Tohoku J. exp. Med., 1984, **142**, 437-443

Biochemical Studies on the Collagen of the Palmar Aponeurosis Affected with Dupuytren's Disease

TADAMASA HANYU,* TATSUYA TAJIMA, TOHRU TAKAGI,† SATOSHI SASAKI,† DAISABURO FUJIMOTO,‡ MAMORU ISEMURA§ and ZENSAKU YOSIZAWA§

Department of Orthopedic Surgery, Niigata University School of Medicine, Niigata 951, † Department of Biochemisty, School of Dentistry, Tokyo Medical and Dental University, Tokyo 133, ‡ Department of Chemistry, Hamamatsu University School of Medicine, Hamamatsu 431-31, and § Department of Biochemistry, Tohoku University School of Medicine, Sendai 980

HANYU, T., TAJIMA, T., TAKAGI, T., SASAKI, S., FUJIMOTO, D., ISEMURA, M. and YOSIZAWA, Z. *Biochemical Studies on the Collagen of the Palmar Aponeurosis Affected with Dupuytren's Disease.* Tohoku J. exp. Med., 1984, **142** (4), 437-443 —— The palmar aponeurosis of patients affected with Dupuytren's disease was examinded for collagen characteristics with regard to extractability, polymorphism, and posttranslational modifications, and the results were compared with those from normal subjects. The increased proportion of type III collagen relative to type I collagen in the affected tissue confirmed the previous findings in this disease. A slight but significant increase in a ratio of glucosylgalactosylhydroxylysine to galactosylhydroxylysine in the Dupuytren's tissue may be interpreted by the increase in the content of type III collagen. The affected tissue contained increased amounts of dihydroxylysinonorleucine as the reducible cross-link of collagen. These data support the view that Dupuytren's tissue contains collagen resembling that in granulation and embryonic tissues. Pyridinoline was shown to occur in normal and affected aponeurosis. No change in its content suggests that this cross-link is not involved in the pathogenesis of contracture in this disease. ———— collagen; aponeurosis; Dupuytren's disease; pyridinoline; hydroxylysine

Dupuytren's contracture is a human disease in which there is progressive and irreversible flexion of the fingers (Dupuytren 1834). In Japan only a limited

Received for publication, August 24, 1983.

Abbreviations: Glc-Gal-Hyl, glucosylgalactosylhydroxylysine; Gal-Hyl, galactosylhydroxylysine; DHLNL, dihydroxylysinonorleucine; HLNL, hydroxylysinonorleucine; DeDHLNL, dehydrodihydroxy lysinonorleucine.

* Present address: Niigata Prefectural Hospital for Rheumatic Disease at Senami, Murakami, Niigata 958. All correspondences should be addressed to this author.

number of cases of this disease has been reported (Maeda et al. 1979). Since several studies have suggested the biochemical changes in the collagen in this disease (Bailey et al. 1977; Bazin et al. 1980; Gelberman et al. 1980; Brickley-Parsons et al. 1981), we also examined biochemical characteristics of collagen in the tissue of our patients.

In the present paper, we report a comparison between normal and affected aponeurosis collagens with respect to extractability, polymorphism, and posttranslational modifications.

Materials and Methods

Materials. Pepsin was obtained from Sigma Chemical Co. (Saint Louis, Missouri). Glucosylgalactosylhydroxylysine (Glc-Gal-Hyl) and Gal-Hyl were prepared as described previously (Isemura et al. 1974). Authentic preparation of pyridinoline was prepared as described previously (Fujimoto and Moriguchi 1978). NaB^3H_4 was purchased from New England Nuclear (Boston, Massachusetts).

Tissues. Specimens of the palmar aponeurosis were sugically obtained from six male patients with Dupuytren's disease. Specimens from five patients with no evidence for this disease were used as normal aponeurosis samples.

Crude collagen fraction. The dissected tissues were homogenized with Polytron (Kinematica Co. Switzerland) in phosphate-buffered saline and the insoluble materials were collected by centrifugation. After washing thoroughly with water, the residue was freeze-dried to give a crude collagen fraction.

