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Modification of Proteoglycan Synthesis by Corneal Stromal Cells on Co-Culture with Either Epithelial or Endothelial Cells

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Corneal stromal cells, prepared from 2-day-old chicks and embedded in collagen gel on an insert dish, were co-cultured with corneal epithelial cells or endothelial cells on a fibronectin-coated companion plate. Cell growth and proteoglycan synthesis, as determined as the incorporation of [^{35}S]sulfate and [^{3}H]glucosamine, were examined for each cell type in both combinations. In comparison with single cultures, growth was affected little, while proteoglycan synthesis was appreciably altered in both combinations. Stromal and epithelial cells stimulated proteoglycan synthesis by each other, while endothelial cells stimulated the synthesis by stromal cells, but stromal cells reduced that by endothelial cells to some extent. Endothelial cells alone synthesized proteoglycans much more actively than did epithelial or stromal cells. Analysis of the radiolabeled proteoglycans revealed that endothelial and epithelial cells had different effects on proteoglycan synthesis by stromal cells: the former tended to increase keratan sulfate synthesis by stromal cells, while the latter tended to increase chondroitin sulfate/dermatan sulfate synthesis. Proteoglycan synthesis in the corneal stroma *in vivo* might thus be controlled by the balance between the antipodal actions of the epithelial and endothelial cell layers.

Key words: chondroitin sulfate/dermatan sulfate, co-culture, corneal cells, keratan sulfate, proteoglycan synthesis.

Mesenchymal-epithelial interactions are known to be of great importance during embryonic development (*1-3*). During organogenetic secondary induction, the mesenchyme induces patterns of branching morphogenesis (*4*), specifies the forms and spatial organization of epidermal derivatives (*5*), and elicits specific programs of epithelial cytodifferentiation and functional activity (*6, 7*). Mesenchymal-epithelial interactions are usually reciprocal, and the epithelial signals to the mesenchyme are involved in many fundamental developmental mechanisms (*7-9*). Such controls can extend into adulthood, and it is clear that mesenchymal interactions also play an important role in the maintenance of adult organs (*10*). For example, the synthesis of site- and appendage-specific cytokeratins by epithelial cells is specified by the underlying adult dermis (*11, 12*). Furthermore, it has been demonstrated that epithelial proliferation is stimulated by estrogen in mixed cultures of mammary epithelial and stromal cells in regions where epithelial cells are in contact with stromal cells (*13*).

The adult cornea is composed of three layers: epithelial, stromal, and endothelial. The stromal-epithelial or stromal-endothelial interaction is also of importance for maintaining the morphology and function of the cornea, as for the other tissues described above. Wilson *et al.* (*14*) have reported the effects of growth factors, which are synthesized and secreted by human corneal stromal cells, on the proliferation, motility, and differentiation of corneal epithelial cells. Sotozono *et al.* (*15*) have also described a paracrine role of keratinocyte growth factor in rabbit corneal epithelial cell growth. Furthermore, the stroma of the adult cornea, through the action of the extracellular matrix synthesized by stromal cells, is of importance in the regulation of corneal epithelial differentiation and proliferation (*16-18*). In addition, Samples *et al.* (*19*) have reported the effects of growth factors on the proliferation of human corneal endothelial cells. Probably, the endothelial-stromal interaction is involved in maintenance of the corneal function, like the epithelial-stromal interaction.

Proteoglycan (PG) and type I collagen constitute the major components of the corneal stroma. PGs occupy the region between collagen fibrils (*20*), and influence fibril assembly and stromal organization (*21-23*). Thus, PGs may play an important role in the maintenance of corneal transparency. The biosynthesis of PG in the corneal stroma readily changes under various conditions *in vivo* and *in vitro*. Corneal scarring results in alteration of the expression type of PG (*24, 25*). When corneal stromal cells are cultured on a plastic dish *in vitro*, the expression pattern of PG also changes markedly (*26-28*). Furthermore, it is expected that the PG biosynthesis in the corneal stroma *in vivo* is influenced by the corneal epithelial and endothelial layers. Previously, we (*29*) reported that when each constituent layer (epithelial, stromal, and endothelial) of the

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Abbreviations: CS/DS, chondroitin sulfate/dermatan sulfate; Endo, endothelial cells; Epi, epithelial cells; FBS, fetal bovine serum; HS, heparan sulfate; KS, keratan sulfate; PG, proteoglycan; Str, stromal cells.

chick cornea was separately cultured, PG synthesis by each layer decreased markedly compared with that in the corresponding layer cultured as part of a whole intact cornea. This suggests that the corneal constituent layers interact with each other in PG synthesis. To clarify this interaction, in this study, chicken epithelial and stromal cells or endothelial and stromal cells were co-cultured on insert dishes and companion plates, and then the radiolabeled PGs synthesized were analyzed.

MATERIALS AND METHODS

Materials—White Leghorn male chicks (2 days old) were obtained from Hattori Youkei-en, Nagoya. [^{35}S]Sulfuric acid (carrier-free) was purchased from the Japan Isotope Association, Tokyo, and D-[6-^{3}H]glucosamine hydrochloride from Amersham International, UK. Ham's F-12 medium was purchased from Nissui Seiyaku, Tokyo, and fetal bovine serum (FBS) from Boehringer Mannheim, Tokyo. Fibronectin (bovine plasma) and chondroitin 4-sulfate (whale cartilage) were purchased from Seikagaku, Tokyo. The following enzymes were obtained from the commercial sources indicated: chondroitinase ABC (from *Proteus vulgaris*; Seikagaku), keratanase (from *Pseudomonas* sp.; Seikagaku), collagenase (from *Clostridium histolyticum*; Wako Pure Chemicals, Osaka), dispase (grade II, from *Bacillus polymyxa*; Boehringer Mannheim, Tokyo), and pronase (actinase A) (from *Streptomyces griseus*; Kaken Kagaku, Tokyo). Type I collagen (Cellmatrix I-A, 3.0 mg/ml HCl solution, pH 3.0; from porcine tendon) was purchased from Nitta Gelatin, Osaka. PD-10 (prepacked disposable Sephadex G-25 column), DEAE-Sephacel and Sepharose CL-4B were purchased from Pharmacia Biotech, Tokyo. Cell culture insert dishes and 6-well companion plates (Falcon) were purchased from Becton Dickinson Labware, USA.

