Biochemical Pharmacology, Vol. 32, No. 11, pp. 1753–1757, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + .00
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REVERSIBILITY OF D-PENICILLAMINE INDUCED COLLAGEN ALTERATIONS IN RAT SKIN AND GRANULATION TISSUE

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(*Received* 1 *June* 1982; *accepted* 13 *December* 1982)

Abstract—Granulation tissue was produced in rats by subcutaneous implantation of Visella® sponges. D-penicillamine (D-pen) 100 or 500 mg/kg was administered daily for 42 days by gastric tubing. Pairfed, placebo treated animals were included as controls. Half of the groups were kept for additionally 28 days without medication. The inhibitory effect of D-pen on cross-link formation in newly synthesized collagen was readily reversible. By contrast, cross-link deficiency lasting beyond the observation period was observed in the higher polymeric collagen variants released by dilute acid, heat exposure or limited pepsin proteolysis as estimated by solubility, α/β chain ratio and/or aldehyde content. By SDS-polyacrylamide gel electrophoresis on gels containing 3.6 M urea it was shown that purified dermal acid soluble collagen from treated animals consisted of a mixture of type I and III collagen, whereas only type I collagen was detected in controls. The band pattern was identical in reduced and unreduced collagen samples. Four weeks after D-pen discontinuance type III collagen had disappeared from the acid extract. Moreover, the ratio of type III to type I collagen in the pepsin digest from both granulation tissue and skin showed a persistent rise with D-pen. These observations indicate that D-pen destabilized type III collagen in particular by interference with its disulfide linkages. The amount of granulation tissue remained unaffected throughout the experiment, whereas the skin collagen content decreased at the higher dose level. The regeneration was not completed by the end of the observation period. Modulation of the molecular stability of granuloma collagens may be of relevance for the antirheumatoid effect of D-pen, but the sustained effect on normal tissues may imply a long standing impairment of their supportive capacity.

D-penicillamine (D-pen) is a potent, remission inducing agent in rheumatoid arthritis [1]. The mechanism of this effect is unknown. The drug has a well established ability to inhibit the biosynthesis of collagen cross-links by trapping aldehydic functional groups in a thiazolidine complex and to labilize Schiff base type cross-links [2]. Recent observations have shown, that the principal effect of D-pen is to inhibit the formation of polyfunctional cross-links [3], and that the interchain disulfide bonds of type III collagen in rat skin are cleaved *in vivo* by D-pen [4].

In patients with rheumatoid arthritis treated with D-pen a correlation has been demonstrated between clinical improvement and reduction in skin collagen synthesis [5] and cross-linking [6], suggesting that interference with the metabolic pathway of collagen may be of importance to the control of the disease activity. On the other hand, lesions such as increased skin friability [4, 7], cutis laxa [8] and elastosis perforans serpiginosa and lung cysts [9] are probably attributable to the lathyrogenic activity of D-pen.

Complete reversal of the D-pen effect on collagen cross-linking has been reported to occur in rat skin after withdrawal of the drug as estimated by solubility and biomechanical parameters [10–14]. These observations are extended in the present investigation by the simultaneous study of skin and granulation tissue and by additional analysis of polymeric collagen in terms of maturation and collagen polymorphism.

MATERIALS AND METHODS

Ninety-six SPF male Sprague–Dawley rats weighing about 200 g at the start of the experiment were randomly allocated to treatment and pairfed control groups each consisting of 12 animals. Only rats which gained weight constantly during one week of adaptation were included. Under ether anesthesia two viscose–cellulose (Visella®) sponges were inserted into the subcutaneous space via a transverse lumbar incision and placed symmetrically around the spine in the infrascapular region. The sponges measured 10 × 10 × 20 mm and had an average dry weight at 85 mg (range 80–90 mg). Before implantation the sponges were sterilized by boiling for 30 min in physiological saline. The treatment groups received D-pen dissolved in two ml of water via a gastric tube in daily doses of 100 or 500 mg/kg. The animals were housed six together with free access to rat pills and water. The control animals were given an equal volume of water by the same route of administration. Pairfeeding was accomplished by restricting the food supply according to the average daily consumption of the corresponding treatment group. The control animals were kept singly in the cages with free water intake. After 42 days half of the animals were killed by decapitation, the other half after additionally 28 days without medication.

