The Synthesis of Glycosaminoglycans by Cultures of Rabbit Corneal Endothelial and Stromal Cells

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Confluent monolayer cultures of rabbit corneal endothelial and stromal cells were incubated independently with [^{35}S]sulphate and [^3H]glucosamine for 3 days. After incubation, labelled glycosaminoglycans were isolated from the growth medium and from a cellular fraction. These glycosaminoglycans were further characterized by DEAE-cellulose column chromatography and by sequential treatment with various glycosaminoglycan-degrading enzymes. Both endothelial and stromal cultures synthesized hyaluronic acid as the principal product. The cell fraction from the stromal cultures, however, had significantly less hyaluronic acid than that from the endothelial cultures. In addition, both types of cells synthesized a variety of sulphated glycosaminoglycans. The relative amounts of each sulphated glycosaminoglycan in the two cell lines were similar, with chondroitin 4-sulphate, chondroitin 6-sulphate and dermatan sulphate as the major components. Heparan sulphate was present in smaller amounts. Keratan sulphate was also identified, but only in very small amounts (1–3%). The presence of dermatan sulphate and the high content of hyaluronic acid are similar to the pattern of glycosaminoglycans seen in regenerating or developing tissues, including cornea.

The cornea is composed of five distinct layers: an external epithelium, Bowman's membrane, a stroma (which comprises over 90% of the thickness of the cornea), Descemet's membrane and an endothelium. Both membranes are collagenous. The corneal endothelium produces Descemet's membrane and controls many transport functions of the cornea (Davson, 1972; Maurice & Riley, 1970).

Glycosaminoglycans of the mammalian corneal stroma are thought to play a role in water binding, which in turn determines the degree of hydration and thickness of the cornea (Maurice & Riley, 1970; Cotlier, 1975). It is generally believed that the mutual repulsion by the negatively charged groups of glycosaminoglycan molecules plus the interaction of glycosaminoglycans with collagen fibrils contribute to the maintenance of the characteristic regular spacing of these fibrils. This regular spacing in turn contributes to the transparency of the cornea (Davson, 1972; Borcherding *et al.*, 1975). Thus the nature of the glycosaminoglycans in the cornea is important.

Glycosaminoglycans in normal corneal stroma

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have been shown to consist of keratan sulphate and chondroitin sulphates in a molar ratio of approx. 3:2 (Meyer *et al.*, 1953; Anseth & Laurent, 1961; Saltermik-Girant & Berman, 1970; Handley & Phelps, 1972). Histochemical studies have revealed the presence of glycosaminoglycans in the corneal endothelium (Maurice & Riley, 1970), but there has previously been no detailed analysis of glycosaminoglycans in this single cell layer because of the analytical limitations imposed by its small mass. There has been a suggestion that the endothelium may be the source of hyaluronic acid during development of chick cornea (Meier & Hay, 1973; Trelstad *et al.*, 1974).

We have previously described the mass culture of pure rabbit corneal endothelial cells (Perlman & Baum, 1974a) and have shown by electron microscopy that these cells maintain their normal morphology (Perlman *et al.*, 1974). We have also shown that the cultured endothelial cells produce a basement membrane *in vitro* (Perlman & Baum, 1974b) which morphologically and chemically resembles Descemet's membrane, the product of corneal endothelial cells *in vivo*. These cultures of rabbit corneal endothelium, which simulate the endothelial cells *in vivo*, thus provide a system in which glycosaminoglycan synthesis can be studied,

The present investigation was undertaken to examine the synthesis of glycosaminoglycans by rabbit corneal endothelial cells and to establish the nature of glycosaminoglycans synthesized. For comparison, parallel experiments were carried out with rabbit corneal stromal-cell cultures. These experiments extend previous studies (Dahl *et al.*, 1974; Gnadinger & Schwager-Hubner, 1975a,b).