Cell Culture and Labeling with Radioactive Precursors—Corneas were dissected from 2-day-old male chicks, and trimmed free of the limbus and scleral ossicles. The corneas were separated into the epithelial, stromal, and endothelial layers as described previously (*29*). The stromal layers were digested with collagenase, and the resultant cell suspension was mixed with a type I collagen solution as described in the previous paper (*30*). Stromal cells ($1.4-2.0\times10^5$ cells) embedded in collagen gel (1.2 ml) were placed on an insert dish (4.30 cm^2; pore size of filter membrane, 0.45 μm). The epithelial and endothelial layers were digested with dispase, respectively, and each resultant cell suspension (epithelial cells, 8.5×10^5; endothelial cells, 1.2×10^5) was placed in the wells (9.6 cm^2) of a companion plate which had been coated with 100 μg of fibronectin in advance. Then, 2.0 ml of Ham's F-12/10% FBS was added to each well. The insert dish with stromal cells embedded in collagen gel was overlaid on the medium in a well and then 1.0 ml of Ham's F-12/10% FBS was added to the insert dish. Co-cultures were conducted with stromal cells in collagen gel on the insert dish and epithelial cells or endothelial cells on the companion plate. As controls, stromal cells in collagen gel on the insert dish were cultured with a companion plate without cells, and collagen gel without cells on the insert dish was cultured with epithelial cells or endothelial cells on the companion plate. The cultures were performed in duplicate in a CO_2 incubator (95% air-5% CO_2) at 37°C, and the medium was replaced daily. One of the duplicates was used for the isolation of PGs, and the other for determining the cell number with a hemocytometer.

On the 9th day of co-culture of stromal cells/epithelial cells, and the 8th day of co-culture of stromal cells/endothelial cells, the medium was removed. Then 1.0 ml of Ham's F-12 containing 0.3% FBS, 250 μCi of sodium [^{35}S]sulfate and 25 μCi of [^{3}H]glucosamine-HCl was added to each insert dish, and 2.0 ml of the medium containing 0.3% FBS, 500 μCi of sodium [^{35}S]sulfate and 50 μCi of [^{3}H]glucosamine-HCl was added to each well of the companion plate. The insert dishes were placed on the corresponding wells of the companion plates, and the cultures were kept in the CO_2 incubator at 37°C for 18 h for radiolabeling.

Isolation and Analysis of Proteoglycans—After radiolabeling, each culture was separated into four parts: medium in the insert dish (hereafter referred to as "Medium I"), collagen gel with embedded stromal cells [Gel (Str)], medium on the epithelial or endothelial cell layer in the wells of the companion plate (Medium II), and epithelial cell layer [Cell layer (Epi)] or endothelial cell layer [Cell layer (Endo)] in the wells. The Medium I and Medium II fractions were chromatographed on PD-10 to remove the unincorporated radioactive precursors. The gel and cell layer fractions were extracted with 4 M guanidine-HCl/protease inhibitors (*30*). The chromatographed Medium I and Medium II, and all the extracts were dialyzed against 7 M urea solution, followed by chromatographies on DEAE-Sephacel columns as described in the previous paper (*30*).

To determine the glycosaminoglycan compositions of PGs, portions of the PG fractions obtained above were digested with 0.5 unit (μmol of unsaturated disaccharide/min) of chondroitinase ABC at 37°C for 2 h or with 0.5 unit (μmol of reducing end/h) of keratanase at 37°C for 4 h in 0.05 M Tris-HCl, pH 7.5/protease inhibitors (*30*). The digests were chromatographed on Sepharose CL-4B columns (1.0×100 cm) equilibrated with 0.1% SDS/10 mM EDTA/0.05 M Tris-HCl (pH 7.5) as described in the previous paper (*30*). To each of the remaining portions of the PG fractions, chondroitin 4-sulfate was added, as a carrier, to 1 mg/ml, followed by 3 volumes of ethanol containing 1.3% potassium acetate. The resultant precipitates were dried in a vacuum, and then digested with 500 μg of pronase in 0.1 M Tris-HCl, pH 8.0. The digests were treated with sodium nitrite for analysis of heparan sulfate (HS) as described in the previous paper (*29*).

RESULTS

Co-Culture of Epithelial Cells and Stromal Cells—Co-cultures were performed as described under "MATERIALS AND METHODS." The cell numbers and the incorporation of radioactive precursors into each fraction are shown in Table I. A considerable number of the inoculated epithelial cells did not adhere to the fibronectin-coated plate and thus were lost on the first day of culture. The remaining cells did not grow markedly and the growth was not stimulated on co-culture with stromal cells. Similarly, the growth of stromal cells was not stimulated on co-culture with epithelial cells. However, the incorporation of radioactive precursors (PG synthesis) in both cell types increased markedly on co-

culture (total ^{3}H and ^{35}S incorporation by each culture in Table I). In particular, the incorporation of ^{35}S in stromal cells was increased on co-culture [compare the Gel (Str) fractions or Medium I fractions for cultures Str and Str/Epi], while ^{3}H incorporation into both Gel (Str) fractions was similar, suggesting that PG synthesis by stromal cells may be specifically stimulated on co-culture. In epithelial cells, the incorporation of ^{3}H and ^{35}S increased markedly on co-culture with stromal cells [compare the Cell layer (Epi) fractions for cultures Epi and Str/Epi]. The increase in incorporation into the epithelial fraction exceeded that into the stromal fraction, showing that PG synthesis by epithelial cells is more strongly stimulated on co-culture than that by stromal cells. Although the total incorporation of ^{3}H and ^{35}S by epithelial cells alone is much less than that by stromal cells, the incorporation into Medium II of Epi is similar to that of Str. Therefore, a comparable part of PG in Medium II of co-culture Str/Epi must have been derived from that synthesized by epithelial cells (see below). Because the incorporation in the Medium I and Gel fractions of culture Epi and in the Well bottom of culture Str was very low, these fractions were not analyzed further.

