EFFECT OF EXTRACELLULAR CHONDROITIN SULFATE ON CULTURED CHONDROCYTES

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INTRODUCTION

All cells have, to some degree, extracellular matrix immediately exterior to the cell membrane proper (Rambourg and Leblond, 1967; Martinez-Palomo, 1970). Cartilage cells have extended matrices. Much is known about the biochemistry of some of the matrix components (Balazs, 1970; Slavkin, 1972), but the biological role of these macromolecules is not well understood (Ogston, 1970).

The cartilage matrix is a complex composite of several macromolecules such as chondroitin sulfates, glycoproteins, and collagen. Intuitively, it may be possible to dissect and selectively modify the extracellular matrix of living cartilage cells by treatment with enzymes, such as chondroitinases, hyaluronidases, and collagenase, to remove various matrix components. The opposite approach is to add matrix components in replacement or in excess. Even though removal and addition of matrix components do not represent true dissection and reconstruction, a comparison of the effects of these approaches may serve as the beginning of a systematic investigation of the biological significance of some of the components that form the complex microenvironment of the cell.

This report presents the following results: Treatment of cultured chondrocytes with enzymes, such as chondroitinase, bovine testicular hyaluronidase, or trypsin, which remove chondroitin sulfate from the matrix, resulted in lower sulfate incorporation by the cells. On the other hand, cultures treated with enzymes such as streptococcal hyaluronidase or collagenase, which do not remove sulfate-labeled material from the cells, maintained control levels of sulfate incorporation. Conversely, when exogenous chondroitin sulfate was added to the

culture medium, the cultures responded by an increased incorporation of labeled sulfate. Neither the enzyme treatments nor addition of chondroitin sulfate affected the overall synthesis of DNA, RNA, collagen, or total cell proteins. It is concluded that extracellular chondroitin sulfate plays a regulatory role in the production of sulfated mucopolysaccharides by cultured chondrocytes.

MATERIALS AND METHODS

Cell culture procedures were similar to those described by Chacko et al. (1969). Aliquots of 5×10^5 cells were plated onto 100-mm tissue culture dishes with 10 ml of culture medium (Ham's F-10 with twice concentrated amino acids, 10% fetal calf serum, 1% bovine serum albumin, 0.1% L-glutamine, 0.1% antibiotic-antimycotic). Incubation was carried out at 37°C in 95% air and 5% CO_2. On the 5th day, chondrogenic floating cells were collected from the medium, briefly trypsinized (0.25% trypsin, 15 min), and plated onto 60-mm tissue culture dishes at 4×10^4 cells per 3 ml fresh medium. All observations were performed within the first 2 wk of culture, during which time the cultures remained at a clonal density and were chondrogenic.

DNA, RNA, and protein syntheses were monitored by the respective incorporation of [^{3}H]thymidine (40 Ci/mmol, 5 μCi/ml), [^{3}H]uridine (25 Ci/mmol, 5 μCi/ml), and [^{3}H]leucine (30 Ci/mmol, 5 μCi/ml) (New England Nuclear, Boston, Mass.) into TCA-precipitable materials. Chondromucopolysaccharide synthesis was monitored by pulse labeling with carrier-free $Na_2{}^{35}SO_4$ (Amersham/Searle Corp., Arlington Heights, Ill., 5–10 μCi/ml). The labeled mucopolysaccharides were isolated and assayed by methods similar to those described by Nameroff and Holtzer (1967). Standard chondroitin sulfate was obtained from Miles Laboratories Inc., Elkhart, Ind. Collagen synthesis was monitored by pulse labeling with [^{3}H]proline (10 Ci/mmol, 5 μCi/ml, New England Nuclear) followed by proline/hydroxyproline determination by the methods of Lukens (1965) modified by Schiltz et al. (1973).

