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Stimulation of Extracellular Matrix Synthesis in the Developing Cornea by Glycosaminoglycans

(epithelium/tissue interaction)

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ABSTRACT **Previously, it was demonstrated that the embryonic corneal epithelium produces the chondroitin sulfate and heparan-sulfate-like compounds and the collagen of the primary corneal stroma. Synthesis of all of these extracellular materials is greatly enhanced *in vitro* when isolated epithelium is grown on collagenous substrata instead of Millipore filters. We report here that chondroitin sulfate, heparin, and heparan sulfate added to the culture medium at a concentration of 200 μg/ml enhance the synthesis by the epithelium of chondroitin sulfate and heparan-sulfate-like compounds 2-fold, whether or not collagenous substrata are employed. Collagen synthesis is unaffected by adding glycosaminoglycan to the medium. Chondroitin sulfate proteoglycan (chondromucoprotein) has the same stimulatory effect as chondroitin sulfate, but dermatan sulfate and hyaluronate have no measurable effect on glycosaminoglycan production by epithelial cells. Keratan sulfate however, seems to depress glycosaminoglycan synthesis. Thus, in this system, only sulfated polyanions like those produced by the corneal epithelium have a stimulatory effect on glycosaminoglycan synthesis. The results are discussed in terms of how the tissues of the cornea (epithelium, endothelium, keratocytes) may interact by changing the composition of the stromal extracellular matrix.**

Modulation of extracellular matrix (ECM) production by constituents of the extracellular milieu has been demonstrated so far in at least four embryonic systems. Nevo and Dorfman (1) showed that synthesis of chondromucoprotein by chondrocytes in suspension culture is enhanced by the addition of sulfated polysaccharides to the culture medium. Solursh and Meier (2) grew chondrocytes on tissue culture dishes and found that the cells produce conditioned medium factor(s) that promote both chondromucoprotein and collagen synthesis by chondrocytes. Kosher *et al.* (3) found that chondromucoprotein enhances the synthesis of sulfated glycosaminoglycans (GAG) by pre-chondrogenic somites and Meier and Hay (4) showed that collagen stimulates the synthesis and deposition of collagen and sulfated GAG by corneal epithelium. In this report, we examine the influence on corneal morphogenesis of the glycosaminoglycans which are present together with collagen in the corneal stroma.

The developing cornea is a particularly interesting embryonic system for the study of extracellular matrix synthesis because in the early stages of embryogenesis the epithelium makes the connective tissue stroma (4–6); subsequently, changes in the hydration and chemical composition of the ECM are correlated with the initiation of important new morphogenetic events (7, 8). The corneal epithelium is the first nonchondrogenic tissue to be employed in a study of the effect of GAG on the production of ECM by differentiating cells. The corneal stroma, however, resembles cartilage in its high content of sulfated polyanions. Of the total GAG synthesized by the 5-day-old-chick corneal epithelium, 75% is chondroitin sulfate and 25%, heparan sulfate-like material (9); the collagen occurs in the form of striated fibrils and basal lamina (6). The evidence to be presented here suggests that chondroitin and heparan sulfate synthesis by the corneal epithelium may be regulated by the adjacent ECM.

Abbreviations: GAG, glycosaminoglycan; ECM, extracellular matrix.

MATERIALS AND METHODS

Corneas were dissected from 5-day-old White Leghorn chick embryos (SPAFAS, Norwich, Conn.) and epithelia, isolated by a 7.5 min enzymatic digestion, were placed on either frozen-killed lens or Millipore filter (type HA, pore size 0.45 μm) as described previously (4, 5). Eight intact epithelia were used for each culture. Tissue plus substratum was placed on the metal support grid of an organ culture dish (Falcon, no. 3010) and incubated at 38° in a humidified gas mixture (95% air, 5% CO_2). The culture medium was Ham's F-12, supplemented with 10% fetal-calf serum (Grand Island Biological Co.), 0.25% whole embryo extract and antibiotics (9). DNA synthesis was measured by the incorporation of [*methyl*-^{3}H]-thymidine (6.7 Ci/mmol) at 5 μCi/ml of medium into hot perchloric-acid-soluble material while protein synthesis was monitored by the incorporation of L-[*G*-^{3}H]amino-acid mixture at 5 μCi/ml of medium into trichloroacetic-acid-precipitable material. All isotopes and scintillators were purchased from New England Nuclear Corp.