PGs were isolated from each extract and medium by DEAE-Sephacel chromatography as described under "MATERIALS AND METHODS." The PG fractions were

TABLE I. **Growth of stromal and epithelial cells, and incorporation of [^{35}S]sulfate and [^{3}H]glucosamine into macromolecules synthesized by both cell types and co-cultured cells.**[a]

Culture[b]	Cell number ($\times 10^5$ cells/dish or well)	Fraction	Incorporation ^{3}H (dpm$\times 10^{-3}$/dish or well)	Incorporation ^{35}S (dpm$\times 10^{-3}$/dish or well)
Str	10.4	Medium I	546	3,630
		Medium II	263	1,390
		Gel (Str)	2,520	17,900
		Well bottom	11	36
		Total	3,329	22,956
Epi	1.22	Medium I	18	26
		Medium II	337	1,311
		Gel	51	66
		Cell layer (Epi)	349	1,260
		Total	755	2,663
Str/Epi	Str 10.6	Medium I	779	4,900
	Epi 1.21	Medium II	1,290	7,200
		Gel (Str)	2,580	24,200
		Cell layer (Epi)	1,220	5,700
		Total	5,869	42,000

[a]The table is a composite of two separate experiments; standard errors are <10% of the means. [b]Str, stromal cells; Epi, epithelial cells; Str/Epi, stromal and epithelial cells.

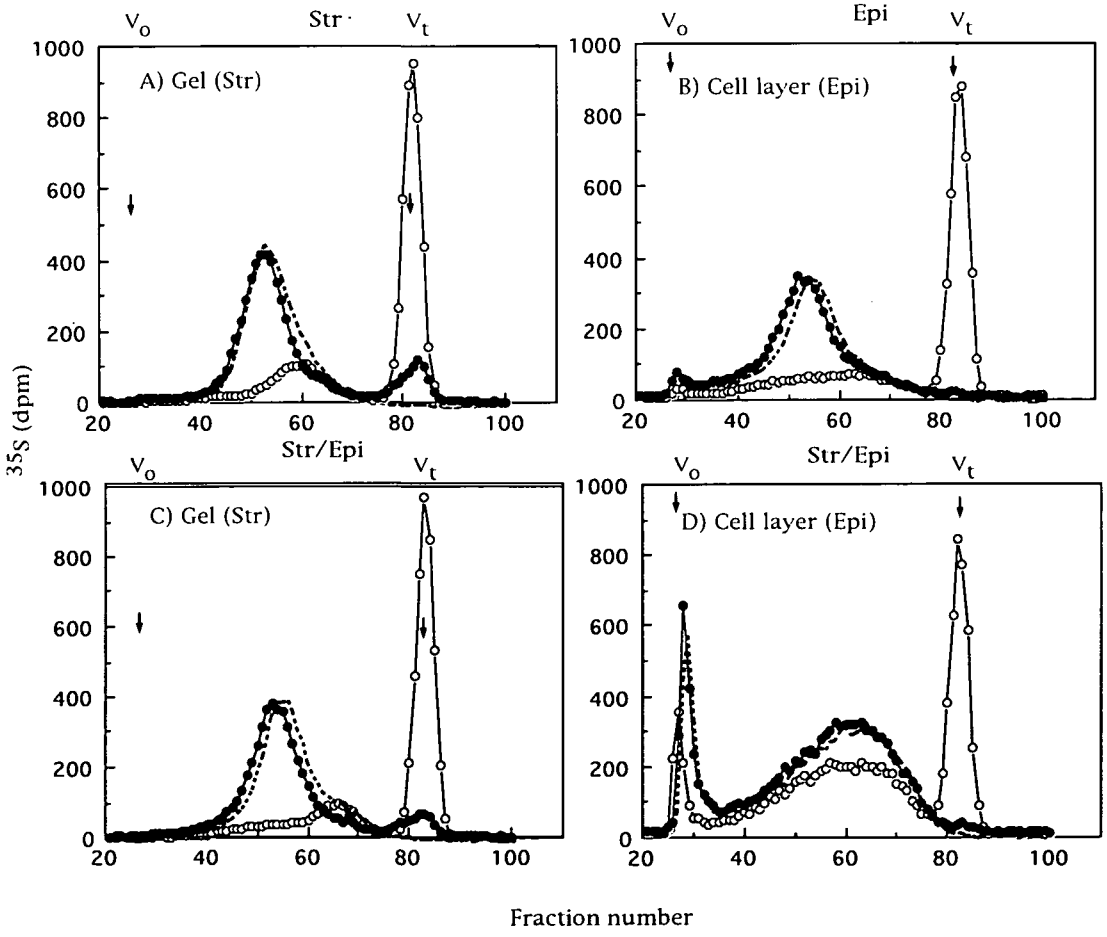

Fig. 1. **Sepharose CL-4B chromatography of cell layer and gel proteoglycans from cultures of stromal cells (Str), and epithelial cells (Epi), or a co-culture of both cell types (Str/Epi), and their enzymatic digests.** Dotted line, intact proteoglycans; open circles, chondroitinase ABC digests; closed circles, keratanase digests. Only ^{35}S activity is shown in the panels. V_o, void volume; V_t, total volume.

