ELEVATION OF GLUCOSAMINE 6-PHOSPHATE SYNTHETASE ACTIVITY IN BLEOMYCIN-INDUCED PULMONARY FIBROSIS IN HAMSTERS

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The single intratracheal instillation of bleomycin sulfate (0.5 mg (potency) per 100 g body weight) in hamsters rapidly increased the activity of glucosamine 6-phosphate synthetase (EC 2.6.1.16) of the lung, a major regulatory enzyme for the synthesis of acidic glycosaminoglycan (AGAG). The activity increased as early as day 2, reached maximum level at day 10, then decreased and returned to the control level at day 45. The content of AGAG was also in creased by bleomycin treatment, but the increase of AGAG followed the elevation of the enzyme activity. These results suggest that the early elevation of glucosamine 6-phosphate synthetase activity is closely related to the accumulation of AGAG in the fibrosing lung caused by bleomycin.

Bleomycin, a glycopeptide antibiotic isolated from *Streptomyces verticillus*[1] has been used clinically for the treatment of several types of cancers[2]. A major complication of this agent is dose-related pulmonary fibrosis, which is characterized by the excessive deposition of connective tissue components[3,4]. However, the mechanism by which bleomycin induces this disease remains uncertain. Numerous investigators have suggested that the alteration of collagen metabolism is involved in the process of pulmonary fibrosis. It has been reported that bleomycin stimulates collagen synthesis[5,6] and enhances prolyl hydroxylase activity[7,8] in the lung of experimental animals.

Acidic glycosaminoglycan (AGAG) is a major ground substance of connective tissues, which is known to be closely related to the formation of collagen fibers.[9,10] Some investigators have reported the elevation of AGAG content in the lung with pulmonary fibrosis.[11,12] OTSUKA *et al.*[13] have shown the stimulatory effect of bleomycin on the synthesis of AGAG in cultured fibroblasts. In this study, we examined the effect of bleomycin on the activity of glucosamine 6-phosphate synthetase (EC 2.6.1.16), a major enzyme for AGAG synthesis, in the lung of hamsters.

Materials and Methods

Animals and Treatment

Male Golden hamsters weighing 100~110 g were lightly anesthetized with sodium pentobarbital. After tracheotomy[14], the animals received intratracheal instillation of bleomycin sulfate (Nippon Kayaku Co. Ltd., Tokyo), 0.5 mg (potency) in 0.3 ml sterile saline per 100 g of body weight.

Control animals received instillation of sterile saline. On days 2, 5, 10, 15 and 45 after bleomycin treatment, animals were killed by exsanguination and their lungs were removed.

Assay of Glucosamine 6-Phosphate Synthetase

The lung from each animal was homogenized at 0~2°C with 9 volumes of 20 mM phosphate buffer (pH 7.0) containing 2 mM dithiothreitol and 1 mM EDTA in a glass-Teflon homogenizer. The homogenate was centrifuged at 15,000×*g* for 30 minutes and the supernatant was used for enzyme assay and protein determination.

Glucosamine 6-phosphate synthetase activity was assayed by the method of POGELL and GRYDER[15] in a reaction mixture containing 40 μmole phosphate buffer (pH 7.5), 10 μmole glutathione (reduced form), 1 μmole EDTA, 15 μmole L-glutamine, 10 μmole fructose 6-phosphate, an appropriate amount of enzyme solution and water to give a total volume of 1 ml. The incubation was carried out at 37°C for 1 hour and the reaction was stopped by adding 1 ml of cold 0.4 N trichloroacetic acid. The mixture was centrifuged at 5,000$\times g$ for 10 minutes and the amount of glucosamine in the supernatant was determined by the method of BLIX.[16] One unit of enzyme was defined as the amount causing the release of 1 nmole product per minute under the standard assay condition.

Protein content was determined by the method of LOWRY *et al.*[17] with bovine serum albumin as standard.

Determination of AGAG Content

The lung from each animal was cut into small pieces and defatted with several changes of acetone for 3 days. The defatted-dried lung was suspended in 3 ml of 0.1 M tris-HCl buffer (pH 8.0) containing 5 mM $CaCl_2$, and Pronase E (Kaken Chemical Co., Tokyo) was added in a ratio of 5 mg to the 100 mg of dry weight. The mixture was incubated for 48 hours at 40°C, then trichloroacetic acid was added to give a final concentration of 10%, and the mixture was centrifuged at 5,000$\times g$ for 10 minutes. The supernatant was neutralized with NaOH, dialyzed overnight against running water, and then mixed with 3 volumes of ethanol containing 1% CH_3COOK. The mixture was kept overnight at 2~3°C, then centrifuged at 5,000$\times g$ for 10 minutes. The pellet was dissolved in a small volume of distilled water, and the content of uronic acid in the sample was determined by the method of BITTER and MUIR[18] with glucuronic acid as standard.

