Glycosaminoglycan synthesis and secretion by the retinal pigment epithelium: polarized delivery of hyaluronan from the apical surface

Preenie deS Senanayake¹, Anthony Calabro², Kazutoshi Nishiyama¹, Jane G. Hu², Dean Bok³ and Joe G. Hollyfield¹,*

¹Cole Eye Institute and ²Biomedical Engineering, The Cleveland Clinic Foundation, Cleveland, OH 44195, USA
³Jules Stein Eye Institute, Brain Research Institute and Department of Neurobiology, University of California Los Angeles, Los Angeles, CA 90095, USA

*Author for correspondence (e-mail: hollyfj@ccf.org)

Accepted 30 October; published on WWW 11 December 2000
Journal of Cell Science 114, 199-205 © The Company of Biologists Ltd

SUMMARY

Hyaluronan and chondroitin sulfate glycosaminoglycan secretion from retinal pigment epithelial cells was established in confluent cultures with high transepithelial resistance. Cell cultures were maintained on Millicell-PCF culture plates, which allow separation of culture medium exposed to apical and basal epithelial surfaces. Following various times in culture, apical and basal culture media were sampled at three day intervals and the glycosaminoglycan content was quantified. Samples were digested with proteinase K to free the glycosaminoglycans from their core proteins, the glycosaminoglycans were ethanol precipitated, and subjected to hyaluronidase SD and chondroitinase ABC digestion to release hyaluronan and chondroitin sulfate disaccharides. Disaccharides were fluorotagged with 2-aminoacridone, separated on polyacrylamide gels and the molar fluorescence in each disaccharide band quantitated. Hyaluronan in the apical medium was significantly higher than in the basal medium (5-12 times) at all recovery intervals (P<0.0001). In contrast, the distribution of unsulfated chondroitin, 4-sulfated chondroitin and 6-sulfated chondroitin disaccharides in apical and basal media was non-polar. Confocal microscopy of cultures probed with a hyaluronan-specific fluorotag established that the HA evident in these cultures is restricted to the apical border of the RPE cultures. Collectively, these data indicate that hyaluronan synthesized by the retinal pigment epithelium is secreted preferentially from the apical surface, suggesting that this tissue is an important source of hyaluronan present in the interphotoreceptor matrix.

Key words: Retinal pigment epithelium, Hyaluronan, Chondroitin sulfate, Polarity

INTRODUCTION

The retinal pigment epithelium (RPE) is a neuroepithelium derived from the outer wall of the optic cup during early organogenesis. In the adult eye, the RPE is positioned between the photoreceptor layer of the neurosensory retina and the choriocapillaris of the choroid. The apical and basolateral surfaces of the RPE are in contact with distinctly different extracellular matrices. The apical surface interacts with the interphotoreceptor matrix (IPM) and the distal tips of the photoreceptor outer segments, whereas the basal surface abuts Bruch’s membrane, a pentalaminate matrix separating the RPE from the capillary endothelial cells of the choroid.

Proximal to the apical surface, the lateral borders of the RPE cells are linked to each other via complex junctional attachment zones (Hudspeth and Yee, 1973). Tight junctions (zonula occludens) that are a part of this complex function to prevent the passive diffusion of molecules between the apical and basal compartments separated by the RPE. Since the capillary endothelial cells below Bruch’s membrane are fenestrated, macromolecules of the serum bathe the matrix below the basolateral borders of the RPE. The barrier function of the RPE prevents the passive movement of extracellular molecules between basal (choroidal connective tissue stroma) and apical (IPM) compartments (Cunha-Vaz et al., 1966; Shiose, 1970), which in turn allows the transport machinery of the RPE to control the volume and composition of fluid in the IPM (Hughes et al., 1998).