digested with keratanase or chondroitinase ABC, and the digests were chromatographed on Sepharose CL-4B columns to determine their glycosaminoglycan compositions. Figure 1 shows Sepharose CL-4B chromatography of the cell layer and gel PG fractions, and their enzymatic digests. The elution profiles of Gel (Str) proteoglycans and their digests were similar between culture Str and co-culture Str/Epi (A and C in Fig. 1). In contrast, those of Cell layer (Epi) changed on co-culture with stromal cells (B and D); a prominent peak appeared at the void volume for the Cell layer (Epi) fraction from co-culture Str/Epi. A comparable part of this peak was resistant to both chondroitinase ABC and keratanase, showing that the peak contains a large sulfated glycoprotein (*29*). When epithelial cells were cultured alone, the peak at the void volume was found in Medium II (not shown) rather than Cell layer (Epi). This suggests that the secretion of a large PG and the large sulfated glycoprotein by epithelial cells is inhibited on co-culture with stromal cells.

From the results of Sepharose CL-4B chromatographies and of nitrite treatment of glycosaminoglycan fractions, the proportions of keratan sulfate (KS), chondroitin sulfate/dermatan sulfate (CS/DS), and heparan sulfate (HS) were calculated, and are summarized in Table II. When stromal cells were cultured alone, a considerable amount of HS was found in the Medium I, Medium II, and Gel (Str) fractions. As described in the previous paper (*29*), when stromal tissues were cultured for 5 h, only a very small amount of HS was found (4.4% for medium; 1.5% for stroma). It appears that HS synthesis by stromal cells in culture increases when the cells are cultured within gels, as it is when the cells are cultured on plastic dishes (*26*). Table II also reveals that epithelial cells hardly synthesize KS. In view of the results of Sepharose CL-4B chromatography described above, it appears likely that Others for the Cell layer (Epi) fraction of Str/Epi and Others for the Medium II fraction of Epi both include a comparable amount of the large sulfated glycoprotein. The percentages of KS in the Medium I and Gel (Str) fractions of Str/Epi are clearly less than that in Str. On the other hand, the percentages of CS/DS are similar in the corresponding fractions. That the overall PG synthesis by stromal cells increased on co-culture with epithelial cells suggests that epithelial cells stimulate CS/DS synthesis by stromal cells rather than KS synthesis. From the similar percentages of HS, it appears that epithelial cells also stimulate HS synthesis by stromal cells. The low percentage of KS in Medium II of Str/Epi can be accounted for by the CS/DS and HS secreted by epithelial cells. The CS/DS value in parenthesis on the bottom line of Table II was determined by chondroitinase ABC digestion of the isolated glycosaminoglycan fraction. Although the reason is unkown, the percentage of epithelial CS/DS detected on analysis of the intact PG fraction was frequently lower than that on analysis of the glycosaminoglycan fraction, which was isolated after pronase digestion of the PG (*29*).

Co-Culture of Endothelial and Stromal Cells—Cell culture, isolation and analysis of PGs were performed as described above, except that the cultures were stopped on the 8th day and radiolabeled, because it was observed in a separate experiment that a fair part of the endothelial cells was detached from the plates on the 9th day of culture. The growth of endothelial and stromal cells, and their incorporation of radioactive precursors are summarized in Table III. The cell number and incorporation for stromal cells alone (culture Str) are considerably less than the corresponding values in Table I. The decrease in total ^{35}S incorporation is much more than the decrease in ^{3}H incorporation, suggesting that a change occurred in the PG metabolism in stromal cells in this experiment. This difference may have been caused by the stopping of the culture on the 8th day rather than on the 9th day in the above experiment, although the precise reason is unknown. As can be seen in Table III, the growth of endothelial cells was not stimulated on co-culture with stromal cells, whereas the growth of stromal cells was stimulated to some extent by endothelial cells. The ^{35}S incorporation into the Medium I and Gel (Str) fractions in culture Str/Endo is about twice as high as that in Str (although the difference in the ^{3}H incorporation is relatively small). Even if the higher

TABLE II. **Glycosaminoglycan compositions of proteoglycans synthesized by stromal cells, epithelial cells, and co-cultured cells.**[a]

Culture	Fraction	Percent of total ^{35}S incorporation			
		KS[b]	CS/DS[b]	HS[b]	Others
Str	Medium I	24.8	52.8	14.3	8.1
	Medium II	24.3	42.5	13.5	19.7
	Gel (Str)	12.9	74.2	7.7	5.2
	Well bottom	—[c]	—	—	—
Epi	Medium I	—	—	—	—
	Medium II	1.3	50.1	19.3	29.3
	Gel	—	—	—	—
	Cell layer (Epi)	1.1	69.8	17.1	12.0
Str/Epi	Medium I	19.5	50.2	15.1	15.2
	Medium II	10.7	43.6	23.1	22.6
	Gel (Str)	9.3	74.5	10.1	6.1
	Cell layer (Epi)	1.4	39.1 (48.6)[d]	24.7	34.8 (25.3)

[a]The table is a composite of two separate experiments; standard errors are < 5% of the means. [b]KS, keratan sulfate; CS/DS, chondroitin sulfate/dermatan sulfate; HS, heparan sulfate. [c]Not determined. [d]The value in parenthesis was determined by chondroitnase digestion of the isolated glycosaminoglycan fraction, and the corresponding value of Others is indicated in parenthesis in the last column.

