Regulation of Gelatinase B Production in Corneal Cells Is Independent of Autocrine IL-1α
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PURPOSE. The matrix metalloproteinase gelatinase B is synthesized by cells at the leading edge of the corneal epithelium migrating to heal a wound. Recent data from the authors’ laboratory suggest that excessive synthesis contributes to repair defects. The goal of the study reported here was to investigate mechanisms controlling gelatinase B production by corneal epithelial cells.

METHODS. Freshly isolated cultures of corneal epithelial cells and early passage stromal fibroblasts from rabbit were used for these studies.

RESULTS. In a previous study, it was found that the cytokine interleukin (IL)-1α is released into the culture medium of corneal epithelial cells more efficiently when they are plated at low density with limited cell-cell contact than when plated at high density. In this study, we show that production of gelatinase B by these cells is similarly affected by cell plating density. However, it is further demonstrated that these two events are not dependent on one another but occur in parallel: IL-1α does not regulate gelatinase B production (synthesis), nor was there evidence that any other secreted autocrine cytokine acts as a mediator. Instead, our data suggest that gelatinase B production is downregulated directly by high cell density and indicate a connection to the level of protein kinase C activity. Nevertheless, the anticancer agent suramin, which blocks collagenase synthesis by interfering with autocrine cytokine-receptor interactions, still inhibits synthesis of gelatinase B.

CONCLUSIONS. Unlike collagenase synthesis by corneal stromal fibroblasts, production (synthesis) of gelatinase B does not appear to be controlled by secreted autocrine cytokines but can still be inhibited by suramin. Suramin may make an effective therapeutic agent for controlling pathologic overproduction of gelatinase B in corneal ulcers.


Resurfacing of the cornea by the epithelium after injury normally occurs rapidly and without incident; however, after certain types of injury, such as chemical or thermal burn, reepithelialization is inhibited. Failure of resurfacing in these cases is thought to be due to degradation of adhesive molecules in the wound bed, which prevents attachment and migration of the epithelial cells. Studies from this laboratory using the thermal injury model have provided evidence that failure to reepithelialize is due to excessive activity of enzymes of the matrix metalloproteinase (MMP) family. Results specifically implicate the 92-kDa MMP gelatinase B (gelB; MMP-9), which catalyzes cleavage of basement membrane components. Synthesis of gelB is stimulated at the leading edge of the epithelium migrating to resurface a corneal wound; this is normal. However, it appears that situations that cause overproduction of gelB can lead to pathology. Therefore, inhibition of gelB production might be used as a therapeutic strategy to facilitate repair of pathologic corneal wounds.

Of all the MMPs, the one whose synthesis has been most studied is fibroblast collagenase (MMP-1), because it was the first MMP family member to be discovered. A model of normal diploid fibroblasts in culture, including corneal fibroblasts, has typically been used for these investigations. These studies have lead to the emergence of a new paradigm for understanding collagenase synthesis that emphasizes the importance of signal routing through autocrine cytokines. For example, autocrine cytokine interleukin-1α (IL-1α) mediates synthesis of collagenase in untreated fibroblast cultures and stimulation in response to several stimulatory conditions. Routing of signals through autocrine cytokines offers a convenient point for therapeutic intervention. For example, IL-1ra, the naturally occurring antagonist of IL-1, can be used to specifically interfere with collagenase synthesis by interfering with the binding of IL-1 to its receptor at the cell membrane. The anticancer drug suramin, which interferes with cytokine-receptor interactions in general, can also be used in this way.

In the fibroblast model, synthesis of gelB is often regulated coordinately with collagenase; for example, both proteins are stimulated by the tumor promoter phorbol myristate acetate (PMA). Reports in the literature have indicated that synthesis of gelB can be stimulated by exogenous IL-1 in some cell types, suggesting that stimulation by PMA might also occur via the IL-1α mediator. On the other hand, stimulation of gelB mRNA levels by PMA occurs with much different kinetics than collagenase, suggesting some independence of mechanism.

Like fibroblasts, epithelial cells in culture synthesize MMPs; however, regulatory mechanisms appear to have some unique features in this cell type. An important characteristic of epithelial cells that makes them different from fibroblasts is their capacity for close adhesive interactions, mediated by cell-cell junctions containing integrins and cadherins. In vivo, this characteristic produces a tissue of tightly packed cells, creating a barrier at body surfaces such as skin and cornea. Several reports have demonstrated regulation of gelB and other MMPs by cell density in epithelial cell cultures (i.e., higher production in low-density cultures than high). Evidence suggests that this is mediated by cell-adhesive interactions, as transfection of human colon carcinoma cells with a CDNA for E-cadherin decreased gelB synthesis. A higher percentage of cells at lower plating density are dividing, and gelB production was found to occur only in the cycling cells of these cultures. However, further mechanisms of cell density regulation have not yet been elucidated.