A number of studies document the synthesis by cultured RPE cells of extracellular matrix molecules; including laminin, fibronectin, integrin, heparin sulfate proteoglycan, type IV collagen, tissue inhibitor of matrix metalloproteinase-3 (TIMP-3), pigment epithelial derived factor (PEDF) and hyaluronan (HA) (Edwards, 1982; Philip and Nachmius, 1987; Stramm, 1987; Newsome et al., 1988; Campochiaro et al., 1989; Ruiz et al., 1996). Recent analysis of matrix molecules present on the apical side of the RPE (within the IPM) identify several molecules, including HA, matrix metalloproteinases (MMPs), interphotoreceptor matrix retinoid binding protein (IRBP), PEDF, and the novel IPM molecules SPACR and SPACRCAN (also called IPM150 and IPM200) (Acharya et al., 1998; Acharya et al., 2000). The finding that some of these molecules bind HA, led us to propose that HA may function as the primary scaffold of the insoluble IPM (Hollyfield, 1999).
Although a number of previous studies indicate that RPE cells in culture synthesize HA (Edwards, 1982; Hewitt and Newsome, 1987; Straam, 1987), the polarity of HA secretion has not been addressed. Because of the potential role of HA in the organization of the IPM, it is critical to define the origin of HA in this compartment and to understand the dynamics of its synthesis and turnover. In the present study we establish the distribution of HA and chondroitin GAGs in apical and basal medium recovered from confluent RPE cultures that have established high resistance junctions.

MATERIALS AND METHODS

Reagents

2-aminoacridone HCl (AMAC) was from Molecular Probes (Eugene, Oregon USA). D-Glucose, D-mannose, mercuric acetate, glacial acetic acid (99.99+%), dimethylsulfoxide (DMSO, 99.9%), sodium cyanoborohydride (95%), and glycerol (99.5%) were from Aldrich-Sigma (PO Box 355, Milwaukee, WI 53201, USA). Proteinase K and phenol red (0.5% w/v) were from Gibco (Grand Island, NY 14072, USA). MONOTM composition gels (#60100) and MONOTM gel running buffer (#70100) were from Glyko Inc (81 Digital Drive Novato, California 94949, USA). Dowex AG50W-X8 (200-400 mesh) Bio-Rad Laboratories (2000 Alfred Nobel Drive, Hercules, CA 94547, USA). Hyaluronidase SD, chondroitinase ABC, and unsaturated hyaluronan, the chondroitin sulfate disaccharide standards and the biotin-conjugated HA binding protein (bHABP) were from Seikagaku, America (Ijamsville, MD 21754, USA). High purity hyaluronan (Healon®, the trade name for highly purified HA, Pharmacia, Maersk Medical Ltd, UK).

Preparation of disaccharide standards for fluorescent derivatization

The AMAC derivatized HA and chondroitin disaccharide standards (with and without mercuric acetate treatment) were prepared as previously described (Calabro et al., 2000b). For quantitation, AMAC derivatized ADi2S standards were calibrated with AMAC derivatized glucose standards as previously described (Calabro et al., 2000a).

RPE cell cultures

The use of all human tissues followed the tenets of the Declaration of Helsinki, and the donors or their guardians gave consent for donation of the tissues. Institutional human experimentation committee approval was obtained for the use of human tissues. The human RPE culture conditions have been described previously (Frambach et al., 1990; Hu et al., 1994) and are summarized as follows: human RPE cell cultures have been described in detail (Calabro et al., 2000a; Calabro et al., 2000b). The basis of this analysis is a novel fluorophore-assisted carbohydrate electrophoresis (FACE) procedure for the qualitative and quantitative analysis of glycosaminoglycans. The method uses enzymes that cleave glycosaminoglycans to create products, primarily mono-and disaccharides, characteristic of the enzyme specificity. Each cleavage exposes a new reducing terminus on the carbohydrate that is fluorotagged by reductive animation with 2-aminoacridone. The tagged products are then displayed by electrophoresis, identified by their characteristic migration, and quantitated by their molar fluorescence.

Culture medium and cell matrix preparation

The methods used for sample preparation and carbohydrate analysis have been described in detail (Calabro et al., 2000a; Calabro et al., 2000b). The culture medium from apical and basal compartments were recovered on days 14, 17, 56, 59, 84, and 87 days of culture for carbohydrate analysis.

Microscopy

Cell density measurements were performed on representative cultures as previously described (Bok et al., 1992). Briefly, cultures on polycarbonate supports were fixed with 4% formaldehyde in phosphate buffer, dehydrated, mounted with glass coverslips and viewed with a ×20 objective and ×10 ocular lenses. One of the oculars was fitted with a calibrated micrometer square, and the cells within the area counted. Counts from 10 fields in four separate cultures were determined and the mean values were expressed as the cell density extrapolated to the total area occupied by the RPE on the Millicell plate.

