Sulfated Proteoglycan Synthesis by Confluent Cultures of Rabbit Costal Chondrocytes Grown in the Presence of Fibroblast Growth Factor

YUKIO KATO and DENIS GOSPODAROWICZ
Cancer Research Institute and the Departments of Medicine and Ophthalmology, University of California, Medical Center, San Francisco, California 94143

ABSTRACT We examined the effect of fibroblast growth factor (FGF) on proteoglycan synthesis by rabbit costal chondrocyte cultures maintained on plastic tissue culture dishes. Low density rabbit costal chondrocyte cultures grown in the absence of FGF gave rise at confluency to a heterogeneous cell population composed of fibroblastic cells and poorly differentiated chondrocytes. When similar cultures were grown in the presence of FGF, the confluent cultures organized into a homogeneous cartilage-like tissue composed of rounded cells surrounded by a refractile matrix. The cell ultrastructure and that of the pericellular matrix were similar to those seen in vivo. The expression of the cartilage phenotype in confluent chondrocyte cultures grown from the sparse stage in the presence vs. absence of FGF was reflected by a fivefold increase in the rate of incorporation of [^{35}S]sulfate into proteoglycans. These FGF effects were only observed when FGF was present during the cell logarithmic growth phase, but not when it was added after chondrocyte cultures became confluent. High molecular weight, chondroitin sulfate proteoglycans synthesized by confluent chondrocyte cultures grown in the presence of FGF were slightly larger in size than that produced by confluent cultures grown in the absence of FGF. The major sulfated glycosaminoglycans associated with low molecular weight proteoglycan in FGF-exposed cultures were chondroitin sulfate, while in cultures not exposed to FGF they were chondroitin sulfate and dermatan sulfate. Regardless of whether or not cells were grown in the presence or absence of FGF, the 6S/4S dissacharide ratio of chondroitin sulfate chains associated with high and low molecular weight proteoglycans synthesized by confluent cultures was the same. These results provide evidence that when low density chondrocyte cultures maintained on plastic tissue culture dishes are grown in the presence of FGF, it results in a stimulation of the expression and stabilization of the chondrocyte phenotype once cultures become confluent.

Chondrocytes are highly specialized cells producing large amounts of cartilage matrix proteoglycans and type II collagen. A number of hormones and growth factors have been shown to influence the growth of chondrocytes and their phenotypic expression (1–9). Among these factors, fibroblast growth factor (FGF)[1] is the most potent mitogen for cultured chondrocytes. Its mitogenic effect can be observed even in the presence of high concentrations of serum (3, 7) and its intraarticular administration results in a substantial stimulation of the proliferation of chondrocytes in vivo (10). However, the effect of FGF on phenotypic expression by chondrocytes remains unclear.

In the present study, we examined the effect of FGF on phenotypic expression by chondrocytes in confluent culture by comparing proteoglycan synthesis in rabbit costal chondrocyte cultures grown in the presence vs. absence of FGF. The results show that the rate of the incorporation of [^{35}S]-sulfate into proteoglycans in confluent cultures grown in the presence of FGF is much higher than that of cultures grown

[1] *Abbreviations used in this paper:* ECM, extracellular matrix; FGF, fibroblast growth factor; PBS, phosphate-buffered saline.

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in its absence. Furthermore, this increase in the incorporation of [^{35}S]sulfate was due to an increased synthesis of proteoglycan characteristic of cartilage matrix.

MATERIALS AND METHODS

Materials: FGF was purified as described from bovine pituitary (11). Dulbecco's modified Eagle's medium (DME, H-16) was obtained from Gibco Laboratories, Grand Island, NY. Fetal calf serum was obtained from Hyclone Sterile System Inc., Logan, UT. Tissue culture dishes were obtained from Falcon Labware, Oxnard, CA. Gentamicin was obtained from Schering Corp., Kenilworth, NJ, and fungizone from E. R. Squibb & Sons, Princeton, NJ. [^{35}S]Sulfate (1,280 Ci/mmole) and aquasol were purchased from New England Nuclear, Boston, MA; chondroitinase AC, chondroitinase ABC, and chondroitin sulfate were from Seikagaku Kogyo Co., Tokyo, Japan; pronase and papain were from Sigma Chemical Co., St. Louis, MO; Sepharose CL-2B and CL-6B were from Pharmacia Fine Chemicals, Piscataway, NJ.

Preparation of ECM-coated Dishes: Cultures of bovine corneal endothelial (BCE) cells were established from bovine eyes as previously described (12). BCE-ECM–coated dishes were prepared by treatment of confluent corneal endothelial cell cultures with 0.02 M NH_4OH in distilled water for 5 min followed by washing with 0.01 M sodium phosphate (pH 7.4), 0.9% NaCl (phosphate-buffered saline [PBS]) as described previously (13).

Chondrocyte Culture: Chondrocytes were isolated from rib cartilage of 3–4-wk-old male New Zealand rabbits as described by Shimomura et al. (14). Growth cartilage was dissected out and cut into small pieces (1–3 mm^3) with a scalpel. The pieces were incubated for 15 min at 37°C in 20 ml of 0.1% EDTA in PBS and then centrifuged at ~1,100 rpm in an International clinical centrifuge (Model CL, No. 221 head) for 5 min. The pellet was resuspended in 20 ml of 0.125% trypsin in PBS. After 30 min at 37°C, the tissue fragments were centrifuged as described above, and the pellet resuspended in 0.2% collagenase (Sigma Chemical Co., type IA) in PBS. After 2.5 h at 37°C, the tissue fragments and cell aggregates were aspirated up and down a 10-ml plastic pipette. The resulting cell suspension was filtered through a nylon sieve (pore size = 45 μm). The filtrate was centrifuged and the pellet was washed three times with DME supplemented with 50 μg/ml gentamicin and 0.25 μg/ml fungizone. In some experiments (see Table III), chondrocytes were isolated from cartilage fragments by prolonged (3-h and 15-min) collagenase treatment.

Cell Growth Determination and Staining with Alcian Green: Chondrocytes were seeded at a density of 3×10^3 cells to 1×10^4 cells per 35-mm dish in 2 ml DME supplemented with 50 μg/ml ascorbic acid, 50 μg/ml gentamicin, 0.25 μg/ml fungizone, and 10% fetal calf serum. Cultures were maintained on plastic tissue culture dishes either in the absence or presence of various concentrations of FGF (ranging from 12 pg/ml to 12 ng/ml and added every other day). After 2–19 d in culture, triplicate plates were incubated for 2 h in 1 ml PBS containing 0.1% collagenase and 0.1% pronase. When cells were detached from the substratum, cell number was determined with a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL). The morphological appearance of the cultures was analyzed by phase-contrast microscopy and pictures were taken on day 19. In addition, the dishes were fixed and stained with alcian green. This dye stains the strongly anionic sulfated proteoglycans present in large amounts in cartilage matrix (15). After fixation in 10% formalin, the dishes were rinsed with water and treated with 3% acetic acid for 10 min. The cells were then stained for 10 min with a solution of 0.5% alcian green in 3% acetic acid. They were then rinsed with water, treated for 3 min with 3% acetic acid, and rinsed again in water for 3 min (3).