Experimental

Cell cultures

New Zealand White albino rabbits (1.5–2.0 kg body wt.) were killed by intravenous injection of pentobarbital. Under sterile conditions, corneas were excised and Descemet's membrane and endothelium were dissected from the underlying stroma (Stocker *et al.*, 1958). The isolated Descemet's membrane-endothelium layer was placed in a 30 ml Falcon tissue-culture flask and incubated in a humidified incubator containing CO₂+air (5:95) at 35°C in the following culture medium (Perlman & Baum, 1974a): Eagle's minimum essential medium, supplemented with 10% (v/v) foetal calf serum, 5% (v/v) calf serum, 2% (v/v) of 200 mM-glutamine, 2% (v/v) of MEM essential (50X, cat. no. 13-606), and 1% of non-essential, amino acids (100X, cat. no. 13-114), NaHCO₃ (7.5%, w/v) (all from Microbiological Associates, Bethesda, MD, U.S.A.), gentamicin (10 µg/ml; Schering Co., Port Reading, NJ, U.S.A.) and amphotericin B (1.2 µg/ml; E. R. Squibb and Sons, Princeton, NJ, U.S.A.).

After 2 weeks in culture, the cells grown from the explants were removed with 0.0005% trypsin (Perlman & Baum, 1974b) and transferred to a new flask for subculture. At 10–14 days after subculture, cultures of endothelium had reached a saturation density of approx. 10⁶ cells/flask. In this same time-period, corneal stromal cultures reached a saturation density of approx. 6 × 10⁵ cells/flask.

Production of glycosaminoglycans

In a typical experiment, two flasks of approx. 1-month-old cultures of corneal endothelium and stroma were exposed to medium containing 30 µCi of [³⁵S]sulphate/ml for 3 days. When [³H]glucosamine was used, 40 µCi was added to 10 ml of medium.

At the end of the labelling period, the medium was removed from each flask and combined, the cultures were washed six times with 3 ml of Dulbecco's phosphate-buffered saline (1X, cat. no. 419, GIBCO, Grand Island, NY, U.S.A.), and cells harvested with a rubber 'policeman' or by treatment with trypsin. Cells obtained were broken twice with a Branson sonicator at setting 2 for 20 s. This fraction was called the 'cellular fraction' even though it contained both

intracellular products and pericellular products from the matrix and external cell membrane.

Identification and assay of glycosaminoglycans

After assay for radioactivity with a Beckman liquid-scintillation spectrometer in 10 ml of Beckman Ready-Solv solution VI (Beckman Instruments Inc., Lincolnwood, IL, U.S.A.) (all further radioactivity assays were performed in the same manner), the cell and growth-medium fractions were chromatographed on a column (1 cm × 20 cm) of Sephadex G-50 with 0.1 M-LiCl to separate labelled macromolecular material from radioactive precursors. The total radioactivity in the macromolecular product was used to calculate the amount of glycosaminoglycans produced per mg of cell protein. The protein associated with the cellular fraction of corneal cultures was assayed with fluorescamine (Roche, Nutley, NJ, U.S.A.) by using bovine serum albumin as standard (Udenfriend *et al.*, 1972).

Samples of labelled glycosaminoglycans were chromatographed on a DEAE-cellulose column (1 cm × 4 cm) with 3 mg of hyaluronic acid (Calbiochem, San Francisco, CA, U.S.A.), 5 mg of chondroitin 4-sulphate (Miles Laboratories, Elkhart, IN, U.S.A.) and 5 mg of heparin (Calbiochem) as internal standards. A logarithmic gradient of LiCl as described previously (Lewis *et al.*, 1973) was used. Fractions were assayed for radioactivity. Carrier standards were located by the carbazole method (Bitter & Muir, 1962) for measurement of uronic acids.

To distinguish between different groups of sulphated glycosaminoglycans produced by the cell cultures, samples of the labelled products plus appropriate standards were sequentially incubated with various glycosaminoglycan-degrading enzymes as follows:

1. Chondroitinase AC (Miles), 0.75 unit (Suzuki, 1972); chondroitin 4-sulphate, 0.5 mg; enriched Tris buffer (Saito *et al.*, 1968), pH 8.0, 0.01 ml; in a total volume of 0.1 ml for 1.5 h at 37°C. This enzyme only degrades hyaluronic acid, chondroitin 4- and 6-sulphate.