When epithelium is wounded, the close contact between cells is disrupted and might provide a signal for changes in
production of proteins required for aspects of tissue repair. Therefore, studies on cell density regulation of gelB production might yield insight into the mechanisms stimulating production in the corneal wound. Recently we reported that IL-1α is produced by corneal epithelial cells in culture, as it is in fibroblast cultures.12 Interestingly, we found that IL-1α is released from epithelial cells more efficiently when they are plated at lower cell density. This suggests the hypothesis that IL-1α might act as an autocrine intermediate to control gelB synthesis as it does collagenase and, thus, determines the cell density dependence of gelB synthesis in epithelial cell cultures. In this study we investigated this hypothesis.

**Materials and Methods**

Primary corneal epithelial cells or early passage corneal fibroblasts were prepared from New Zealand White rabbits as described previously.6-12 Animal use was conducted with adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. SIRC cells, a spontaneously arising line of rabbit corneal epithelial cells, were obtained from the American Type Culture Collection (Rockville, MD). For an experiment, cells were plated in 24-well cluster dishes in minimum essential medium (MEM; Life Technologies, Gaithersburg, MD) containing 10% supplemented calf serum (HyClone, Utah, NY) to facilitate attachment and spreading on the plastic. The next day, plating medium was replaced with serum-free MEM, an equivalent volume per well (usually 350 μl), and appropriate treatment agents were added. For biosynthetic-labeling of proteins, [35S]-methionine was included at 80 μCi/ml. Cell-conditioned media were collected for analysis of MMPs after 24 hours. The following treatment agents were used: PMA at 10−6 M (Sigma, St. Louis, MO), the isouquinolinone-sulfonamide derivatives H7 and HA1004 (Seikagaku, Tokyo, Japan), at doses between 10 μM and 50 μM, human recombinant IL-1α (R&D Systems, Minneapolis, MN) at 10 ng/ml, human recombinant IL-1ra (R&D Systems) at doses between 10 ng/ml and 100 ng/ml, anti-rabbit IL-1α neutralizing antibody (Cytokine Sciences, Boston, MA) at a dose ranging from 3 × 10−8 M to 3 × 10−6 M. According to manufacturer’s directions, IL-1α and IL-1ra stocks were reconstituted in sterile saline with 0.1% bovine serum albumin at 1 μg/ml to 10 μg/ml.

Zymography was one method used to analyze MMPs secreted into culture medium. With this technique, proteolytic species are separated on the basis of molecular size by electrophoresis through a nonreducing sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) within which a substrate for the enzyme of interest is copolymerized. The position of each enzyme in the gel is visualized by its capacity to degrade the substrate. Equal volume samples of conditioned medium from each cell culture well (usually 15 μl) were compared by zymography against gelatin or β-casein as previously described.7-4 Standard MMP preparations were run alongside experimental lanes to aid in identification of zymographic bands with specific MMPs. Quantification of gelatinase bands on zymograms was performed by laser scanning densitometry (Molecular Dynamics, Sunnyvale, CA). Band densities were then corrected to a standard curve to convert density values to relative enzyme quantities.

Immunoprecipitation analysis of biosynthetically labeled proteins provided a second means to analyze secreted MMPs. To perform this analysis, we used equal volume samples of conditioned medium from each culture well (usually about half of each sample). When replicate treatments were performed, conditioned media from replicate culture wells receiving identical treatment were pooled, and then equal-sized samples from each pool were analyzed. MMP immunoprecipitation was performed on each sample according to our standard method.6-7,9 The antibody used in this study was a dog antiserum against rabbit gelB.9 Immunoprecipitates were displayed by SDS-PAGE, and gels were dried and autoradiographed to display immunoprecipitated proteins. Quantification of gel bands from autoradiographs was performed by laser scanning densitometry (Molecular Dynamics, Sunnyvale, CA). For both zymographic and biosynthetic MMP analysis, the total 35S-labeled secreted protein in samples was also routinely examined by SDS-PAGE to ensure that treatments did not affect overall protein synthesis.

