CALCITONIN AND PARATHYROID-HORMONE STIMULATION OF ACID MUCOPOLYSACCHARIDE SYNTHESIS IN CULTURED CHONDROCYTES ISOLATED FROM GROWTH CARTILAGE

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1. Introduction

During endochondral ossification, proteoglycans and glycosaminoglycans (acid mucopolysaccharides) are known to be important in the transformation of epiphyseal cartilage into bone [1–4]. However, the regulation of glycosaminoglycan synthesis in cartilage and the exact relation of glycosaminoglycan to the process of calcification are unknown.

Previous results in this laboratory indicated the remarkable osteogenic potential of growing cartilage cells in cooperation with certain host cells, when transplanted into rats, even after cultivation of these chondrocytes in vitro [5]. However, resting cartilage cells had no osteogenic activity under similar conditions [5].

This paper reports studies on the effects of calcitonin, parathyroid hormone, and vitamin D on cultured chondrocytes isolated from the rib of young rats. Both calcitonin and parathyroid hormone stimulated the uptake of labeled sulfate into acid mucopolysaccharides synthesized in the growing cartilage-cell system. 1α-Hydroxyvitamin D_3 had no consistent effect on sulfate uptake. However, resting cartilage cells did not respond at all either hormone or vitamin D on sulfate uptake. In the immediate neighborhood of areas of active endochondral calcification, there is a rapid and high concentration of sulfated glycosaminoglycans. The increase in sulfate uptake into glycosaminoglycans in growing cartilage cells may implicate these chondrocytes in calcification and calcitonin and parathyroid hormone may be involved in this mechanism.

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2. Materials and methods

2.1. *Materials*

Powdered Ham's F-12 medium was obtained from Nissui Pharmaceutical Co., Tokyo, fetal calf serum from GIBCO, New York, and plastic dishes (35 mm) from Lux Scientific Corp., USA $Na_2\ ^{35}SO_4$ (19.6 mCi/mmol) was obtained from New England Nuclear Corp., Boston. Bovine calcitonin (73 MRC U/mg, Armour Pharmaceutical Co.) and parathyroid hormone (Beckman, synthetic 1–34 peptide) were generously provided by Mr M. Okazaki (Yamanouchi Pharmaceutical Co., Tokyo) and Dr S. Sakakibara (Protein Research Foundation, Mino, Osaka), respectively. Salmon calcitonin (SMC 20-251, 4000 MRC U/mg) was kindly given by Dr H. Friedli (Sandoz, A. G., Basel). 1α-Hydroxyvitamin D_3 was a gift from Dr Y. Nishii (Chugai Pharmaceutical Co., Tokyo). Pronase was purchased from Kaken Pharmaceutical Co., Tokyo.

2.2. *Cells and cell culture*

The costochondral junction was removed aseptically from the ribs of young male Sprague-Dawley rats, weighing 80–90 g. After removing adhering soft tissue, the growing cartilage (GC) and resting cartilage (RC) were separated and GC- and RC-chondrocytes

were each dissociated, as described previously [5]. The isolated chondrocytes were suspended in Ham's F-12 medium containing 10% fetal calf serum (previously heated at 56°C for 30 min), 50 μg/ml of ascorbic acid, 32 000 mU/ml of penicillin, 40 μg/ml of streptomycin, and 5 μg/ml of fungizone. Volumes of 2 ml of medium containing approximately 2×10^5 cells were placed in 35 mm plastic dishes and incubated for 4–7 days at 37°C in an air–CO_2 atmosphere (95:5).

2.3. *Measurement of mucopolysaccharide synthesis*

Mucopolysaccharide synthesis was monitored by measuring incorporation of $Na_2{}^{35}SO_4$ using the following modification of the procedure of Solursh and Meier for chick embryo chondrocytes [6]. The attached cells were washed three times with $MgSO_4$-free Earle's solution and then pre-incubated with 3 ml of the same buffer for 15 min at 37°C. Then 2 ml of $MgSO_4$-free Earle's solution containing $Na_2{}^{35}SO_4$ (2 μCi/ml) with or without hormone or vitamin were added, and the cultures were incubated for an appropriate period at 37°C in an air–CO_2 atmosphere (95:5). The reaction was stopped by adding 5% trichloroacetic acid containing 1 mM $MgSO_4$ and the cells and insoluble extracellular materials were scraped off the dish with a rubber policeman. This insoluble material was washed five times with 5% trichloroacetic acid, suspended in 0.2 M Tris–HCl buffer, pH 8.0, and digested with 1 mg/ml of pronase for 10 h at 55°C. Then 3 ml of water containing 3 mg of chondroitin sulfate were added. Polysaccharides were precipitated by addition of 0.9 ml of 1% cetylpyridinium chloride and then the mixture was allowed to stand for 1 h at 37°C. The precipitate was washed twice with cold water, dissolved in 10 ml of Insta-Gel emulsifier (Packard) and its radioactivity was determined.