All polysaccharides were added to the culture medium at the concentration of 200 μg/ml used by Nevo and Dorfman (1) because this seemed to be the lowest chondromucoprotein dose with a maximum stimulatory effect on embryonic cartilage. It would be preferable to work with "physiological" doses, but we have no data on the physiological concentration of these various substances in the embryonic cornea. Chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, keratan sulfate-1 (isolated from bovine corneas), heparin (molar N-sulfate to hexosamine ratio of 0.89), heparan sulfate (molar N-sulfate to hexosamine ratio of 0.51) and hyaluronic acid were a gift of Drs. J. A. Cifonelli, M. B. Mathews, and L. Rodén. These are the purified acid mucopolysaccharide reference standards prepared under Grant 5R01HE11083 at the University of Chicago, Department of Pediatrics. Chondroitin sulfate (mixed isomers) and hyaluronic acid obtained from Sigma were used at the same concentration and were

TABLE 1. *The effect of stromal polysaccharides on collagen and sulfated GAG production by 5-day-old corneal epithelium cultured on Millipore or lens capsule**

Polysaccharide added	Substratum	Collagen synthesis (cpm)		Sulfated GAG synthesis (cpm)	
		Medium	Tissue	Medium	Tissue
Control	Millipore	962	1091	403	496
	Lens	954	4384	385	1437
Chondroitin-4-sulfate	Millipore	941	1043	842	878
	Lens	959	4400	803	2528
Chondroitin-6-sulfate	Millipore	914	1104	821	858
	Lens	982	4388	809	2491
Heparin	Millipore	923	1012	744	795
	Lens	978	4303	719	2295
Heparan sulfate	Millipore	933	1077	811	896
	Lens	961	4299	789	2544
Hyaluronate	Millipore	948	1084	416	502
	Lens	955	4260	392	1434

* One experiment typical of 12. Each of the 12 experiments has its own control in order to circumvent variations in sulfated GAG synthesis due to variations in the age of the epithelium (9). The per cent change in total polysaccharide production relative to the control of Millipore and lens for the 12 experiments is +87.0% ± 4.5 for chondroitin-4-sulfate, +84.9% ± 3.1 for chondroitin-6-sulfate, +68.3% ± 3.8 for heparin, +86.2% ± 4.2 for heparan sulfate, and +1.2% ± 2.2 for hyaluronate. Here and below Table 2, values are expressed as the mean ± the standard deviation about the mean. Cultures were labeled for 24 hr with 2.5 μCi/ml [^{3}H]proline or 10 μCi/ml $^{35}SO_4$.

found to have the same effects as the purified standards. Unless specified, the tables show data utilizing the Chicago reference standards. Polysaccharides (chondroitin sulfate, hyaluronate, and keratan sulfate) dialyzed against F-12 stock were as effective as GAG dissolved directly in F-12 medium. Purified adult chick sternal chondromucoprotein (mixed isomers of chondroitin-4 and 6-sulfate, glycosidically bound to a peptide) was a gift of Dr. Michael Solursh.

The term heparan sulfate will be used with following connotation in this report. The heparan-sulfate-like material which is said to be present in the corneal stroma at the embryonic period studied here is defined as sulfated GAG susceptible to nitrous acid degradation (9). Nitrous-acid-degradable material includes heparan sulfate, the more highly sulfated heparin and other possible variations of heparan sulfates (10); these products will be referred to collectively as heparan sulfate-like compounds. On the other hand, the purified standards added to the culture medium will be referred to as heparin, heparan sulfate, etc.

