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EFFECT OF D-PENICILLAMINE ON GLYCOSAMINOGLYCAN CHANGES DURING RABBIT TENDON REGENERATION*

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Abstract—Tendon regeneration was studied in rabbits treated with 70 mg/kg/day of D-penicillamine by subcutaneous injection. After 1 day of treatment a 1-cm piece of Achilles tendon was excised surgically and regeneration allowed to proceed for periods of time ranging from 4 days to 4 months, while continuing penicillamine treatment. Regeneration tissue was then collected and analyzed for collagen solubility, hexosamine–hydroxyproline ratio, glycosaminoglycan composition and solubility profile of the CPC complexes of the glycosaminoglycans. Collagen solubility of regeneration tissue was generally increased 2 to 4-fold compared with untreated control values. The patterns of change of glycosaminoglycan composition and the CPC complex solubility profiles were generally quite similar in the control and penicillamine-treated tissues except for a systematic departure in the chromatographic fraction corresponding to dermatan sulfate. The dermatan sulfate fraction in untreated controls rose steadily from 40 per cent of total after 1 month of regeneration to 54 per cent at 4 months; this fraction in penicillamine-treated rabbits remained constant at about 40 per cent of total for the same period. Analogous changes in composition were observed in mature tendon from penicillamine-treated rabbits. Increasing the dose of penicillamine to 140 mg/kg/day caused excessive toxicity.

WE HAVE recently reported on time related changes in glycosaminoglycan composition associated with regeneration of rabbit tendon.[1] Briefly, we found that a hyaluronate-like glycosaminoglycan is most abundant during the early regeneration process and that a dermatan-sulfate-like glycosaminoglycan is most abundant late in the regeneration process. Chondroitin-4-sulfate-like material is most abundant at intermediate regeneration periods and a small fraction of chondroitin-6-sulfate-like material makes up a remarkably constant percentage of the total glycosaminoglycans regardless of regeneration time. The possibility was entertained that these patterns of change might have some bearing on the maturation process. Thus, if maturation of regeneration tissue were arrested, changes in glycosaminoglycan composition reflecting a less mature connective tissue might result. Administration of D-penicillamine to experimental animals is known[2] to prevent collagen cross-linking and result in the accumulation of large amounts of soluble collagen. This treatment was used as a means of preventing tendon maturation. Glycosaminoglycan changes indicative of a less mature regeneration tissue resulted from penicillamine treatment but the changes in composition were rather subtle and appeared only after long term regeneration.

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MATERIALS AND METHODS

The methods for the preparation of regeneration tissue and for its analysis have been previously published in detail.[1,3,4] Briefly, 1-cm pieces of rabbit Achilles tendon were excised surgically, and regeneration was allowed to proceed for 4, 7, 10, 14, 21, 28, 42, 56 and 112 days (four animals per time period). Regeneration tissue was stored deep frozen and cut into 10μ slices by means of a cryostat prior to analysis. Aliquots were used to determine collagen solubility, total hexosamine content, glycosaminoglycan composition and solubility profile of CPC*—complexes of the glycosaminoglycans. The use of a cryostat in the preparation of tissue samples represents a modification of the previous method.

Bulk D-penicillamine was obtained from E. R. Squibb and Co. It was used as a 10 per cent solution in physiological saline, sterilized by filtration through a suitable cellulose acetate membrane, and administered by daily subcutaneous injection in the back. Penicillamine treatment was initiated one day prior to tendon excision. The dose in most cases was 70 mg D-penicillamine/kg body weight/day. Some animals were maintained on 140 mg/kg/day. The animals were weighed once weekly.

RESULTS

The results of collagen solubility studies on regeneration tissues of various ages from rabbits treated with a daily dose of 70 mg/kg body weight of D-penicillamine are listed in Table 1. The data represent the percentage of the total hydroxyproline

TABLE 1. HYDROXYPROLINE AND HEXOSAMINE CONTENT OF TENDON REGENERATION TISSUE FROM RABBITS TREATED WITH 70 mg/kg/day OF D-PENICILLAMINE AFTER VARIOUS PERIODS OF REGENERATION

	Regeneration time (days)								
	4	7	10	14	21	28	42	56	112
Total hydroxyproline* (mg)	6·6	11·0	13·6	11·5	12·8	27·0	39·8	46·6	33·1
Neutral salt-soluble hydroxyproline (% of total)	0·9	1·7	1·4	2·1	2·2	2·1	2·3	1·7	1·8
Acid citrate-soluble hydroxyproline (% of total)	9·6	9·8	4·8	5·5	4·8	8·2	12·4	8·8	7·1
Total hexosamine* (mg)	1·2	1·8	1·1	1·3	0·8	1·9	2·4	1·9	2·0
Hexosamine/hydroxyproline	0·18	0·16	0·08	0·11	0·06	0·07	0·06	0·04	0·06
Sample size (mg dry wt.)	303	402	292	316	227	699	842	798	735

