Synthesis of sulphated proteoglycans by rheumatoid and normal synovial tissue in culture

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SUMMARY Synthesis of sulphated proteoglycans by cell lines derived from explants of 7 rheumatoid and 9 normal specimens of synovial tissue, as well as by 7 lines of skin fibroblasts from non-rheumatoid patients, was examined. Cells of all 3 types were cultured as monolayers. They were then disaggregated and their capacity to synthesise proteoglycan estimated in cell suspensions by the incorporation of [³⁵S]-sulphate into CPC-precipitable material during 2 hours of incubation. Cell suspensions incorporated somewhat more [³⁵S]-sulphate than corresponding duplicate monolayers. Synovial cells from rheumatoid patients incorporated 2 to 3 times as much [³⁵S]-sulphate as synovial cells from normals. Skin fibroblasts, however, incorporated less [³⁵S]-sulphate than rheumatoid or normal synovial cells up to the fifth passage. Thereafter their incorporation gradually increased to overtake that of synovial cells. About one-half to one-third of the total [³⁵S]-sulphate labelled material was closely associated with cells from synovial tissues and fibroblasts respectively.

Urine, plasma, and synovial fluid from patients with rheumatoid arthritis have been shown to contain increased levels of chondroitin sulphate when compared with similar fluid obtained from non-rheumatoid sources (Badin *et al.*, 1955; Di Ferrante, 1957; Barker *et al.*, 1966).

Since the supernatants of normal synovial cell cultures appeared to contain little or no sulphated proteoglycans when examined by either critical electrolyte concentration methods or moving boundary electrophoresis (Kling *et al.*, 1955; Fraser *et al.*, 1965; Baxter *et al.*, 1973), it was generally believed that the elevated levels of sulphated proteoglycans in the body fluids of rheumatoid patients did not arise from the synovium but from the breakdown of the matrix of articular cartilage (Badin *et al.*, 1955).

However, with the use of [³⁵S] sulphate the present

studies have shown that both normal and rheumatoid synovial cells maintained in long term culture synthesised proteoglycans. There were significant effects of passage and age of the cultures on proteoglycan synthesis.

Materials and methods

CELL CULTURES

Cell cultures were derived by means of techniques involving explant culture (Marsh *et al.*, 1978) from 7 rheumatoid and 9 normal human synovial tissue specimens (see Table 1 for clinical features). Seven normal synovial specimens were obtained at meniscectomy and 2 were obtained at necropsy. All rheumatoid synovial tissue was obtained at surgical operations. For comparative purposes 7 lines of skin fibroblasts were cultured from skin excised at the time of abdominal surgery from non rheumatoid patients.

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Table 1 Clinical features of donors of synovial tissue

Tissue	Number	Age (years)		Sex		Source		ARA category		RF	
		Mean	Range	M	F	Knee	Other	Class.	Def.	≥ 1:16	0
Rheumatoid synovium	7	62	54-68	1	6	5	2	5	2	6	1
Normal synovium	9	38	19-64	4	5	9	0	-	-	0	9
Skin fibroblasts	7	47	1-66	5	2	0	7	-	-	0	7

The synovial tissue was grossly dissected to remove as much subintimal connective tissue as possible and then cut into small pieces, 1–2 mm³. The small pieces of tissue (explants) were placed in Petri dishes containing nutrient medium (Dulbecco's modification of Eagle's minimum essential medium, Flow Laboratories Scotland) supplemented with ascorbic acid (50 µg/ml), L-glutamine (1 mg/ml), penicillin (50 µg/ml), streptomycin (50 µg/ml), and 30% heat inactivated fetal calf serum. The concentration of the fetal calf serum was gradually reduced to 20% when a confluent monolayer of cells had developed and were ready to be subcultured. Incubation was carried out at 37°C in an atmosphere of 10% CO₂ and room air.

When sufficient numbers of cells had grown out of the explants to form a monolayer, the cell strains were subcultured as follows: cells were disaggregated by incubating at 37°C with 0.125% crystalline trypsin (type I, Sigma Chemical Co., Kingston, Surrey, UK) in 0.004M EDTA for 30 seconds. The trypsin-EDTA solution was removed and the cells incubated for another 30 seconds. The cells were then suspended in fresh medium containing 20% fetal calf serum. This cell suspension was subcultured directly or used in the [³⁵S]-sulphate incorporation studies, after an aliquot was enumerated in a haemocytometer or electronic particle counter (Coulter Counter, Model DN, Coulter Electronics). Each such disaggregation and subculturing procedure was regarded as a passage. Between 10 and 14 days after the first passage, cells had again grown in a confluent monolayer and were then termed 'cell lines.' The cultures studied were grouped into those of the first to the fifth passage inclusive, and those of later passages as previous investigations (Marsh *et al.*, 1978) have shown that cell cultures alter significantly by the sixth passage.

