Inhibition of Growth Factor Effects in Retinal Pigment Epithelial Cells

Katrinka H. Leschey, John Hines, Jeff H. Singer, Sean F. Hackett, and Peter A. Campochiaro

Several agents were examined for their effect on growth factor-stimulated processes in retinal pigment epithelial (RPE) cells. DNA synthesis was assessed by $^3$H-thymidine incorporation in density-arrested cells using previously determined maximally effective concentrations of various growth factors with and without test substances. Cell migration was assessed in Boyden chamber assays. For each test substance, trypan blue exclusion was used to determine nontoxic concentrations, and the effect of several concentrations were assessed on selected growth factors. The most effective, nontoxic concentration was then used for comparisons. Two cationic proteins, protamine and histone type II B, caused inhibition of RPE chemotaxis and $^3$H-thymidine incorporation induced by several growth factors, but a cationic polypeptide, polylysine, did not. Protamine and histone were particularly effective inhibitors of acidic and basic fibroblast growth factors (FGF) but not if they were exposed to cells and then removed before growth factor addition. They had no effect on serum-stimulated chemotaxis or $^3$H-thymidine incorporation even when used in the presence of serum. Three anionic substances, heparin, pentosan polysulfate, and suramin, also inhibited RPE chemotaxis and $^3$H-thymidine incorporation induced by several different growth factors. They were less effective inhibitors of the FGFs than protamine and histone but were better inhibitors of serum-induced effects. Also unlike protamine and histone, the anionic substances maintained their inhibitory effect even when removed before growth factor addition. Since migration and proliferation of RPE cells are important processes in the pathogenesis of proliferative vitreoretinopathy, these agents and their mechanism of action deserve further study for potential therapeutic applications.

Proliferation and migration of retinal pigment epithelial (RPE) cells occurs after retinal detachment, and these processes have been implicated in the pathogenesis of proliferative vitreoretinopathy (PVR). Several studies focused on inhibition of RPE proliferation as a potential therapy for PVR. They generally used drugs that inhibit intracellular functions, such as inhibitors of DNA or RNA synthesis or microtubule function, that are required for cellular proliferation but are also needed in nonproliferating cells. As a result, these agents are associated with significant toxicity.

There is mounting evidence to suggest that cellular migration and proliferation are controlled in large part by growth factors, proteins that act through cell-surface receptors. Those that are involved in retinal wound healing and PVR are not known with certainty, but it is likely that serum-derived growth factors and those produced locally by ocular cells are involved. We recently demonstrated that several growth factors, including epidermal growth factor (EGF), acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and insulin-like growth factor 1 (IGF-1), are able to act synergistically to stimulate DNA synthesis in RPE cells.

Inhibition or modulation of growth factor–receptor interactions has a potential advantage for prophylaxis and therapy of PVR since little effect on nontarget cells would be anticipated. In this study we investigated several agents that, based on the literature, might be expected to interfere with growth factor binding or transduction of the signal from the cell surface, for their effect on $^3$H-thymidine incorporation and migration in RPE cells. The rationale for these studies was not only to try and identify potentially useful pharmacologic agents for PVR, but also to try and learn more about growth regulation in RPE.

Materials and Methods

Both b- and aFGF were obtained from R&D Systems (Minneapolis, MN), EGF from BRL (Gaithers-
burg, MD), IGF-1 from Chemicon (El Segundo, CA), insulin from Sigma (St. Louis, MO), and recombinant PDGF from Amgen (Thousand Oaks, CA). Protamine (molecular weight, 7000), histone type II B (molecular weight, 13,774), polylysine (molecular weight, 3800), suramin (molecular weight, 1429), heparin (mean molecular weight, 12,000), and pentosan polysulfate (molecular weight, 1500-5000) were obtained from Sigma.

