Fos Expression and Growth Regulation in Bovine Corneal Endothelial Cells

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The authors evaluated the effects of stimulation (by serum, wounding, and three peptide growth factors: fibroblast growth factor [FGF], insulin, and transforming growth factor-β [TGF-β]) on the expression of the protein product of the immediate early gene, c-fos in bovine corneal endothelial (BCE) cells. These results were compared with those of cells which were made quiescent by serum starvation. They also examined the effect of these same growth factors or wounding on DNA synthesis. Quiescent cells expressed low levels of c-fos protein. Serum was the most potent stimulator, whereas FGF and insulin were modest stimulators. TGF-β1 did not significantly stimulate c-fos protein production. The results from DNA synthesis were different. Serum and FGF were still the most potent stimulators; insulin and TFG-β1 were weak stimulators. These data suggest that growth factors induce c-fos protein in BCE cells and that this may in part regulate the downstream event, cellular proliferation. Further investigation into the regulation of this and other protooncogene products may provide insight into the mechanisms which modulate corneal endothelial cell growth in humans. Invest Ophthalmol Vis Sci 33:3307–3314, 1992

The corneal endothelium is responsible for the maintenance of corneal optical clarity. During postnatal development, endothelial cells undergo hypertrophy. Significant proliferation, however, is not observed in humans.1 This probably accounts for the decline in endothelial cell density observed with increasing age2–5 and after surgical injury.6 Contrary to their behavior in vivo, corneal endothelium can be made to proliferate in vitro by adding growth factors to the tissue culture medium and coating plastic culture dishes with extracellular matrix protein.7–10 Numerous studies have focused on the regulation of cellular proliferation in corneal endothelial cells;11,12 however, in these cells, little is known about the molecular mechanisms involved in this biologic process.

In fibroblasts, a number of genes have been identified whose protein products are thought to play a pivotal role in the regulation of cellular growth and differentiation.13 Some of them, the immediate early genes, are induced very rapidly and transiently by serum growth factors, such as platelet-derived growth factor.14,15 Activation of these genes is among the first detectable responses of fibroblasts to growth stimulation signals. Some of the immediate early gene products have been identified as transcription factors,16 and it is likely that they are necessary to regulate a “second wave” of gene expression that will convert these short-term events into long-lasting consequences, such as proliferation.

One of the best characterized immediate early genes is the c-fos protooncogene. Its protein product is located in the nucleus17 and forms a heterodimeric transcription complex with another immediate early gene product, c-jun.18,19 Numerous studies have demonstrated that the c-fos protein is necessary for cells to proliferate. For example, when c-fos expression is blocked by antisense c-fos sequences, or microinjection of fos antibodies, the ability of cells to proliferate is greatly reduced.20–22 However, overexpression of c-fos has been implicated in unrestricted growth and is able to transform fibroblasts.24 To learn more about the control of proliferation in corneal endothelial cells, we analyzed Fos expression in these cells. In this study, we demonstrate that c-fos protein synthesis is induced in response to different peptide factors, fetal calf serum, or wounding. Furthermore, application of these growth factors also results in increased DNA synthesis.

Materials and Methods

Cell Culture

Bovine corneal endothelial (BCE) cells were established in culture according to previously published
methods. \textsuperscript{11,23} The medium used for primary and maintenance cultures contained a mixture of Ham's F12 and TC199 media supplemented with 3 mg/ml glutamine, antibiotics (100 U/ml penicillin and 100 U/ml streptomycin), and 15% fetal calf serum. Endothelial cells from one subclone (passage 2-3) were plated onto glass cover slips and used at 80% confluence. These cultures were determined to be endothelial by morphologic criteria because no specific cell markers have currently been identified. A phase-contrast micrograph of the BCE cells that was used is depicted in Figure 1.

DNA Synthesis

DNA synthesis in BCE cells was assessed by the incorporation of bromodeoxyuridine (BrdU; Amersham International, Arlington Heights, IL) into DNA. BrdU was added to the media of either starved, serum-, or growth factor-stimulated cells for 24 hr. Cells incorporating BrdU were determined by indirect immunofluorescence.

Antibodies

A mouse monoclonal antibody directed against the c-fos nuclear protein (Oncogene Science, Manhasset, NY) was used to identify this protein in BCE cells. The antibody (0.1 \( \mu \)g/ml) was diluted (1:30) with phosphate-buffered saline (PBS) containing bovine serum albumin (BSA) before use. A mouse monoclonal antibody directed against BrdU (Amersham International) was used to identify cells which incorporated BrdU.

Indirect Immunofluorescence

BCE cells were grown onto cover slips until approximately 80% confluent and were made quiescent by incubation in media containing low serum (0.5% fetal calf serum) for 24–48 hr. Growth-promoting or inhibiting factors or 20% fetal calf serum were added to the media of starved cells. Alternatively, injury to the monolayer was created by wounding a confluent area. The growth factors utilized included fibroblast growth factor (FGF, 100 ng/ml; Collaborative Research, Bedford, MA), insulin (5 \( \mu \)g/ml; Sigma, St. Louis, MO), and transforming growth factor-\( \beta \) (TGF-\( \beta \), 1 ng/ml; Sigma).

