Growth Factor Responsiveness of Human Retinal Pigment Epithelial Cells

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Growth factor effects on DNA synthesis in density-arrested human retinal pigment epithelial cells were assessed by [3H]-thymidine incorporation. Acidic and basic fibroblast growth factor and epidermal growth factor were potent stimulators, whereas platelet-derived growth factor, insulinlike growth factor-1, and insulin were weak or modest stimulators when used alone. When used in combination, each of the above growth factors caused a significant enhancement of [3H]-thymidine incorporation regardless of its effect when used alone. The combination of all four growth factors was significantly more effective than all other combinations, demonstrating synergism in their action. Similar results were found in cell proliferation assays. In contrast to this, transforming growth factor-beta inhibited the ability of each of the other growth factors and serum-containing media to stimulate [3H]-thymidine incorporation. These data suggest that DNA synthesis in human retinal pigment epithelial cells can be modulated by several growth factors, some in a stimulatory or synergistic manner and at least one in an inhibitory manner. A better understanding of these complex interactions may provide insights relevant to normal and abnormal ocular wound healing. Invest Ophthalmol Vis Sci 31:839-846, 1990

The retinal pigment epithelium (RPE) forms a monolayer of cells beneath the sensory retina that is normally mitotically inactive. However, several types of insult may result in initiation of RPE proliferation, manifested clinically by hyperpigmented retinal scars. This suggests that the RPE participates in ocular wound healing, a contention that is supported by histologic studies of cryosurgical and photocoagulation lesions. Proliferation of RPE cells also occurs after retinal detachment and has been implicated in the pathogenesis of proliferative vitreoretinopathy (PVR). Therefore, a better understanding of the factors that control RPE cell proliferation may provide information that is relevant to normal and abnormal ocular wound healing.

A previous study has demonstrated that epidermal growth factor (EGF) stimulates proliferation of human RPE in culture. In the current study, we investigated several growth factors (GFs) known to participate in wound healing for their ability to stimulate DNA synthesis in human RPE cells in culture.

Materials and Methods

Basic and acidic fibroblast growth factor (FGF) and transforming growth factor-beta (TGF-beta) were obtained from R&D Systems (Minneapolis, MN), EGF from Bethesda Research Labs (Gaithersburg, MD), insulinlike growth factor-1 (IGF-1) from Chemicon (El Segundo, CA), insulin and transferrin from Sigma (St. Louis, MO), and recombinant platelet-derived growth factor (PDGF) from Amgen (Thousand Oaks, CA).

Primary cultures of human RPE cells were established from eyes obtained from the Old Dominion Eye Bank (Richmond, VA), by a previously published technique. The three cell lines used in the current study were from donors of age 27 years, 6 months, and 26 years. Immunohistochemical staining for cytokeratins was performed on each cell line. Cells were plated in triplicate on wells on collagen-coated glass slides and grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 15% fetal bovine serum (FBS) at 37°C, 5% CO₂. After 2 days, the cells were washed in 0.05 M Tris-buffered saline, pH 7.6 (TBS), and fixed in methanol. The fixed cells were covered with normal rabbit serum (NRS; Arnel) in TBS for 30 min at room temperature in a humidified chamber to block nonspecific binding. Excess serum was removed, and the cells were washed in 0.05 M Tris-buffered saline, pH 7.6 (TBS), and fixed in methanol. The fixed cells were covered with 10% normal rabbit serum (NRS; Arnel) in TBS for 30 min at room temperature in a humidified chamber to block nonspecific binding. Excess serum was removed without washing, and the cells were covered with primary antibodies diluted in TBS containing 1% NRS and incubated overnight at 4°C. Primary antibodies consisted of a cocktail of three mouse...
monoclonal antibodies to keratin including a 1:250 dilution of a 20:1 ratio of antibody from clones AE1 and AE3 (Hybritech, San Diego, CA) and a 1:50 dilution of antibody from clone CAM 5.26 (Becton Dickinson, Mountain View, CA). After incubation with primary antibodies, the cells were warmed to room temperature, washed with TBS/NRS, and incubated for 30 min in a 1:25 dilution of rabbit antiserum globulins (Arnel, Brooklyn, NY) in TBS/NRS. Cells then were washed and incubated for 30 min with a 1:400 dilution of a mouse peroxidase-antiperoxidase (PAP) complex diluted in TBS/NRS. Cells were washed twice in 0.05 M Tris buffer, pH 7.6, and incubated in the dark for 15 min with a freshly made and filtered solution of 0.07% 3,3'-diaminobenzidine-4HCl (DAB) in Tris buffer containing 0.0185% H2O2.

