

Effect of Ascorbic Acid on Arylsulfatase Activities and Sulfated Proteoglycan Metabolism in Chondrocyte Cultures

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ABSTRACT A correlation between increased arylsulfatase activities and decreased sulfated proteoglycan content in human osteoarthritic articular cartilage suggested a possible interrelationship between these parameters. Since we had previously shown that ascorbate caused a decrease in levels of arylsulfatase A and B activities in normal chondrocyte cultures, the validity of the above relationship was examined by measuring the effect of vitamin C on the biosynthesis and distribution of ³⁵S-labeled proteoglycans and arylsulfatase A and B activities in cell extracts of chondrocytes derived from normal and osteoarthritic tissue.

Arylsulfatase A and B activities were found to be reduced in the presence of ascorbic acid in all normal and osteoarthritic cell lines examined when measured 3, 6, 10, and 13 days after the introduction of the vitamin in the culture medium. Acid phosphatase activity, on the other hand, was found to be elevated in the presence of ascorbate.

The inhibitory effect by ascorbic acid on arylsulfatase activities could be reversed by withdrawing the vitamin from the nutrient medium. Addition of EDTA to the cell extracts before assay also reversed the inhibition.

Sulfated proteoglycan biosynthesis as reflected in ³⁵S-sulfate uptake per milligram of DNA was significantly increased in the presence of ascorbic acid. The distribution of the newly synthesized molecules between the cell layer and medium fractions was altered. In the presence of ascorbate, more deposition into the cell layer of newly synthesized macromolecules occurred.

These data suggest an inverse relationship between arylsulfatase activities and the stability of the newly synthesized sulfated proteoglycans in the extracellular matrix.

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INTRODUCTION

Osteoarthritis is characterized in part by the erosion of articular cartilage in the afflicted joint. Based on work from several laboratories, a possible model for the pathogenesis of the disease includes an initial metabolic or mechanical insult on the cartilage which alters the cellular environment so as to increase tissue degradation by an accelerated activation or derepression of lysosomal enzymes. The loss of extracellular components from the matrix, in turn, stimulates the anabolic pathways of the surrounding chondrocytes to replicate DNA and synthesize new matrix material at a more rapid rate than normal. Increased cell replication and sulfated proteoglycan biosynthesis, which characterize this attempted repair process, cannot control the disease process, however, and eventually cellular destruction is so rapid that biosynthetic activities cease and all cartilage is lost from the joint surface (1-6).

Previous studies from this laboratory have shown that arylsulfatases A and B, lysosomal enzymes involved in the breakdown of sulfated macromolecules, are elevated in osteoarthritic human articular cartilage when compared with age matched controls (7).

In addition to its role as a cerebroside sulfatase (8), arylsulfatase A catalyzes the desulfatation of other glycolipids containing a galactose-3-sulfate moiety (9) as well as ascorbate sulfate (10). Arylsulfatase B, the defective enzyme in Maroteaux-Lamy disease (11-13), has been shown to catalyze the desulfation of *N*-acetyl-galactosamine 4-sulfate groups such as are found in dermatan sulfate and chondroitin-4-sulfate (14).

We have recently described the isolation, purification, and characterization of arylsulfatases A and B from human articular cartilage (15). These enzymes have now also been isolated and characterized from

human articular chondrocyte cultures.¹ The enzymes isolated from chondrocytes had essentially identical biochemical and physico-chemical properties as those from the intact tissue. This further substantiates the integrity of the chondrocyte system in use.

During the course of the isolation procedure, it was noted that the activities of arylsulfatases A and B in extracts of chondrocytes were reduced when ascorbic acid was included in the culture medium. Further investigations revealed that increasing concentrations of ascorbic acid reduced cellular levels of arylsulfatase A and B activities in normal human chondrocyte cultures on a dose-response basis.

These studies have been extended and in this report we demonstrate that arylsulfatase A and B activities in osteoarthritic chondrocytes are also modulated by inclusion of ascorbic acid in the culture medium. Furthermore, the biosynthesis and distribution between the cell layer and medium fractions of newly synthesized sulfated proteoglycans in normal and osteoarthritic chondrocytes were also found to be dependent upon ascorbate concentrations in the culture fluid. The effect of ascorbic acid on these metabolic parameters could be reversed in chondrocytes by removal of the vitamin from the nutrient fluid.

In addition, a positive correlation between cellular acid phosphatase activity and ascorbic acid concentration in the growth medium was present in both normal and osteoarthritic chondrocytes. The higher levels of acid phosphatase activity found in osteoarthritic cells even after repeated cell passage will be discussed.

METHODS

Culture of chondrocytes. Chondrocytes derived from surgical specimens of normal or osteoarthritic human articular cartilage were maintained at pH 7.6 in complete Ham's F-12 medium containing glutamine (58.5 $\mu\text{g/ml}$), MgSO_4 (200 $\mu\text{g/ml}$), alpha-ketoglutarate (16.8 $\mu\text{g/ml}$), ascorbic acid (50 $\mu\text{g/ml}$), fetal calf serum (10%), penicillin (100 U/ml), and streptomycin sulfate (100 $\mu\text{g/ml}$). Cells used in these experiments were either in second or third passage. On day 1 of the experimental period, cells from two Falcon flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) (75 cm^2) were isolated by trypsinization, combined, suspended in complete F-12 medium, and inoculated into 44 to 58 Leighton tubes (6.5 cm^2 window area). The passed cells were fed with the same complete medium on days 4 and 6. On days 8 and 10, medium B (complete medium minus ascorbic acid) was used to feed all the cells. Beginning on day 12, and continuing every other day until the completion of the experiment, medium C (complete medium modified to contain ascorbic acid [70 $\mu\text{g/ml}$]) was used to feed one-half of the Leighton tubes whereas the remaining tubes continued to be maintained on medium B.

