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Keratan Sulfate Synthesis by Corneal Stromal Cells within Three-Dimensional Collagen Gel Cultures

Kiyoshi Nakazawa,[1] Ayako Morita, Hiroe Nakano, Chizuko Mano, and Nahoko Tozawa

Section of Radiochemistry, Faculty of Pharmacy, Meijo University, 150 Yagotoyama, Tempaku-ku, Nagoya 468

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The corneal stromal cells from 2-day-old chicks were cultured on plastic dishes or within three-dimensional collagen gel in the presence or absence of growth factor (EGF, bFGF, PDGF, TGF-β1, or their combinations). The cells were labeled with [^{35}S]sulfate and [^{3}H]-glucosamine, and the radio-labeled proteoglycans were examined. Keratan sulfate was synthesized to some extent (15.4-16.9% of total synthesis for medium fraction; 8.0% for cell layer fraction) in a primary culture even when the cells were cultured on plastic dishes, although the values were very much lower than that (42.7%) in the stromal fraction of organ culture of corneal explants. The primary culture in collagen gel showed some increase in the proportion of keratan sulfate synthesis as compared with the culture on plastic. Among growth factors, addition of EGF to the culture in gel caused a further increase in the proportion of keratan sulfate synthesis. bFGF and TGF-β1 increased proteoglycan synthesis as a whole to some extent, but chondroitin sulfate/dermatan sulfate synthesis was increased preferentially and, consequently, the proportion of keratan sulfate synthesis to total synthesis was decreased. PDGF also caused some decrease in the proportion. In the culture after one passage (secondary culture), the keratan sulfate synthesis decreased markedly (8.6-8.3% of total synthesis for medium fraction; 2.7% for cell layer or gel fraction) and a large chondroitin sulfate/dermatan sulfate proteoglycan appeared whether the cells were cultured on plastic or in collagen gel. But, when the medium was changed to CG medium (serum-free medium) in the middle of either primary or secondary cultures, the keratan sulfate synthesis (27.8% for medium fraction; 15.6% for gel fraction) was maintained at the level of that of the primary culture in gel. EGF and bFGF were not additive to the effect of CG medium on the keratan sulfate synthesis in the secondary culture. Instead, EGF and bFGF stimulated hyaluronic acid synthesis in the culture. The mechanism of these changes in the expression type of proteoglycan and their significance remain to be clarified.

Key words: corneal stromal cells, culture in collagen gel, growth factors, keratan sulfate, proteoglycans.

Proteoglycan and type I collagen constitute the major components of corneal stroma. Proteoglycans occupy the region between collagen fibrils (*1, 2*) and influence fibril assembly and stromal organization (*3-9*). The maintenance of constant fibril diameter, regular packing of fibrils and organization of these fibrils into orthogonal lamellae are important in the development of corneal transparency (*10-12*). Thus, proteoglycans may play an important role in the maintenance of corneal transparency.

The corneal stroma contains two classes of small proteoglycans: one with chondroitin sulfate/dermatan sulfate side chains, which has been shown to be decorin (*13*), and the other with keratan sulfate side chains, which has recently been termed lumican (*14*). In normal corneal stroma of most mammals and birds, the two proteoglycans are present in almost the same amounts (*15-20*). But the accumulation and biosynthesis of the two major proteoglycans in corneal stroma readily change under various conditions *in vivo* and *in vitro*. Corneal scars result in an alteration of the expression type of proteoglycan. Reductions in the amount of keratan sulfate proteoglycan and its sulfation, as well as the appearance of a highly sulfated large chondroitin sulfate/dermatan sulfate proteoglycan and hyaluronic acid, are observed in the scar regions (*21, 22*). When corneal fibroblasts are cultured on a plastic dish *in vitro*, the expression pattern of proteoglycan also changes markedly (*23-28*). In those studies, the authors have reported that keratan sulfate biosynthesis mostly disappeared in cell cultures, whereas the proportions of chondroitin sulfate/dermatan sulfate and heparan sulfate biosyntheses increased and hyaluronic acid biosynthesis appeared. Doane *et al.* (*29, 30*) attempted to culture chick corneal fibroblasts within three-dimensional collagen gels, which can be easily manipulated, yet resemble the environment encountered by the fibroblast *in vivo*. Cells grown in

[1] To whom correspondence should be addressed.

Abbreviations: CG, CG medium; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; CS/DS, chondroitin sulfate/dermatan sulfate; EGF, epidermal growth factor; FBS, fetal bovine serum; F-12, Ham's F-12 medium; bFGF, basic fibroblast growth factor; KS, keratan sulfate; NEM, *N*-ethyl maleimide; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PMSF, phenylmethyl-sulfonyl fluoride; TGF-α and -β1, transforming growth factor-α and -β1.

collagen gels, however, synthesized no keratan sulfate proteoglycan.

Many investigators have reported that various growth factors influence the synthesis and metabolism of proteoglycans in many organ cultures and cell cultures, and have described many different effects on them. Transforming growth factor-β (TGF-β) increases the syntheses of chondroitin sulfate/dermatan sulfate proteoglycans (*31-36*), heparan sulfate proteoglycan (*37*), and hyaluronic acid (*38, 39*) in various cultures; basic fibroblast growth factor (bFGF) increases chondroitin sulfate proteoglycan (*40, 41*); platelet-derived growth factor (PDGF) increases chondroitin sulfate proteoglycan (*36*) and hyaluronic acid (*38*); epidermal growth factor (EGF) increases hyaluronic acid (*38*) and dermatan sulfate proteoglycan (*39*). In addition, the growth factors have various effects on the size, sulfation and epimerization of glycosaminoglycan chains of proteoglycans (*32-36, 40*). EGF, TGF-α, TGF-β1, and interleukin-1 were detected by immunohistochemistry in all three layers of the human cornea (epithelial, stromal, and endothelial layers), while EGF and bFGF were detected in the three types of cells by immunocytology (*42*). Further, TGF-β1 induced changes in the distribution of EGF receptors in the three types of rabbit corneal cells (*43*). But nothing is known about the effects of growth factors on corneal proteoglycan synthesis.

