Effect of Vanadate on Cartilage-Matrix Proteoglycan Synthesis in Rabbit Costal Chondrocyte Cultures

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Abstract. The effect of vanadate on proteoglycan synthesis by cultured rabbit costal chondrocytes was examined. Rabbit chondrocytes were seeded at low densities and grown to confluency in medium supplemented with 10% fetal bovine serum, and then the serum concentration was reduced to 0.3%. At the low serum concentration, chondrocytes adopted a fibroblastic morphology. Addition of 4 μM vanadate to the culture medium induced a morphologic differentiation of the fibroblastic cells to spherical chondrocytes, and increased by two- to threefold incorporation of [^{35}S]sulfate and [^{3}H]glucosamine into large, chondroitin sulfate proteoglycans. The stimulation of incorporation of labeled precursors reflected real increases in proteoglycan synthesis, in that chemical analyses showed increases in the accumulation of macromolecules containing hexuronic acid and hexosamine in vanadate-maintained cultures. However, vanadate had only a marginal effect on [^{35}S]sulfate incorporation into small proteoglycans and [^{3}H]glucosamine incorporation into hyaluronic acid and chondroitinase AC-resistant material. These results provide evidence that vanadate selectively stimulates the synthesis of proteoglycans characteristically found in cartilage by rabbit costal chondrocyte cultures.

CHONDROCYTES are highly specialized cells that produce large amounts of chondroitin sulfate proteoglycan and type II collagen. Previous studies have shown that hormones (14, 16, 34), growth factors (14, 17, 34), vitamin A (33), cyclic AMP analogues (8, 25, 36), cytoskeleton-disrupting agents (37), and extracellular matrices (18) affect chondroitin sulfate proteoglycan synthesis by chondrocytes. However, there have been no studies showing that vanadium compounds stimulate proteoglycan synthesis by chondrocytes.

Vanadium is a transition metal required for normal growth, and found in many tissues (4, 11, 40). Its oxidized form, vanadate, has a number of metabolic effects, including mitogenic actions in fibroblasts (6, 22, 32) and insulinlike actions in adipocytes (9, 38). Although the mechanisms of vanadate actions have not been fully understood, it is interesting that vanadate inhibits phosphotyrosyl protein phosphatase activity (23, 35). Vanadate has little if any significant effect on phosphoserine-specific protein phosphatase activity (23, 35). In addition, recent studies demonstrated that the addition of vanadate to the medium of normal rat kidney (NRK)[1] cells resulted in increased levels of tyrosine phosphorylation of cellular proteins (22). These observations suggest that vanadate produces metabolic effects at least in some cases through the inhibition of phosphotyrosine phosphatase, although vanadate affects, besides phosphotyrosine phosphatase, several enzymatic reactions concerned with phosphorylation and dephosphorylation, such as Na^+/K^+ ATPase (5, 6), alkaline phosphatase (30), and adenylate cyclase (29).

In the present study, we tested the effect of vanadate on proteoglycan synthesis by chondrocytes, because tyrosine phosphorylation may play a role in the regulation of chondrocyte phenotypic expression. Our results indicate that vanadate, at concentrations ranging from 0.1 to 6 μM, stimulates the synthesis of cartilage-matrix proteoglycans by rabbit costal chondrocyte cultures. Although vanadate significantly enhanced DNA synthesis in the chondrocytes, the stimulation of proteoglycan synthesis was not due to a mitogenic response by the chondrocytes to vanadate.

Materials and Methods

Materials

Sodium orthovanadate and pronase E (protease, type XIV) were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine pituitary fibroblast growth factor (basic FGF) (12) was generously supplied by Dr. Denis Gospodarowicz (University of California, San Francisco). Dulbecco's modified Eagle's medium (DME, H-16) and fetal bovine serum were obtained from Gibco (Grand Island, NY). Plastic tissue culture dishes (35-mm diam), and 6- and 16-mm diam microwell plates were obtained from Terumo Co. (Tokyo) and Falcon Labware (Oxnard, CA), respectively. Sepharose CL-2B was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Chondroitinase AC was from Seikagaku Kogyo Co. (Tokyo). Collagen (type I) was from Nitta Co. (Osaka). [^{35}S]sulfate (carrier-free) was purchased from Japan Atomic Energy Research Institute (Tokyo); D-[6-^{3}H]glucosamine (27 Ci/mmol), L-[3-^{3}H]serine (28 Ci/mmol), and [6-^{3}H]thymidine (2 Ci/mmol) were from the Radiochemical Centre (Amersham, United

1. *Abbreviations used in this paper*: FGF, fibroblast growth factor; NRK, normal rat kidney.

Kingdom). Monomeric proteoglycan was extracted from rabbit costal cartilage, as previously described (16).

Chondrocyte Culture

Chondrocytes were obtained from rib cartilage of 3-wk-old male New Zealand rabbits as described by Shimomura et al. (31). Resting cartilage cells were used throughout this study. Cells were seeded at low densities (13–25 cells/mm^2) and grown in the presence of FGF on plastic tissue culture dishes or in its absence on collagen (type I)-coated dishes. Alternatively, cells were seeded at high density (90 cells/mm^2) and grown in the absence of FGF in plastic microwells. These cultures yielded, at an early confluent stage, a homogeneous cell population composed of fibroblastic cells, and at a late confluent stage (3–10 d after cultures became confluent), the fibroblastic cells converted into spherical chondrocytes (17, 18, data not shown). To examine the effect of vanadate on the chondrocyte's phenotypic expression and proteoglycan synthesis, we used early confluent cultures, because cells in late confluent cultures had already reexpressed the chondrocyte phenotype and showed high levels of basal proteoglycan synthetic activity. When chondrocytes were seeded at low density and grown in the absence of FGF on plastic dishes, the majority of them irreversibly lost the ability to reexpress the chondrocyte phenotype in confluent cultures (17, 18), and hardly increased their rate of proteoglycan synthesis in response to vanadate.