¹Oguchi, H., and E. R. Schwartz. Enzymes from human articular chondrocytes. Isolation of arylsulfatases A and B. In preparation.

Metabolic studies. In those experiments where reversibility of the effects of ascorbic acid was studied, cells were passed into Falcon flasks (25 cm^2) on day 1 with medium D (complete F-12 medium minus both ascorbic acid and alpha-ketoglutarate) at pH 7.6. This medium was also used for feeding on day 4. The cells were now divided into two categories. On days 6 and 8, medium B was used as culture fluid for one-half of the cells and medium C for the remainder. Representative flasks from each category had their medium replaced on day 9 with identical medium which also contained $\text{Na}_2^{35}\text{SO}_4$ (30 $\mu\text{Ci/ml}$). The labeled cells were harvested on day 10 (see below).

The flasks containing cells not labeled at this time were maintained for an additional 6 days (days 10–16). Some of the cells were switched from medium C to medium B for this period. Labeling media containing ^{35}S -sodium sulfate were introduced into all the flasks on day 15, exactly 24 h before cell harvest on day 16.

Cell harvest was initiated by removal of the labeling medium which was heated at 100°C for 5 min, and then dialyzed for 6 h against 0.1 M ammonium sulfate. This was followed by extensive dialysis against 0.001 M EDTA, pH 7.4 before the determination of radioactive content.

The cell layer was washed with ice-cold F-12 medium containing Na_2SO_4 (1 mg/ml). It was then washed two times each with Gey's Balanced Salt Solution and isotonic saline before being subjected to mild trypsinization. The chondrocytes were collected by centrifugation. The supernatant or trypsin fraction was inactivated with fetal calf serum and heated at 100°C before analysis for radioactive content.

The pelleted cell fraction was washed with isotonic saline, suspended in 3 ml of distilled water, subjected to 10 cycles of freeze-thawing, and then homogenized in an all-glass apparatus. Aliquots of this solution were used for determination of enzyme activities and DNA, ascorbic acid, protein, and radioactive content.

Coordinated studies of sulfated proteoglycan metabolism and arylsulfatase activities in long-term cultures were also conducted in Falcon flasks (25 cm^2). The cells had been maintained routinely for 18 days in complete F-12 medium before being deprived of ascorbic acid (Medium B) on day 1 of the experimental period. Medium C was used to feed one-half of the cells on days 5 and 8 while the other half continued to be maintained with medium B. All flasks had their culture fluid replaced by medium containing $\text{Na}_2^{35}\text{SO}_4$ on day 9 for a 24-h period at which time the cells were harvested as described above.

Biochemical methods. Arylsulfatase activities were measured by a modification of the procedure described by Roy (16) and Allen and Roy (17) with 4-nitrocatechol sulfate as substrate. The incubation mixture for arylsulfatase A contained 1.0 mM 4-nitrocatechol sulfate, 0.125 M sodium acetate buffer, pH 4.8, and cell extract in a final volume of 0.3 ml. For arylsulfatase B, the incubation mixture contained 12 mM 4-nitrocatechol sulfate, 0.5 M sodium acetate buffer, pH 5.6, and cell extract in a final volume of 0.3 ml. The reaction was stopped by the addition of 0.7 ml of 1 N NaOH after 4 h of incubation at 37°C. The absorbance of the supernate after centrifugation was monitored at 515 nm. Control experiments contained equivalent volumes of heat-killed cell extracts. The assay mixtures were modified to include 0.5 mM EDTA in those experiments where reversibility of the inhibition of arylsulfatase activities by the chelating agent was examined (18).

Acid phosphatase activity was determined by incubating 0.03 ml of cell extract with 4 mM *p*-nitrophenyl phosphate in a final volume of 0.23 ml at pH 4.8. The reaction was stopped after 30 min by the addition of 0.22 ml of 1 N

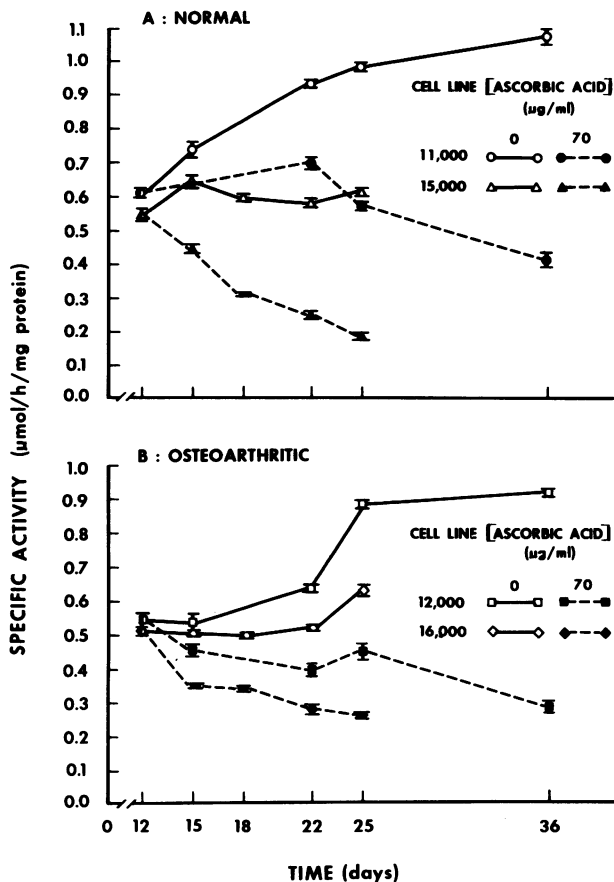


FIGURE 1 Effect of ascorbic acid on the specific activity of arylsulfatase A. (A) Aliquots of the homogenates of normal chondrocytes grown in the absence of ascorbate (○,△) and in the presence of 70 µg/ml of the vitamin in the culture medium (●,▲) were assayed for arylsulfatase A activity and protein content as described in Methods. All the cells had been on complete medium up to day 8 and had been deprived of ascorbic acid between days 8 and 12. (B) Osteoarthritic chondrocytes grown in the absence of (□,◇) and in the presence of 70 µg/ml ascorbic acid (■,◆) were analyzed for enzyme activity and protein content as described above.