In this study, we have cultured chick corneal stromal cells within three-dimensional collagen gels in the presence or absence of various growth factors, and examined the changes in expression type of proteoglycan under various conditions. In particular, we sought conditions under which the synthesis of keratan sulfate proteoglycan would be restored to the level seen in corneal stroma *in vivo*.

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 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 CG medium (modified Iscove's medium; Serotec, UK) from Dai-nihon Seiyaku, Osaka. Fetal bovine serum (FBS) was purchased from Boehringer Mannheim, Tokyo. EGF (murine recombinant; Mallinckrodt, USA) and bFGF (human recombinant; Mallinckrodt, USA) were purchased from Seikagaku, Tokyo: TGF-β1 (human recombinant; King Jozo, Japan) and PDGF (human platelet; Becton Dickinson, USA) were from Cosmo Bio, Tokyo. The following enzymes were obtained from the commercial sources indicated: chondroitinase ABC (from *Proteus vulgaris*; Seikagaku, Tokyo), keratanase (from *Pseudomonas* sp.; Seikagaku), hyaluronidase (from *Streptomyces hyaluronolyticus*; Seikagaku), collagenase (from *Clostridium histolyticum*; Wako Pure Chemical, Osaka), and trypsin (for cell culture; Boehringer Mannheim, 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. YM-10 (43 mm, for ultrafiltration; Amicon) was obtained from Grace Japan, Tokyo.

Cell Culture and Labeling with Radioactive Precursors—Corneas were dissected from 2-day-old male chicks and trimmed free of limbus and scleral ossicles. Then, the corneas (from 80 eye balls) were treated in 0.1% EDTA/phosphate-buffered saline (PBS: 1.15 g of $NaHPO_4$, 8.0 g of NaCl, 0.2 g of KCl, 0.2 g of KH_2PO_4, 0.5 g of streptomycin sulfate, and 10^5 U of penicillin G in 1,000 ml) at room temperature for 20 min to weaken the adhesion of epithelial and endothelial layers to stroma. The epithelial and endothelial sheets were scraped off the treated corneas. The corneal stromas were cut into small pieces and incubated with 0.2% collagenase in 2.0 ml of PBS at 37°C for 1.5 h. The digest was centrifuged and the pellet was suspended in 3.0 ml of Ham's F-12/10% FBS medium. The cell suspension was washed with the same medium three times more. Finally, the cell pellet was resuspended in 2.8 ml of Ham's F-12/10% FBS and cultured in the following three ways. Portions of cell suspensions were used to count the cell numbers with a hemocytometer.

(i) Primary culture for 8 days: Type I collagen solution (3 mg/ml HCl, pH 3.0) was mixed with 10-fold concentrated Ham's F-12 medium and HEPES solution (0.477 g of HEPES and 0.22 g of $NaHCO_3$ in 10 ml of 0.05 M NaOH) with the volume ratio of 8 : 1 : 1 in an ice bath. Then, 1.8 ml of the cell suspension obtained above was mixed with 10.2 ml of the collagen/Ham's F-12/HEPES in an ice bath, and 2.0 ml of the cell suspension/collagen mixture (final concentration of collagen; 2 mg/ml) was added to each 35 mm plastic culture dish, where 1 ml of the collagen/Ham's F-12/HEPES had been added without cells in advance and gelatinized at 37°C for 30 min. After that, the cell suspension/collagen mixture on the dish was gelatinized at 37°C for 30 min, and 1.5 ml of Ham's F-12/10% FBS was overlaid thereon. Each dish (4.5×10^5 cells) was incubated at 37°C in an atmosphere of 5% CO_2 for 7 days. In parallel with these cultures, 0.3 ml of the cell suspension (4.5×10^5 cells) was directly plated on 35 mm plastic culture dishes and cultured in 3.0 ml of Ham's F-12/10% FBS for 7 days. Culture media were changed for fresh media every other day. Thereafter, media of all the cultures were changed for 1.0 ml of Ham's F-12/0.3% FBS each and the cultures were continued at 37°C for 8 h. Then, the cultures were labeled in 1.0 ml of Ham's F-12 medium containing 0.3% FBS, 250 μCi of sodium [^{35}S]sulfate, and 25 μCi of [^{3}H]glucosamine in the presence or absence of one growth factor (EGF, 20 ng; bFGF, 20 ng; TGF-β1, 20 ng; PDGF, 40 ng) at 37°C for another 16 h. After labeling, proteoglycans were isolated from the media and gels (or cell layer) as described below. Simultaneously, corresponding cultures were incubated under the same conditions, but without radioactive precursors. Then, the cultures on plastic dish were treated with 0.25% trypsin/PBS and those in collagen gel with 0.2% collagenase/PBS at 37°C for 20 min to count the cell number. Of the two dishes cultured under each condition, one dish was used for the isolation of labeled proteoglycan and the other for counting of the cell number.

