Effects of Insulin and EGF on DNA Synthesis in Bovine Endothelial Cultures: Flow Cytometric Analysis

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Purpose. To investigate the effects of insulin, epidermal growth factor (EGF), and the corneal storage media—DexSol—at 24 and 48 hours on DNA synthesis in confluent primary cultures of bovine corneal endothelial cells.

Methods. Flow cytometry was used to measure changes in DNA synthesis. This technique allows a large number of cells to be counted and sorted into G1, S, and G2/M phases of the cell cycle.

Results. Changing the normal culture media to DexSol had no effect on the cell cycle at 24 or 48 hours. The addition of insulin, EGF, or insulin + EGF to DexSol increased DNA synthesis within 24 hours. The mitotic indices for DexSol, DexSol + insulin, and DexSol + EGF were 0.134 (SE = 0.022), 0.207 (±0.027), and 0.205 (±0.052), respectively. Adding insulin + EGF to the DexSol resulted in the most significant change in S and G2/M, increasing the mitotic index to 0.300 (±0.072) (P = 0.0116). At 48 hours, the presence of the growth factors no longer had any effect.

Conclusions. Flow cytometry was a useful technique in separating cultured bovine corneal endothelial cells according to their DNA content. Analysis of the cultures after the addition of insulin and EGF showed an increase in DNA synthesis. The synergistic effects of the growth factors on corneal endothelial cells suggest that they stimulate mitotic activity by different mechanisms. The addition of mitogens to eye bank storage media may increase corneal endothelial cell densities in donor corneas.

The corneal endothelium is necessary for the maintenance of transparency in the cornea.1 This function is accomplished by transporting ions from the stroma, preventing stromal edema.2,3 When human endothelium is damaged as a result of ocular disease, trauma, or surgery, this barrier function is lost. Human corneal endothelium has limited proliferative capacity and does not regenerate;4 therefore, wound repair occurs by enlargement and migration of adjacent cells.5 If the injured area is small, cell migration is adequate to reestablish monolayer integrity. Extensive injuries may not heal sufficiently by cell enlargement and migration, resulting in irreversible corneal edema and corneal clouding.6 In these instances, corneal transplantation may be necessary. The success of corneal transplantation is dependent on the maintenance of a confluent cell monolayer in donor corneas. Factors that promote wound healing in vivo or that promote increased cell densities in stored corneas before transplantation may aid in maintaining this monolayer.

Epidermal growth factor (EGF) has been shown to be mitogenic for corneal endothelial cells in vitro and is used to maintain and grow the cells in tissue culture for prolonged periods of time without increased levels of serum in the media.7 In cultured corneal endothelial cells, EGF has also been shown to significantly stimulate DNA synthesis compared to controls.8,9

Epidermal growth factor has been identified in various human tissues and fluids, including corneal endothelium and tears.10,11 Wilson and Lloyd have shown that senescent cells continue to express EGF-receptor mRNA, suggesting that these cells might respond to exogenous EGF.10 EGF has also been reported to stimulate migration of corneal endothelial cells from the wound edge, inducing cellular elongation and promoting redistribution of actin filaments.

Flow Cytometric Analysis of Corneal Endothelial Cultures

which led to an increase in wound closure rate.12-14 When added together, insulin and EGF have a synergistic effect on the proliferation of corneal endothelial cells, as shown by an increase in DNA synthesis and an increase in the number of mitotic figures.15

Corneal storage techniques, which involve storage of donor corneas in culture media, provide a means to expose the endothelium to mitogenic factors such as EGF and insulin.19 Introduction of EGF, insulin, or both may provide a means to increase the density of donor corneal endothelial cells through an increase in mitotic activity.

DNA synthesis and mitotic activity have been studied using 3H-thymidine uptake,7,13,15,16, specular microscopy,17 and cell proliferation assays.7,18 Flow cytometry is another technique used to measure DNA synthesis. This technique allows the measurement of the percentage of cells in the different phases of the cell cycle and calculation of the mitotic index. In this study, we used flow cytometry to determine the mitotic activity of bovine corneal endothelial cells in culture after the addition of EGF, insulin, and EGF + insulin.