Ultrastructure of Chondrocyte Cultures: Chondrocyte cultures in 35-mm plastic dishes were fixed for 1–2 h with 0.5% paraformaldehyde and 1% glutaraldehyde, buffered with a 0.1-M sodium cacodylate containing 0.2 mM $CaCl_2$ (pH 7.4). The cultures were postfixed with 1% osmium in 0.07 M veronal acetate for 1 h, dehydrated in graded ethanol 50–100%, and embedded in Araldite 502. A JEOL 100C electron microscope was used to photograph thin sections of cell cultures that were stained with uranyl acetate and lead citrate (16).

Determination of the Rate of Proteoglycan Synthesis: After 5–19 d in culture, we exposed cells for 12 h at 37°C to 10 μCi/ml of [^{35}S]sulfate in 1 ml DME supplemented with 50 μg/ml ascorbic acid, 50 μg/ml gentamicin, 0.25 μg/ml fungizone, and 10% fetal calf serum in the presence or absence of FGF. Alternatively, 15- or 18-d-old cultures were exposed for 3 h at 37°C to 2 μCi/ml of [^{35}S]sulfate in 0.8 ml DME. The rate of proteoglycan synthesis was determined by measuring the incorporation of [^{35}S]sulfate into material precipitated with cetyl pyridinium chloride, as previously described (16).

In another series of experiments, 15-d-old cultures were exposed for 3 h at 37°C to 175 μCi of [^{35}S]sulfate in 0.8 ml DME with 10% fetal calf serum. The medium was kept frozen at −30°C until analyzed. The cell layers were overlaid with 1.0 ml of buffer containing 4 M guanidine HCl, 0.1 M ε-amino-*n*-caproic acid, 20 mM EDTA, 1 mg/ml of benzamidine HCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetic acid, and 50 mM Tris HCl (pH 8.0). The culture dishes were then put on a shaker for 24 h at 4°C. After clarification by centrifugation, the cell extracts were stored at −30°C until analyzed.

Preparation of Rabbit Costal Cartilage Proteoglycan: Proteoglycans were extracted from rabbit costal cartilage tissue fragments by incubating them on a shaker platform for 24 h at 4°C in the presence of 4 M guanidine HCl in 50 mM Tris-HCl (pH 8.0) with protease inhibitors. Insoluble material was removed by centrifugation, and aliquots (2 ml) of the cartilage extracts were applied on a Sepharose CL-2B column (1.6 × 97 cm) equilibrated in 4 M guanidine HCl, 50 mM Tris-HCl buffer (pH 8.0) with protease inhibitors. 3-ml fractions were collected. Fractions 28–38, corresponding to the proteoglycan monomer, were pooled, and used as proteoglycan carrier.

Relative Hydrodynamic Sizes of Proteoglycans: 0.4-ml aliquots of the medium were mixed with an equal volume of 8 M guanidine HCl in water. 0.3 ml of a 4-M guanidine HCl solution containing 1.5 mg rabbit costal cartilage proteoglycan was then added. Samples were applied onto a Sepharose CL-2B column (1.6 × 97 cm) equilibrated in 4 M guanidine HCl, 50 mM Tris-HCl buffer (pH 8.0) with protease inhibitors. Fractions were collected and counted as described in Fig. 5.

0.5-ml aliquots of the cell layer fractions were mixed with 0.3 ml of a 4-M guanidine HCl solution containing 1.5 mg rabbit costal cartilage proteoglycan carrier, and directly applied onto a Sepharose CL-2B column (1.6 × 97 cm) equilibrated in 4 M guanidine HCl, 50 mM Tris-HCl buffer (pH 8.0) with protease inhibitors. Fractions were collected and counted as described in Fig. 5.

Sepharose CL-6B Chromatography of Papain-digested Proteoglycan Monomer: Aliquots (4.5 ml) of the pooled fractions of the high molecular weight proteoglycan monomer eluting from Sepharose CL-2B were mixed with 9 ml of a water solution containing 2 mg chondroitin sulfate. 27 ml of 95% ethanol solution containing 1.3% potassium acetate was then added. After 2 h at 0°C, the suspension was centrifuged. The precipitate was washed with 70% ethanol, then dissolved with 0.15 ml of distilled water. Aliquots (100 μl) of the samples were mixed with 100 μl of water containing 100 μg of papain, 0.2 M sodium acetate, (pH 7.0), 10 mM EDTA, and 10 mM cysteine hydrochloride, and incubated at 65°C for 5 h. The papain-digest was analyzed by exclusion gel chromatography on a Sepharose CL-6B column (1.6 × 87 cm) that was equilibrated and eluted with 2 M guanidine HCl, 50 mM Tris-HCl (pH 8.0). 2.5 ml of fractions were collected and counted.

Chondroitinase Digestion: Aliquots (4.5 ml) of the pooled fractions of high and low molecular weight proteoglycans (Fractions 31–41 and 46–60, respectively) eluting from Sepharose CL-2B were mixed with 9 ml of a water solution containing 2 mg chondroitin sulfate. 27 ml of 95% ethanol solution containing 1.3% potassium acetate was then added. After 6–8 h at 0°C, the suspension was centrifuged. The precipitate was solubilized with 4.5-ml aliquots of the appropriate pooled fractions. 9 ml of water was also added. The solution was again mixed with 27 ml of 95% ethanol solution containing 1.3% potassium acetate. After 6–8 h at 0°C, the suspension was centrifuged. This precipitation step was repeated four times. The final precipitate was solubilized with 0.2 ml of water. Aliquots of the samples (150 μl) were mixed with an equal volume of 0.2 M sodium acetate (pH 7.0), 10 mM EDTA, 10 mM cysteine HCl containing 150 μg of papain, and incubated at 65°C for 5 h. The reaction was stopped by boiling for 10 min, and insoluble material was removed by centrifugation. The supernatant was mixed with threefold excess of 95% ethanol/1.3% potassium acetate. The samples were kept at 0°C for 1 h, and the resulting precipitate was collected by centrifugation. The precipitate was solubilized with 0.3 ml of water, and glycosaminoglycans were again precipitated by adding 3 ml of 95% ethanol/1.3% potassium acetate. This precipitation step was repeated three times. The final precipitate was solubilized with 0.15 ml of water.

The ^{35}S-labeled glycosaminoglycan prepared as described above were digested with chondroitinase (17). Aliquots (10–35 μl) of samples were mixed with 10 μl of a water solution containing chondroitinase AC (0.2 U) or chondroitinase ABC (0.2 U), and 5 μl of 1 M Tris-HCl buffer (pH 7.6) containing 0.05 M sodium acetate and 0.01 M NaF. The reaction mixture containing 50 μl of the solution was incubated at 37°C for 5 h. After incubation, the mixture was heated at 100°C for 2 min. Aliquots (30 μl) of the solution were applied to a cellulose thin layer sheet (20 × 20 cm). A chromatography paper (Whatman No. 1) (Whatman Chemical Separation Inc., Clifton, NJ) extension was taped to the upper edge of the thin layer sheet to allow the solvent to rise 21–23 cm above the base line (18). After a 24-h desalting step using *n*-butanol:ethanol:water (52:32:16, vol/vol), chromatograms were developed with *n*-butanol:acetic acid:2 M NH_4OH (2:3:1) (18). Radioactivity in each fraction (0.5 cm) was eluted from the cellulose by addition of 0.5 ml of 0.1 M HCl followed by heating for 24 h at 65°C. Aquasol (5 ml) was added and radioactivity determined.