(ii) Primary culture for 11 days: The stromal cells were cultured in collagen gel or on plastic dishes for 10 days and 8 h the same way as described in (*i*). Then, each culture was labeled in 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 with EGF (20 ng), the combination of EGF (20 ng) and

TGF-β1 (20 ng), the combination of EGF (20 ng) and bFGF (20 ng) or no growth factor at 37°C for another 16 h. In addition to these cultures, media of some cultures in collagen gel were changed for 1.5 ml of Ham's F-12/0.3% FBS/30 ng EGF or 1.5 ml of CG medium (modified Iscove's medium) 5 days after the cultures were started. The cultures were continued for another 5 days and 8 h. The media were changed for fresh media every day. After that, the cultures were labeled with the same media containing the radioactive precursors.

(iii) Secondary culture for 9 days: Collagen mixture for culture was prepared in the same way as described in (*i*), 5.5×10^5 stromal cells per dish were mixed with the collagen mixture and, after the gelation of collagen, cultured at 37°C in an atmosphere of 5% CO_2. One group of cultures in gel was incubated in 1.5 ml of Ham's F-12/10% FBS for 9 days. Other groups of cultures in gel were incubated in 1.5 ml of the same medium for 5 days and then in 1.5 ml of CG medium or CG medium with growth factor (30 ng EGF or 30 ng bFGF) for another 4 days. The media of all the cultures were changed for fresh media every day. After that, the media were removed from cultures and each gel containing cells was treated with 1.0 ml of 0.2% collagenase/PBS at 37°C for 20 min. The resultant cell suspensions were centrifuged and washed with Ham's F-12/10% FBS three times. Finally, the pellets were resuspended in Ham's F-12/10% FBS, and the resultant cell suspensions ($\sim6.7\times10^5$ cells/dish) were cultured for 8 days under the same conditions as used for the respective primary cultures. Then, each culture was labeled in 1.0 ml of the same medium containing 250 μCi of sodium [^{35}S]-sulfate and 30 μCi of [^{3}H]glucosamine for 17.5 h. In parallel with these cultures, the stromal cells (5.5×10^5 cells/dish) were directly plated on plastic dishes and cultured in 3.0 ml of Ham's F-12/10% FBS for 9 days. Thereafter, each cell layer on the dish was treated with 1.0 ml of 0.25% trypsin/PBS at 37°C for 20 min and the resultant cell suspension was plated on plastic dishes (6.6×10^5 cells/dish). The secondary cultures were continued for 8 days and labeled the same way as described above.

Isolation of Proteoglycans—Because, in the experiments on corneal explant culture (*44*), the glycosaminoglycan composition was very different between explant and medium fractions, medium and gel (or cell layer) fractions were also analyzed separately for the glycosaminoglycan composition in this study. The medium was removed from each radio-labeled culture. Solid guanidine hydrochloride was added to each medium to make 4 M. Each was then applied to a PD-10 column equilibrated with 0.05 M Tris-HCl (pH 7.5) containing 4 M guanidine hydrochloride, 0.2 M NaCl, 5 mM PMSF, 10 mM NEM, 10 mM EDTA, 0.1 M 6-amino-hexanoic acid, and 0.5% CHAPS (hereafter referred to as Solution I) to remove unincorporated radioactive precursors. The excluded fractions were pooled as medium macromolecular fraction. Each of the gels and cell layers on dishes was extracted in 2 ml of Solution I at 4°C overnight. Following centrifugation for 15 min at 4°C and $24,000\times g$, the supernatant was transferred and the resultant residue was re-extracted in 2 ml of Solution I at 4°C overnight. After centrifugation, each supernatant was combined with the first supernatant. The combined supernatant and medium macromolecular fractions were dialyzed against 0.05 M Tris-HCl (pH 7.5) containing 7 M urea, 0.5 mM PMSF, 1.0 mM NEM, 1.0 mM EDTA, 10 mM 6-aminohexanoic acid, and 0.1% CHAPS. Portions of the dialyzed extract and medium fractions were assayed for ^{3}H and ^{35}S activities. The remaining portions were applied to a DEAE-Sephacel column (1.0×13 cm) equilibrated with 0.05 M Tris-HCl (pH 7.5) containing 7 M urea and 0.1% CHAPS. The chromatography was performed as described previously (*44*). Portions of fractions were assayed for ^{3}H and ^{35}S activities and peaks with both ^{3}H and ^{35}S activities were pooled as proteoglycan fractions. Proteoglycan fractions from DEAE-Sephacel chromatography (materials eluted in the range of 0.075 to 2.0 M NaCl) were concentrated by ultrafiltration with a YM-10 membrane at 4°C and then dialyzed against 50 mM Tris-HCl (pH 7.5) containing 50 mM sodium acetate and 50 mM NaCl (*45*).

Enzymatic Treatments and Molecular Sieve Chromatography—To analyze the glycosaminoglycan composition of proteoglycans, portions of the dialyzed proteoglycan fractions 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 50 mM Tris-HCl (pH 7.5) containing 0.1 M 6-aminohexanoic acid, 10 mM EDTA, 5 mM PMSF, and 10 mM NEM (to inhibit the protease activities which contaminate the commercial enzyme preparations). After digestion, one-ninth volume of 1.0% SDS/0.1 M EDTA/0.05 M Tris-HCl (pH 7.5) was added and each mixture was applied to a Sepharose CL-4B column (1.0×100 cm) equilibrated with 0.1% SDS/10 mM EDTA/0.05 M Tris-HCl (pH 7.5). Sepharose CL-4B chromatography was performed as reported previously (*44*). The proportions of keratan sulfate and chondroitin sulfate/dermatan sulfate were calculated from the activities of degradative products which were eluted around the column volume. Other portions of the proteoglycan fractions were digested with 10 TRU (turbidity reducing unit) of hyaluronidase at 60°C for 5 h in 10 mM acetate buffer (pH 6.0) containing the same protease inhibitors as described above. The digests were chromatographed on a Sepharose CL-4B column as described above.

