; MSI, Westboro, MA) by capillary transfer with 20 × SSC.24 DNA was fixed to the filter by baking at 80°C for 2 hr. Methods for oligonucleotide 5' radiolabelling and hybridization were described previously.13,25,26

Results

The morphology of the rabbit corneal epithelial cells in the primary culture is shown in Figure 2. Figure 3 shows the PCR products for EGF, FGFb, TGFβ1, and beta actin that were amplified from the two cDNA samples produced from the primary corneal epithelial cell cultures. Amplification products of the expected length were detected for each of the growth factors in both of the primary rabbit epithelial cultures. A faint band approximately 800 base pairs in size also was present on the gel for beta actin. Additional faint bands that are larger than the expected products also were present for EGF, FGFb, and TGFβ1. Except for beta actin, however, these bands were too short to be amplification products from the genomic DNA sequences.

Residual epithelial defects at the time of removal of the epithelial specimens for RNA isolation were estimated to be approximately 80%, 50%, and 20% of the original 4 mm diameter wound at 6, 24, and 72 hr, respectively, after wounding. PCR products for EGF, FGFb, TGFβ1, and beta actin amplified from central and peripheral corneal epithelium of wounded and unwounded corneas are provided in Figure 4. This figure shows that amplified sequences of the appropriate size for each growth factor were detected in ex vivo corneal epithelial samples. The number of ex vivo corneal samples in the control group and each of the wounded groups in which each growth factor and beta actin mRNA was detected is provided in Table 2.

In each group, results are provided for central and peripheral corneal epithelium. The PCR technique used in this study was not quantitative. Also, the amount of epithelium included in the RNA extractions probably varied between groups, especially for the central specimens, where the amount of epithelium included in the 6 mm harvesting varied with the extent of wound closure. Therefore, the only information that can be provided is whether or not the growth factors were detected by PCR. EGF and TGFβ1 mRNAs each were detected in at least one of the central and peripheral unwounded control samples. FGFb mRNA was not detected in any of the central or peripheral unwounded control samples. Amplification products for all three growth factors were detected in central and peripheral epithelial samples.
from wounded corneas. Amplification products for FGFb mRNA, however, were detected only in the 24 hr (not shown) and 72 hr wounded samples. In general, growth factor mRNA detection for EGF, FGFb, and TGFβ1 was similar in central and peripheral epithelial samples at individual time points. Faint bands that were larger than the expected amplification products can be detected in some lanes.

Figure 5 shows that amplification bands of the appropriate sizes were detected for EGF, FGFb, and TGFβ1 in the sample produced by pooling the epithelium of four unwounded rabbit corneas. Thus, FGFb could be detected in unwounded corneal epithelium if a larger mass of tissue was included in the RNA extraction. PCR products that were larger than expected were in the amplifications for EGF, FGFb, and TGFβ1.

Hybridization of Southern blots of each of the PCR products with oligonucleotide probes complementary to 30 nucleotide intervals within the amplified EGF, FGFb, and TGFβ1 sequences (Table 1) detected molecules of the expected sizes for in vitro corneal epithelium of four unwounded rabbit corneas. Thus, FGFb could be detected in unwounded corneal epithelium if a larger mass of tissue was included in the RNA extraction. PCR products that were larger than expected were in the amplifications for EGF, FGFb, and TGFβ1.

Hybridization of Southern blots of each of the PCR products with oligonucleotide probes complementary to 30 nucleotide intervals within the amplified EGF, FGFb, and TGFβ1 sequences (Table 1) detected molecules of the expected sizes for in vitro corneal epithelium.
Fig. 4. PCR products amplified from the cDNA samples generated from the unwounded and 72-hr wounded ex vivo rabbit corneal epithelial specimens. The lanes marked $\Phi$ contain PhilX 174/HAE111 size markers. The lengths of selected markers in base pairs are provided to the left. PCR reactions with heavy bands were selected so that they would be easily observed. C and P above each lane indicate central or peripheral epithelium, respectively. The primers used to generate the PCR products (Table 1) are indicated above each lane. More than one band can be seen in some of the lanes. The PCR product of the predicted size was detected for each growth factor or for beta actin in these specimens (arrowheads).