NaOH and the absorbance was measured at 405 nm. Control assays were conducted with equal aliquots of cell extract previously heated at 100°C for 5 min (19).

DNA was determined by a slight modification of the fluorometric micro method described by Switzer and Summer (20). Aliquots of 0.18 ml of cell extract, heated at 37°C in 1 N NH₄OH, were lyophilized and resuspended in 0.075 ml diamminobenzoic acid with heating at 60°C. The percentage of fluorescence of 25-µl aliquots of the reacted DNA dissolved in 2.5 ml of 0.6 N perchloric acid was determined in a Turner spectrofluorometer (Turner Associates, Palo Alto, Calif.) with an excitation wave length of 440 nm and an emission wave of 550 nm.

Protein was determined by the method of Lowry et al. with bovine serum albumin as a standard (21).

Ascorbic acid levels were measured by the method of Zannoni et al. (22). Aliquots of 0.1 ml of cell extract were deproteinized in a final volume of 0.4 ml of 5% TCA. Frac-

tions of 0.15 ml of the supernate, removed after centrifugation, were reacted in a final volume of 0.2 ml containing 0.4% alpha, alpha dipyridil, 0.15% FeCl₃, and 4.25% H₃PO₄. Absorbance of the resulting solution was monitored at 525 nm.

Calculations. All experiments were conducted in quadruplicate tubes or flasks. Radioactive content was determined in triplicate for each fraction from a given Leighton tube or flask by analyzing aliquots of 0.05 or 0.1 ml in Aquasol (New England Nuclear, Boston, Mass.) contained in plastic vials. The total radioactivity incorporated per tube or flask was calculated by adding ³⁵S counts per minute in the dialyzed medium to those found in the dissolved cell solution and trypsin fraction. That part attributable to the cell layer equals the total radioactivity minus the amount contributed by the dialyzed medium fraction. In other words, the cell layer consists of the cells per se plus the extracellular material which is sensitive to the action of trypsin and is therefore found in the trypsin fraction.

RESULTS

Arylsulfatase activities. Inclusion of ascorbic acid in the culture medium resulted in a decrease in the specific activity of arylsulfatase A in normal and osteoarthritic chondrocytes when compared with identical cells grown in the absence of vitamin C (Figs. 1A and B). The vitamin C-dependent decrease was measurable as early as 3 days subsequent to the introduction of ascorbic acid into the cell culture fluid (day 15 of the experimental period) and remained evident throughout the duration of the experiment.

The decrease has been measured as late as day 50 (data not shown). The slopes of the regression curves describing the specific activities of arylsulfatase A as a function of time in normal cell line (15,000) were 1.844 and -26.843 in the absence and presence (70 µg/ml) of ascorbic acid, respectively (Fig. 1A). Values of 7.746 and -17.021 were calculated to describe the comparable slopes for the osteoarthritic cell line (16,000) in the absence and presence of vitamin C, respectively (Fig. 1B).

Without exception, in all normal and osteoarthritic cell lines examined, the slopes of regression curves which described arylsulfatase A specific activity as a function of time were positive in the absence of ascorbate and negative when the vitamin was present. Although variations in the specific activity of arylsulfatase A were found inherent in the different cell lines at the outset of the experimental period, addition of ascorbic acid always caused a relative decrease in the measurable activity of this enzyme.

The ascorbate-dependent decrease in the specific activity of arylsulfatase B in normal and osteoarthritic chondrocytes as a function of time is shown in Fig. 2. Slopes of 6.623 and 6.196 for changes in arylsulfatase B activity with time in the absence of ascorbate and -43.573 and -46.420 in the presence of ascorbate for normal (15,000) and osteoarthritic (16,000) cell lines, respectively, were evaluated by multiple regression

analysis (Figs. 2A and B). These values are highly significant with multiple correlation coefficients of 0.841, 0.790, 0.833, and 0.921, respectively. Comparable ascorbate-dependent decreases in arylsulfatase B activities were observed in all other cell lines studied. It appears that normal and osteoarthritic human chondrocytes respond in a like manner to the modulating effect of ascorbic acid on arylsulfatase activities.

Some overlap of arylsulfatase A activity in the assay for arylsulfatase B and overlap of arylsulfatase B activity in the assay for arylsulfatase A does occur. Experiments conducted on partially purified enzymes from chondrocyte cultures subsequent to their separation by ion-exchange chromatography on DEAE Sephadex (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.)