RESULTS

Effect of FGF on the Growth of Cultured Rabbit Costal Chondrocytes

The ability of FGF to stimulate the growth of rabbit costal chondrocytes was evaluated. Low density chondrocyte cultures were exposed for 6 d to DME supplemented with 10% fetal calf serum and increasing concentrations of FGF ranging from 12 pg to 12 ng/ml (Fig. 1*A*). A FGF mitogenic effect was detectable at concentrations as low as 12 pg/ml, and was maximal at 0.5 ng/ml, resulting in a 12-fold increase in cell number over controls.

Fig. 1*B* shows the growth curve of rabbit chondrocytes seeded at low density (3×10^3 cells/35-mm dish) and exposed either to DME supplemented with 10% fetal calf serum alone or DME supplemented with 10% fetal calf serum and FGF (0.6 ng/ml). After a 2-d lag-time, chondrocytes started to proliferate actively regardless of whether or not they were exposed to FGF. The average doubling time during the logarithmic growth phase in cultures exposed to FGF was 16 h, while that of cultures not exposed to FGF was 35 h. Cultures became confluent on day 7 for those exposed to FGF and on day 15 for those not exposed to it. The final cell density (1.8×10^6 cells/35-mm dish) of cultures exposed to FGF was 90% higher than that (9.5×10^5 cells/35-mm dish) of cultures not exposed to the mitogen.

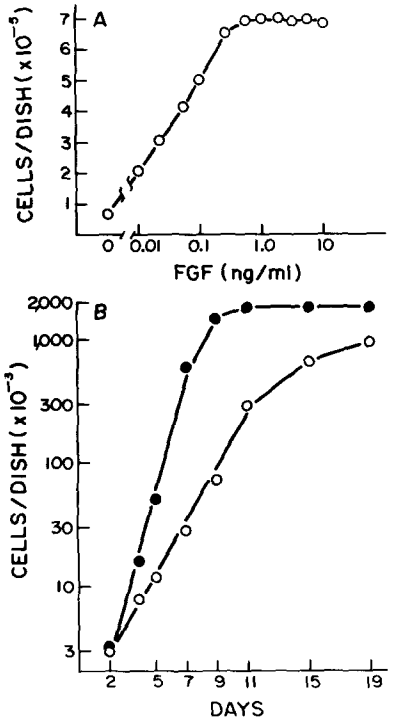

FIGURE 1 Effect of FGF on the proliferation of low density rabbit costal chondrocytes. (*A*) Rabbit costal chondrocytes were seeded at low density (1×10^4 cells per 35-mm plastic tissue culture dish) and exposed to DME supplemented with 10% fetal calf serum and increasing concentrations of FGF. FGF was added every other day in 10-μl aliquots of medium. After 6 d in culture, triplicate dishes were harvested and counted as described in Materials and Methods. The standard deviation in the different determinations did not exceed 5% of the mean. (*B*) Rabbit costal chondrocytes were seeded at low density (3×10^3 cells/35-mm plastic tissue culture dish) and exposed to DME supplemented with 10% fetal calf serum in the presence (●) or absence (○) of FGF (0.6 ng/ml). FGF was added every other day. After 2–19 d in culture, triplicate dishes were harvested and counted as described in Materials and Methods. The standard deviation in the different determinations did not exceed 5% of the mean.

Histological Characterization of Chondrocyte Cultures Grown in the Presence or Absence of FGF

When low density (3×10^3 cells/35-mm dish) cultures of rabbit costal chondrocytes were grown in DME supplemented with 10% fetal calf serum and in the absence of FGF, the majority of the cells (>80%) in confluent cultures adopted a fibroblastic or epithelioid configuration (Fig. 2*A*). No conversion of the cells to overtly differentiated spherical chondrocytes was observed even after 19 d in culture. Cultures grown in the absence of FGF did stain poorly with alcian green and only in the areas where cartilage nodules were observed (Fig. 2*C*). In contrast, almost all cells (>99%) grown in the presence of FGF, although they had adopted a fibroblastic conformation during their proliferative stage, at confluence reassumed the spherical conformation characteristic of chondrocytes (Fig. 2*B*). These spherical cells were surrounded by a refractile matrix which stained intensely with alcian green (Fig. 2*C*). Transmission electron microscope analysis of confluent chondrocyte cultures grown in the presence of FGF showed an ultrastructure characteristic of chondrocytes in vivo and cells were embedded in a well-developed three-dimensional cartilaginous matrix in which abundant collagen fibrils were observed (Fig. 3). The ultrastructure of the cartilage-like tissue that was expressed by confluent cultures grown in the presence of FGF was strikingly similar to that seen in vivo (19), suggesting that FGF stimulates the accumulation of cartilage-matrix macromolecules in the cell layer-matrix.

In another series of experiments, it was observed that even when seeded at a clonal cell density (10^2 cells/35-mm dish), chondrocyte cultures maintained on plastic tissue culture dishes and grown in the presence of FGF for over 19 d did develop at confluency into a homogeneous cartilage-like tissue (data not shown).

Effect of FGF on Proteoglycan Synthesis by Cultured Rabbit Costal Chondrocytes

For quantitative estimation of sulfated proteoglycan synthesis, the incorporation of [^{35}S]sulfate into material precipitated with cetylpyridinium chloride was measured. Fig. 4*A* shows the changes in the level of [^{35}S]sulfate incorporation into proteoglycans per dish when rabbit chondrocytes were maintained for 5–19 d in the presence or absence of FGF. The level of [^{35}S]sulfate incorporation in cultures exposed or not to FGF increased with the age of the culture, reaching a maximum on day 15. The level of [^{35}S]sulfate incorporation per dish in cultures exposed to FGF was higher than that of cultures not exposed to FGF during the whole experimental period (from day 5 to 19). The maximal level of incorporation of [^{35}S]sulfate in cultures grown in the presence of FGF was ninefold higher than that reached in cultures grown in its absence.

