Effects of Adenosine 3':5'-Cyclic Monophosphate and Serum on Synthesis of Hyaluronic Acid in Confluent Rat Fibroblasts

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A small amount of hyaluronic acid is synthesized in confluent cultures of rat fibroblasts, which have a high content of cyclic AMP. Addition of calf serum caused a rapid decrease in the cellular cyclic AMP content and large increases in hyaluronic acid synthetase activity and hyaluronic acid production. Addition of cyclic AMP also caused a marked increase in hyaluronic acid synthetase activity within 2h and then increased hyaluronic acid production. The effects of cyclic AMP and serum on hyaluronic acid synthesis were additive. Prostaglandin E₂, which increased the cyclic AMP by stimulating adenylate cyclase, was as effective as cyclic AMP in increasing hyaluronic acid synthetase activity, but AMP was far less effective than cyclic AMP. These results indicate that cyclic AMP itself stimulates the mucopolysaccharide synthesis and that the effect of serum is not due to a decrease in cyclic AMP in the cells.

Hyaluronic acid is usually synthesized only during the exponential growth phase in cultured cells capable of its synthesis (Koyama & Ono, 1970; Tomida *et al.*, 1974). We showed previously that this was explained partly because the syntheses of DNA and hyaluronic acid in cultured cells were both dependent on factors in the calf serum added to the medium. In fact, readdition of serum to confluent cultures of an established cell line SEN (fiber in Japanese), in which serum factors had probably been exhausted or inactivated, induced DNA synthesis and hyaluronic acid synthase activity directly involved in hyaluronic acid synthesis (Tomida *et al.*, 1975).

It has been reported that addition of serum to a variety of quiescent cells results in a rapid decrease in the cellular cyclic AMP concentration (Burger *et al.*, 1972; Otten *et al.*, 1972; Oey *et al.*, 1974) and in a variety of metabolic changes, such as increased uptake of metabolites, increased protein and RNA synthesis and the onset of DNA synthesis and cell division. Addition of N⁶O²-dibutyryl cyclic AMP, a derivative of cyclic AMP, has been shown to inhibit these effects of serum. Therefore it has been suggested that the effects of serum are triggered off by a decrease in the cyclic AMP concentration inside the cells.

We have found that cyclic AMP or N⁶O²-dibutyryl cyclic AMP enhances the production of hyaluronic acid by SEN cells during the exponential growth

phase (Koyama *et al.*, 1976). In the present work, we studied the effect of cyclic AMP on confluent cultures of SEN cells, with special reference to the relation between the effects of serum and cyclic AMP.

Materials and Methods

Cells and culture methods

The rat fibroblast cell line SEN (Koyama *et al.*, 1976) was maintained in 60mm Petri dishes containing modified Eagle's minimal essential medium (Yamane *et al.*, 1968; commercial product of Nissui Seiyaku Co., Tokyo, Japan), supplemented with 10% (v/v) calf serum and 0.1% (w/v) Bacto-Peptone (Difco, Detroit, MI, U.S.A.), at 37°C under CO₂/O₂ (1:19). Confluent cultures were prepared by growing the cells up to confluency in minimal essential medium with 2.5% calf serum and 0.1% Bacto-Peptone for 6 or 7 days without removal of the medium.

Analysis of acid mucopolysaccharides

Our previous studies (Koyama *et al.*, 1976) have demonstrated electrophoretically that SEN cells can produce three kinds of mucopolysaccharides: hyaluronic acid, chondroitin 4-sulphate and heparan sulphate. In the present work, synthesis of mucopolysaccharides was monitored by measuring incorporation of [³H]glucosamine. Cells in two Petri dishes were labelled with 1μCi of [³H]-glucosamine (2.41 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.)/ml for 12h. The medium and the cells were then used for extraction of mucopolysaccharides, as described previously (Koyama *et al.*,

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1976). The purified samples and authentic hyaluronic acid (Boehringer Mannheim, Tokyo, Japan) and chondroitin 4-sulphate (Seikagaku Kogyo, Tokyo, Japan) were subjected to electrophoresis on cellulose acetate strips (Sartorius, Göttingen, West Germany) in pyridinium acetate buffer (5ml of pyridine and 50ml of acetic acid made up to 1 litre with water, pH 3.5) at a constant current of 0.5mA per cm for 50min (Seno & Meyer, 1963). The strips were stained with 0.5% (w/v) Alcian Blue 8GX (Searle, High Wycombe, Bucks., U.K.) in 3% (v/v) acetic acid and decolorized in 1% acetic acid. The strips were dried and cut into 2.5mm lengths, and the radioactivity of each piece was counted in 5ml of scintillation fluid [4g of 2,5-diphenyloxazole and 0.3g of 1,4-bis-(5-phenyloxazol-2-yl)benzene per litre in toluene] in a Beckman LS-250 type liquid-scintillation counter.

