ET\textsubscript{B} and Epidermal Growth Factor Receptor Stimulation of Wound Closure in Bovine Corneal Epithelial Cells

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**Purpose.** To determine if there is a heterogeneous pattern of endothelin (ET) receptor subtype (i.e., ET\textsubscript{A} and ET\textsubscript{B}) gene expression in the bovine corneal epithelium (BCE). To determine if ET receptor subtype stimulation increases the effectiveness of epidermal growth factor (EGF) to accelerate wound closure in a primary culture of bovine corneal epithelial cells (BCEC).

**Methods.** In situ hybridization histochemistry was used to characterize ET\textsubscript{A} and ET\textsubscript{B} gene expression in the BCE. A wound closure assay evaluated wound healing rates in BCEC after 4 to 7 days in culture. \[^{3}H\] thymidine incorporation and MTT assay measured proliferation.

**Results.** ETA gene expression was appreciably higher in the basal cells than in the suprabasal cells, whereas the pattern for ETB was reversed. Epidermal growth factor (5 ng/ml) maximally increased wound closure by 145\% above the control. With 5 ng/ml EGF, either 10\textsuperscript{-9} M ET-1 or 10\textsuperscript{-8} M sarafotoxin-6-c (s-6-c) increased wound closure by an additional 39\% (P < 0.001) above that measured with 5 ng/ml EGF alone. BQ123 (10\textsuperscript{-7} M) did not alter any of these effects of ET-1 or s-6-c. Epidermal growth factor stimulated wound closure through a selective increase in proliferation. Neither ET-1 nor s-6-c alone had any effect on proliferation or migration.

**Conclusions.** Both ET\textsubscript{A} and ET\textsubscript{B} genes are expressed in BCE. However, in BCEC only, ET\textsubscript{B} stimulation increases the effectiveness of EGF to stimulate wound closure. This response was caused by an increase in cell migration rather than proliferation because, after treatment with mitomycin C, neither ET-1 nor EGF stimulated wound closure.

The endothelins (ETs) are a novel group of three different 21-amino acid peptide local hormones (ET-1, ET-2, and ET-3).\textsuperscript{1,2} Their effects on cell function can be mediated through the stimulation of three different ET receptor subtypes—ET\textsubscript{A}, ET\textsubscript{B}, and ET\textsubscript{C}.\textsuperscript{3,5} This assignment is in agreement with pharmacologic binding studies that also demonstrated three different receptor subtypes.\textsuperscript{6-15} ET\textsubscript{A} has a higher affinity for ET-1 than for the other ET isopeptides.\textsuperscript{5,14} On the other hand, ET\textsubscript{B} is nonselective because its affinity for each of the three ET isopeptides is approximately equal.\textsuperscript{15} ET\textsubscript{C} has a higher affinity for ET-3 than ET-1 or ET-2.\textsuperscript{4,16} In a host of tissues, each of these ET receptor subtypes is involved in the regulation of a diverse variety of functions through different cell signaling mechanisms. There is approximately 63\% homology between human ET\textsubscript{A} and ET\textsubscript{B}, whereas interspecies sequence similarity between each subtype is approximately 83\%.\textsuperscript{17}

Epidermal growth factor (EGF) and ET-1 are two of an emerging number of cytokines that can stimulate corneal epithelial cell proliferation. In a primary culture of the rabbit corneal epithelium, ET-1 stimulated cell proliferation and accelerated wound closure in vivo.\textsuperscript{18,19} Its effect on cell proliferation appears to be mediated through a stimulation of ET\textsubscript{A}, whereas the ET receptor subtype responsible for hastening wound closure was not identified. In the bovine, neither ET-receptor gene expression nor a functional role for any of the ETs was identified. Nevertheless, the mitogenic effects of EGF were documented in the bovine and...
other mammalian species.\textsuperscript{20–30} Because the ETs and EGF mediate their effects through different cell signaling pathways, it is conceivable that ET-1 or another ET homolog could interact with a specific ET receptor subtype to increase the effectiveness of EGF in stimulating corneal epithelial proliferation and migration.