Fig. 4*B* shows the changes in the level of [^{35}S]sulfate incorporation into proteoglycans per cell. Between days 5 and 9, the level of proteoglycan synthesis as reflected by [^{35}S]sulfate incorporation per cell in cultures exposed to FGF was ~50%

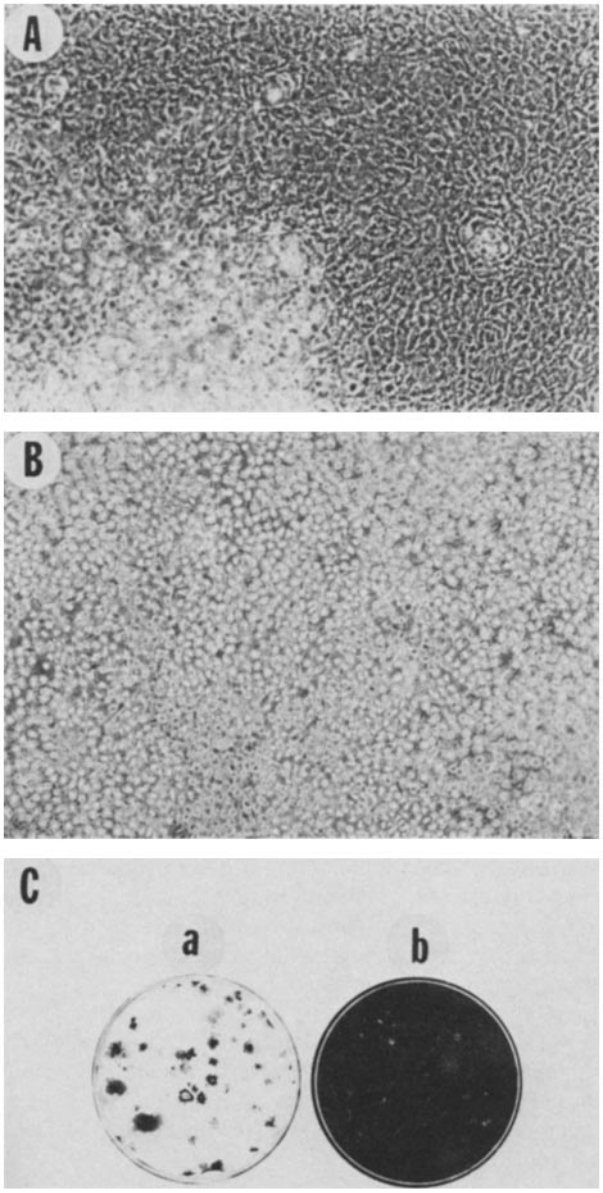

FIGURE 2 Morphological appearance of rabbit costal chondrocytes maintained on plastic culture dishes and exposed to DME supplemented with 10% fetal calf serum in the presence of FGF. (*A* and *B*) Rabbit costal chondrocytes were seeded at 3×10^3 cells/35-mm plastic tissue culture dish and exposed to DME supplemented with 10% fetal calf serum in the presence (*B*) or absence (*A*) of FGF. FGF (0.6 ng/ml) was added every day. Pictures were taken on day 19 with a Nikon phase-contrast photomicroscope (× 100). (*C*) Comparison of alcian green staining of rabbit costal chondrocyte cultures grown in the absence (*a*) or presence (*b*) of FGF. Culture conditions were as described above. After 19 d in culture, one set of plates were fixed with 10% formalin and stained with alcian green, as described in Materials and Methods.

lower than that of cultures not exposed to FGF. This is consistent with previous studies where brief exposure of sparse actively growing cultures of rabbit articular chondrocytes to FGF results in a significant decrease in incorporation of [^{35}S]sulfate into proteoglycans (2, 7). However, the level of [^{35}S]sulfate in cultures not exposed to FGF decreased after day 11. In contrast, the level of [^{35}S]sulfate incorporation in cultures exposed to FGF markedly increased between days 11 and 15, after which it stayed stable. This resulted, on day 15,

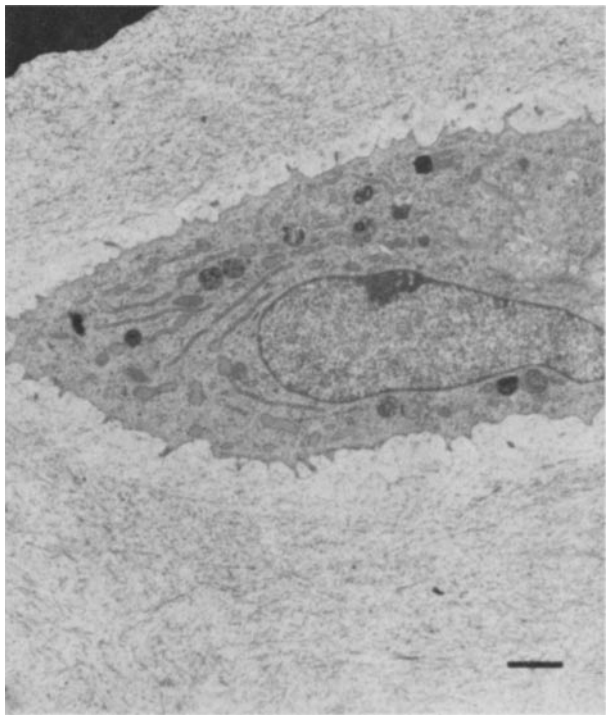

FIGURE 3 Transmission electron microscopy of chondrocytes cultures maintained in the presence of FGF. A cross-section of the cell layer of cultures maintained for 19 d in the presence of FGF is shown. Culture conditions were as described in Fig. 2. Bar, 2 μm. × 3,850.

in a fourfold increase in the incorporation of [^{35}S]sulfate in cultures exposed to FGF relative to those not exposed to the mitogen (Fig. 4*B*).

Next, we examined the effect of increasing concentrations of FGF on the incorporation of [^{35}S]sulfate into proteoglycans in confluent cultures. As shown in Table I, addition of FGF to exponentially growing cells resulted in confluent cultures in a dose-dependent increase in the rate of the incorporation of [^{35}S]sulfate into proteoglycans. This increase could be observed at concentrations as low as 4 pg/ml, and was maximum (four- to fivefold increase) at 0.4–0.6 ng FGF/ml. This increase in the incorporation of [^{35}S]sulfate into proteoglycans was paralleled by an increase in the number of spherical cells surrounded by a refractile matrix (data not shown). These results taken together indicate that when chondrocytes are grown in the presence of FGF, it results in an increase in the net synthesis of sulfated proteoglycans in confluent cultures.

In another series of experiments, we examined whether the stimulatory effect of FGF on the incorporation of [^{35}S]sulfate in confluent cultures could be observed when FGF was included in the medium before or after cultures became confluent. As shown in Table II, the rate of [^{35}S]sulfate incorporation, measured on day 18, in cultures exposed to FGF from day 0 to day 8 or 12 was six- to eightfold higher than that of cultures never exposed to the mitogen. When cultures were exposed to FGF from days 0 to 18, the rate of [^{35}S]sulfate incorporation was ninefold higher than that of cultures never exposed to it. In contrast, when cultures were exposed to FGF after reaching confluence (days 12 to 18), FDF had little effect on the rate of [^{35}S]sulfate incorporation measured at day 18 (Table II).