Polysaccharide production was monitored by measurement of the incorporation of $H_2{}^{35}SO_4$ (carrier free) at 5–10 μCi/ml of medium or D-[*U*-^{3}H]glucosamine (7.3 Ci/mM) at 10 μCi/ml of medium into enzyme-sensitive (8) or nitrous-acid-sensitive material (11) as described previously (9). Collagen production was measured by the incorporation of [*U*-^{3}H]proline (6.0 Ci/mmole) at 2.5 μCi/ml of medium into hot-trichloroacetic-acid-soluble material as described before (9). Since the rate of degradation of newly synthesized collagen and GAG produced by epithelia grown on Millipore filter or lens capsule is similar and is relatively insignificant (4), the total amount of collagen or GAG accumulated after the 24-hr culture period will be referred to as synthesis even though total production is a slight underestimate of total synthesis.

TABLE 2. *The effect of sulfated polysaccharides on collagen and sulfated GAG production by 5-day-old corneal epithelium cultured on Millipore or lens capsule**

Polysaccharide added	Substratum	Collagen synthesis (cpm)		Sulfated GAG synthesis (cpm)	
		Medium	Tissue	Medium	Tissue
Control	Millipore	923	1142	1095	1088
	Lens	966	4569	1015	2817
Sternal chondromucoprotein	Millipore	962	1221	1952	1984
	Lens	1022	4624	1883	5187
Dermatan sulfate	Millipore	919	1177	1121	1101
	Lens	979	4492	1086	2852
Keratan sulfate-1	Millipore	923	1185	886	884
	Lens	956	4505	839	2229

* One experiment typical of 8. The per cent change in total polysaccharide production relative to the control of Millipore and lens for the eight experiments is +84.5% ± 4.2 for sternal chondromucoprotein, +1.5% ± 3.7 for dermatan sulfate and −21.1% ± 3.6 for keratan sulfate-1. Cultures were labeled for 24 hr with 2.5 μCi/ml [^{3}H]proline or 10 μCi/ml $^{35}SO_4$.

RESULTS

In order to determine whether or not the types of glycosaminoglycans found in the primary corneal stroma could influence the production of extracellular materials by the corneal epithelium, we exposed cultures of corneal epithelium to 5 μCi/ml of $^{35}SO_4$ or 2.5 μCi/ml of [^{3}H]proline for 24 hr in the presence of chondroitin sulfate or heparan sulfate. The labeled GAG produced by the epithelia were isolated by cetylpyridinium chloride precipitation, while collagen was assayed by hot trichloroacetic acid extraction. As can be seen from Table 1, none of the polysaccharides added to the medium had any influence on collagen production on either of the substrata employed. It was shown previously (4) that on an "inductive" substratum (lens capsule), corneal epithelium produces over three times as much collagen as on a "noninductive" substratum (Millipore filter). The sulfated polysaccharides, chondroitin sulfate, heparin, and heparan sulfate, stimulate the production of sulfated GAG by the corneal epithelium cultured either on Millipore filter or on lens capsule. Remarkably, the stimulatory effect of sulfated polysaccharides added to the culture medium is relatively the same regardless of the substratum. About a 2-fold increase occurs (Table 1) on both Millipore filter and lens capsule in both medium and tissue. Hyaluronate, a nonsulfated polysaccharide produced by the endothelium of the cornea (9, 12), has no effect on either collagen or sulfated GAG synthesis (Table 1).

Since it is known that cartilage cells in suspension culture (13) produce more chondromucoprotein when any large sulfated polyanion is added to the medium (1), we attempted to determine if there was any specificity in the response of the corneal epithelium to sulfated polysaccharides. The results in Table 2 indicate that the highly charged polyanion, dermatan sulfate, has no effect on matrix synthesis by corneal epi-

TABLE 3. *Identification of the polysaccharides produced by the 5-day-old corneal epithelium cultured on Millipore filter in the presence of sulfated polysaccharides**