The cultured RPE cells, along with the polycarbonate membranes, were cut out from their chambers and fixed for 24 hours in 1% formaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C and then in 1% OsO4 in the same buffer for 30 minutes. The monolayers and their supports were sequentially dehydrated in 70-100% ethanol, treated with propylene oxide and embedded in Araldite 502 (Ted Pella Inc., Redding, CA). Ultrathin sections were cut with a diamond knife, stained with uranium and lead salts and imaged in a Zeiss 910 electron microscope.

For confocal microscopy, RPE cultures with high resistance junctions were fixed in chilled methanol for ten minutes following a brief rinse of the RPE/filter complex in 1× PBS. RPE cells were blocked in 5% (w/v) bovine serum albumin. Filters were then incubated with biotin-conjugated HA binding protein overnight at 4°C. This incubation was followed by sequential incubation with FITC-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature. The immunostained filters were mounted on glass slides using an antifading medium (Vector, Burlington, CA) containing DAPI, a dye specific for nuclei. The cultures were observed using a TCS-SP/Leica confocal microscope (Heidelberg, Germany). The optimal dilutions for the bHABP and secondary antibody were 1:100 and 1:200, respectively. To control for probe specificity and eliminate possible autofluorescence in RPE cells, a neighboring portion of the filter was stained identically except that the bHABP was omitted.
containing the low molecular mass molecules such as glucose and salts were aspirated to waste. The pellet was washed with 1 ml of −20°C absolute ethanol and centrifuged at 10,000 g for 15 minutes at 4°C. The supernatant solution was aspirated to waste, and the precipitate dried for 5 minutes on a vacuum concentrator. The lyophilized precipitate was re-suspended in 100 μl, 0.0005% phenol red, 100 nM ammonium acetate, pH 7 (Calabro et al., 2000a; Calabro et al., 2000b).

GAG digestions
The ammonium acetate extracts were digested for 1 hour at 37°C with 100 mU/ml of hyaluronidase SD followed by 3 hours at 37°C with the addition of 100 mU/ml of chondroitinase ABC. The enzyme digest were dried on a vacuum concentrator prior to derivatization with AMAC as described below. Representative samples were mercury treated to distinguish internal disaccharides with their unsaturated hexuronic acid from non-reducing terminal disaccharides with saturated hexuronic acid residues or hexosamines (Calabro et al., 2000a).

Fluorotagging procedures
Hyaluronidase SD and chondroitinase ABC digestion products were derivatized by addition of 20 μl of 12.5 mM AMAC (500 nmoles) in 85% DMSO/15% acetic acid followed by incubation for 15 minutes at room temperature. Then 20 μl of 1.25 M sodium cyanoborohydride (50,000 nmoles) in ultrapure water was added followed by incubation for 16 hours at 37°C. After derivatization, 10 μl of glycerol (20% final concentration) was added to each sample prior to electrophoresis. All derivatized samples were stored in the dark at −70°C (Calabro et al., 2000a).

Electrophoresis, imaging and quantitation
The derivatized unknown samples to be run in separate lanes were spiked with one AMAC-derivatized ΔDi2S standard containing 62.5, 31.3, 15.6, 7.8, 3.9, or 1.9 pmoles per 5 μl as an internal standard. Samples were run on MONO™ composition gels with MONO™ gel buffer. The samples were electrophoresed at 4°C for 80 minutes at a constant 500 V with a starting current of 25 mA /gel, and a final current of 10 mA /gel. The gels in their glass supports were illuminated with UV light (365 nm) from an Ultra Lum Transilluminator, and imaged with a Quantix cooled CCD camera (Roper Scientific/Photometrics). The images were analyzed using the Gel-Pro Analyzer™ program version 3.0 (Media Cybernetics). The digital images shown in the results section depict oversaturated pixel intensity for the major derivatized structures in order to allow visualization of less abundant derivatized carbohydrates. Quantitation was performed on gel images having all pixels within a linear 12-bit intensity depth range. The pixel density of the ΔDi2S standard concentrations listed above were 2.86, 1.31, 0.8, 0.45, 0.36, and 0.31, respectively. The efficiency of recovery of HA, 0S, 4S and 6S glycosaminoglycans using this protocol was 68%, 74%, 72% and 70%, respectively.