We have also observed that rabbit costal chondrocytes maintained on extracellular matrix (ECM) produced by bo-

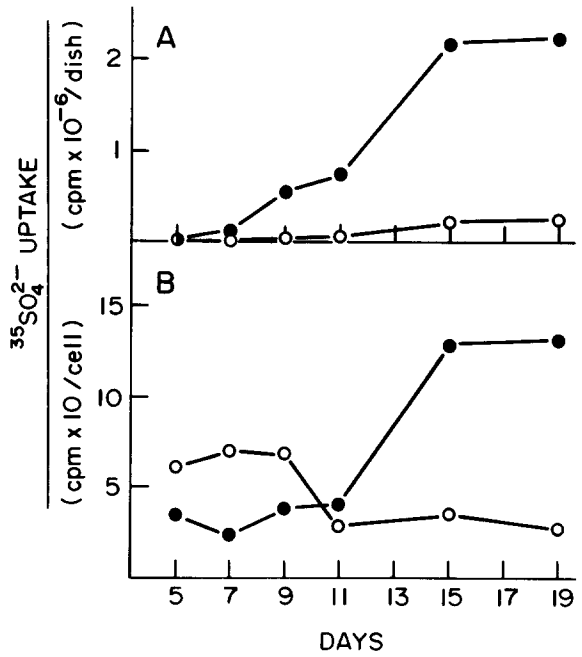

FIGURE 4 Changes in the rate of [^{35}S]sulfate incorporation into proteoglycans in low density chondrocyte cultures maintained in the presence or absence of FGF. Rabbit chondrocytes were seeded at 3 × 10^3 cells/35-mm dish on plastic culture dishes and exposed to DME supplemented with 10% fetal calf serum alone (○) or DME supplemented with 10% fetal calf serum and FGF (0.6 ng/ml) (●). FGF was added every other day. After 5–19 d in culture, triplicate plates representing each condition were exposed for 12 h to 10 μCi/ml of [^{35}S]sulfate in 1.0 ml DME supplemented with 10% fetal calf serum in the presence or absence of FGF (0.6 ng/ml). The rate of [^{35}S]sulfate incorporation into proteoglycans present in the cell layer and in the medium per dish (*A*) or per cell (*B*) was determined as described in Materials and Methods. The standard deviation in the different determinations did not exceed 10% of the mean.

TABLE I

Effect of Increasing Concentrations of FGF on Proteoglycan Synthesis by Rabbit Costal Chondrocytes in Culture

		$^{35}SO_4^{2-}$ Uptake	
FGF	Cells/dish	cpm × 10^{-3}/dish	cpm × 10^3/cell
ng/ml	*× 10^{-4}*		
0	66	16 ± 4	24
0.004	73	27 ± 1	37
0.04	142	84 ± 9	59
0.1	168	145 ± 8	87
0.4	165	162 ± 6	98
0.6	169	177 ± 11	104
1.0	169	159 ± 10	94
4.0	166	171 ± 12	103

Rabbit costal chondrocytes were seeded at 3 × 10^3 cells per 35-mm plastic tissue culture dish and exposed to DME supplemented with 10% fetal calf serum and increasing concentrations of FGF. FGF was added every other day. After 15 d in culture, triplicate plates were exposed for 3 h to 2 μCi/ml of [^{35}S]sulfate in 0.8 ml of DME. Values represent averages ± standard deviation for triplicate determinations.

vine corneal endothelial cells synthesize a larger amount of sulfated proteoglycans in confluent cultures than those maintained on plastic (Kato and Gospodarowicz, manuscript submitted for publication). Consistent with this observation, the rate of [^{35}S]sulfate incorporation into proteoglycans in 18-d-old cultures maintained on ECM-coated dishes was eightfold higher than that of similar cultures maintained on plastic tissue culture dishes. When FGF was added to actively growing cultures maintained on ECM-coated dishes, it had little effect on the incorporation of [^{35}S]sulfate into proteoglycans in confluent cultures (Table II). This may imply that the maximal level of [^{35}S]sulfate incorporation can be achieved even in the absence of FGF by maintaining chondrocytes on ECM-coated dishes.

TABLE II

Effect of Length of Exposure to FGF on the Rate of Proteoglycan Synthesis by 18-d cultures of Chondrocytes Maintained on Plastic and ECM-coated Dishes

			$^{35}SO_4^{2-}$ uptake	
Substrate	Addition of FGF	Cells/dish	cpm × 10^{-3}/dish*	cpm × 10^3/cell*
	Periods	*× 10^{-5}*		
Plastic	None	10.5 ± 0.3	9 ± 1 (56)‡	9
	days 0–8	19.2 ± 1.2	111 ± 15 (90)	58
	days 0–12	19.5 ± 0.3	133 ± 8 (92)	68
	days 0–18	19.6 ± 0.7	150 ± 6 (88)	77
	days 12–18	12.9 ± 0.2	8 ± 1 (63)	6
ECM	None	17.1 ± 0.8	125 ± 4 (94)	73
	days 0–18	21.9 ± 0.5	149 ± 4 (92)	68

Rabbit costal chondrocytes were seeded at 3 × 10^3 cells per 35-mm plastic tissue culture dish and exposed to DME supplemented with 10% fetal calf serum. FGF (0.6 ng/ml) was added every other day during the indicated culture periods. After 18 d in culture, triplicate plates were exposed for 3 h to 2 μCi/ml of [^{35}S]sulfate in 0.8 ml DME. Values represent averages ± standard deviation for triplicate determinations.

* Incorporation of $^{35}SO_4^{2-}$ was measured on day 18 as described in Materials and Methods.

‡ Percent of the radioactivity incorporated into proteoglycans present in the cell layer vs. radioactivity incorporated into proteoglycans present in the cell layer and in the culture medium.

The stimulatory effect of FGF on proteoglycan synthesis (Tables I and II) could result from the higher final cell density reached by cultures grown in the presence of FGF than in its absence. To examine this question, we have taken advantage of the fact that FGF does not increase the final saturation density of cultures prepared with chondrocytes isolated from costal cartilage fragments by prolonged collagenase-treatment. Such cells when grown on plastic tissue culture dishes in the absence of FGF adopted a fibroblastic configuration with extremely elongated cellular processes and grew on top of each other forming an irregular multilayer in confluent cultures. This resulted in about a twofold increase in the final cell density over that reached by chondrocyte cultures seeded initially with cells that had been prepared by the conventional method. When FGF was added to actively growing cultures seeded initially with cells that had been prepared by prolonged collagenase treatment, ~50% of the cells reassumed a spherical configuration characteristic of overtly differentiated chondrocytes at confluence. This was reflected by a fourfold increase in the level of [^{35}S]sulfate incorporation into proteoglycans on day 18 in FGF-exposed cultures. However, FGF did not significantly increase the final cell density of such cultures (Table III). Therefore the stimulatory effect of FGF on proteoglycan synthesis is not due to an increase in final cell density of confluent cultures.