2. Chondroitinase ABC (Miles), 0.75 unit (Suzuki, 1972); chondroitin 4-sulphate, 0.5 mg; enriched Tris buffer, pH 8.0, 0.01 ml; in a total volume of 0.1 ml for 1.5 h at 37°C. This enzyme degrades hyaluronic acid, dermatan sulphate, chondroitin 4- and 6-sulphate, but has no activity on heparin, heparan sulphate and keratan sulphate.

3. Crude heparinase, 0.1 mg; heparin, 1 mg; sodium acetate buffer, pH 6.0, 10 µmol; in a total volume of 0.1 ml for 22 h at 20°C. Crude heparinase was prepared from *Flavobacter heparinum* (Linker, 1966) by sonication in a Branson sonifier at setting 4, followed by centrifugation at 20000g for 10 min. The crude enzyme obtained in the supernatant de-

grades all types of glycosaminoglycans except keratan sulphate.

4. Endo- β -galactosidase (keratanase; a gift from Dr. S. Suzuki), 0.3 unit (Nakajawa & Suzuki, 1975); keratan sulphate (a gift from Dr. G. Armand), 0.1 μ mol (as galactose); Tris/HCl buffer, pH 7.2, 5 μ mol; in a total volume of 0.1 ml for 3 h at 37°C. This enzyme degrades keratan sulphate.

After incubation, the total reaction mixtures were chromatographed on a column (1 cm $\times$ 20 cm) of Sephadex G-50 with 0.1 M-LiCl solution, and radioactivity was determined for each fraction. The amount of material present in the excluded fractions represented the macromolecular glycosaminoglycans resistant to the particular enzyme. Degradation products of labelled and standard glycosaminoglycans were located by the carbazole assay (Bitter & Muir, 1962) for determination of uronic acids or with anthrone (Calbiochem) for measurement of hexoses (Trevelyan & Harrison, 1952) in the case of keratan sulphate.

Disaccharide products of chondroitinase digestion were identified by chromatography on Whatman no. 1 paper, together with disaccharide standards Δ Di-4S, * Δ Di-6S, Δ Di-0S and unsaturated hyalobiuronic acid, overnight in butan-1-ol/acetic acid/aq. 1.0 M-NH₃ (2:3:1, by vol.) (Saito *et al.*, 1968). After chromatography, the carrier disaccharides were detected with u.v. light. The chromatograms were then cut into 1 cm strips which were counted separately for radioactivity after elution with water.

The specific radioactivity of [³⁵S]sulphate was calculated from the amount of [³⁵S]sulphate added to the incubation medium and the amount of inorganic sulphate present in the medium (determined by the method of Antonopoulos, 1962). The specific radioactivity of [³H]glucosamine had to be calculated indirectly (Kleinman *et al.*, 1975), since it would be diluted with unknown amounts of other precursors such as glucose in the course of incorporation into glycosaminoglycans. This was as follows: portions of ³H,³⁵S-labelled sulphated glycosaminoglycans, freed from hyaluronic acid by DEAE-cellulose column chromatography (Kleinman *et al.*, 1975), were subjected to chondroitinase ABC digestion. Paper chromatography (Saito *et al.*, 1968) was carried out for the resulting degradation products and the radioactive spot corresponding to the standard Δ Di-4S was removed and assayed for radioactivity. Since Δ Di-4S has a 1:1 molar ratio of sulphate to hexos-

* Abbreviations: Δ Di-4S, 2-acetamido-2-deoxy-3-O-(β -D-gluc-4-enepyranosyluronic acid)-4-O-sulpho-D-galactose; Δ Di-6S, 2-acetamido-2-deoxy-3-O-(β -D-gluc-4-enepyranosyluronic acid)-6-O-sulpho-D-galactose; Δ Di-0S, 2-acetamido-2-deoxy-3-O-(β -D-gluc-4-enepyranosyluronic acid)-D-galactose; unsaturated hyalobiuronic acid, 2-acetamido-2-deoxy-3-O-(β -D-gluc-4-enepyranosyluronic acid)-D-glucose.

amine, the specific radioactivity of [³H]glucosamine could be calculated from the known [³⁵S]sulphate specific radioactivity.