Determination of Rates of Proteoglycan and Protein Synthesis

Chondrocytes were seeded at a density of 2 × 10^4 cells per 16-mm plastic microwell and grown to confluency in 0.5 ml of DME supplemented with 10% fetal bovine serum, 0.4 ng/ml of FGF, 50 μg/ml of ascorbic acid, 32 U/ml of penicillin, and 40 μg/ml of streptomycin (growth medium). FGF and ascorbic acid were added every other day until cultures became confluent. The cells were preincubated in 0.5 ml of DME with 0.3% fetal bovine serum for 24 h. They were then transferred to 0.5 ml of fresh DME with 0.3% fetal bovine serum for 3–40 h. Vanadate (0.06–60 μM) was added to the medium, as described in Fig. 2 and Tables II and IV. In some experiments, insulin (3 μg/ml) was also added to the medium (see Table VI). The cells were then exposed for 3 h to 2 μCi/ml of [^{35}S]sulfate, 5 μCi/ml of [^{3}H]glucosamine, or 5 μCi/ml of [^{3}H]serine in 0.3 ml of DME (serum-free). We estimated the rate of proteoglycan synthesis by measuring the incorporation of [^{35}S]sulfate, [^{3}H]glucosamine, and [^{3}H]serine into material precipitated with cetylpyridinium chloride after treatment with pronase E (19), and the rate of total protein synthesis by measuring the incorporation of [^{3}H]serine into 5% trichloroacetic acid-insoluble cell precipitate (20).

Relative Hydrodynamic Sizes of Proteoglycans

Chondrocytes were seeded at a density of 5 × 10^4 cells per 35-mm plastic dish and grown to confluency in 2 ml of DME supplemented with 10% fetal bovine serum, 0.4 ng/ml of FGF, 50 μg/ml of ascorbic acid, and antibiotics. FGF and ascorbic acid were added every other day. They were then incubated in the presence or absence of 4 μM vanadate in 3 ml of DME with 0.3% FBS. After 24 h, the cells were exposed for 6 h to 50 μCi [^{35}S]sulfate in 0.7 ml of DME. 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 6-amino-*N*-caproic acid, 20 mM EDTA, 1 mg/ml benzamidine HCl, 1 mM iodoacetic acid, 1 mM phenylmethylsulfonyl fluoride, 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 (4,000 *g* for 15 min), the cell extracts were stored at −30°C until analyzed. 0.47-ml aliquots of the medium were mixed with 0.67-ml aliquots of the appropriate cell layer fraction and 0.47 ml of 8 M guanidine HCl in water. 0.1 ml of 4 M guanidine HCl solution containing 0.5 mg of rabbit costal cartilage proteoglycan was then added. Samples were applied onto a Sepharose CL-2B column (0.9 × 110 cm) equilibrated in 4 M guanidine HCl, 50 mM Tris-HCl buffer, pH 8.0, with protease inhibitors. Fractions were collected as described in Fig. 3.

Chondroitinase Digestion

Low-density chondrocyte cultures were grown to confluency in the presence of FGF on plastic culture dishes. The cultures were incubated for 24 h in the presence or absence of 4 μM vanadate in 3 ml of DME with 0.3% fetal bovine serum. They were then exposed for 6 h to 30 μCi/ml of [^{3}H]glucosamine in 1.0 ml of DME with or without vanadate. The ^{3}H-labeled glycosaminoglycans were extracted from the cell layers and from the medium, and then digested with chondroitinase AC as previously described (16, 18). The resulting disaccharides were analyzed by thin-layer chromatography (16, 18, 24).

Determination of the Rate of DNA Synthesis

DNA synthesis was estimated by measuring incorporation of [^{3}H]thymidine into 5% trichloroacetic acid-insoluble cell precipitate (20). Chondrocytes were seeded at a density of 1 × 10^4 cells per 6-mm plastic microwell and grown to confluency in 0.1 ml of the growth medium without FGF. The cells were then preincubated in 0.1 ml of DME supplemented with 0.3% fetal bovine serum. After 24 h, the medium was replaced by 0.1 ml of DME supplemented with 0.3% fetal bovine serum and various concentrations of vanadate, and the incubation continued for 24 h. In some experiments, insulin (3 μg/ml) was also added to the medium. The cells were then exposed to 13 μCi/ml of [^{3}H]thymidine in 50 μl of DME for 3 h.

Determination of DNA, Protein, Hexuronic Acid, and Hexosamine

DNA content was determined by a fluorometric procedure (13, 39). Total protein was estimated by a dye-binding procedure (2). Hexuronic acid was estimated by the method of Bitter and Muir (1). Total hexosamine content of the cell matrix was estimated by the method of Elson and Morgan (10) after treatment with papain and hydrolysis in 4 M HCl for 6 h at 100°C.

Results

Vanadate-induced Morphologic Differentiation of Chondrocytes

Rabbit chondrocytes were seeded (10^5 cells per 35-mm collagen-coated dish) in DME supplemented with 10% fetal bovine serum, 50 μg/ml of ascorbic acid and antibiotics. When cultures became confluent, the serum concentration was reduced to 0.3%. In the low serum concentration, cells adopted a fibroblastic configuration (Fig. 1 *A*). Addition of 2 and 6 μM vanadate induced morphologic differentiation of the fibroblastic cells to spherical chondrocytes (Fig. 1, *B* and *C*). Within 24 h, they began to round and after 3 to 4 d 50% and 90% of the 2 and 6 μM ion-treated cells assumed a spherical configuration, respectively (Fig. 1, *B* and *C*). The spherical cells were surrounded by a refractile matrix. On the other hand, vanadate, at concentrations above 20 μM, induced spindlelike cells (Fig. 1, *D* and *E*). This morphologic alteration was best seen with 60 μM vanadate (Fig. 1 *E*). The morphology of chondrocytes treated with 20–60 μM vanadate was similar to that of Rous sarcoma virus-transformed chondrocytes (26).