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Tissue sampling and preparation. The back skin was shaved with an animal clipper immediately after decapitation, and two circular skin biopsies each measuring 5.3 cm^2 were collected symmetrically around the spine in the lumbar region at least one cm apart from the wound edge. Subcutaneous fat was scraped off by a scalpel. Perispongial tissue was carefully removed from the granulomas, and a sample was taken for histological examination. After weighing, the tissues were freeze dried to constant weight, powdered by means of a Wiley Micro Mill and stored at −20°.

Collagen was estimated by the hydroxyproline (Hyp) content of tissue samples and extracts by the method of Stegemann [15] after hydrolysis in 6 M hydrochloric acid at 118° for 18 hr. Neutral salt soluble collagen (NSC) was extracted by horizontal shaking overnight at 4° of pulverized tissue samples in 0.5 M NaCl + 0.02 M Tris–HCl, pH = 7.4. Three serial extractions were performed and following each extraction, the soluble compounds were isolated by centrifugation for 30 min at 4° and 20,000 rpm in a Sorvall refrigerated centrifuge. The extracts were passed through Frisenette filters and pooled. Acid soluble collagen (ASC) was extracted in a similar manner using 0.5 M acetic acid, pH = 3.4. An aliquot of each extract was hydrolysed in an equal volume of 12 M hydrochloric acid for the estimation of Hyp. Acid soluble collagen was purified according to Heikkinen [16]. Heat soluble collagen (HSC) was extracted from the acid insoluble tissue residue by dissolution in 0.2 M phosphate buffer, pH = 6.4 containing 0.25 M NaCl and shaking for 1 hr at 60° [17]. After centrifugation for 30 min at room temperature and 12,000 rpm, an aliquot of the supernatant was taken for Hyp analysis. Collagen was precipitated from the remaining extract by dialysis against disodiumphosphate and demineralized water at 4° and freeze dried. The yield from granulomas was too small for further analysis. Pepsin soluble collagen (PSC) was released by limited pepsin proteolysis according to Sykes [18].

Analyses on purified collagen. Aldehyde estimation was performed on aliquots of purified HSC from skin by the colorimetric method of Paz [19]. Acetic aldehyde was used as standard. The α/β chain ratio of ASC and PSC from both tissues and from dermal HSC was measured by SDS polyacrylamide gel electrophoresis followed by densitometric scanning of the gels according to Furthmayr and Timpl [20]. In order to study the molecular heterogeneity of skin ASC, samples were electrophoresed on polyacrylamide gels containing 3.6 M urea by which the migration rate of α1(III) chains is retarded so that the otherwise co-migrating α1(I) and α1(III) chains are resolved [21]. Unreduced and 2-mercaptoethanol reduced samples were run in parallel in order to investigate whether reduction of interchain disulfide bonds of type III collagen had occurred *in vivo* with D-pen. The relative amount of type I and III collagen in PSC from both tissues was determined as the ratio between their constituent α1(III) and α1(I) + α2 chains by interrupted gel electrophoresis as described by Sykes [18]. The intra-assay variation was 5.2%.

Statistical method. Treatment and control groups were compared by means of Students *t*-test. Only P values < 0.05 at a two tailed test were considered to be statistically significant. In case of inhomogeneity of variance (*F*-test) the Mann–Whitney *U*-test was used.