We report here on the relative levels and localization of ET receptor subtype gene expression in the intact bovine corneal epithelium (BCE). A functional wound closure assay was used to determine the possible relevance of their expression to corneal epithelial renewal. This was accomplished by using a primary culture of the bovine corneal epithelium (BCEC) to determine if there is any interaction between EGF and ET receptor subtype stimulation on wound closure.

METHODS

In Situ Hybridization Histochemistry

Fresh bovine corneas were fixed in isopentane on dry ice and mounted in a Lipshaw embedding matrix (Shandon, Pittsburgh, PA). Sections of 12- to 16-μm thickness were cut and placed on slides that were stored at −70°C for later use. The riboprobes were prepared by in vitro transcription reactions using linearized pBSII KS clones of templates. DIG-UTP (Boehringer Mannheim, Indianapolis, IN) was used to label the riboprobes. Linearization of the templates was made with Xba I and Kpn I for ET\textsubscript{a} antisense and sense transcripts and Kpn I and Xba I for ET\textsubscript{b} antisense and sense transcripts, respectively. The sizes of the antisense riboprobes for ET\textsubscript{a} and ET\textsubscript{b} were 139 and 191 nt, respectively. The labeling reaction was prepared at room temperature and was incubated at 37°C for 2 hours. After incubation with 20 U of RNase-free DNase I for 15 minutes at 37°C, the riboprobes were characterized by identifying them in 5% polyacrylamide denaturing gels, calculating OD ratios at the 260/280 nm, performing a colorimetric assay.

Frozen sections were fixed with 4% paraformaldehyde for 10 minutes, dehydrated through a graded series of ethanol, and permeabilized with 1 μg/ml proteinase K in 10 mM Tris HCl, 5 mM ethylenediaminetetraacetic acid, and 0.5% sodium dodecyl sulfate at 37°C for 10 minutes.\textsuperscript{31} The slides were washed twice with diethyl pyrocarbonate-treated phosphate-buffered saline and fixed again with 4% paraformaldehyde for 5 minutes. After washing twice with 2 X SSC for 5 minutes, the sections were prehybridized at 42°C for 3 hours in prehybridization solution (50% formamide, 1X Dehardt’s, 5% dextran sulfate, 500 μg/ml SS DNA, 250 μg/ml yeast tRNA) and then hybridized with a riboprobe (200 to 400 ng/ml) in prehybridization solution at 42°C overnight. For a negative control, the appropriate sense riboprobe was used. The unbound riboprobes were removed with a series of shaking washes at room temperature in 1 X SSC for 5 minutes and again for another 15 minutes. Subsequently, they were treated with 0.5 X SSC for 30 minutes and then in 1 X SSC for 30 minutes at 37°C. At room temperature, the tissues were blocked with buffer 1 containing 0.05% Triton X-100 and 2% sheep serum for 30 minutes and then incubated with antidigoxigenin antibody conjugated with alkaline phosphatase (1:400 dilution in buffer 1 containing 100 mM Tris HCl, pH 7.5, 150 mM NaCl, Triton X-100, and 1% sheep serum) for 3 hours. After washing twice in buffer 1 for 30 minutes, tissues were equilibrated with buffer 2 (100 mM Tris HCl, pH 9.5, 150 mM NaCl, and 50 mM MgCl\textsubscript{2}) for another 2 minutes and then incubated with nitro-blue-tetrazolium-chloride/5-bromo-4-chloro-3-indolyl phosphate solution containing 200 μg/ml levamisol (Sigma, St. Louis, MO) for 2 to 4 hours. The color development was monitored under a microscope every 30 minutes and stopped with buffer 3 (1 M Tris HCl, pH 8.0, and 0.5 M ethylenediaminetetraacetic acid) when the tissue sections displayed staining sufficient for evaluation.