Hydrodynamic Sizes of Newly Synthesized Proteoglycans

The results presented in Table I showed that confluent cultures grown from the sparse stage in the presence of FGF

increase their rate of incorporation of [^{35}S]sulfate into proteoglycans. However, these results do not eliminate the possibility that the increased incorporation of [^{35}S]sulfate is due to production of a proteoglycan that is not characteristic of cartilage matrix. To test this possibility, we analyzed the hydrodynamic sizes of ^{35}S-labeled proteoglycans synthesized by confluent chondrocyte cultures grown in the presence or absence of FGF by exclusion gel chromatography on Sepharose CL-2B. The elution profiles are shown in Fig. 5. As can be seen in Fig. 5, *A* and *C*, confluent cultures grown in the absence of FGF produced two proteoglycan species (high and low molecular weight). The high molecular weight proteoglycan (K_{av} = 0.29) was similar in size to cartilage proteoglycan synthesized by high density cultures of rat chondrosarcoma cells (20) and chick embryo chondrocytes (21). The low molecular weight proteoglycan (K_{av} = 0.64 to 0.67) was similar in size to that produced by fibroblasts and called ubiquitous or fibroblastic proteoglycan (20–26). On the other hand, cultures grown in the presence of FGF produced one major sulfated proteoglycan species with high molecular weight (K_{av} = 0.21 to 0.29). Only a small amount of low molecular weight proteoglycan species (K_{av} = 0.64) was observed in these cultures (Fig. 5, *B* and *D*). The high molecular weight proteoglycan (K_{av} = 0.21) present in the cell layer-matrix in FGF-treated cultures was larger than that (K_{av} = 0.29) present in the medium of the same cultures (Fig. 5*B* vs. 5*D*). On the other hand, the high molecular weight proteoglycan (K_{av} = 0.29) present in the cell layer-matrix of control cultures was similar in size to that (K_{av} = 0.29) present in the medium of the control cultures (Fig. 5*A* vs. 5*C*). It was observed that the size (K_{av} = 0.21) of the high molecular weight proteoglycan present in the cell layer-matrix in cultures exposed to FGF was slightly larger than that (K_{av} = 0.29) of high molecular weight proteoglycan present in the cell layer-matrix of cultures not exposed to the mitogen (Fig. 5*B* vs. 5*A*).

Table IV shows the relative distribution of ^{35}S-radioactivity incorporated into high and low molecular weight proteoglycans present in the cell layer-matrix and in the medium of confluent cultures grown from the sparse stage in the presence vs. absence of FGF. The levels of [^{35}S]sulfate incorporated into high molecular weight proteoglycan present in the cell

TABLE III

Effect of Length of Exposure to FGF on the Rate of Proteoglycan Synthesis After 18 d in Culture by Chondrocytes Isolated from Rabbit Costal Cartilage by Prolonged Collagenase Treatment

Substrate	Addition of FGF	Cells/dish	$^{35}SO_4^{2-}$ uptake: cpm × 10^{-3}/dish*	$^{35}SO_4^{2-}$ uptake: cpm × 10^3/cell*
	Periods	*× 10^{-5}*		
Plastic	None	19.6 ± 1.7	18 ± 1 (78)‡	9
	days 0–10	22.3 ± 0.4	78 ± 14 (95)	35
	days 0–14	21.7 ± 0.3	76 ± 8 (95)	35
	days 0–18	21.4 ± 0.4	85 ± 7 (92)	40

Experimental and culture conditions were as described in Table II, except that rabbit costal chondrocytes isolated from cartilage segments by prolonged collagenase treatment were used.

* Incorporation of $^{35}SO_4^{2-}$ was measured on day 18 as described in Materials and Methods.

‡ Percent of the radioactivity incorporated into proteoglycans present in the cell layer vs. radioactivity incorporated into proteoglycans present in the cell layer and in the culture medium.

TABLE IV

Incorporation of [^{35}S]Sulfate into High and Low Molecular Weight Proteoglycans

Fraction	FGF	[^{35}S]Sulfate incorporation (cpm/cell): High molecular weight proteoglycans	[^{35}S]Sulfate incorporation (cpm/cell): Low molecular weight proteoglycans
Cell layer	−	0.91	0.31
	+	5.08	0.71
Medium	−	0.32	0.18
	+	0.66	0.15

The levels of ^{35}S-radioactivity incorporated into high molecular weight proteoglycan (Fractions 25–45) and low molecular weight proteoglycan (Fractions 46–60) eluted from a Sepharose CL-2B column are shown. Culture conditions were as described in Fig. 5. The elution profiles of ^{35}S-labeled proteoglycans present in the cell layer and the medium of cultures grown in the presence of absence of FGF are also as shown in Fig. 5.

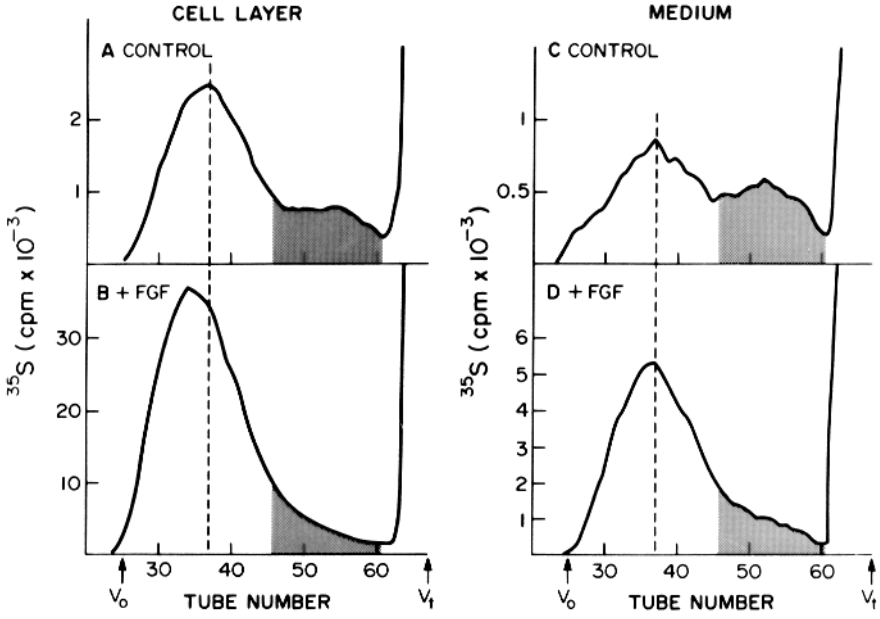

FIGURE 5 Sepharose CL-2B chromatography of proteoglycans present in the cell layer-matrix and in the medium from cultures grown in the presence or absence of FGF. Cultures were seeded and maintained for 15 d as described in Fig. 2. Aliquots of the 4-M guanidine HCl extract of the cell layer-matrix (*A* and *B*) and the medium (*C* and *D*) from cultures grown in the presence (*B* and *D*) or absence (*A* and *C*) of FGF were applied onto a Sepharose CL-2B column that was equilibrated in 4 M guanidine HCl, 50 mM Tris-HCl (pH 8.0) with protease inhibitors as described in Materials and Methods. 3-ml fractions were collected. Aliquots (0.3 ml) of each fraction were mixed with 0.3 ml ethanol and 10 ml Aquasol. Radioactivity was measured in a Beckmann LS-8000 scintillation counter. Free unincorporated radioactivity elutes at *Vt*. *Vo* was determined with high molecular weight hyaluronic acid synthesized by rabbit costal chondrocytes.

layer-matrix and in the medium of confluent cultures grown in the presence of FGF was six- and twofold higher than those of cultures grown in its absence, respectively. In contrast, the amount of ^{35}S incorporated into low molecular weight proteoglycan present in the cell layer-matrix of confluent cultures grown in the presence of FGF was only twofold higher over controls, while the amount of ^{35}S incorporated into low molecular weight proteoglycans released into the medium of FGF-grown cultures was almost the same as the control levels. Similar results were obtained with different batches of chondrocytes (data not shown). These results suggest that FGF selectively stimulates the synthesis of high molecular weight proteoglycan and its preferential incorporation into a cartilaginous matrix.

