Effect of multiplication-stimulating activity (MSA) on the cyclic AMP level and proteoglycan synthesis in cultured chondrocytes

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Abstract. Multiplication-stimulating activity (MSA), a somatomedin purified from conditioned medium of Buffalo rat liver cells, had little effect on the intracellular level of cyclic AMP when it markedly enhanced the synthesis of sulphated glycosaminoglycans in rabbit chondrocytes in culture. In addition, MSA did not inhibit prostaglandin E$_1$- or parathyroid hormone-induced accumulation of cyclic AMP in the chondrocytes. On the contrary, MSA slightly decreased stimulation of cyclic AMP accumulation by prostaglandin in human fibroblasts. Dibutyryl cyclic AMP and MSA increased the incorporation of $^{35}$Ssulphate and $^3$Hserine into proteoglycans synthesized by rabbit chondrocytes, and their effects were additive. These findings suggest that somatomedin and dibutyryl cyclic AMP enhance sulphate proteoglycan synthesis through different mechanisms. The lack of inhibitory effect of MSA on cyclic AMP accumulation may be favourable for producing additive effects with cyclic AMP on proteoglycan synthesis and DNA synthesis in chondrocytes.

Somatomedin seems to play a basic role in the control of cartilage growth and metabolism (Hall et al. 1975). We showed that multiplication-stimulating activity (MSA), a family of polypeptides purified from conditioned medium of a rat liver cell line, stimulates the synthesis of sulphated proteoglycans by a specific mechanism when it enhances cell division of chondrocytes (Kato et al. 1981, 1982). Rechler et al. (1978) showed that MSA binds to specific plasma membrane receptors in various cells, suggesting that the initial event in the action of somatomedin is an alteration in membrane function or in production of an intracellular second messenger.

Tell et al. (1973) reported that high concentrations of partially purified somatomedin C inhibit adenylate cyclase activity in crude membranes from a variety of cells. They also showed that somatomedin inhibits adenylate cyclase activity stimulated by epinephrine, prostaglandin E$_1$ (PGE$_1$) and parathyroid hormone. Anderson et al. (1979) showed that MSA has little effect on the basal intracellular pool of cyclic AMP but inhibits increase in cyclic AMP levels induced by PGE$_1$ in chick embryo fibroblasts. These findings suggest that the decrease in cyclic AMP level may be related to the action of somatomedin on macromolecule synthesis.

Cyclic AMP analogues have been shown to be potent stimulators of proteoglycan synthesis (Miller et al. 1979; Takigawa et al. 1981), RNA synthesis (Drezner & Lebovitz 1979) and DNA synthesis (Bomboy & Salmon 1980) in chondrocytes. Takigawa et al. (1981) showed that parathyroid hormone (PTH) increases ornithine decarboxylase activity and proteoglycan through increase of cyclic AMP. Therefore, it would be contrary to the findings by Takigawa et al. (1982) if somatomedin, a
potent stimulator of cartilage growth and metabolism, were to inhibit basal or hormone-induced cyclic AMP accumulation in chondrocytes. To clarify this problem, we examined whether MSA inhibits basal, PGE
subscript{1} or PTH-induced cyclic AMP accumulation in chondrocytes and fibroblasts. In addition, we examined whether MSA and dibutyryl cyclic AMP ([Bu
subscript{2}c-AMP] have additive effects on the synthesis of sulphated proteoglycans in rabbit costal chondrocytes.

Materials and Methods

Cell culture
The costochondral junction was removed aseptically from the ribs of young New Zealand rabbits (300–500 g). Chondrocytes were dissociated from the growth cartilage as described in the previous paper (Shimomura et al. 1975). The isolated chondrocytes were suspended in Eagle’s minimum essential medium (MEM; Nissui Pharmaceutical Co., Tokyo) containing 10% foetal calf serum (FCS; GIBCO, USA). Volumes of 1 ml of the medium containing 4 x 10⁶ cells were placed in plastic microwells (16 mm multiwell plate, Linbro Scientific Inc., USA) and incubated at 37°C under 5% CO₂ in air.

Fibroblasts were obtained from the skin of a 3-month-old child and used at the 4th to 10th passage.

Preparation of MSA
MSA was purified from serum-free medium conditioned by cloned Buffalo rat liver cells (Kato et al. 1981). In the present study, we used this MSA preparation or a pure MSA peptide purchased from Collaborative Research Inc., USA. The two MSA preparations produced the same results.

Assay of proteoglycan and protein syntheses
Proteoglycan synthesis was monitored by measuring the incorporation of H₂¹⁵SO₄ or [³H]serine into material precipitated with cetylpyridinium chloride after treatment with Pronase E (Kaken Kagaku Co., Tokyo) as described previously (Kato et al. 1981). Protein synthesis was determined by measuring incorporation of [³H]serine into the trichloroacetic acid-insoluble fraction of cells (Kato et al. 1981).