* Pool of four tendons.

extractable with neutral salt and acid citrate solutions. Total hydroxyproline and total hexosamine values in mg per pool of four tendons are also listed as are the ratios of hexosamine–hydroxyproline. The outstanding difference between these data and comparable ones for regeneration tissue from untreated rabbits[1] lies, as expected, in collagen solubility. This is illustrated in Fig. 1 which represents a comparison of the percentage of soluble collagen (sum of neutral salt-soluble and acid citrate-soluble fractions) extractable from regeneration tissue of untreated and penicillamine treated rabbits. It is clear despite a great deal of variation that the soluble collagen values in

* Abbreviations used: CPC, cetyl pyridinium chloride; BAPN, β-aminopropionitrile.

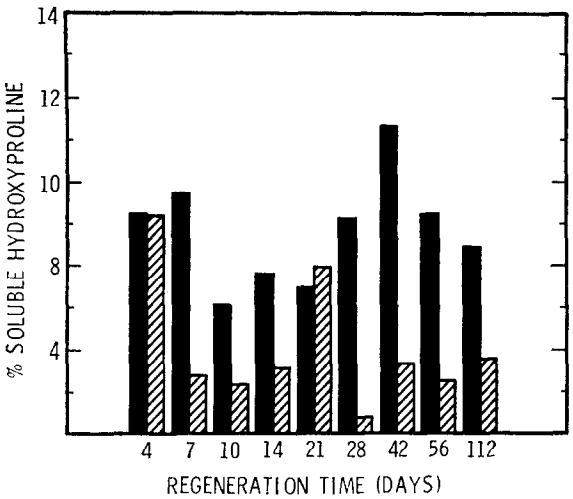

FIG. 1. Effect of penicillamine treatment on collagen solubility of rabbit tendon regeneration tissue after various periods of regeneration. The height of the bars represents the sum of neutral salt-soluble and acid citrate-soluble hydroxyproline as a percentage of total hydroxyproline. Dark bars, penicillamine treated (70 mg/kg/day), stipled bars, untreated controls.

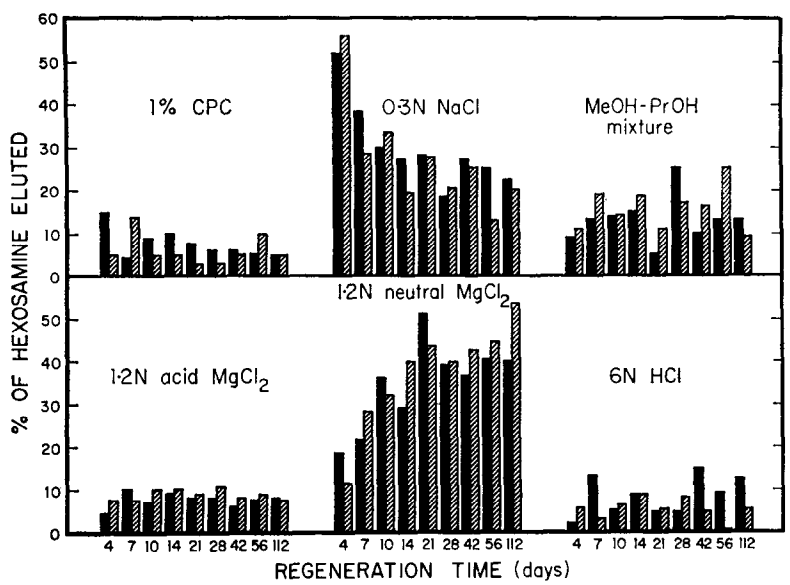

FIG. 2. CPC-column chromatography of the glycosaminoglycans of rabbit tendon regeneration tissue grown for various periods of time in penicillamine-treated and control animals. The height of the bars represents the amount of hexosamine eluted by a solvent expressed as a percentage of the hexosamine eluted by all solvents from a particular sample. Data from each solvent system are grouped together to facilitate comparisons. Stipled bars, untreated controls from Ref. 1; dark bars, penicillamine treated, 70 mg/kg/day.

penicillamine treated rabbits are generally two- to fourfold higher than in untreated rabbits. The remainder of the data listed in Table 1 are not very different from those previously published. The values for total collagen at 42 and 56 days are substantially higher than those in the untreated control study, but the hexosamine values are similarly elevated and the hexosamine–hydroxyproline ratios do not differ greatly. The trend of a change from higher hexosamine–hydroxyproline ratios in early regeneration to lower ratios later in the regeneration period, which was already noted in the control study is apparent in the penicillamine study as well. The fact that the hexosamine–hydroxyproline ratios in late regeneration of penicillamine treated rabbits appear slightly elevated compared with corresponding controls may be fortuitous, since values generally show substantial variations.

