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STIMULATORY EFFECT OF BLEOMYCIN ON THE HYALURONIC ACID SYNTHETASE IN CULTURED FIBROBLASTS

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Abstract—The mechanism of the stimulatory effect of bleomycin on the production of hexosamine-containing substances by cultured fibroblasts was studied by measuring changes in the activity of hyaluronic acid (HA) synthetase. HA synthetase activity was increased by 1 μg bleomycin/ml, and its stimulatory effect could be detected 4 days after the start of bleomycin treatment and was enhanced by lengthening the time of treatment, i.e. by 96 per cent for an 8-day treatment. In cell-free systems, however, bleomycin showed no stimulatory effect on this enzyme activity. The enhancement of HA synthetase activity by bleomycin was inhibited by the addition of cycloheximide or actinomycin D. The synthesis of HA was much less influenced by cycloheximide than was that of glycoprotein and sulfated glycosaminoglycan. From our results it can be concluded that HA synthetase activity, induced when the old medium of stationary cultures was renewed with fresh medium containing 5% fetal bovine serum, was significantly enhanced by the addition of bleomycin. However, the detailed mechanism of enhancement of HA synthetase activity has not yet been elucidated.

It is well known that bleomycin, an antitumor antibiotic isolated from a strain of *Streptomyces verticillus* [1], has a variety of actions such as single-strand breaks of DNA, inhibition of DNA-dependent DNA polymerase, and arrest of cells just prior to mitosis [2–7].

In previous papers [8, 9], we reported that bleomycin significantly increased the synthesis of acidic glycosaminoglycans (AGAG), glycoproteins and collagen in cultured fibroblasts. However, since these experiments were performed in the steady state, it was possible that the incorporation of labeled thymidine into DNA was inhibited in only a small percentage of cells, whereas AGAG was synthesized in about 95 per cent of cells. Accordingly, we suggested that inhibition of DNA synthesis is independent of the stimulatory effect of bleomycin on AGAG synthesis.

In this report, the effect of bleomycin on HA synthetase was studied by using cultured fibroblasts known to produce primarily a large amount of HA [9].

EXPERIMENTAL

Culture conditions. Cloned fibroblasts (clone C5 from rat carrageenin granuloma) were used [10, 11]. The cells were grown in a Petri dish at 37° in a CO_2 atmosphere with Ham's F-12 medium (Nissui Seiyaku Co., Tokyo) supplemented with 10% fetal bovine serum (Microbiological Associates, Bethesda, MD) and antibiotics (penicillin, 100 units/ml, and streptomycin, 100 μg/ml). All experiments started just after the cells reached their stationary phase, when the concentration of serum was lowered to 5% so that a constant cell number was maintained. A more detailed description is given in a previous paper [9].

Treatment of cells. A sterilized aqueous solution of bleomycin hydrochloride (Lot No. 1600AC01, a gift from Nippon Kayaku Co., Tokyo) was stored as a stock solution (1 mg/ml) at −20° until use. Just before use, the stock solution was diluted with Ham's F-12 medium, containing 5% fetal bovine serum, to the appropriate concentrations. The cells were exposed to various concentrations of bleomycin (0.1 to 10 μg/ml) during their stationary phase for 8 days. The medium was changed every 2 days. When cells reached their stationary phase, the medium was changed to a fresh medium containing 5% fetal bovine serum and cycloheximide (1 μg/ml) or actinomycin D (1 μg/ml). After an 8-day treatment with bleomycin, the cells were labeled with 0.5 μCi/ml of D[6-^{3}H]glucosamine hydrochloride (sp. act., 29 Ci/m-mole) for 6 hr.

Extraction of AGAG and glycoproteins. After cultivation, the medium and harvested cells were treated separately with Pronase E (Kaken Chemicals Co., Tokyo). The digest was added, with trichloroacetic acid, to Pronase E, and the precipitated protein was removed by centrifugation. The supernatant fraction was dialyzed and the dialysate was designated as a hexosamine-containing substance. AGAG was precipitated by adding cetylpyridinium chloride to the dialysate. The supernatant fraction was designated as the glycoprotein fraction. AGAG samples were subjected to electrophoresis on cellulose acetate strips (Separax, Joko Sangyo Co., Tokyo) with 0.3 M calcium acetate. The location of HA and sulfated GAG was detected by staining the strip with alcian blue. Both electrophorograms were cut out and counted in a scintillation counter.

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More details are described in previous papers [9, 10].