Effect of Vanadate on [^{35}S]Sulfate Incorporation into Proteoglycans

Because vanadate (2–6 μM) increased the number of spherical chondrocytes that were surrounded by a refractile matrix (Fig. 1), the effect of vanadate on proteoglycan synthesis by chondrocytes was examined. When rabbit costal chondrocytes were incubated with vanadate (0.06–6 μM) for 24 h, the incorporation of [^{35}S]sulfate into proteoglycans increased in a concentration-dependent manner (Fig. 2 *A*). The effect of vanadate on [^{35}S]sulfate incorporation into proteoglycans could be observed at concentrations as low as 0.06 μM and was maximal at 4–6 μM. The maximal level of [^{35}S]sulfate incorporation into proteoglycans was 2.6-fold higher than that of cultures without vanadate. At concentrations above 12 μM, vanadate suppressed [^{35}S]sulfate incorporation into proteoglycans (Fig. 2 *A*).

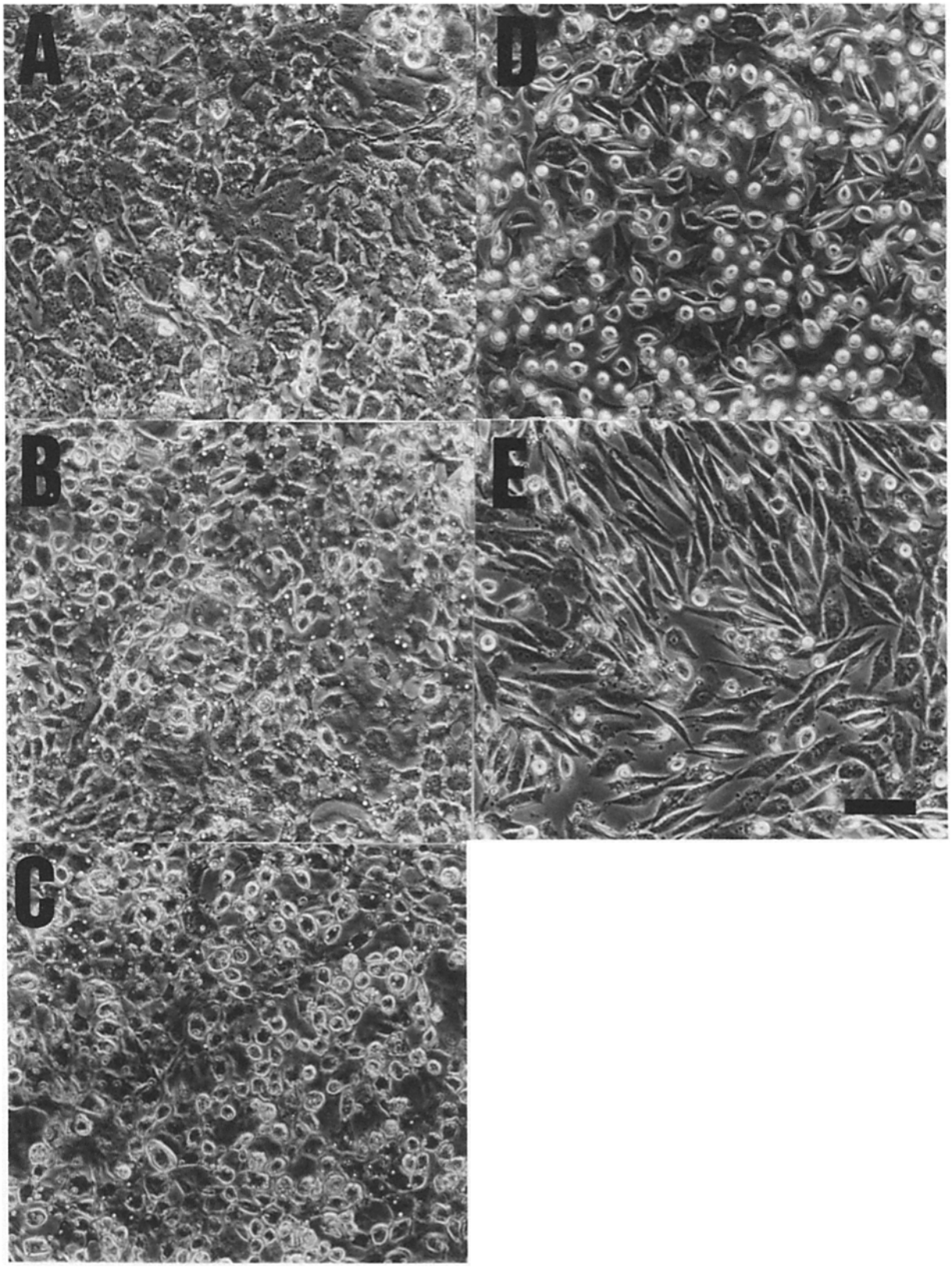

Figure 1. Morphologic appearance of rabbit costal chondrocytes treated with or without vanadate. Rabbit chondrocytes were seeded (10^5 cells per 35-mm collagen-coated dish) and grown to 90%–95% confluency in DME supplemented with 10% fetal bovine serum, 50 μg/ml of ascorbic acid, and antibiotics. They were then exposed either to DME supplemented with 0.3% fetal bovine serum (*A*) or DME supplemented with 2 (*B*), 6 (*C*), 20 (*D*), or 60 μM (*E*) vanadate and 0.3% fetal bovine serum. The medium was replaced every day by the appropriate medium. After 4 d, pictures were taken with an Olympus phase-contrast photomicroscope. Bar, 20 μm.