METHODS

Culturing

Calf eyes were obtained from an abattoir shortly after death. The eyes were kept in cold phosphate-buffered saline with penicillin, streptomycin, and amphotericin B until dissection.19 After incubating the corneas in 0.25% trypsin for 10 minutes, the endothelial cells were scraped from the Descemet's layer using forceps and then pooled. The cells were pelleted (10 minutes, 500g), resuspended in culture medium consisting of 1:1 mixture of DMEM/Ham's F-12, 10% fetal bovine serum, insulin (5 µg/ml), hydrocortisone (1 µg/ml), and gentamicin (50 µg/ml), and plated on 35 mm tissue culture dishes. Cultures were fed three times weekly and maintained at 37°C in a 5% CO2 incubator until the endothelial monolayer was confluent (7 to 10 days).

Experimental Conditions and Flow Cytometry

After the cultures became confluent, the endothelial culture media was changed to DexSol (Chiron Intratec, Irvine, CA) or to DexSol containing epidermal growth factor (20 ng/ml mouse, Sigma, St. Louis, MO), insulin (10 µg/ml beef, Lilly, Indianapolis, IN), or EGF + insulin. Each of these groups had a minimum of 3 plates per experiment, and each experiment was done on cultures from the same day. Cultures were prepared for flow cytometric analysis at 24 or 48 hours. The cultures were rinsed twice with Ca2+/Mg2+-free Hanks balanced salt solution and incubated in 0.25% trypsin at 37°C for approximately 30 to 45 minutes. Cell suspensions were centrifuged (500g, 10 minutes) and then washed twice in phosphate-buffered saline to remove excess trypsin. After the final wash, the cells were fixed and permeabilized in a 2:1 mixture of 0.5 ml cold 95% ethanol and 0.3 ml citrate buffer (250 mM sucrose, 40 mM trisodium citrate, 5% DMSO, pH 7.6) and 0.1 ml RNase (2.0 mg/ml, Sigma), and incubated at 37°C for 30 minutes. The cells were stained with propidium iodide (50 µg/ml) for 15 minutes at room temperature and centrifuged (500g, 10 minutes). The pellet was resuspended in 1.0 ml phosphate-buffered saline and filtered through a 30-µ nylon mesh screen.20

Fresh bovine corneal endothelial tissue was also collected for analysis by scraping the endothelium from the cornea upon enucleation. The tissue was prepared for flow cytometry using the same methods as those used for corneal endothelial cultures.

Samples were analyzed on an Epics Profile Cytometer (Epics Division, Coulter, Hialeah, FL). Propidium iodide-stained nuclei were excited with a 488-nm air-cooled argon laser, and the fluorescence emission greater then 680 nm was collected on a linear scale. A minimum of 15,000 nuclei were counted per sample. Doublets and clumps were excluded from the analysis by gating on a bivariate distribution of the peak fluorescence versus the integral fluorescence signal.

Standards

For an external DNA standard, polymorphonuclear leukocytes were isolated from bovine blood using the procedure of Weiss.21 Cells were counted and stored at −70°C at 1 X 106 cells per vial in citrate buffer (250 mM sucrose, 40 mM trisodium citrate, 5% DMSO, pH 7.6). Polymorphonuclear leukocytes were run immediately before and after the endothelial samples and compared to ascertain whether the mean G1 channels were within ± 5 channels and the coefficient of variation (CV) remained under 5. Daily quality control of the instrument was performed using fluorescent beads (DNA-Check, Epics Division, Coulter). A total of 5 X 104 beads were analyzed per sample.

Data Analysis

All histograms were analyzed using a cell cycle analysis program (Multicycle, Phoenix Flow Systems, San Diego, CA). This program used the polynomial model by Dean and Jett22 with a single cell cycle model (diploid cycle). The mitotic index (MI) was calculated using:

\[
MI = \frac{S + G2/M}{G1}
\]

For statistical analysis, a one-way analysis of variance was used. Statistical significance was assumed at P < 0.05 level.
Fluorescence Intensity

FIGURE 1. DNA histogram of bovine corneal endothelial cells in culture media for 48 hours. G0/G1 mean peak channel is 60 and G2/M mean peak channel is 120. Y-axis scale x1.