Soluble and insoluble collagen fractions. The crude collagen fraction was extracted with 0.5M acetic acid at 4°C overnight, and the extraction was repeated twice. The residue was then suspended in 0.5M acetic acid and digested with pepsin at 4°C for 48h. A ratio of pepsin to the crude collagen fraction was about 1 : 10 (by dry weight). The digestion was repeated once more. Acetic acid-soluble and pepsin-solubilized fractions were combined and purified by repeated precipitation with NaCl according to the method described previously (Sykes et al. 1976; Isemura et al. 1981). After exhaustive dialysis against 0.05M acetic acid, the preparation was freeze-dried, and termed as soluble collagen. The residue of the pepsin digestion was washed with water, and freeze-dried. This fraction was termed as insoluble collagen.

The degree of solubilization was expressed in % determined from a ratio of a dry weight of soluble collagen to a dry weight of soluble plus insoluble collagens.

Collagen polymorphism. Soluble collagen was subjected to interrupted sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Sykes et al. 1976). Gels were stained with Coomassie brilliant blue R-250 and a ratio of type III to type I collagens was determined densitometrically according to the method described previously (Sykes et al. 1976; Isemura et al. 1981). This ratio was calculated on the assumption that type I and type III collagens have compositions of $[\alpha_1(I)]_2\alpha_2(I)$ and $[\alpha_1(III)]_3$, respectively.

Hydroxylysine and its glycosides. The crude collagen fraction was hydrolyzed with 2.5N NaOH at 110°C for 20h, and hydroxylysine and its glycosides were analyzed as described previously (Hanyu et al. 1979; Isemura et al. 1981).

Pyridinoline. The crude collagen fraction was hydrolyzed with 6N HCl at 110°C for 24h. The hydrolysate was subjected to chromatography on a phosphocellulose column (1.2×7 cm) with a linear gradient elution from 0 to 0.5M HCl. The effluent was monitored for fluorescence with a Hitachi 244 fluorescence spectrophotometer and a fluorescent compound cerresponding to pyridinoline was isolated as described previously (Fujimoto and Moriguchi 1978). Identification of the compound with pyridinoline was carried out by fluorescence spectrophotomeric analysis (Fujimoto and Moriguchi 1978) and by high voltage

paper electrophoresis at pH 3.6 The pyridinoline content was determined spectrophotometrically by reading absorbance at 295 nm (Fujimoto and Moriguchi 1978). The total collagen content of the crude collagen fraction was calculated from the hydroxyproline content which was determined by amino acid analysis.

Reducible cross-links. DHLNL and HLNL in insoluble collagen were analyzed after reduction with NaB^3H_4 as described previously (Kuboki et al. 1977; Isemura et al. 1981).

Statistical analysis. The significance of a difference was tested by unpaired t-test and data for an average are presented as mean±standard deviation.

Results

Extractability of soluble collagen

The degree of solubilization of aponeurosis collagen with acetic acid and by proteolysis with pepsin from the crude collagen fraction was 32-75% with an average of 61±15% in the six cases of Dupuytren's disease and 5-24% with an average of 14±8.2% in the four normal cases. Thus, the solubilization of collagen of affected aponeurosis was significantly higher than that of normal collagen ($p<0.001$).

Collagen polymorphism

A ratio of type III to type I collagen in Dupuytren's soluble collagen (0.36±0.08, $n=5$) was higher than that in the normal specimen (0.23±0.06, $n=3$) ($p<0.05$).

Hydroxylysine and its glycosides

Hydroxylysine and its glycosides were analyzed for 6 affected and 3 normal subjects. The Dupuytren's crude collagen fraction had the higher content of the total hydroxylysine (1.22±0.31 residues per 1,000 amino acid residues) than the normal fraction did (0.91±0.11 residue) ($p<0.05$). The degree of glycosylation of hydroxylysine was significantly elevated ($p<0.01$) in the affected subjects (26.9±2.0%) as compared with that in the normal subjects (22.6±2.9). The mean value of a ratio of Glc-Gal-Hyl to Gal-Hyl in the Dupuytren's fraction was 2.65±0.26, which was significantly higher than that in the normal fraction, 2.18±0.22 ($p<0.05$).