RESULTS

Two animals treated with D-pen 500 mg/kg/day were excluded because of granuloma infection. A transiently increased weight gain at maximally 9% was observed between day 9 and 24 in the D-pen 100 mg/kg group (P values <0.05–0.01). In rats treated with D-pen 500 mg/kg the body weight was reversibly decreased by about 9% as compared to controls ($P < 0.01$), and the skin was easily torn leading to slowly healing dilacerations.

The collagen concentration remained unchanged in both tissues. In the high dose group the total collagen content per skin biopsy showed a partially reversible decline by about 20% below the control values, whereas the amount of granuloma collagen remained unaffected (results not listed).

The proportion of skin and granuloma collagen extractable by neutral salt solvent and dilute acetic acid was increased at both dose levels (Fig. 1). The increased solubility was associated with higher α/β chain ratio in purified acid soluble collagen (Table 1). Except for the acid soluble skin collagen the values had become normal within the drug free interval.

When purified, dermal ASC was electrophoresed on polyacrylamide gels containing 3.6 M urea, two additional bands appeared migrating behind the β11(I) and α1(I) bands respectively in the experimental but not in the control groups (Fig. 2). The band pattern was identical in reduced and unreduced samples, and the effect had vanished by the end of the experiment indicating a reversible molecular heterogeneity of dermal ASC with D-pen.

The 60° labile collagen pool decreased in skin and increased in granulation tissue after D-pen in both doses (Fig. 1). Except for skin HSC at the highest dose level this effect was fully reversible. By contrast, the increased aldehyde content of dermal HSC was still elevated four weeks after cessation of the treatment, whereas the α/β ratio showed a reversible augmentation (Table 1).

The proportion of skin collagen extractable by limited pepsin digestion showed a partially reversible decrease with D-pen, whereas barely significant increases were found in granulation tissue (Fig. 1). The α/β ratio of skin and granuloma PSC increased, in the latter only at the highest dose level. In contrast to the alteration in skin, this effect could still be detected in granulation tissue after the treatment had been stopped (Table 1).

The type III to type I collagen ratio of dermal PSC showed a lasting rise at both dose levels, and the same effect was observed in granulation tissue in the 100 mg/kg group (Table 1).

DISCUSSION

D-pen inhibited the cross-link formation in both newly synthesized and polymeric subsets of skin and granuloma collagen as reflected by decreased dermal