Measurement of glucosamine uptake

The cells, attached to 60mm dishes, were washed three times with warm minimal essential medium or glucose-free Hanks balanced salt solution (Hanks, 1948) and incubated for 10min at 37°C in 1ml of minimal essential medium or glucose-free Hanks solution containing 2.5 μ Ci of [3 H]glucosamine (2.41 Ci/mmol). The uptake of the sugar was linear with time for at least 20min. The radioactive solution was removed; cells were washed three times with 10ml of ice-cold Hanks solution and then dried. The dried cells were dissolved in 1ml of Soluen TM-100 (Packard Instrument Co., Zurich, Switzerland), neutralized by addition of 50 μ l of acetic acid, taken up in 10ml of toluene scintillation fluid and counted for radioactivity in a liquid-scintillation counter.

Cyclic AMP assay

Cyclic AMP was extracted and purified by the procedure of Maganiello & Vaughan (1972). It was measured by using a Cyclic AMP Assay Kit (The Radiochemical Centre, Amersham, Bucks., U.K.) by the method of Gilman (1970). The specificity of the cyclic AMP-binding assay was controlled by phosphodiesterase digestion.

Assay of hyaluronic acid synthetase activity

The synthetase was assayed as described in our previous papers (Tomida *et al.*, 1974, 1975). Its specific activity was expressed as pmol of [14 C]-glucuronic acid (290mCi/mmol; The Radiochemical Centre) incorporated into hyaluronic acid/h per mg of protein. The protein content was determined by the method of Lowry *et al.* (1951), with bovine albumin as standard.

Chemicals

Cyclic AMP, ADP, adenosine and theophylline were obtained from Sigma Chemical Co., St. Louis,

MO, U.S.A. AMP, ATP, UTP, and 8-bromo cyclic AMP were obtained from Boehringer Mannheim Yamanouchi, Tokyo, Japan. Prostaglandin E₂ was purchased from Ono Pharmaceutical Co., Osaka, Japan, GMP from Nutritional Biochemical Co., Cleveland, OH, U.S.A., UMP from Wako Pure Chemical Industries, Osaka, Japan, and adenine from The Daiichi Pure Chemical Co., Tokyo, Japan.

Results

Effects of cyclic AMP and serum on synthesis of acid mucopolysaccharides

Cyclic AMP and calf serum, at concentrations of 3mM and 20% (v/v) respectively, were added directly to confluent SEN cells in depleted medium. The cells were labelled with [3 H]glucosamine for 12h, and then acid mucopolysaccharides were extracted from the medium and the washed cells and analysed by electrophoresis. Acid mucopolysaccharides synthesized by the cells were rapidly secreted into the medium, so only results obtained with samples of medium are shown in Fig. 1. Identification of the mucopolysaccharides was reported previously (Koyama *et al.*, 1976). Untreated confluent cells incorporated a small amount of [3 H]-glucosamine into hyaluronic acid and a large amount of the labelled sugar into chondroitin 4-sulphate (Fig. 1a). Addition of serum to these cells led to a large increase in the incorporation of the sugar into hyaluronic acid alone (Fig. 1c). This effect of serum was similar whether the serum was added to cultures in depleted medium or in fresh one. In later experiments, serum was added at the same time as the medium was changed. Addition of cyclic AMP had a similar effect to that of serum, although it caused less increase in the incorporation into hyaluronic acid (Fig. 1b).

We studied the effects of serum and cyclic AMP on sugar uptake into the cells (Table 1). [3 H]Glucosamine was efficiently transported in spite of the presence of glucose. The rate of sugar uptake was decreased by replacing medium with minimal essential medium alone. Addition of either cyclic AMP or serum, or both, all caused an increase in the sugar uptake. Therefore the increase in incorporation of labelled sugar into acid mucopolysaccharides (Fig. 1) could be due in part to enhanced uptake of [3 H]-glucosamine by increasing intracellular pools of the labelled sugar. However, the degree and specificity of increase in [3 H]glucosamine incorporation, which is shown in Fig. 1, indicate that synthesis of hyaluronic acid is in fact stimulated by treatment with either serum or cyclic AMP. This effect of cyclic AMP is consistent with the results obtained by chemical determination of mucopolysaccharides in growing SEN cells (Koyama *et al.*, 1976).

Fig. 1. Comparison of effects of cyclic AMP and serum on acid mucopolysaccharide synthesis in confluent cultures
Cyclic AMP and calf serum were added to confluent SEN cells to final concentrations of 3 mM and 20% (v/v) respectively, without changing the medium. Treated cultures and untreated cultures were labelled with [³H]glucosamine for 12h. Acid mucopolysaccharides were extracted from 10ml of the media and subjected to electrophoresis in pyridinium acetate buffer. (a) —Cyclic AMP, —serum; (b) +cyclic AMP, —serum; (c) —cyclic AMP, +serum. The positions of the standards hyaluronic acid (HA) and chondroitin 4-sulphate (CS) are indicated at the top of the Figure.