RESULTS
The RPE cultures
A diagram of the chamber used for culturing RPE is presented in Fig. 1A. As is evident in this illustration, RPE cultures established on the porous support of the inner chamber allows isolation of the two medium compartments, with one exposed only to the apical surface of the RPE (the inner compartment) and the other exposed to the basaolateral surface via the open channels in the supporting membrane. The key factor in separation of the apical and basal culture media requires cell-cell interaction with the establishment of high resistance junctions. Chambers containing no cells or RPE cells prior to
reaching confluence have no resistance to current passage between electrodes placed in the inner and outer chambers. The resistance of RPE cultures used in these studies ranged between 800-1500 ohms/cm², indicating the physical separation by the RPE of medium on apical and basal borders.

The RPE cells at confluence become heavily melanized (Fig. 1B) and when viewed with low magnification microscopy, showed a cobblestone appearance with most cells forming a hexagonal border (Fig. 1C). Cell counts made from representative cultures reflect an average density of 395,000 ± 28,800 RPE cells/mm² (mean ± s.d.). When examined with transmission electron microscopy, the RPE exists as a single, low cuboidal layer, with apical microvillae and prominent melanin granules in the apical cytoplasm (Fig. 1D). The cultures illustrated were prepared on identical Millicell supports used for the culture medium assays. Below the basal plasma membrane, a basal lamina usually separates the RPE from the supporting membrane. Pores in the supporting membrane, which communicate with the basal compartment, are evident in longitudinal profile (Fig. 1D).

Carbohydrate analysis
Electrophoretic patterns of AMAC-labeled carbohydrates from apical and basal media preparations are presented in Fig. 2A. The patterns of carbohydrates that accumulated in the medium over the 3 day period between culture medium changes can be compared directly to the authentic standards present in the lane labeled MW. Note the prominent hyaluronan disaccharide band (ΔDiHA) in the apical medium lanes (A1, A2 and A3) relative to the density of the ΔDiHA band in the basal medium (B1, B2 and B3). In contrast, the unsulfated chondroitin disaccharide bands (ΔDi0S) show similar density in apical and basal media samples. The single prominent broad band midway between the ΔDi4S and ΔDi6S standards, represents these two chondroitin disaccharides, which do not separate well in these preparations. The composition of the unresolved broad band was determined to consist of ΔDi4S and ΔDi6S by mercuric acetate treatment of representative apical and basal RPE culture media as previously described (Calabro et al., 2000a). The most prominent bands in these preparations represent glucose remaining in the culture medium. Usually, the basal medium contained more remaining glucose than the apical medium.

The carbohydrate composition of cells from representative RPE cultures was also analyzed. As is evident in Fig. 2B, no ΔDiHA or ΔDi0S is evident in the cellular material. In contrast, some ΔDi4S and ΔDi6S is evident above control levels. Three unknown bands of variable intensity (x1, x2 and x3) are also present. Band x1 was novel to the cellular carbohydrates and was not seen in the medium samples (compare with Fig. 2A).

The first quantitative assessment of the medium samples was performed on a single culture maintained for 99 days. The medium from apical and basal compartments recovered at three day intervals from day 54 through day 99 of this culture period was processed and the ΔDiHA, ΔDi0S and combined ΔDi4S and ΔDi6S disaccharides quantified (Fig. 3). The amount of ΔDiHA in the apical medium is consistently 5 to 10 times higher than the amount present in the basal medium. In contrast, the amount of ΔDi0S is similar in apical and basal medium throughout the period of analysis, while ΔDi4S-ΔDi6S levels are slightly more abundant in the apical medium.

