Retinal Pigment Epithelium-Stromal Interactions Modulate Hyaluronic Acid Deposition

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Hyaluronic acid (HA) is a major component of the extracellular matrix (ECM) and is particularly prominent in various structures of the eye. Choroidal mesenchymal fibroblasts (CHM) and fetal retinal pigment epithelial (fRPE) cells were cultured individually and in cocultivation, as a paradigm for ocular stromal-epithelial interactions. Such interactions are thought to be a key mechanism for the modulation of the ECM and of HA deposition in the region of Bruch's membrane and related structures. In cocultivation, increased levels of HA production were observed, more than the sum of the two cell types grown individually. Conditioned medium from fRPE cultures placed over CHM cells was able to enhance production in such cells several-fold, demonstrating that cell-cell contact was not needed for this enhanced production. On the other hand, when conditioned medium from CHM fibroblasts was added to the fetal RPE cells, no increase in HA production was observed. A soluble HA-stimulating factor apparently released by fRPE cells in a paracrine manner enhanced HA production in CHM cells. The fRPE cell-conditioned media was unable to exert this effect on the fRPE cells themselves. The fRPE cells may lack the appropriate receptor. Alternatively, they may not have the biosynthetic machinery for augmented HA production.

Hyaluronic acid (HA), a high molecular weight glycosaminoglycan (GAG), is a component of the extracellular matrix (ECM). It contains alternating units of glucuronic acid and N-acetylglucosamine. As a highly charged anion, HA takes on a large volume of water of hydration. It plays an important role in fetal growth and development, in wound healing, and in tumorigenesis. In all of these basic biological processes, the presence of HA opens up tissue spaces and creates an environment that promotes cell motility.

Hyaluronic acid is found at many sites in the eye, particularly in the vitreous. Kaneko demonstrated that HA makes up 21.9% of retinal pigment epithelium and 8% of the interphotoreceptor matrix. However, it is absent in the neuroretina. Despite the obvious importance of HA in the eye, the sites of HA production and its metabolic fate are unclear. We are attempting to elucidate the mechanism of HA production in the eye and to understand the reactions that modulate that process. We postulate that stromal-epithelial interactions play a major role, as has been shown in cocultivation of tumor epithelial cells and stromal fibroblasts. Toward this end, cultured human choroidal mesenchymal (CHM) fibroblasts and fetal retinal pigment epithelial (RPE) cells were examined separately and in cocultivation as a paradigm for ocular stromal-epithelial interactions.

We demonstrated that cocultivation of these two cell types dramatically increased levels of HA production. As expected, the stromal CHM cells were the main source of HA secretion. However, conditioned medium alone from fRPE cells was able to stimulate production of HA several-fold in CHM cells. The fRPE cells apparently secreted an HA-stimulating factor (HASA) into the culture medium. Thus, cell-cell contact was not needed for this HA-enhanced production.

Materials and Methods

Materials

Bacterial protease (pronase type XIV) was obtained from Sigma Chemical (St. Louis, MO), the specific hyaluronidase from the mold Streptomyces hyalurolyticus was obtained from Calbiochem (San Diego, CA), and the [3H]glucosamine (44.8 Ci/mmol) was obtained from New England Nuclear Corporation (Wilmington, DE). Umbilical cord HA was obtained from ICN ImmunoBiologicals (Lisle, IL). Basic fibro-
blast growth factor (bFGF) was isolated as described by Gospodarowicz et al.6

