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SELECTIVE INHIBITION OF COLLAGEN SYNTHESIS BY D-PENICILLAMINE IN CARRAGEENIN-INDUCED INFLAMMATION IN RATS

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Abstract—The effect of D-penicillamine on syntheses of collagen and noncollagen protein in inflammed tissued was studied by incubating minced granulation tissue with [^{3}H]proline and various amounts of D-penicillamine. D-Penicillamine selectively inhibited the incorporation of [^{3}H]proline into collagen hydroxyproline when 30 mM D-penicillamine was present in the incubation medium, and the accumulation of [^{3}H]proline-labeled protocollagen was found in the minced granulation tissue incubated with 30 mM D-penicillamine. In addition, administration of D-penicillamine (800 mg/kg) into granuloma pouch every 12 hr for 4 days, selectively inhibited the collagen synthesis and increased the collagen solubility in granulation tissue. When partially purified prolyl hydroxylase was incubated with [3,4-^{3}H]proline-labeled protocollagen substrate in the presence of D-penicillamine, the enzyme activity was decreased in proportion to the concentration of D-penicillamine. This inhibition was restored by increasing the amount of ferrous iron in the incubation medium. These results suggest that the selective inhibition of collagen synthesis *in vivo* and *in vitro* may be mainly due to the ability of D-penicillamine to chelate ferrous iron, a cofactor of prolyl hydroxylase.

D-Penicillamine (β,β-dimethylcysteine) has been reported to be effective on the basis of a double-blind test in the treatment of rheumatoid arthritis [1,2], although the mechanism by which the drug exerts its effect in rheumatoid arthritis is unknown.

As to the effect of D-penicillamine on collagen solubility, Nimni *et al.* [3,4] have reported that administration of D-penicillamine to animals caused an accumulation of soluble collagen in skin. They [4] suggested that D-penicillamine reversibly interacts with the aldehydes present in the collagen molecule to form a thiazolidine type complex and this interaction results in the inhibition of intra- and inter-molecular cross-linking of collagen. In contrast to skin collagen, we [5] and Henneman [6] demonstrated that D-penicillamine did not affect the solubility of collagen in the granulation tissue induced by carrageenin.

Hydroxylation of proline residues in protocollagen, a proline-rich precursor of collagen, is a specific process of collagen biosynthesis. Halme *et al.* [7] and Chvapil *et al.* [8] reported that D-penicillamine did not affect the prolyl hydroxylase activity, while Keiser *et al.* [9] demonstrated that high doses of D-penicillamine inhibited prolyl hydroxylase activity in the skin of rats. In addition, Uitto *et al.* [10] demonstrated that D-penicillamine inhibited prolyl hydroxylase activity in a mouse embryo cell-free system.

The present experiments were designed to investigate further the effect of D-penicillamine on the synthesis and the solubility of collagen in inflamed tissue, with the aid of an experimental model for chronic proliferative inflammation such as rheumatoid arthritis.

MATERIALS AND METHODS

A granuloma pouch was induced in male rats of the Donryu strain, weighing 130–170 g, by subcutaneous injection of a 2% (w/v) solution of Seakem 202 carrageenin (Marine Colloid Inc., Springfield, NJ. U.S.A.) according to the procedure of Tsurufuji *et al.* [11].

Treatment in vitro with D-penicillamine. Granulation tissue was harvested and minced immediately after sacrifice of rats bearing 8-day-old granuloma. The minced granulation tissue (1.0 g) was incubated under an atmosphere of 95% O_2–5% CO_2 at 35° with various amounts (10–40 mM) of D-penicillamine in 10 ml of Krebs' saline serum substitute (KSSS) [12] containing 1 mg each of potassium penicillin-G and dihydrostreptomycin sulfate. After 6 hr incubation of 10 μCi of L-[^{3}H]proline (generally labeled, 63 Ci/m-mole) was added to the incubation medium and the incubation was continued for a further 1 hr. At the end of the incubation, 50% trichloroacetic acid was added to give a 10% concentration and the flasks were chilled in ice-water. The contents were centrifuged and the precipitate was washed with ethanol five times. Collagen was extracted twice as gelatin by autoclaving the precipitate with distilled water, and the resulting collagen-free residue was referred to as the "noncollagen protein fraction". The amounts and radioactivities of collagen and noncollagen protein fractions were measured according to the procedure described previously [13].

