Rabbit corneas cultured in a serum-free, dextran-supplemented medium maintained a high rate of incorporation of $^3$H-glucosamine into glycosaminoglycans (GAG) for several weeks in culture. The labeled GAG secreted by cultured corneas was identified as keratan sulfate, galactosaminoglycan, and heparan sulfate. Corneal GAG labeled in vivo consisted primarily of keratan sulfate and galactosaminoglycan. Changes in the composition of the culture medium affected the rate of accumulation of $^3$H-GAG in cultured corneas but had little effect on the relative abundance of the different GAG types. Physical or enzymatic damage to the cultured corneas resulted in marked changes in the ratio of labeled GAG types secreted. Physical damage to the epithelial or endothelial surfaces and/or mild trypsin treatment of the corneas before culture resulted in a stimulation of galactosaminoglycan secretion and a consequent decrease in the relative abundance of keratan sulfate. The presence of soy trypsin inhibitor prevented these changes. Treatment of the corneas with collagenase resulted in a decrease in all GAG secretion, particularly keratan sulfate. When corneas were removed from chilled, enucleated eyes and rinsed with solutions of soy trypsin inhibitor before incubation, the GAG secreted in culture had a similar ratio of keratan sulfate to galactosaminoglycan as GAG labeled in vivo. This ratio was maintained 1 week in culture. These results suggest that secretion of the characteristic connective tissue of the corneal stroma by keratocytes is dependent on interaction between the cells and components of the stromal extracellular matrix. Invest Ophthalmol Vis Sci 24:208-213, 1983

In adult animals, corneal connective tissue is secreted by the stromal keratocytes, flattened mesenchymal cells located between the stromal lamellae. For short periods of time in organ culture, keratocytes continue to secrete characteristic stromal connective tissue components. After 48 hours in organ or cell culture, marked changes take place in the nature of these secretions. A well-characterized aspect of this change is the alteration in corneal glycosaminoglycan synthesis. There are two major corneal types of glycosaminoglycan (GAG), keratan sulfate and galactosaminoglycan (a chondroitin sulfate-dermatan sulfate hybrid). In vivo, more than half of the total GAG secreted by keratocytes is keratan sulfate, but after 48 hours in organ or cell culture, this level decreases to 10% or less of the total. Keratocytes grown in monolayer cultures exhibit a fibroblastic morphology and a GAG synthesis similar to cells from other connective tissues, with keratan sulfate making up 2% or less of the total GAG.

Standard culture conditions, therefore, appear to induce a dedifferentiation of the keratocyte, both in morphology and connective tissue synthesis. In this study, we have used GAG synthesis as a measure of normal corneal connective tissue synthesis to examine culture conditions under which differentiated function can be maintained. Conditions are defined in which keratan sulfate synthesis is retained for several weeks in organ cultures.

Materials and Methods

Cultures

Corneas were excised from the eyes of adult New Zealand white rabbits immediately after death or from unwashed whole eyes shipped on ice from Pel-Freeze Biologicals (Rogers, AK) and used within 48 hours of enucleation. Corneas were removed with a scleral rim, rinsed in GKN (135 mM NaCl, 5 mM KCl, 5 mM glucose), trimmed to the limbus, and rinsed in several portions of cold Eagle's minimal essential medium (MEM) with antibiotics. Corneas were incubated in MEM with additions of 10% (v/v)
fetal bovine serum (Microbiological Associations, Bethesda, MD), 5% (w/v) dextran (molecular weight 40,000, Sigma Chemical Co., St. Louis, MO), or with a micronutrient-trace element supplement. This supplement contained a mixture of compounds in concentrations determined by McKeohan et al to be optimal for mammalian connective tissue cells in culture. The final concentrations were the following: 0.25 μg/ml CuSO₄ · 5H₂O; 0.24 μg/ml MnSO₄ · 5H₂O; 1.24 μg/ml (NH₄)₆Mo₇O₂₄ · 4H₂O; 3.8 μg/ml H₂SeO₃; 0.58 μg/ml NH₄VO₃; 144 μg/ml ZnSO₄ · 7H₂O; 1.4 mg/ml FeSO₄ · 7H₂O; 15 mg/ml asparagine · H₂O; 34.5 mg/ml l proline; 10.5 mg/ml serine; 5 mg/ml niacinamide; 10 mg/ml choline · HCl; 0.10 mg/ml thymidine; 16 mg/ml i inositol; 1.35 mg/ml adenine. The first seven of these compounds (trace elements) were combined in a 500-fold concentrated stock solution, acidified with HCl. The final eight compounds (micronutrients) were combined in a 100-fold concentrated stock solution. The two solutions were sterile-filtered through 0.45-micron membrane filters and added directly to MEM. To the supplemented MEM was also added sterile 1 M HEPES buffer, pH 7.0, to make a final concentration of 10 mM. The supplemented medium was designated MEMS.