Results and Discussion

The intratracheal instillation of bleomycin in hamsters significantly increased the wet weight of the lung at days 2, 5, and 10 after the treatment (Fig. 1). Gross and histological evidence of pulmonary injury such as hemorrhage, edema, and mononuclear and polymorphonuclear cellular infiltration was observed in the lung of bleomycin-treated animals, as reported by SNIDER *et al.*[19] As shown in Fig. 2, bleomycin treatment rapidly increased the activity of glucosamine 6-phosphate synthetase in the lung; the specific activity increased significantly as early as day 2, reached maximum level (220 per cent of the control activity) at day 10, then decreased and returned to the control level at day 45.

On the other hand, the content of AGAG in the lung remained unchanged at day 2, but significantly increased at days 5, 10 and 15 after bleomycin treatment (Fig. 3). The increase of AGAG con-

Fig. 1. Change in the lung weight after bleomycin treatment.

○, control; ●, bleomycin. Each point shows the mean±S.E. of 4~9 animals. *, $P<0.05$.

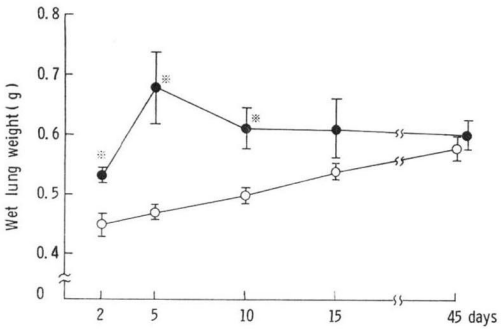
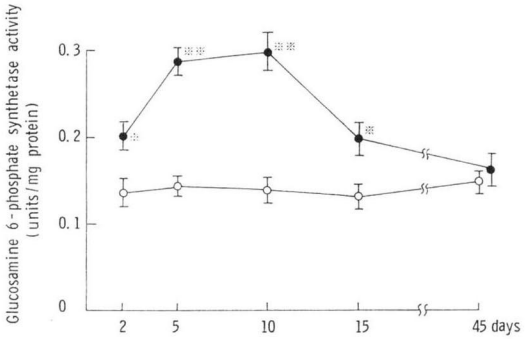


Fig. 2. Change in the glucosamine 6-phosphate synthetase activity after bleomycin treatment.

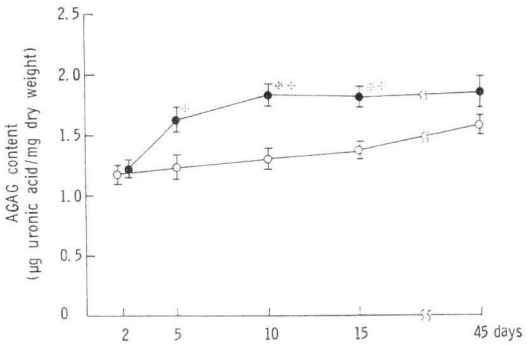
○, control; ●, bleomycin. Each point shows the mean±S.E. of 4 animals. *, $P<0.05$; **, $P<0.01$.



tent followed the elevation of glucosamine 6-phosphate synthetase activity, suggesting that the early elevation of the enzyme activity is involved in the accumulation of AGAG in the bleomycin-induced lung injury.

Fig. 3. Change in the AGAG content after bleomycin treatment.

○, control; ●, bleomycin. Each point shows the mean±S.E. of 4 animals. *, $P<0.05$; **, $P<0.01$.



Glucosamine 6-phosphetate synthase activity is known to be particularly high in fetal liver,[20] regenerating liver[21] and some kinds of tumors.[22,23] TSUIKI and MIYAGI[24] have suggested a possible participation of this enzyme in cell replication. This enzyme catalyzes the transfer of the amide group of glutamine to fructose 6-phosphate to form glucosamine 6-phosphate, and this reaction is the rate-limiting step in the formation of UDP-*N*-acetylglucosamine, which is a precursor for the biosynthesis of AGAG.

Many studies have shown a close association of AGAG with the formation of collagen fibers; the addition of chondroitin sulfate proteoglycan to tropocollagen modifies the kinetic of collagen fibril formation from collagen molecules.[25] Soluble collagen has been suggested to be precipitated as insoluble collagen by AGAG during the course of wound healing.[26]

Since the accumulation of collagen and AGAG is a prominent feature of pulmonary fibrosis, our result on the early elevation of glucosamine 6-phosphate synthetase activity may be important in considering one of the mechanism of fibrosis caused by bleomycin. Further study is necessary to clarify the intracellular distribution of the enzyme in fibrotic lung.

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