Measurement of [^{3}H]*proline-labeled protocollagen.* Minced granulation tissue (2.0 g) obtained from rats bearing 9-day-old granuloma was incubated under an atmosphere of 95% O_2–5% CO_2 at 37° with 30 mM D-penicillamine in 20 ml of KSSS. After 30 min incubation, 30 μCi of L-[^{3}H]proline was added to the incubation medium and the incubation was continued for 90 min. At the end of the incubation protein synthesis was stopped by the addition of 1 ml of the cycloheximide solution (400 μg/ml) and the flasks were chilled in ice-

water. The contents were centrifuged at 100,000 *g* for 15 min at 4° and the precipitate was washed with the solution containing cycloheximide (20 μg/ml) and α,α′-dipyridyl (1 mM). After centrifugation the precipitate was homogenized and the [^{3}H] proline-labeled protocollagen fraction was extracted by the procedure described previously [14].

Prolyl hydroxylase for the measurement of protocollagen was partially purified from 8-day-old granuloma according to the procedure of Rhoads and Udenfriend [14]. Briefly, the homogenate of granulation tissue was fractionated with $(NH_4)_2SO_4$ (30–60 percent saturation) and purified by DEAE-Sephadex A-50 column chromatography.

An aliquot of the [^{3}H]proline-labeled protocollagen fraction was incubated with the partially purified hydroxylase. The basic system for assay of [^{3}H]proline-labeled protocollagen substrate in a 5 ml volume contained: [^{3}H]proline-labeled protocollagen fraction, 1.2–1.8 mg protein in 2 ml; partially purified hydroxylase, 1.14 mg protein in 1 ml; α-ketoglutarate, 0.5 mM; $FeSO_4$, 0.05 mM; ascorbic acid, 2 mM; dithiothreitol, 0.1 mM; catalase, 0.1 mg/ml; bovine serum albumin, 2 mg/ml; Tris–HCl (pH 7.8), 50 mM. Incubation was carried out in air at 37° for 90 min. At the end of the incubation, conc. HCl was added to give a 6 N concentration and the samples were hydrolyzed at 105° for 16 hr. The specific activity of collagen hydroxyproline in the hydrolysate was measured by the method described previously [13]. The presence of [^{3}H]proline-labeled protocollagen in the minced granulation tissue was proved by the increase in the specific activity of collagen hydroxyproline during the incubation of [^{3}H]proline-labeled protocollagen fraction with partially purified prolyl hydroxylase.

Measurement of prolyl hydroxylase activity. The substrate, [3,4-^{3}H]proline-labeled protocollagen, for the prolyl hydroxylase was prepared by incubating minces (30 g) of granulation tissue with L-[3,4-^{3}H]proline (1 mCi, 41 Ci/m-mole) in the presence of 1.5 mM α,α′-dipyridyl according to the method used previously to prepare [^{3}H]proline-labeled substrate [13].

Prolyl hydroxylase activity was assayed by measurement of tritiated water released during hydroxylation of [3,4-^{3}H]proline-labeled protocollagen according to the method of Hutton *et al.* [16]. The incubation mixture contained the same components described above, except that [3,4-^{3}H]proline-labeled protocollagen (1 ml, 6×10^4 d.p.m.) replaced [^{3}H]proline-labeled protocollagen. Incubation was carried out in air at 37° for 90 min. At the end of incubation, 50% trichloroacetic acid was added to give a 5% concentration. Radioactivity of tritiated water (3 ml) collected by vacuum distillation was determined in a liquid scintillation spectrometer using Bray's solution (17 ml) [17].

Treatment in vivo with D-*penicillamine.* The treated group was given D-penicillamine (400 mg or 800 mg/kg body weight) with pyridoxine·HCl (1 mg/rat) into the granuloma pouch every 12 hr from day 6 to day 9, while the control rats were injected with 0.9% NaCl. A dose of 10 μCi/kg body weight of L-[^{14}C(U)]proline (uniformly labeled, 247 mCi/m-mole) was injected intravenously 3 hr after the last injection of D-penicillamine and 12 hr later (on day 10) the rats were killed. Collagen and noncollagen proteins of granulation tissue were fractionated and their amounts and radioactivities were determined as described above.

The collagen solubility of granulation tissue was determined as follows: An aliquot (1.0 g) of minced granulation tissue was homogenized in 30 ml of ice-cold 1 M NaCl–50 mM Tris·HCl (pH 7.4) with a Vir-Tis homogenizer. The homogenate was shaken for 2 days at 4° and then centrifuged at 8,000 *g* for 30 min at 1°. The precipitate was suspended in 30 ml of 1 M NaCl–50 mM Tris·HCl (pH 7.4) and the extraction was repeated three times. The supernatants were pooled and filtered. The filtrate was referred to as "neutral salt-soluble collagen". Insoluble collagen was obtained as gelatin by autoclaving the residue remaining after neutral salt-soluble collagen extraction with 30 ml of distilled water at 120° for 60 min. The gelatinization was repeated twice.