Corneas were cultured in 60-mm plastic tissue culture dishes, each containing two corneas and 5 ml of medium. Incubation was at 37°C in a humidified CO₂ incubator. Medium was changed weekly. Preincubation of cultures was carried out after removal of the epithelial and endothelial cell layers by scraping the corneas with a steel spatula in cold MEMS containing 0.5 mg/ml soy trypsin inhibitor (Sigma) as noted. The tissue was rinsed and incubated in the same medium for 30 min at 37°C with trypsin (0.05 mg/ml) or bacterial collagenase (Sigma, Type II) 0.5 mg/ml. The cultures were then rinsed three times for 15 min in supplemented MEM with trypsin inhibitor and three times without inhibitor before incubation in MEMS with 5% dextran (MEMDS).

Rabbit stromal monolayer cell cultures were established by outgrowth if fibroblasts from minced cornea incubated in MEM with 10% fetal calf serum. Cultures were divided 1:2 weekly. Labeling and isolation of GAG was carried out as described. Corneal GAG was labeled during the last 7 days of culture by a single addition of 2 μCi/ml 3H-glucosamine HCl (20 Ci/mmole, New England Nuclear, NET 190A) unless otherwise noted. For each experiment, corneas from two to three identical cultures were rinsed and pooled. The labeled corneas were digested with protease as described elsewhere after initial digestion with collagenase 0.5 mg/ml at 55°C for 4–12 hours. The collagenase treatment aided solubilization of the corneas. It had no measurable effect on the size or type of GAG recovered. GAG was purified on DEAE cellulose columns as described previously, omitting precipitation with trichloracetic acid. Digests were passed over 0.9 × 4 cm columns of DEAE cellulose (Sigma) equilibrated with Tris-HCl buffer, pH 8. Columns were washed with 20 ml of 0.15 M NaCl, 0.02 M Tris, pH 8, and GAG was eluted with 5 ml of 2 M NaCl 0.02 M Tris, pH 8. The GAG was dialysed and lyophylized.

CPC-Cellulose Fractionation of GAG

To 0.9 ml of 3H-GAG with added carrier (0.6 mg each hyaluronic acid and chondroitin sulfate) was added 0.1 ml 10% cetylpyridinium chloride (CPC) solution. After 30 min, this mixture was passed over a 0.9 × 3 cm column of cellulose (Sigma cellulose 100, Sigma) equilibrated with 1% CPC. The column was rinsed with 2 ml 1% CPC (fraction I), then eluted with 3 ml 0.3 M NaCl in 0.05% CPC (fraction II), followed by 3 ml 1.2 M MgCl₂ in 0.05% CPC (fraction III). Fractionation of standards showed that keratan sulfate eluted in fraction I, hyaluronic acid eluted in fraction II, and chondroitin sulfate, dermatan sulfate, and heparan sulfate all eluted in the third fraction.

Degradative Analysis

Radioactive GAG was degraded with leech hyaluronidase (Biometrics, Boston, MA) 0.5 mg/ml in 0.02 M Tris, pH 7.2, with 0.5 mg/ml chondroitin sulfate and hyaluronic acid. After 16 hours at 37°C, the sample was heated to 100°C for 3–5 min, chromatographed on Sephadex G-50 superfine (12.5 × 0.7 cm, Sigma), and eluted with 0.2 M pyridinium acetate, pH 6. Degradation was defined as the proportion of radioactive material no longer eluting in the column excluded volume after enzyme treatment.