In another series of experiments, the molecular weight of newly synthesized glycosaminoglycans was also examined. The ^{35}S-labeled glycosaminoglycans, cleaved from the intact high molecular weight proteoglycan by treatment with papain were analyzed by exclusion gel chromatography on a Sepharose CL-6B column equilibrated in 2 M guanidine HCl, 50 mM Tris-HCl (pH 8.0). The elution profiles showed that the length (K_{av} = 0.54–0.56) of the glycosaminoglycan chains associated with high molecular weight proteoglycan present in the confluent cell-layer matrix of FGF-grown cultures was not significantly different from that of control cultures (data not shown). Therefore, the increased incorporation of [^{35}S]-sulfate into high molecular weight proteoglycans present in the confluent cell layer-matrix of FGF-grown cultures is not due to an increase in glycosaminoglycan chain size.

Chondroitinase Digestion

The ^{35}S-labeled glycosaminoglycans, cleaved from high and low molecular weight proteoglycans by treatment with papain were digested with chondroitinase AC or ABC, and the released disaccharides were analyzed by thin layer chromatography, as described in Materials and Methods.

In samples from confluent cultures grown in the presence of FGF, >98% of the ^{35}S-labeled glycosaminoglycans associated with high molecular weight proteoglycan were digested to disaccharides by chondroitinase AC (Table V). This indicates that the high molecular weight proteoglycans produced by confluent rabbit costal chondrocyte cultures grown in the presence of FGF are chondroitin sulfate proteoglycan. On the other hand, in samples from confluent cultures grown in the absence of FGF, 85–90% and 93–94% of the ^{35}S-labeled glycosaminoglycans associated with high molecular weight proteoglycan were digested by chondroitinase AC and ABC, respectively (Table V), suggesting that a small proportion (4–7%) of dermatan sulfate associates with high molecular weight proteoglycans produced by confluent cultures of chondrocytes grown in the absence of FGF.

In samples from confluent cultures grown in the presence of FGF, 87–93% and 96–98% of the ^{35}S-labeled glycosaminoglycans associated with low molecular weight proteoglycans were digested by chondroitinase AC and ABC, respectively (Table V). On the other hand, samples from confluent cultures grown in the absence of FDF, 35–53% and 88–93% of the ^{35}S-labeled glycosaminoglycans associated with low molecular weight proteoglycans were digested by chondroitinase AC and ABC, respectively (Table V). These results indicate that the major sulfated glycosaminoglycans associated with low molecular weight proteoglycans in FGF-exposed cultures were chondroitin sulfate, while in cultures not exposed to FGF they were chondroitin sulfate and dermatan sulfate. The dermatan sulfate was primarily present as the 4-sulfated isomer (data not shown).

Table V also shows that the 6S/4S disaccharide ratio of the chondroitin sulfate chains associated with high and low molecular weight proteoglycans were 1.9:2.1 and 1.3:1.6, respectively. Exposure of the cells to FGF had little effect on the 6S/4S disaccharide composition of the chondroitin sulfate glycosaminoglycans associated with high and low molecular weight proteoglycans produced by confluent cultures (Table V). Similar results were obtained with different batches of chondrocytes. Although cultured chondrocytes synthesized oversulfated chondroitin sulfate chains under certain experimental conditions (26, 27), disulfated disaccharides were not observed at significant levels in samples from rabbit costal chondrocyte cultures used in the present study. Therefore, the increased incorporation of ^{35}S-radioactivity in confluent cultures grown from the sparse stage in the presence of FGF was not the result of synthesis of oversulfated chondroitin sulfate chains.

TABLE V

Disaccharide Analysis of High and Low Molecular Weight Proteoglycans Eluted from Sepharose CL-2B

Proteoglycans Fraction	FGF	Chondroitinase AC				Chondroitinase ABC dermatan sulfate	
		Origin (a)	Di6S	Di4S	(6/4)	Origin (b)	(a-b)
		%	%	%		%	%
High molecular weight proteoglycans							
Cell layer	−	10.3	54.1	25.4	(2.1)	6.1	4.2
	+	1.9	59.3	30.9	(1.9)	ND	1.9
Medium	−	14.5	50.2	25.3	(2.1)	7.3	7.2
	+	1.5	59.3	28.5	(2.1)	ND	1.5
Low molecular weight proteoglycans							
Cell layer	−	47.4	28.7	20.4	(1.4)	11.9	35.5
	+	7.2	48.3	36.3	(1.3)	2.2	5.0
Medium	−	65.1	14.7	57.0	(1.3)	7.1	57.0
	+	12.8	48.7	31.2	(1.6)	4.1	6.7

[^{35}S]Sulfate-labeled glycosaminoglycans from the cell layer-matrix and the culture medium of confluent cultures grown in the presence or absence of FGF were digested with chondroitinase AC or ABC, as described in Materials and Methods.

DISCUSSION

It has repeatedly been reported that chondrocytes grown on plastic tissue culture dishes progressively transform into motile cells that are morphologically indistinguishable from fibroblasts (28–30). A similar observation was done in the present study since rabbit costal chondrocytes seeded at low densities on plastic culture dishes and exposed to DME supplemented with 10% fetal calf serum lost their phenotypic expression, as shown by their fibroblastic morphology and decreased incorporation of [^{35}S]sulfate into proteoglycans, after proliferating for eight generations and becoming confluent. In contrast, chondrocytes maintained on plastic culture dishes in the presence of FGF retained their ability once confluent to express their cartilage phenotype even after proliferating for nine generations. This was reflected by the ability of chondrocytes to reorganize into a homogeneous cartilage-like tissue in vitro, as shown by increases in the number of spherical cells, the extent of staining with alcian green, and the incorporation of [^{35}S]sulfate into proteoglycans. Furthermore, the ultrastructure of a cartilage-like tissue in cultures maintained in the presence of FGF was similar, when analyzed by transmission electron microscopy, to that of the cartilage in vivo. These observations establish that FGF stimulates and stabilizes the phenotypic expression of low density chondrocyte cultures maintained on plastic tissue culture dishes.