2.4. *Determination of protein*

Protein was measured by the method of Lowry et al. [7] on precipitates. A dish inoculated with approximately 2×10^5 GC-chondrocytes and cultivated for 4 days contained about 200–250 μg of protein. Protein content per dish of RC-chondrocytes was slightly higher than for GC-chondrocytes.

3. Results

Figure 1 is a typical experiment of the time course of $^{35}SO_4{}^{2-}$ uptake into acid mucopolysaccharides synthesized by GC- and RC-chondrocytes and the effects of calcitonin and parathyroid hormone on incorporation of sulfate. Addition of calcitonin (10 mU/ml incubation medium) to GC-chondrocyte cultures significantly increased $^{35}SO_4{}^{2-}$ uptake by these cells: the uptake was 30% more after 12 h, and 50% more after 24 h than that in control cultures. Bovine and salmon calcitonin had similar stimulatory effects. Parathyroid hormone (0.1 μg/ml incubation medium) had a marked stimulatory effect on the incorporation of labeled sulfate in this system.

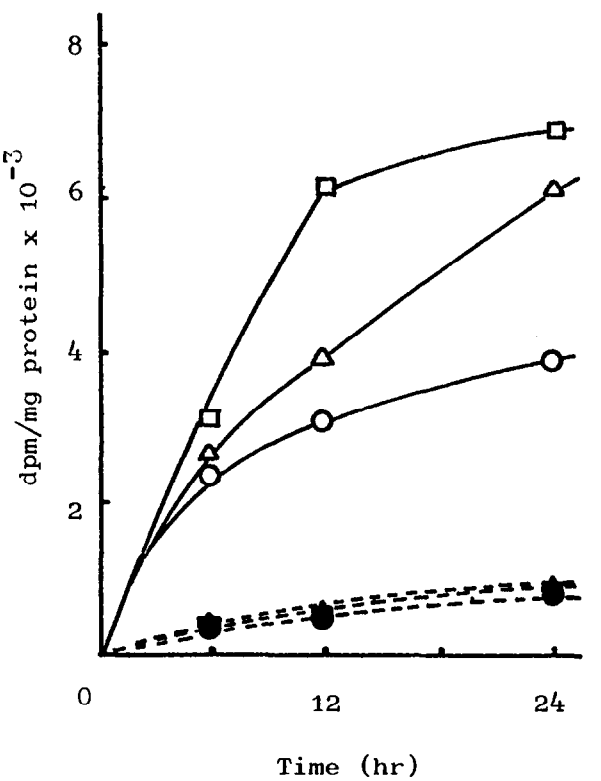

Fig.1. Effects of calcitonin and parathyroid hormone on $^{35}SO_4{}^{2-}$ uptake into mucopolysaccharides. GC-chondrocytes, open symbols, solid lines; RC-chondrocytes, solid symbols, dotted lines. Circles, control; triangles, calcitonin (10 mU/ml); squares, parathyroid hormone (0.1 μg/ml). Both chondrocytes were cultivated for 7 days. Values are the average of two cultures.

Table 1
Effect of calcitonin on $^{35}SO_4^{2-}$ uptake into mucopolysaccharides (GC-chondrocytes[a])

Calcitonin (mU/ml)	$^{35}SO_4^{2-}$ uptake[b,c] (dpm/mg protein)	% of control
0	3906	100
1	4745	121
10	6065	155
100	6588	169

[a] 6 day cultivation.
[b] 24 h incubation at 37°C.
[c] Average of two cultures.

Reproducible stimulatory effects by these two hormones were obtained in different batches of chondrocytes. By contrast, uptake of radioactivity by RC-chondrocytes was found to be only one-quarter to one-third as much as that by GC-chondrocytes after incubation for 24 h under the same conditions (fig.1). Moreover, the $^{35}SO_4^{2-}$uptake of the cultured RC-chondrocytes was not affected by either calcitonin or parathyroid hormone. The morphological characteristics of both chondrocytes used in uptake studies were found to be well preserved after 4–7 days' incubation plus interruption and washing with $MgSO_4$-free medium plus re-incubation for 24 h.

In the GC-chondrocyte system, the effects of both calcitonin and parathyroid hormone on acid mucopolysaccharide labeling were dose-dependent, as shown in tables 1 and 2.

1α-Hydroxyvitamin D_3 (0.2 μg/ml incubation medium) had no consistent effect on sulfate uptake by either type of chondrocyte (fig.2).

Table 2
Effect of parathyroid hormone on $^{35}SO_4^{2-}$ uptake into mucopolysaccharides (GC-chondrocytes[a])

Parathyroid hormone (μg/ml)	$^{35}SO_4^{2-}$ uptake[b,c] (dpm/mg protein)	% of control
0	3250	100
0.01	4930	152
0.1	5388	166
1	11 873	365

[a] 4-day cultivation.
[b] 24 h incubation at 37°C.
[c] Average of two cultures.