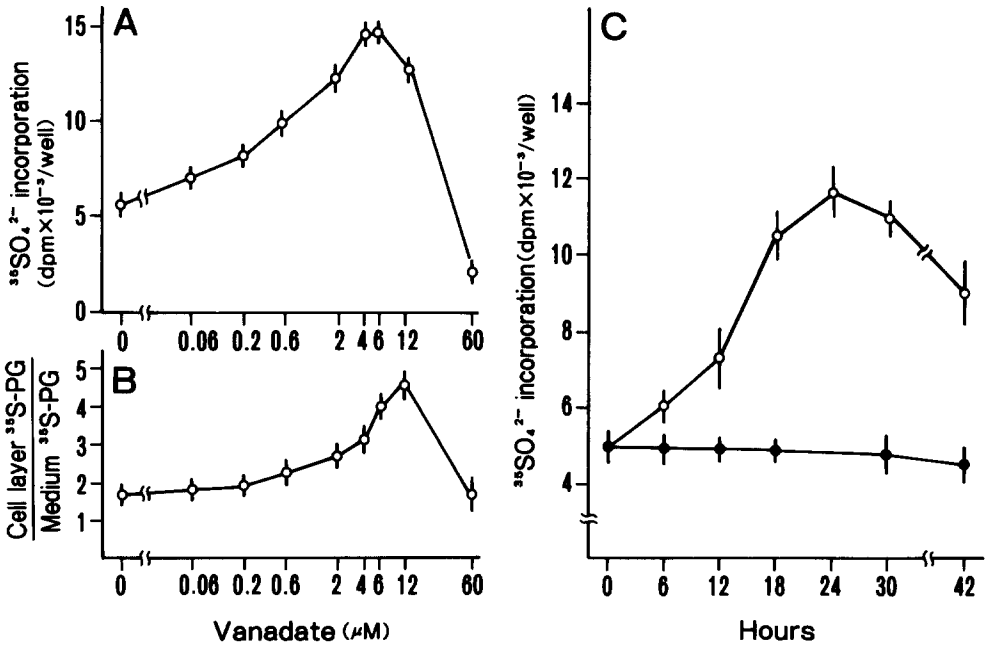

Figure 2. Effect of vanadate on [^{35}S]sulfate incorporation into proteoglycans by rabbit costal chondrocyte cultures. (*A* and *B*) Confluent cultures maintained in 16-mm plastic wells were exposed for 24 h to 0.5 ml of DME supplemented with 0.3% fetal bovine serum and increasing concentrations of vanadate (0.06–60 μM). The cultures were then incubated for 3 h in 0.3 ml of DME containing 2 μCi/ml of [^{35}S]sulfate. (*A*) The rate of [^{35}S]sulfate incorporation into proteoglycans was measured as described in Materials and Methods. (*B*) The distribution ratio between ^{35}S-labeled proteoglycans present in the cell layers vs. those released into the medium. (*C*) Confluent cultures maintained in 16-mm plastic wells were exposed for the indicated hours either to 0.5 ml of DME supplemented with (*filled circle*) 0.3% fetal bovine serum or (*open circle*) DME supplemented with 4 μM vanadate and 0.3% fetal bovine serum. The cultures were then incubated for 3 h in 0.3 ml of DME containing 2 μCi/ml of [^{35}S]sulfate. Points and bars are averages ± standard deviation for triplicate determinations.