Assay of HA synthetase. After cultivation, the cells were harvested with a teflon spatula and then mixed vigorously in a Thermo-Mixer (Thermonics Co., Tokyo). The cell lysates obtained were frozen and then thawed two times and centrifuged at 10,000 *g* for 15 min. The pellet was washed three times with cold saline and stored at −20°. The sample was thawed by the addition of a measured amount of cold saline just before enzyme assay. Aliquots were taken and the protein contents measured by the method of Lowry *et al.* [12]. The rest of the sample was used as an enzyme source. HA synthetase activity was assayed according to the method of Ishimoto *et al.* [13]. This enzyme reaction was based on the incorporation of UDP[^{14}C]*N*-acetylglucosamine (sp. act., 300 mCi/m-mole) into HA in the presence of Tris–HCl buffer (pH 7.2), UDP-glucuronic acid, $MgCl_2$, and the enzyme (about 50 μg protein). The reaction mixture was incubated at 37° for 60 min and the reaction stopped by putting the reaction tubes into boiling water for 2 min. The reaction mixture was spotted on Whatman No. 3MM filter paper and subjected to descending paper chromatography with the following system: isobutylic acid–1 M NH_4OH (5:3, v/v). After 48 hr of development, the radioactivity in the immobile material remaining at the origin was measured with 10 ml of scintillation fluid (4 g PPO, 0.3 g POPOP and 1 l. toluene)* by a liquid scintillation counter. The radioactivity at the origin was reduced to the background level when the reaction mixture was chromatographed after digestion with hyaluronidase from *Streptomyces hyalurolyticus*. The specific activity of this enzyme was expressed as pmoles of [^{14}C]*N*-acetylglucosamine incorporated into HA/hr/mg of protein.

Reagents. Cycloheximide was purchased from Wako Pure Chemical Industries Ltd., Tokyo. Actinomycin D was purchased from Makor Chemicals Ltd., Jerusalem. UDP-glucuronic acid was purchased from Boeringer Mannheim, Tokyo. All the radioactive materials were purchased from the Radiochemical Center, Amsterdam.

RESULTS AND DISCUSSION

Effect of bleomycin on HA synthetase activity in cultured fibroblasts. HA synthetase is known to act at the final step of the pathway of HA synthesis, and this enzyme polymerizes an equal molality of UDP-glucuronic acid and UDP-*N*-acetylglucosamine to produce HA. As expected, the HA synthetase activity in the cultured fibroblasts was enhanced by bleomycin. A marked increase (96 per cent over control) in the activity of this enzyme was seen in cells treated with 1 μg bleomycin (Fig. 1), and this stimulation was enhanced by lengthening the time of treatment, i.e. by 48 per cent with a 6-day treatment and by 96 per cent with an 8-day treatment

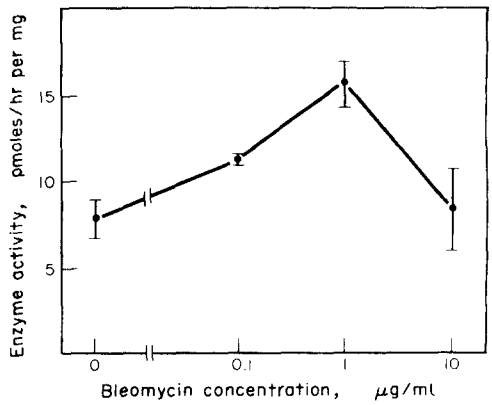

Fig. 1. Dose–response curve of cultured fibroblasts treated with bleomycin. Enzyme activity was assayed after an 8-day treatment with bleomycin. Medium transfer was performed every 2 days. Cultures were harvested 6 hr after the last renewal of media, and hyaluronic acid synthetase in the cells was assayed. Details are described in the experimental section. Each point indicates mean ± S.E. (N = 3). The results indicate the pmoles/hr/mg of protein.

(Fig. 2). It seems necessary for the cells to contact 1 μg bleomycin/ml for more than 4 days in order to elevate significantly the enzyme activity, since higher doses of bleomycin failed to shorten the lag time (not shown).