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<tr>
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Discussion

The sensitive PCR method for amplification of specific sequences from cDNA generated from total cellular RNA has been used to demonstrate that rabbit corneal epithelial cells produce mRNAs coding for EGF, FGFb, and TGF$\beta$1. Messenger RNAs coding for these growth factors were detected in rabbit corneal epithelial cells in primary culture and corneal epithelial tissue that was removed from the rabbit cornea and used immediately for the isolation of total cellular RNA (ex vivo). These results confirm those obtained with human ex vivo and in vitro corneal epithelial cells. In that study, PCR was used to demonstrate that cultured and ex vivo human corneal epithelial cells produce mRNA coding for EGF, EGF receptor, FGFb, TGF$\beta$1, and IL-1a. The PCR primers that were designed from the human EGF receptor and IL-1a sequences do not amplify the rabbit mRNA because of nucleic acid sequence differences.

EGF, FGFb, and TGF$\beta$1 mRNAs were detected ex vivo in the epithelium from wounded and unwounded rabbit corneas. It should be noted, however, that the technique used for removal of the epithelium produces an immediate wounded state. Therefore, EGF, FGFb, and TGF$\beta$1 mRNA levels could be different in the unwounded in vivo corneal epithelium. FGFb mRNA was not detected in the unwounded corneal epithelial samples from individual corneas but was detected in the sample prepared by pooling the corneal epithelium from four corneas. Therefore, given sufficient material for RNA extraction, FGFb mRNA can be detected in the previously unwounded cornea. Although the PCR technique used in this study is not quantitative, the lack of detection of
FGFb in the individual unwounded cornea and its detection in wounded corneas suggests there could be an upregulation of FGFb mRNA expression in response to injury. Quantitative PCR methods should allow more precise measurements to be performed of alterations in the expression of the growth factor mRNAs after wounding.

For EGF, FGFb, and TGFβ1, the bands on the Southern blots that were larger than the expected PCR amplification products could represent alternative RNA splicing products. Products on the agarose gel that were not the expected length and were not identified on the Southern blot may represent nonspecific PCR products from mRNA sequences similar to the growth factors being studied. Alternatively, they could represent growth factor specific alternative splicing products that lost the probing sequence during the splicing process. Precise identification of these products will require nucleic acid sequencing.

Studies in animals4,5 and humans6 have suggested that the process of epithelial cell proliferation after corneal wounding occurs predominantly at the limbus. This mitotic activity of the corneal epithelial cells has been attributed to limbal stem cells.31,32 Differences in differentiation and proliferative potential could be paralleled by differences in the expression of regulatory factors. We could not detect a difference in the qualitative production of mRNAs coding for EGF, FGFb, and TGFβ1 between central and peripheral samples of epithelium. Messenger RNAs for all three growth factors were detected in central and peripheral epithelial specimens from rabbit corneas. Because this study did not use quantitative methods, however, undetected differences could exist in the levels of the mRNAs coding for the EGF, FGFb, and TGFβ1 growth factors in different areas of the corneal epithelium.

Ex vivo samples that were unwounded and 72-hr postwounding are shown. C and P designate central and peripheral corneal epithelial samples, respectively. Since FGFb was not detected by PCR in the single unwounded cornea, the unwounded C lane contained PCR product from the unwounded ex vivo sample pooled from four rabbit corneas (indicated by asterisk). The primers used to generate the PCR products (Table 1) were indicated above each lane. The predicted product (Table 1) was detected for each growth factor in each of the samples (arrowheads). Larger alternative bands were noted for EGF (approximately 490 base pairs), FGFb (approximately 525 base pairs), and TGFβ1 (approximately 330 base pairs). Ex vivo and in vitro product patterns were similar for each growth factor.
The lack of detection of each of these growth factor mRNAs in some ex vivo samples is attributable to the difficulty in transferring the epithelium from the cornea to the container used for RNA extraction, losses that occur during RNA extraction, and the low concentration of the sequences of interest in the target samples prior to amplification. In future quantitative studies, each sample should contain larger quantities of the epithelium. For example, all of the epithelium from each cornea could be included in a single RNA extraction. Alternatively, the diameter of the central corneal epithelial sample could be increased and the peripheral samples could include the epithelium from all four quadrants.

