ANALYSIS OF THE MECHANISM OF BLEOMYCIN-INDUCED CUTANEOUS FIBROSIS IN MICE

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Mouse skin assays for collagen, determined as hydroxyproline, were carried out on a bleomycin-treated group and on untreated controls. Animals receiving bleomycin showed cutaneous collagen levels approximately 1.75 time higher than those exhibited by the untreated controls. With respect to the ratios of hydroxyproline in soluble collagen fraction to cutaneous total hydroxyproline content, the bleomycin-treated group displayed a roughly 50 per cent decrease in neutral salt soluble fraction and a slight diminution of the acid soluble fraction, as compared with the untreated controls. The findings indicate a significant increase in insoluble collagen content in the skin of mice receiving bleomycin.

In an attempt to clarify the mechanism of collagenolysis by the anticancer antibiotic, cutaneous tissue specimens taken from animals treated with the antibiotic were grown *in vitro*, and crude collagenase preparations isolated from tissue cultures assayed radiologically, using ^{14}C-glycine labelled collagen as the substrate. Lower cutaneous collagenolytic activity was observed in mice treated with bleomycin.

Developed recently as a unique anticancer drug virtually devoid of adverse side effects on the hematopoietic system, bleomycin[1,2] is used at present primarily in the treatment of squamous cell carcinoma. Clinical as well as laboratory studies[3,4], however, have demonstrated the development of pulmonary fibrosis[5] and changes in nail tissue[6] as an occasional consequence of bleomycin administration, thus presenting some intricate problems in this anticancer chemotherapy.

Meanwhile, there is as yet a dearth of information of particular note as to the mechanism of fibrotic response to bleomycin, with the exception of a report by TEZUKA[7] that bleomycin is capable of enhancing the activity of protocollagen proline hydroxylase which is involved in the biosynthesis of collagen.

In view of this, the present study was undertaken to attempt an analysis of the fibrotic response of the skin of mice treated with bleomycin and, at the same time, to determine the course of biotransformation of the drug in cutaneous fibrosis in order to clarify the underlying mechanism.

Materials and Methods

(1) Bleomycin.

Solutions of sterile bleomycin powder, supplied in vials containing the equivalents of 15 mg pure bleomycin base (Nippon-Kayaku Co., Ltd.), were used.

(2) Laboratory animals.

Animals used were 14-week-old male mice of the ICR strain with an average body weight of 25 g.

(3) Mouse skin assay for hydroxyproline following administration of bleomycin.

1) Drug administration: Bleomycin solution was administered to mice intraperitoneally in doses of 10 mg/kg daily for 10 days and, on the 20 th day after the conclusion of the 10-day dosing,

the animals were sacrificed and the skin of the back removed to be used in the assay for hydroxyproline.

2) Preparation of soluble collagen from the skin of treated mice: Cutaneous tissue specimens, 150~400 mg, were minced with scissors and suspended in 10 ml of 0.45 M sodium chloride solution, followed by homogenization by means of a glass homogenizer. The tissue homogenate was allowed to stand at 4°C for 48 hours for extraction, and was then centrifuged at 17,500 r.p.m. for 30 minutes. The resulting supernate was separated, the sediment extracted again in 10 ml more of 0.45 M NaCl solution and centrifuged in the same manner as above. The combined saline extracts were designated as the neutral salt soluble fraction. The sedimented material was resuspended in 10 ml of 0.5 M acetic acid, processed for extraction and centrifuged in the same manner as the foregoing. This supernate and the sediment were designated as the acid soluble fraction and the insoluble collagen fractions,[8] respectively.

3) Hydroxyproline assay: Each fraction was concentrated to 1.0~1.5 ml, and an adequate amount of 6~12 N hydrochloric acid was added to a 6 N concentration. Each fraction was hydrolyzed at 120°C for 18 hours and analyzed for hydroxyproline content by the procedure of KIVIRIKKO *et al.*[9]

(4) Preparation of collagenase from mouse skin.

After hair clipping and subsequent application of a depilatory, the skin of the dorsal region of each mouse was sterilized with tincture of iodine, washed with sodium thiosulfate and a portion of the disinfected skin of the back, 1.5 cm×1.5 cm, was removed and minced into pieces, 2 mm×2 mm approx. The cutaneous tissue fragments were placed on a sterile Millipore filter and incubated in a tissue culture bottle at 37°C for 10 days with a medium consisting of equal parts of EAGLE's MEM and Medium 199. During the 10-day period of incubation, the medium was replaced at 24-hour intervals and the fluids harvested were spun at 17,500 r.p.m. for 30 minutes. The supernates were stored at −20°C. All the supernates were combined, dialyzed at 4°C in distilled water, and lyophilized. Immediately prior to assay for collagenase activity, the lyophilized powder was dissolved in 0.2 ml of Tris buffer and 0.1 ml aliquots used in each determination.