RESULTS

A representative DNA histogram for bovine corneal endothelial cell cultures appears in Figure 1. The control cultures were defined as those in culture media for 48 hours before flow cytometry. The average values for the percentage of cells in the cell cycle phases for control cultures were: G0, 89.3% (range, 85% to 93%); S, 6.1% (range, 2% to 10%); and G2/M, 4.6% (range, 3% to 6%) (Table 1). The calculated mitotic index for control corneal endothelial cells was 0.120 (SE = ±0.020). Addition of fresh culture media was sufficient to cause an increase in DNA synthesis. The mitotic index rose to 0.235 (±0.026) at 24 hours but returned to control levels by 48 hours.

Replacement of the culture media with DexSol, a serum-free defined medium, did not have a significant effect on DNA synthesis. The percentages of cells in each phase of the cell cycle were comparable to the control values for endothelial cell cultures at 48 hours (Table 1). The addition of insulin or EGF to the DexSol stimulated an increase in DNA synthesis within 24 hours. The DNA histograms showed increases in both G2/M and S peaks under these conditions (Fig. 2). At 24 hours, the mitotic indices for DexSol, DexSol + insulin, and DexSol + EGF were 0.134 (±0.022), 0.207 (±0.027), and 0.205 (±0.052), respectively. The addition of both insulin and EGF to the DexSol had the greatest effect on proliferation of the endothelial cells, as indicated by a mitotic index of 0.300 (±0.072). At 48 hours, the presence of growth factors was no longer effective in maintaining this higher level of DNA synthesis, with mitotic indices similar to controls.

DISCUSSION

Flow cytometry provides a unique tool to assess the mitotic activity of corneal endothelium. Flow cytometry has an advantage over 3H-thymidine uptake in that it is a rapid and direct measure of the cell cycle. A large number of cells can be counted, and changes in cell proliferation can be monitored by changes in the cell cycle phases. With 3H-thymidine uptake, similar measurements are much more time consuming. Also, there may be discrepancies between 3H-thymidine uptake.

TABLE 1. Determination of Mean Percentage and Standard Error of the Mean of Cells in G1, S, and G2/M at 24 Hours by Flow Cytometry and Calculation of the Mitotic Index

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>% G1</th>
<th>% S</th>
<th>% G2/M</th>
<th>Mitotic Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control†</td>
<td>5</td>
<td>89.32 (±1.58)§</td>
<td>6.10 (±1.45)</td>
<td>4.58 (±0.53)§</td>
<td>0.120 (±0.020)</td>
</tr>
<tr>
<td>Culture Media (24 hours)</td>
<td>4</td>
<td>80.02 (±1.51)</td>
<td>10.92 (±1.81)</td>
<td>7.78 (±0.43)§</td>
<td>0.255 (±0.026)</td>
</tr>
<tr>
<td>DexSol</td>
<td>5</td>
<td>88.34 (±1.69)§</td>
<td>7.60 (±1.68)</td>
<td>4.68 (±0.37)§</td>
<td>0.134 (±0.022)</td>
</tr>
<tr>
<td>DexSol + Insulin</td>
<td>6</td>
<td>83.10 (±1.89)</td>
<td>9.98 (±1.84)</td>
<td>7.53 (±0.57)§</td>
<td>0.207 (±0.027)</td>
</tr>
<tr>
<td>DexSol + EGF</td>
<td>5</td>
<td>83.55 (±3.21)</td>
<td>8.88 (±1.84)</td>
<td>7.62 (±1.58)§</td>
<td>0.205 (±0.025)</td>
</tr>
<tr>
<td>DexSol + Insulin + EGF</td>
<td>5</td>
<td>77.80 (±4.08)</td>
<td>10.20 (±2.46)</td>
<td>12.00 (±1.67)</td>
<td>0.300 (±0.072)</td>
</tr>
</tbody>
</table>

* The n value represents the number of replicates for each condition.
† Control cultures were defined as those in culture media for 48 hours before flow cytometry.
§ Significant compared to culture media (24 hours) (P < 0.05).
§§ Significant compared to control (P < 0.01).
†† Significant compared to DexSol (P < 0.001) and control (P = 0.0071).
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Fluorescence Intensity