Results

A DEAE-cellulose chromatogram of ³H,³⁵S-labelled glycosaminoglycans from the cellular fraction of rabbit corneal endothelial cultures is shown in Fig. 1. There was a large amount of [³H]hyaluronic acid present which chromatographed near a standard hyaluronic acid peak. ³H,³⁵S-labelled sulphated glycosaminoglycans were found in the area of standard chondroitin 4-sulphate. Glycosaminoglycans found in the growth media from endothelial cultures and from stromal cultures appeared to have elution profiles similar to those obtained from the cellular fraction of endothelial cultures. The cellular fraction of stromal cultures, however, had a somewhat different DEAE-cellulose chromatogram pattern, which is shown in Fig. 2; there was much less hyaluronic acid. In all cases, the [³H]hyaluronic acid and the

Fig. 1. Chromatography on DEAE-cellulose of glycosaminoglycans from cell fraction of rabbit corneal endothelial culture

Samples, together with standards of hyaluronic acid (3 mg), chondroitin 4-sulphate (5 mg) and heparin (5 mg), were eluted from a DEAE-cellulose column (1 cm $\times$ 4 cm) with an exponential gradient of LiCl. The mixing flask contained 125 ml of water and the reservoir contained 1 M-LiCl. Fractions (2.5 ml) were collected. The reservoir was changed from 1 M-LiCl to 2 M-LiCl after fraction 85. Standard glycosaminoglycans were located by the assay for uronic acids. Band 1, hyaluronic acid; band 2, chondroitin 4-sulphate. ●, ³⁵S radioactivity; ○, ³H radioactivity.

^3H , ^{35}S -labelled sulphated glycosaminoglycans were eluted from the DEAE-cellulose column in the early portions of the standard hyaluronic acid and chondroitin 4-sulphate peaks, suggesting that the labelled glycosaminoglycans were smaller in size with a lower total charge content than the standards. No heparin was found in any fractions of any cultures. In all DEAE-cellulose chromatograms there was some material which was eluted before hyaluronic acid, but this was not studied further. It may represent glycoproteins.

The production of glycosaminoglycans by confluent monolayer cultures of rabbit corneal endothelium and corneal stroma are shown in Tables 1, 2 and 3. Table 1 shows the total amount of glycosaminoglycans (both sulphated and non-sulphated) produced based on ^3H -labelled glucosamine incorporation. With both endothelial and stromal cell lines, more labelled material was found in the nutrient medium than within the cells. The amount of ^3H -labelled glycosaminoglycans produced by endothelial cell cultures was considerably less than that produced by stromal cells grown under the same conditions.

Table 2 shows the hyaluronic acid content of endothelial and stromal cultures. The amount was determined by DEAE-cellulose column chromatography and identification of unsaturated hyalobiuronic acid after chondroitinase ABC digestion. Hyaluronic acid was the principal product of all fractions from the endothelial cell cultures, and also represented the dominant amount in the medium of stromal cell cultures. However, hyaluronic acid represented only a relatively small proportion of the glycosaminoglycans found in the cell fraction of stromal cultures.

Table 3 shows the total amount of sulphated glycosaminoglycans produced based on ^{35}S -sulphate incorporation. Distribution between the media and the cell fractions was similar for endothelial and stromal cultures. However, on total cell-protein basis there was less ^{35}S -sulphate-labelled material produced by

the endothelial cell cultures than by the stromal cultures.

Table 4 shows the relative amounts of the various sulphated glycosaminoglycans produced by the cultures. Chondroitin 4- and 6-sulphate, constituents of normal corneal stroma, always made up a major portion of the sulphated macromolecular material. Dermatan sulphate, which is not a constituent of normal cornea but has been found during corneal wound healing (Anseth & Fransson, 1969) and in some corneal disorders (Anseth, 1969), was also present in large amounts. Heparan sulphate, a substance found to be produced by many cell lines (Kraemer, 1971; Buonassisi & Ozzello, 1974; Kleinman *et al.*, 1975) and suggested to be a component of all cells (Kraemer, 1971), comprised a small proportion

Fig. 2. Chromatography on DEAE-cellulose of glycosaminoglycans from cell fraction of rabbit corneal stromal-cell culture

Details are as described for Fig. 1.