Primary Culture of Bovine Corneal Epithelial Cells

The methods used for primary culture of bovine corneal epithelial cells were essentially the same as those described, and they adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.\textsuperscript{32,33} Cow eyes were obtained at the local slaughterhouse and were transferred within 30 minutes to the laboratory on ice. They were sterilized with 70% EtOH for 20 to 30 seconds, followed by flushing with sterile saline. The corneas were isolated surgically, and the endothelium and a large part of the stroma were removed by peeling during microscopic dissection. The thinned corneas were placed epithelial side down in sterilized scintillation caps and incubated in serum-free DMEM/F-12 with 5% Dispase II (Boehringer Mannheim) at 37°C for 30 to 40 minutes. Enzymatic digestion was terminated by replacement with complete medium containing 10% fetal bovine serum (HyClone, Logan, UT). The loosened epithelial cells were scraped off and further dispersed by passing them approximately 20 times through a syringe attached to a 23-gauge needle. The cells were washed twice by resuspension and centrifugation and were seeded into Falcon Primaria multiwell plates (5 X 10\textsuperscript{5} cells/well in a 24-well plate, 1 X 10\textsuperscript{5} cells/well in a 96-well plate), and incubated at 37°C in a 5% CO\textsubscript{2} humid incubator. Unless otherwise indicated, the routine culture medium was DMEM/F12 containing 10% fetal calf serum, 1 μg/ml of hydrocortisone, 100 U/ml pen-
A combination of penicillin, and 100 μg/ml streptomycin. The medium was changed every 2 to 3 days.

**Wound Closure Assay**

A functional wound closure assay was used to resolve the contributions by proliferation and cell migration to wound healing. Three to 4 days after the cells reached confluence in Primaria 24-well plates (Falcon), the culture medium was drained away. With the end of a stainless steel probe (6 mm in diameter), a 8-mm wide Whatman paper filter disc was pressed down centrally and firmly and rotated back and forth against...
the top of the culture. The wells were washed four times with Hank’s balanced salt solution to remove remaining cellular debris and serum. Serum-free DMEM/F12 medium containing 0.5% dimethyl sulfoxide and 0.2 mg/ml bovine serum albumin with or without the agents of interest was then added. This medium was exchanged daily from 4 to 7 days after wounding. Bovine corneal epithelial cells were incubated with 2 μg/ml of mitomycin C for 24 hours before wounding to evaluate the contribution by proliferation to wound closure. To evaluate the extent of wound closure, the cells were fixed with 4% paraformaldehyde and stained with Giemsa. The extent of wound closure was digitized with an optical scanner, Scangal5, (Jandel, San Rafael, CA), and quantified with a computer program, SigmaScan (Jandel). For statistical analysis, each test group was replicated at least in triplicate, and each experiment was repeated three times. Statistical significance was determined with the indicated appropriate analyses (see figure legends).

MTT Assay

Hansen’s method was used for the MTT (3-(4,5-dimethylthiazol-2-yl)-diphenyl tetrazolium bromide) assay, and the reaction was monitored with a scanning multiwell spectrophotometer (enzyme-linked immunoassorbent assay reader). The BCECs were isolated from BCE as described above and were seeded in Primaria 96-well plates (Falcon) in DMEM/F12 medium containing 10% fetal bovine serum at a density of 1 × 10⁵ cells/well. Once the growing cells covered approximately 40% to 50% of the surface of a well, they were washed with Hank’s balanced salt solution four times to remove the serum and were replaced with serum-free medium containing 0.5% dimethyl sulfoxide, 200 μg/ml bovine serum albumin, 100 U/ml penicillin, 100 μg/ml streptomycin, with or without the agents of interest (same medium used in wound closure assay). The medium was changed daily. When subconfluence was reached, 25 μl of a 5 mg/ml MTT solution was added to each well (with 100 μl of medium). After 2 to 3 hours of incubation at 37°C, 100 μl of lysis buffer (20% sodium dodecyl sulfate, 50% N,N-dimethyl formamide, pH 4.7) was added to each well. After another 3 hours of incubation at 37°C in the dark, the OD values were measured with the THERMOMax Microplate Reader (Molecular, Menlo Park, CA) at a test wavelength of 570 nm and a reference wavelength of 630 nm. Each experiment was repeated five times.