TABLE III. **Growth of stromal and endothelial cells, and incorporation of [^{35}S]sulfate and [^{3}H]glucosamine into macromolecules synthesized by both cell types and co-cultured cells.**[a]

Culture[b]	Cell number ($\times 10^5$ cells/dish or well)	Fraction	Incorporation (^{3}H (dpm$\times 10^{-3}$/dish or well))	Incorporation (^{35}S (dpm$\times 10^{-3}$/dish or well))
Str	7.80	Medium I	199	598
		Medium II	250	277
		Gel (Str)	1,560	5,550
		Well bottom	11	22
		Total	2,020	6,447
Endo	4.47	Medium I	46	29
		Medium II	3,100	14,300
		Gel	185	410
		Cell layer (Endo)	2,600	13,000
		Total	5,931	27,739
Str/Endo	Str 11.4	Medium I	329	1,380
	Endo 4.50	Medium II	3,130	13,000
		Gel (Str)	2,490	11,200
		Cell layer (Endo)	1,980	8,950
		Total	7,929	34,530

[a]The table is a composite of two separate experiments; standard errors are < 10% of the means. [b]Str, stromal cells; Endo, endothelial cells; Str/Endo, stromal and endothelial cells.

number of stromal cells in the co-culture is taken into account, PG synthesis by stromal cells was clearly stimulated on co-culture with endothelial cells. Conversely, PG synthesis by endothelial cells was reduced to some extent on co-culture with stromal cells, as shown by the ^{3}H and ^{35}S incorporation of the Cell layer (Endo) fractions. Interestingly, it appears that endothelial cells alone in culture synthesize PGs much more actively than do epithelial or stromal cells. Because the incorporation in the Well bottom fraction of culture Str and the Medium I fraction of culture Endo was very low, these fractions were not analyzed further. But the Gel fraction of culture Endo was analyzed as to PGs.

PGs were isolated from the respective fractions by DEAE-Sephacel chromatography, and the PG fractions and their enzymatic digests were chromatographed on Sepharose CL-4B columns as described above. Figure 2 shows Sepharose CL-4B chromatography of the gel and cell layer fractions, and their digests. The profiles of Cell layer (Endo) in Fig. 2C revealed that a considerable amount of a large CS/DS PG eluted at the void volume is present in the endothelial cell layer fraction. The large CS/DS PG was also found in the Medium II fractions of Str/Endo and Endo (not shown). The profiles of the Gel (Str) and Cell layer (Endo) fractions hardly changed on the co-culture of stromal and endothelial cells (Fig. 2, A and D; Fig. 2, C and E).

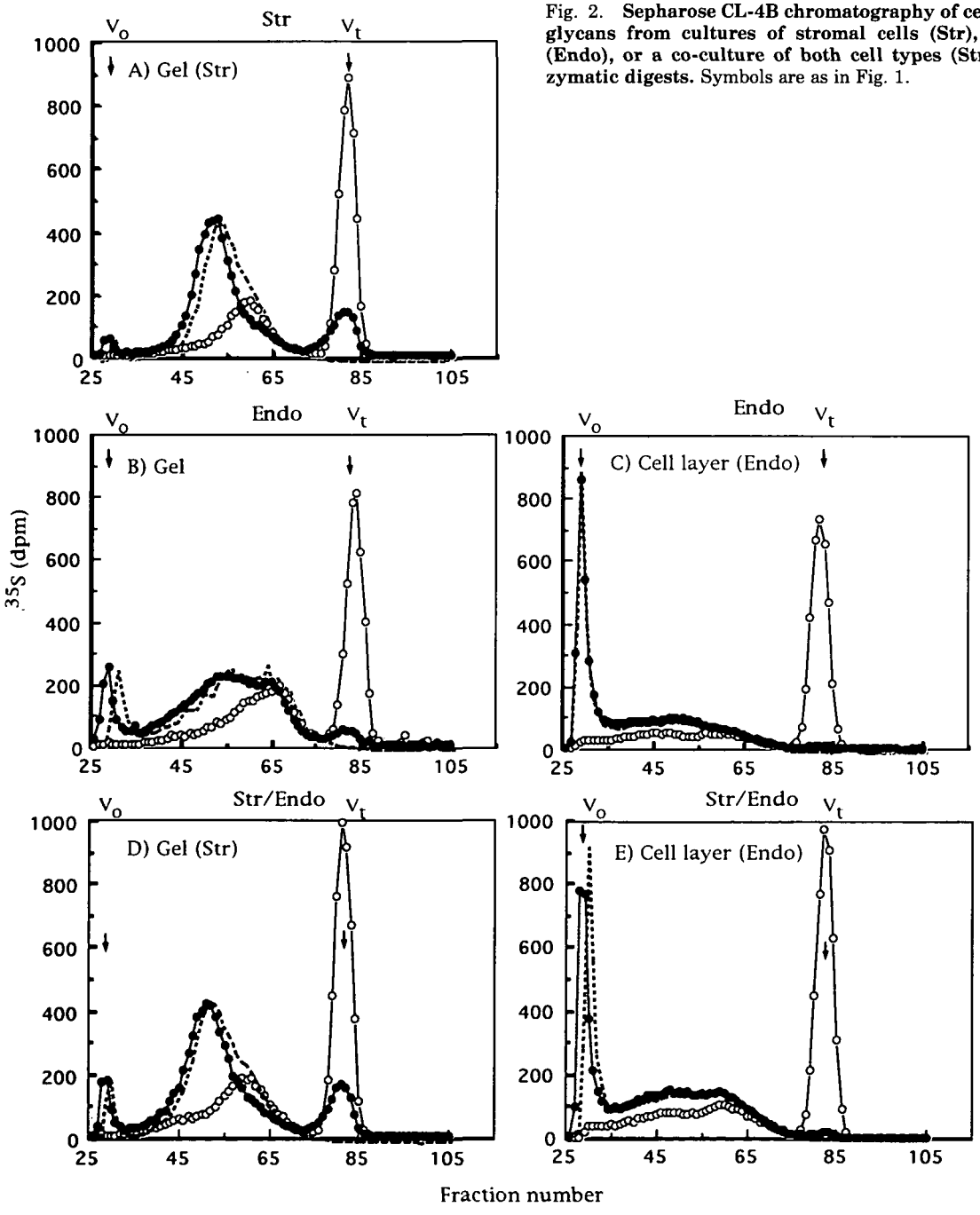

Fig. 2. **Sepharose CL-4B chromatography of cell layer and gel proteoglycans from cultures of stromal cells (Str), and endothelial cells (Endo), or a co-culture of both cell types (Str/Endo), and their enzymatic digests.** Symbols are as in Fig. 1.