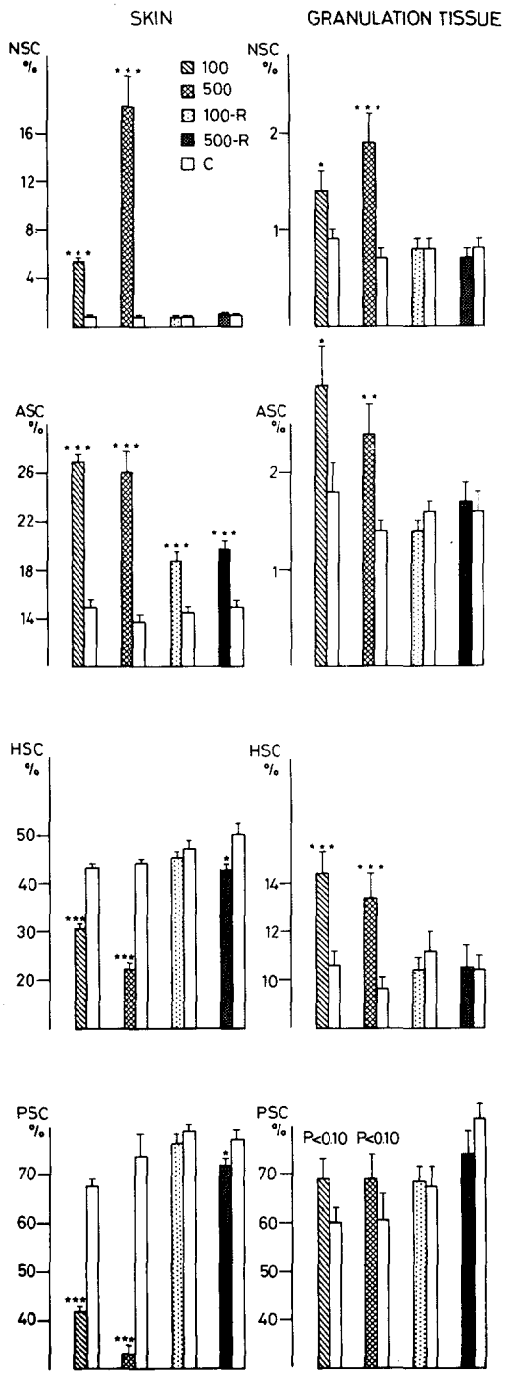

Fig. 1. Collagen solubility profile during and after D-pen treatment. Values are expressed in per cent of total tissue collagen (mean ± S.E.M.). Abbreviations: 100 and 500, D-pen dose in mg/kg/day; R, reversibility group; NSC, neutral salt soluble collagen; ASC, acid soluble collagen; HSC, heat soluble collagen; PSC, pepsin soluble collagen. *, **, *** = $P < 0.05$, 0.01 and 0.001, respectively.

Table 1. Reversibility of D-pen effects on purified soluble collagen fractions from skin and granulation tissue

		100 $n = 12$	C $n = 12$	500 $n = 10$	C $n = 12$	100-R $n = 12$	C $n = 12$	500-R $n = 12$	C $n = 12$
Aldehyde × 10^3/Hyp ($\mu g/\mu g$)									
HSC	S	2.7 ± 0.1*** (11)	2.0 ± 0.1 (11)	2.4 ± 0.1***	1.7 ± 0.1	2.1 ± 0.2**	1.5 ± 0.1	1.8 ± 0.1** (11)	1.6 ± 0.01
α/β ratio									
ASC	S	1.60 ± 0.08***	0.86 ± 0.02 (11)	1.85 ± 0.26**	0.89 ± 0.04 (10)	1,15 ± 0.09* (9)	0.92 ± 0.03 (9)	1.05 ± 0.05** (9)	0.89 ± 0.03 (9)
HSC	S	1.00 ± 0.03***	0.71 ± 0.02	1.22 ± 0.07***	0.69 ± 0.02	0.67 ± 0.02* (11)	0.60 ± 0.02 (11)	0.71 ± 0.03	0.70 ± 0.02
PSC	S	2.42 ± 0.03***	2.09 ± 0.05	2.36 + 0.05*** (9)	2.03 ± 0.06	2.19 ± 0.04	2.17 ± 0.03	2.32 ± 0.05	2.19 ± 0.04
ASC	G	2.16 ± 0.12***	1.29 ± 0.07	3.09 ± 0.17***	1.49 ± 0.05	1.64 ± 0.04	1.55 ± 0.09 (11)	1.58 ± 0.07	1.45 ± 0.09
PSC	G	1.88 ± 0.04	1.94 ± 0.07 (11)	2.52 ± 0.09**	2.14 ± 0.06 (11)	1.89 ± 0.07	2.04 ± 0.08	2.45 ± 0.07*	2.24 ± 0.07
III/I ratio									
PSC	S	0.28 ± 0.02*	0.23 ± 0.01	0.29 ± 0.01** (8)	0.24 ± 0.01 (11)	0.30 ± 0.01***	0.22 ± 0.01	0.30 ± 0.02* (11)	0.23 ± 0.01
PSC	G	0.60 ± 0.02*	0.52 ± 0.02 (11)	0.53 ± 0.02	0.53 ± 0.03 (11)	0.65 ± 0.03* (10)	0.52 ± 0.03 (11)	0.50 ± 0.02	0.53 ± 0.01

Values are mean ± S.E.M. Group size other than indicated at the top of the table is given in brackets. Abbreviations: S, skin; G, granulation tissue; Hyp, hydroxyproline; III/I ratio, ratio of type III to type I collagen. Further details are given in legend to Fig. 1.