RESULTS

Primary Cultures for 8 Days—The stromal cells in collagen gel or on plastic dishes were cultured and labeled as described under "MATERIALS AND METHODS." Growth of the cells and incorporation of the ^{35}S precursor into macromolecules by them are shown in Table I. The incorporation in Table I shows only ^{35}S activity, not ^{3}H. As can be seen from the second column ("Cell number") in Table I, it seems that growth factors did not stimulate the cell growth. The incorporations of ^{35}S precursor by cultures on plastic dishes, especially those into the medium fraction, are very high as compared to those of the cultures in gel. This may mean that, on culture in gel, the radioactive precursor was diluted with medium inside the gel and/or that the properties of the cells changed greatly during culture on plastic dishes. TGF-β1 and bFGF slightly increased the ^{35}S incorporation as compared to the culture in gel with no growth factor (53.7% by TGF-β1; 22.2% by bFGF): many investigators have reported that both growth factors stimulate proteoglycan synthesis for various types of cells. EGF and PDGF had no effect on the incorporations.

As described under "MATERIALS AND METHODS," the

proteoglycan fractions obtained from DEAE-Sephacel chromatography of the extracts and media were digested with chondroitinase ABC or keratanase and the digests were chromatographed on a Sepharose CL-4B column. The proportions of keratan sulfate (KS) and chondroitin sulfate/dermatan sulfate (CS/DS) were calculated from the chromatograms. The results are shown in Table II. Even when the stromal cells were cultured on plastic dishes, they synthesized keratan sulfate to some extent [although the values are very low as compared to 42.7%, the proportion of KS synthesized in the stromal fraction of the cultured chicken corneal stromal explant and to 83.6% in the medium fraction (*44*)]. Other investigators (*23-28*) have reported almost complete loss of keratan sulfate synthesis by cells on plastic. This discrepancy between their and our results may be accounted for by the fact that they used cells which had been passaged several times. "Others" in the table could consist mainly of heparan sulfate proteoglycan and sulfated glycoprotein, because heparan sulfate synthesis and sulfated glycoprotein synthesis were stimulated in the culture of stromal cells in gel in separate experiments (the data will be presented elsewhere). When the cells were cultured in gel with no growth factor, the proportion of KS synthesis increased by 1.9-fold for the gel fraction and 2.4-fold for the medium fraction as compared to those by the cells on plastic. The culture within three-dimensional collagen gel, which resembles the environment *in vivo*, resulted in some restoration of the proportion of KS synthesis. EGF increased the proportion of KS synthesis, especially in that of the medium fraction. bFGF, TGF-β1, and PDGF tended to decrease the proportion of KS synthesis as compared to those by cells in gel with no growth factor. This means that both bFGF and TGF-β1 may stimulate preferentially the syntheses of CS/DS in the gel and "Others" in the medium, because both growth factors stimulated proteoglycan synthesis overall (Table I).

Primary Cultures for 11 Days—Next, we examined how the proteoglycan synthesis changed after longer culture (11 days). Table III shows growth of the cells and the incorporation of ^{35}S into macromolecules in the 11-day cultures. As in Table I, the incorporation of ^{35}S by the cells on plastic is very high as compared to that by the cells in gel. On comparison between the cultures in gel, the combination of EGF and TGF-β1 ("F-12/EGF+TGF-β1") showed some stimulation of proteoglycan synthesis, but either EGF and bFGF or EGF and TGF-β1 showed no synergetic effect. When the medium of F-12/10% FBS was changed for F-12/0.3% FBS/EGF in the middle of culture and the culture was continued in the latter medium ["in gel, F-12/EGF (continuous)" in Table III], the cells grew poorly. This means that EGF has no effect on growth of corneal stromal cells and factors present in FBS may be necessary for growth of the cells. In contrast, the cells grew comparably in gel with CG medium (modified Iscove's medium; serum-free medium; *46-48*). The reason why the incorporation of ^{35}S by the cells in CG medium was much lower than those by the cells in other media, may be much higher concentration of sulfate salt as a component of CG medium, so that the specific activity of [^{35}S]sulfate is reduced (Ham's F-12, 2.5 μg of cupric sulfate, 863 μg of zinc sulfate and 834 μg of ferrous sulfate/liter; CG, 97.7 mg of Mg_2SO_4/liter). On the other

TABLE I. **Growth of stromacytes in 8-day primary cultures under different conditions and incorporation of [^{35}S]sulfate and [^{3}H]glucosamine into macromolecules by them.**[a]

Conditions[b]	Cell number ($\times 10^6$/dish)	^{35}S activity			
		Gel or cell layer	Medium (dpm$\times 10^{-3}$/dish)	Total	Total/cell (dpm/cell)
On plastic					
F-12	3.38	14,210	51,156	65,366	19.3
In gel					
F-12	4.28	5,517	1,334	7,051	1.65
F-12/EGF	4.05	5,591	1,411	7,002	1.73
F-12/bFGF	4.20	6,706	1,908	8,614	2.05
F-12/TGF-β1	4.31	7,687	2,700	10,837	2.51
F-12/PDGF	4.55	5,642	1,613	7,255	1.59

[a]The table is a composite of two separate experiments; standard errors are <10% of the means. [b]Cells were cultured as described in the text and labeled in F-12/0.3% FBS/radioactive precursors in the presence or absence of the growth factor indicated.