Thymidine Incorporation

After 24 hours of serum starvation, the BCECs were labeled for an additional 24 hours with 1 μCi/ml [³H] thymidine (3.3 to 4.8 TBq/mmol, Amersham, Arlington, IL) with or without the agents of interest in serum-free medium. Twenty-four hours later, the cells were lysed with 0.2 N NaOH, and the lysates were harvested on Filter Mat (Skatron, Sterling, VA) with or without the agents of interest in serum-free medium. Twenty-four hours later, the cells were lysed with 0.2 N NaOH, and the lysates were harvested on Filter Mat (Skatron, Sterling, VA) with or without the agents of interest in serum-free medium. Twenty-four hours later, the cells were lysed with 0.2 N NaOH, and the lysates were harvested on Filter Mat (Skatron, Sterling, VA) with or without the agents of interest in serum-free medium. Twenty-four hours later, the cells were lysed with 0.2 N NaOH, and the lysates were harvested on Filter Mat (Skatron, Sterling, VA) with or without the agents of interest in serum-free medium. Twenty-four hours later, the cells were lysed with 0.2 N NaOH, and the lysates were harvested on Filter Mat (Skatron, Sterling, VA) with or without the agents of interest in serum-free medium.

To monitor cell growth under wound closure conditions, [³H] thymidine was added 24 hours after wounding. After an additional 24 hours of incubation, the cells were lysed with NaOH. The lysates were transferred from 24-well to 96-well plates for harvesting. All chemicals were purchased from Sigma.

RESULTS

Localization of Endothelin Receptor Subtype Expression in Bovine Corneal Epithelium

In situ hybridization histochemistry was used to determine any localization of ET receptor subtype expression in the BCE. Using Southern blot analysis, it was demonstrated that each of the designed riboprobes selectively bound to the intended target cDNA for either ETA or ETB (data not shown). Equivalent amounts of nonradioactive antisense DIG-riboprobes were applied to different frozen sections of BCE, along with the corresponding sense riboprobes as negative controls. As shown in Figs. 1A and 1C, the level of ETα gene expression detected with the appropriate antisense riboprobe was higher in the proliferating basal layer than in the differentiating suprabasal layers. In contrast, a reverse pattern was found for ETβ; namely, the level of ETβ gene expression was higher in the suprabasal layers than in the basal layers. Note that in Figures 1B and 1D (negative controls), there was no staining with the corresponding sense riboprobes, indicating that the antisense riboprobes had the appropriate nucleotide sequences to distinguish between ETA and ETB gene expression.

FIGURE 1. In situ hybridization histochemistry of ETA and ETB gene expression in bovine corneal epithelium. 1 = superficial layers; 2 = suprabasal layers; 3 = basal layer; 4 = stroma. (A,C) Results of hybridization with ETA and ETB antisense riboprobes, respectively. (B,D) Results of hybridization with ETA and ETB sense riboprobes as negative control, respectively.
FIGURE 2. Wound closure assay (WCA). (A) Dose-dependent effects of ET-1 on wound closure after 7 days in cell cultures treated with mitomycin C (2 µg/ml) for 24 hours before wounding. Assay was repeated three times, and no difference was observed. (B) WCA after 5 days in bovine corneal epithelial cell cultures not treated with mitomycin C non-treated cells but with different [ET-1]s. Assay was repeated three times, and no significant effect by ET-1 on extent of wound closure was observed.