Cell Culture

Four- to five-month-old human fetal eyes were obtained after therapeutic abortion and stored refrigerated in McCarey-Kaufman medium. Dissections were performed within 12 hr in a laminar flow hood. The exteriors of the eyes were rinsed with Neosporin (Burroughs Wellcome, Research Triangle Park, NC) and an equatorial cut was made. The vitreous was removed and the retina was excised. Tissue containing RPE cells were dissected free from choroidal mesenchymal cell contamination using a Zeiss (Oberkochen, Germany) operating microscope. Bruch's membrane at this stage (4–5 mo gestation) separates RPE cells from choroidal fibroblasts. Careful dissection using the dissecting microscope made it possible to remove the RPE layer in small fragments (2–3 mm²) free from any choroidal fibroblasts. These cells were placed on the ECM-coated dishes. These cells rapidly formed colonies, and after 1–2 wk they were passaged into larger culture dishes. At confluence, these cells formed closely opposed, hexagonal monolayers that very much resembled their in vivo counterparts. They began to regain pigmentation within 2 wk after they reached confluency. At no time did they lose their monolayer configuration. No contaminating fibroblasts were observed. The RPE tissues were transferred to 35 mm ECM-coated7 plates containing M199 Earle's basic salt solution supplemented with 15% fetal calf serum (FCS). Choroidal mesenchymal fibroblasts also were harvested and placed in separate culture. This preformed ECM was derived from bovine corneal endothelial cells. Such cells were grown on the tissue culture plates for 1 wk. Cells then were removed with ammonium hydroxide, leaving a secreted layer of basement membrane-containing ECM on the plate. This preformed matrix then was rinsed five times with phosphate buffered saline before the RPE cells were fed.

After primary outgrowth of RPE cells was observed, the medium was changed and 1 ng/ml bFGF was added every other day. When a sufficient number of cells had migrated from the tissue, they were passaged. The cells were dissociated from the dish by STV solution (0.05% trypsin, 0.02% EDTA in 0.9% NaCl, pH 7.4), and the enzyme activity was neutralized with serum-supplemented medium. Cell number was determined in a Coulter counter, and the cells were seeded on new extracellular matrix-coated plates for further propagation.

Subsequent cultures of RPE cells were maintained in DME-H16 (Dulbecco's modified Eagle's medium) supplemented with 15% FCS, 300 μg/ml glutamine, 50 μg/ml gentamicin, 100 U/ml penicillin-streptomycin, and 2.5 μg/ml fungizone. Every other day, 1 ng/ml bFGF was added to the culture. Confluent plates of RPE cells were passaged at a split ratio of 1:64 for experimental studies or frozen in liquid nitrogen for future stock. CHM cell cultures were maintained in DME-H16 with 10% FCS.

The conditioned medium was collected and centrifuged in Eppendorf microcentrifuge tubes (Fisher Scientific, Santa Clara, CA) at 12,000 rpm to remove floating cells and debris. This medium, used for analyzing HA and HASA levels, was frozen and stored at −20°C.

Measurement of HA Synthesis in Cultured Cells

The human CHM were seeded routinely in 3 ml DME-H16 with 10% FCS, at a density of 2 × 10³ cells in wells of 35 mm diameter (Costar, Cambridge, MA). Cells were 50% confluent at that time. Cells normally achieved 100% confluency after 48 hr in culture. The RPE cells were seeded in 3 ml DME-H16 with 15% FCS and 1 ng/ml FGF, at a density of 2 × 10⁵ in 35 mm wells. Cells were incubated for 5 days, at which time they had reached 100% confluency. The cultures were replenished with 1.5 ml of fresh medium consisting of DME-H21 Special (glucose-free; Gibco, Grand Island, NY), 0.1% glucose, 1% penicillin-streptomycin, 1% glutamine, 1% ascorbate, and 15% FCS. After 20 min, [1,6-³H]glucosamine was added at a final concentration of 50 μCi/ml. After a 48 hr labeling period, the labeled medium was removed and the cell layer was washed with calcium- and magnesium-free PBS. The medium was pooled with this 1 ml wash and stored in a −20°C freezer until it was used to determine levels of HA. After a 24 hr exposure to 1 ml of 4 mol/l guanidium chloride in 50 mmol/l Tris HCl (pH 7.5) at 4°C, cell layers were collected for further analysis.