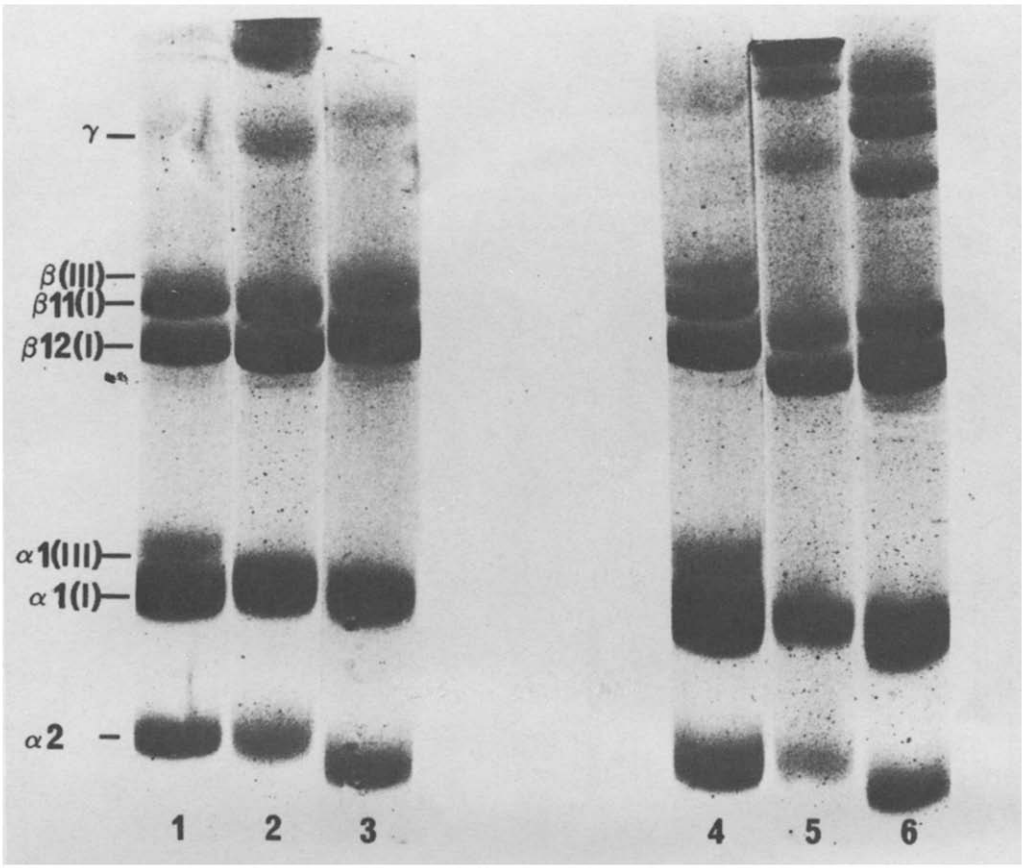

Fig. 2. SDS polyacrylaminde gel electrophoresis of purified, acid soluble skin collagen in the presence of 3.6 M urea [21]. 1–2: D-pen 100 mg/kg/day for 42 days and following withdrawal respectively. 3: Control (42 days). 4–5: D-pen 500 mg/kg/day for 42 days and after drug withdrawal, respectively. 6: Control (70 days). The samples were unreduced. Notice the reversible appearance of type III collagen mono- and dimers in the experimental groups.

breaking strength in the presence of altered solubility properties and increased α/β chain ratio and/or aldehyde content in purified collagen extracts (Fig. 1 and Table 1). Cleavage of labile Schiff base intermediates [2] in the presence of almost complete blocking of the maturation process can explain the depletion of dermal polymeric collagen in contrast to the small increments observed in the same fractions of granuloma collagen, which is primarily stabilized by D-pen insensitive keto-imine cross-links [22]. Both HSC and PSC appeared to be cross-link deficient. Since the major cross-linking regions located in the C and N terminal sequences of the collagen molecule are preserved by heat denaturation and removed by pepsinization it can be concluded, that the D-pen effect is not restricted to the non helical extensions of the molecule, but that additional helical cross-linking sites [23] are also blocked by D-pen.