RESULTS

Incubation of minced granulation tissue with D-*penicillamine in vitro.* Incubation of minced granulation tissue was carried out in the presence of 10–40 mM D-penicillamine in the medium. The results are summarized in Table 1. D-Penicillamine in 10 mM concentration significantly increased the incorporation of [^{3}H]proline into collagen hydroxyproline, whereas the incorporation was markedly inhibited in concentra-

Table 1. Effect of D-penicillamine on the incorporation of [^{3}H]proline into collagen and noncollagen protein during incubation *in vitro* of minced granulation tissue

Concn of D-penicillamine	No. of flasks	Collagen hydroxyproline		Noncollagen protein	
		Sp. act. (dis./min/μg hyp)	Per cent of control	Sp. act. (dis./min/μg protein)	Per cent of control
0 mM (control)	6	1.041 ± 0.059	100	6.329 ± 0.135	100
10 mM	6	1.432 ± 0.120†	138	6.829 ± 0.291	108
20 mM	6	1.286 ± 0.106	124	7.335 ± 0.242*	116
30 mM	6	0.559 ± 0.048*	54	6.145 ± 0.225	97
40 mM	6	0.192 ± 0.010*	18	4.092 ± 0.089*	65

Data are shown as means ± S. E.
*Values are significantly different from control, $P < 0.01$.
†Values are significantly different from control, $P < 0.02$.

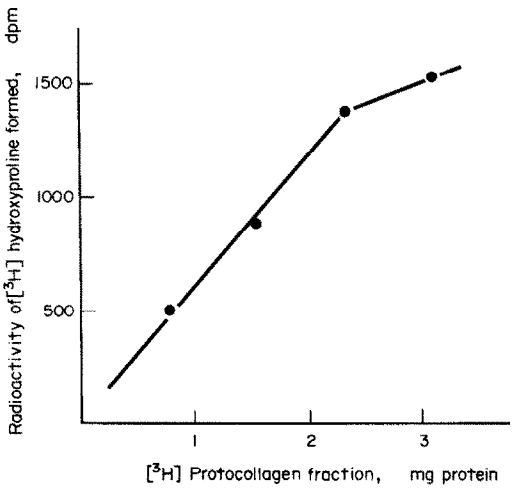

Fig. 1. Relationship between [^{3}H]hydroxyproline formed and the protein content of [^{3}H]proline-labeled protocollagen fraction added to the incubation mixture. Each point is the average of two determinations. Incubation conditions are described in the text.

tions of more than 30 mM of D-penicillamine. On the other hand, D-penicillamine in 20 mM concentration slightly increased the incorporation of [^{3}H]proline into noncollagen protein, while the incorporation was markedly inhibited by 40 mM D-penicillamine. On the whole, protein synthesis was enhanced at a lower concentration and inhibited at higher concentrations of D-penicillamine, although this effect of D-penicillamine on collagen synthesis was found at lower concentrations compared with that on the synthesis of noncollagen protein. At a concentration of 30 mM, D-penicillamine inhibited the [^{3}H]proline incorporation into collagen hydroxyproline by 53.7 per cent, whereas the incorporation into noncollagen protein was not affected. This result suggests that D-penicillamine, at a concentration of 30 mM, inhibits a specific process of collagen biosynthesis such as hydroxylation of protocollagen, a proline-rich precursor of the collagen molecule.

Hydroxylation of [^{3}H]proline-labeled protocollagen. As shown in Figure 1, the radioactivity of [^{3}H]hydroxyproline formed during the incubation of [^{3}H]proline-labeled protocollagen with partially purified prolyl hydroxylase was proportional to the amount of [^{3}H]proline-labeled protocollagen added to the incubation medium, indicating that the amount of labeled protocollagen can be measured in terms of the radioactivity of [^{3}H]hydroxyproline formed in the present incubation procedure. Consequently, 1–2.5 mg protein of [^{3}H]proline-labeled protocollagen fraction was used for the assay of the amount of [^{3}H]proline-labeled protocollagen in granulation tissue.