Table 1. Incorporation of ^3H glucosamine into glycosaminoglycans by cultures of endothelial and stromal cells

Each flask of corneal cultures was labelled with 3 ml of medium containing 1.43×10^6 c.p.m. of ^3H glucosamine for 3 days. The amount of glycosaminoglycans synthesized was calculated from the specific radioactivity of ^3H glucosamine ($8.7 \mu\text{mol/mCi}$). Each line of values is from an independent experiment.

Cell type	$10^{-5} \times ^3\text{H}$ radioactivity incorporated into glycosaminoglycans (c.p.m.)		Total glycosaminoglycans produced (μg)	Total cell protein (mg)	Glycosaminoglycans produced ($\mu\text{g}/\text{mg}$ of cell protein)
	Cell	Medium			
Endothelium	0.8	5.9	2.1	0.5	4.2
	0.9	2.1	0.9	0.3	3.1
Stroma	3.6	58.1	18.5	0.8	22.8
	2.4	46.5	14.7	0.9	16.1

(10–20%) of the total ^{35}S -labelled glycosaminoglycans. As in other cell lines (Buonassisi & Ozzello, 1974; Kleinman *et al.*, 1975), a higher percentage of the glycosaminoglycans was present as heparan sulphate in the cell fractions than in the growth media.

All of the ^{35}S -labelled material from cell fractions of both endothelial and stromal cultures was degraded by heparinase. In the growth media, however, approx. 1–3% of the [^{35}S]sulphate-labelled macromolecular material was resistant to the heparinase. Most of this was shown to be keratan sulphate rather than sulphated glycoproteins by its susceptibilities to digestion by keratan sulphate endo- β -galactosidase. Before the enzyme treatment, standard carrier keratan sulphate and this [^{35}S]sulphate-labelled material appeared in the excluded volume from a column of Sephadex G-50. In the case of the heparinase-resistant ^{35}S -labelled material from the endothelial-cell medium, all of the radioactivity co-chromatographed exactly with degraded carrier keratan sulphate after keratanase treatment. About 40% of the heparinase-resistant material from growth medium of stromal cultures was not degraded, even by endo- β -galactosidase. This material could be sulphated glycoproteins, as previously found in the cornea (Maurice & Riley, 1970).

Discussion

The present paper demonstrates that confluent monolayer cultures of rabbit corneal endothelial cells are capable of synthesizing hyaluronic acid and a spectrum of sulphated glycosaminoglycans, including a small amount of keratan sulphate. Non-confluent cell lines, not examined in the present study, might be expected to produce glycosaminoglycans

different in type and amount from confluent lines. Although the function of endothelial glycosaminoglycans is not clear, they may be related to the formation of collagen and thus to the production of Descemet's membrane.

In common with previous studies on embryonic-chick corneal stromal cells (Conrad & Dorfman,

Table 2. *Hyaluronic acid content*

The amount of hyaluronic acid in each fraction of cell cultures was calculated by one of two methods: 1. DEAE-cellulose column chromatography was performed as described in Figs. 1 and 2. The amount of [^3H]glycosaminoglycans in the peak near standard hyaluronic acid and the amount of [^3H]glycosaminoglycans near standard chondroitin 4-sulphate were compared. 2. Paper chromatography of chondroitinase ABC degradation products was performed as described in the Experimental section. The amount of [^3H]disaccharides co-chromatographing with unsaturated hyalobiuronic acid was used to calculate the hyaluronic acid content. Results shown in the second column were obtained from two separate experiments.

Cell type	Hyaluronic acid (% of total glycosaminoglycans)	
	From DEAE-cellulose column	From reactions with chondroitinase ABC
Endothelium		
Cell fraction	76	66, 67
Medium fraction	70	75, 77
Stroma		
Cell fraction	34	32, 39
Medium fraction	93	90, 89

Table 3. *Incorporation of [^{35}S]sulphate into sulphated glycosaminoglycans by cultures of endothelial and stromal cells*

Each flask of corneal cultures was labelled with 3 ml of medium containing 174×10^6 c.p.m. of [^{35}S]sulphate for 3 days. The amount of [^{35}S]glycosaminoglycans synthesized was calculated from the specific radioactivity of [^{35}S]sulphate ($8.6 \mu\text{mol/mCi}$) by assuming that one sulphate group is present in each repeating disaccharide unit of glycosaminoglycan. Each line of results represents an independent experiment.