Recently, it was reported that HA synthetase activity was induced when calf serum was added to the medium [14]. As shown in Fig. 3, HA synthetase activity was induced when the old medium of the stationary culture was renewed with fresh medium containing 5% fetal bovine serum. The HA syn-

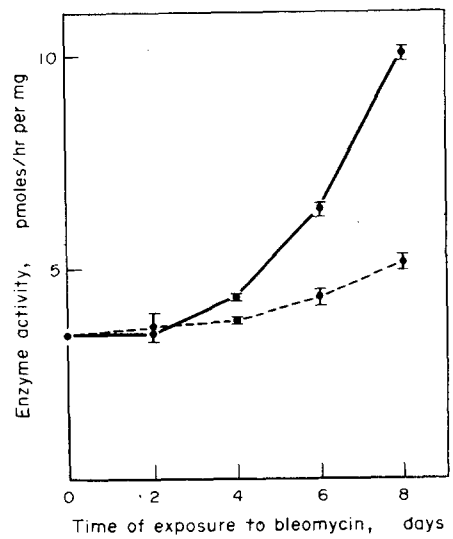

Fig. 2. Effect of bleomycin on hyaluronic acid synthetase activity in cultured fibroblasts. Cells were exposed to 1 μg bleomycin/ml for 0–8 days during the stationary phase. Medium transfer was performed every 2 days. Cultures were harvested 6 hr after the last renewal of media, and the enzyme activity was assayed. Each point indicates mean ± S.E. (N = 3). The results indicate the pmoles/hr/mg of protein: broken line = control cultures; solid line = bleomycin-treated cultures.

* PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene.

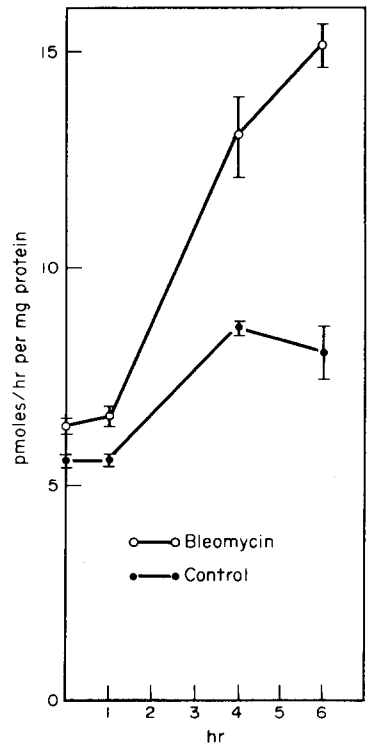

Fig. 3. Effect of medium change on hyaluronic acid synthetase activity in cultured fibroblasts treated with 1 μg bleomycin/ml. Enzyme activity was assayed after an 8-day treatment with bleomycin. Medium transfer was performed every 2 days. Cultures were harvested 0–6 hr after the last renewal of media, and hyaluronic acid synthetase in the cells was assayed. Each point indicates mean ± S.E. (N = 3). The results indicate the pmoles/hr/mg of protein.

thetase activity which was induced reached a maximum level for 4 hr and then decreased gradually. On the other hand, HA synthetase activity in the group pretreated with bleomycin was significantly enhanced and continued to increase during the period of observation, compared with that of the untreated group. Therefore, we suggest that the ability of the fibroblasts for HA synthesis was accelerated by treatment with bleomycin. However, HA synthetase activity in the old medium before renewal did not increase, even when the cells were treated with bleomycin for 8 days.

Direct effect of bleomycin on the HA synthetase activity in cultured fibroblasts. Tetsuka *et al.* [15] have reported that bleomycin enhanced the activity of protocollagen proline hydroxylase in collagen biosynthesis, and that this enhanced activity was due to an allosteric transition of the enzyme. In order to examine whether bleomycin has a direct effect on HA synthetase itself, the effect of bleomycin was examined in a cell-free system. As shown in Table 1, bleomycin added directly to the enzyme reaction mixture showed no stimulatory effect, even at the high dose of 100 μg bleomycin/ml. This result suggests that bleomycin does not act as an allosteric activator of HA synthetase.

Effect of cycloheximide or actinomycin D on the stimulation of HA synthetase activity by bleomycin.

Table 1. Direct effect of bleomycin on hyaluronic acid synthetase activity in a cell-free system*

Dose (μg/ml)	Enzyme activity (pmoles/hr/mg protein)
0	10.75 ± 0.07†
1	10.03 ± 1.49
10	11.07 ± 0.07
100	11.24 ± 0.67

* Cultures were harvested 6 hr after the last renewal of medium and the enzyme activity was measured. Enzyme fraction was obtained from control cultures. Bleomycin was added directly to the enzyme reaction mixture.

† Results are expressed as mean ± S.E. (N = 3).