TABLE II. **Glycosaminoglycan compositions of proteoglycans synthesized by stromacytes in 8-day primary cultures under different conditions.**[a]

Conditions[b]	Fractions	Percent of total ^{35}S-activity		
		KS	CS/DS	Others
On plastic				
F-12	Medium	15.4	61.7	22.9
	Cell layer	8.0	56.1	35.9
In gel				
F-12	Medium	36.9	33.0	30.1
	Gel	15.4	61.7	22.9
F-12/EGF	Medium	47.7	16.7	35.6
	Gel	16.4	61.5	22.1
F-12/bFGF	Medium	30.1	25.5	44.4
	Gel	12.6	68.9	18.5
F-12/TGF-β1	Medium	32.3	21.9	45.8
	Gel	10.4	66.1	23.5
F-12/PDGF	Medium	27.3	37.2	35.5
	Gel	12.7	70.6	16.7

[a]The table is a composite of two separate experiments; standard errors are <5% of the means. [b]Categories are as defined in Table I. KS, keratan sulfate; CS/DS, chondroitin sulfate/dermatan sulfate.

TABLE III. **Growth of stromacytes in 11-day primary cultures under different conditions and incorporation of [^{35}S]sulfate and [^{3}H]glucosamine into macromolecules by them.**[a]

Conditions	Cell number ($\times 10^6$/dish)	^{35}S activity			
		Gel or cell layer	Medium (dpm$\times 10^{-3}$/dish)	Total	Total/cell (dpm/cell)
On plastic					
F-12	3.50	23,642	103,026	126,668	36.2
In gel					
F-12	4.48	7,703	5,995	13,698	3.06
F-12/EGF[b]	5.02	11,070	5,227	16,297	3.25
F-12/EGF +bFGF[b]	4.25	11,156	3,722	14,878	3.50
F-12/EGF +TGF-β1[b]	4.58	13,960	4,905	18,865	4.12
F-12/EGF (continuous)[c]	1.51	6,101	1,316	7,417	4.19
CG[c]	3.24	1,256	319	1,575	0.49

[a]The table is a composite of two separate experiments; standard errors are <10% of the means. [b]After cells were cultured as described in the text, the cultures were labeled in F-12/0.3% FBS/radioactive precursors with the growth factor or the combination indicated. [c]The medium was changed for F-12/EGF or CG medium in the middle of culture as described in the text.

hand, growth of the cells on plastic was very weak in CG medium.

As described above, the proportions of KS and CS/DS were calculated from the results of Sepharose CL-4B chromatography (in Table IV). The cells on plastic still synthesized KS to an extent similar to that of the corresponding 8-day culture, even after culture for 11 days. The effects of culture in gel and growth factors on KS synthesis were similar to those in 8-day cultures. The cells in gel (with no growth factor) synthesized a greater proportion of KS than the cells on plastic. Addition of EGF also increased further the proportion of KS in the medium fraction. bFGF and TGF-β1 were not synergetic with EGF on KS synthesis, but they tended to reduce it. The effect of continuous addition of EGF on KS synthesis was not more than that of single addition on the last day of culture. The culture in CG medium showed an effect similar to that of "F-12/EGF" on KS synthesis.

Secondary Culture for 9 Days—Because CG medium increased KS synthesis, like EGF, the stromal cells were cultured in CG medium or CG medium with growth factor through one passage and the proteoglycans synthesized by them were compared with those by the cells cultured in Ham's F-12/10% FBS through one passage. Table V shows the cell growth and the incorporations of ^{3}H and ^{35}S by the cells in secondary culture under the different conditions. Obviously, the incorporations of ^{3}H and ^{35}S by the cells in "CG" or "CG/growth factor" were much lower than those by the cells in "F-12/FBS." The reason why the incorporation of ^{3}H by the cells in CG medium was lower may be the much higher concentration of glucose (F-12, 1.80 g/liter; CG, 4.50 g/liter) in CG medium. When cultures in CG and CG/growth factor are compared (three lower lines in Table V), both EGF and bFGF stimulated cell growth in secondary culture in CG medium and, consequently, increased the incorporation of ^{35}S a little, but EGF decreased the incorporation of ^{3}H, although the reason is unknown. These effects are different from the action of EGF on primary culture in F-12 medium ["F-12/EGF (continuous)" in Table III].

Proteoglycan fractions and their enzymatic digests were chromatographed on a Sepharose CL-4B column. The results for gel and cell layer proteoglycan fractions are shown in Fig. 1. Although the result for the gel fraction of culture in gel with CG/FGF is not shown, it was very similar to the result for culture in gel with CG/EGF (panel D). Chromatographies of the proteoglycans synthesized by the cells both on plastic and in gel with F-12/FBS (dotted lines in panels A and B) showed prominent peaks which were eluted near the void volume. Because these peaks disappeared on digestion with chondroitinase ABC (open circles in panels), they represent a large chondroitin sulfate/dermatan sulfate proteoglycan (CS/DSPG). This large proteoglycan could be the same species as the large CSPG which was found in corneal opaque scars (*21*) and in cultures of stromal cells (*23-28, 30*) by many investigators. In contrast, the large proteoglycan was not found in the culture in gel with CG or with CG/EGF (panels C and D). Also, the large proteoglycan was not found in the culture in gel with CG/FGF or in primary cultures as a prominent peak (data not shown). When the prominent peaks (dotted lines) are compared in Fig. 1, the peak of panel A is larger in molecular size than the others. This difference in size may be ascribed to a difference in glycosaminoglycan chain size, because the glycosaminoglycans isolated from these proteoglycans were eluted from a Sepharose CL-6B column in the same order as the respective proteoglycans (unpublished data, Nakazawa, K. and Ando, H.). Many investigators have reported that the glycosaminoglycan size of proteoglycans is increased by the addition of TGF-β1 (*32, 34, 36*) or bFGF (*40*) to the cultures. In our experiments,