In some experiments, cells were treated with conditioned media prepared from cultured epithelial cells. According to our standard method to prepare this reagent,12 epithelial cells collected from 6 corneas are plated to 100-mm culture dishes. For the present study, a modification was made whereby epithelial cells were plated at 1:2 and 1:16 dilutions of the standard plating density, that is, three corneal equivalents per plate (2/6 or 0.4 corneal equivalents per plate (6/16). Cells were plated in MEM containing 10% supplemented calf serum to promote cell attachment and spreading. The next day the medium was removed, the cells were washed, and the medium was replaced with 8 ml serum-free MEM. Cells were allowed to condition this medium for 48 hours before collection for use in an experiment.

Protein kinase C (PKC) activity in lysates of corneal epithelial cells was assayed by measuring the incorporation of 32P, from [γ-32P]-ATP into a synthetic peptide derived from myelin basic protein, using a kit purchased from Life Technologies. Trichloroacetic acid-precipitable counts per minute (cpm) were subtracted from background which was determined as the cpm obtained when a pseudosubstrate inhibitor peptide was included in the reaction. Background-subtracted cpm were then normalized to total protein concentration as determined by the dye-binding assay (Bio-Rad, Hercules, CA).

**Results**

To determine whether epithelial cell plating density affects gelB production, equal-sized samples of conditioned medium from cultures plated at serially increasing dilutions, and then incubated for 24 hours, were analyzed by zymography (Fig. 1A, left and middle). The total amount of gelB (92-kDa gelatinase) in each sample (which is a relative measure of the total amount produced by each culture) increased hardly at all over a four-fold increase in cell density and only 1.4-fold over a 16-fold increase in cell density. In contrast, the amount of 65-kDa gelatinase (gelatinase A; MMP-2; gela) produced by each culture increased by approximately 7.9-fold over the 16-fold cell density.
FIGURE 1. Effects of plating density on MMP expression by corneal cells and role of autocrine IL-1α. (A, left) Corneal epithelial cells were plated on 24-well cluster plates by serially increasing two-fold dilution from 3 × 10⁵ cells/well (1:1) to 0.75 × 10⁵ cells/well (1:4), and half the wells were treated with PMA. Cell-conditioned medium was collected from each well after 24 hours for analysis. Gelatinases secreted into equal volume samples of the medium from each well were displayed by gelatin zymography. Arrows indicate gelA (65 kDa) and gelB (92 kDa), as identified by comparison to a standard MMP preparation run alongside (not shown). Center: corneal epithelial cells were plated by serially increasing two-fold dilution, as at left, and allowed to condition their medium for 24 hours. Gelatin zymography was used to analyze equal volume samples of cell-conditioned media. Zymographic band densities were quantified and then corrected to a standard curve as described in the Materials and Methods section. Bars, SD from the mean; n = 4. Right: corneal epithelial cells were plated by serially decreasing two-fold dilution from 3 × 10⁵ cells/well (1:1) to 0.37 × 10⁵ cells/well (1:8), as at left. ³⁵S-Methionine was added to label proteins biosynthetically. Cells were allowed to condition their medium for 24 hours, then an equal volume sample of each conditioned medium was electrophoresed on a polyacrylamide gel. The migration positions of reduced molecular size standards are indicated in kilodaltons (kDa). Relative amounts of labeled protein in each lane were quantified by densitometry, and relative mean values for each cell plating density are indicated beneath the lanes. (B) Corneal epithelial cells were plated in a 24-well culture dish at 2 × 10⁵ cells/ml and were then left untreated (−) or were treated with either the stimulator IL-1α (10 ng/ml) or the inhibitors, IL-1α-neutralizing Ab or IL-1ra. Treatments were performed in the presence or absence of PMA. Conditioned media was collected after 24 hours, and equal volume samples were analyzed by gelatin zymography. Arrows indicate the migration position of 92-kDa (gelB) and 65-kDa (gelA) gelatinases. (C, left) Corneal epithelial cells were plated as in (B). Culture wells were either left untreated (−) or treated with PMA (10⁻⁶ M), IL-1α (10 ng/ml), or IL-1ra (1000 ng/ml). ³⁵S-Methionine was added to label proteins biosynthetically. Cells were allowed to condition their medium for 24 hours. Conditioned media from replicate culture wells receiving identical treatment were then pooled. We then used equal volume samples of pooled conditioned medium from each treatment group (usually about half of each sample). The relative level of gelB accumulated in the conditioned medium was then determined by immunoprecipitation analysis. GelB was detected as the characteristic 92-kDa doublet band (arrow) on the resultant autoradiogram. Right: corneal fibroblasts were plated in a 24-well cluster dish at equal density (2 × 10⁵ cells/well). Duplicate wells were left untreated (−) or were treated with either IL-1α-neutralizing Ab, IL-1ra, PMA, or IL-1ra. Conditioned media were analyzed by casein zymography. Arrows indicate the zymogram bands corresponding to collagenase/stromelysin (MMP-1/MMP-3) at 50 kDa, gelA at 65 kDa, and gelB at 92 kDa, as determined by comparison to MMP standards run in adjacent lanes (not shown).
density range, much more in keeping with the change in cell number. This suggested that there was a selective "plating density effect" on gelB production, that is, each cell was producing less gelB as the cell plating density increased. The synthesis of total secreted proteins also increased relative to the cell number, further indicating the selectivity of the plating density effect (Fig. 1A, right). Interestingly, when cells were treated with PMA, the inhibition of gelB production at higher cell densities was relieved (Fig. 1A, left). Thus, at a low cell density (1:4), PMA had little effect on gelB production, but at high cell density (1:1), PMA strongly stimulated gelB.