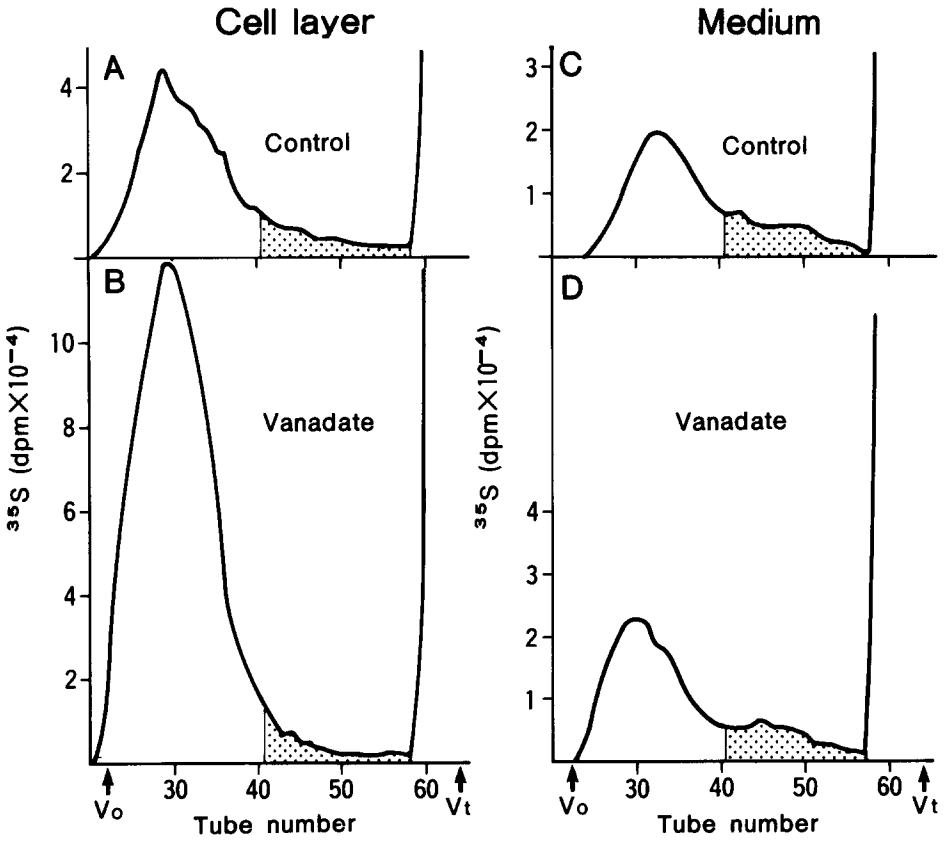

Figure 3. Sepharose CL-2B chromatography of proteoglycans located in the cell layers and in the medium from cultures exposed or not to vanadate. Confluent cultures were exposed for 24 h either (*A*, *C*) to DME supplemented with 0.3% fetal bovine serum alone or (*B*, *D*) DME supplemented with 4 μM vanadate and 0.3% fetal bovine serum. Cultures were then incubated for 6 h in the presence or absence of 4 μM vanadate in DME containing 50 μCi/ml of [^{35}S]sulfate. Aliquots of the 4 M guanidine HCl extract of the cell layers (*A*, *B*) or aliquots of the medium (*C*, *D*) from cultures exposed or not to vanadate were applied on a column of Sepharose CL-2B, which was equilibrated in 4 M guanidine HCl, 50 mM Tris HCl, pH 8.0, with protease inhibitors. 1-ml fractions were collected. Aliquots (0.25 ml) of each fraction were mixed with 0.25 ml of ethanol and 10 ml of Aquasol. Radioactivity was measured in a LKB 1215 RACKBETA liquid scintillation counter. Free unincorporated radioactivity elutes at V_t. V_o was determined with high molecular weight hyaluronic acid synthesized by rabbit costal chrondrocytes. Shaded area represents low molecular weight proteoglycans.

Fig. 2 *B* shows the effect of vanadate on the distribution of newly synthesized proteoglycans incorporated into the cell layers vs. those released into the medium. When cultures were incubated with vanadate (2–12 μM), the ratio between [^{35}S]proteoglycans present in the cell layers and those present in the medium increased in a concentration-dependent manner (Fig. 2 *B*). This increase was accompanied by the appearance of extensive refractile matrices, suggesting an increased deposition of proteoglycans in a cartilaginous matrix. The highest ratio was observed at a vanadate concentration of 12 μM and was 2.7 times of the basal ratio (Fig. 2 *B*). These observations suggest that vanadate increases the efficiency in the incorporation of newly synthesized proteoglycans into a cartilaginous matrix.

Fig. 2 *C* shows the time course for the vanadate (4 μM)-induced stimulation of proteoglycan synthesis. The rate of [^{35}S]sulfate incorporation into proteoglycans began to increase 6 h after the addition of vanadate and reached a maximum at 24 h. Thereafter, it decreased gradually, probably because the medium pH decreased in the vanadate-treated cultures. When the vanadate-treated cultures were transferred to fresh medium with vanadate at 24 h, no decreases in [^{35}S]sulfate incorporation were observed during the subsequent incubation (data not shown).

Effect of Vanadate on Syntheses of Cartilage-characteristic Proteoglycan and Low Molecular Weight Proteoglycan

The results presented in Fig. 2 show that vanadate increases the 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 a cartilage matrix. To test this possibility, we analyzed the hydrodynamic sizes of ^{35}S-labeled proteoglycans synthesized in cultures exposed or not to vanadate by gel exclusion chromatography on Sepharose CL-2B. The elution profiles are shown in Fig. 3. Chondrocytes exposed or not to 4 μM vanadate produced two proteoglycan species (high and low molecular weight) (Fig. 3). The high molecular weight proteoglycan was similar in size to cartilage-specific proteoglycan synthesized by high-density cultures of rat chondrosarcoma cells (34) and chick embryo chondrocytes (7). The low molecular weight proteoglycan was similar in size to that produced by fibroblasts, and called ubiquitous or fibroblastic proteoglycans (21, 27, 28). The levels of [^{35}S]sulfate incorporated into high molecular weight proteoglycan present in the cell layers and in the medium of vanadate-exposed cultures were 2.8- and 1.3-fold higher than those of cultures without vanadate, respectively (Fig. 3 and Table I). In contrast, the levels of [^{35}S]sulfate incorporated into low molecular weight proteoglycan in the cell layers and in the medium of vanadate-exposed cultures were similar to those of cultures without vanadate (Fig. 3 and Table I). These observations suggest that vanadate selectively stimulates the synthesis of high molecular weight proteoglycan and its preferential incorporation into a cartilaginous matrix.

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

Fraction	Vanadate	[^{35}S]Sulfate incorporation	
		High molecular weight proteoglycan	Low molecular weight proteoglycan
		dpm/10 cells	
Cell layer	−	2.56	0.49
	+	7.18	0.47
Medium	−	1.06	0.36
	+	1.35	0.39

The levels of ^{35}S radioactivity incorporated into high molecular weight proteoglycan (fractions 21–40) and low molecular weight proteoglycan (fractions 41–57, shaded area) eluted from a Sepharose CL-2B column are shown. Culture conditions were as described in Fig. 3. The elution profiles of ^{35}S-labeled proteoglycans present in the cell layer and the medium of cultures exposed or not to 4 μM vanadate are also shown in Fig. 3.

Effect of Vanadate on [^{3}H]Glucosamine Incorporation into Glycosaminoglycans

To examine whether vanadate increases not only [^{35}S]sulfate incorporation but also that of [^{3}H]glucosamine into proteoglycans, we incubated chondrocytes with or without 4 μM vanadate for 24 h. The cells were then exposed to [^{3}H]glucosamine for 3 h. The level of radioactivity incorporated into glycosaminoglycans in cultures treated with vanadate was 2.4-fold higher than that of cultures without vanadate (Table II).