TABLE IV. **Glycosaminoglycan composition of proteoglycans synthesized by stromacytes in 11-day primary cultures under different conditions.**[a]

Conditions	Fractions	Percent of ^{35}S-activity		
		KS	CS/DS	Others
On plastic				
F-12	Medium	16.9	60.6	22.5
	Cell layer	8.0	47.3	44.7
In gel				
F-12	Medium	21.0	50.3	28.7
	Gel	15.8	65.3	18.9
F-12/EGF[b]	Medium	29.8	37.9	32.3
	Gel	15.1	69.3	15.6
F-12/EGF+bFGF[b]	Medium	17.6	50.0	32.4
	Gel	13.2	73.3	13.5
F-12/EGF+TGF-β1[b]	Medium	20.8	51.9	27.3
	Gel	12.3	74.5	13.2
F-12/EGF (continuous)[c]	Medium	23.3	39.7	37.0
	Gel	16.1	72.7	11.2
CG[c]	Medium	24.4	42.5	33.1
	Gel	13.0	69.0	18.0

[a]The table is a composite of two separate experiments; standard errors are <5% of the means. [b,c]Categories are as defined in Table III.

TABLE V. **Growth of stromacytes in secondary cultures under different conditions and incorporation of [^{35}S]sulfate and [^{3}H]-glucosamine into macromolecules by them.**[a]

Conditions	Cell number ($\times 10^{6}$/dish)	Activities							
		Gel or cell layer		Medium		Total		Total/cell	
		^{35}S	^{3}H	^{35}S	^{3}H	^{35}S	^{3}H	^{35}S	^{3}H
		(dpm$\times 10^{-3}$/dish)						(dpm/cell)	
On plastic									
F-12/FBS	4.29	11,100	3,020	26,200	6,580	37,300	9,600	8.69	2.24
In gel									
F-12/FBS	6.29	8,140	1,910	2,620	722	10,760	2,632	1.71	0.42
CG[b]	3.24	1,219	635	407	175	1,626	810	0.50	0.25
CG/EGF[b]	5.15	1,650	438	479	240	2,129	678	0.41	0.13
CG/bFGF[b]	4.65	1,660	791	426	291	2,086	1,082	0.45	0.23

[a]The table is a composite of two separate experiments; standard errors are <10% of the means. [b]The medium was changed for the indicated medium in the middle of primary and secondary culture as described in the text.