Figure 2 presents data on CPC column chromatography of the glycosaminoglycans of tendon regeneration tissue from penicillamine-treated rabbits compared with untreated controls. Despite fluctuations in some of the fractions, and particularly in the fraction eluted with methanol–propanol mixture, the general trend of the time-related patterns of change of the penicillamine-treated and control tissue glycosaminoglycans are remarkably similar. The only systematic deviation from this general finding was encountered in the 1·2 N neutral magnesium chloride fraction. After an initial rise to a peak at 3 weeks very similar to the control curve it fell off to approximately 40 per cent and did not increase again, whereas the control eventually rose to over 50 per cent of the total.

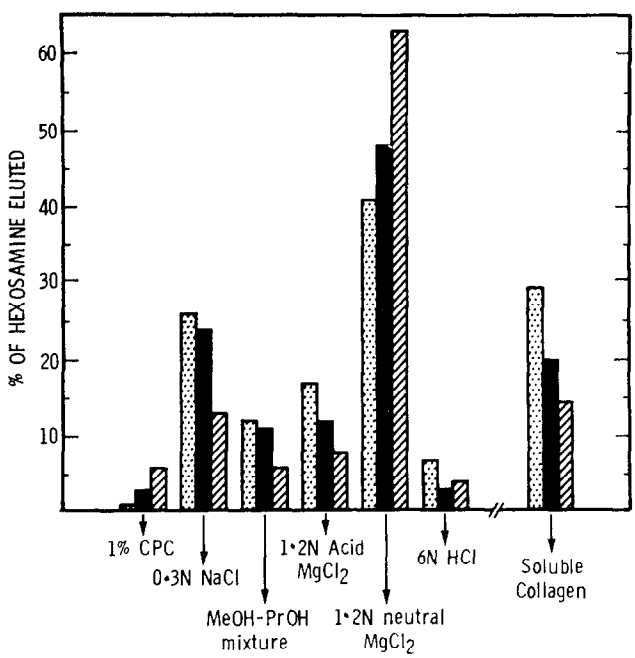

FIG. 3. Effect of penicillamine treatment on glycosaminoglycan composition of mature, non-wounded rabbit tendon as analyzed by CPC column chromatography. Solvents listed under each group of bars, ordinate on left as in Fig. 2. Also included are data on collagen solubility. Ordinate on right as in Fig. 1. Stipled bars, untreated control; dark bars, penicillamine treated (70 mg/kg/day for 7 days); dotted bars, penicillamine treated (140 mg/kg/day for 10 days).

Mature tendons from the hind legs not subjected to surgical procedures but of course also exposed to penicillamine were occasionally analyzed as well. These differed from untreated mature tendon, mainly by a fairly substantial decrease in the percentage of the total glycosaminoglycans eluted in the 1·2 N neutral magnesium chloride fraction and corresponding increases in some of the other fractions, particularly the fraction eluted with 0·3 N sodium chloride. Figure 3 shows the relative glycosaminoglycan composition of mature rabbit tendon subjected to penicillamine *in vivo* for 10 days at 140 mg/kg, or for 7 days at 70 mg/kg compared with that of untreated mature tendon.

Figure 4 shows solubility profiles of the CPC-complexes of the glycosaminoglycans from rabbit tendon regeneration tissues grown for various periods of time while the animal was treated with a daily dose of 70 mg/kg body weight of D-penicillamine.