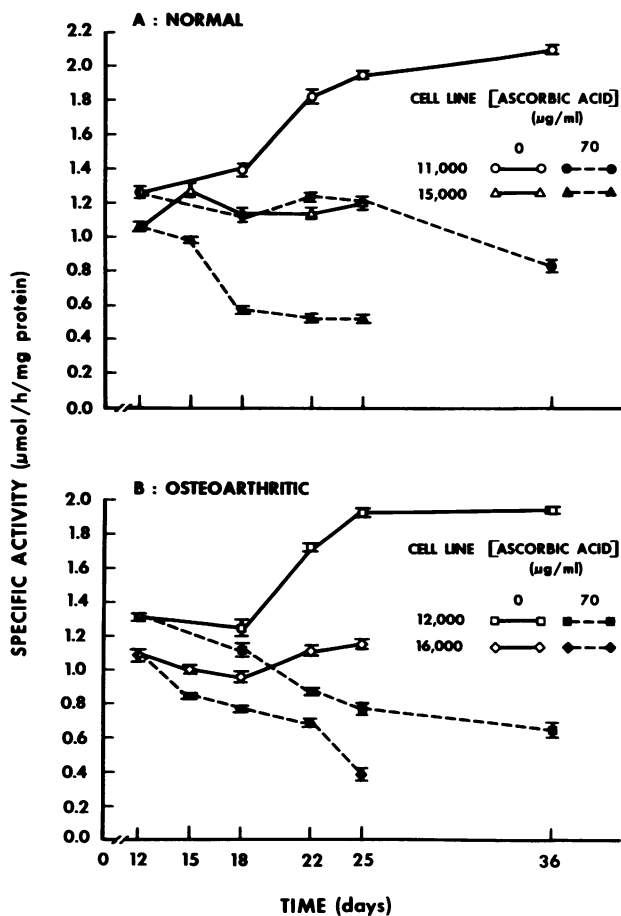


FIGURE 2 Effect of ascorbic acid on arylsulfatase B activity. (A) Aliquots of the homogenates of normal chondrocytes grown without (O, Δ) or with 70 μg/ml ascorbic acid (●, ▲) were assayed for arylsulfatase B activity and protein content as described in the legend to Fig. 1. (B) Osteoarthritic chondrocytes were assayed for arylsulfatase B activity and protein content as described above. Cells had been grown in the absence of (□, Δ) or presence of 70 μg/ml ascorbic acid (■, ◆).

TABLE I
Effect of EDTA on the Inhibition of Arylsulfatase A and B Activities by Ascorbate

Ascorbic acid μg/ml	Specific activity*			
	Arylsulfatase A§		Arylsulfatase B§	
	EDTA concentration (mM)†			
	0	0.5	0	0.5
0	0.404	0.396	1.028	1.052
70	0.268	0.472	0.828	1.060

* Specific activity is defined as micromoles 4-nitrocatechol formed per hour per milligram of protein.

† Concentration of vitamin C in the culture medium.

§ Average of duplicate determinations. Enzyme activities were determined as described under Methods.

|| EDTA was added immediately before assay to the extracts of normal cells grown in the presence (70 μg/ml) or absence of ascorbic acid.

indicated less than 10% overlap of activities. Although the absolute values of the control and inhibited enzyme levels might be affected by this cross-reaction, the slopes would not be significantly altered. The above data, therefore, indicate that both enzymes are inhibited by inclusion of ascorbate in the culture medium.

Addition of EDTA (0.5 mM) to the cell extracts before assay resulted in the reversal of the ascorbate-induced inhibition of arylsulfatase A and B activities (Table I). The chelating agent had no effect on extracts of cells grown in the absence of the vitamin.

The inhibition by ascorbate of the activity of purified arylsulfatase A from ox liver as well as the reversal of this inhibition by EDTA had been shown previously by Roy (18). In this system, 60 and 50% inhibition was observed when the purified enzyme was preincubated for 1 h with 0.5 mM and 0.1 mM ascorbate, respectively.

To evaluate whether a similar mechanism was perhaps operative in chondrocyte cultures, the intracellular levels of the vitamin were determined in the cell extracts.

Intracellular ascorbic acid concentrations found in extracts of normal chondrocytes grown in a medium containing vitamin C (70 μg/ml) and harvested on experimental day 10, ranged from 8.0 to 11.2 μg/ml of cell extract. Arylsulfatase A and B activities were inhibited an average of 25 and 32%, respectively, in these extracts. Continued growth on the ascorbate-supplemented medium to experimental day 16, increased the inhibition of arylsulfatase A and B activities slightly to average values of 31 and 34%, respectively. In contrast, the intracellular levels of

ascorbate decreased to an average value of 3.4 $\mu\text{g/ml}$ of cell extract (range: 3.0–3.8 $\mu\text{g/ml}$).

No ascorbate was measurable in the solutions used to wash the cells prior to their rupture. This indicates that the ascorbate present in the cell extracts had intracellular origins rather than arising from mechanically bound material on the cell surface.

Although the ascorbate concentration in the medium was constant (70 $\mu\text{g/ml}$), the level of the vitamin found within the cells showed substantial variation. The relative inhibition of arylsulfatases A and B, on the other hand, increased in a regular manner with time. This supports the premise that an additional factor (perhaps a metal ion) is involved in the inhibitory process.

Cellular protein content. Both normal and osteoarthritic chondrocytes exhibited an increase in cellular protein with increasing time in culture. This reflects normal growth. In all instances, however, the increase in cellular protein for a given cell line was greater when the chondrocytes were grown in the presence of vitamin C. The particular increase per unit time was specific for each cell line and was dependent not only on the particular growth pattern of that cell line but also on the number of cells used to inoculate the Leighton tubes initially. Total cellular protein values found on days 15 and 25 for two different normal and osteoarthritic cell lines are summarized in Table II. Similar increases in protein content of 16.10 and 18.31% in the absence of and 30.73 and 29.37% in the presence of ascorbic acid were calculated for the 10-day interval for the two different osteoarthritic cell lines. The increases in protein for the two normal cell lines, on the other hand, were

widely divergent. In the absence of ascorbic acid, 14.94 and 44.62% increases were measured over the 10-day period. When the vitamin was included in the culture medium, 44.36 and 60.99% increases were observed. Since the media used in all these experiments were identical except for the presence or absence of ascorbic acid, it appears that the precise quantitative increase found for both the normal and osteoarthritic chondrocytes was a function of the cells themselves. The increase was always greater in the presence of ascorbate. The rate of increase of cellular protein was greatest between days 15 and 18 of the experimental period. After this time, it occurred at a decreasing rate.