DexSol + EGF + Insulin

FIGURE 2. DNA histograms of bovine corneal endothelial cells. (a) DexSol, (b) DexSol + 10 
μg/ml insulin, (c) DexSol + 20 ng/ml EGF, (d) DexSol + insulin + EGF. Go/G1 mean peak 
channel is 60, and G2/M mean peak channel is 120. Y-axis scale ×5.

take and cell proliferation. For example, Jozan et al\textsuperscript{23} stated that in certain breast cancer cells, there was an 
estradiol-induced increase in incorporation of \(^{3}\text{H}\)-thymidine into DNA without any change in cell multiplication due to thymidine incorporation during repair 
of damaged DNA. These repair processes occur in both proliferating and nonproliferating cells; therefore, uptake of \(^{3}\text{H}\)-thymidine would not necessarily be 
correlated with cell division.\textsuperscript{24}

In this study, flow cytometry was used to examine the effect of EGF and insulin on corneal endothelial 
cells in culture. It was found that in defined media, the addition of either insulin or EGF stimulated an increase in DNA synthesis and mitosis in endothelial 
cells. When insulin and EGF were added together, they appeared to have a synergistic effect. The mitotic 
index for DexSol + insulin + EGF was significantly higher when compared to DexSol + insulin, DexSol + 
EGF, or DexSol alone. At 48 hours, the presence of growth factors no longer had any effect, with mitotic 
indices returning to control values. There was a similar effect on the mitotic index after fresh media was
added to the cell cultures. The culture media is normally changed on a 2-day schedule. Within 24 hours of adding fresh media, there was an increase in the mitotic index comparable to that with DexSol + insulin + EGF. The mitotic index returned to control levels within 2 days. This increase was likely due to the fetal calf serum used in the media, which contains a variety of mitogenic factors including insulin and EGF.

The mitogenic effects of EGF on bovine corneal endothelial cultures were also studied by Gospodarowicz et al. EGF added to the cultures was shown to be effective in stimulating the initiation of DNA synthesis. After incubation with $1.5 \times 10^{-13}$ M EGF for 12 to 28 hours, the labeling index increased from 10% for controls to 55% to 60%, as measured by $^3$H-thymidine incorporation. Woost et al. also reported that bovine corneal endothelial cells responded to EGF treatment with large increases in DNA synthesis, whereas with insulin the response was less.

These results suggest that the mitogenic effects of these growth factors are time dependent, that the EGF and insulin may be depleted from the media, or that the cells become refractory to it within 48 hours. Several studies have shown that chronic exposure to insulin induces downregulation of the receptors in various cell types. Downregulation was complete after a 10-hour exposure to insulin in 3T3 to L1 cells. Cells do have the capacity to regulate the number of receptors they possess in response to a variety of physiological conditions.

There has been some controversy as to whether growth factors such as EGF are normally present in aqueous humor. Parelman et al. have found EGF in human aqueous humor in contrast to other studies in which EGF was undetectable. However, cultured human corneal cells were shown to produce mRNA for EGF and EGF receptors. Downregulation of the receptors may also cause low endothelial proliferative capacity. A combination of factors is most likely responsible for the regulation of mitotic activity of the endothelium in vivo.

Human corneal endothelial cells have the capacity for cell division when grown in cell cultures. Therefore, the addition of growth factors in eye bank media may be of value by increasing donor corneal endothelial cell densities before transplantation. It has been shown that when a human globe was exposed to culture medium containing EGF (100 ng/ml), 4.3 ng/ml of EGF reached the anterior chamber and accumulated in the aqueous humor. However, the half-life of EGF in the aqueous humor was only 0.6 ± 0.5 hours. The low level of uptake and the short half-life of EGF in the aqueous humor may limit the effect of EGF. Our results also indicate that the stimulation of DNA synthesis and mitosis occurs within 24 hours. This suggests that further work is needed to determine the optimal mitogen concentrations and exposure time before transplantation. Multiple additions of growth factors to the media during corneal storage or the addition of mitogens shortly before using the donor corneas may be required.

Key Words
flow cytometry, insulin, EGF, mitotic index, endothelial cells

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References
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