Synthesis of glycosaminoglycans by cultures of normal human corneal endothelial and stromal cells

Beatrice Y. J. T. Yue, Jules L. Baum, and Jeremiah E. Silbert

Monolayer cultures of normal human corneal endothelial and stromal cells were incubated with [35S]sulfate and [3H]glucosamine for 4 hr. The labeled glycosaminoglycans resulting from this incubation were isolated from the cell layer and the growth medium and further characterized. Both endothelial and stromal cell cultures synthesized a variety of sulfated glycosaminoglycans, with chondroitin 6-sulfate as the major product. Chondroitin 4-sulfate, derrnan sulfate, and heparan sulfate were present in smaller amounts. Keratan sulfate was produced only in minimal amounts. Both cell types also synthesized hyaluronic acid. The hyaluronic acid production in stromal cell strains derived from donors of different ages was similar. The demonstration that the endothelial cell strain derived from a 1-day-old baby contained more hyaluronic acid than cultures from older donors suggests a possible age-related phenomenon as seen in developing tissues.

Key words: synthesis, sulfated glycosaminoglycans, hyaluronic acid, human, cornea, stroma, endothelium, cell cultures

Glycosaminoglycans of the mammalian cornea are thought to play an important role in regulating its hydration, thickness, and transparency. It has been shown that keratan sulfate normally comprises about 60% of the glycosaminoglycans in cornea, with chondroitin sulfates being the remaining component.1, 2

The cellular metabolism and function of corneal glycosaminoglycans have not been well defined, mainly because of the analytical limitations imposed by the small mass of corneal tissue. The utilization of tissue cultures seems to be a reasonable method to obviate this limitation. Previously, this laboratory3 and several other research groups4–7 have studied the in vitro glycosaminoglycan synthesis by cultures of rabbit and chick corneal cells. Recently, we have established the mass culture of normal human corneal endothelial cells8 and have shown by electron microscopy that these endothelial cells in culture maintain their normal morphology and produce a basal lamina. This human corneal endothelium in culture serves as a better model for human corneal metabolism than do animal corneal cultures. In particular, cultured human corneal endothelial cells provide baseline information toward a better understanding of various corneal diseases primarily involving endothelium.

The present study describes the synthesis of glycosaminoglycans by confluent cultures...
Table I. Incorporation of $[^{35}S]$sulfate into sulfated glycosaminoglycans by cultures of endothelial and stromal cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Age of donors</th>
<th>$[^{35}S]$sulfate incorporated into sulfated glycosaminoglycans (cpm X $10^4$)</th>
<th>Total $[^{35}S]$glycosaminoglycans produced (ng)</th>
<th>Total cell protein (mg)</th>
<th>ng of $[^{35}S]$glycosaminoglycans produced per mg of cell protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelium</td>
<td>1 day</td>
<td>28.8</td>
<td>249.1</td>
<td>3.1</td>
<td>80.4</td>
</tr>
<tr>
<td></td>
<td>5 months</td>
<td>3.9</td>
<td>42.1</td>
<td>0.9</td>
<td>52.6</td>
</tr>
<tr>
<td></td>
<td>17 years</td>
<td>1.7</td>
<td>10.1</td>
<td>0.2</td>
<td>50.5</td>
</tr>
<tr>
<td>Stroma</td>
<td>16 years</td>
<td>17.4</td>
<td>84.2</td>
<td>1.7</td>
<td>49.5</td>
</tr>
<tr>
<td></td>
<td>60 years</td>
<td>27.4</td>
<td>149.8</td>
<td>2.3</td>
<td>65.1</td>
</tr>
</tbody>
</table>

Each flask of corneal cultures was incubated with 3 ml of medium containing $4 \times 10^4$ dpm of sodium $[^{35}S]$sulfate for 4 hr. The amount of $[^{35}S]$glycosaminoglycans produced was calculated from the specific activity of sodium $[^{35}S]$sulfate (713.8 nmol/mCi) by assuming that one sulfate group is present in each disaccharide unit of glycosaminoglycans and that the intracellular pool of sulfate and 3'-phospho-adenosine 5'-phosphate was negligible (since from 1 to 4 hr of labeling period the total radioactivity incorporated increased linearly with the labeling time).

of normal human corneal endothelial cells and identifies those glycosaminoglycans synthesized. For comparison, parallel experiments were also performed with normal human corneal stromal cultures.