Galactosaminoglycan (i.e., chondroitin sulfate and/or dermatan sulfate) was degraded with chondroitinase ABC (Sigma) 0.1 unit/ml in 0.2 M Tris–HCl, pH 8, with 1 mg/ml bovine serum albumin. Incubation and fractionation was carried out as described above. Chondroitinase-resistant GAG was isolated from the G-50 excluded material, dried, and used for determination of heparan sulfate and keratan sulfate.
Week of incubation and tissue GAG isolated as described in Methods. The proportion of GAG degraded by chondroitinase during organ culture in different culture media is trace nutrients as described in Methods.

Phadex G-50 as described above, or on S-200 as described below. Galactosaminoglycan content was defined as the proportion of GAG degraded by hyaluronidase.

Heparan sulfate was degraded by butyl nitrite treatment of chondroitinase-resistant GAG at room temperature. Degradation of GAG was assayed on Sephadex G-50 as described above, or on S-200 as described below. Keratan sulfate was degraded by two 24-hour treatments with 0.125 units/ml of endo-β-galactosidase from *Escherichia freundii* (provided by Dr. M. Fukuda). Degradation was analyzed by chromatography on Sephacryl S-200 (28 × 0.7 cm, Sigma) eluted with 4 M guanidinium chloride, 0.02 M Tris-HCl, pH 7.2. Degradation was defined as the proportion of radioactive material that shifted to a lower molecular size after enzyme treatment. Degradation of desulfated keratan sulfate was analyzed on Sephadex G-50 as above. Desulfation of chondroitinase-resistant GAG was carried out in dimethyl sulfoxide with 10% water at 80°C for 5 hours as described. Desulfated GAG was recovered after dialysis and lyophilization.

Results

Rabbit corneas in organ culture maintained an active incorporation of 3H-glucosamine into GAG for several weeks. Initial experiments showed that the maximum rate of incorporation during 1- to 2-week incubations was obtained with serum-free culture medium containing 5% dextran. Addition of trace nutrients (Table 1) further increased the rate of incorporation, as well as allowing the tissue to continue the high rate of 3H-GAG secretion for at least 4 weeks of culture. The supplemented medium was chosen therefore for subsequent organ culture experiments.

When purified 3H-GAG was fractionated by CPC-cellulose chromatography (Table 2), 15-25% of the total label was eluted from fraction I, the CPC-soluble fraction. By comparison, 55% of GAG labeled in vivo and only 2% of GAG from monolayer cultures of stromal fibroblasts were soluble in CPC. Keratan sulfate is the only GAG standard that elutes in this CPC fraction. These results suggest that corneas in organ culture secrete amounts of cultured keratan sulfate intermediate between cornea in vivo and cultured stromal fibroblasts.

The presence of keratan sulfate in organ culture GAG was confirmed when the GAG fractions eluted from CPC-cellulose chromatography were analyzed with specific degradative enzymes. As shown in Table 3, 16% of the total 3H-GAG from organ culture GAG was identified as keratan sulfate by its susceptibility to endo-β-galactosidase, an enzyme that hydrolyses the bonds of keratan sulfate and no other type of GAG. The data in Table 3 also demonstrate the effectiveness of CPC-cellulose chromatography in identifying corneal keratan sulfate. Seventy-five percent of all keratan sulfate eluted in CPC fraction I, while 95% of all other GAG eluted in fractions II and III.

The supplemented medium used in these experiments did not support keratan sulfate secretion by primary keratocytes after isolation from cornea with proteolytic enzymes (data not shown). To investigate the point at which keratan sulfate synthesis was lost, we exposed denuded stromae to a 30-min incubation
with trypsin or collagenase, rinsed them extensively in medium containing soy trypsin inhibitor, then incubated them with $^3$H-glucosamine for 2 days. The enzyme preincubation, as shown in the first experiment in Table 4, resulted in a decrease in the relative proportion of CPC fraction I GAG from 24% in the control to 10% in the enzyme-treated stromae. Interestingly, the control cultures preincubated without trypsin inhibitor also showed a decreased proportion of CPC fraction I, suggesting that trypsin inhibitor was partially blocking the decrease in keratan sulfate synthesis that occurs in the transition from in vivo to culture.