Table 1. Rate of glucosamine uptake in confluent cultures
Cyclic AMP was added to confluent SEN cells to 3 mM, or the medium was changed with fresh Eagle's minimal essential medium containing either 3 mM-cyclic AMP or 20% calf serum, or both. Then, 4h later, the rate of [³H]glucosamine uptake was measured in 1 ml of Eagle's medium, which contained 5.6 mM-glucose, or in 1 ml of glucose-free Hanks balanced salt solution. Data are expressed as c.p.m. of [³H]glucosamine incorporated/10 min per 10⁶ cells.

Experimental conditions	Incorporation	
	+Glucose	—Glucose
1. No medium change		
No addition	1154	1983
Addition of cyclic AMP	1326	2832
2. Medium change with:		
Eagle's medium alone	641	1131
Eagle's medium+cyclic AMP	1025	1495
Eagle's medium+serum	1904	4603
Eagle's medium+cyclic AMP+serum	2095	5666

Fig. 2. Cyclic AMP concentration in confluent cultures treated with serum
At zero time the medium of confluent SEN cells was replaced by fresh Eagle's minimal essential medium containing 20% calf serum. Then cyclic AMP was extracted at the times indicated and its concentration (pmol/mg of acid-insoluble protein) was assayed.

(Fig. 2). The cyclic AMP decreased to one-third of the initial value within 10min and remained at this low value throughout the observation period of 22h. This result differs from that of Burger *et al.* (1972), who observed a transient fall in the cyclic AMP concentration in 3T3 cells, followed by rapid return to the initial high value. However, our results are similar to those of Oey *et al.* (1974) on 3T3 cells.

Comparison of the effect of cyclic AMP on hyaluronic acid synthetase activity with those of other nucleotides and reagents

Previously we observed production of hyaluronic acid by B-6 cells, a clone of a hybrid between mouse

Cellular cyclic AMP after addition of serum

We measured the concentration of cyclic AMP in confluent SEN cells after treatment with serum

Fig. 3. Induction of hyaluronic acid synthetase activity by cyclic AMP in confluent cultures

Cyclic AMP was added to confluent SEN cells to a final concentration of 3 mM and cells were harvested at the times indicated, and hyaluronic acid synthetase activity was measured.

Table 2. Effect of actinomycin D and cycloheximide on the induction of hyaluronic acid synthetase activity by cyclic AMP in confluent cultures

Cyclic AMP with or without an inhibitor of RNA or protein synthesis was added to confluent SEN cells, and 3 h later the cultures were assayed for hyaluronic acid synthetase activity. The enzyme activity is expressed as pmol of [14 C]glucuronic acid incorporated into hyaluronic acid/h per mg of protein.

Additions	Final concentration (M)	Hyaluronic acid synthetase activity
None	—	2.66
Cyclic AMP	3×10^{-3}	21.0
Cyclic AMP+	3×10^{-3}	1.48
actinomycin D	4×10^{-6}	
Cyclic AMP+	3×10^{-3}	0.76
cycloheximide	3.6×10^{-5}	

mammary carcinoma cells and Chinese-hamster cells, and their two parental lines (Koyama & Ono, 1970). We found that its production was proportional to the activity of hyaluronic acid synthetase in a cell-free assay system (Tomida *et al.*, 1974; Ono *et al.*, 1974). Assay of this enzyme is simpler and more sensitive than measurement of [3 H]glucosamine incorporation into hyaluronic acid. We examined the effect of cyclic AMP on hyaluronic acid synthetase activity in SEN cells. Confluent cells had a very low activity at zero time (Fig. 3). After addition of cyclic AMP, the enzyme activity was rapidly induced without any apparent lag time, reached a maximum in 2–4 h, and then returned to the basal value after 12 h. The effect of cyclic AMP was concentration-dependent, being optimal at a concentration of 3 mM. This increase in

Table 3. Effects of various substances on hyaluronic acid synthetase activity in confluent cultures

The medium of confluent SEN cells was replaced by fresh Eagle's minimal essential medium containing 0.4% calf serum and the substances indicated, and 3 h later the cultures were assayed for hyaluronic acid synthetase activity. Values are averaged from two or three independent experiments and expressed relative to the value in the cells grown in Eagle's medium and 0.4% calf serum. Results are means $\pm$ S.E.M.