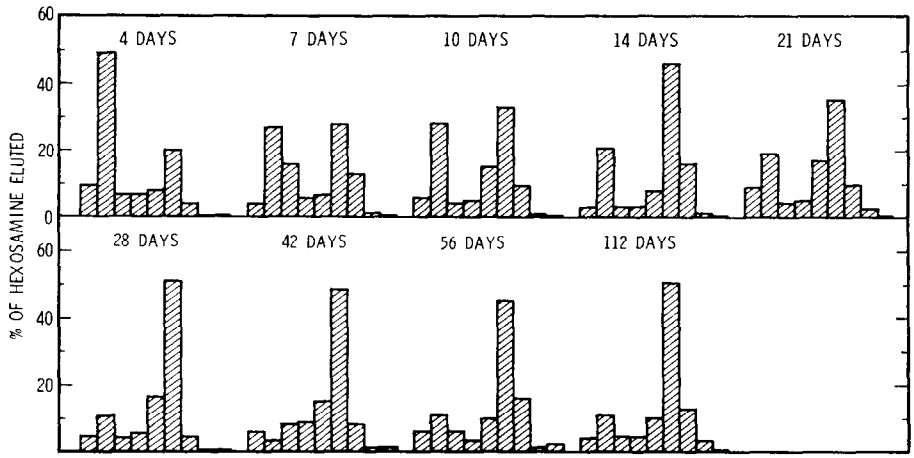

FIG. 4. Solubility profiles of the CPC-complexes of glycosaminoglycans from rabbit tendon regeneration tissues grown for various periods of time while the animal was on 70 mg/kg/day of D-penicillamine. The height of the bars represents the amount of hexosamine eluted by a solvent expressed as a percentage of the hexosamine eluted by all solvents. The solvents are (from left to right) 1% CPC; the following normalities of magnesium chloride in 0·05% CPC: 0·2, 0·4, 0·6, 0·8, 1·0, 1·2 and 1·4 N. The last solvent is 6 N hydrochloric acid.

The elution patterns are not identical in every detail with the ones previously published for untreated controls, but they are very similar and in view of the lack of specificity of the solvents employed, no definite change can be related to penicillamine treatment.

A group of rabbits were given penicillamine at a daily dose of 140 mg/kg body weight in an attempt to accentuate the changes observed in the glycosaminoglycan composition of tendon regeneration tissues on the lower dose of penicillamine. At the 140 mg/kg dose penicillamine was found to be acutely toxic. A number of rabbits died, while the remaining ones appeared to be severely ill. The diet of these rabbits was not fortified with pyridoxal and the toxicity observed may have been the result of induced pyridoxal deficiency. The results of this study will not be presented in detail since it appears that penicillamine at the higher dose exerts a profound systemic effect rather than to act as a specific means to prevent connective tissue maturation. The data obtained had no similarity with the previously established patterns of change. Thus,

the CPC fraction eluted with 0·3 N sodium chloride made up only about 10 per cent of the total glycosaminoglycan hexosamine at 4 days and increased slowly to about 30 per cent of total over a period of 4 weeks. On the other hand the fraction eluted with 1·2 N neutral magnesium chloride made up over 30 per cent of the total hexosamine eluted at 4 days and fluctuated around that value for the entire 4-week period of the experiment. Solubility profiles similarly reflected the paucity of material soluble at the low salt concentration in young regeneration tissue from rabbits treated at a high dose of penicillamine. Interestingly, analysis of mature tendon from rabbits on 140 mg/kg/day of penicillamine reflected the changes anticipated from the experiments with low dosage penicillamine, namely, a reduction in the fraction representing dermatan sulfate and an increase in the hyaluronate fraction along with an elevated level of soluble collagen (Fig. 3).

DISCUSSION

The present study represents an attempt to obtain information on possible functions of the glycosaminoglycan constituents of connective tissue. Previous work[1] has permitted establishing definite temporal patterns of change of glycosaminoglycan composition during tendon regeneration in the rabbit. The possibility was entertained that these patterns of change might have some bearing on the maturation process, and that if maturation of tissue were arrested, a glycosaminoglycan pattern reflecting a less mature connective tissue might result.

Penicillamine and other lathyritic agents do not appear to impair the synthetic activity of the fibroblast. Rather, these agents block connective tissue maturation through their inhibitory effect on collagen crosslinking. It is for this reason that penicillamine was used in this study. As expected from reports in the literature,[2, 5, 6] its principal effect was an increase in soluble collagen in the regeneration tissue. It was of some concern that none of the values for soluble collagen markedly exceeded 10 per cent, whereas data in the literature indicate much higher values for soluble collagen of skin from penicillamine treated rats. In order to investigate this discrepancy, skin from a rabbit treated with penicillamine for 14 days was analyzed. It had 37 per cent soluble collagen. Thus, it would appear that penicillamine treatment was basically successful in increasing collagen solubility but that tendon regeneration tissue is relatively more resistant to the action of penicillamine than skin. Such a differential effect of penicillamine could be due to an intrinsic difference in the tissue or due to the inability of penicillamine to reach the particular extravascular connective tissue compartment. If the latter were the case *in vitro* treatment of the respective, finely divided, tissues with penicillamine should yield similar collagen solubilities. This is not so since rabbit skin and tendon yielded collagen solubilities of 29 and 13 per cent after *in vitro* penicillamine treatment for 24 hr (0·5 mg/ml in saline at 4°). Tendon collagen appears relatively resistant to the action of penicillamine. Similar tissue differences might explain Nimni's[2] finding that penicillamine affects predominantly soft connective tissues and is very much less effective than BAPN in producing lathyritic changes in the bones.