Primary cultures of human RPE cells were established from eyes obtained from the Old Dominion Eye Bank (Richmond, VA) by a previously published technique. Three cell lines were used in these experiments, one from a 26-year-old donor (Figs. 1A–C and Tables 1 and 2), one from a 35-year-old donor (Figs. 1D–3), and one from a 39-year-old donor (Table 3 and Fig. 4). Each of the cell lines contained pure populations of RPE cells by immunohistochemical stain-
Table 1. The effect of combinations of various agents on [3H]thymidine incorporation in retinal pigment epithelial cells

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Acidic FGF</th>
<th>Insulin</th>
<th>5% FBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13,124 ± 951</td>
<td>13,301 ± 2322</td>
<td>15,384 ± 4090</td>
</tr>
<tr>
<td>Heparin</td>
<td>8057 ± 586</td>
<td>13,885 ± 2040</td>
<td>11,733 ± 2835</td>
</tr>
<tr>
<td>10 μg/ml</td>
<td>5192 ± 455</td>
<td>10,808 ± 2172</td>
<td>11,711 ± 2748</td>
</tr>
<tr>
<td>Suramin</td>
<td>8352 ± 1044</td>
<td>6399 ± 1668</td>
<td>15,641 ± 3569</td>
</tr>
<tr>
<td>10 μg/ml</td>
<td>3800 ± 417</td>
<td>2292 ± 630</td>
<td>11,026 ± 1987</td>
</tr>
<tr>
<td>Protamine</td>
<td>277 ± 37</td>
<td>2620 ± 506</td>
<td>20,304 ± 4544</td>
</tr>
<tr>
<td>10 μg/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ suramin</td>
<td>6543 ± 914</td>
<td>7834 ± 2354</td>
<td>11,445 ± 3213</td>
</tr>
<tr>
<td>Heparin</td>
<td>2067 ± 366</td>
<td>1819 ± 617</td>
<td>6860 ± 2399</td>
</tr>
<tr>
<td>50 μg/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ protamine</td>
<td>5697 ± 474</td>
<td>12,737 ± 3110</td>
<td>12,425 ± 3296</td>
</tr>
<tr>
<td>Suramin</td>
<td>4112 ± 718</td>
<td>3468 ± 983</td>
<td>10,103 ± 2894</td>
</tr>
<tr>
<td>50 μg/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ protamine</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Retinal pigment epithelial cells were incubated for 16 hr in acidic fibroblast growth factor (acidic FGF, 1 μg/ml), insulin 5 μg/ml, or 5% fetal bovine serum (FBS) in the presence and absence of the inhibitor combinations listed above. Listed values represent the mean ±SEM number of disintegrations per minute (DPM) per well calculated from eight experiments performed in duplicate. The mean DPM per well for cells incubated in media alone in these experiments was 2041 ± 433.

Table 2. The effect of heparin, suramin, and protamine on EGF- and serum-stimulated cell growth: cell number per well (×10^3)

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>EGF as stimulant</th>
<th>5% FBS as stimulant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.1 ± 1.4</td>
<td>16.0 ± 1.6</td>
</tr>
<tr>
<td>Stimulant</td>
<td>23.2 ± 2.2*</td>
<td>246.9 ± 5.7</td>
</tr>
<tr>
<td></td>
<td>18.5 ± 1.6§</td>
<td>213.2 ± 4.8§</td>
</tr>
<tr>
<td>+ suramin</td>
<td>13.3 ± 2.0ı</td>
<td>105.6 ± 7.3ı</td>
</tr>
<tr>
<td>+ protamine</td>
<td>24.5 ± 2.4</td>
<td>263.7 ± 9.2</td>
</tr>
</tbody>
</table>

Retinal pigment epithelial cells were plated in 24-well plates and the media were replaced with Dulbecco’s Modified Eagle’s Media containing 10 ng/ml of EGF or 5% fetal bovine serum with or without (control media) heparin (50 μg/ml), suramin (50 μg/ml), or protamine (10 μg/ml). The media were changed on days 2 and 4, and on day 6 the cells were washed, trypsinized, and counted in a Coulter electronic cell counter. The listed values represent the mean ±SEM calculated from six experiments. Trypan blue exclusion on parallel wells showed no difference between control and experimental wells with less than 4% stained cells.

* P < 0.05, §P < 0.001 by paired Student’s t-test for difference from control; ıP < 0.02, ııP < 0.002, ıııP < 0.001 by paired Student’s t-test for difference from stimulant alone.