![Fig. 2. Fluorograms showing the separation of saccharides recovered from the culture media (A) and RPE cultures/matrices (B). The images shown depict oversaturated pixel intensity for the major derivatized components in order to allow visualization of less abundant derivatized components. The lane labeled MW shows the separation of a standard mixture of 12 AMAC derivatized saccharides that gives a wide separation of well-resolved bands. (A) Saccharides present in apical (A1-A3) and basal (B1-B3) medium from three separate cultures (1-3). Control lane shows a derivatized medium sample without cell cultures for background corrections. ΔDi2S standards at progressive dilutions from 62.5-1.9 pmol (as determined by hexuronic acid analysis) were added to the second (labeled A1) through the seventh lane (labeled B3) to generate an internal concentration curve in each gel (R=0.999, P=0.0001) for quantification of resolved disaccharides. (B) Saccharides present in RPE cultures-matrices (1-3) and a control polycarbonate filter with medium only maintained for the same period of time as the RPE cultures.](image-url)
Hyaluronan synthesis by the RPE

To provide quantitative data on additional cultures that would allow more definitive statistical comparison, the carbohydrate profiles from seventeen separate RPE cultures were analyzed (Fig. 4A). Data from the two cultures designated A and B were from medium samples collected at three day intervals over 100 days of culture. Those in cultures designated C-K were individual medium samples from single collection times. Although the amounts of carbohydrates vary between the profiles shown, Di4S-Di6S are most abundant, followed by Di0S, and then DiHA. The amount of DiHA in apical medium is 4 to 6 times higher than that present in the basal medium. Although the magnitude of the difference varied from culture to culture, in each of these comparisons, the differences in HA concentration was highly significant (P<0.0001). The levels of Di4S-Di6S and Di0S in the apical medium as compared to the basal concentrations were not statistically significant.

The differences in relative amounts of the Δ-disaccharides in apical and basal medium are more evident when the data is expressed as an apical/basal concentration ratio (Fig. 4B). The data used in this figure are the same as was plotted by concentration in Fig. 4A. Equal concentration in apical and basal medium would reflect a ratio of 1, which is near the level of Di4S-Di6S and Di0S ratios. In contrast, the ΔDiHA ratios range from 5 to 12, indicating 5 to 12 times the amount of HA disaccharides in the medium exposed to the apical surface of the RPE cultures as compared to that present in the basal compartment.

Hyaluronan distribution

The use of the biotinylated HA-binding fragment derived from aggrecan, in conjunction with confocal microscopy, allows the direct visualization of HA associated with the RPE cells (Fig. 5). When viewed en face, green fluorescence, from the streptavidin-FITC that binds to bHABP, was readily evident on the cultures, but the staining was not uniformly distributed (Fig. 5A). In some regions the whole of the epithelial surface appeared labeled in either a uniform or a punctate pattern (Fig. 5B). In other areas, FITC fluorescence was associated with bright strands or cables of HA. Some areas of the cultures were free of FITC fluorescence, with only the DAPI-stained nuclei evident. In viewing the en face images, it was evident that much of the FITC-fluorescence was above the DAPI-labeled nuclei. When Z-axis images were generated from the cultures, all FITC-fluorescence was found at the level of the apical surface of the RPE (Fig. 5C). No areas were observed with FITC-fluorescence below the DAPI-labeled nuclei.
DISCUSSION

The data reported in this study indicate that HA secreted by the RPE in confluent cultures with high resistance junctions is highly polarized, with 82-95% of the HA delivered into the apical medium. To our knowledge this is the first demonstration in any epithelium of polarized HA secretion.

HA is unique among GAGs in that it is synthesized at the cell surface as a free GAG (Prehm, 1984; Weigel et al., 1997). In contrast, all other GAGs are covalently linked to a core protein, and their biosynthetic pathway involves the Golgi apparatus, followed by vesicular transport to the cell surface for delivery into the extracellular compartment (Hascall and Hascall, 1981). Hyaluronan synthase (HAS), the enzyme responsible for polymerization of HA, is located in the plasma membrane. As UDP-precursor sugars (glucuronic acid and N-acetylglucosamine) are brought to the cytoplasmic side of the enzyme, the assembled HA polymer is extruded to the exterior of the cell. HAS has been cloned in human and mouse. In both species three distinct genes are present (HAS1, HAS2 and HAS3), which are located at three different positions in the genome (chromosomes 19q13.3, 8q24.12, and 16q22.1, respectively, for human; and chromosomes 17, 15 and 8, respectively, for mouse) (Spicer et al., 1997). The finding that the bulk of the HA synthesized by the RPE is present in the apical medium suggests that most of the hyaluronan synthase in these cells resides in the apical plasma membrane.

