THE INHIBITION OF SULPHATE INCORPORATION IN ISOLATED ADULT CHONDROCYTES BY HYALURONIC ACID

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1. Introduction

Proteoglycans in the matrix of adult cartilage are metabolised quite rapidly [1, 2], nevertheless adult cartilage has a limited capacity for repair. Embryonic cartilage on the other hand, is able to replace the proteoglycans of the matrix within a few days after they have been largely depleted by the action of enzymes [3, 4], which suggests that the macromolecules in the environment exert some influence over the synthetic or secretory functions of chondrocytes. The possible influence of a variety of macromolecules on the biosynthesis of sulphated glycosaminoglycans has therefore been examined using suspensions of non-dividing adult chondrocytes, obtained from pig laryngeal and articular cartilage.

Cartilage contains small amounts of hyaluronic acid [5], low concentrations of which have important biological effects [6]. The effect of low concentrations of hyaluronic acid on the incorporation of $^{35}SO_4^{2-}$ into material precipitable by cetylpyridinium chloride (CPC) is reported here.

2. Materials and methods

2.1. *Media*

Hank's balanced salt solution and Leibovitz L-15, both in powder form, and foetal calf serum were all supplied by Flow Laboratories Ltd., Victoria Park, Heatherhouse Road, Irvine, Ayrshire, Scotland. Tyrode's salt solution, also in powder form, was purchased from Difco Laboratories, P.O. Box 14B, Central Avenue, East Molesley, Surrey, U.K. Carrier free [^{35}S]sulphate was added to Tyrode's salt solution at 0.5 or 0.2 mCi/ml. [^{3}H]thymidine was supplied at 18.4 Ci/mM and diluted in Leibovitz L-15 medium appropriately. All radioisotopes were obtained from the Radiochemical Centre, Amersham, Bucks., U.K. Isolated cells were maintained in Leibovitz medium enriched with 10% foetal calf serum to which streptomycin (100 μg/ml) and penicillin (100 iu/ml) were added.

2.2. *Chemicals*

Bacterial collagenase (EC 3.4.4.19, *Clostridium histolyticum*) 125–200 units/mg was obtained from Sigma Chemical Co., Ltd., Norbiton Station Yard, Kingston-upon-Thames, Surrey, U.K. To isolate cells from matrix, the enzyme (2.5 mg/ml) was dissolved in sterile Leibovitz medium containing 10% foetal calf serum. Hyaluronic acid was obtained from British Drug Houses Ltd., Poole, Dorset, U.K. and diluted appropriately in Tyrode's solution containing radioactive sulphate. Scintillation fluid (toluene–2 methoxyethanol, 3:2 v/v), contained per litre 80 gm of naphthalene and 4 gm of 2,3-bis-(5-t-butylbenzoaxazol-2-yl) thiophen. Ficoll was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

2.3. *Methods*

2.3.1. Culture preparation

Thyroid cartilages were dissected from fresh adult pig larynges, and freed of soft tissue and perichondrium. The cartilage was chopped with a scalpel into 0.5–1.0 mm cubes in a sterile Petri dish and washed several times in Hank's solution; chilled conditions were not adopted. The cartilage from each larynx was treated separately with 5–10 ml of collagenase solution, (about 1 ml/1 g wet weight of tissue) and incubated

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overnight. The digestion was sometimes not complete. The suspension of cells thus released was diluted 10-fold and shaken. After standing for 3 min, large partially digested pieces of cartilage and debris settled out and the suspension of cells and small particles decanted. The cells were centrifuged (1000 rpm) and the pellets washed three times in Hank's solution. Cells were then completely freed of all debris as follows:

Solutions of Ficoll at the various concentrations described below were made in Hank's solution at pH 6.5. Pellets of washed cells were prepared and resuspended in 5 ml of 21% Ficoll and placed into a discontinuous gradient in 25 ml polycarbonate centrifuge tubes. The gradient consisted of 5 ml of 32% Ficoll at the bottom as a cushion; then layered with 5 ml each of 25% and 21% Ficoll, the latter containing the cells. This was overlaid with 5 ml each of 16% and 12% Ficoll solution. The cells were then centrifuged for 30 min, at 20 000 *g* on an MSE Superspeed 65. The specific gravity of the gradient of 12% to 32% Ficoll ranged between 1.04–1.12. Fractions were collected by displacement from below with each fraction being diluted 1:4 with 4 vol of Hank's solution. The number of cells in each fraction was measured using a Coulter counter and mean cell volumes estimated from a threshold graph. The Ficoll fractions containing cells were normally pooled, leaving debris to be discarded. The cells were washed twice to remove Ficoll and resuspended in Leibovitz L-15 medium, plus 10% foetal calf serum.