(5) ^{14}C-Glycine labelled collagen.

These were prepared by extracting neutral salt soluble and acid soluble fractions of dorsal skin tissue collagen from guinea pigs given ^{14}C-glycine and by diluting the fractions so that they contained 1,200~1,400 cpm of radioactivity per mg, in accordance with the method of NAGAI *et al.**

(6) Collagenase assay.

To 0.2 ml of 0.4% ^{14}C-glycine collagen solution, 0.2 ml of 0.1 M Tris buffer containing 10 mM $CaCl_2$ and 0.4 M NaCl, pH 7.5, was added and the mixture agitated at 2~4°C. It was then incubated at 37°C for 2 hours to allow it to gel. Then 0.1 ml of the collagenase solution was added into the gel and mixed thoroughly. After 16 hours at 37°C,. the mixture was centrifuged and the resulting supernate combined with BRAY's solution was assayed for collagenase activity using a liquid scintillation counter (Pachard Model 3320).

RESULTS

(1) Hydroxyproline in the Skin of Bleomycin-treated Mice

Table 1 shows the data concerning the hydroxyproline contents per unit weight of wet skin tissue of mice in the untreated group and of those in the bleomycin-treated group. The sum of the hydroxyproline contents in the three collagen fractions (neutral salt-soluble, acid-soluble, and insoluble) was taken as an indication of the total collagen level in the skin. As can be seen, the average hydroxyproline level in the skin was shown to be 1.5±0.11 mcg/ml in the untreated group

* Personal communication from Prof. YUTAKA NAGAI, Institute of Hard Tissue Research, Tokyo Medical School and Dental University.

Table 1. Content of hydroxyproline in mouse skin (total hydroxyproline mcg per mg skin weight)

Non-treated mice	Hydroxyproline (mcg)	Bleomycin treated mice	Hydroxyproline (mcg)
1	1.61	1	2.60
2	1.56	2	1.82
3	1.50	3	2.19
4	1.31	4	2.86
Average	1.50± .11	Average	2.62± .39

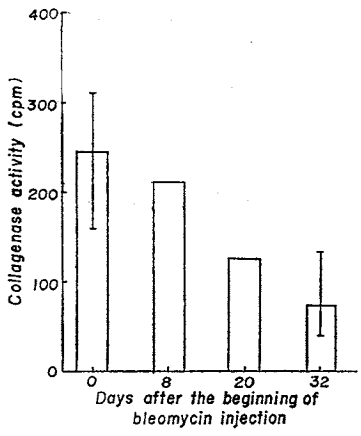
Table 2. Hydroxyproline content of mouse skin (Non-treated)

Number	$\frac{\text{neutral soluble}}{\text{total}} \times 100$	$\frac{\text{acid soluble}}{\text{total}} \times 100$
1	7.21	11.21
2	4.20	7.64
3	4.49	10.93
4	3.53	6.73
Average	4.83	9.31

Table 3. Hydroxyproline content of mouse skin (Bleomycin-treated)

Number	$\frac{\text{neutral soluble}}{\text{total}} \times 100$	$\frac{\text{acid soluble}}{\text{total}} \times 100$
1	1.82	7.98
2	5.20	4.51
3	1.55	2.94
4	1.55	13.57
5	1.18	7.38
Average	2.26	7.28

Fig. 1. Collagenase activity of bleomycin-treated mouse skin.



and 2.62±0.39 mcg/ml in the bleomycin-treated group, respectively (Table 1). The cutaneous collagen content in mice of the latter group exceeded that in the untreated controls by 75%, indicating a significant increase following administration of bleomycin.

Comparison of the ratios of hydroxyproline contents in respective soluble fractions to total hydroxyproline level between the untreated group (Table 2) and the bleomycin-treated group (Table 3) indicated an approximately 50% decrease in the neutral salt soluble fraction of the treated group. A slight diminution of the collagen content in the acid soluble fraction was also evident with the bleomycin-treated group.

(2) Collagenase Activity in the Skin of Mice after Bleomycin

Dorsal cutaneous tissue samples obtained from three untreated mice, a mouse sacrificed on day 8 of bleomycin administration, another sacrificed on the 20 th day after initiation of the 10-day bleomycin dosing and three mice on the 32 nd day after bleomycin treatment were assayed for collagenase activity. As can be noted from Fig. 1, the untreated control group exhibited a radioactivity count of 162 cpm or more for skin collagenase whereas the average values displayed by the bleomycin-treated group were 68 cpm on day 32 and recognizably lower on day 8 and day 20 when compared with the untreated group. This finding indicates diminution of cutaneous collagenase activity following administration of the drug.