In another series of experiments, the ^{3}H-labeled glycosaminoglycans from cultures treated with or without vanadate were digested with chondroitinase AC, and the resulting disaccharides were analyzed by thin-layer chromatography. The results are shown in Table III. The proportions of ^{3}H radioactivity incorporated into hyaluronic acid and chondroitinase AC-resistant material in samples from vanadate-treated cultures were significantly (40%–60%) lower than those of samples from cultures without vanadate. On the other hand, the proportions of ^{3}H radioactivity incorporated into chondroitin 6-sulfate, chondroitin 4-sulfate, and unsulfated chondroitin in samples from vanadate-treated cultures were similar to those for cultures without vanadate. Because vanadate (4 μM) increased by 140% the incorporation of [^{3}H]glucosamine into total glycosaminoglycans (Table II), the results obtained with chondroitinase AC suggest that vanadate (4 μM) selectively stimulates the synthesis of chondroitin sulfate glycosaminoglycans. However, high concentrations (20–60 μM) of vanadate decreased the incorporation of [^{3}H]glucosamine into chondroitin sulfate and increased the incorporation into hyaluronic acid (Kato, Y., et al., unpublished observations), probably because of cells' transformation (see Fig. 1, *D* and *E*).

Table II. Effect of Vanadate on [^{3}H]Glucosamine Incorporation into Glycosaminoglycans

Addition	[^{3}H]Glucosamine incorporation	
	dpm per well	%
None	8,336 ± 1,894	100
Vanadate	20,090 ± 3,230	241

Confluent cultures maintained in 16-mm plastic wells were exposed for 24 h to 0.5 ml of DME supplemented with 0.3% fetal bovine serum, with or without 4 μM vanadate. The cells were then incubated for 3 h in 0.3 ml of DME containing 5 μCi/ml of [^{3}H]glucosamine. Values are averages ± standard deviation for quadruplicate determinations.

Table III. Disaccharide Analysis of Chondroitinase AC-digested Glycosaminoglycans

Fraction	Vanadate	Thin-layer chromatography disaccharide analysis				
		Hyaluronic acid	Chondroitin 6-sulfate	Chondroitin 4-sulfate	Chondroitin	Chondroitinase AC-resistant
				%		
Cell layer	−	4.4	56.9	20.0	10.2	8.5
	+	2.3	63.3	21.8	7.7	4.9
Medium	−	10.4	45.8	20.4	13.3	10.2
	+	4.0	56.0	22.3	11.5	6.2

[^{3}H]Glucosamine-labeled glycosaminoglycans from the cell layer and the medium of confluent cultures exposed or not to 4 μM vanadate were digested with chondroitinase AC, as described in Materials and Methods.

Effect of Vanadate on [^{3}H]Serine Incorporation into Total Protein and Proteoglycans

Rabbit chondrocytes were exposed to 4 μM vanadate for 3–33 h in DME supplemented with 0.3% fetal bovine serum and then labeled with [^{3}H]serine for 3 h. To explore a possible relation between the vanadate-induced stimulation of proteoglycan synthesis and that of total protein synthesis, we simultaneously measured, in separate culture dishes, the incorporation of [^{3}H]serine into proteoglycans and into total protein. The level of [^{3}H]serine incorporation into total protein began to increase 3 h after the addition of vanadate (Table IV). The maximal increase was observed in cultures exposed to vanadate for 21–27 h. The maximal level of [^{3}H]serine incorporation into total protein was 1.9-fold higher than the basal level. Vanadate also increased the incorporation of [^{3}H]serine into proteoglycans (Table IV). This increase could be observed 9 h after the addition of vanadate, and the maximal increase was observed at 27 h. The maximal level of [^{3}H]serine incorporation into proteoglycans was 2.9-fold higher than the basal level. Note that the extent of the stimulation of proteoglycan synthesis after 3 h is consistently (1.5- to 2.6-fold) higher than that of the stimulation of total protein synthesis (Table IV). This suggests that vanadate selectively stimulates proteoglycan synthesis when it increases total protein synthesis.

Effect of Vanadate on [^{3}H]Thymidine Incorporation

Since vanadate enhanced DNA synthesis in fibroblasts (6, 22, 32) and bone cells (3), we examined the effect of vanadate on [^{3}H]thymidine incorporation in rabbit chondrocytes. Addition of vanadate, at concentrations ranging from 0.06 to 0.6 μM, had little effect on [^{3}H]thymidine incorporation in serum-deficient chondrocyte cultures (Fig. 4), although it already increased [^{35}S]sulfate incorporation into proteoglycans (Fig. 2 *A*). When chondrocytes were exposed to 2–6 μM vanadate, [^{3}H]thymidine incorporation increased and the maximal increase observed was 2.6-fold (Fig. 4). In contrast, 5% and 10% fetal bovine serum induced 10- and 19-fold increases, respectively (Fig. 4, data not shown). Vanadate is, therefore, a weak mitogen for chondrocytes.

Table IV. Effect of Vanadate on the Incorporation of [^{3}H]Serine into Total Protein and Its Incorporation into Proteoglycans

Length of exposure of chondrocyte cultures to vanadate	[^{3}H]Serine in protein	[^{3}H]Serine attached to glycosaminoglycans
h	*dpm × 10^{-3} per well*	
0	243 ± 9 (0)*	4.9 ± 0.7 (0)
3	301 ± 26 (24)	5.6 ± 0.8 (14)
9	364 ± 39 (49)	8.5 ± 0.7 (73)
15	366 ± 15 (51)	8.8 ± 0.5 (80)
21	460 ± 19 (89)	12.1 ± 2.0 (147)
27	429 ± 25 (77)	14.1 ± 2.0 (188)
33	380 ± 29 (58)	12.4 ± 1.3 (153)

Confluent cultures maintained in 16-mm plastic wells were exposed for 33 h to 0.5 ml of DME supplemented with 0.3% fetal bovine serum. Vanadate, at a concentration of 4 μM, was added to the cultures for the indicated hours before the end of the incubation. The cultures were then exposed to 5 μCi/ml of [^{3}H]serine in 0.3 ml of DME for 3 h. Values are averages ± standard deviation for three to six determinations.
* Values in parentheses are percent increase.