To determine if ET and EGF receptors affect wound closure through an effect on proliferation, mitomycin C was used to inhibit cell proliferation selectively. In preliminary experiments, 2 µg/ml of mitomycin C inhibited [3H]thymidine incorporation by 87% without cytotoxicity (data not shown). This concentration of mitomycin C was used throughout to separate the contributions by proliferation and migration to wound closure. ET-1 (10^{-11} - 10^{-7} M) did not have any significant effects on wound closure, irrespective of whether the BCC cultures had been treated with mitomycin C (Figs. 2A, 2B). On the other hand, EGF (0.1 to 20 ng/ml) increased the rate of wound closure only in those BCC cultures not treated with mitomycin C (Fig. 3). The maximum response to EGF occurred at 5 ng/ml, but, at higher concentrations, the response declined slightly but not significantly (Fig 3). As indicated in Figures 4A and 4B (i.e. 10^{-8} M and 10^{-7} M ET-1, respectively), neither concentration of ET-1 had any significant effect on the wound closure rate, whereas EGF (1 and 5 ng/ml, respectively) significantly stimulated closure by 77% to 145% above the control (P < 0.0001), respectively. When EGF (1 or 5 ng/ml) was combined with ET-1 (10^{-8} or 10^{-9} M, respectively), as indicated in Figures 4A and 4B, its effect on wound closure increased further by either 32% or 99% (P < 0.001), respectively.

To determine if an increase in the effectiveness of EGF to stimulate wound closure is dependent on the selective stimulation of an ET receptor subtype, we determined if the selective agonist for ET_{b}, sarafotoxin-6-c (s-6-c), could increase the effectiveness of EGF and if preincubation with a specific antagonist for ET_{a}, BQ-123, could suppress any of the effectiveness of EGF in stimulating wound closure. Figure 5A shows that s-6-c (10^{-8} M) with EGF (1 ng/ml) was as effective as ET-1 (10^{-8} M) with EGF (1 ng/ml) in stimulating wound closure. Similarly, the effect with 10^{-9} M s-6-c, in combination with 5 ng/ml EGF on wound closure, was the same as when ET-1 (10^{-9} M) was combined with EGF (5 ng/ml) (see Fig. 5B). As also shown, in Figure 5B, wound closure occurred as rapidly with BQ-123 (10^{-7} M), ET-1 (10^{-9} M), and EGF (1 ng/ml) as when 10^{-8} M ET-1 was combined with EGF (1 ng/ml). These effects were indistinguishable from those obtained with EGF (5 ng/ml) when used in combination with 10^{-9} M s-6-c or 10^{-9} M ET-1 (Fig. 5B). Taken together, ET-1 or s-6-c interact with ET_{b} to enhance the effectiveness of EGF in stimulating the rate of wound closure.

To determine in the wound closure assay whether increases in proliferation explain why ET agonists in-
FIGURE 4. Extent of wound closure with ET-1, EGF, ET-1/EGF, in bovine corneal epithelial cells cultures not treated with mitomycin C (n = 5; mean ± SD, four replicates in each group). (A) Seven-day culture that contained $10^{-8}$ M ET-1, 1 ng/ml EGF alone or in combination. (B) Five-day culture that contained $10^{-9}$ M ET-1, 5 ng/ml EGF alone or in combination. In both conditions, the randomized block analysis of variance shows a significant difference between the control and EGF or ET-1/EGF group, and between EGF and ET-1/EGF group ($P < 0.05$, indicated by asterisks). There was no significant difference between the control and ET-1 groups. ET = endothelin; EGF = epidermal growth factor.