No significant difference in collagenase activity was noted to exist between the medium of mouse skin tissue culture containing 0.1 mcg /ml bleomycin and that not containing the drug. An *in vitro* test of the effect of bleomycin on the activity of collagenase also failed to reveal any conspicuous influence of the drug on the enzyme from *Clostridium histolyticum* or on that from murine skin.

Summary and Discussion

Bleomycin, with proven remarkable efficacy in squamous cell carcinoma, pulmonary carcinoma and systemically disseminated malignant growths is extensively used at present. Side effects such as cutaneous and pulmonary fibrosis as well as histologic changes of the connective tissue of the blood vessel wall present practical problems, *e.g.* elucidation of the mechanism of fibrillogenesis caused by the drug and the search for an effective method of controlling further reactions.

The study reported herein was initiated in an attempt to analyse the fibrotic response to bleomycin by dorsal skin assays for hydroxyproline (collagen content) in mice. There revealed significant increase in the hydroxyproline content per unit weight of the skin following administration of bleomycin, providing evidence for fibroplasia of the skin of animals treated with the drug. In skins of bleomycin-treated mice, the neutral salt-soluble collagen exhibited consistently lower levels as compared with skins of controls. Contents of the acid-soluble collagen did not differ significantly between the drug-treated and the untreated control groups.

It was deduced from these findings that the following mechanisms appear to be operating in the genesis and progress of fibrosis associated with bleomycin administration: A prompt conversion of the synthesized and secreted neutral salt-soluble collagen to insoluble collagen, a rapid destruction of the neutral salt-soluble collagen, and/or diminution of tissue collagenolytic activity.

Experiments were then undertaken to pursue the time-course of tissue collagenolytic activity in mice receiving bleomycin. Tissue cultures of the drug-treated mouse skin displayed considerable depletion of collagenolytic enzyme in their media as compared with those of the skin of the untreated mice. Bleomycin suppression of the DNA synthesis results in inhibition of RNA and protein biosynthesis consequently. Our observations of suppresion of collagenase activity can be said to be in accord with the mode of action of bleomycin for protein synthesis. Collagenase has, however, been shown to normally occur in tissues as an inactive complex,[11,12] masked with collagenase inhibitors.[10] Accordingly, the possibilities in this regard suggest that the anticancer chemotherapeutic agent may in some way affect the masking of inhibitors with collagenase or that the drug itself may function as a collagenase inhibitor. The latter possibility is ruled out by our observation that the drug exerts no appreciable effect *in vitro* upon the activity of bacterial collagenase or mouse skin collagenase. Whatever the response to bleomycin, it seems to follow from these expeimental findings that the diminution of tissue collagenase activity observed in mice following administration of the drug cannot be conclusively interpreted as representing any absolute decrease of the enzyme.

Whilst it would risk a great danger to take the experimental findings as immediately applicable to the metabolism of insoluble collagen which accounts for a considerable portion of total tissue collagen since the substrate employed in the tissue collagenase assay was nothing but ^{14}C-glycine-labelled soluble collagen. It seems highly plausible that collagenase does take part in the tissue collagen metabolism in some way or other as has been suggested by various reported facts, *e.g.* a pronounced elevation of tissue collagenase activity in the tail fin of metamorphosing tadpole (NAGAI *et al.*[13] and USHUKU *et al.*[14]), a marked increase in collagenase activity with concomitant rapid depletion of collagen in the uterine tissue of post-parturient women (JEFFERY *et al.*[15]) and enhancement of collagenase activity during bone resorption.[16~18] The experimental results obtained in the study reported herein, *viz.* the significant increase in the hydroxyproline content per unit weight

of wet skin tissue and the demonstrable relative decrease of hydroxyproline in the neutral salt soluble collagen fraction after bleomycin, are satisfactorily explicable on the hypothesis that the drug administration gave rise to diminution of tissue collagenase activity and hence to suppression of the insoluble collagen metabolism.

Bleomycin thus has been found to bring about an increase in collagen content of the skin of mice and the results of the present investigation suggest a vital role of the drug in lowering the tissue collagenolytic enzyme activity in some way or other in the genesis of bleomycin-induced fibrosis, though effects of the antitumor drug on collagen biosynthesis in the cutaneous tissue remain to be studied.

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