Pyridinoline

Since there has been no report describing the occurrence of pyridinoline in the aponeurosis, we examined if the tissue contains this compound. The fluorescent compound isolated from the normal or Dupuytren's aponeurosis showed the same fluorescence spectrum as authentic pyridinoline (Fig. 1). High voltage paper electrophoresis also indicated that the isolated compound migrated at the same rate as authentic pyridinoline (Fig. 2). These results indicated that the normal and affected aponeurosis contained pyridinoline. The pyridinoline content in the

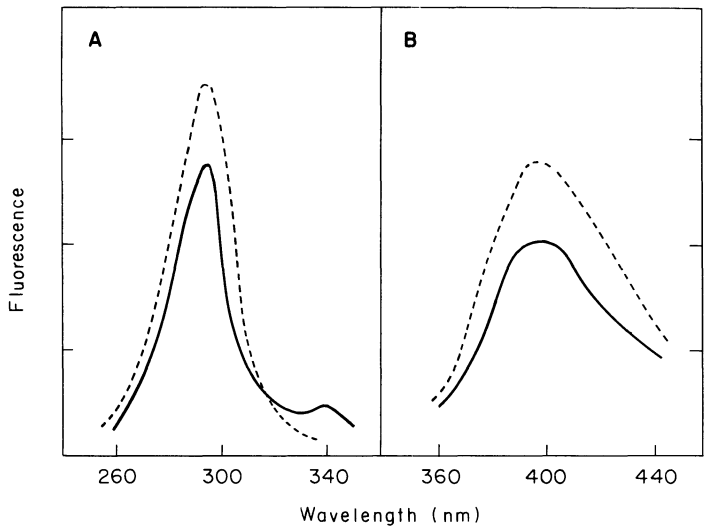

Fig. 1. Excitation and fluorescence spectra of the fluorescent compound isolated from Dupuytren's crude collagen fraction. Spectra were measured in 0.1 N HCl and intensity of fluorescence is expressed in arbitrary units in the ordinate. (A) Excitation spectra with fluorescence at 395 nm. (B) Fluorescence spectra with excitation at 295 nm. (——), Dupuytren's aponeurosis fluorescent compound; (------), authentic pyridinoline isolated from bovine Achilles tendon collagen.

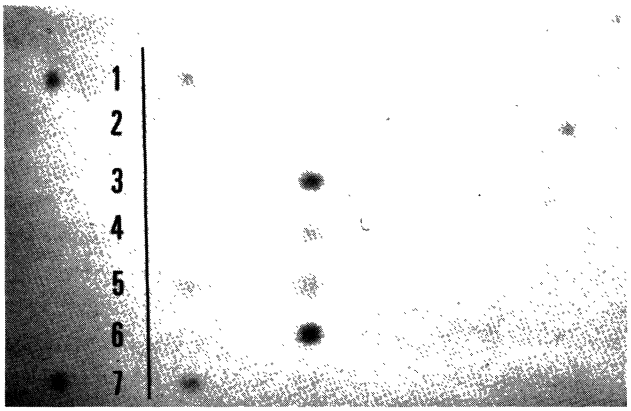

Fig. 2. High voltage paper electrophoresis of the fluorescent compound isolated from the aponeurosis crude collagen fraction. Electrophoresis was carried out at 50 V/cm for 1 h in pyridine : acetic acid : water (1 : 10 : 89 by volume), pH 3.6.
Spots are visualized with ninhydrin staining. The cathode is to the right. 1 and 7, glycine (right) and glutamic acid; 2, arginine; 3 and 6, authentic pyridinoline isolated from bovine Achilles tendon; 4, fluorescent compound derived from the Dupuytren's aponeurosis (case 4); 5, fluorescent compound derived from the normal aponeurosis (case 5).

crude collagen fractions derived from the six Dupuytren's patients was 0.059–0.072 residue per 1,000 amino acids with an average of 0.064 ± 0.002 residue and that from four normal subjects was 0.059–0.064 residue per 1,000 amino acids with an

average of 0.061 ± 0.001 residue. There was no significant difference in this content between normal and affected subjects.