Assay of cyclic AMP
When chondrocytes became confluent, they were preincubated for 24 h in 1 ml of Dulbecco’s modified Eagle’s medium (DMEM). The medium was then replaced by 0.4 ml of fresh medium and after incubation for 60 min MSA (1 µg/ml), parathyroid hormone (2.2 IU/ml) or PGE
subscript{1} (10 µg/ml) was added. We confirmed that the medium change did not affect the level of cyclic AMP. After incubation with hormones, the medium was removed and mixed with 0.4 ml of cold trichloroacetic acid. Simultaneously, chondrocytes were overlayed with 1 ml of cold 5% trichloroacetic acid. The cells were collected with a rubber policeman and the dishes were washed with 1 ml of the same acid. The cells and the washing solution were combined, sonicated at 20 KHz for 60 s, and centrifuged at 1600 x g for 10 min at 4°C. The mixture containing medium was also centrifuged to remove protein. Trichloroacetic acid in the supernatant was removed by extraction three times with 3 volumes of ethylether saturated with distilled water, and then the remaining solution was used for cyclic AMP assay. The precipitate from the cell fraction was dissolved in 1 N NaOH and used for protein determination. Protein was determined by the method of Lowry et al. (1951). More than 90% of the radioactive cyclic AMP added to cultures with trichloroacetic acid was recovered by this procedure. Cyclic AMP was determined by the method of Honma et al. (1977) using a Yamasa cyclic AMP assay kit. Alternatively, after incubation, the medium was removed and the cells were promptly treated with 0.8 ml of cold trichloroacetic acid (5%; W/V) for 15 min at 0°C to extract cyclic AMP. The two procedures gave the same results.

Results and Discussion
As reported previously, 0.2–1 µg/ml of MSA markedly increases proteoglycan synthesis in rabbit costal chondrocytes in culture (Kato et al. 1981). However, MSA at 1 µg/ml had essentially no effect on the cyclic AMP level in the chondrocytes during the whole experimental period (Fig. 1). Takigawa et al. (1981) showed that PTH greatly increased the level of cyclic AMP in rabbit chondrocytes. To study the effect of MSA on PTH-stimulated cyclic AMP accumulation in chondrocytes, we added MSA and PTH simultaneously to the culture medium and measured the intra- and extracellular cyclic AMP concentrations (Fig. 1). MSA had little effect on PTH-stimulated cyclic AMP accumulation during the whole experimental period. The results were highly reproducible at the doses of MSA (0.01–10 µg/ml, results not shown) and times examined.

PGE
subscript{1} has been shown to stimulate cyclic AMP accumulation in chick embryo fibroblasts (Anderson et al. 1979). Similarly, PGE
subscript{1} markedly increased the intracellular concentration of cyclic AMP in cultured rabbit chondrocytes (Fig. 2). Its effect was rapid, being evident within 5 min and reaching a
maximum in 5 to 20 min. The time course of PGE₁ stimulation of cyclic AMP accumulation was quite different from that of PTH stimulation. However, MSA had little effect on PGE₁ stimulation of cyclic AMP accumulation in chondrocytes, as in the case with PTH. Anderson et al. (1979) showed that MSA markedly depresses the PGE₁-induced increase in the cyclic AMP level in chick embryo fibroblasts. We also found that MSA decreased PGE₁-induced accumulation of cyclic AMP in human fibroblasts at 20 and 30 min, although it had less inhibitory effect than in chick embryo fibroblasts (Fig. 3). These findings suggest that with respect to cyclic AMP metabolism, the response of chondrocytes to MSA differs from that of fibroblasts.

Effects of MSA and PGE₁ on the intracellular cyclic AMP level in rabbit chondrocytes. After preincubation, the medium was replaced by 0.4 ml of serum-free medium 60 min before addition of PGE₁ (10 µg/ml) or MSA (1 µg/ml). PGE₁ and MSA were added to the cultures and cells were harvested at the times shown. Intracellular cyclic AMP was measured by radioimmunoassay. Points are averages ± SD for 5 dishes.

Recently, Bomboy & Salmon (1980) showed that analogues of cyclic AMP and cyclic nucleotide phosphodiesterase inhibitor markedly potentiate the effect of somatomedin on DNA synthesis in cultured cartilage from hypophysectomized rats. Analogues of cyclic AMP stimulate DNA synthesis in chondrocytes in organ culture (Bomboy & Salmon 1980), but they suppress DNA synthesis in monolayer cultures (Miller et al. 1979; Takigawa et al. 1981). In the present study, we examined the effects of MSA, [Bu]₂c-AMP and both on the synthesis of sulfated proteoglycans by cultured chondrocytes. Addition of MSA to rabbit chondrocyte cultures increased [³⁵S]sulfate uptake by these cells: the uptake was 30% more after 7 h and 110% more after 14 h than that in control cultures (Fig. 4). Addition of [Bu]₂c-AMP also increased the uptake: the uptake was 60% more after 7 h and 100% more after 14 h. When MSA and [Bu]₂c-AMP were added simultaneously, the uptake was 190% more after 7 h and 170% more after 14 h.
suggest that somatomedin and long acting cyclic AMP analogues enhance the synthesis of sulphated proteoglycans in chondrocytes through different mechanisms.