FIGURE 5. Functional endothelin receptor subtype responsible for wound closure in bovine corneal epithelial cells cultures not treated with mitomycin C (n = 5; mean ± SD, four replicates in each group). (A) Seven-day culture in which 1 ng/ml EGF, $10^{-8}$ M ET-1, $10^{-9}$ M s-6-c, and $10^{-7}$ M BQ-123 were used alone or in combination. (B) Five-day culture in which $10^{-9}$ M ET-1, 5 ng/ml EGF, $10^{-9}$ M s-6-c, and $10^{-8}$ M BQ-123 were used alone or in combination. In both conditions, the randomized block analysis of variance shows a significant difference between the EGF group and the ET-1/EGF, BQ423/ET-1/EGF, and s-6-c/EGF groups ($P < 0.05$, indicated by asterisks). ET = endothelin; EGF = epidermal growth factor.

Growth Factor Stimulation of Wound Closure in Corneal Epithelium

Endothelin-1 stimulates rabbit corneal epithelial wound closure in vivo.19 This effect could be a consequence of increases in both proliferation and migration based on its stimulation of [3H]thymidine incorporation in a primary culture of rabbit corneal epithelial cells.18 With regard to the effect of ET-1 on proliferation, it appeared to be a result solely of stimulating ET$_X$. Because its stimulatory effect was dependent on the presence of serum, ET-1 acted as a comitogen. This dependence of ET-1 on serum is in agreement with the current study using the bovine because we also found that neither ET-1 nor s-6-c accelerated wound closure in a serum-free medium. Instead, either of these agonists increased the effectiveness of EGF to stimulate wound closure. Their effects on the wound closure response to EGF were solely a}

crease the effectiveness of EGF, either the MTT assay or [3H]thymidine incorporation were performed in parallel with the wound closure assay. Epidermal growth factor, in combination with either ET-1 or s-6-c, had the same effect on cell proliferation as 1 ng/ml EGF alone: [3H]thymidine incorporation increased by 42% whereas neither $10^{-9}$ M ET-1 nor s-6-c ($10^{-9}$ M) had any additional effect (data not shown). These negative effects of either ET-1 or s-6-c on proliferation suggest that they increase the effectiveness of EGF to stimulate wound closure solely by an enhancement of cell migration.

DISCUSSION

Endothelin-1 stimulates rabbit corneal epithelial wound closure in vivo.19 This effect could be a consequence of increases in both proliferation and migration based on its stimulation of [3H]thymidine incorporation in a primary culture of rabbit corneal epithelial cells.18 With regard to the effect of ET-1 on proliferation, it appeared to be a result solely of stimulating ET$_X$. Because its stimulatory effect was dependent on the presence of serum, ET-1 acted as a comitogen. This dependence of ET-1 on serum is in agreement with the current study using the bovine because we also found that neither ET-1 nor s-6-c accelerated wound closure in a serum-free medium. Instead, either of these agonists increased the effectiveness of EGF to stimulate wound closure. Their effects on the wound closure response to EGF were solely a
A consequence of a stimulation of migration because, in BCEC cultures treated with mitomycin C, their effectiveness was eliminated, and because neither agonist enhanced the stimulatory effects of EGF on mitotic activity as measured with [3H] thymidine incorporation or the MTT assay. This difference between the effects of ET receptor subtype stimulation on proliferation and migration in the rabbit and the bovine is consistent with each receptor subtype being linked to the regulation of a different response. In the bovine, ETβ is linked to migration, whereas in the rabbit, ETα is linked to proliferation.

There is a heterogeneous pattern of distribution in the corneal epithelium of the levels of gene expression for ETα and ETβ because ETα expression is higher in the proliferating basal cells, whereas ETβ is higher in the differentiating suprabasal cells. There are several possible interpretations of the functional significance of this pattern of gene expression for ETα and ETβ. The stimulation of each of these receptor subtypes could mediate functions of the basal and suprabasal layers; the appearance of ET isoform and receptor subtype gene expression is instead merely a consequence of functions mediated by these cell layers. Even though ETβ gene expression was low in the basal proliferating cell layer of BCE, ETβ stimulation in BCEC increased the effectiveness of EGF to stimulate wound closure. One explanation for this apparent contradiction is that it may not be possible to extrapolate between the two systems regarding the level of ETβ gene expression. Alternatively, the level of gene expression is not reflective of functional ETβ receptor density in the BCE and BCEG.