For [3H]-thymidine incorporation,14 RPE cells at passage 2-4 were trypsinized lightly and plated in 16-mm wells of 24-well plates at 6 X 10⁴ cells per well in DMEM supplemented with 15% FBS. They were grown for 3-4 days until confluent, and then washed and changed to serum-free DMEM containing various GF or control media. After a 16-hr incubation, the cells were pulsed with 2 μCi/ml [3H]-thymidine (sp act, 6.7 Ci/mM; New England Nuclear, Boston, MA) for 2 hr. The cells were washed three times with PBS and five times with ice-cold 5% trichloroacetic acid (TCA). One milliliter 0.1 NaOH containing 0.1% SDS was then added to each well, and after 1 hr, a 100-μl aliquot was added to 4.5 ml scintillation fluid and counted in a Beckman scintillation counter.

To examine for possible synergism between GFs requiring comparison of the effects of several GF combinations, certain precautions were taken. GF concentrations at or near the peak of their dose-response curve were used. To control for potential variability related to culture conditions, such as time from last subculture and cell density, comparisons were performed using the same cells plated identically on a single 24-well plate. In this manner, duplicates were used to generate a single experimental value for 12 different combinations. These experiments were repeated to obtain from 8 to 12 experimental values, which then were entered into a database and examined by analysis of variance using the

Fig. 1. Immunohistochemical staining for cytokeratins demonstrates a pure population of RPE in three cell lines. (A) Donor age 27 yr (×400). (B) Donor age 6 months (×400). (C) Donor age 26 yr (×400). (D) Same as (a) with nonimmune serum substituted for primary antibodies.
Fig. 2. Single GF stimulation of [\textsuperscript{3}H]-thymidine incorporation in three RPE cell lines. [\textsuperscript{3}H]-Thymidine incorporation was performed as detailed in Methods, using RPE cell lines established from donors age 27 yr (A), 6 months (B), and 26 yr (C). Optimal concentrations were selected from preliminary experiments and were 10 ng/ml PDGF, 50 ng/ml IGF-1, 10 ng/ml EGF, 5 μg/ml insulin, 10 ng/ml acidic FGF, 10 ng/ml basic FGF, and 5 μg/ml transferrin. Each bar represents the mean ± SEM from at least 10 experiments. *, P < 0.001; **, P < 0.005; ****, P < 0.02 by the student unpaired t-test for difference from control.
SAS statistical software package. Possible differences arising from experimental techniques were adjusted by including plate number in the analysis of variance. Comparisons between various growth factor combinations were made by calculating student t statistics for the least square means from the analysis of variance using a pooled standard error.

In some experiments, cells were plated in clip-on wells on collagen-coated glass slides and grown in DMEM supplemented with 15% FBS until confluent. They then were washed and changed to serum-free DMEM containing various GFs or control media. After a 16-hr incubation, the cells were pulsed with 2 µCi/ml [3H]-thymidine for 2 hr, washed three times with PBS, and air-dried after removal of the clip-on wells. The slides were dipped in Kodak (Rochester, NY) NTB2 liquid emulsion, and after 7 days they were developed according to the manufacturer’s instructions.