Acid phosphatase activity. In contrast to decreasing levels of arylsulfatase activities associated with increasing concentrations of ascorbic acid there appeared to be an increase in acid phosphatase activity when vitamin C was included in the culture medium. This increase, usually not observed until 5 to 7 days after the introduction of the vitamin enriched medium on day 12, was apparent in both normal and osteoarthritic cell lines. No significant difference was seen on experimental day 15. On day 25, on the other hand, the average specific activity of acid phosphatase in normal cells from eight different tubes representing two different cell lines was 3.983 and 4.610 in the absence and presence of ascorbate, respectively. An increase from 5.349 to 5.581 in the average specific activity was found in osteoarthritic cells when these were grown in the presence of ascorbate.

Furthermore, a significant elevation ($P < 0.001$) in the level of acid phosphatase in osteoarthritic cells

TABLE II
Effect of Ascorbic Acid on Cellular Protein in Human Chondrocyte Cultures

Cell line§	Cell type	Total cellular protein ($\mu\text{g/tube}$)*					
		0			Ascorbic acid ($\mu\text{g/ml}$)†		
		Day 15	Day 25	Increase %	Day 15	Day 25	Increase %
11,000	Normal	76.05±1.17	87.41±0.26	14.94	79.26±0.38	114.42±0.90	44.36
15,000	Normal	55.22±0.28	80.05±0.32	44.62	60.49±0.29	97.38±0.44	60.99
12,000 ^{**}	OA	84.32±0.61	97.90±0.61	16.10	87.46±0.56	114.34±0.39	30.73
16,000 ^{‡‡}	OA	56.16±0.08	66.44±0.22	18.31	56.72±0.08	73.39±0.36	29.37

OA, osteoarthritis.

* Determinations were done in quadruplicate in Leighton tubes with a surface area of 6.5 cm^2 .

† Concentration of ascorbic acid introduced on day 12. All cells had been grown on ascorbic acid (50 $\mu\text{g/ml}$) from day 1 to day 8 and had been deprived of ascorbic acid between days 8 and 12.

§ All cells in second passage.

^{||} Patient M. A.

^{||} Patient B. E.

^{**} Patient W. E.

^{‡‡} Patient C. C.

TABLE III
*Effect of Ascorbic Acid on Cellular Enzyme Activities and DNA and Protein Content in Long-Term Confluent Cultures of Normal Human Chondrocytes**

Ascorbic acid $\mu\text{g/ml}$	Specific activity						Concentration				
	Arylsulfatase† A		Arylsulfatase† B		Acid† phosphatase		Protein§		DNA§		
	$\mu\text{mol/hmg cell protein}$						$\mu\text{g/flask}$				
0	0.617		1.177		3.742		219.11		7.06		
70	0.381		0.808		4.007		246.78		6.90		
	SE	0.018	0.002	0.036	0.002	0.047	0.023	4.99	0.71	0.30	0.31
	P	0.002		0.002		0.003		0.002		0.71	

* Cells derived from normal articular cartilage had been passed 18 days before the beginning of the experimental period (day 1) and had been maintained on standard medium containing ascorbic acid (50 $\mu\text{g/ml}$) in Falcon flasks (25 cm^2) during that interval. The cells were deprived of ascorbic acid between days 1 and 6 at which time one-half of the cells were placed on media containing ascorbic acid (70 $\mu\text{g/ml}$) and were so maintained until they were harvested (day 10) as described in Methods. The remaining cells were maintained on ascorbic acid-free medium until they were harvested.

† Average of four flasks. Enzyme determinations were performed in duplicate for each flask exactly as described under Methods. § Average of four determinations. Chondrocytes, grown in Falcon flasks (25 cm^2), were harvested and suspended in 3.0 ml of distilled water. Aliquots were removed for protein and DNA determinations as described under Methods.

^{||} Standard Student *t* test.

when compared with normal chondrocytes grown under identical conditions was observed. This increase in acid phosphatase activity, previously noted by Ehrlich and co-workers (2) in human osteoarthritic articular cartilage slices, was expressed by the osteoarthritic chondrocytes regardless of whether they were grown in the presence or absence of ascorbic acid.

Long-term cultures. That the modulating effects of ascorbic acid were not only evident during periods of cell division occurring shortly after cell passage but were also evident after confluency had been well established is demonstrated in Tables III and IV. In addition, these data present evidence supporting an interrelationship between sulfated proteoglycan metabolism and arylsulfatase activities.

These experiments were carried out in Falcon flasks (25 cm^2) rather than Leighton tubes. Confluency had been established before the beginning of the experimental period which lasted a total of 10 days. Whereas one-half of the cells were deprived of ascorbic acid for the entire experimental period, the cells in the remaining flasks were exposed to ascorbic acid between days 6 and 10. Cells in all flasks were harvested on day 10. Although no difference in DNA content per flask was noted in the two groups, the cellular protein content was increased significantly in the presence of the vitamin (Table III). This increase was analogous to what had been observed for protein changes in the experiments conducted in Leighton tubes. From the data presented in Table III, the ascorbate-dependent decrease in arylsulfatase A activity was calculated to be 38.25%. A decrease of 31.35% was calculated for arylsulfatase B activity.

Acid phosphatase activity increased 7.08% in the presence of vitamin C. These results were also consistent with those observed in the time-study experiments described above. It appears, therefore, that the modulating effect of ascorbic acid on enzyme activities and protein content was neither transitory nor dependent on simultaneous cell multiplication but was present at

TABLE IV
*Effect of Ascorbic Acid on the Biosynthesis and Distribution between Medium and Cell Layer Fractions of Newly Synthesized Sulfated Proteoglycans (PG) in Long-Term Chondrocyte Cultures**

Ascorbic acid $\mu\text{g/ml}$	Total incorporation†§		[³⁵ S]PG in cell layer†		[³⁵ S]PG in cells†		
	Total [³⁵ S]PG		Total [³⁵ S]PG		[³⁵ S]PG in cell layer		
	$(^{35}\text{S-cpm/mg DNA}) \times 10^{-7}$		%		%		
0	4.561		67.82		2.297		
70	7.903		84.21		0.606		
	SE	0.238	0.395	2.75	0.76	0.071	0.016
	P	0.0003		0.0009		0.0001	

* Chondrocyte cultures used were as indicated in legend to Table III. Medium containing $\text{Na}_2^{35}\text{SO}_4$ was introduced exactly 24 h before cell harvest on day 10.