The mRNAs coding for EGF, FGFβ, and TGFβ1 could have been derived from cell types other than the epithelium in the samples obtained directly from ex vivo rabbit corneas. For example, the corneal epithelium is known to contain Langerhans cells. However, because the ex vivo detection of the growth factors in the epithelium paralleled the results obtained from cultured epithelial cells, we believe that the detected mRNA molecules probably are produced in the epithelium. In addition, Langerhans cells are concentrated at the limbus and decrease to nearly zero in the central corneal epithelium. Because we detected the EGF, FGFβ, and TGFβ1 mRNAs in the central and peripheral epithelium with similar efficiency, Langerhans cells are unlikely to be the only source. In addition, mRNAs coding for FGFβ35 and TGFβ36 recently have been identified in skin keratinocytes—cells similar to corneal epithelial cells.

The highest order of gene expression control in higher organisms occurs at the level of synthesis of mRNA (transcription). Therefore, identification of the messenger RNA coding for a particular protein usually indicates that the protein is synthesized within the tissue. The level of a protein within the tissue, however, also may be influenced by RNA processing, translational regulatory mechanisms, and varying rates of protein degradation. Therefore, additional studies with methods such as immunoblotting will be needed to demonstrate conclusively that EGF, FGFβ, and TGFβ1 growth factor proteins are produced in corneal epithelial cells. Immunodetection of the protein alone would not have provided sufficient evidence that the growth factors were synthesized in the corneal epithelium, however, because the proteins could have been sequestered within the epithelium from the tears or other sources. For example, a recent study noted intense immunostaining for retinol-binding protein (RBP) in the corneal endothelium. In situ hybridization studies detected RBP mRNA only in the retinal pigmented epithelial cells, however. This suggests that the RBP that localized to the endothelium did so by a mechanism other than synthesis.

If the corresponding growth factor and cytokine proteins are identified, further work will be needed to determine whether precursor proteins for each modulator are processed into the mature protein in corneal epithelial cells. EGF, for example, is synthesized as a large precursor protein that is processed to the smaller mature EGF molecule. Recent studies, however, have demonstrated that the EGF precursor molecule can compete with mature EGF for the EGF receptor and stimulate proliferation of EGF-dependent cells. Therefore, lack of processing of the precursor proteins does not necessarily indicate that the precursor itself has no function in the tissue.

One previous study that used radioimmunoassay techniques reported that EGF protein was not detected in human corneal epithelium or tears. Because subsequent studies have identified EGF in the tears of humans and mice, however, the epithelium should be reexamined for EGF protein.

What are the possible functions of the EGF, FGFβ, and TGFβ1 produced by the corneal epithelium? EGF receptor protein is known to be present in the plasma membrane of corneal epithelial cells. Although receptors for FGFβ and TGFβ have not been identified on corneal epithelial cells, they probably are present because exogenous FGFβ1,2,6 and TGFβ3,5 have been shown to have specific effects on these cells. Therefore, the growth factors produced by the corneal epithelial cells probably have autocrine or paracrine epithelial functions. It also is plausible that these growth factors could have effects on keratocytes or other cells of the cornea. Thus, the role of these epithelial cell-produced growth factors in maintaining the corneal surface and the healing response to corneal wounding remains to be elucidated. The relationship between tear growth factors such as EGF, that are thought to be produced by the lacrimal gland, and growth factors produced by corneal epithelial cells also is uncertain. It is possible that a portion of the EGF identified in tears is derived from the corneal epithelium. The exogenous growth factors might have on the expression of these epithelial cell-produced growth factors and the physiologic processes they mediate is also unknown. Further work is needed to understand the role of growth factors produced by the corneal epithelium.

Key words: corneal epithelium, epidermal growth factor, basic fibroblast growth factor, transforming growth factor beta-1, wound healing, polymerase chain reaction

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