For cell growth experiments, RPE cells were plated in 16-mm wells of 24-well plates at 6 x 10⁴ cells per well in DMEM supplemented with 15% FBS. They were grown until confluent; washed; and changed to serum-free DMEM containing various growth factors, DMEM containing 5% FBS, or control media. After 48 hr, the cells were washed, trypsinized, and counted in a Coulter electronic cell counter. Each growth factor was tested in duplicate wells in eight experiments, and statistical comparisons were made using the student paired t-test.

Results

Each of the cell lines from three different donors showed immunohistochemical staining for cytokeratins in all of the cells examined, demonstrating that each of the cell lines consists of a pure population of RPE cells (Fig. 1).

In initial experiments, a range of concentrations of each GF was tested for [3H]-thymidine incorporation. Each showed a typical dose–response curve, except for transferrin, which did not stimulate [3H]-thymidine uptake.
thymidine incorporation at any concentration tested (data not shown). Using a concentration resulting in activity at or very near the peak of the dose–response curve for each GF, each of the three cell lines showed a similar pattern of single GF-induced stimulation of [3H]-thymidine incorporation, although there were some differences in the magnitude of the stimulation (Fig. 2). Acidic and basic FGF and EGF caused marked stimulation in each cell line, whereas PDGF, IGF-1, and insulin caused only modest stimulation.

In some experiments, [3H]-thymidine incorporation was performed as noted above, but was assessed by radioautography instead of liquid scintillation spectroscopy. This demonstrated that the GF-induced stimulation of [3H]-thymidine incorporation measured by liquid scintillation spectroscopy was associated with a marked increase in the frequency of nuclear labeling with [3H]-thymidine (Fig. 3). A high rate of nuclear labeling occurred also in the presence of serum-containing media (Fig. 3).

To examine for possible synergism between GFs, experiments were performed in paired fashion, as detailed in Methods. Since IGF-1 and insulin were very similar in the amount of stimulation they caused in single GF experiments, insulin was used for combination experiments. The same was true for acidic and basic FGF, with basic FGF somewhat less effective in two cell lines; therefore, basic FGF was used in combination experiments. Each GF showed strong synergism with each of the other GFs tested (Fig. 3, Table 1), regardless of its potency in single GF experiments. Insulin was particularly effective in enhancing the activity of other GFs, despite its lack of potency when used alone (Fig. 4, Table 1). Sequential addition of GFs resulted in significant enhancement of [3H]-thymidine incorporation in almost all cases, with the combination of all four GFs tested (PDGF, EGF, basic FGF, and insulin) significantly better than any other combination (Table 1). This combination resulted also in [3H]-thymidine incorporation that was higher than that stimulated by media containing 10% FBS (data not shown).

The effect of selected GFs was also examined in cell growth experiments. After 48 hr, each of the GFs caused a statistically significant increase in RPE cell number compared to cell number in control media (Fig. 5). As was the case with [3H]-thymidine incorporation, insulin caused the smallest increase in cell number, while EGF and acidic FGF (even at 1 ng/ml) caused the largest (Fig. 5). Also similar to the situation with [3H]-thymidine incorporation, the combination of all four GFs caused a significantly greater increase in cell number than did each of the GFs alone (Fig. 5; \( P < 0.01 \) by analysis of variance).