† Average of four determinations.

§ Total $^{35}\text{SO}_4$ incorporation is sum of $^{35}\text{SO}_4$ -labeled material found in dialyzed medium, extracellular, and cellular fractions. The extracellular and cellular fractions comprise the cell layer.

^{||} Standard Student *t* test.

varying stages after cell passage in chondrocyte cultures (Table III).

Sulfated proteoglycan biosynthesis was also studied in these well-established cultures. The chondrocytes were labeled with $\text{Na}_2^{35}\text{SO}_4$ for the 24-h period before cell harvest on day 10. The biosynthesis as reflected in sulfate uptake as well as the distribution of newly synthesized sulfated proteoglycans amongst the different cell layer and the medium fractions were evaluated. These data are summarized in Table IV.

Addition of ascorbic acid to the culture medium resulted in a 70% increase in the biosynthesis of sulfated proteoglycans per Unit of DNA. Furthermore, in the presence of ascorbic acid, a larger proportion of the newly synthesized molecules was deposited in the cell layer. In this experiment, where cells were grown in the presence of vitamin C for the last 4 days of a 10-day experimental period, the increased deposition of ^{35}S -proteoglycans was approximately 25% (column 2, Table IV). This corresponds to a decrease from 32.18 to 15.79% of ^{35}S -proteoglycans in the medium fraction.

The distribution of newly synthesized sulfated proteoglycans between extra- and intracellular sites within the cell layer was also affected by ascorbic acid. The net intracellular accumulation of labeled sulfated molecules was threefold greater in the absence of ascorbic acid (column 3, Table IV). This difference was highly significant with ($P < 0.0001$).

Thus, as the intracellular content of ^{35}S -labeled material decreased from 7.150×10^5 to 4.033×10^5 cpm per mg DNA, the total amount of ^{35}S -proteoglycans in the medium plus trypsin-sensitive fractions increased from 4.490×10^7 to 7.863×10^7 cpm per mg DNA. Increased catabolism within secondary lysosomes, decreased transport across the cell membrane or a combination of these factors could account for the higher intracellular content in the absence of the vitamin.

It is evident from the data shown in Tables III and IV that concomitant with lower activities of arylsulfatase A and B, higher levels of newly synthesized sulfated proteoglycans were found in human chondrocytes when grown in culture in the presence of ascorbic acid. Although the deposition of these macromolecules into the cell layer was substantially increased under these conditions, the amount of ^{35}S -labeled material found intracellularly was greatly reduced. This suggests that ascorbate, through interactions with cellular factors which result in lower arylsulfatase activities, promotes sulfated proteoglycan deposition in the extracellular matrix. The reduced enzyme levels would cause less breakdown of the sulfated macromolecules.

Reversibility of the effects by ascorbic acid. The modulating effects by ascorbic acid can be reversed by removal of the vitamin from the culture fluid. Absence of the vitamin for the last 6 days of a

16-day period resulted in restoration of enzyme activities and sulfated proteoglycan metabolic parameters to values approaching those measured in cells which had been grown in an ascorbate-free environment. This is illustrated in Fig. 3. Chondrocytes, grown entirely in the absence of ascorbate, were used as reference cells for the data presented in ratio form in this figure. The variable cells were those which were grown in the presence of ascorbate for the entire time (Group 1) or those which were withdrawn from an ascorbate environment for the latter half of the experimental period (Group 2). As the value of a given parameter of the variable cells approaches that of the reference cells, the ratio approaches unity.

Arylsulfatase A and B specific activities in cells grown with ascorbate (Group 1) were substantially lower than in those cells which had ascorbate withdrawn after the first two-thirds of the growth period (Group 2). This is reflected in the higher values of the ratios of the activities in the group 2 cells when compared with control chondrocytes (Fig. 3).

The ascorbate-dependent increase in acid phosphatase activity was reversed when the vitamin was removed from the culture medium. The chondrocytes responded by showing a sufficiently large decrease in acid phosphatase activity so that a ratio of less than unity was calculated (Fig. 3).

The reversibility of the effect by ascorbic acid was also observed when the biosynthesis of sulfated proteoglycans and their distribution between medium and cell layer fractions were examined. When the cells were removed from a medium containing ascorbic acid and grown in the absence of the vitamin for 6 days, the fraction of radioactively labeled sulfate incorporated per microgram of cell protein approached that found for cells grown entirely in the absence of ascorbic acid. Those cells which were retained on a medium containing ascorbic acid continued to show higher levels of sulfate incorporation. The ratio of sulfate incorporation for these cells (Group 1) when compared to the reference cells was 1.448. The difference between this ratio and that of the former group (Group 2) 1.080 was statistically significant ($P < 0.001$).