Two hours after growth factor or serum stimulation or wounding, the cells were washed with PBS and fixed with 3.7% formaldehyde. Their membranes were permeabilized with 0.3% Triton X-100 in PBS. The cells were incubated with the primary antibody, c-fos antibody (1:30 dilution), in PBS with 0.5% NP40 (Sigma, St. Louis, MO) and 2 mg/ml BSA (1 hr at 37°C); then the second antibody, biotinylated goat anti-rabbit immunoglobulin (Ig) G (1:400 dilution; Vector, Burlingame, CA), in PBS with 0.5% NP40 and 1 mg/ml BSA (1 hr at 37°C); and then the fluorochrome, streptavidin Texas red (1:100 dilution; Vector), in PBS. Between all incubation steps, the cover slips were washed with several changes of PBS with 0.5% NP40. Finally, the cover slips were washed.
briefly in distilled water and mounted onto microscope slides with Gelvatol (Air Products and Co., Allentown, PA). The time course of c-fos protein synthesis was examined by fixing quiescent or serum-stimulated cells at 0.5, 1, 2, and 4 hr poststimulation and staining for fos using the methods described.

Cells undergoing DNA synthesis were determined by adding BrdU along with the growth factor or serum. The cells were fixed 24 hr later with methanol (−20°C). The detection antibody was anti-BrdU (undiluted); the secondary antibody was biotinylated goat antimouse IgG (1:400 dilution; Vector). The fluorochrome remained the same.

Fluorescent staining was viewed on a Zeiss Axio phot microscope (Max Erb Instruments, Burbank, CA), and photographs were taken with T-Max film (Eastman Kodak, Rochester, NY). An independent observer examined each slide under high-power oil magnification (×630) for the presence of nuclear staining of either fos or BrdU. For fos staining, only cells exhibiting brightly staining nuclei were counted as positive. A total of 150–200 cells on each cover slip were counted. Duplicate cover slips were used in each experiment. Each experiment was repeated three times. Statistical analysis was performed with Statview 512+ program (Brainpower, Calabasas, CA). Analysis of variance was used to determine effectiveness between groups. A P value <0.05 was considered significant.

Results

Fos Expression

To analyze for Fos expression in BCE cells, they were grown on glass cover slips, fixed with 3.7% formaldehyde, and stained with c-fos antibodies for immunofluorescence. First, we compared the level of c-fos protein in quiescent versus serum-stimulated cells. As shown in Figure 2A, few quiescent cells showed nuclear staining (10.9 ± 4%: mean ± standard error of the mean). However, in serum-stimulated cells, many of the cells exhibited strong nuclear staining (83.7 ± 6%) indicative of the expression of c-fos protein (Fig. 2B). We next analyzed Fos expression in response to different growth factors. The growth-promoting peptides,25,26 FGF (at a concentration of 100 ng/ml, Fig. 3A) and insulin (at a concentration of 5 μg/ml) stimulated Fos expression (Fig. 3B) but to a lower level than serum (in 50.8 ± 6% and 49.4 ± 7% of cells, respectively). Fos expression by TGF-β1, a peptide that inhibits some cell types in vitro, was slightly greater (20.5 ± 6%) than the control (P > 0.05, Fig. 3C). Surgical injury to corneal endothelium, such as may happen in a penetrating corneal laceration, was stimulated by scratching a line with a Bard Parker no. 15 blade across the monolayer. This procedure induced fos protein most strongly in the cells lining the wound (Fig. 4). The differential rates of staining after growth factor or serum stimulation are summarized in Figure 5. Staining was significantly greater than controls for all growth factors tested except TGF-β1.

To analyze the time course of the fos protein production, cover slips of quiescent or serum-stimulated (20% fetal calf serum) BCE cells were harvested after 0.5, 1, 2 and 4 hr. The cells then were stained with c-fos antibodies. Expression of the c-fos protein was noted as early as 1 hr after serum stimulation. Levels peaked at 2 hr and were diminishing at 4 hr (Fig. 6).

DNA Synthesis

To determine optimal conditions for measuring DNA synthesis in BCE cells, cultures of such cells plated onto glass cover slips were starved for 24 hr and incubated with media containing 0.5% or 20% fetal calf serum for varying time intervals (24–72 hr). Under these conditions, maximal DNA synthesis (as measured by BrdU incorporation) occurred after 24 hr of incubation. A 24-hr incubation was therefore used in all subsequent experiments.
Fig. 3. Fluorescent photograph of FGF (A), insulin (B), and TGF-β1-stimulated cells (C) (original magnifications ×400). Few strongly staining nuclei are noted in response to TGF-β1.

Fig. 4. Fluorescent photograph depicting bright, Fos staining at the edge of the wound. Arrow depicts wound margin.

Fig. 5. Bar graph depicting the percentage of cells staining positively for c-fos nuclear protein after induction by growth factors or serum. Note that serum is the most potent stimulator of c-fos protein.