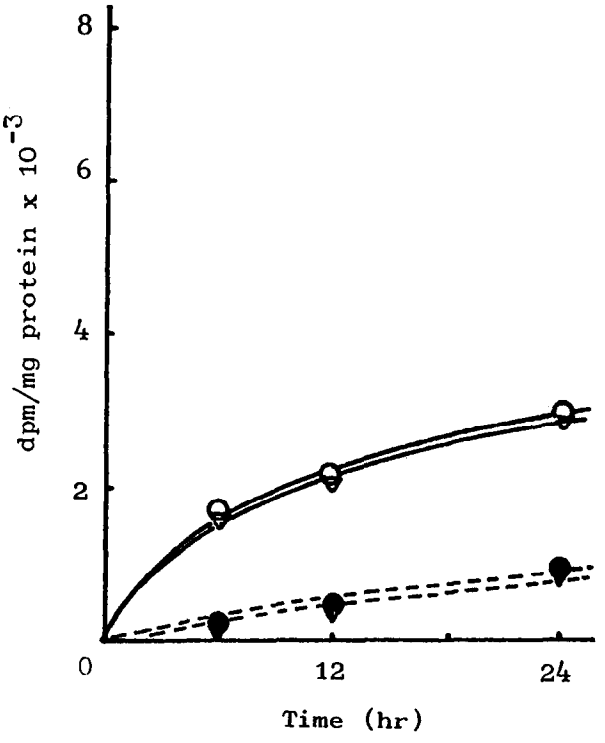

Fig.2. Effect of 1α-hydroxyvitamin D_3 on $^{35}SO_4^{2-}$ uptake into mucopolysaccharides. GC-chondrocytes, open symbols, solid lines; RC-chondrocytes, solid symbols, dotted lines. Circles, control; triangles, 1α-hydroxyvitamin D_3 (0.2 μg/ml). Both chondrocytes were cultivated for 4 days. Values are the average of two cultures.

4. Discussion

As reported previously from this laboratory, when grown in Ham's F-12 medium supplemented with 10% fetal calf serum (heated at 56°C for 30 min) for 4–7 days in vitro, GC-chondrocytes are polygonal and epithelial-like [5]. They exhibit the properties of well-differentiated cartilage cells, forming a refractile matrix and staining metachromatically with toluidine blue [5]. On the other hand, under the same conditions RC-chondrocytes are spindle-shaped like fibroblasts and they show less intense metachromasia than GC-chondrocytes [5]. Moreover, when transplanted into the peritoneal cavity of rats, GC-chondrocytes have a remarkable osteogenic potential in cooperation with certain host cells, whereas RC-chondrocytes show no osteogenic activity [5].

The present results indicated that GC-chondrocytes synthesize glycosaminoglycans more actively than RC-chondrocytes, quantitatively confirming our previous observations on the difference in metachromasia of the two types of cultured chondrocytes. It is very interesting that both calcitonin and parathyroid hormone, which regulate calcium metabolism in bone tissue or cells in opposite ways, stimulated glycosaminoglycan synthesis in GC-chondrocytes. However, neither hormone affected sulfate uptake by RC-chondrocytes. As reported previously from this laboratory, calcium uptake by cultured GC-chondrocytes, as measured by ^{45}Ca incorporation, was stimulated by calcitonin [8]. Parathyroid hormone or 1α-hydroxyvitamin D_3, however, strikingly decreased calcium uptake [8]. On the contrary, calcium uptake by cultured RC-chondrocytes was only slightly stimulated by calcitonin, but no effect occurred with parathyroid hormone or 1α-hydroxyvitamin D_3 [8]. The stimulatory effect of calcitonin on acid mucopolysaccharide synthesis in GC-chondrocytes was also observed in metachromatic staining of the cultured cell system with toluidine blue (unpublished). The details of the characteristics of these cells will be published in another paper.

Kawashima et al. reported that growth of femur tissue isolated from chick embryos is stimulated by parathyroid hormone in vitro [9]. Bone hyaluronate synthesis in organ culture is also stimulated by parathyroid hormone [10]. Calcitonin has a stimulatory effect on the incorporation of labeled glucose into the glycosaminoglycans synthesized in calf-embryo bone cells [11]. However, little is known about the exact effects of calcitonin and parathyroid hormone on the synthesis of acid mucopolysaccharides, which are macromolecular components of the epiphyseal cartilage matrix with important roles in the early stages of calcification. There remains to be investigated in more detail the relationship between calcium uptake and/or release and glycasaminoglycan synthesis. The implication of cyclic AMP in calcitonin and parathyroid hormone action in glycosaminoglycan synthesis is also unknown. 1α-Hydroxyvitamin D_3 has no effect on acid mucopolysaccharide synthesis, although it has a marked effect on calcium metabolism in GC-chondrocytes [8].

The results indicate that GC- and RC-chondrocytes seem to have striking differences in morphology, osteogenic potentiality, activity for glycosaminoglycan synthesis, and responsiveness to calcitonin and parathyroid hormone. These findings suggest that GC-chondrocytes have important roles in bone formation and that there is a close relationship between growth cartilage cells and bone cells.

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