Because more IL-1α on a per cell basis is also released into the culture medium of corneal epithelial cells when they are plated at low density compared with high density, we investigated the hypothesis that IL-1α regulates gelB production. However, as assayed by gelatin zymography (Fig. 1B), treatment with exogenous IL-1α did not alter the amount of gelB produced by corneal epithelial cells. Furthermore, as demonstrated by both zymography (Fig. 1B) and immunoprecipitation analysis (Fig. 1C, left), the addition of IL-1α neutralizing antibody (Ab), or the less selective inhibitor IL-1ra (which antagonizes both IL-1α and IL-1β), did not alter the production (synthesis) of gelB. Similarly, PMA-stimulated gelB production was unaffected by either of the IL-1 inhibitors (Fig. 1B). We also examined regulation of gelB synthesis by IL-1 in fibroblasts to learn whether our findings were epithelial cell-specific. Collagenase and stromelysin (MMP-1 and MMP-3) were produced by the untreated fibroblast cultures shown in Figure 1C (right). In cells supplied with IL-1α Ab or IL-1ra, collagenase/stromelysin production was selectively blocked; synthesis of gelA was unaffected, as we have previously shown. This confirms that the IL-1α autocrine is present and active; nevertheless, gelB is not produced by these cells. PMA treatment resulted in a coordinate stimulation in the production of both collagenase/stromelysin and gelB (Fig. 1C, right). In contrast, IL-1α treatment enhanced collagenase/stromelysin production to a high level without affecting gelB production (Fig. 1C, right). We conclude that in both corneal epithelial cells and fibroblasts production of gelB is independent of control by autocrine IL-1α.

To begin to investigate the possibility of a role for other autocrine cytokines other than IL-1α in controlling gelB production, corneal epithelial cells were treated with suramin, an inhibitor of cytokine:receptor interactions. Treatment with suramin resulted in a dose-dependent reduction in gelB synthesis (Fig. 2A) in cultures that were otherwise untreated, or in cultures that were treated with PMA to stimulate levels of gelatinase B synthesis. The effects of suramin were selective, because the total secreted protein profile was unaffected (data not shown). Encouraged by these results, we directly assayed for gelB synthesis-stimulating cytokines in epithelial cell-conditioned media. Conditioned media collected from epithelial cells plated at 1:2 cell dilution or 1:16 cell dilution each stimulated synthesis of total secreted proteins by approximately 3.5-fold (Fig. 2B, left). Immunoprecipitation analysis revealed a similar stimulation of gelB synthesis of about fourfold (Fig. 2B, left). These data indicate that epithelial cells produce a cytokine or cytokines that stimulate gelB synthesis as part of a general stimulation of total protein synthesis; however, they provide no evidence for the production of a cytokine or cytokines that selectively stimulate synthesis of gelB. These data suggest that gelB synthesis is regulated directly by cell plating density, without the requirement for an autocrine intermediate.

Because treatment with PMA, an activator of PKC, relieved the inhibitory effect of high cell density on gelB production,
we hypothesized that production of gelB might be regulated by PKC, other protein kinases, or both. To investigate this hypothesis, cells were treated with specific protein kinase inhibitors (Fig. 3, top). As assayed by zymography, H-7, a relatively selective inhibitor of both PKC and cyclic nucleotide-dependent protein kinases, almost completely inhibited production of gelB when used at 10 μM. A second inhibitor, HA1004, which is very effective against cyclic nucleotide-dependent protein kinases but fairly ineffective against PKC, was less potent an inhibitor at 10 μM. However at 50 μM, HA1004 and H-7 both inhibited. As a control, we examined the effects of these inhibitors on PMA-stimulated gelB production. Although H-7 completely inhibited PMA-stimulated gelB production, HA1004 only showed a slight inhibitory effect. The effects of the inhibitors were selective for gelB, total [35]S-labeled protein profiles were unchanged by these agents (data not shown). These results suggest that gelB production by corneal epithelial cell cultures is regulated by both PKC and cyclic nucleotide-dependent protein kinases.