Experiment B in Table 4 explores this possibility. Corneas were removed from chilled enucleated eyes, with care to minimize damage to the epithelial and endothelial surfaces. One surface was scraped, then the tissues were preincubated and washed as before. This handling of the tissue resulted in a greater incorporation into $^3$H-GAG than with previous culture techniques, as well as a much increased proportion of fraction I GAG. Omission of trypsin inhibitor and damage to the corneal surface both resulted in decreased proportion of CPC fraction I GAG. The data in Table 4 show that the relative decrease in fraction I GAG, due to tissue damage or from low levels of trypsin, resulted from a moderate decrease in the absolute amount of $^3$H-GAG in fraction I and a marked increase in the $^3$H-GAG in fractions II and III. Collagenase, on the other hand, strongly inhibited all $^3$H-GAG secretion.

Table 5 presents degradative analysis of $^3$H-GAG secreted under conditions where tissue damage was minimized and corneas were rinsed with trypsin inhibitor before culture. After 2 and 7 days in culture, $^3$H-GAG from the cultured corneas closely resembled that labeled in vivo. After 3 weeks in culture, the $^3$H-GAG exhibited a relative decrease in keratan sulfate content and increases primarily in heparan sulfate and unidentified GAG. All of the GAG in this experiment contained material not degraded by the enzyme treatments; however, much of this material in the sample made in vivo was susceptible to endo-$eta$-galactosidase after desulfation (data in parentheses). This is probably due to the fact that this enzyme does not hydrolyze the sulfated galactose moieties found in corneal keratan sulfate.

About 15% of $^3$H-GAG made in the 1- and 3-week cultures was not identified by degradative treatments used for Table 5. Subsequent analyses indicated that this material consisted mostly of heparan sulfate. Butyl nitrite treatment was found to degrade 20–25% of the $^3$H-GAG from culture when this degradation was measured with Sephacryl S-200 gel chromatography (data not shown). Only about half as much degradation was detected using Sephadex G-50, indicating a large molecular size for the fragments produced by butyl nitrite. The use of the smaller pore G-50 gel, therefore, underestimated the proportion of heparan sulfate in GAG in Table 5. Assuming that all of the identified material in Table 5 was heparan sulfate leads to the conclusion that this GAG made up about 25% of the total labeled GAG in all three cultures. The cultures incubated 2 and 7 days in Table 5 had hyaluronic acid, keratan sulfate, and galactosaminoglycan present in ratios almost identical to those found in vivo. The primary change in GAG made during 1 week of culture, therefore, appears to be the increase in accumulation of heparan sulfate.

Table 4. Effect of proteolytic treatment and tissue damage on corneal GAG accumulation in vitro

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Total GAG (cpm/cornea $\times 10^{-2}$)</th>
<th>CPC Fractions (% Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II &amp; III</td>
</tr>
<tr>
<td>Experiment A—Scraped stromae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypsin inhibitor</td>
<td>25</td>
<td>6.0 (24%)</td>
</tr>
<tr>
<td>No inhibitor</td>
<td>52</td>
<td>4.2 (8%)</td>
</tr>
<tr>
<td>No inhibitor and Trypsin</td>
<td>43</td>
<td>4.3 (10%)</td>
</tr>
<tr>
<td>Trypsin inhibitor collagenase</td>
<td>12</td>
<td>0.9 (8%)</td>
</tr>
<tr>
<td>Experiment B—Whole cornea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypsin inhibitor</td>
<td>89</td>
<td>60 (67%)</td>
</tr>
<tr>
<td>No inhibitor</td>
<td>89</td>
<td>55 (62%)</td>
</tr>
<tr>
<td>No inhibitor—epithelium scraped</td>
<td>115</td>
<td>55 (48%)</td>
</tr>
<tr>
<td>No inhibitor—endothelium scraped</td>
<td>96</td>
<td>30 (45%)</td>
</tr>
</tbody>
</table>

Corneas were preincubated 30 minutes as described in Methods, and incubated 48 hours with 4 $\mu$Ci/ml $^3$H-glucosamine in MEMDS.