Chemical Analyses of the Cell Layer

Vanadate (4 μM) increased the incorporation of [^{35}S]sulfate, [^{3}H]glucosamine, and [^{3}H]serine into proteoglycans. However, this might reflect changes in pool sizes rather than real increases in cells' synthetic activity. Therefore, chemical analyses were carried out to confirm the effect of vanadate stimulation of proteoglycan synthesis. Rabbit chondrocytes in confluent cultures were incubated with or without vana-

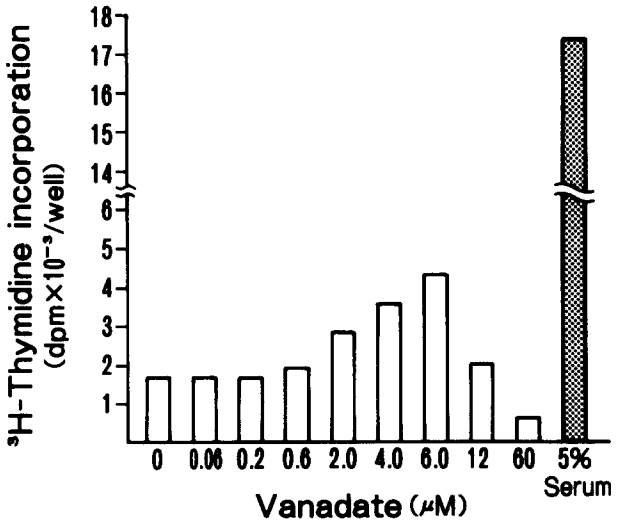

Figure 4. Effect of increasing concentrations of vanadate on [^{3}H]-thymidine incorporation in rabbit chondrocyte cultures. Quiescent cultures maintained in 6-mm wells were exposed to 0.1 ml of DME supplemented with 0.3% or 5% fetal bovine serum. Vanadate was added at concentrations ranging from 0.06 to 60 μM. After 24 h, they were labeled with [^{3}H]thymidine (0.65 μCi in 50 μl DME) for 3 h. Values are averages for three determinations. The standard deviation in the different determinations did not exceed 10% of the average.

date in DME supplemented with 0.3% fetal bovine serum for 3 d. Increases of 1.5- to 1.8-fold in hexuronic acid and hexosamine were observed in the cell layers for cultures treated for 3 d with 3–10 μM vanadate compared with the low serum medium alone (Table V). In contrast, no increases in protein and DNA were observed upon treatment with 3–10 μM vanadate (Table V). The vanadate-induced increases in hexuronic acid and hexosamine were comparable to those obtained by treatment of chondrocyte cultures with 5% fetal bovine serum (Table V).

Lack of Synergism between the Effect of Vanadate and Insulin on Proteoglycan Synthesis

Because the effect of vanadate on DNA synthesis in 3T3 cells was greatly potentiated by insulin (32), it is interesting to determine whether vanadate and insulin produce an additive or synergistic effect on chondrocyte proteoglycan synthesis. When chondrocytes were incubated with insulin (3 μg/ml) in DME supplemented with 0.3% fetal bovine serum for 24 h, the incorporation of [^{35}S]sulfate into proteoglycans increased 2.8-fold (Table VI). On the other hand, vanadate (6 μM) induced a 2.0-fold increase. Combination of insulin with vanadate produced no more additive increases in [^{35}S]sulfate incorporation into proteoglycans (Table VI). In contrast, insulin and vanadate produced a synergistic stimulation of [^{3}H]thymidine incorporation in serum-deficient chondrocyte cultures (Table VI), as in the case of the experiments with 3T3 cells (32). These observations suggest that the vanadate stimulation of [^{35}S]sulfate incorporation into proteoglycans is not directly linked to the ion stimulation of DNA synthesis.

Discussion

Vanadate increased the incorporation of [^{35}S]sulfate and [^{3}H]glucosamine into large chondroitin sulfate proteoglycans synthesized by cultured rabbit costal chondrocytes. The stimulation of incorporation of labeled precursors reflected real increases in proteoglycan synthesis, in that chemical analyses showed increases in the accumulation of macromolecules containing hexuronic acid and hexosamine in vanadate-maintained cultures. However, vanadate had only a marginal effect on [^{35}S]sulfate incorporation into small proteoglycans, and [^{3}H]glucosamine incorporation into hyaluronic acid and chondroitinase AC-resistant material. These results provide evidence that vanadate selectively stimulates the synthesis of proteoglycans characteristically found in cartilage in rabbit costal chondrocyte cultures.

Three possibilities may account for the stimulatory effect of vanadate on proteoglycan synthesis. First, the increase in proteoglycan synthesis is due to a mitogenic response by chondrocytes to vanadate. This is not likely, however, because the maximal increase in [^{3}H]thymidine incorporation obtained by treatment with vanadate was only 2.6-fold, and because no increases in DNA were observed upon treatment of quiescent chondrocyte cultures with vanadate for 3 d. Secondly, the increase in proteoglycan synthesis is secondary to increased overall protein synthesis. This is also unlikely, because the extent of the vanadate-induced stimulation of proteoglycan synthesis was consistently (1.5- to 2.6-fold) higher than that for stimulation of total protein synthesis. Third, vanadate increases proteoglycan synthesis by stimulating the conversion in poorly differentiated chondrocyte cultures from a "fibroblastic" expression to a "chondrocyte" expression. This possibility appears to be likely, because in early confluent cultures composed of fibroblastic cells vanadate induced morphologic differentiation to spherical chondrocytes and increased [^{35}S]sulfate incorporation into proteoglycans. In late confluent cultures, vanadate had little effect on [^{35}S]sulfate incorporation into proteoglycans, probably because cells had already reexpressed the spherical phenotype.