As determined by exclusion of Trypan Blue, the suspension of cells remained viable for about three weeks but were usually used between 3–8 days after isolation. No mitotic figures were observed. The lack of mitotic activity was also established by the lack of incorporation of [^{3}H]thymidine, 2 μCi being added to representative cultures. After 18 hr-incubation cells were washed twice with cold Hank's solution, twice with cold 5% trichloroacetic acid and finally with 5 ml of methanol. The pellets were dissolved in either 0.5 ml of 0.5 N NaOH or 0.25 ml of 'Hyamine', to which 10 ml of scintillant was added and radioactivity measured.

Suspensions of primary cultures of skin fibroblasts obtained from human scrotum and female abdominal wall, were prepared by scraping with vigorous agitation fully confluent monolayers from Petri dish cultures. Synovial cells were prepared similarly.

2.3.2. Inorganic sulphate incorporation

Cell suspensions (chondrocytes, fibroblasts or synovial cells) were divided into aliquots containing 5×10^5 cells/ml and pairs selected appropriately as controls and experimental samples. Each pair of cultures came from the same larynx or culture and each experiment was done in quadruplicate. Test solutions were prepared by appropriate dilution of hyaluronic acid with Tyrode's solution containing $^{35}SO_4^{2-}$. Control solutions contained no hyaluronic acid.

Cells were incubated at 37°C for 2 hr in Tyrode's solution prepared as above. They were then spun free of their media, washed twice in Tyrode's solution containing 0.05 M Na_2SO_4. Washings and media were pooled. To both cells and media, 2% CPC was added separately to a final concentration of 1%. The mixtures were stirred vigorously with kieselguhr and incubated overnight at 37°C, and then shaken and centrifuged. The pellets were washed free of non-precipitated sulphate with 2% CPC by resuspending and centrifuging and the radioactivity of the washings measured. Complete disruption of the cells was confirmed by microscopic examination. All the pellets were quantitatively transferred with 70% propanol into vials containing 10 ml of scintillation fluid. The kieselguhr was allowed to settle for at least 15 min and radioactivity measured in a Packard Tricarb liquid scintillation spectrophotometer. Counting rates were corrected for quenching using a channels–ratio method with an external standard.

3. Results and discussion

The amount of radioactive $^{35}SO_4^{2-}$ incorporated during a 2 hr incubation by isolated control cells was unaffected by – whether the cells were in contact as in a pellet, or in suspension, hence all the results described here refer to suspensions of cells.

At concentrations above 5×10^{-2} μg/ml, table 1 shows that hyaluronic acid approximately halved the incorporation of $^{35}SO_4^{2-}$ into CPC-precipitable material by adult chondrocytes from laryngeal cartilage. At lower concentrations of hyaluronic acid, from 5×10^{-3} to 1×10^{-4} μg/ml there was some inhibition, whereas below 1×10^{-5} μg/ml, incorporation of sulphate was

Table 1
The effect of hyaluronic acid on the incorporation of $^{35}SO_4^{2-}$ into cetylpyridinium chloride-precipitable material by suspension cultures of chondrocytes.

Concentration of hyaluronic acid (μg/ml)	Total incorporation of $^{35}SO_4^{2-}$ (medium + cells) (% of Controls)	Proportion of $^{35}SO_4^{2-}$ associated with cells (% of Total incorporation)
100.0	43.0 ± 6.3	48.0 ± 5.2
50.0	57.0 ± 9.0	49.0 ± 3.0
8.0	33.8 ± 15.6	50.5 ± 7.2
5.0	57.0 ± 18.0	53.2 ± 1.2
1.0	48.0 ± 1.0	50.3 ± 3.0
0.8	44.0 ± 7.4	48.5 ± 5.3
8×10^{-2}	62.9 ± 3.4	39.6 ± 4.7
5×10^{-2}	55.0 ± 10.0	39.0 ± 6.0
5×10^{-3}	72.25 ± 11.6	35.0 ± 6.0
1×10^{-3}	85.75 ± 11.2	32.0 ± 3.5
1×10^{-4}	85.00 ± 13.5	38.0 ± 7.0
1×10^{-5}	101.6 ± 8.2	32.0 ± 0.8
1.35×10^{-6}	130.0 ± 18.2	35.0 ± 2.7
Control	= 100	32.8 ± 3.5

similar to that of controls (table 1). This effect appeared to be specific to chondrocytes since hyaluronic acid did not inhibit sulphate incorporation by various primary cultures of fibroblasts or synovial cells (table 2). These primary cultures were selected for their long generation time indicating that most of the cells would be in G1 and G2 as were the chondrocytes.

None of the other compounds tested in this system had any effect at the concentrations at which hyaluronic acid inhibited sulphate incorporation. At higher concentrations however, some compounds reduced the proportion of radioactivity associated with cells and stimulated sulphate incorporation, but none inhibited it (table 3).

The isolated chondrocytes usually separated into two major populations on the density gradient. Hyaluronic acid inhibited sulphate incorporation by cells in both of the major fractions. Three sub-fractions of cells obtained from the upper less dense part of the gradient were inhibited by low concentrations of hyaluronic acid, whereas concentrations above 1×10^{-2} μg/ml were needed to inhibit sulphate incorporation by denser cells.