The 3H-thymidine incorporation was done by a previously described technique. Briefly RPE cells at confluence were lightly trypsinized and plated in 16-mm wells of 24-well plates at 6 × 10^4 cells per well in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% fetal bovine serum (FBS). They were grown for 3–4 days until confluent and then washed and changed to serum-free M199 containing various growth factors or 5% FBS with or without test drugs. After a 16-hr incubation, the cells were pulsed with 2 μCi/ml of 3H-thymidine (6.7 Ci/mMol; New England Nuclear, Boston, MA) for 2 hr. The cells were washed three times with phosphate-buffered saline and five times with ice cold 5% trichloroacetic acid. One ml of 0.1 M NaOH containing 0.1% sodium dodecyl sulfate was then added to each well, and after 1 hr, a 100-μl aliquot was added to 4.5 ml of scintillation fluid and counted in a Beckman scintillation counter (Irvine, CA). Statistical comparisons were made with student paired t-test.

Cellular migration assays were done in a 48-well Boyden chamber apparatus by modification of a previously described technique. Briefly, RPE cells were trypsinized and resuspended in serum-free medium at a density of 5 × 10^5 cells per ml. Twenty-five microliters of test drug diluted in DMEM was added to the bottom wells of the apparatus and covered with a polycarbonate membrane containing 8-μm pores previously coated with type I collagen. Fifty microliters of cell suspension was then added to the top wells and incubated at 37°C for 6 hr. The apparatus was then disassembled, the porous membrane was removed, and all cells were scraped from the top surface, leaving only cells that had migrated through the pores. The cells were fixed in methyl alcohol and stained with modified Wright’s stain. The number of cells in ten (×400) fields were counted for each well. Each concentration of the test drug was tested in triplicate in at least four different assays. Since there was a moderate amount of variability in baseline migration from assay to assay, the results were expressed as a
Fig. 2. Effect of three anionic agents on growth factor- or serum-induced \([^{3}H]\) thymidine incorporation in retinal pigment epithelial cells. Cells at confluence were washed and then media containing one of the listed growth factors on fetal bovine serum (FBS), and (a) heparin, (b) pentosan polysulfate, or (c) suramin, were added and cells were incubated for 16 hr. \([^{3}H]\) thymidine incorporation was then measured. Growth factor concentrations were the same as those listed in the legend to Figure 1 except that for acidic FGF, which was 1 ng/ml. Each bar represents the mean (±SEM) from at least eight experiments performed in triplicate. Statistical comparisons were made by Student’s paired t-test. (a) * \(P < 0.05\); ** \(P < 0.02\); *** \(P < 0.001\); (b) \(P < 0.05\); ** \(P < 0.02\); *** \(P < 0.001\); (b) * \(P < 0.05\); ** \(P < 0.005\); *** \(P < 0.001\); and (c) * \(P < 0.005\); ** \(P < 0.001\).
percentage of cell migration induced by each chemotactant.

For cellular proliferation assays $2 \times 10^4$ RPE cells were plated in 16-mm wells of 24-well plates in DMEM supplemented with 10% FBS and allowed to attach overnight. The cells were then washed and placed in DMEM containing 10 µg/ml of EGF or 5% FBS with or without heparin (50 µg/ml), suramin (50 µg/ml), or protamine (10 µg/ml). The media were changed on days 2 and 4, and on day 6, the cells were washed, trypsinized, and counted in a Coulter electronic cell counter.

Results

Several basic proteins compete with radiolabeled PDGF for binding to cell-surface receptors.\textsuperscript{15,16} Protamine sulfate is a very effective competitive inhibitor,\textsuperscript{15,16} and therefore we examined its effect on PDGF-stimulated $^3$H-thymidine incorporation in RPE cells. At 10 µg/ml, its most effective nontoxic concentration, it inhibited the PDGF response by 60% (Fig. 1A). It was also a potent inhibitor of several other growth factors and was particularly effective in antagonizing the responses of α- and bFGF...
Table 3. Effect of cationic and anionic agents on retinal pigment epithelial cell migration: percent of stimulation by chemoattractant alone

<table>
<thead>
<tr>
<th></th>
<th>ECCM PDGF FBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protamine</td>
<td>78 ± 9*</td>
</tr>
<tr>
<td>Histone IIb</td>
<td>83 ± 7*</td>
</tr>
<tr>
<td>Polylysine</td>
<td>92 ± 5</td>
</tr>
<tr>
<td>Suramin</td>
<td>35 ± 2†</td>
</tr>
<tr>
<td>Heparin</td>
<td>44 ± 1†</td>
</tr>
</tbody>
</table>