The physiologic significance of the in vitro effect of vanadate on chondrocyte proteoglycan synthesis remains unclear. However, the stimulatory effect of vanadate on proteoglycan synthesis was observed at concentrations (0.06–6 μM) similar to those found in a number of tissues (4, 11, 40). Furthermore, vanadyl was as potent as vanadate in stimulating [^{35}S]sulfate incorporation into proteoglycans in rabbit chondrocyte cultures (data not shown). These observations suggest that vanadium compounds play a role in vivo in supporting cartilage proteoglycan synthesis. The nutritional requirements for vanadium for normal growth are also consistent with the physiologic significance of the present observation.

Because vanadate is a powerful inhibitor of phosphotyrosine phosphatase (23, 35), it is interesting to consider that tyrosine phosphorylation and dephosphorylation might be involved in vanadate actions in chondrocytes. Recent studies demonstrated that added vanadate increased the level of phosphotyrosine in NRK cell proteins (22). We have also

Table V. Chemical Analyses of the Cell Layer Matrix

Addition	DNA	Protein	Uronic acid	Hexosamine
	μg/dish	*μg/μg DNA*	*μg/μg DNA*	*μg/μg DNA*
None	15.6	59.8	3.69	2.68
Vanadate 3 μM	15.7	61.9	5.43	4.22
6 μM	15.6	64.1	6.46	4.59
10 μM	16.0	62.3	5.67	4.63
5% serum	19.8	64.5	5.50	4.38

Rabbit chondrocytes were seeded (10^5 cells per 35-mm plastic dish) and grown to confluency in DME supplemented with 10% fetal bovine serum, 0.4 ng/ml FGF, 50 μg/ml ascorbic acid, and antibiotics. They were then exposed to 3 ml of DME supplemented either with 0.3% fetal bovine serum and various concentrations (0–10 μM) of vanadate or 5% fetal bovine serum. The medium was replaced by the appropriate medium every day. After 3 d, contents of DNA, protein, hexuronic acid, and hexosamine in the cell layers of cultures were measured. Values are averages for triplicate determinations. Range of DNA variation was ±12%; protein variation ±8%; uronic acid variation ±16%; and hexosamine variation ±18%.

Table VI. Effect of Vanadate and Insulin on Proteoglycan Synthesis and DNA Synthesis

Addition	[^{35}S]Sulfate incorporation	[^{3}H]Thymidine incorporation
	dpm per well	
None	7,214 ± 405	1,318 ± 186
Vanadate	14,503 ± 1,389	2,816 ± 195
Insulin	19,938 ± 1,465	3,792 ± 262
Insulin + vanadate	21,065 ± 1,811	15,865 ± 1,308

Rabbit chondrocytes were preincubated for 24 h in DME supplemented with 0.3% fetal bovine serum. The cells were then incubated for 24 h in DME supplemented with 0.3% fetal bovine serum in the presence of vanadate (6 μM), insulin (3 μg/ml), or both. [^{35}S]Sulfate incorporation into proteoglycans and [^{3}H]thymidine incorporation into DNA were measured as described in Materials and Methods. Values are average ± standard deviation for four wells.

found that addition of 6 and 60 μM vanadate to chondrocyte cultures resulted in 8- and 31-fold, respectively, increases in the level of phosphotyrosine in chondrocyte protein (K. Owada and Y. Kato, manuscript in preparation). The vanadate-induced increase in the level of phosphorylation on tyrosine may lead to stimulation of chondrocyte phenotypic expression, inasmuch as vanadate (0.06–6 μM) increased the synthesis of cartilage-matrix proteoglycans in rabbit costal chondrocyte cultures. This notion is supported by the findings in previous studies that insulinlike growth factor-I enhances tyrosine phosphorylation (15), and stimulates chondrocyte proteoglycan synthesis (41). The present study also shows, however, that vanadate, at 20–60 μM, decreases cartilage proteoglycan synthesis by chondrocytes and induces morphologic transformation. The configuration of the vanadate-transformed cells was similar to that of chondrocytes transformed by Rous sarcoma virus (26) whose transforming protein is a tyrosine kinase. These observations suggest that although moderate increases in the level of phosphotyrosine are required for the expression of the chondrocyte phenotype, excess increases cause cells to express the transformed phenotype. Klarlund (22) has also shown that vanadate (37 μM) induces transformation of NRK cells as evidenced by generation of a highly refractile morphology and growth in the absence of a solid support, when vanadate increases 40-fold the level of phosphotyrosine in cell protein. We have also found that although vanadate, at concentrations above 20 μM, does not stimulate the growth of chondrocytes in soft agar culture because of its cytotoxicity, vanadate at a concentration of 6 μM induces low levels of soft agar colony formation by chondrocytes (Kato, Y., unpublished data). However, vanadate is known to affect, besides phosphotyrosine phosphatase, a variety of enzymatic reactions concerned with phosphorylation and dephosphorylation, such as Na^+/K^+ ATPase (5, 6), alkaline phosphatase (30) and adenylate cyclase (29). Therefore, further studies are needed to prove a direct relation between tyrosine phosphorylation and vanadate actions in chondrocytes.

In summary, vanadate (0.06–6 μM) stimulated the synthesis of cartilage-matrix proteoglycans in rabbit costal chondrocyte cultures and induced morphologic differentiation. The extent of expression of the differentiated phenotype could be controlled by the concentration of vanadate added to the medium. Therefore, vanadate is a novel tool for studying the mechanism involved in chondrocyte phenotypic expression.

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