Table 2
The effect of hyaluronic acid on the incorporation of $^{35}SO_4^{2-}$ into cetylpyridinium chloride precipitable material by suspensions of primary cultures of fibroblasts and synovial cells.

Type of cell	Concentration of hyaluronic acid (μg/ml)	Total incorporation of $^{35}SO_4^{2-}$ (medium + cells) (% of Controls)	Proportion of $^{35}SO_4^{2-}$ associated with cells (% Total incorporation)
Synovial cell (first passage fully confluent)	1×10	117.46 ± 16.65	63.49 ± 7.2
	1×10^{-1}	100.16 ± 22.73	56.28 ± 8.2
	1×10^{-2}	120.65 ± 20.99	65.08 ± 9.2
	1×10^{-3}	98.5 ± 15.46	53.20 ± 7.3
	Control	= 100	54.69 ± 3.1
Skin fibroblast (first passage fully confluent)	1×10	69.66 ± 15.2	25.84 ± 5.2
	1×10^{-1}	97.43 ± 17.2	36.14 ± 7.2
	1×10^{-2}	114.12 ± 13.2	42.33 ± 5.3
	1×10^{-3}	104.98 ± 20.2	38.94 ± 8.3
	Control	= 100	37.09 ± 9.2
Skin fibroblast (third passage fully confluent)	1×10	93.3 ± 13.22	39.5 ± 1.2
	1×10^{-1}	82.14 ± 10.27	34.76 ± 5.7
	1×10^{-2}	161.09 ± 2.44	67.80 ± 9.3
	1×10^{-3}	120.62 ± 3.06	50.6 ± 7.5
	Control	= 100	42.12 ± 3.3

Table 3
The effect of various macromolecular compounds on the incorporation of $^{35}SO_4^{2-}$ into cetylpyridinium chloride-precipitable material by suspension cultures of chondrocytes.

Additive	Concentration of additive (μg/ml)	Total concentration of $^{35}SO_4^{2-}$ (medium + cells (% of Control)	Proportion of $^{35}SO_4^{2-}$ associated with cells (% of Total incorporation)
Chondroitin sulphate	6×10^3	135.8 ± 19.0	7.0 ± 7.2
	6×10^2	102.5 ± 7.7	6.6 ± 2.3
	2– 3×10^2	137.2 ± 20.1	7.6 ± 0.7
	1– 2×10^2	103.3 ± 6.8	19.7 ± 6.5
	80.0	115.8 ± 5.8	79.0 ± 10.0
	40.0	111.5 ± 9.1	86.0 ± 14.0
	20.0	100 ± 10	39.0 ± 5.10
	10.0	110 ± 1.2	37.7 ± 9.3
Lysozyme (egg white)	17×10^3	215 ± 7.3	20.3 ± 3.2
	2– 6×10^3	166 ± 13.2	28.8 ± 5.1
	1– 2×10^2	155 ± 11.1	24.5 ± 3.3
	10–20	164 ± 2.3	42.8 ± 3.2
	1.0	112 ± 6.1	39.0 ± 3.3
(Cartilage)	1×10^2	102 ± 15.2	37.5 ± 7.2
	50.0	115 ± 12.2	27.1 ± 8.7
	1.0	88 ± 3.3	37.14 ± 6.2
Protamine	14×10^3	83 ± 13	42.6 ± 3.3
	8– 9×10^3	72 ± 18.2	46.7 ± 4.2
	1.3 $\times 10^2$	106 ± 7.3	35.6 + 7.1
	1.0 $\times 10^2$	140 ± 2.0	45.67 ± 13.2
	1.3	117 ± 3.9	45.0 ± 11.2
	1.0	90 ± 16	54.78 ± 3.2
	0.05	87 ± 16	32.39 ± 8.2

After incubation of the entire population of cells for 2 hr, approximately one third of the total radioactivity incorporated into CPC-precipitable material was still associated with the cells in control cultures (table 1). In the presence of higher concentrations of hyaluronic acid this proportion increased to about half. Thus hyaluronic acid, as well as inhibiting total incorporation at higher concentrations, also reduced the proportion of labelled material appearing in the media during the 2 hr of the experiment.

Toole has shown that during chondrogenesis in chick embryos, the hyaluronic acid content of the tissue diminished [7]. Moreover, at concentrations comparable with those effective here (table 1) hyaluronic acid inhibited chondrogenesis [8]. Since hyaluronic acid is present at low concentrations in adult cartilage [5] and is the component which brings about aggregation of proteoglycans [9], hyaluronic acid would appear to have several important biological functions that could not have been deduced from its known chemical properties. The present results and those of Hardingham, et al. [10] indicate that the microenvironment of chondrocytes influences the biosynthesis of matrix constituents.

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