[³⁵S]-SULPHATE INCORPORATION TECHNIQUES
The incorporation of [³⁵S]-sulphate into cetylpyridinium chloride (CPC)-precipitable macromolecules by skin fibroblasts, by normal synovial cells, and by rheumatoid synovial cells was determined under 2 separate conditions: (a) in short-term suspension cultures, and (b) in monolayer cultures, by a modification of the technique described by Wiebkin and Muir (1973).

(a) *Short-term suspension cultures.* Suspension cultures of cells were prepared by disaggregating cells from fully confluent monolayer cultures, as described above. The disaggregated cells were placed in 5 ml of fresh medium containing 20% fetal calf serum and an aliquot taken from enumeration in the Coulter counter. After centrifuging the cell suspension at 80 g for 10 minutes the medium

was removed and replaced by 1.5 ml of Tyrode's balanced salt solution. The cells were washed twice in Tyrode's solution and then resuspended in 1 ml of Tyrode's solution containing 10 µCi of carrier-free [³⁵S]-sulphate (Radiochemical Centre, Amersham, Bucks, UK) and incubated for 2 hours at 37°C in 5% CO₂ and air. After incubation the cells were centrifuged free of their media, at 500 g for 10 minutes, and washed twice in Tyrode's solution containing 0.05M Na₂SO₄. Washings and media were pooled and termed 'supernatants'. To both cells and supernatants 5% CPC was added separately to a final concentration of 1%. The mixtures were stirred vigorously with kieselguhr (Hopkins and Williams, Chadwell Heath, Essex, UK), incubated overnight at 20°C, and then stirred again and centrifuged at 500 g for 10 minutes. The pellets were washed free of non-precipitated sulphate with 1% CPC by resuspending and centrifuging 3 times. Pellets were incubated at 56°C for 30 minutes in 4 volumes of 1M hyamine-hydroxide and radioactivity measured. Radioactivity was expressed as dpm/10⁴ cells for both the cell and supernatant samples. The total incorporation of [³⁵S]-sulphate into CPC-precipitable macromolecules was calculated as follows:

$$\text{Total } [^{35}\text{S}]\text{-sulphate incorporation/10}^4 \text{ cells} = \frac{\text{dpm cell pellet} + \text{dpm supernatant}}{10^4 \text{ cells}}$$

A minimum of 3 determinations were made on each cell line at each passage studied. A retention index (R) was also calculated for each culture studied. This index, describing the proportion of cell-associated macromolecules, was expressed as follows:

$$R = \frac{[^{35}\text{S}]\text{-sulphate in cell pellet}}{\text{Total } [^{35}\text{S}]\text{-sulphate incorporated}} \times 100$$

(b) *Monolayer cultures.* Duplicate monolayer cultures of cells originally derived from skin, normal synovium, and rheumatoid synovium were prepared. When the cultures were fully confluent, 1 culture of each duplicate was taken into suspension, and the incorporation of [³⁵S]-sulphate into CPC-precipitable macromolecules was determined by the method described for the short-term suspension culture. The other duplicate culture was retained in the monolayer state and washed free of medium with 3 washings of Tyrode's solution. Such monolayer cultures were incubated at 37°C in 5% CO₂ and air in 5 ml of Tyrode's solution containing 15 µCi of [³⁵S]-sulphate for 2 hours. The monolayer cultures were then brought into suspension by means of a 'rubber policeman' and then transferred to test-tubes. The entire contents of the culture were centrifuged and media removed and saved as 'supernatant' sample. Cells were then washed twice

in Tyrode's solution containing 0.05M Na₂SO₄. These washings and media were pooled. CPC-precipitation and measurement of radioactivity was performed on both cells and supernatants, as described above. Duplicate suspension cultures (as described above) were used for both incorporation of [³⁵S]-sulphate and for enumeration of cell numbers. The number of cells per monolayer culture was determined by extrapolation from duplicate suspension culture data.

Results

SHORT-TERM SUSPENSION CULTURES

The total incorporation per 10⁴ cells of [³⁵S]-sulphate into CPC-precipitable macromolecules by short-term suspension cultures of skin fibroblasts and normal and rheumatoid synovial cells were grouped into those from the first to the fifth passage inclusive, and those of later passages (Fig. 1). In the

Fig. 1 The incorporation of [³⁵S]-sulphate into CPC-precipitable macromolecules by suspension cultures. Bars show mean $\pm$ 1 SE for each group. $\square$ = Skin fibroblast cultures. Passage 1-5 = mean of 40 cell cultures; passage 6-10 = mean of 44 cell cultures. $\dots$ = Normal synovial tissue cultures. Passage 1-5 = mean of 51 cell cultures; passage 6-10 = mean of 11 cell cultures. ▨ = Rheumatoid synovial tissue cultures. Passage 1-5 = mean of 23 cell cultures. passage 6-10 = mean of 11 cell cultures.

analysis of the data all differences between mean values were subjected to Cochran and Cox's modification of Student's *t* test.