Chondroitinase AC-I from *Flavobacterium heparinum* (Miles Laboratories, Inc.), bovine testicular hyaluronidase (Worthington Biochemical Corp., Freehold, N. J., chromatographically purified, 8,600 U/mg), streptococcal hyaluronidase (kindly provided by Dr. Paul Bell), and collagenase from *Clostridium histolyticum* (Worthington Biochemical Corp., chromatographically purified, 400 U/mg) were incubated with the following paranitrophenyl substrates for 1 h at 37°C to check for contaminating glycosidase, phosphatase, and sulfatase activities: paranitrophenyl-α-D-mannoside, -*N*-acetyl-β-D-glucosaminide, -α-D-glucopyranoside, -β-D-glucopyranoside, -α-D-galactopyranoside, -β-D-galactopyranoside, -α-L-fucoside, -β-L-fucoside, -phosphate, and -sulfate (kindly provided by Dr. Leonard Warren). Protease activity was monitored by incubation with cell cultures prelabeled with [^{3}H]leucine (5 μCi/ml, 24 h) or in test tubes with heat-denatured [^{14}C]algal proteins (International Chemical and Nuclear Corp., Cleveland, Ohio, 1 mCi/mg, 5 μCi/ml). After 6 h of incubation at 37°C, the mixtures were precipitated with TCA (10% final concentration) and the amount of TCA-soluble radioactivity was determined. Parallel controls contained no enzymes. Protease activity was also assayed for by incubation (20 min, 37°C) with Azocoll (Calbiochem, San Diego, Calif.).

RESULTS

Treatment by Enzymes

Cultures were prelabeled for 24 h with $Na_2{}^{35}SO_4$ (5–10 μCi/ml, carrier-free), washed three times with Simms balanced salt solution (BSS), and given fresh medium containing an enzyme. Table I shows the amount of [^{35}S]sulfate radioactivity remaining with the cells after 6 h of incubation at 37°C with each of the enzymes. It was found that increasing the time of incubation or enzyme concentrations did not significantly affect the results of streptococcal hyaluronidase or collagenase. These two enzymes removed little or no [^{35}S]sulfate radioactivity from prelabeled cells. On the other hand, chondroitinase and bovine testicular hyaluronidase extensively removed sulfate-labeled material from the cells. To minimize the effects of possible contaminants, the lowest effective enzyme

TABLE I

Enzyme Removal of Prelabeled [^{35}S]Sulfate Radioactivity

	Cells	Medium	Total
	cpm/culture		
1.5–2-min treatment:			
Control	22,401	1,851	24,252
Trypsin, 0.08%	4,031	19,880	23,911
6-h treatment:			
Control	20,695	4,319	25,014
Chondroitinase, 0.03 U/ml	6,465	20,173	26,638
Bovine testicular hyaluronidase, 0.5 μg/ml	5,235	20,609	25,844
Streptococcal hyaluronidase, 10 μg/ml	22,065	5,432	27,497
Collagenase, 10 μg/ml	20,393	5,897	26,290

TABLE II

Effect of Treatment by Enzymes

	[^{3}H]Thymidine	[^{3}H]Uridine	[^{3}H]Leucine	[^{35}S]Sulfate
		cpm/μg DNA		
Control	14,091	9,860	234,677	27,753
Chondroitinase	13,683	8,688	228,265	18,792
Testicular hyaluronidase	12,032	8,563	218,734	17,148
Trypsin	14,568	8,975	198,410	14,233
Streptococcal hyaluronidase	11,533	9,686	219,924	25,735
Collagenase	13,350	9,324	214,618	26,480

Results from one representative set of experiments. Each number is the average of three samples. The data on leucine and sulfate incorporations represent the combined radioactivity from cells and media.

concentrations tested were used. All experiments to be described in this section were performed at the enzyme concentrations indicated in Table I with an incubation time of 6 h. Cultures were also treated with 0.08% trypsin (Grand Island Biological Co., Grand Islands, N. Y.) in BSS for 1.5–2 min. This enzyme's concentration and incubation time were used to obtain maximum removal of labeled sulfate radioactivity without detaching the cells from the substrate.

The enzymes were checked for contaminating activities (see Materials and Methods). Chondroitinase was found to be free of protease, glycosidase, phosphatase, or sulfatase activities. Bovine testicular hyaluronidase had no sulfatase, phosphatase, or protease activities but did contain some ($<2 \times 10^{-6}$ μmol/min/mg protein) glycosidase activities. Collagenase, too, contained some ($<2 \times 10^{-5}$ μmol/min/mg protein) glycosidase activities and some ($<1 \times 10^{-5}$ μmol/min/mg protein) sulfatase activity but no phosphatase activity. Collagenase is also known to have contaminating protease activities (unpublished observations; Goldberg and Green, 1967; Peterkofsky and Diegelmann, 1971). Streptococcal hyaluronidase had no glycosidase, sulfatase, or phosphatase activities but did contain some protease activity (by Azocoll incubation at 37°C, 20 min, OD_{530} = 10^{-3} that of collagenase at 30 μg/ml).