The redistribution of sulfated proteoglycans between the cell layer and the medium also showed that the regulatory effects of ascorbic acid could be reversed. As had been noted above, in the presence of ascorbic acid a larger proportion of newly synthesized sulfated proteoglycans was deposited in the cell layer. The proportion of sulfated proteoglycans deposited in the cell layer decreased when the cells were removed from the ascorbic acid environment. Thus, as shown in Fig. 3, the quantity of newly synthesized sulfated proteoglycans deposited in the cell layer for cells which had their initial growth on ascorbic acid and then were

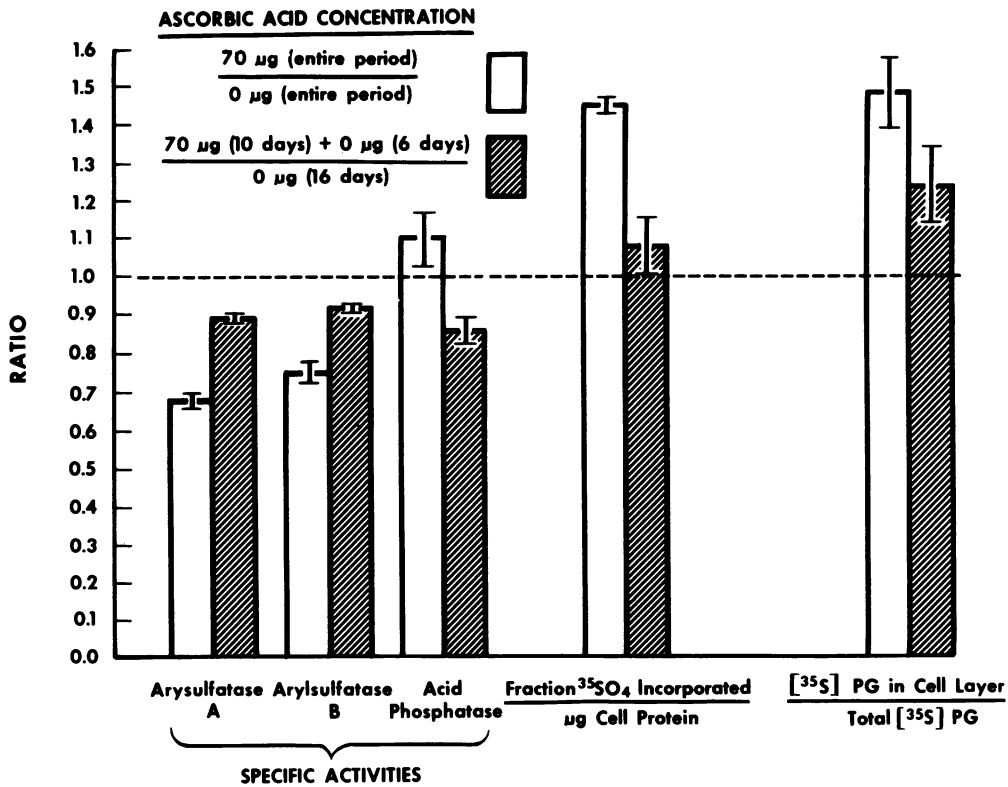


FIGURE 3 Reversibility of the effect of ascorbic acid on arylsulfatase activities and sulfated proteoglycan metabolism. The ratio of values for cells grown in the presence of 70 µg/ml ascorbic acid (Group 1) to those of cells grown in the absence of the vitamin (reference cells) (□), and for cells removed from an ascorbic acid environment for the last 6 days of the experimental period (Group 2) to reference cells (▨) are given in bar graph form. Enzyme activities and radioactivity content were determined as described in Methods.

removed from the vitamin-containing environment (Group 2) was significantly lower than that found in cells which were grown in the presence of the vitamin during the entire period (Group 1).

No ascorbic acid was found in the cell extracts of the reference cells or the cells removed from an ascorbate environment for the latter part of the experimental period (Group 2). The ascorbate level in the cells from Group 1, on the other hand, averaged 5.23 µg/ml of cell extract. These extracts had been frozen at -20°C before these analyses.

DISCUSSION

Progressive depletion of sulfated proteoglycans from the extracellular matrix of articular cartilage is one of the earliest manifestations of osteoarthritis. Higher levels of lysosomal enzymes, shown to be present in the diseased tissue, are thought to be responsible for this loss. In animal systems where arthritic lesions of the articular cartilage were induced by mechanical means, arylsulfatase activities were found to be sig-

nificantly elevated for extended periods of time (23). Increased levels of arylsulfatase A and B were correlated with increasing sulfated proteoglycan depletion as monitored by histological methods in tissue slices from human osteoarthritic articular cartilage (7). These data suggested a possible physiological relationship between arylsulfatase activities and the catabolism of sulfated proteoglycans. Since we had previously shown that ascorbic acid modulates the levels of arylsulfatase A and B activities in normal chondrocyte cultures (24), it was of interest to examine what effect the vitamin might have on this metabolic relationship. Furthermore, if ascorbate was found to have an active regulatory role in normal cells, would this property be evident in osteoarthritic chondrocytes.

Vitamin C has been implicated in the metabolism of collagen, glycosaminoglycans, as well as other sulfated, and lipid complex compounds. In collagen biosynthesis, ascorbic acid has been shown to function as a cofactor for prolyl and lysyl hydroxylases (25-28). This property was proposed to be responsible for the ability of vitamin C to modulate collagen-hydroxy-

proline synthesis in tissue culture systems (29, 30). Ascorbic acid may have multiple mechanisms of action. Addition of ascorbic acid to mouse fibroblasts resulted in an increase in prolyl hydroxylase activity. This increase was attributed to enzyme activity rather than *de novo* synthesis. It was suggested that ascorbate may act by aggregating inactive subunits into an active enzyme (29). Ascorbate has also been shown to increase the rate of hydroxyproline synthesis by cultured tendon cells. In contrast, it appears that ascorbate acts as a cofactor for the prolyl hydroxylase reaction in primary tendon cells (31). In chick embryo fibroblasts, ascorbate was found to increase secretion of collagen (28, 32).

Glycosaminoglycan synthesis and secretion also were affected in fibroblast cultures. An increased accumulation of dermatan sulfate was found in the cell layer of Hurler syndrome fibroblasts when ascorbic acid was added to the culture medium (33). In scorbutic guinea pigs, cartilage cells were found to contain reduced levels of glycosaminoglycans, DNA, and glycogen. These deficiencies were associated with reduced synthesis of galactosamines (34).