Reducible collagen cross-links

Insoluble collagen derived from the Dupuytren's aponeurosis specimens contained substantial amounts of DHLNL and HLNL, while no or little DHLNL was detected in normal insoluble collagen. Since these compounds were measured only after being radio-labeled, the results are given in a ratio of DHLNL to HLNL rather than in their absolute contents. A ratio of DHLNL to HLNL ranged from 2.3 to 5.1 with an average of 3.4 ± 0.38 in six cases of Dupuytren's disease while that ranged from zero to 1.1 in three normal cases. These data are consistent with the previous report that DHLNL is virtually absent in normal aponeurosis collagen (Bailey et al. 1977; Bazin et al. 1980; Brickley-Parsons et al. 1981).

Discussion

The present study confirmed the previous results reported by several authors that Dupuytren's aponeurosis collagen shows increased proportions of type III collagen (Bailey et al. 1977; Bazin et al. 1980; Gelberman et al. 1980; Brickley-Parsons et al. 1981) and DHLNL as a reducible collagen cross-link as compared with age-matched normal aponeurosis tissues (Bailey ett al. 1977; Bazin et al. 1980; Brickley-Parsons et al. 1981). These characteristics are typical of embryonic and granulation collagens (Bailey et al. 1977), and suggest that the Dupuytren's tissue is abundant in newly and rapidly synthesized immature collagen (Brickley-Parsons et al. 1981). A higher solubilization of the Dupuytren's collagen observed here is consistent with other reports (Bailey et al. 1977; Bazin et al. 1980), although absolute percentages of solubilization were somewhat lower in this study.

It was shown that slight but significant difference in a relative content of two hydroxylysine glycosides. This is at least partly because of the relative increase in type III collagen, since type III collagen has a higher value for this ratio than type I collagen (Shinkai and Yonemasu 1979; Takagi et al. 1984). In contrast to our finding, no significant difference in this ratio was observed in the experiments of Brickley-Parsons et al. (1981). The reason for this difference is not clear at present.

The degree of glycosylation of hydroxylysine was elevated in the affected tissues. Since hydroxylysine-linked carbohydrates are suggested to play important roles in collagen fibril formation (Morgan et al. 1970; Schofield et al. 1971) and in collagen turnover (Isemura et al. 1976), the changes in hydroxylysine glycosides observed here may be related to the abnormal collagen architecture in the Dupuytren's aponeurosis tissue.

Pyridinoline is one of newly discovered collagen cross-linking amino acids

(Fujimoto et al. 1977) and has been shown to distribute in several tissues including costal cartilage, Achilles tendon, rib, and femoral bone (Fujimoto and Moriguchi 1978). This study demonstrated the occurrence of pyridinoline in normal and Dupuytren's aponeurosis. Since several tissues show an age-related change in its content (Moriguchi and Fujimoto 1978), and since we found a change in the content of DeDHLNL which is a possible precursor of pyridinoline, it was expected that Dupuytren's collagen might contain more of this cross-link. However, the present data indicated that there was no difference in this content between normal and affected collagens. Therefore, it seems unlikely that pyridinoline is involved in the pathogenesis of this disease. This finding also has an implication that DeDHLNL is not a direct precursor of pyridinoline or that the Dupuytren's tissue may have a system which maintains the pyridinoline level constant irrespective of the high concentration of DeDHLNL.