A single molecule of HA can have a molecular mass approaching 10 million Daltons, which would contain 25,000 repeat disaccharides, supporting an extended length of around 25 μm (Hascall and Hascall, 1981). One might argue that because of its large size, it may be difficult for HA secreted from the basolateral RPE surface to move through the pores in the Millicell plate and have access to the basal medium. Evidence against this possibility comes from the finding that chondroitin sulfate GAGs show near equal distribution in apical and basal culture medium. It is also unlikely that HA could be trapped below the RPE or be restricted from entering the basal medium if secreted from the basolateral borders of the RPE cells. Since the GAG content of the RPE cells and adherent matrix was extracted directly from the filter, any HA present in or on the filter should have been detected, but this analysis indicates that no detectable HA is associated with the filter (see Fig. 2B). The final evidence excluding the possibility that HA is restricted from moving through the filter comes from the observation that HA was always present in the basal medium, albeit in small amounts (Fig. 3). We conclude that the distribution of HA observed in apical and basal medium reflects the amounts of HA secreted from apical and basolateral borders of the RPE during the period between culture medium changes.

bHABP was useful in independently defining the distribution of HA in the RPE cultures. After application of bHABP, all streptavidin-FITC fluorescence was associated with the apical surface of the RPE (Fig. 5). Unexpected was the variation in distribution of HA in different regions of the cultures. The absence of HA fluorescence in some areas could be due to retraction of the HA in response to the methanol fixation or in regional loss of HA by the brief PBS rinse prior to fixation. The punctate areas of HA fluorescence present in many areas on the apical surface of the RPE were particularly intriguing (Fig. 5A-B). It is possible that these may represent short segments of HA that remain associated with single or small clusters of HAS at the apical surface of the epithelium. Additional studies using HAS antibodies and bHABP are currently underway to evaluate this possibility.

In the eye, HA delivered from the RPE apical surface would become a part of the IPM. Early biochemical studies demonstrate that small amounts of HA can be rinsed from the IPM (Berman, 1964; Berman and Bach, 1968; Berman, 1969; Adler and Klucznik, 1982), suggesting that HA may be only a minor component of this matrix. More recently we have experimentally demonstrated that the glycoprotein SPACR and the proteoglycan SPACRCAN bind HA (Acharya et al., 1999; Acharya et al., 2000), which lead us to propose that HA may function in the IPM as the basic scaffold to which these and other IPM molecules are anchored. It is likely that this binding to HA is mediated through RHAMM-type HA-binding motifs present in the primary sequence of the SPACR and SPACRCAN polypeptide (Hollyfield, 1999). Thus the presence of HA in the IPM and the associations made by HA with other
molecules present in and around this matrix may be of fundamental importance to the stability of macromolecules comprising the insoluble IPM as well as the attachment of the retina to the RPE.

Although our analysis clearly identifies the RPE as a potential source for HA in the IPM, it is possible that the retina may also contribute HA to this compartment. Recent studies on isolated retinal cells indicate that HA is synthesized by the radial glial cells of the retina (Müller cells), but not by retinal neurons (Normand et al., 1998). Müller cells traverse the full thickness of the retina with their apical border abutting the IPM and their basal end feet terminating at the vitreal surface (Hogan et al., 1971). Additional studies of the polarity of retinal HA secretion will be required to define the destination of HA synthesized by Müller cells.

In summary, FACE analysis of HA and chondroitin sulfate GAGs delivered into apical and basal culture medium by the RPE was established. HA was 5-12 times more abundant in the medium exposed to the apical surface of the epithelium than in the medium exposed to the basolateral border. In contrast, the amount of chondroitin sulfate was similar in both compartments. These data indicate that hyaluronan synthesized by the RPE is secreted preferentially from the apical surface.

Supported by grants from The Foundation Fighting Blindness (D.B. and J.G.H.), Hunt Valley, Maryland; Retina Research Foundation (J.G.H.), Houston, Texas; and the National Institutes of Health (D.B. and J.G.H.), Bethesda, Maryland. D.B. is also supported by the Dolly Green Chair at UCLA.

REFERENCES


Hyaluronan synthesis by the RPE 205