Cell Counts

Duplicate cultures for cell counts were seeded at the same time and grown under conditions identical to their radiolabeled counterparts. Cells were incubated in a solution of 0.05% trypsin and 0.02% EDTA for 1 min to dissociate them from the wells. After dilution, triplicate wells were counted using a Coulter counter.

Determination of HA

HA levels were measured as described by Huey et al.6 Briefly, to release free glycosaminoglycans from the samples, proteins, including proteoglycan core proteins, were digested with pronase XIV at 37°C for
2 hr. A second digest with or without hyaluronidase at 37°C for 2 hr then was performed. Both digestions were terminated by heating samples at 90°C for 10 min. Samples were precipitated with 7.5% cetylpyridinium chloride (CPC) in the presence of carrier HA and chondroitin sulfate (type A) on GF/A glass fiber filters (Whatman International, Maidstone, UK) using a 10-well vacuum-assisted manifold suction apparatus (Hoefer Scientific, San Francisco, CA). The filters were placed into scintillation vials, dried in a 55°C oven, and soaked for 24 hr in Optifluor (Packard, Downers Grove, IL). Levels of radioactive label were determined in a Beckman (San Ramon, CA) LS 7500 counter. HA levels were derived from the difference in radiolabeling of triplicate samples digested in the presence and absence of hyaluronidase.

**Statistical Analysis**

Data are expressed as mean values ± standard deviations for three samples at each experimental point.

**Results**

Cultured CHM fibroblasts and fRPE cells were used in this model to study ocular stromal-epithelial interactions. These two cell types were examined individually and in cocultivation. Measurements of cell-layer and media compartments were made. The CHM cells were the major producers of HA (Fig. 1), synthesizing over five times the levels of HA compared to fRPE cells. In each cell line, the major proportion of HA was present in the medium, whereas the cell-layer contained only minor amounts of total HA.

The relative proportion of HA synthesis compared to other GAGs was examined next. HA was the major GAG synthesized in the medium of CHM and fRPE cells, making up 90% and 80% of the totals, respectively. However, in the cell-layer-associated GAGs, entirely different profiles were observed. In stromal CHM cells, the cell-layer contained approximately equal levels of HA and other GAGs, whereas in the fRPE cultures, HA represented only 2% of total GAGs (Fig. 2).

CHM fibroblasts and fRPE cells in cocultivation then were examined. Because the medium contained...
Fig. 4. Levels of hyaluronic acid were determined after exposure of cells to conditioned medium in various combinations: fRPE-CM over CHM (R/R) showed the highest levels of HA in any of the combinations examined. The fRPE-CM induced HA production in the stromal CHM cells, suggesting the presence of a soluble factor underlying the stimulation. C/R, CHM-CM over fRPE. C/C, CHM-CM over CHM. R/C, fRPE over CHM.

The predominant portion of HA, only medium is reported in the subsequent experiments. A greater than threefold increase in HA production was observed compared to the sum of each cell line cultivated individually (Fig. 3). Because only half the number of cells of each cell type were plated in these cocultivation experiments—assuming that the stromal CHM cells remained the predominant source of HA—a sixfold stimulation actually occurred on a per cell basis. As predicted, the interaction between the stromal and epithelial cells produced a marked stimulation in the total HA production.

The levels of HA in the cell layers were routinely studied at the time the media was collected. The deposition of HA in the cell layer was one-tenth to one-twentieth the levels secreted into the culture medium in each of the cell types examined. It was not established whether a longer cocultivation period of RPE cells and CHM fibroblasts would increase the level of pericellular and matrix deposition, but it did appear that most of the HA produced by cells was secreted apically into the culture medium. Of course, it would be intrinsically interesting to determine whether, in long-term cultures of either cell type, enhanced matrix deposition would occur, reflecting basal or bipolar secretion.