TABLE IV. **Glycosaminoglycan compositions of proteoglycans synthesized by stromal cells, endothelial cells, and co-cultured cells.**[a]

Culture	Fraction	Percent of total ^{35}S incorporation			
		KS	CS/DS	HS	Others
Str	Medium I	12.9	64.5	12.8	9.8
	Medium II	24.2	32.0	13.1	30.7
	Gel (Str)	15.8	63.4	9.1	11.7
	Well bottom	–	–	–	–
Endo	Medium I	–	–	–	–
	Medium II	1.5	59.4	8.5	30.6
	Gel	5.3	54.9	14.4	25.4
	Cell layer (Endo)	1.1	68.4	17.4	13.1
Str/Endo	Medium I	32.5	30.7	8.8	28.0
	Medium II	4.9	61.2	14.7	19.2
	Gel (Str)	14.7	60.3	12.7	12.3
	Cell layer (Endo)	1.7	61.1	23.1	14.1

[a]The table is a composite of two separate experiments; standard errors are <5% of the means. Abbreviations are as in Tables II and III.

The Gel fraction of Endo (Fig. 2B) must be the PGs which were synthesized and secreted into medium by endothelial cells. The profile of the intact fraction (dotted line) (Fig. 2B) showed much less of the large CS/DS PG in comparison with the Cell layer (Endo) fraction (Fig. 2C), and was also different from that of the Medium II fraction of Endo (not shown). Most of the PGs in the Gel fraction were of a smaller molecular size than those in the Cell layer (Endo) fraction and the Medium II fraction of Endo. Furthermore, the profile of the keratanase digest (closed circles) (Fig. 2B) showed the presence of a small amount of KS in the Gel fraction.

The glycosaminoglycan compositions were determined by Sepharose CL-4B chromatography and HS analysis, and are summarized in Table IV. The glycosaminoglycan composition of each fraction of Str in Table IV is different from that of the corresponding fraction of Str in Table II. As described above, this discrepancy may be related to the ending of the culture on the 8th day. However, the percentage of KS in the Medium I fraction of co-culture Str/Endo (32.5%) exceeds that of culture Str not only in Table IV (12.9%) but also in Table II (24.8%). This shows that, in contrast to epithelial cells, endothelial cells tend to increase KS synthesis by stromal cells. Endothelial cells themselves also seem to synthesize a small amount of KS, as seen from the KS percentage (5.3%) in the Gel fraction of Endo (*31*), although it was hardly found in the Medium II fraction of Endo. The low percentage of KS (4.9%) in the Medium II fraction of Str/Endo can be accounted for by the active synthesis and secretion of PGs other than KSPG by endothelial cells.

DISCUSSION

In this study, co-cultures were performed with insert dishes and companion plates. Collagen gel containing stromal cells on an insert dish was readily separable from the epithelial or endothelial cell layer on a companion plate, allowing the growth of each cell type to be examined. In addition, the PGs in each gel, cell layer or medium fraction could be analyzed separately. The fact that cell growth was hardly stimulated in any cell type on co-culture of stromal and epithelial cells or stromal and endothelial cells is not consistent with the findings of other authors (*14, 15, 17*). The reason for this is unknown, but it may be related to the fact that the medium was replaced daily during the cultures. It seems that the stimulatory effect of co-culturing on cell growth is not so great.

On the other hand, PG synthesis by each cell type changed markedly on co-culture. Because ^{3}H and ^{35}S incorporation in the Medium I and Gel fractions of cultures Epi and Endo was very low compared to the corresponding incorporation of culture Str (Tables I and III), most of the PGs in the Medium I and Gel (Str) fractions of co-cultures Str/Epi and Str/Endo are regarded as having been synthesized by stromal cells, and those in the Cell layer (Epi) and Cell layer (Endo) fractions of the co-cultures are regarded as having been synthesized by epithelial and endothelial cells, respectively. Thus, based on the results in Table I, stromal and epithelial cells stimulated the overall PG synthesis by each other. Endothelial cells also stimulated the synthesis by stromal cells, while stromal cells reduced that by endothelial cells to some extent (Table III). Previously, we (*29*) reported that PG synthesis by each corneal constituent layer cultured alone decreased markedly for all three layers compared with that in the corresponding layer cultured as part of an intact whole cornea. This result of the previous study is consistent with the results described above except that for endothelial cells. But it should be considered that the data for the endothelial layer in the previous paper were inaccurate because of the very low radioactive incorporation into the layer. In this study, the cultured endothelial cells themselves synthesized and secreted PGs (especially a large CS/DS PG) much more actively compared with stromal and epithelial cells, unlike the endothelial layer in the previous paper. The PG synthesis by endothelial cells could be stimulated during cell culture.