After enzyme treatment, the cultures were washed three times with BSS and fed fresh medium with labeled sulfate. After a labeling period of 6 h, the cultures and media were collected for assay of label incorporation. Results from one set of experiments are summarized in Table II. Most of the sulfate-labeled material from both control and treated cultures migrated in electrophoresis like chondroitin sulfate. Although results varied among different preparations and lots of enzymes, in eight different sets of experiments there were consistent decreases in labeled sulfate incorporation when the cultures were treated with chondroitinase (32 ± 11%), bovine testicular hyaluronidase (35 ± 9%), or trypsin (48 ± 10%). During the 6-h period after washing, little or no residual chondroitinase or hyaluronidase activity was found in the cultures as monitored by further release of prelabeled [^{35}S]sulfate radioactivity or ability of the cultures to break down exogenously added labeled chondroitin sulfate.

Streptococcal hyaluronidase-treated cultures demonstrated control levels of sulfate incorporation. Streptococcal hyaluronidase breaks down hyaluronic acid and chondroitin but is inactive toward chondroitin sulfate (Linker et al., 1956). The enzyme did not remove prelabeled sulfate radioactivity from cultured chondrocytes even though enzyme activity was demonstrable since 12% of glucosamine-labeled materials from parallel cultures was rendered dialyzable upon enzyme treatment and the viscosity of hyaluronic acid (Worthington Biochemical Corp.) was reduced upon incubation with the enzyme.

Like streptococcal hyaluronidase-treated cultures, collagenase-treated cultures showed control levels of sulfate incorporation. Collagenase also did not remove [^{35}S]sulfate-labeled material from the cells. Increasing this enzyme concentration to 0.3 mg/ml gave essentially the same results. Cultures prelabeled with [^{3}H]proline lost only 5–10% more radioactivity from the cells upon treatment for 6 h with collagenase than controls. Collagenase activity per se was demonstrable in that most of the proline-labeled material removed from the cells was rendered TCA soluble; and with respect to those counts remaining associated with

the cells, the treated cells had 60% more TCA-soluble counts than controls. Furthermore, in the TCA-precipitated material, the controls had 50% more labeled hydroxyproline than the collagenase-treated cultures.

Table II also indicates that after treatment by the enzymes, overall cell growth and synthetic processes monitored by incorporation of labeled precursors of proteins and nucleic acids remained the same as those of control cultures. Treatment with the enzymes also had little effect on total collagen synthesis as measured by conversion of labeled proline to hydroxyproline (Table III).

Addition of Chondroitin Sulfate

Stimulation of [^{35}S]sulfate incorporation was observed after addition of 0.3 mg/ml chondroitin sulfates A and C (Miles Laboratories Inc., super special grade) to normal chondrocyte cultures (Table IV). Similar stimulatory effects have been found with chondromucoprotein, chondroitin sulfates, and other negatively charged macromolecules by Nevo and Dorfman (1972) and with chondromucoprotein by Kosher et al. (1974). By using labeled chondroitin sulfate, it was found that the added chondroitin sulfate remained intact in the medium and was not taken up by the cells. Furthermore, in the presence of added chondroitin sulfate, incorporation of labeled thymidine, uridine, and leucine remained at control levels.

Chondroitin sulfate, when added to enzyme-treated cultures, also stimulated their levels of labeled sulfate incorporation. Surprisingly, cultures treated with chondroitinase, testicular hyaluronidase, or trypsin incorporated as much [^{35}S]sulfate as control cultures similarly stimulated by added chondroitin sulfate (Table IV). It is also intriguing that in both control and enzyme-treated cultures the increase in sulfate-labeled material was found mostly in the medium rather than associated with the cell matrix.

TABLE III

Collagen Synthesis

	Hypro		Pro	
	Cells	Medium	Cells	Medium
	cpm/culture			
Control	2,136	1,048	12,205	57,174
Chondroitinase	1,952	1,068	11,493	41,652
Testicular hyaluronidase	2,195	1,312	12,438	40,076
Streptococcal hyaluronidase	2,790	1,273	13,127	50,435
Trypsin	2,045	1,091	10,432	44,729

0.05 mg/ml ascorbic acid was added to the cultures.