Additions	Hyaluronic acid synthetase activity
Eagle's medium alone	0.96 ± 0.04
Eagle's medium+0.4% calf serum	1.00
Calf serum (10%)	7.99 ± 0.63
Prostaglandin E ₂ (10 μ g/ml)	7.39 ± 0.31
Theophylline (1 mM)	2.22 ± 0.49
Adenine (0.1 mM)	1.51 ± 0.63
Adenine (3 mM)	1.00 ± 0.20
Adenosine (0.1 mM)	1.59 ± 0.55
Adenosine (3 mM)	0.67 ± 0.16
UMP (0.1 mM)	1.31 ± 0.31
GMP (0.1 mM)	1.20 ± 0.23
GMP (3 mM)	2.71 ± 0.33
AMP (0.1 mM)	2.18 ± 0.64
AMP (3 mM)	2.00 ± 0.14
ADP (0.1 mM)	5.77 ± 0.57
ATP (0.1 mM)	3.18 ± 1.02
ATP (3 mM)	4.21 ± 0.07
UTP (3 mM)	1.30 ± 0.18
Cyclic AMP (0.1 mM)	2.55 ± 0.09
Cyclic AMP (3 mM)	6.89 ± 0.62
8-Bromo cyclic AMP (0.1 mM)	2.63 ± 0.55
8-Bromo cyclic AMP (3 mM)	6.72 ± 0.52
Calf serum (10%)+cyclic AMP (3 mM)	11.6 ± 1.80

hyaluronic acid synthetase activity was completely blocked by simultaneous addition of actinomycin D or cycloheximide (Table 2), so new syntheses of RNA and protein both seem to be required, as in induction on addition of serum (Tomida *et al.*, 1975).

To determine whether the effect on enzyme activity was specific to cyclic AMP, the effects of various nucleotides and reagents that would affect the intracellular concentration of nucleotides were examined (Table 3).

Other compounds besides cyclic AMP increased hyaluronic acid synthetase activity, and ADP and ATP were both more effective than cyclic AMP at a concentration of 0.1 mM. However, cyclic AMP was more effective than other nucleotides at a concentration of 3 mM. AMP was less effective than cyclic AMP or 8-bromo cyclic AMP at any concentration tested. Prostaglandin E₂ (10 μ g/ml), which increased the intracellular concentration of cyclic AMP (Maganiello & Vaughan, 1972), was as effective as 3 mM-cyclic AMP. These results indicate that cyclic AMP

itself increases hyaluronic acid synthetase activity and that its effect is not due to AMP formed by its breakdown.

Discussion

Addition of calf serum to confluent SEN cells causes a decrease in cellular cyclic AMP and induction of hyaluronic acid synthesis. However, addition of cyclic AMP or prostaglandin E_2 to these cells also leads to stimulation of hyaluronic acid synthesis. This indicates that induction of hyaluronic acid synthesis by serum is not due to a decrease in the intracellular cyclic AMP. An analogous example was reported by Hogan *et al.* (1974), namely that ornithine decarboxylase in quiescent baby-hamster kidney cells is induced by either calf serum or N^6O^2 -dibutyryl cyclic AMP. Further, uptake of glucosamine by SEN cells is stimulated by either calf serum or cyclic AMP. It was confirmed that uptake of [^{14}C]glucose, as well as of glucosamine, is similarly stimulated by either cyclic AMP or N^6O^2 -dibutyryl cyclic AMP or prostaglandin E_2 (results not shown). Kuroki & Yamakawa (1974) also reported that the uptake of 2-deoxy-D-glucose but not 2-aminoisobutyric acid by hamster-embryo cells is stimulated by preincubation with N^6O^2 -dibutyryl cyclic AMP and theophylline. Thus serum and cyclic AMP are not necessarily antagonistic, although opposite effects on growth regulation have been reported (Rozengurt & Pardee, 1972; Kram *et al.*, 1973).

Goggins *et al.* (1972) reported that treatment of virus-SV40-transformed 3T3 cells with N^6O^2 -dibutyryl cyclic AMP enhances the synthesis of chondroitin sulphates. It is also reported that synthesis of chondroitin sulphates in cartilage is stimulated by a serum factor called somatomedin (Daughaday *et al.*, 1972). SEN cells can synthesize chondroitin 4-sulphate. However, addition of serum or of cyclic AMP to cells in the exponential phase or the stationary phase of the growth cycle did not have any significant effect on their chondroitin sulphate synthesis. It is likely that synthesis of chondroitin sulphate is constitutive in SEN cells.

The capacity of cells to produce hyaluronic acid is always correlated with their hyaluronic acid synthetase activity (Ono *et al.*, 1974). A variety of nucleotides besides cyclic AMP was found to cause some increase in hyaluronic acid synthetase activity; in particular ADP and ATP gave a significant increase (over fourfold of the control value). These

data may merely show that the stimulation of the synthetase activity is not specific to cyclic AMP among a variety of nucleotides. Otherwise it may be possible that the effective nucleotides induce the enzyme activity by raising the intracellular concentrations of cyclic AMP.

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