Sulfate formation is an important pathway for the biotransformation of phenolic drugs. Concomitant administration of vitamin C and salicylamide, a widely used analgesic and antipyretic, caused a decrease in the conversion of the latter to salicylamide sulfate. This inhibition was prevented by the administration of a sulfate donor suggesting that the competitive formation of ascorbate sulfate was the basis of the observed inhibition (35). Ascorbic acid 2-sulfate had been shown to be a metabolite in rat tissue (36) and in human urine (37) as well as a substrate for mammalian arylsulfatase A (10).

In epiphyseal cartilage, vitamin C has been shown to prevent loss of matrix components from embryonic chick limb bud rudiments which are in a state of hyperoxia caused by exposure to 85% oxygen. The protection, thought to occur via a cooperative effort with vitamin E, is postulated to result from the prevention of lipid peroxidation of lysosomal membranes (38). In other words, the action of ascorbic acid is attributable to its reducing capabilities. More recently, vitamin C has been shown to inhibit ACTH-induced cyclic AMP formation in adrenal cells. Again, the inhibiting effect was reversible by vitamin E. It was proposed that the membrane-bound enzyme adenylate cyclase was the site of interaction between ACTH, ascorbic acid, and alpha-tocopherol (39).

Lipid involvement is also seen in the accumulation of cholesterol in the livers and other tissues of guinea pigs maintained on a vitamin C deficient diet (40). The benefits of vitamin C may extend much beyond improving "the common cold" since its administration to hyperlipidemic individuals has been shown to de-

crease the plasma cholesterol levels in these subjects (41).

On examination, arylsulfatase A and B activities were found to be reduced to a similar degree in normal and osteoarthritic human chondrocytes when the cells were grown in culture in the presence of ascorbic acid. In neither case did this reflect a general loss of activity for all lysosomal enzymes since beta-glucuronidase and *N*-acetyl-glycosaminidase activities were not affected by the vitamin. Acid phosphatase activity, on the other hand, was found to be significantly higher in both normal and pathological cells when ascorbate was included in the growth medium.

Of additional interest was the finding that acid phosphatase activity was elevated in osteoarthritic cells when compared to normal cells grown under identical conditions. An increase in acid phosphatase activity with increasing severity of the disease had been demonstrated previously in articular cartilage from normal and osteoarthritic human hip joints (2). Similar increases had been found in the cartilage of osteoarthritic joints in experimental animals (23). The cells used in the experiments reported herein were either in second or third passage. It is most significant that these data imply that the information for the stimulus responsible for the heightened phosphatase levels was retained on cell passage. The stimulus acted independently of ascorbic acid.

The biosynthesis of sulfated proteoglycans per unit of DNA as well as their distribution amongst the different medium and cell layer fractions have been found to be a function of pH, presence or absence of serum and (or) ascorbic acid, type of serum used, and the ratio of surface area to culture fluid (42, 43). Incorporation of ³⁵S-inorganic sulfate and the subsequent distribution of the labeled molecules was also found to be dependent upon the time course of the experiment in relation to the time of last cell passage. Greater ³⁵S-sulfate incorporation by chondrocytes from rabbit articular cartilage had been found in Dulbecco's Modified Eagles' medium than in F-12 medium (44).

Purified proteoglycans added to a suspension culture of chondrocytes stimulated the rate of synthesis of proteochondroitin sulfate (45). These observations re-emphasize the necessity for rigid control of experimental design when comparing parameters for different cell types under varying culture conditions.

Ascorbic acid has been shown to stimulate sulfated proteoglycan biosynthesis in chick embryo fibroblasts (46), have no effect in rabbit chondrocytes (44), and to cause decreased sulfate incorporation in human articular cartilage chondrocytes (47).

In the experiments reported here, we have found that the net biosynthesis of sulfated proteoglycans per unit of DNA as reflected in sulfate uptake was significantly stimulated by ascorbic acid in both normal and

osteoarthritic human chondrocytes. It is likely that, in part, the increase in cell protein found in the presence of ascorbate also reflects this increased biosynthesis. The difference in the distribution of the newly synthesized molecules was indicative of increased stabilization within the extracellular matrix when vitamin C was present. The finding of increased accumulation in the extracellular space together with the observed reduced levels of arylsulfatase activities suggest decreased catabolism occurring intracellularly within secondary lysosomes. This was compatible with the observed reduction in radioactive content found within the cells.

The mechanism by which ascorbic acid acts to produce the described changes is not clear from these experiments although the ability to reverse the effects by removal of the vitamin substantiate the premise that the effects are not the result of permanent damage to the cell membrane. Studies performed on arylsulfatase A from ox liver suggested that ascorbate inhibited the enzyme by reducing traces of copper to Cu^+ which, in turn, acted as the true inhibitor (18). Whether this was the mechanism operative in chondrocytes is not known. The increase in inhibition with longer periods of time in culture, concomitant with rather variable intracellular concentrations of ascorbate, implies the involvement of an additional cellular factor such as a metal ion.

The mechanism by which ascorbate acts to increase the net synthesis as well as to stabilize sulfated proteoglycans of the extracellular matrix has also not yet been defined. A possible mechanism would involve an initial reduction in the activity of the lysosomal enzymes which, in turn, would result in less breakdown of the components comprising the extracellular matrix. Alternatively, increased deposition of collagen, known to occur in the presence of ascorbic acid, could provide a more stable environment for deposition of newly synthesized proteoglycans.

Cysteine residues are known components of the protein core of proteoglycans. Ascorbate in its role as a reducing agent, has been found to increase the ratio of cysteine:cystine in physiological solutions. The synthesis of the core and other proteins which contain the sulfhydryl amino acid may therefore be promoted in the presence of vitamin C. Additional experiments that also monitor collagen and glycoprotein synthesis will have to be conducted to elucidate the mechanism by which ascorbic acid acts in the chondrocyte culture system.

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