We also reported in the previous paper (*29*) that the sulfated macromolecules synthesized by epithelial layers in tissue culture consisted mostly of HSPG and a large sulfated glycoprotein. In this study, the cultured epithelial cells synthesized mainly CS/DS PG (see the fractions of Epi in Table II). But the glycosaminoglycan composition of the Cell layer (Epi) fraction approached that of epithelial layers in tissue culture on co-culture with stromal cells (Table II), showing that stromal cells affect the expressed type of PG in epithelial cells. On the other hand, it appears that stromal cells hardly influence the expressed type of PG in endothelial cells (Table IV). A small amount of KS was present in the Gel fraction of Endo, but not in its Medium II fraction. Because KSPG is of a much smaller molecular size and more diffusible than the large CS/DS PG and the large sulfated glycoprotein, KSPG synthesized by endothelial cells could more easily reach the gel layer on the insert dish and adhere thereto. Similar phenomena may occur in the cornea *in vivo*; the small PGs synthesized by endothelial cells may pass through Descemet's membrane and penetrate the stroma.

In conclusion, endothelial cells showed a different effect from epithelial cells on PG synthesis by stromal cells: endothelial cells tended to increase KS synthesis, while epithelial cells did not affect KS synthesis, but tended to increase CS/DS synthesis. PG synthesis by the stroma *in vivo* could be controlled by the balance between the antipodal actions of epithelial and endothelial cells. For exam-

ple, when the epithelial or endothelial layer is wounded, the balance could be destroyed and the expressed type of PG in the stroma could change markedly. Furthermore, it is well-known that the expressed type of PG in the stroma of the developing embryonic cornea changes with advancing age (*32-34*). This change in the expressed type of stromal PG could also be caused by the actions of maturing epithelial and endothelial cells. In this experiment, stromal cells did not come into contact with epithelial or endothelial cells during a co-culture. Therefore, their mutual effects on each other as to PG synthesis must be mediated by water-soluble and diffusible compound(s). These might be growth factor(s), because many investigators have reported that various growth factors and their receptors are present in the cornea (*14, 15, 35, 36*), and that they influence the synthesis and metabolism of PGs in many organ cultures and cell cultures (*30, 37-41*).