Polysaccharide added	Sample source	Total counts in glycosaminoglycans: Cetylpyridinium-chloride-precipitable	Testicular-hyaluronidase-sensitive (%)	Nitrous-acid-sensitive (%)	Leech-hyaluronidase-sensitive (%)
Control	Medium	805	76.4	24.1	0.4
	Tissue	796	72.1	27.8	0.2
Chondroitin sulfate	Medium	1549	75.8	23.4	1.2
	Tissue	1657	73.9	25.8	0.8
Heparan sulfate	Medium	1459	75.5	24.3	0.5
	Tissue	1531	73.6	25.7	1.1
Keratan sulfate	Medium	641	80.4	18.5	0.5
	Tissue	649	81.2	17.9	0.8

* One experiment typical of 6. Cultures were labeled with 10 μCi/ml [^{3}H]glucosamine.

thelium. Chondromucoprotein (chondroitin sulfate proteoglycan isolated from chick sterna) stimulates sulfated GAG production almost 2-fold, essentially to the same extent as did chondroitin sulfate. Keratan sulfate isolated from bovine cornea, however, has a moderate inhibitory effect on sulfated GAG synthesis by the corneal epithelium and here again there seems to be no effect on collagen synthesis (Table 2).

In order to determine whether or not the stimulatory or inhibitory effects of chondroitin sulfate, heparan sulfate, and keratan sulfate on GAG synthesis were specific for a particular polysaccharide made by the corneal epithelium, cultures were labeled with 10 μCi/ml of [^{3}H]glucosamine and the labeled polysaccharides were identified by enzyme extraction (9). Chondroitin sulfate and heparan sulfate stimulate the synthesis of both chondroitin sulfate (testicular-hyaluronidase-sensitive material, Table 3) and heparan-sulfate-like material (nitrous-acid-sensitive material, Table 3). The 3:1 ratio of chondroitin sulfate to heparan sulfate, which is characteristic of the secretion produced by unstimulated epithelium (9), was maintained regardless of which of these two stimulatory polysaccharides were added to the medium. However, the inhibitory effect of keratan sulfate may be largely on heparan sulfate synthesis rather than on chondroitin sulfate synthesis, since the ratio of labeled chondroitin sulfate to heparan sulfate was raised to 4:1 from 3:1. The addition of polysaccharides to the culture medium never resulted in the production of hyaluronate by the corneal epithelium; the evidence is that there was no significant glucosamine-labeled material susceptible to leech hyaluronidase, hyaluronate being the only glycosaminoglycan known to be sensitive to this enzyme (8).

Polysaccharides added to the culture medium failed to alter protein synthesis (as measured by the incorporation of tritiated amino acids into trichloroacetic-acid-precipitable material) or DNA synthesis (as measured by the incorporation of tritiated thymidine into hot-perchloric-acid-soluble material) in the 24-hr period under study. The DNA data obtained on these cultures (Table 4 of ref. 4) thus can be used to express the stimulatory effect on GAG synthesis on a per cell (per DNA) basis. When either chondroitin or heparan sulfate is added to epithelium cultured on Millipore filter, cpm/μg DNA are increased 2-fold, from 1083 $\pm$ 127 to 2056 $\pm$ 241, and when lens capsule is the substratum cpm/μg DNA are increased from 1822 $\pm$ 91 to 3333 $\pm$ 166. Interestingly enough, agar, a sulfated polysaccharide, was unsuitable as a substratum, for it caused extensive cell death (4). Finally, we can point out that enhanced incorporation of sulfate into polysaccharides by GAG is not just the result of oversulfation of polysaccharide molecules. The data in Table 3 indicate that nearly a 2-fold stimulation of polysaccharide production by added GAG occurred when glucosamine was the label, just as when $^{35}SO_4$ was the label (Table 1).

DISCUSSION

The most significant finding of this study is the demonstration of a similar stimulatory effect of chondroitin sulfate, heparin, and heparan sulfate on chondroitin and heparan sulfate synthesis by 5-day-old corneal epithelium grown on a natural substratum (lens capsule) or on Millipore filter. We showed earlier that either pure collagen or lens capsule stimulates corneal epithelium to make two to three times as much collagen and glycosaminoglycan (GAG) as the epithelium produces on Millipore filter (4). Even when GAG production is not optimal, as on Millipore filter, the stimulatory effect of either chondroitin sulfate or the heparan sulfate class of compounds is to double the amount of GAG that each cell is producing. Moreover, the level of GAG stimulation achieved by collagen (4) can be further amplified by these two types of mucopolysaccharides.