### Table 1. Effect of growth factor combinations on the stimulation of [3H]-thymidine incorporation in RPE cells

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Increase (%) by addition of PDGF</th>
<th>Increase (%) by addition of EGF</th>
<th>Increase (%) by addition of basic FGF</th>
<th>Increase (%) by addition of insulin</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>125.3</td>
<td>96.7</td>
<td>142.6</td>
<td>161.8</td>
<td>0.0001</td>
</tr>
<tr>
<td>EGF</td>
<td>153.3</td>
<td>120.0</td>
<td>153.3</td>
<td>172.0</td>
<td>0.0001</td>
</tr>
<tr>
<td>PDGF + EGF</td>
<td>172.0</td>
<td>125.3</td>
<td>172.0</td>
<td>192.0</td>
<td>0.0001</td>
</tr>
<tr>
<td>PDGF + basic FGF</td>
<td>172.0</td>
<td>125.3</td>
<td>172.0</td>
<td>192.0</td>
<td>0.0001</td>
</tr>
<tr>
<td>PDGF + insulin</td>
<td>172.0</td>
<td>125.3</td>
<td>172.0</td>
<td>192.0</td>
<td>0.0001</td>
</tr>
<tr>
<td>EGF + basic FGF</td>
<td>172.0</td>
<td>125.3</td>
<td>172.0</td>
<td>192.0</td>
<td>0.0001</td>
</tr>
<tr>
<td>EGF + insulin</td>
<td>172.0</td>
<td>125.3</td>
<td>172.0</td>
<td>192.0</td>
<td>0.0001</td>
</tr>
<tr>
<td>basic FGF + insulin</td>
<td>172.0</td>
<td>125.3</td>
<td>172.0</td>
<td>192.0</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

*Note: 10 experiments caused by addition of the GF in the column head to the GF or GF combination listed in columns 1, 4, 7, and 10. Statistical comparisons were made by analysis of variance.*
Fig. 4. Synergism in GF stimulation of [3H]-thymidine incorporation in RPE cells. [3H]-Thymidine incorporation was performed as detailed in Methods. Concentrations of GFs used were the same as those listed in the legend to Figure 2. Each bar represents the mean (±SEM) calculated from at least nine experiments. *, P < 0.0001; **, P < 0.0004; ***, P < 0.0301 for differences from single GFs alone by analysis of variance.

In contrast to the marked synergistic effect demonstrated by the other GFs, TGF-beta showed an antagonistic effect. When used alone, it had no significant effect, but in combination with other GFs or FBS-containing media, it significantly blunted their ability to enhance [3H]-thymidine incorporation in RPE cells (Fig. 6).

Discussion

Trauma results in the initiation of a sequence of cellular events through which the traumatized tissue is repaired. There is mounting evidence to suggest that these events are controlled in large part by GFs, proteins that act through cell surface receptors to stimulate cellular migration and proliferation.15,16 In experimental skin wounds, cell participation occurs in an orderly sequence: recruitment and proliferation of inflammatory cells occurs first, followed by that of fibroblastic cells, endothelial cells, and finally epithelial cells.17 These events may be orchestrated by target-tissue and producing-tissue specificity of GFs, such that each wave of cells produces a GF specific for the next required participant.17 Therefore, the GF responsiveness of a particular cell might provide insight into its role(s) in wound healing processes.

In the current study, we have demonstrated that DNA synthesis in RPE cells is stimulated by several different GFs, including EGF, IGF-1, insulin, acidic and basic FGF, and PDGF. This enhanced DNA synthesis results in an increase in cell number after 48 hr of incubation. Insulin and IGF-1 had almost identical effects in all experiments, suggesting that they may operate through the same receptor. Effects of insulin may be mediated through its own receptor or through the IGF-1 receptor.18 The high concentration of insu-

Fig. 5. GF stimulation of cell proliferation in RPE cells. Cells were plated, grown to confluence; washed; and incubated for 48 hr in the presence of serum-free media containing 0.2% bovine serum albumin (media alone), media containing 5% FBS, media containing 1 ng/ml acidic FGF, media containing 10 ng/ml EGF, media containing 5 μg/ml insulin, media containing 10 ng/ml PDGF, or media containing 1 ng/ml aFGF plus 10 ng/ml EGF plus 5 μg/ml insulin plus 10 ng/ml PDGF. The cells then were washed, trypsinized, and counted in a Coulter electronic counter. Each bar represents the mean (±SEM) from eight experiments performed in duplicate. *, P < 0.05; **, P < 0.02; ***, P < 0.01; ****, P < 0.001 by the student paired t-test.
Fig. 6. Effect of TGF-beta on GF and serum stimulation of [3H]-thymidine incorporation in RPE cells. [3H]-Thymidine incorporation was performed as detailed in Methods, using the above-listed growth factors in the presence and absence of 2 ng/ml TGF-beta. The concentration of other GFs used were the same as those listed in the legend to Figure 2. Each bar represents the mean (±SEM) calculated from at least six experiments. *, P < 0.02; **, P < 0.005; ***, P < 0.001 by analysis of variance.