As in vivo, BCEC repopulation of a wounded area is dependent on the rate of cell proliferation and on migration.20,21,25,36–39 Because EGF was identified in an organ culture of the bovine cornea to stimulate epithelial proliferation, we determined if ET-1 can interact with EGF to stimulate further the rate of wound closure above that measured with EGF alone.28 By determining the inhibitory effects of mitomycin C, it was possible to test if ET and EGF stimulation hastened wound closure through a selective effect on proliferation or migration. Irrespective of the presence or absence of mitomycin C, selective ET receptor subtype stimulation had no effect on wound closure. Therefore, by itself, ET-1 does not stimulate either cell proliferation or migration, but EGF alone stimulated wound closure in the absence of mitomycin C. This result with EGF is consistent with its inability to stimulate rat corneal epithelial cell migration in culture and confirms that EGF is a mitogen in the corneal epithelium.29

An interaction between EGF and ET-1 in the stimulation of wound closure was sought by determining if ET-1 affected the rates of wound closure measured in the presence of submaximal (i.e., 1 ng/ml) and maximal (5 ng/ml) doses of EGF. In both instances, ET-1 enhanced the rates of closure. Without mitomycin C, 1 ng/ml of EGF significantly stimulated wound closure by 77% above control, and, in the presence of 10−8 M ET-1, it increased by an additional 33% (P < 0.001) from that measured in the presence of EGF alone (Fig. 4A). With 5 ng/ml of EGF, the rate of wound closure increased by 145% above the control, whereas with 10−9 M ET-1, it increased another 39% (P < 0.0001) above that measured in the presence of EGF alone (Fig. 4B). The ET receptor subtype involved in increasing the effectiveness of EGF to stimu-
late wound closure through an effect on migration was determined by using the selective antagonist of ET$_A$, BQ123, and the selective agonist of ET$_B$, s-6-C. Their specificities were demonstrated in ligand-binding studies, which showed that the $K_I$ value for BQ-123 was 17 nM for ET$_A$ compared to 11,100 nM for ET$_B$. Conversely, the $K_I$ value for s-6-c was 2800 nM for ET$_A$ and 0.29 nM for ET$_B$. We found that ET$_B$ receptor subtype stimulation was responsible for hastening wound closure because s-6-c was as effective as ET-1 in enhancing the effect of EGF on wound closure whereas BQ123 didn’t change the effect of ET-1.

It is only possible to speculate at this point about how ET$_B$ and EGF receptor stimulation interact to regulate cell migration. A similar interaction between FGF and ET-1 was reported in cultured bovine endothelial cells. There is evidence suggesting a possible link between EGF receptor stimulation and its contribution to an increase in cell migration. Epidermal growth factor receptor stimulation may lead to a change in actin polymerization through the phosphoinositide cascade. A protein that affects the state of actin polymerization is profilin, which is bound to an intermediate in the phosphoinositide cascade (i.e., PIP$_2$). Epidermal growth factor receptor stimulation results in profilin release, allowing it to interact with actin, which affects its polymerization and cytoskeletal filament formation. Such changes promote the formation of adhesion plaques, pseudopods, and gelation. These effects can be necessary but are not sufficient to increase cell motility and corneal epithelial cell migration. A change in corneal epithelial cell migration may also require solation and contraction of the tail portion of the cell, which is how the endothelins mediate contraction of vascular smooth muscle cells. Therefore, in BCEC, ET$_B$ receptor stimulation may increase the effectiveness of EGF to stimulate wound closure through effects different from those of EGF on the cytoskeleton.

**Key Words**
cornea, cytokines, endothelin, epithelium, gene expression

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