Material and methods

Corneas were excised from human eyes at autopsy from donors ranging in age from 1 day to 72 years. Under sterile conditions, Descemet's membrane with the attached endothelium was stripped from the underlying stroma, and the remaining stroma was then separated from epithelium. The isolated Descemet's membrane–endothelium and the stromal layers were each cultured as described elsewhere in 30 ml Falcon flasks (Falcon Plastics, Los Angeles, Calif.) at 35°C in 5% CO2-95% air.

With rare exceptions, the successful growth of human endothelial cells could only be achieved with corneas from young donors. As the age of donors increased to over 20 years, the potential for growth dropped significantly. Thirteen of 19 cultures derived from donors under the age of 20 could be grown to confluency in 8 to 12 weeks. The saturation density was $1.8 \times 10^6$ cells per flask. From older donors, however, only three (ages 26, 45, and 72) of 62 cultures reached confluency. In contrast, stromal cultures from older donors generally grew well. Confluent cultures of these cells could be obtained in 6 to 8 weeks with approximately $1.4 \times 10^6$ cells per flask.

To label glycosaminoglycans, confluent human corneal cultures were exposed to growth medium supplemented with 200 μCi/ml sodium $[^{35}S]$sulfate and 16 μCi/ml $[^1H]$glucosamine (both obtained from New England Nuclear Corp., Boston, Mass.) After incubation for 4 hr, the medium fraction was separated from the cell layer fraction (containing both intracellular and pericellular material), and the newly synthesized glycosaminoglycans were isolated as previously described by chromatography on a column of Sephadex G-50. The radioactivity associated with the labeled macromolecular material was used to calculate the amount of glycosaminoglycans produced per milligram of cell protein. The protein associated with the cell layer of corneal cultures was determined with fluorescamine, with bovine serum albumin used as standard. The specific activity of the $[^{35}S]$sulfate in the incubation medium was calculated directly from the amount of sodium $[^{35}S]$sulfate added to the medium and the amount of inorganic sulfate present.

To characterize the sulfated glycosaminoglycans produced by the human corneal cultures, radioactively labeled products plus appropriate standards were sequentially incubated with chondroitinase AC (Miles Laboratories, Inc., Kankakee, Ill.), chondroitinase ABC (Miles), crude heparinase (prepared from Flavobacterium heparinum$^{[3]}$) and endo-β-galactosidase (a gift from Dr. S. Suzuki) as previously described.$^{[3]}$ Carrier standards used were chondroitin 4- and 6-sulfates (Miles), heparin (Calbiochem, La Jolla, Calif.) and keratan sulfate (a gift from Dr. G. Armand, purified from human nucleus pulposus$^{[1]}$). After each enzyme digestion, the total reaction mixture was boiled for 4 min and chromatographed on a column of Sephadex G-50 with a solution of 0.1M LiCl. Fractions were assayed for radioactivity in liquid scintillation spectrometer (Beckman Instruments, Inc., Palo Alto, Calif.). In order to confirm the activity of the en-
Table II. Relative percent of sulfated glycosaminoglycans produced by cultures of human endothelium and stroma*

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Age of donor</th>
<th>Source</th>
<th>Chondroitin 6-sulfate</th>
<th>Chondroitin 4-sulfate</th>
<th>Dermatan sulfate</th>
<th>Heparan sulfate</th>
<th>Keratan sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelium</td>
<td>1 day</td>
<td>Cell</td>
<td>41</td>
<td>21</td>
<td>36</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1 day</td>
<td>Medium</td>
<td>47</td>
<td>24</td>
<td>20</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>5 months</td>
<td>Cell</td>
<td>39</td>
<td>21</td>
<td>23</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>5 months</td>
<td>Medium</td>
<td>49</td>
<td>27</td>
<td>19</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>17 years</td>
<td>Cell</td>
<td>42</td>
<td>24</td>
<td>21</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>17 years</td>
<td>Medium</td>
<td>46</td>
<td>26</td>
<td>17</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Stroma</td>
<td>16 years</td>
<td>Cell</td>
<td>53</td>
<td>18</td>
<td>10</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>16 years</td>
<td>Medium</td>
<td>56</td>
<td>20</td>
<td>20</td>
<td>3</td>
<td>1</td>
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<td>2</td>
</tr>
<tr>
<td></td>
<td>60 years</td>
<td>Medium</td>
<td>66</td>
<td>16</td>
<td>12</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

*Each value represents the mean of 3 to 5 experiments.

zymes, degradation products of standard glycosaminoglycans were located by the carbazole assay\textsuperscript{12} for determination of uronic acids or with anthrone (Calbiochem) for measurement of hexoses\textsuperscript{13} in the case of keratan sulfate.