Discussion

The importance of the integrity of corneal endothelium in the maintenance of a transparent cornea is...
well known. Because only limited (if any) proliferation occurs in vivo after surgical trauma or with advancing age, bullous keratopathy (resulting in poor vision) is sometimes a complication of intraocular surgery.

Recently, studies about the regulation of cellular growth of corneal endothelium by an increasing number of growth-promoting or inhibiting peptides have increased our knowledge in this area. In the current study, we measured DNA synthesis in BCE cells to determine the mitogenic effect of growth factors and to explore the role of Fos as a potential regulator of cellular proliferation. We chose to examine the effect of stimulation by serum, FGF, insulin, and TGF-β1. Serum was utilized as a stimulating agent because it contains many growth factors and hormones which may also act as growth factors. The other polypeptides were chosen for specific reasons. FGF, originally isolated from pituitary extracts, was chosen as an example of a competence growth factor. This type of growth factor acts to prime quiescent cells to enter G1 of the cell cycle. FGF has been shown to induce proliferation of corneal endothelial cells isolated from cows, rabbits, and baboons, and in vivo, in cats, when injected intracamerally. Insulin was chosen as an example of a progression growth factor. This type of growth factor enables competent cells to progress through the cell cycle and begin DNA synthesis. Insulin alone may stimulate proliferation of BCE cells; however, it may further enhance the effect of epidermal growth factor (EGF). The combination of insulin and EGF is being investigated as an additive to corneal preservation medium in an effort to increase endothelial cell number before corneal transplantation. The third growth factor, TGF-β1, was chosen because this family of peptides exhibits a wide range of biologic effects in different cell types. In some cell types, specifically skeletal myoblasts and preadipocytes, TGF-β1 inhibits differentiation, whereas in epithelial cells, TGF-β1 stimulates differentiation. TGF-β1 also contrasts with other growth factors in its ability to act as a mitogen in different cell types; it stimulates proliferation in fibroblasts but inhibits proliferation in epithelial cells. In BCE cells, TGF-β1 has been shown not to increase DNA synthesis. Lastly, TGF-β1 increases production of extracellular matrix proteins, which have been implicated in the regulation of endothelial cell growth.

As currently understood, mitogenic factors are thought to control cellular proliferation by inducing the expression of growth-related genes. Approximately 50-100 genes have been identified in fibroblasts. One such gene is c-fos, which codes for a nuclear protein which acts as a transcription factor that then controls other genes required for cellular proliferation. To elucidate possible molecular mechanisms which modulate corneal endothelial cell growth, we examined whether the c-fos nuclear protein was expressed in this cell type and if it was induced by mitogens.

Our results demonstrate that the c-fos nuclear protein is not constitutively expressed in quiescent corneal endothelial cells; however, the growth factors, FGF and insulin, induced synthesis of this nuclear protein. This is similar to results from other cell types, including endothelium of vascular origin, where Fos is induced by many mitogens. Twenty percent fetal calf serum was the most effective stimulus tested. It induced 83.7% of the cells to express the c-fos protein.
Serum, which contains many growth factors and hormones, also was more effective than other growth factors alone in stimulating Fos production in fibroblasts. This suggests that the effects of growth factors may be additive or even synergistic. However, TGF-β1, a growth-inhibiting peptide in many cell types, failed to induce significant amounts of c-fos protein. In some cell types, e.g., NIH3T3 cells, TGF-β1 stimulates expression of c-fos messenger RNA in a sustained rather than transient fashion and subsequent translation of the protein does not occur.43 Whether c-fos messenger RNA is transcribed at all or in a transient or sustained pattern in response to TGF-β1 in BCE cells remains unknown. Wounding of a monolayer of BCE cells by scratching a line also induced fos in cells lining and close to the wound.

The effect of these growth factors on DNA synthesis in BCE cells produced results different than that of c-fos protein production. Twenty percent serum was not more effective than FGF in stimulating DNA synthesis. Contrary to the literature, TGF-β1 stimulated DNA synthesis to a level similar to insulin but not...
statistically different from quiescent uninduced cells. These results may be explained in part by the different times required for maximal DNA synthesis by each growth factor. Unlike c-fos protein production, DNA synthesis did not uniformly occur in cells lining the wound unless serum was also given. Growth factors are probably necessary for DNA synthesis to be observed. The percent of cells undergoing DNA synthesis did not correlate linearly with the percent of cells expressing the c-fos protein, supporting the concept that the early event of Fos production is related to, but does not always result in, DNA synthesis.

In summary, our immunofluorescent studies have demonstrated that the c-fos protein is induced in corneal endothelial cells by stimulating growth factors, serum, or wounding. Minimal expression of Fos was observed during quiescence or after treatment with TGF-β1. Our results suggest that growth-promoting factors may stimulate cellular proliferation of corneal endothelial cells at least in part by the regulation of the c-fos gene and its nuclear protein. Further investigation into the regulation of this and other protooncogene products may give insight into mechanisms responsible for the limited proliferative capacity of corneal endothelial cells and may lead to the ability to regulate the growth of these cells in vivo.

Key words: c-fos protooncogene, corneal endothelium, DNA synthesis, fibroblast growth factor, insulin, transforming growth factor-β

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