Minced granulation tissue was incubated with 30 mM D-penicillamine and [^{3}H]proline. Protocollagen labeled with [^{3}H]proline was extracted and incubated

Table 2. Effect of D-penicillamine on the accumulation of [^{3}H]proline-labeled protocollagen during incubation *in vitro* of minced granulation tissue

	No. of flasks	Sp. act. of [^{3}H]hydroxyproline (dis./min/μg hyp)		(B) / (A)
		(A) Before incubation	(B) After incubation	
Control	6	88.554 ± 2.685	87.923 ± 2.761	0.993 ± 0.008
D-penicillamine (30 mM)	7	19.955 ± 0.803	30.563 ± 0.711	1.541 ± 0.049*

Data are shown as means ± S. E.
*Values are significantly different from control, $P < 0.001$.

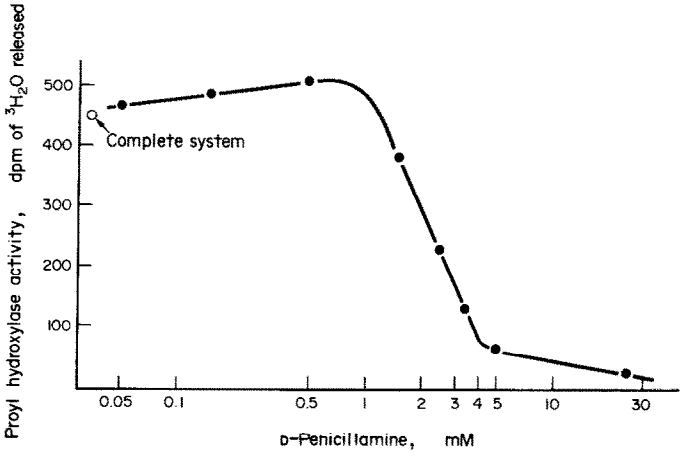

Fig. 2. Effect of D-penicillamine on the prolyl hydroxylase activity. Partially purified prolyl hydroxylase was incubated with [3,4-^{3}H]proline-labeled protocollagen in the presence of various amounts of D-penicillamine. Each point is the average of two determinations. Incubation conditions are described in the text.

Table 3. Effect of ferrous iron on the prolyl hydroxylase activity inhibited by D-penicillamine

	Prolyl hydroxylase activity* (d.p.m. of ^3H_2O released)	Per cent inhibition
Complete system	480	—
Complete system containing		
5 mM D-penicillamine	86	82
5 mM D-penicillamine and 0.5 mM $FeSO_4$	383	20
5 mM D-penicillamine and 1.5 mM $FeSO_4$	475	1
5 mM D-penicillamine and 3.0 mM $FeSO_4$	362	25
5 mM D-penicillamine and 5.0 mM $FeSO_4$	294	39

*Each value is the average of two determinations.

with partially purified prolyl hydroxylase. The specific activity of collagen hydroxyproline increased in the group incubated with D-penicillamine, while no increase was found in the control group (Table 2). The results indicate that D-penicillamine, at a concentration of 30 mM, causes accumulation of protocollagen in the minced granulation tissue.

Effect of D-penicillamine on prolyl hydroxylase activity. Effect of D-penicillamine on prolyl hydroxylase activity was studied in a cell-free system. Partially purified prolyl hydroxylase activity slightly increased in the presence of 0.2–0.5 mM D-penicillamine and decreased in proportion to the concentration (1–5 mM) of D-penicillamine in the present assay system (Fig. 2).

As shown in Table 3, the inhibited activity of prolyl hydroxylase by 5 mM D-penicillamine was completely restored by the addition of 1.5 mM $FeSO_4$, but the enzyme activity was partially inhibited at higher concentrations (3 and 5 mM) of $FeSO_4$. The results suggest that D-penicillamine inhibits prolyl hydroxylase activity through a mild chelating action on ferrous iron, a cofactor of the enzyme.

Treatment in vivo with D-penicillamine. A high dose (800 mg/kg) of D-penicillamine inhibited the [^{14}C]proline incorporation into collagen hydroxyproline, whereas the incorporation of [^{14}C]proline into noncollagen protein was not affected (Table 4). It seems that these results correspond to the results obtained by incubation *in vitro* of minced granulation tissue with 30 mM D-penicillamine. The increase in the solubility of granulation tissue collagen was relatively slight by the treatment of 400 mg of D-penicillamine per kg of body weight, which D-penicillamine in a high dose (800 mg/kg) markedly increased the solubility of the tissue collagen (Table 4).