Cyclic AMP is a common second messenger for the actions of several protein hormones. In addition, it has been implicated in regulation of cell growth in a number of systems (Friedman 1976). However, Richman et al. (1980) showed that the action of MSA on L6 myoblast growth is not correlated with change in cyclic nucleotides. In the

(Fig. 4). Therefore, MSA and [Bu]2c-AMP had additive effects in stimulating sulphated glycosaminoglycan synthesis in rabbit chondrocytes. In another experiment, we examined the effects of MSA, [Bu]2c-AMP and both on the incorporation of [3H]serine into total protein and proteoglycans in rabbit chondrocytes (Table 1). Rabbit chondrocytes were incubated for 7 h in the presence of [3H]serine. As in the case of the experiment with [35S]sulphate, MSA and [Bu]2c-AMP had additive effects on the incorporation of [3H]serine into proteoglycans in the medium and cell fractions. Although MSA increased the incorporation of [3H]serine into total protein, the extent of stimulation was less than that of stimulation of [3H]serine uptake into proteoglycans (Table 1). [Bu]2c-AMP did not increase the incorporation of [3H]serine into total protein, but stimulated [3H]serine uptake into proteoglycans (Table 1). These findings sug-

Effects of MSA and cyclic AMP on the synthesis of sulphated proteoglycans in cultured chondrocytes. When chondrocytes became subconfluent, cultures were started in 0.4 ml of MEM containing 1 μCi of H2[35S]O4, MSA (1 μg/ml) and [Bu]2c-AMP (1 mM) and incubated for the indicated hours. The values of control cultures at 7 h and 14 h were 4762 DPM/dish and 14 233 DPM/dish, respectively. Points are averages ± SD for 5 dishes. a: significance of difference from saline control (P < 0.01) b: significance of difference from MSA (P < 0.05) c: significance of difference from both MSA + [Bu]2c-AMP (P < 0.05). Data were analyzed by the unpaired Student's t-test.

![Fig. 3](image-url)

**Fig. 3.** Effects of PGE1 and MSA on the intracellular cyclic AMP level in human fibroblasts. When fibroblasts became subconfluent, they were preincubated for 24 h in serum-free MEM. The medium was renewed 60 min before addition of hormones. PGE1 (10 μg/ml) and MSA (1 μg/ml) were added to the cultures. After incubation, intracellular cyclic AMP was extracted at the times shown and its level in the cell layer was measured by radioimmunoassay. Points are averages ± SD for 5 dishes.
Effects of MSA, [Buβc-AMP and both on [3H]serine incorporation into total cellular protein and proteoglycans synthesized by rabbit chondrocytes. When rabbit chondrocytes became confluent, they were preincubated for 24 h in 0.4 ml of DMEM. Then the cells were further incubated from 7 h in 0.4 ml of the same fresh medium containing 5 µCi of [3H]serine in the presence or absence of MSA (1 µg/ml), [Buβc-AMP (1 mM) or both. The incorporation of [3H]serine into proteoglycans in the medium and cell fraction were measured separately. Values are averages ± SD for 4 dishes.

<table>
<thead>
<tr>
<th>Addition</th>
<th>[3H]serine incorporation (dpm/dish)</th>
<th>Proteoglycan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total protein</td>
<td>Medium</td>
</tr>
<tr>
<td>None</td>
<td>587 ± 180 ± 779</td>
<td>3626 ± 337</td>
</tr>
<tr>
<td>[Buβc-AMP</td>
<td>591 ± 090 ± 4725</td>
<td>4737 ± 263</td>
</tr>
<tr>
<td>MSA</td>
<td>670 ± 990 ± 3543</td>
<td>4702 ± 360</td>
</tr>
<tr>
<td>[Buβc-AMP + MSA</td>
<td>722 ± 320 ± 1389</td>
<td>5833 ± 434</td>
</tr>
</tbody>
</table>

Acknowledgments

This work was supported in part by grants-in-aid from the Ministry of Education, Science and Culture, and grants from the Yamada Science Foundation, Naito Foundation, and the Foundation of Growth Science. We wish to thank Drs. K. Igarashi and Y. Sugino (Takeda Pharmaceutical Co., Osaka) for cloning Buffalo rat liver cells, Mrs. Elizabeth Ichihara for assistance in the preparation of this manuscript, and Miss Sayuri Kitagawa for valuable secretarial help.

References


Received on November 15th, 1982.