References

1) Bailey, A.J., Sims, T.J., Gabbiani, G., Bazin, S. & LeLous, M. (1977) Collagen of Dupuytren's disease. *Clin. Sci. mol. Med.*, **53**, 499-502.
2) Bazin, S., LeLous, M., Duance, V.C., Sims, T.J., Bailey, A.J., Gabbiani, G., Dandiran, G., Pizzolato, G., Browski, A., Nicoletis, C. & Delaunay, A. (1980) Biochemistry and histology of the connective tissue of Dupuytren's disease lesions. *Eur. J. clin. Inv.*, **10**, 9-16.
3) Brickley-Parsons, D., Glimcher, M.J., Smith, R.J., Albin, R. & Adams, J.P. (1981) Biochemical changes in the collagen of the palmar fascia in patients with Dupuytren's disease *J. Bone Joint Surg.*, **63A**, 787-797.
4) Dupuytren, B.G. (1834) Permanent retraction of the fingers, produced by an affection of the palmar fascia. *Lancet*, II, 222-225.
5) Fujimoto, D., Akiba, K. & Nakamura, N. (1977) Isolation and characterization of a fluorescent material in bovine Achilles tendon collagen. *Biochem. biophys. Res. Commun.*, **76**, 1124-1129.
6) Fujimoto, D. & Moriguchi, T. (1978) Pyridinoline, a non-reducible crosslink of collagen: Quantitative determination, distribution, and isolation of a crosslinked peptide. *J. Biochem.*, **83**, 863-867.
7) Gelberman, R.H., Amiel, D., Rudolph. R.M. & Vance, R.M. (1980) Dupuytren's contracture: an electron microscopic, biochemical, and clinical correlative study. *J. Bone Joint Surg.*, **62A**, 425-432.
8) Hanyu, T., Takahashi, H. & Isemura, M. (1979) Analysis of human urinary hydroxylysine and its glycosides in Paget's disease and primary hyperparathyroidism. *Connective Tissue*, **11**, 55-61. (in Japanese with English abstract)
9) Isemura, M., Ikenaka, T., Mega, T. & Matsushima, Y. (1974) On the structure of galactosylhydroxylysine and glycopeptides derived from bovine tracheal cartilage. *Biochem. biophys. Res. Commun.*, **57**, 751-757.
10) Isemura, M., Takahashi, K. & Ikenaka, T. (1976) Comparative study of carbohydrate-protein complexes II. Determination of hydroxylysine and its glycosides in human skin and scar collagens by an improved method. *J. Biochem.*, **80**, 653-658.
11) Isemura, M., Hanyu, T., Ono, T., Igarashi, R., Sato, Y., Gejyo, F., Nakazawa, R., Miyakawa, T., Takagi, T., Kuboki, Y. & Sasaki, S. (1981) Studies on prolidase deficiency with a possible defect in collagen metabolism. *Tohoku J. exp. Med.*, **134**, 21-28.
12) Kuboki, Y., Ogushi, K. & Fusayama, T. (1977) Collagen biochemistry of the two

layers of carious dentin. *J. dent. Res.*, **56**, 1233-1237.
13) Maeda, K., Miura, T., Nakamura, R., Inoue, G., Araki, S. & Ohshima, T. (1979) Dupuytren's contracture : Clincal study of 68 cases in our series. *Orthop. Surg.*, **30**, 1809-1818. (in Japanese)
14) Morgan, P.H., Jacobs, H.G., Segrest, J.P. & Cunningham, L.W. (1970) A comparative study of glycopeptides derived from selected vertebrate collagens. A possible role of the carbohydrate in fibril formation. *J. biol. Chem.*, **245**, 5042-5048.
15) Moriguchi, T. & Fujimoto, D. (1978) Age-related changes in the content of the collagen crosslink, pyridinoline. *J. Biochem.*, **84**, 933-935.
16) Schofield, J.D., Freeman, I.L. & Jackson, D.S. (1971) The isolation and amino acid and carbohydrate composition of polymeric collagens prepared from various human tissues. *Biochem. J.*, **124**, 467-473.
17) Shinkai, H. & Yonemasu, K. (1979) Hydroxylysine-linked glycosides of human complement subcomponent Clq and of various collagens. *Biochem. J.*, **177**, 847-852.
18) Sykes, B., Puddle, B., Francis, M. & Smith, R. (1976) The estimation of two collagens from human dermis by interrupted gel electrophoresis. *Biochem. biophys. Res. Commun.*, **72**, 1472-1480.
19) Takagi, T., Araki, C., Yoshioka, H. & Usuku, G. (1984) Difference in hydroxylysine glycosides between type I and type III collagens. *Connective Tissue*, **15**, 112-113. (in Japanese)