TABLE IV

Effect of Exogenously Added Chondroitin Sulfate

	−CS*		+CS*	
	Cells	Medium	Cells	Medium
	cpm/μg DNA			
Control	18,504	2,364	19,173	27,042
Chondroitinase	10,851	1,576	16,482	29,128
Testicular hyaluronidase	9,373	1,673	17,986	25,332
Trypsin	8,207	2,201	15,345	25,049
Streptococcal hyaluronidase	16,018	2,824	15,597	30,635
Collagenase	17,593	3,081	21,693	23,792

* Without (−CS) or with (+CS) exogenously added chondroitin sulfate. Results are from one representative set of experiments. Each number is the average of three samples. All samples were parallel plated sister cultures.

DISCUSSION

Attempts were made by biochemical dissection to gain information on the significance of the chondrocyte extracellular matrix. Removal of chondroitin sulfate from the matrix resulted in decreased [^{35}S]sulfate incorporation by the cells. On the other hand, addition of exogenous chondroitin sulfate to the cultures resulted in increased [^{35}S]sulfate incorporation. Most of the sulfate-labeled material isolated from both the treated and control cultures migrated in electrophoresis as chondroitin sulfate. It is concluded that chondroitin sulfate of the extracellular matrix plays a regulatory role in the production of sulfated mucopolysaccharide by cultured chondrocytes.

An increase in total hexosamine has been observed in organ cultures of tibial rudiments treated with bovine testicular hyaluronidase (Fitton-Jackson, 1970) or papain (Bosmann, 1968) and of hyaluronidase-treated cartilage pieces (Hardingham et al., 1972). It would be of interest to know how the increase in total hexosamine relates to the synthesis and turnover of sulfated mucopolysaccharides.

The mechanism of the cell's response to its extracellular matrix is not known. The exogenously added chondroitin sulfate is not actively taken up into the cell or degraded into ethanol-soluble fragments. Furthermore, the stimulatory effect was elicited not only by chondroitin sulfate

but also by a number of other large, negatively charged macromolecules (Nevo and Dorfman, 1972). Therefore, extracellular chondroitin sulfate itself may not be involved metabolically in a positive feedback manner. On the other hand, since not all negatively charged molecules elicited a stimulatory response (Nevo and Dorfman, 1972), charge alone may not be the factor. Recently it has been reported that the secondary structures of mucopolysaccharides can vary significantly (Arnott et al., 1973; Isaac and Atkins, 1973). This level of variation, in addition to variations in sulfate distribution (Kimata et al., 1973), can very likely have consequential effects on the tertiary and quaternary conformations of sulfated mucopolysaccharides (Dea et al., 1972) and their interactions with collagen molecules (Lowther et al., 1970; Mathews, 1970; Hanada and Anan, 1973; Obrink, 1973). Therefore the integrity of the cartilage matrix involves several levels of variables any or all of which may play a role in the mechanism of the cell's response.

Cartilage cells are intimately surrounded by their extracellular matrix mucopolysaccharides as demonstrated by the appearance of metachromasia around the cells after toluidine blue staining, and by the fact that neither extensive washing nor EDTA treatment cannot remove the matrix. There are many conceivable ways in which a matrix of highly charged and hydrated macromolecules can physiologically affect a cell. For instance, the matrix may act as an ion exchange barrier that regulates the diffusion of various molecules to and from the cell (Ogston, 1970). Mucopolysaccharides have strong affinities for cations (Phillips, 1970; Mathews, 1970; Macgregor and Bowness, 1970; Grant et al., 1973). Altering the amount of matrix mucopolysaccharides may therefore alter local pH and ion concentrations. Such alterations may significantly affect the synthetic systems by altering transport and thus available pool sizes of the necessary precursors. Furthermore, local alterations of cell surface molecules may profoundly affect the cell membrane proper, as has been widely reported in instances of protease treatment of cell surfaces (see review by Wallach, 1972).

The cartilage cell is specialized in synthesizing extracellular mucopolysaccharides. Many other cells also possess mucopolysaccharides on their surfaces (Kojima and Yamagata, 1971; Kraemer, 1971). Furthermore, virally transformed cells have varying amounts of extracellular mucopolysaccharides (Goggins et al., 1972; Satoh et al., 1973). It would be of interest to see how these cells are affected by alterations in the amount of their extracellular mucopolysaccharides.

SUMMARY

Treatment of clonal chondrocyte cultures with enzymes that remove chondroitin sulfate from the cells resulted in decreased [^{35}S]sulfate incorporation by the cultures. Addition of chondroitin sulfate to chondrocyte cultures resulted in increased sulfate incorporation. The results suggest a regulatory role for extracellular chondroitin sulfate in the production of sulfated glycosaminoglycans by cartilage cells.

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