The unsaturated disaccharides resulting from chondroitinase ABC treatment were identified as previously described\textsuperscript{14} by chromatographing on Whatman No. 1 paper in butan-1-ol/acetic acid/1.0M ammonium hydroxide (2:3:1, by volume). This method was used to determine the hyaluronic acid content, since in this system, unsaturated hyalobiuronic acid\textsuperscript{*} (a product of hyaluronic acid after chondroitinase ABC digestion) could be separated from the comparable unsaturated disaccharides (ΔDi-4S, ΔDi-6S, and ΔDi-OS) from chondroitin sulfates.

Results

The production of sulfated glycosaminoglycans by confluent monolayer cultures of normal human corneal endothelium and stroma based on the [\textsuperscript{35}S]sulfate incorporation is shown in Table I. Generally more labeled material was present within the cells than in the growth medium. The amount of glycosaminoglycans produced by endothelial and stromal cell cultures based on cell mass (milligrams of cell protein) was similar, independent of the age of donors.

The relative amounts of various sulfated glycosaminoglycans produced by the human corneal cultures are shown in Table II. Each cell strain was analyzed separately. As can be seen, there were no significant variations with cell strains derived from donors of different ages. Chondroitin 6-sulfate was always the principal product; chondroitin 4-sulfate, dermatan sulfate, and heparan sulfate were present in smaller quantities (10\% to 25\%). As in other cell lines,\textsuperscript{15, 16} a higher percentage of the sulfated glycosaminoglycans was present as heparan sulfate in the cell layer than in the growth medium. Keratan sulfate was also identified but was present only in very small amounts (1\% to 3\%). Analyses of chondroitinase ABC degradation products by paper chromatography indicated that less than 1\% of [\textsuperscript{3}H]hexosamine-
labeled disaccharides co-chromatographed with ΔDi-OS. Since the disaccharide is the product from degradation of nonsulfated chondroitin, we can conclude that there was essentially no nonsulfated chondroitin present. The hyaluronic acid content obtained by identifying [3H]unsaturated hyalobiuronic acid is shown in Table III. Hyaluronic acid represented only a minor portion of the total glycosaminoglycans in all fractions of human endothelial and stromal cultures. The percentage of glycosaminoglycans present as hyaluronic acid was similar in the cell layer and in the growth medium.

No significant variations were found in the amount of hyaluronic acid produced by different cell strains derived from donors ranging in age from 6 weeks to 72 years. Interestingly, however, there was more hyaluronic acid present in one of the endothelial cell strains (derived from a 1-day-old donor) than in the other cell strains.

Discussion

The present study demonstrates that confluent monolayer cultures of human corneal endothelial and stromal cells synthesize hyaluronic acid and a variety of sulfated glycosaminoglycans. The pattern of sulfated glycosaminoglycans found in human corneal cultures is analogous to that found in rabbit corneal cultures; both are considerably different from that seen in normal cornea.1, 2

The production of keratan sulfate, the major product of corneal tissue, is minimal, and dermatan sulfate and heparan sulfate are produced in addition to chondroitin 4-sulfate and chondroitin 6-sulfate, which are both normal constituents of cornea. Dermatan sulfate has been detected in cornea during wound healing,17 and heparan sulfate has been shown15, 16, 18 to be produced by many cell lines.

The amount of sulfated glycosaminoglycans produced by normal human corneal endothelial and stromal cultures is comparable. In contrast, rabbit endothelial cells in culture were found to produce much smaller amounts of sulfated glycosaminoglycans than do rabbit stromal cells.3

Human corneal cells in culture synthesize only small amounts of hyaluronic acid, which distributes uniformly in the cell layer and in the growth medium. Rabbit corneal cells, on the other hand, produce hyaluronic acid as a principal product, most of which is in the medium.3

Despite previous reports1, 2, 19 that nonsulfated chondroitin is present in corneal tissue, less than 1% of the [3H]galactosamine-labeled glycosaminoglycans found in corneal cell cultures was unsulfated.

The relative amounts of various sulfated glycosaminoglycans and hyaluronic acid produced by corneal cell strains derived from donors of different ages (>1 day) appear to be similar. It is of note that the endothelial cultures derived from a single newborn baby contained more hyaluronic acid than did cultures derived from older donors. The synthesis of hyaluronic acid has been associated with embryonic or developing tissues.20, 21 It has been shown that in human skin,22 hyaluronic acid content diminished with age during fetal life and during childhood growth. Our experiments suggest that in a tissue culture system the hyaluronic acid production may still be related to an early age or an early stage of development of the cells.

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REFERENCES