SIRC cells are an immortalized line of rabbit corneal epithelial cells that produce gelB only at very low levels. Coplating of SIRCs with corneal epithelial cells inhibited gelB production by corneal epithelial cells in a cell density-dependent manner (Fig. 3, bottom). Because PMA relieves the inhibitory effect of high cell density on gelB production, we hypothesized that high cell density might act by repressing PKC activity. To test this hypothesis, we plated cells at a high density (10^7 cells per 100-mm plate) so that they were confluent in the cell culture plate and at a 1:10 dilution of this density. We then measured PKC activity in each lysate and normalized for equal protein, so that the data would reflect activity on a per cell basis. PKC activity was easily detectable in lysates from both high- and low-density cultures. However, in two of three repetitions of this experiment, we found considerably higher activity in lysates prepared from low-density cultures than in high-density cultures (Table 1). These data are consistent with the hypothesis that production of gelB in cells from high-density cultures might be reduced through inhibition of PKC activity.

**DISCUSSION**

An important characteristic of epithelial cells is their capacity for close adhesive interactions mediated by cell-cell junctions containing integrins and cadherins. When epithelium is
wounded, this close contact is disrupted, and this could provide a signal for changes in gene expression required for aspects of tissue repair. In a previous study, we found that the cytokine IL-1α is released into the culture medium of corneal epithelial cells more efficiently when they are plated at low density than when plated at high density. In this study, we show that production of gelB by these cells is similarly affected by cell plating density, suggesting that IL-1α may regulate gelB. However, we go on to demonstrate that these two events are not dependent on one another but rather occur in parallel. Thus, IL-1α does not control untreated or PMA-stimulated gelB production in either epithelial cells or in fibroblasts, and we could find no evidence that another autocrine cytokine acts as mediator either. In addition, gelB is not stimulated by cytochalasin B, which stimulates collagenase and stromelysin expression via the IL-1α intermediate. Instead, our data suggest that gelB production is downregulated directly by increased cell density and suggest a connection to downregulation of PKC activity.

Suramin is a new anticancer drug that, among other functions, inhibits ligand receptor interactions. A previous report demonstrated suramin inhibition of collagenase mRNA levels stimulated by UV light, an induction that was subsequently shown to be mediated by autocrine cytokines. In our study, suramin caused a significant reduction in untreated and PMA-stimulated gelB production in corneal epithelial cells, despite the fact that we could find no evidence for regulation through secreted cytokines. Possibly, suramin inhibits gelB production by disrupting interaction of cell surface-bound ligands with their cell surface-bound receptors. It is also known that suramin blocks cells in the G₀ stage of the cell cycle; considering the reported connection between gelB mRNA levels and the cell cycle in epithelial cell cultures, this could also explain our findings with suramin. Whatever the mechanism, our findings could be important in the clinic because suramin might be used to inhibit gelB production during corneal reepithelialization. Suramin would probably not be beneficial during the later cell replication phase of reepithelialization, because it inhibits cell division. However, during the initial period in which epithelial cells are migrating to close the corneal defect, no cell replication occurs. Thus, timely application of suramin might enhance the rate or success of reepithelialization in normal and pathologic wound-healing situations.

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References


TABLE 1. Relative PKC Activity in Cells Plated at High or Low Density

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<th>Experiment</th>
<th>Cell Density</th>
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<th>1:10</th>
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<tr>
<td>1</td>
<td>3,762</td>
<td>2,873</td>
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<td>2</td>
<td>446</td>
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</tr>
<tr>
<td>3</td>
<td>3,765</td>
<td>26,130</td>
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Values are cpm. Cells were plated in 100-mm plates at 10⁷ cells/100-mm plate (1:1) and at 10⁶ cells/100-mm plate, a 1:10 dilution. A density of 10⁷ cells/100-mm plate is roughly equivalent to 3 X 10⁵ cells/well in the 24-well cluster dish format. Cell lysates were then assayed for activity in transferring a phosphate group from ³²P-ATP to a synthetic peptide from myelin basic protein. Indicated are trichloroacetic acid-precipitable cpm after subtraction of background and normalization of the cpms from the low-density cell lysates to account for the lower protein concentration of the lysates.