The patterns of change of tendon glycosaminoglycans in early regeneration during penicillamine treatment were generally unaffected by the treatment. During late regeneration a slight but definite change was observed in the chromatographic fraction corresponding to dermatan sulfate. This fraction in treated as in control tissues made

up a rapidly increasing part of the total glycosaminoglycans during the first 21 days, and in both treated and control tissues it was somewhat reduced again at 28 days. However, in the absence of penicillamine the dermatan sulfate-like fraction increased again subsequently and eventually reached 53 per cent of total at 112 days. In the penicillamine-treated tissues on the other hand, the dermatan sulfate-like fraction was reduced to about 40 per cent of total after 28 days and remained at that level for the next 3 months. That this is not likely to be a fortuitous finding can be inferred from the observation that mature, non-wounded Achilles tendon from rabbits treated with penicillamine showed changes of a similar nature, namely reduction of the dermatan sulfate-like fraction to about 40 per cent of total and corresponding increases in other fractions. It has been observed by Loewi and Meyer[7] that dermatan sulfate appears to be associated with thick, insoluble collagen fibers of pig skin. Anseth and Fransson[8] and Anseth[9] found dermatan sulfate associated with corneal scar tissue and corneal grafts of poor optical quality but not in grafts of good quality. This again may be due to the fact that dermatan sulfate is often associated with mature coarse collagen fibers of greatly varying diameter.[10] Similarly, our control data[1] on unhampered tendon regeneration indicated that high levels of dermatan sulfate were most closely associated with late tendon regeneration and mature tendon. The present data support these concepts by showing a decrease in dermatan sulfate fraction under circumstances where collagen crosslinking and tendon maturation is prevented.

When an attempt was made to accentuate the relatively mild changes in the dermatan sulfate-like fraction by increasing the dose of penicillamine to 140 mg/kg/day, acute toxicity resulted. The patterns of change of the glycosaminoglycan fractions during the regeneration process were radically altered. However, in view of the systemic changes it is unlikely that the experiments with high doses of penicillamine contribute to the question of the role of glycosaminoglycans in tendon regeneration.

The medical and surgical applications of lathyrism have been reviewed recently by Peacock, Madden and Smith.[11] Penicillamine has been prescribed on an experimental basis in the treatment of patients with rheumatoid arthritis. The dose of 70 mg/kg/day penicillamine used here corresponds to the highest recorded dose used in patients with connective tissue diseases (5 g/day) and is several fold higher than the more conservative dose of 1–2 g/day used in our clinical studies.[12]

Investigations of the effect of lathyritic agents on glycosaminoglycan composition of connective tissues have been undertaken before. Levene, Kranzler and Franco-Browder[13] subjected chick embryo cartilage to BAPN and concluded that BAPN did not affect the glycosaminoglycan composition of that tissue. Glimcher, Seyer and Brickley[14] obtained quantitatively different results in the same system but did not study the glycosaminoglycan composition in detail. Conclusions in accord with those of Levene *et al.* were reached by Chakrabarti, Sengupta and Aikat[15] after treating rats in which granulomata had been induced by means of polyvinyl sponge implants, with BAPN. These results do not conflict with our present data since neither study was carried out sufficiently long so that one would expect any changes on the basis of the present findings and in fact the chick cartilage is apparently devoid of dermatan sulfate. On the other hand Pedrini-Mille and Pedrini[16] and Lenzi and Pedrini-Mille[17] found that the lathyrogen aminoacetonitrile had a specific effect on the chondroitin sulfates of skin and bone fracture callus from rats. Most of these data are difficult to interpret since individual glycosaminoglycans were not usually separated. An

attempt at exploring the effect of lathyrism on wound healing by electron microscopy revealed no difference from controls (Jacques[18]). It is presently difficult to reconcile the diversity of these results with a uniform role of each glycosaminoglycan fraction in the architecture of connective tissues although the diversity is not entirely unexpected in view of the variety of organ systems and lathyrogens used by various workers.

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