Cell type	$10^{-5} \times ^{35}\text{S}$ radioactivity incorporated into sulphated glycosaminoglycans (c.p.m.)		Total [^{35}S]glycosaminoglycans produced (μg)	Total cell protein (mg)	[^{35}S]Glycosaminoglycans produced ($\mu\text{g}/\text{mg}$ of cell protein)
	Cell	Medium			
Endothelium	2.7	12.1	2.9	3.1	0.9
	3.3	5.6	2.0	1.0	2.0
	0.6	1.9	0.8	0.5	1.6
	0.7	0.8	0.5	0.3	1.7
Stroma	3.2	23.5	5.8	0.7	8.4
	0.4	5.5	2.0	0.3	6.1
	4.9	13.8	6.2	0.8	7.6
	1.5	7.1	2.8	0.9	3.1

Table 4. Percentages of total sulphated glycosaminoglycans produced by cultures of endothelium and stroma

Results are means $\pm$ S.D. obtained from five experiments.					
Cell type	Chondroitin 4-sulphate	Chondroitin 6-sulphate	Dermatan sulphate	Heparan sulphate	Keratan sulphate
Endothelium					
Cell fraction	17 $\pm$ 2	48 $\pm$ 4	11 $\pm$ 1	24 $\pm$ 2	—
Medium fraction	7 $\pm$ 1	43 $\pm$ 3	37 $\pm$ 4	10 $\pm$ 1	3
Stroma					
Cell fraction	13 $\pm$ 2	32 $\pm$ 6	40 $\pm$ 5	15 $\pm$ 5	—
Medium fraction	16 $\pm$ 1	35 $\pm$ 3	39 $\pm$ 2	8 $\pm$ 3	1

1974), the present study shows that rabbit corneal stromal cells produce dermatan sulphate and heparan sulphate in addition to chondroitin 4- and 6-sulphate, the normal products of stroma. Rabbit stromal cells synthesize more dermatan sulphate and less heparan sulphate than do chick stromal cells. This variance may be due to differences in species and/or the stage of corneal development.

Previous studies have shown some disagreement regarding the presence of keratan sulphate in corneal stromal-cell cultures. One report (Dahl *et al.*, 1974) stated that keratan sulphate constitutes as much as 10% of the total glycosaminoglycans of rabbit stromal-cell cultures. Another (Gnadinger & Schwager-Hubner, 1975b) also reported that a small amount of keratan sulphate was present. However, in both cases this material was not specifically identified as keratan sulphate. Still another study with chick stromal cells (Conrad & Dorfman, 1974) did not demonstrate keratan sulphate in long-term cultures. Our sensitive and quantitative technique did show production of a small amount of keratan sulphate in confluent monolayer cultures of stroma.

Our studies indicate that rabbit stromal cells produce a large amount of hyaluronic acid, most of which is found in the growth medium. This agrees with previous findings by Dahl *et al.* (1974) and Gnadinger & Schwager-Hubner (1975b). The former group showed that 60–70% of glycosaminoglycans from rabbit stromal-cell cultures contain glucosamine and have properties similar to those of hyaluronic acid.

Comparison between rabbit corneal endothelial- and stromal-cell cultures indicate that, under these conditions, endothelial cells produce smaller amounts of glycosaminoglycans than do stromal cells. It is noteworthy that both types of cells in culture produce the same type of sulphated glycosaminoglycans and that the pattern of glycosaminoglycans found in culture is markedly different from that found in normal corneas. These differences indicate that metabolism of glycosaminoglycans is affected by the environmental condition of cell cultures.

Dermatan sulphate is produced in the cornea during early stages of wound healing (Anseth & Fransson, 1969) and synthesis of hyaluronic acid has been associated with embryonic development and reparative processes in cornea (Toole & Gross, 1971b) and other tissues (Toole & Gross, 1971a; Manasek *et al.*, 1973). Our demonstration of large amounts of both hyaluronic acid and dermatan sulphate in corneal-cell cultures may indicate that the conditions of tissue culture mimic a developing or regenerating system as suggested previously (Gnadinger & Schwager-Hubner, 1975a,b).

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