Cell migration assays were performed using the stimulating agents listed across the top horizontal column with and without the inhibitors listed along the vertical column. Values represent the mean ± (SEM) percentage of migrated cells for each listed stimulating agent calculated from at least four experiments performed in triplicate. The values for media alone and each stimulating agent alone in cells per 400x field were: media alone, 30.6 ± 6.9; endothelial cell-conditioned media (ECCM, a 1:20 dilution), 119.7 ± 18.3; platelet-derived growth factor (PDGF, 10 ng/ml), 138 ± 15.2; 10% fetal bovine serum (FBS), 83.2 ± 21.4.

Protamine added with growth factor

Fig. 4. Effect of protamine added simultaneously or 2 hr after growth factor stimulation on [3H]thymidine incorporation in retinal pigment epithelial cells. Cells at confluence were washed and then incubated for 16 hr in media containing 5% fetal bovine serum (FBS), 5 µg/ml of insulin, or 1 ng/ml of basic fibroblast growth factor (FGF). Parallel wells were treated with no protamine (open bars, control) 10 µg/ml of protamine added simultaneously with growth stimulators (bars with slanted lines) [3H]Thymidine incorporation was measured. Each bar represents the mean (±SEM) for eight experiments performed in triplicate. * P < 0.05; ** P < 0.01; *** P < 0.001 by Student’s paired t-test for difference from control.
in antagonizing the effective of aFGF (Table 1, \( P < 0.0001 \) and \( P < 0.078 \), respectively).

We examined the effect of heparin, suramin, and protamine on sustained cell growth stimulated by EGF or 5% FBS (Table 2). Unaffected by protamine, EGF caused a modest increase in cell number over 6 days that was completely inhibited by heparin and suramin. Serum caused a large increase in cell number that was partially inhibited by heparin and suramin and slightly enhanced by protamine.

Each of the agents was examined in RPE migration assays using PDGF (10 ng/ml), 10% FBS, or endothelial cell-conditioned media (ECCM, 5%) as stimulants for migration. Endothelial cell-derived growth factors, a significant portion of which is PDGF, account for most of the RPE-migration stimulating activity in ECCM. Suramin, heparin, and protamine, effective antagonists of PDGF-stimulated \(^{3}H\)-thymidine incorporation, also antagonized PDGF- and ECCM-stimulated migration (Table 2). Heparin was the only effective inhibitor of FBS-stimulated migration. Polysine and histone type II B were poor inhibitors of each of the stimulants (Table 2), paralleling their lack of effect on PDGF- or FBS-stimulated \(^{3}H\)-thymidine incorporation.

The mechanism of the inhibitory effects were investigated by preincubating cells for 6 hr with inhibitors and then removing them before the addition of growth factors or serum. Heparin and suramin were equally effective inhibitors when used in this manner as when they were present only during growth factor incubation (Fig. 3). They were most effective when kept on the cells after a 6-hr preincubation. By contrast, protamine had no inhibitory effect when removed before growth factor addition (Fig. 3C); however, it was equally effective when it was added at the same time as the growth factors or 2 hr after (Fig. 4).

**Discussion**

In this study, as in a previous study, we found that several growth factors were able to stimulate DNA synthesis in RPE cells. Three cell lines were used, and in one, the relative potencies of the growth factors tested were identical to that seen in the previous report. In the other two lines, the potencies were similar except that PDGF was somewhat more effective in one and insulin was somewhat more effective in the other. This is interesting and suggests that particular growth-factor receptors might be expressed at higher levels than usually seen in some cell lines, but it has little bearing on the main focus of these studies since all experiments were done and analyzed in pairs.