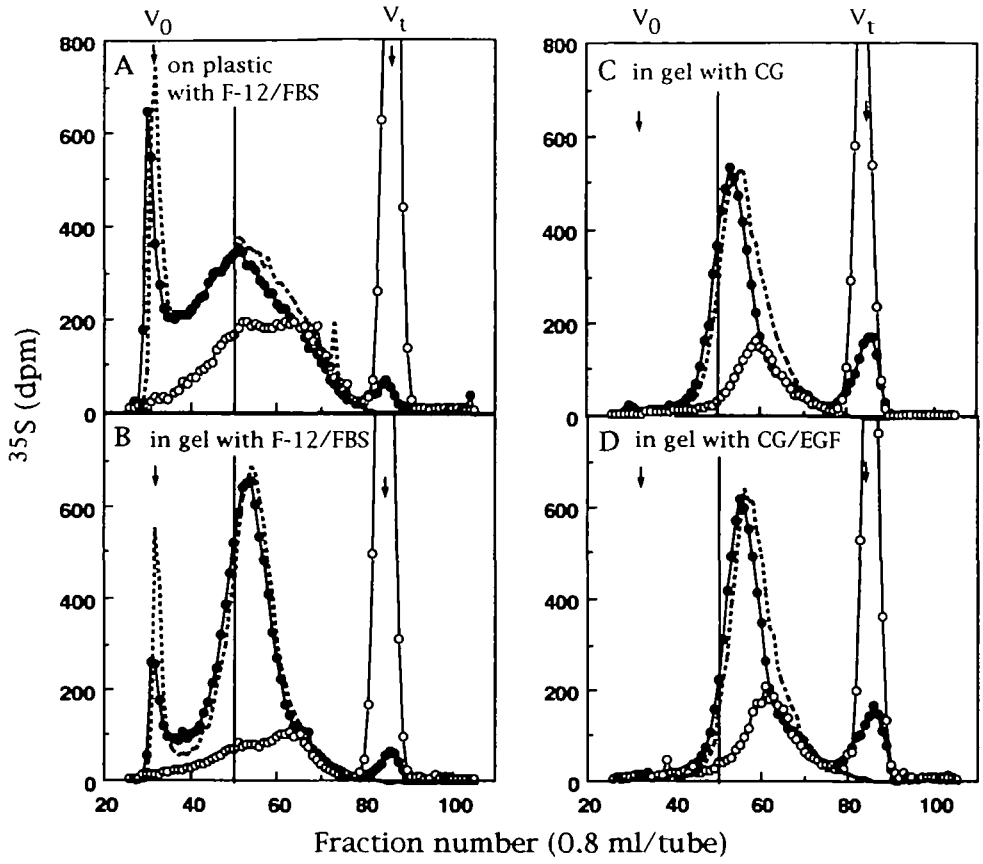

Fig. 1. **Sepharose CL-4B chromatography of proteoglycans from gel and cell layer fractions of different secondary cultures and their enzymatic digests.** Dotted lines, intact proteoglycans; closed circles (●), keratanase digests; open circles (○), chondroitinase ABC digests. A is the fraction from culture on plastic with F-12/FBS and its enzymatic digests; B, the fraction from culture in gel with F-12/FBS and the digests; C, the fraction from culture in gel with CG and the digests; D, the fraction from culture in gel with CG/EGF and the digests. Only ^{35}S activity is shown in the panels. The vertical lines in the panels express the position of fraction number 50. V_o, void volume; V_t, total volume. The proportion of CS/DS or KS was calculated from the counts of the latest-eluting peaks (Table VI).

the amounts of proteoglycans synthesized increased a little in the presence of EGF or bFGF, but no increase in glycosaminoglycan size was found. What this discrepancy means remains to be clarified. The elution profiles of medium fractions (data not shown) were similar to those of the respective gel or cell layer fractions, except that the former PGs were smaller than the latter and the amounts of keratanase-degradative products eluted at the column volume were greater in the former fractions.

The proportions of KS and CS/DS were calculated from the results of Sepharose CL-4B chromatography, and are shown in Table VI. When the cells were cultured on plastic with F-12/FBS through one passage, the proportion of KS synthesis decreased markedly. That marked decrease in KS synthesis was also observed even when the cells were cultured in gel with F-12/FBS. But, when the medium was changed to CG medium in the middle of primary and secondary cultures, the proportions of KS synthesis (27.8% for medium and 15.6% for gel in Table VI) were maintained at levels similar to those in primary culture (24.4% for medium and 13.0% for gel in Table IV). The addition of EGF or bFGF to CG medium showed no additive effect on KS synthesis and even caused some decrease in KS synthesis. Schrecengost *et al.* (*49*) have reported that, because enzymes for the sulfation of KS might be absent in corneal stromacytes cultured on plastic, unsulfated KS was produced in the primary culture, instead of KS. Thus we digested the samples with endo-β-galactosidase (from *Escherichia freundii*), which is able to degrade unsulfated and undersulfated KS, unlike keratanase, and determined the glycosaminoglycan composition. The results (not shown) showed that the proportion of KS was increased only 2-3% more for all of the samples than the values from keratanase digests in Table VI. It is likely that undersulfated KS is not present in these cultures, but unsulfated KS was not determined in this study.

TABLE VI. **Glycosaminoglycan compositions of proteoglycans synthesized by stromacytes in secondary cultures under different conditions.**[a]

Conditions	Fractions	Percent of total ^{35}S-activity		
		KS	CS/DS	Others
On plastic				
F-12/FBS	Medium	8.6	61.9	29.5
	Cell layer	2.7	55.9	41.4
In gel				
F-12/FBS	Medium	8.3	55.8	35.9
	Gel	2.7	79.6	17.7
CG[b]	Medium	27.8	43.5	28.7
	Gel	15.6	69.3	15.1
CG/EGF[b]	Medium	25.6	39.1	35.3
	Gel	14.3	68.6	17.1
CG/bFGF[b]	Medium	18.3	59.8	21.9
	Gel	10.1	73.1	16.8

[a]The table is a composite of two separate experiments; standard errors are <5% of the means. [b]Categories are as defined in Table V.

Both EGF and bFGF stimulated hyaluronic acid synthesis in the secondary cultures. As can be seen in Fig. 2, Sepharose CL-4B chromatography of intact medium fractions from the cultures with growth factor showed sharp ^{3}H peaks at the void volume (dotted lines in panels B and C). These ^{3}H peaks disappeared upon digestion with *Streptomyces* hyaluronidase (open circles in panels B and C). The