Cells were next cultured in the presence and absence of conditioned media in various combinations to determine whether a soluble factor was responsible for this augmentation in HA synthesis. Conditioned media were prepared by exposing confluent cells to fresh or conditioned media for 48 hr. Levels of HA synthesis were obtained as shown in Figure 4. The conditioned media from the fRPE cells stimulated nearly three times the level of HA production in the CHM cells. This suggested that a soluble factor, HASA, with the ability to stimulate production of HA in the CHM culture was being produced by the fRPE cells. Cell-cell contact was not needed for this enhancement to occur.

The preformed ECM was derived from bovine corneal endothelial grown on the tissue culture plates for 1 wk. Cells were removed, leaving a secreted layer of basement membrane material on the plate. This ECM stimulated the growth of the RPE cells. This matrix also enhanced the morphology of the fRPE, giving them a more differentiated or orderly cuboidal "cob-
blestone” appearance (Fig. 5). However, the level of HASA secretion into the culture medium was the same from cells grown on ECM-coated and plastic culture plates, indicating that ECM did not modulate production of the factor.

Discussion

Hyaluronic acid is a major component of the vitreous. It also is prominent in the interphotoreceptor matrix and RPE. Berman was the first investigator to demonstrate GAG production in bovine RPE cells. Edwards reported the secretion of GAGs, including HA, by cultured human RPE cells. Recently, Azuma et al presented histochemical evidence that Müller cells also produce HA. This biosynthetic capacity, however, is transient and occurs in the fetus only from 12 wk gestation up to the neonatal stage. Although not in a human system, Threlkeld et al indicated that retinal glial cells (Müller cells) derived from developing chick retina produce HA in culture.

The production and composition of GAGs is altered in a variety of disorders that involve the posterior portion of the eye. Prominent among these are retinitis pigmentosa. A number of degenerative retinal diseases also have been described in animal models. Despite the obvious importance of HA in the normal eye and its perturbation in a variety of disease processes, the physiology of HA production and deposition still are not understood. Studies such as those described here are an attempt to define such mechanisms.

To examine the nature of stromal-epithelial interaction in the eye, RPE cells and CHM cells were cultured. Both cell types synthesized HA and secreted it into their media. In agreement with the finding of Stramm, the cell layer retained only small amounts of HA, while the major portion was secreted into the medium. CHM cells produced far greater quantities of HA than did epithelial cells, suggesting that stromal cells may be a chief source of HA in the eye. By cocultivating tumor epithelial cells and stromal fibroblasts, Knudson et al observed a marked stimulation of HA production. In the present experiments, cocultivation also stimulated levels of HA production, underlining the importance of interactions between different cell types in the modulation of HA. The stimulation of HA deposition in the present report did not require cell-cell contact, unlike that described by Knudson. Because the highest levels of HA were obtained by adding conditioned medium from RPE cells to CHM cells, we propose that RPE cells produced a soluble factor that stimulated HA production in stromal cells. This factor appeared to work in a paracrine fashion, because the factor was not able to stimulate HA production in the RPE cells themselves. The RPE cells may not have the necessary receptor or the requisite biosynthetic machinery to respond to this factor. The factor may be analogous to HASA, described previously in this laboratory. Experiments are underway to elucidate this further.

Bruch’s membranes are developed early in fetal development and are well established at 4–5 mo gestation. In fact, the entire eye is well formed by this stage of development. The RPE cells from adult donors also have been studied for their ability to induce HA formation in CHM fibroblasts, and similar effects were observed in the production of HASA activity. Therefore, it is conceivable that, at least in cells from young adults, stromal-epithelial interactions in culture reflect the in situ situation. In the older age group, however, it is not clear whether the phenomenon remains unchanged. Additional experiments are needed, using cells from older individuals, to establish this phenomenon unequivocally.

Key words: choroidal fibroblasts, extracellular matrix, hyaluronic acid, retinal pigment epithelium, stromal-epithelial interactions

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References