The fact that corneal epithelial cells react differently to different ECM molecules is of considerable interest. Any type of collagen (4) seems to stimulate both GAG and collagen synthesis by corneal epithelium. Hyaluronate, a nonsulfated GAG, which has an inhibitory effect on chondrogenesis (1, 17), has no effect on matrix production by corneal epithelium. Minimizing the possibility that the corneal epithelium is capable of being stimulated by any sulfated polysaccharide is the evidence that dermatan sulfate fails to influence corneal extracellular matrix production and keratan sulfate depresses GAG synthesis by corneal epithelium. The fact that chondroitin sulfate proteoglycan is capable of stimulating GAG synthesis indicates that the peptide portion of the chondromucoprotein does not interfere with the interaction of the epithelium and the polysaccharide portion of the molecule. Only the classes of GAG that the corneal epithelium produces seem to be able to enhance synthesis of GAG by the epithelium at the concentrations used here and by Nevo and Dorfman (1). In what way could chondroitin sulfate and heparan-sulfate-like compounds interact with corneal epithelial cells to bring about such a striking stimulation of GAG synthesis?

Nevo and Dorfman (1) postulated that chondromucoprotein interacts with components of the cell membrane to activate preexisting metabolic machinery of chondrocytes. There was no evidence of induction of glycosyltransferases and, as in the present study, collagen synthesis was not affected by GAG. There is biochemical (10) and morphological (12, 14) evidence of a close relation between GAG and the cell surface, just as extracellular collagen also seems to be in close contact with, and is perhaps part of, the cell membrane (15). If such postulated sites of interaction of GAG and collagen with the corneal cell membrane exist, however, they are likely

to be different because collagen affects both GAG and collagen synthesis, whereas GAG affects only GAG synthesis. Nevo and Dorfman (1) suggested an alternative hypothesis, namely, that large polyanions might bind to and inactivate an inhibitory factor for GAG synthesis in the chondrocyte cytoplasm or culture medium.

Bernfield *et al.* (14) proposed that surface-associated mucopolysaccharides may control salivary gland branching by regulating calcium access to the plasmalemma of cells. Similarly, Kosher *et al.* (3) suggested that somite chondrogenic expression may be controlled by the ability of chondromucoprotein to bind, sequester, or regulate cations found in the cellular microenvironment. Lippman (16) proposed that glycosaminoglycans on the cell surface might act as cation traps and Toole *et al.* (17) emphasize the calcium-binding capacity of hyaluronate as an explanation for its ability to inhibit cartilage nodule formation *in vitro*. Since all of the acid mucopolysaccharides have a high negative charge, moreover, it is difficult to rule out the possibility that cations or other contaminants tightly bound to the GAG contribute to their reported morphogenetic effects.

The results of the *in vitro* work reported here seem to have relevance to the understanding of tissue interaction during corneal morphogenesis *in vivo*. The newly invaginated lens induces the overlying epithelium to produce the primary corneal stroma (18) and it has been suggested that the collagen and GAG of the lens capsule may influence epithelial differentiation (15). The corneal epithelium subsequently synthesizes chondroitin sulfate, heparan-sulfate-like compounds, and collagen. The fact that these classes of compounds promote their own synthesis may explain the continued production of corneal stroma by the epithelium even after it becomes separated from the lens by the endothelium (4, 7). There is no evidence to suggest that endothelium influences epithelial differentiation. However, the principal product of the endothelium, hyaluronate (9, 12), may play a role in the formation of the secondary stroma. By the end of the fifth day of development the hyaluronate-rich stroma begins to swell and then is invaded by fibroblasts (keratocytes) which synthesize most of the keratan sulfate, heparan sulfate, chondroitin sulfate, and collagen (19) found in the secondary stroma. It is tempting to speculate that the changing composition of the extracellular matrix not only is responsible for the dynamic structural remodeling taking place during embryogenesis, but also is informational in directing and regulating cell differentiation.

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