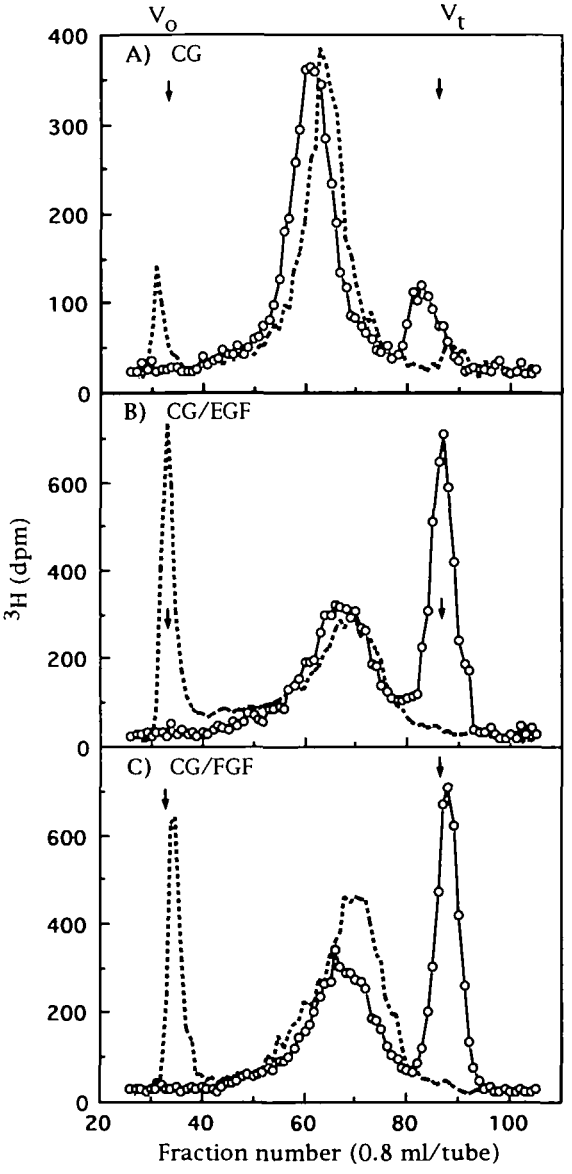

Fig. 2. **Sepharose CL-4B chromatography of medium proteoglycan fractions from different secondary cultures and their hyaluronidase digests.** Dotted lines, intact fractions; open circles, hyaluronidase digests. Only ^{3}H activity is shown in the panels. A is the fraction from culture in CG medium (CG) and its hyaluronidase digest; B, the fraction from culture in CG medium with EGF (CG/EGF) and the digest; C, the fraction from culture in CG medium with bFGF (CG/FGF) and the digest. V_o, void volume; V_t, total volume.

elution profiles of ^{35}S were unchanged before and after digestion (data not shown). The sharp ^{3}H peaks eluted at the void volume were also observed on chromatography of gel fractions from cultures in CG/EGF and CG/bFGF (data not shown). But no *Streptomyces* hyaluronidase-degradative ^{3}H peak was found on chromatography of medium and gel (or cell layer) fractions from other secondary cultures, or any of primary cultures (data not shown).

DISCUSSION

Many investigators (*23-28, 30*) have reported that KS synthesis decreased markedly when corneal stromal cells were cultured on plastic dishes or in collagen gel. Our study has shown that KS synthesis by the cells was maintained to some extent in primary culture even when they were cultured on plastic dishes for 11 days. Furthermore, when the cells were cultured in three-dimensional collagen gel, the proportion of KS synthesis became greater than that by the cells on plastic (Tables II and IV). The three-dimensional collagen gel may provide an environment similar to that encountered by the cells *in vivo*; that may have resulted in some restoration of the proportion of KS synthesis in the culture. But, whenever the cells were cultured on plastic with Ham's F-12/10% FBS or in gel with Ham's F-12/10% FBS through one passage (secondary culture), their KS synthesis decreased markedly and, instead, a high molecular CS/DSPG appeared in the cultures. The properties of the cell may change very much during one passage ("dedifferentiation") even if the cells are cultured in gel. These results are consistent with those reported by other investigators (*23-28, 30*). Those investigators cultured the cells on plastic or in collagen gel after several passages and then analyzed the proteoglycans synthesized. Thus it is very likely that the cells dedifferentiated during the passages and mostly lost the ability to synthesize KS. Interestingly, when the culture medium was changed to CG medium (modified Iscove's medium) in the middle of primary and secondary cultures in collagen gel, the KS synthesis by the cells in secondary culture was maintained at a level similar to that by the cells in the corresponding primary culture (Tables IV and VI). CG medium may contain a component that suppresses the change in expression type of proteoglycan during cell culture, or Ham's F-12 and FBS may contain one that stimulates the change. CG medium contains human transferrin (32.0 mg/liter), soybean lecitin (100 mg/liter) and bovine serum albumin (1.0 g/liter) (*46*). These substances may participate in suppressing the change in the expression type *in vitro*. We cannot completely exclude the possibility that the very low concentration of sulfate in Ham's F-12 medium may be related to the results described above; for example, the sulfation of CS/DS could predominate over that of KS owing to the very low concentration of sulfate. But other investigators (*23, 25-28, 30, 49*) have observed the loss of KS synthesis in cultures where Dulbecco's modified Eagle's medium or Eagle's minimum essential medium was used as the culture medium; both media contain 97.7 mg of Mg_2SO_4/liter (this concentration is the same as that in CG medium).

Concerning the effects of growth factors on proteoglycan synthesis, EGF seems to have some effect on the increase in the proportion of KS synthesis in primary culture in gel (Tables II and IV). As in reports on other types of cells by many investigators, TGF-β1 and bFGF increased proteoglycan synthesis as a whole in our cultures: that resulted from preferential stimulation of CS/DS, HS or sulfated glycoprotein synthesis and, consequently, the proportion of KS synthesis decreased. PDGF decreased the proportion of KS synthesis in primary culture. In secondary cultures, the effects of EGF and bFGF were not additive with that of CG medium on the maintenance of KS synthesis. Instead, both growth factors stimulated hyaluronic acid synthesis in secondary culture. Many investigators have noted hyaluronic acid synthesis in cultures of corneal stromal cells on

plastic or in collagen gel (*22-24, 26, 27, 29*). In their experiments, appearance of hyaluronic acid was accompanied with a marked decrease in KS synthesis and an appearance of a high molecular CS/DSPG. But, in our experiment, appearance of hyaluronic acid did not accompany with these. The reason for this discrepancy is unknown.

In conclusion, KS synthesis decreased markedly in culture after one passage when the cells were cultured on plastic or in collagen gel with Ham's F-12/10% FBS. But, when the medium was changed for CG medium in the middle of culture, the KS synthesis was maintained at the level of that in primary culture even in the absence of any growth factor. What factor(s) in CG medium is responsible for this effect is not yet known.

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