REFERENCES

1. Takaya, H. (1978) Dynamics of organizer. A. Morphogenic movements and specificities in induction and differentiation of the organizer in *Organizer: Milestone of a Half-Century from Spemann* (Nakamura, O. and Toivonen, S., eds.) pp. 49-70, Elsevier/North Holland, New York
2. Sharpe, P.M. and Ferguson, M.W.J. (1988) Mesenchymal influences on epithelial differentiation in developing systems. *J. Cell Sci. Suppl.* **10**, 195-230
3. Mizuno, T. and Yasugi, S. (1990) Susceptibility of epithelia to directive influences of mesenchymes during organogenesis: Uncoupling of morphogenesis and cytodifferentiation. *Cell Differ. Dev.* **31**, 151-159
4. Bernfield, M.R., Banerjee, S.D., Koda, J.E., and Rapraeger, A.C. (1984) Remodeling of the basement membrane as a mechanism of morphogenetic tissue interaction in *The Role of Extracellular Matrix in Development* (Trelstad, R.L., ed.) pp. 545-596, AR Liss, New York
5. Sengel, P. (1976) *Morphogenesis of Skin*, pp. 1-227, Cambridge University Press, New York
6. Cunha, G.R., Chung, L.W.K., Shannon, J.M., Taguchi, O., and Fuji, H. (1983) Hormone-induced morphogenesis and growth: Role of mesenchymal-epithelial interactions. *Rec. Prog. Horm. Res.* **39**, 559-598
7. Ekblom, P. (1984) Basement membrane proteins and growth factors in kidney differentiation in *The Role of Extracellular Matrix in Development* (Trelstad, R.L., ed.) pp. 173-206, AR Liss, New York
8. Spooner, B.S. (1973) Microfilaments, cell shape changes and morphogenesis of salivary epithelium. *Am. Zool.* **13**, 1007-1022
9. Thesleff, J. and Hurmerinta, K. (1981) Tissue interactions in tooth development. *Differentiation* **18**, 75-88
10. Cunha, G.R., Bigsby, R.M., Cooke, P.S., and Sugimura, Y. (1985) Stromal-epithelial interaction in adult organs. *Cell Differ.* **17**, 137-148
11. Sawyer, R.H., O'Guin, W.M., and Knapp, L.W. (1984) Avian scale development. X. Dermal induction of tissue specific keratins in extraembryonic ectoderm. *Dev. Biol.* **101**, 8-18
12. Schweizer, J., Winter, H., Hill, M., and Mackenzie, I. (1984) The keratin polypeptide patterns in heterotypically recombined epithelia of skin and mucosa of adult mouse. *Differentiation* **26**, 144-153
13. McGrath, C.M. (1984) Augmentation of the response of normal mammary epithelial cells to estradiol by mammary stroma. *Cancer Res.* **43**, 1355-1360
14. Wilson, S.E., He, Y.-G., Weng, J., Zieske, J.D., Jester, J.V., and Schultz, G.S. (1994) Effect of epidermal growth factor, hepatocyte growth factor, and keratinocyte growth factor, on proliferation, motility and differentiation of human corneal epithelial cells. *Exp. Eye Res.* **59**, 665-678
15. Sotozono, C., Kinoshita, S., Kita, M., and Imanishi, J. (1994) Paracrine role of keratinocyte growth factor in rabbit corneal epithelial cell growth. *Exp. Eye Res.* **59**, 385-392
16. Gospodarowicz, D., Greenburg, G., and Birdwell, C.R. (1978) Determination of cellular shape by the extracellular matrix and its correlation with the control of cellular growth. *Cancer Res.* **38**, 4155-4171
17. Chan, K.Y. and Haschke, R.H. (1983) Epithelial-stromal interactions: Specific stimulation of corneal epithelial cell growth *in vitro* by a factor(s) from cultured stromal fibroblasts. *Exp. Eye Res.* **26**, 231-246
18. Reid, L.M. and Jefferson, D.M. (1984) Cell culture studies using extracts of extracellular matrix to study growth and differentiation in mammalian cells in *Mammalian Cells in Mammalian Cell Culture* (Mather, J.P., ed.) pp. 239-280, Plenum Press, New York
19. Samples, J.R., Binder, P.S., and Nayak, S.K. (1991) Propagation of human corneal endothelium *in vitro* effect of growth factors. *Exp. Eye Res.* **52**, 121-128
20. Scott, J.E. (1988) Proteoglycan-fibrillar collagen interactions. *Biochem. J.* **252**, 313-323
21. Birk, D.E. and Lande, M.A. (1981) Corneal and scleral collagen fiber formation *in vitro*. *Biochim. Biophys. Acta* **670**, 362-369
22. Scott, J.E. (1987) Molecules for strength and shape: Our fibre-reinforced composite bodies. *Trends Biochem. Sci.* **12**, 318-321
23. Rada, J.A., Cornuet, P.K., and Hassell, J.R. (1993) Regulation of corneal collagen fibrillogenesis *in vitro* by corneal proteoglycan (lumican and decorin) core proteins. *Exp. Eye Res.* **56**, 635-648
24. Hassell, J.R., Cintron, C., Kublin, C., and Newsome, D.A. (1983) Proteoglycan changes during restoration of transparency in corneal scars. *Arch. Biochem. Biophys.* **222**, 362-369
25. Funderburgh, J.L., Cintron, C., Covington, H.I., and Conrad, G.W. (1988) Immunoanalysis of keratan sulfate proteoglycan from corneal scars. *Invest. Ophthalmol. Vis. Sci.* **29**, 1116-1124
26. Conrad, G.W., Hamilton, C., and Haynes, E. (1977) Differences in glycosaminoglycans synthesized by fibroblast-like cells from chick cornea, heart, and skin. *J. Biol. Chem.* **252**, 6861-6870
27. Dahl, I.M.S. and Cöster, L. (1978) Proteoglycan biosynthesis in cultures of corneas and corneal stroma cells from adult rabbits. *Exp. Eye Res.* **27**, 175-190
28. Dahl, I.M.S. (1981) Biosynthesis of proteoglycans and hyaluronate in rabbit corneal fibroblast cultures. Variation with age of the cell line and effect of foetal calf serum. *Exp. Eye Res.* **32**, 419-433
29. Nakazawa, K., Isomura, T., and Shimoeda, S. (1995) Proteoglycan synthesis by chicken corneal explants. *J. Biochem.* **117**, 697-706
30. Nakazawa, K., Morita, A., Nakano, H., Mano, C., and Tozawa, N. (1996) Keratan sulfate synthesis by corneal stromal cells within three-dimensional collagen gel cultures. *J. Biochem.* **120**, 117-125
31. Yue, Y.J.T., Baum, J.L., and Slibert, J.E. (1976) The synthesis of glycosaminoglycans by cultures of rabbit corneal endothelial and stromal cells. *Biochem. J.* **158**, 567-573
32. Hart, G.W. (1976) Biosynthesis of glycosaminoglycans during corneal development. *J. Biol. Chem.* **251**, 6513-6521
33. Funderburgh, J.L., Caterson, B., and Conrad, G.W. (1986) Keratan sulfate proteoglycan during embryonic development of the chicken cornea. *Dev. Biol.* **116**, 267-277
34. Nakazawa, K., Suzuki, S., Wada, K., and Nakazawa, K. (1994) Proteoglycan synthesis by corneal explants from developing embryonic chicken. *J. Biochem.* **117**, 707-718
35. Hongo, M., Itoi, M., Yamaguchi, N., and Imanishi, J. (1992) Distribution of epidermal growth factor (EGF) receptors in rabbit corneal epithelial cells, keratocytes and endothelial cells, and the changes induced by transforming growth factor-β1. *Exp. Eye Res.* **54**, 9-16
36. Wilson, S.E., Schultz, G.S., Chegini, N., Weng, J., and He, Y.-G. (1994) Epidermal growth factor, transforming growth factor alpha, transforming growth factor beta, acidic fibroblast growth factor, basic fibroblast growth factor, and interleukin-1 proteins in the cornea. *Exp. Eye Res.* **59**, 63-72

37. Kato, Y. and Gospodarowicz, D. (1985) Sulfated proteoglycan synthesis by confluent cultures of rabbit costal chondrocytes grown in the presence of fibroblast growth factor. *J. Cell Biol.* **100**, 477-485
38. Bassols, A. and Massague, J. (1988) Transforming growth factor β regulates the expression and structure of extracellular matrix chondroitin/dermatan sulfate proteoglycans. *J. Biol. Chem.* **263**, 3039-3045
39. Dodge. G.R., Kovalszky, I., Hassell, J.R., and Iozzo, R.V. (1990) Transforming growth factor β alters the expression of heparan sulfate proteoglycan in human colon carcinoma cells. *J. Biol. Chem.* **265**, 18023-18029
40. Schönherr, E., Järveläinen, H.T., Sandell, L.J., and Wight, T.N. (1991) Effects of platelet-derived growth factor and transforming growth factor-β1 on the synthesis of a large versican-like chondroitin sulfate proteoglycan by arterial smooth muscle cells. *J. Biol. Chem.* **266**, 17640-17647
41. Westergren-Thorsson, G., Schmidtchen, A., Särnstrand, B., Fransson, L.Å., and Malmström, A. (1992) Transforming growth factor-β induces selective increase of proteoglycan production and changes in the copolymeric structure of dermatan sulphate in human skin fibroblasts. *Eur. J. Biochem.* **205**, 277-286