With respect to cationic agents, we found that protamine, and to a lesser extent histone type II b, inhibit PDGF-induced \(^{3}H\)-thymidine incorporation and chemotaxis in RPE cells. They also inhibit \(^{3}H\)-thymidine incorporation induced by several other growth factors and are particularly potent inhibitors of a- and bFGF. Similar effects have been reported in other cell types including inhibition of \(^{125}\text{I}\)-PDGF binding and mitogenic activity in mouse fibroblasts, inhibition of \(^{125}\text{I}\)-bFGF binding to bovine brain membrane receptors, and inhibition of bFGF-induced mitogenic activity in Chinese hamster lung fibroblasts. Protamine has also been shown to inhibit the mitogenic activities of a- and bFGF in bovine aortic endothelial cells and a baboon kidney cell line (BHK-21). However, the effect of cationic agents on RPE cells differed from those on other cell types in at least two respects. Protamine was found to enhance the \(^{125}\text{I}\)-EGF binding in mouse fibroblasts and EGF-induced mitogenic activity in BHK-21 cells. In human RPE cells, both protamine and histone inhibited EGF-induced \(^{3}H\)-thymidine incorporation to a lesser degree than that induced by the FGFs or PDGF. The second difference was that polysine stimulated the effects of growth factors in RPE cells but was inhibitory in mouse fibroblasts and Chinese hamster lung fibroblasts. In Chinese hamster lung fibroblasts, 1 \( \mu \text{M} \) protamine enhanced the mitogenic activity of bFGF; higher concentrations were inhibitory. Thus, the size and concentration of cationic agents may be important variables that help to determine their effect on mitogenic activity.

Based on the results in other cell types, it is likely that protamine and histone exert their effect through modulation of growth-factor binding, but the nature of the interaction is unknown. The ability to bind to heparin or heparin-like molecules may be an important determinant of growth-factor-modulating activity of cationic agents. Thus, they may act by binding to heparin-like molecules on the cell surface that interact with several growth factor receptors.

In this study, we also showed that three anionic agents inhibit growth factor- and serum-mediated effects in RPE cells. Heparin and pentosan polysulfate had similar activity among the various growth factors; suramin was slightly different. Each retained its inhibitory activity when preincubated with cells and then removed before growth factor addition. This suggests—but does not prove—that their mechanism of action may involve binding to the cell surface. Since they modulate the effects of several different growth factors, they may bind to a site other than a specific growth factor receptor but in some manner may interfere with transduction of the mitogenic and chemotactic signals.

Several studies identified a growth-inhibitory effect of heparin. Most of this work was done in vascular
smooth muscle cells in which heparin, heparin-like proteins, and pentosan polysulfate inhibited serum-induced proliferation. However, heparin enhanced growth factor-induced proliferation in human skin fibroblasts and vascular endothelial cells. In eye-derived (scleral) fibroblasts, heparin inhibited serum-induced proliferation. Recent work may provide an explanation for the different effects of heparin on different cell types. The potentiating effect of heparin on FGF-induced proliferation in vascular endothelial cells occurs as a result of heparin–FGF binding which stabilizes the FGF and increases its affinity for its receptor. The inhibitory effect of heparin is mediated through cell surface receptors that inhibit a protein kinase C-dependent pathway for cell proliferation. It is likely that cells that contain heparin receptors linked to an inhibitory pathway for cell proliferation would be unaffected by heparin-induced growth factor stabilization. This appears to be true in RPE cells and is the likely explanation for why heparin inhibits the effects of a- and bFGF and other growth factors.

Suramin inhibited binding of 125I-PDGF to its receptor and dissociate bound 125I-PDGF. In AKR-2B cells, a continuous clonal cell line derived from AKR mouse embryos, suramin inhibits binding and DNA synthesis induced by several different growth factors. It was least effective in antagonizing the effects of EGF such as we found for RPE in our study. Like heparin, suramin retains its inhibitory effects when removed from the media before growth factor addition, suggesting that it acts through a cell binding site. The somewhat different spectrums of action and additive effects of heparin and suramin suggest that they may act through different cell-surface receptors, but additional work is needed to determine this with certainty.

This study has important implications with respect to PVR. We identified several agents that modulate RPE migration and proliferation, two processes that have been implicated in its pathogenesis. Further work is needed to determine if these agents have similar effects in animal models of PVR. In addition, our results suggest the presence of at least two ways in which the stimulatory effect of growth factors can be modulated in RPE. Elucidation of the mechanism of these modulating effects may suggest a novel approach for treatment and prophylaxis of PVR.

Key words: retinal pigment epithelium, growth factors, retinal wound healing, growth inhibition, proliferative vitiretino-pathy

References