lin required to stimulate DNA synthesis in RPE suggests that this effect is mediated through the IGF-1 receptor. The effects of EGF and the FGFs were dramatic, whether used alone or in combination with other GFs, whereas insulin and PDGF had very modest effects alone and were better stimulators when combined with other GFs. TGF-beta had no significant effect alone, but inhibited the ability of each of the other GFs to stimulate DNA synthesis.

These data suggest that RPE cells are unlike most other epithelial cells with respect to GF responsiveness (though they do show some epithelial characteristics, such as their response to EGF and TGF-beta). This unique responsiveness may reflect a very prominent role in wound healing, not typical of epithelial cells elsewhere in the body, which usually participate only in the latter stages to provide a cover for the wound. However, RPE cells differ from other epithelial cells in that they originate from neural ectoderm, and although they have certain epithelial characteristics, such as polarity and prominent transport functions, they have other functions as well. They are intimately associated with neural cells and serve an accessory function analogous to that served by glia. Glia play a prominent role in wound healing throughout the nervous system and are particularly responsive to PDGF. The RPE also acts as a barrier between the circulation and the avascular outer retina, a function analogous to that of endothelial cells, which are particularly responsive to the FGFs. RPE cells are capable of phagocytosis and have been demonstrated to participate in tissue debridement under certain circumstances, a function usually attributed to macrophages. Perhaps cellular functions correlate better with GF responsiveness than with germ layer origin or cell morphology.

Synergistic responses to GFs have been demonstrated previously in vitro and in vivo. Replication of cultured BALB/c 3T3 cells requires both PDGF and IGF-1, a finding that led to the concepts of competence and progression. Although these concepts are strictly applicable only to mouse fibroblasts, synergism between PDGF and IGF-1 has also been demonstrated in human fibroblasts and porcine smooth muscle cells. Both of these cell types appear to require IGF-1 to initiate DNA synthesis, but in its absence they produce an IGF-1-like protein that can partially compensate. This type of autocrine regulation of growth control may also occur in RPE: RPE have been demonstrated to produce proteins immunologically similar to PDGF, FGFR, and TGF-beta.

TGF-beta is a potent stimulator of fibroblast growth, but epithelial cells are either inhibited or show no response in its presence. The potent inhibition by TGF-beta of DNA synthesis induced by each of the other GFs in RPE is consistent with its effects in other epithelia. The inhibition suggests that the receptor for TGF-beta is linked to a strong inhibitory pathway that is able to override the stimulatory signals of each of the other GFs. Although the mechanism of growth inhibition by TGF-beta is currently unknown, its elucidation may provide a new approach to the problem of uncontrolled proliferation of RPE, such as that which occurs in PVR.

Several studies support the concept that the RPE plays a prominent role in ocular wound healing. Migration and proliferation of RPE cells has been demonstrated to occur after retinal cryopexy, retinal laser, retinal detachment, penetrating trauma, subretinal neovascularization, and retinal degenerations. Migration and proliferation of RPE have also been implicated in the pathogenesis of PVR. Although the stimuli for the RPE response in each of these situations is unknown, the current study demonstrates several GFs that may play a role. It also suggests a complex interaction between GFs as they may interact in a synergistic or antagonistic manner in modulating RPE cell proliferation. A better under-
standing of these interactions may provide insights relevant to normal and abnormal ocular wound healing.

Key words: retinal pigment epithelium, growth factors, wound healing, proliferative vitreoretinopathy, retinal detachment

Acknowledgment

The authors would like to thank Donald Kaiser, Dr. P. H., for his assistance with statistical analysis.

References