The mechanism by which chondrocytes maintained on plastic tissue culture dishes lose their phenotypic characteristics unless FGF is included in the culture medium is unknown. It may reflect an adverse adaption of the cells to the artificial substrate. Recent studies have shown that rabbit costal chondrocytes maintained on dishes coated with ECM retain their phenotypic expression even in the absence of FGF after proliferating for more than 12 generations (Kato and Gospodarowicz, manuscript submitted for publication). Therefore, as in the case of ECM, FGF may stimulate or stabilize chondrocytes' phenotypic expression by circumventing the adverse influence of the plastic substrate.

Although addition of FGF to actively growing cultures resulted in a marked increase in [^{35}S]sulfate incorporation into proteoglycans once cultures became confluent, it also caused a temporal suppression of [^{35}S]sulfate incorporation into proteoglycans during the logarithmic growth phase of the cultures. This is consistent with previous studies (2, 7) that demonstrated that brief exposure of sparse cultures of rabbit articular chondrocytes to FGF resulted in significant decrease in incorporation of [^{35}S]sulfate into proteoglycans. This temporary suppression of sulfated proteoglycan synthesis by FGF may reflect the increase in the growth rate of chondrocytes exposed to the mitogen. This suppression is not unique to sparse cultures exposed to FGF. It has been shown that epidermal growth factor also inhibits sulfated proteoglycan synthesis in sparse cultures of rabbit costal chondrocytes when it stimulates the proliferation of chondrocytes (8). On the other hand, the stimulatory effect of FGF on sulfated proteoglycan synthesis in confluent cultures appears to be unique to this mitogen, since the addition of other growth factors such as epidermal growth factor and insulin to sparse, actively growing chondrocyte cultures did not result in a stimulation of sulfated proteoglycan synthesis once cultures became confluent.

The present study shows that the final effect of FGF, when added to actively growing chondrocyte cultures, is to preserve and enhance the ability of the cells to express their phenotypic expression once culture becomes confluent. The three following possibilities could account for such an effect: (*a*) FGF could have an indirect effect on chondrocytes' phenotypic expression by increasing the final cell density of confluent cultures. The results from experiments with chondrocytes isolated from cartilage by prolonged collagenase-treatment show, however, that FGF can stimulate sulfated proteoglycan synthesis in confluent cultures without a concomitant increase in final cell density under certain culture conditions. Therefore, it is unlikely that the increase in final cell density alone accounts for the stimulation of sulfated proteoglycan synthesis in confluent cultures grown in the presence of FGF. (*b*) FGF, when added after cells have reached confluence, could revert the phenotype of dedifferentiated fibroblastic cells to that of chondrocytes. This is not likely, since FGF, when added to cultures grown to confluency in its absence, did not revert the phenotypes of dedifferentiated cells, nor did it increase the rate of proteoglycan synthesis. (*c*) FGF, when added to actively growing chondrocytes, prevents at each cell cycle the dedifferentiation of a given percentage of the population into an irreversible fibroblastic stage. That this is the most likely possibility is supported by the fact that cells, when confluent, need only to be grown in the presence of FGF to express their proper phenotype. A similar effect of FGF on the stabilization of phenotypic expression and prevention of dedifferentiation of vascular endothelial cells has already been reported (31, 32). It was only when FGF was added to actively growing cells that prevention of their dedifferentiation or reversal to their proper phenotype was observed (31, 32).

The present study also demonstrates that the major proteoglycan produced by rabbit costal chondrocytes grown in the presence or absence of FGF is high molecular weight chondroitin sulfate proteoglycans. The proteoglycan monomer was similar in size to cartilage-characteristic proteoglycan produced by high density cultures of rat chondrosarcoma cells (20) and chick embryo chondrocytes (21), when analyzed by gel filtration on Sepharose CL-2B. Furthermore, the majority of proteoglycans produced by rabbit costal chondrocytes were able to form aggregates even in cultures maintained in the absence of FGF on plastic tissue culture dishes (Kato and Gospodarowicz, manuscript submitted for publication). These results have provided evidence that rabbit costal chondrocytes grown in the presence or absence of FGF synthesize the proteoglycan characteristic of cartilage matrix. In addition, the rabbit costal chondrocyte cultures produced a small population of low molecular weight proteoglycans. Since a neutral protease synthesized by chondrocytes has been shown to be capable of degrading both monomeric and aggregate proteoglycans (33), the low molecular weight proteoglycan could represent a degradation product of high molecular weight cartilage proteoglycan. However, the 6S/4S disaccharide ratio (1.3:1.6) of the chondroitin sulfate chains associated with low molecular weight proteoglycan was significantly lower than that (1.9:2.1) of high molecular weight proteoglycan. In addition, the chemical composition of low molecular weight proteoglycan differed from that of high molecular weight proteoglycan, and especially in the case of cultures grown in the absence of FGF. The major sulfated glycosaminoglycans associated with low molecular weight proteoglycan present in control cultures were chondroitin sulfate-dermatan sulfate. In contrast, the major sulfated glycosaminoglycans associated

with high molecular weight proteoglycan were chondroitin sulfate. These results suggest that low molecular weight proteoglycan represents another species of proteoglycan that is also synthesized by chondrocytes together with high molecular weight proteoglycan. Other studies have also suggested that the low molecular weight proteoglycan in cartilage is not a degradation product of high molecular weight proteoglycan (34, 35).

The levels of incorporation of [^{35}S]sulfate into high and low molecular weight proteoglycans in confluent cultures grown in the presence of FGF were 4.7- and 1.8-fold higher, respectively, than those observed with cultures grown in its absence. This suggests that chondrocyte cultures grown in the presence of FGF favor the production of high molecular weight, cartilage-characteristic proteoglycan, rather than that of low molecular weight proteoglycan. Furthermore, FGF produced qualitative changes in the synthesis of sulfated proteoglycans. The high molecular weight proteoglycan present in the cell layer-matrix of cultures grown in the presence of FGF was slightly larger in size than that synthesized in cultures grown in its absence. The increase in the monomer size of the cell layer and matrix-associated proteoglycan produced by FGF-treated chondrocytes may be due to an increase in the number of glycosaminoglycan chains attached to the core protein. This is consistent with previous studies that demonstrated that insulin and somatomedin increase the proteoglycan monomer size when the hormones increase net synthesis of cartilage-characteristic proteoglycan in rat chondrosarcoma cell cultures (20). In addition, the major sulfated glycosaminoglycan chains associated with low molecular weight proteoglycan produced in confluent cultures grown in the presence of FGF were chondroitin sulfate, as compared with chondroitin sulfate-dermatan sulfate in the case of cultures grown in the absence of FGF. Fibroblastic cells in cultures maintained without FGF may be responsible for the greater proportion of dermatan sulfate, since dedifferentiated chondrocytes, unlike differentiated ones, synthesize a large amount of dermatan sulfate (36).

In summary, the present study shows that FGF stimulates and stabilizes the phenotypic expression of low density chondrocyte cultures maintained on plastic tissue culture dishes. This is reflected in increased synthesis of high molecular weight proteoglycan characteristic of cartilage matrix and changes in the structures of high and low molecular weight proteoglycans.

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