Table 5. Degradative analysis of total $^3$H-GAG secreted in vitro and in vivo

<table>
<thead>
<tr>
<th>GAG type</th>
<th>In vivo</th>
<th>2 day</th>
<th>7 day</th>
<th>21 day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaluronic acid</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Galactosaminoglycan</td>
<td>47</td>
<td>40</td>
<td>41</td>
<td>49</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>2</td>
<td>5</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>Keratan sulfate</td>
<td>34 (41)</td>
<td>33</td>
<td>36 (36)</td>
<td>10 (19)</td>
</tr>
<tr>
<td>Unidentified</td>
<td>11 (5)</td>
<td>19</td>
<td>17 (17)</td>
<td>24 (16)</td>
</tr>
</tbody>
</table>

Corneas were excised from chilled enucleated eyes, rinsed in MEMS containing 1% soy trypsin inhibitor, and cultured in MEMS. GAG was labeled with $^3$H-glycosamine (5 Ci/ml) during the last 2 days of culture.
Discussion

Our experiments demonstrate that intact cornea retains the ability to secrete keratan sulfate in vitro for several weeks. Changes in the composition of the culture medium, while strongly affecting the total 3H-GAG accumulation rate, had no measurable effect on the relative proportion of keratan sulfate. Changes in this proportion were seen as a result of any treatment that disrupted the corneal structure. Physical damage to the cornea resulted in a moderate decrease (0–50%) of the absolute amount of 3H-keratan sulfate accumulating in the culture. The relative proportion of keratan sulfate was decreased even further by a stimulation in the accumulation of other GAG, primarily glycosaminoglycan. The effects of tissue damage were blocked by soy trypsin inhibitor, suggesting that these metabolic changes were induced in the keratocytes by action of a protease. Serine (trypsin-like) proteases are known to act directly as mitigens and to alter differentiated functions of cells in vitro.\(^\text{11,12}\) The work of Weimar et al has implicated such a protease in a number of keratocyte responses to epithelial wounding.\(^\text{13-15}\) Among others, these responses include enzyme induction and transformation of keratocytes to a fibroblastic morphology. The changes in GAG metabolism we have observed may, therefore, be part of a generalized keratocyte response to corneal wounding.

A different alteration in GAG secretion was observed when corneas were digested with collagenase. Under these conditions, keratan sulfate accumulation was reduced greatly (85% or more), and little or no stimulation of other GAG types was seen. Collagenase virtually liquified the tissue, probably solubilizing much of the stromal extracellular matrix. Matrix protein attachment factors have been identified for several cell types that affect their expression of differentiated functions in vitro.\(^\text{19,20}\) Thus, the effect of collagenase in this system might be the solubilization of some stromal component required for keratan sulfate synthesis. This hypothesis is supported by our recent finding that keratocytes secrete a keratan sulfate-like material when cultured on an acellular insoluble stromal matrix preparation.\(^\text{21}\)

Heparan sulfate, a compound normally present as 2–5% of corneal GAG, made up more than 20% of the 3H-GAG. The relative insensitivity of the heparan sulfate to deaminative hydrolysis is presumably due to nonuniform distribution or low content of N-sulfate in the polymer. We have observed this characteristic in heparan sulfate from corneal cells in culture (unpublished data), and it has been seen in other forms of heparan sulfate as well.\(^\text{16}\) The function of heparan sulfate proteoglycan is not well understood, but it is found in cell attachment sites in association with fibronectin.\(^\text{17}\) Fibronectin, like heparan sulfate, is not a normal corneal component, but it is found throughout the stroma after epithelial wounding.\(^\text{18}\) This finding suggests that heparan sulfate and fibronectin may be secreted by the keratocytes as a coordinated response to a common change in the extracellular environment of the stroma, for example, increased stromal hydration.

We have described a culture system in which corneal keratan sulfate synthesis is maintained in vitro for several weeks. This system allows examination of aspects of keratocyte metabolism not previously practicable. In addition to continuing the examination of normal keratocyte function that we have begun here, studies on the metabolism of fetal corneas, corneal scars, and corneal dystrophies might all be approached effectively using this system.

Key words: cornea, organ culture, proteoglycan, connective tissue, keratan sulfate, cell differentiation, glycosaminoglycan.

References