DISCUSSION

It is well known that the administration of D-penicillamine to rats causes an accumulation of soluble collagen in skin [3]. We [5] and Henneman [6] reported that D-penicillamine did not cause any accumulation of soluble collagen in granulation tissue, though the solubility of skin collagen was markedly increased by the D-penicillamine treatment. On the other hand, the present experiment shows that the effect of D-penicillamine on the collagen solubility of granulation tissue is dose-dependent; at a higher dose (800 mg/kg), D-penicillamine increases the solubility of the tissue collagen, while a lower dose (400 mg/kg) of D-penicillamine is ineffective. Nimni *et al.* [4,18] reported that D-penicillamine, at relatively low doses, prevented cross-linking by bind-

Table 4. Effect of D-penicillamine on the solubility of collagen and on the incorporation of [^{14}C]proline into collagen hydroxyproline and noncollagen protein of granulation tissue in rats

		D-Penicillamine	
	Control	400 mg/kg	800 mg/kg
No. of rats	7	7	7
Net body wt (g)	177 ± 7	172 ± 5	152 ± 5†
Exudate (g)	41.3 ± 3.0	40.8 ± 4.1	47.0 ± 4.6
Granulation tissue, wet wt (g)	6.62 ± 0.16	6.91 ± 0.37	7.08 ± 0.39
Collagen content (mg hydroxyproline):			
Total collagen	17.71 ± 0.95	18.77 ± 1.43	17.44 ± 1.75
Neutral salt-soluble collagen	2.22 ± 0.16	3.23 ± 0.75	5.59 ± 0.54‡
Insoluble collagen	15.49 ± 0.90	15.54 ± 1.13	11.85 ± 1.25*
Noncollagen protein (mg) in the whole tissue	135.2 ± 8.7	117.6 ± 6.5	96.2 ± 5.8‡
Incorporation of [^{14}C]proline:			
Radioactivity of collagen hydroxyproline (dis./min/μg DNA)	0.405 ± 0.031	0.337 ± 0.010	0.304 ± 0.025*
Radioactivity of noncollagen protein (dis./min/μg DNA)	1.548 ± 0.118	1.529 ± 0.125	1.308 ± 0.112

Data are shown as means ± S. E.
Values are significantly different from control; *$P < 0.05$, †$P < 0.02$, ‡$P < 0.01$.

ing to the peptide-bound aldehydes on collagen molecule, whereas D-penicillamine, at higher doses, reduced the aldehyde content of collagen molecule by inhibiting an amine oxidase which causes the oxidative deamination of the ε-amino group of lysine to form a peptide bound α-amino adipic-δ-semialdehyde. Therefore, it seems likely that, in contrast to skin collagen, the collagen solubility of granulation tissue is not affected by blocking the aldehyde residues present on the collagen molecule, but the reduction of the aldehyde residues on collagen molecule, by inhibiting amine oxidase activity, causes the increase in the collagen solubility of granulation tissue. In support of this view, we [5] demonstrated that β-aminopropionitrile, an inhibitor of amine oxidase, markedly increased the collagen solubility of granulation tissue.

Chvapil *et al.* [8] reported that D-penicillamine in 10 mM concentration predominantly inhibited the [^{14}C]proline incorporation into collagen hydroxyproline during incubation *in vitro* of skin slices from 12-day-old chick embryos. Their results are not in conflict with our conclusion that the effect of D-penicillamine is dose-dependent; D-penicillamine in a lower concentration (10 mM) increases the [^{3}H]proline incorporation into collagen hydroxyproline and there is a concentration (30 mM) in which D-penicillamine selectively inhibits collagen synthesis without affecting the synthesis of noncollagen protein, although D-penicillamine in higher concentrations (more than 40 mM) inhibits both the synthesis of collagen and noncollagen protein during incubation *in vitro* of minced granulation tissue (Table 1).

D-Penicillamine inhibited prolyl hydroxylase activity (Fig. 2) and this inhibition was restored by the addition of ferrous iron to incubation medium (Table 3). These results are consistent with those of Uitto [10]. Thus, D-penicillamine inhibits prolyl hydroxylase in a cell-free system by binding the ferrous iron necessary for the enzyme. Keiser *et al.* [9] demonstrated that high doses and long duration of administration of D-penicillamine inhibited prolyl hydroxylase activity in the skin of rats. In the present experiment, D-penicillamine (800 mg/kg) inhibited the incorporation of [^{14}C]proline into collagen hydroxyproline, whereas the [^{14}C]proline incorporation into noncollagen protein was not affected. Albergoni *et al.* [19] demonstrated that the administration of D-penicillamine (150 mg/kg) at intervals of 12 hr for 20 days decreased the content of divalent cations including iron in various tissues of rat. The selective inhibition of collagen synthesis *in vivo* and *in vitro* may be due largely to the ability of D-penicillamine to chelate ferrous iron, a cofactor of prolyl hydroxylase, resulting in the inhibition of the enzyme.

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