In order to know whether synthesis of both protein and RNA is involved in the stimulatory effect of bleomycin on HA synthetase, inhibitors of protein and RNA syntheses were used. As shown in Table 2, the activity of this enzyme, in the cells treated with cycloheximide, decreased to about 40 per cent in both control and bleomycin groups, and in the cells treated with actinomycin D, the elevation of HA synthetase activity was also blocked completely. These results suggest that new synthesis of both protein and RNA is required for an increase in HA synthetase activity.

It has been generally recognized that the synthesis of AGAG is inhibited by inhibitors of protein synthesis since concomitant synthesis of the corresponding core protein in the biosynthesis of AGAG is necessary [16–20]. However, detailed data supporting the existence of HA in animal tissue as a proteoglycan are lacking.

In order to determine whether the inhibition of HA synthetase induced in the cultures treated with cycloheximide or actinomycin D was due to direct effects of these reagents on the synthesis of this enzyme itself or to indirect effects caused by a diminished synthesis of core protein, the cultured cells were labeled with [^{3}H]glucosamine for 6 hr in the presence of cycloheximide and their effects on the synthesis of various hexosamine-containing substances were compared. As shown in Table 3, inhibition of the incorporation of [^{3}H]glucosamine into HA by cycloheximide was exceedingly small in the cultured fibroblasts compared with the inhibition of incorporation into chondroitin sulfate and glycoprotein, which have been recognized to have core protein. This effect of cycloheximide was inconsistent with results obtained by Matalon and Dorfman [21] on cultured fibroblast and by Smith and Hammerman [22] on mouse fibroblasts and synovial cells. However, the inhibitory rate for the synthesis of HA synthetase was larger than that for the incorporation of [^{3}H]glucosamine into HA. From these results it seems most reasonable to conclude that inhibition of HA synthetase induced by cycloheximide is related to the diminished synthesis of HA synthetase rather than to the inhibition of core protein synthesis.

The observation that new medium is necessary for stimulation of HA synthetase, as described above, suggests that independent stimulation of HA synthetase by bleomycin is unlikely. Accordingly, it can

Table 2. Effect of cycloheximide or actinomycin D on the enhancement of hyaluronic acid synthetase activity by bleomycin*

Treatment		Enzyme activity (pmoles/hr/mg protein)			
		Control	%	Bleomycin	%
Cycloheximide	Absent	6.36 ± 0.62†	100	12.31 ± 0.15	100
	Present	2.66 ± 0.26	42	4.43 ± 0.43	36
Actinomycin D	Absent	7.01 ± 0.73	100	13.12 ± 0.52	100
	Present	3.19 ± 0.07	46	2.83 ± 0.20	22

* Cells were exposed to 1 μg/ml of cycloheximide or actinomycin D for 6 hr after an 8-day treatment with 1 μg/ml of bleomycin.

† Results are expressed as mean ± S.E. (N = 3).

Table 3. Effect of cycloheximide on the synthesis of hexosamine-containing substances by cultured fibroblasts*

Group	Glycoprotein (cpm/1 × 10^6 cells)	Glycosaminoglycan (cpm/1 × 10^6 cells)		
		Total	Hyaluronic acid	Sulfated glycosaminoglycan
Cell layer				
Control	3,036 ± 118†	17,259 ± 21	8,997 ± 86	1,297 ± 76
Cycloheximide	1,199 ± 13	12,082 ± 146	7,087 ± 428	277 ± 46
(per cent inhibition)	(60)	(30)	(21)	(79)
Medium				
Control	17,934 ± 1,340	11,430 ± 132	6,487 ± 663	2,278 ± 87
Cycloheximide	4,373 ± 335	6,708 ± 96	6,407 ± 184	366 ± 23
(per cent inhibition)	(76)	(41)	(0)	(84)

* Radioactivity of the extracted glycoprotein and total AGAG was measured in a liquid scintillation counter. The scintillator mixture was composed of toluene (1 l.), Triton X-100 (500 ml) and 2,5-diphenyloxazole (6 g). The AGAG obtained was subjected to electrophoresis on cellulose acetate strips, and the radioactivity of HA and sulfated GAG on the electrophorogram developed with authentic standard samples was measured by a liquid scintillation counter in the conventional toluene–PPO–POPOP system by cutting out the individual channels stained with alcian blue.

† Each value indicates mean ± S.E. (N = 3).

be concluded that bleomycin additionally enhances HA synthetase activity induced by the addition of fetal bovine serum. Further experimentation is necessary to explain fully this interesting observation.

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