Rheumatoid synovial cell cultures incorporated significantly more [³⁵S]-sulphate into CPC-precipitable material than did normal synovial cell cultures ($P < 0.01$). On the other hand the incorporation by skin fibroblasts was significantly less than that of normal synovial cells during the first 5 passages ($P < 0.05$). After the fifth passage, however, the incorporation by skin fibroblasts increased significantly ($P < 0.01$), and this incorporation was significantly greater than that of normal synovial cells ($P < 0.01$) or rheumatoid synovial cells ($P < 0.05$). However, rheumatoid cultures continued to incorporate significantly more [³⁵S]-sulphate ($P < 0.05$) than did normal synovial cultures during these later passages.

SULPHATE RETENTION INDEX

The proportion of the [³⁵S]-sulphate associated with normal or rheumatoid synovial cells was 40-50% of the total incorporation (Fig. 2). There was no difference in this respect between normal and rheumatoid cells. With skin fibroblasts, however, about 30% of the total incorporation was associated with the cells, and the difference between synovial cells and skin fibroblasts was statistically significant ($P < 0.01$) and was maintained right through to the tenth passage ($P < 0.05$).

MONOLAYER CULTURES

Fig. 3 compares the incorporation of [³⁵S]-sulphate into CPC-precipitable material by the 3 types of cells at the fourth passage in monolayer and short-term suspension cultures. In all experiments suspension cultures incorporated somewhat more [³⁵S]-sulphate than the corresponding monolayer cultures.

Discussion

The incorporation in vitro of [³⁵S] sulphate into CPC-precipitable material reflects the active synthesis of proteoglycans (Gregory and Robbins, 1960). During the CPC-precipitation the effective electrolyte concentration in the supernatants or cell samples was no greater than 0.14M or 0.029M respectively and hence was well below the critical electrolyte concentration above which sulphated glycosaminoglycans are not precipitated (Scott, 1955).

To maximise labelling the cells were incubated in Tyrode's balanced salt solution, which contains no sulphate, and cells were used in suspension as the incorporation of [³⁵S]-sulphate was greater than in monolayers. Total labelled material precipitable by

Fig. 2 The cell retention indices ($100 \times [\text{S}^{35}\text{S}]$ -sulphate incorporation in cell pellet/total $[\text{S}^{35}\text{S}]$ -sulphate incorporation) for sulphated macromolecules synthesised by suspension cultures. Bars show mean ± 1 SE for each group. □ = Skin fibroblast cultures. Passage 1–5 = mean of 40 cell cultures; passage 6–10 = mean of 44 cell cultures. ▤ = Normal synovial tissue cultures. Passage 1–5 = Mean of 51 cell cultures; passage 6–10 = mean of 11 cell cultures. ▨ = Rheumatoid synovial tissue cultures. Passage 1–5 = mean of 23 cell cultures; passage 6–10 = mean of 11 cell cultures.

CPC in both cells and supernatant was estimated and not that in the supernatants alone. About 40–50% of the labelled material was associated with synovial cells (Fig. 2). This has also been observed with chondrocyte cultures (Srivistava *et al.*, 1974; Wiebkin and Muir, 1977).

Stidworthy *et al.* (1973) has reported that glycosaminoglycan synthesis decreased with age both in vitro and in vivo and that the relative amount of nonsulphated to sulphated glycosaminoglycans also increased. The donors of normal synovial tissue were not matched for age, but the individual cell lines did not show any positive correlation between donor age and levels of sulphated proteoglycan synthesis (Fig. 4).

Changes in morphology of synovial cells with each successive passage in culture have recently been

Fig. 3 The incorporation of $[\text{S}^{35}\text{S}]$ -sulphate into CPC-precipitable macromolecules by monolayers and suspension at the fourth passage. Mean of duplicate experiments on individual cell lines are shown. M = monolayer culture, S = suspension culture.

demonstrated. By the sixth passage cells were markedly uniform in size and buoyant density (Marsh *et al.*, 1978), and it is emphasised that in comparative studies cultures should be matched for passage number. When this was done, it was found that up to the fifth passage skin fibroblasts synthesised less proteoglycan than synovial cells and that rheumatoid synovial cells synthesised 2 to 3 times more than normal synovial cells. Although the standard error differences of the means was large, they were nevertheless statistically significant.

The results suggest that the rheumatoid synovial cells may have undergone a metabolic change that was not reversed in culture after many passages. It also appears that, when comparisons were being made, cells cultured for the same length of time and number of passages should be used. As polyanions bind C1q component of complement (Agnello *et al.*, 1969), the production of increased amounts of sulphated polyanion by rheumatoid synovial tissue in vivo might activate complement locally and so potentiate inflammation in the joint.

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Fig. 4 The incorporation of $[^{35}\text{S}]$ -sulphate by 51 normal synovial cell cultures, passage 1-5 (derived from 9 donors) versus age of donors ($r = +0.035$).

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