The collagen population isolated after pepsinization is commonly considered to be representative of total tissue collagen. However, different yields of collagen obtained by pepsin proteolysis of normal and diseased tissue may be an indication of metabolic as well as structural differences [24–26]. In both skin and granulation tissue small increments in the relative amounts of type III collagen were recorded with D-pen (Table 1). It is not likely that D-pen changes the collagen phenotype of fibroblasts because approximately equal proportions of type I and III collagen are synthesized by rheumatoid synovium in the presence of D-pen [26]. Alternatively D-pen may have a preferentially destabilizing effect on type III collagen polymers by reducing the number of interchain disulfide bonds. This conjecture was supported by the demonstration of type III collagen mono- and dimers in dermal ASC from treated, but not from control animals when the material was electrophoresed on polyacrylamide gels containing 3.6 M urea (Fig. 2) [21]. In unreduced collagen samples type III collagen is normally present as γ-components stabilized by intramolecular disulfide linkages. Since the band pattern was identical in the reduced and unreduced samples, the interchain disulfide bonds of the native type III collagen molecules extracted by dilute acid must have been disrupted *in vivo* by the thiol group of D-pen. Thus, these observations indicate that D-pen acts on type III collagen both by diminishing the number of aldehyde cross-links and by reducing the extent of intra- and possibly intermolecular [27] disulfide bonding leading to re-distribution of type III collagen between different polymeric collagen variants.

The neutral salt extractable collagen fraction of

both skin and granulation tissue was readily restored upon withdrawal from D-pen as reported earlier for rat skin after one to four weeks treatment in comparable and higher doses [10–14]. By contrast, dermal ASC was still defective in cross-links long after cessation of the treatment (Fig. 1, Table 1). Despite almost complete quantitative reversion of the polymeric collagen pools (Fig. 1), increased aldehyde content of dermal HSC and increased α/β ratio of granuloma PSC could still be detected by the end of the recovery period (Table 1). This can explain the observation by Nimni *et al.* [10], that the rate of increase in chemically determined insoluble collagen after withdrawal of D-pen is greater than the gain in tensile strength.

The persistent rise in the type III/I collagen ratio after D-pen had been stopped is presumably a sign of rapid re-polymerization of type III collagen due to re-formation of the disulfide bonds, which render type III collagen insoluble under non denaturing conditions [28]. The concomitant disappearance of type III collagen from dermal ASC in the treatment groups (Fig. 2) is consistent with this assumption.

D-pen is a slowly acting antirheumatic agent suggesting that its disease modifying activity may partially be attributable to effects on mediators of repair processes or on the macromolecules constituting the chronic inflammatory lesions. Thus, removal of excessively deposited collagen defective in cross-links may occur more readily due to its increased degradability by collagenase [29]. Moreover, a shift in the molecular stability of type III collagen, which occurs early at sites of injury [30, 31], may impair the basic lattice for the subsequent granuloma proliferation. On the other hand the lathyrogen like activity of D-pen implies a slowly subsiding hypersensitivity of normal skin to mechanical injury.

Acknowledgements—This study was supported by grants from the Danish Medical Research Council, the Danish League against Rheumatism and the Daell Foundation. The animal experiments were carried out at the Department of Dermatology H, Rigshospitalet, Copenhagen. D-penicillamine was a gift from Dista Co Ltd. The authors wish to thank Miss B. Staalø and Miss A. M. Hildebrandt for competent technical assistance.

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