European Journal of Clinical Investigation (1980) **10**, 9–16

Biochemistry and histology of the connective tissue of Dupuytren's disease lesions

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Received 2 June 1978 and in revised form 3 October 1979

Abstract. When compared to age-matched control aponeurosis, lesions of Dupuytren's disease contain higher contents of water, collagen and chondroitin-sulphate, as well as increased proportions of soluble collagen and of reducible cross-links; these indicate synthesis of new collagen. The lesions show also increased amounts of type III collagen and an increased hydroxylation and glycosylation of the reducible cross-links. All these parameters are characteristic of granulation and scar tissues. Type III collagen was located by means of immunofluorescence on thin argyrophilic fibres and also within the large fibre bundles which appeared to be disrupted into microbundles. The increase of type III collagen and the presence of myofibroblasts in the apparently unaffected aponeurosis show that the disease is widespread and suggest that it is initiated within the aponeurosis and propagated by the cells migrating along the collagen bundles.

Key words. Collagen, fibroblasts, myofibroblasts, fibromatosis.

Introduction

Dupuytren's disease is a human affliction in which there is a progressive irreversible contraction of one or more fingers [1]. The most obvious changes are the shrinkage of parts of the palmar aponeurosis and the development of typical nodules along its length. Histological studies have shown that the most significant changes take place in the nodules, which are essentially masses of densely packed cells embedded in a collagen-rich matrix [2], typical of the so-called 'fibrotic lesions'.

Ultrastructural studies have focused primarily on the extracellular fibrous collagen, mainly because of the long-established belief presumably based on the contraction observed on denaturation, that collagen can shorten *in vivo* [3]. The concept that mature collagen fibres shorten *in vivo* is presently abandoned. Alternatively, Gabbiani & Majno [4] have proposed that the mechanism of shrinkage resides in the contractile ability of myofibroblasts which are the most frequent cellular elements observed in the nodules.

At the present time, five genetically distinct collagens are known to exist. Skin, tendon and bone consist primarily of type I, hyaline cartilage of type II and fetal skin and blood vessels mainly of type III. Basement membranes are referred to as type IV but have not yet been fully characterized. Similarly the molecular composition of type AB, which has been shown to be closely associated with basement membranes, remains to be elucidated.

In a preliminary study [5] type III collagen, which is not present to any significant extent in the normal aponeurosis, was shown to be present in the nodules and contracture as well as in the apparently unaffected aponeurosis of patients with Dupuytren's disease. In this paper, we have attempted to follow the development of the lesion in the aponeurosis by biochemical studies and immunofluorescent localization of the polymorphic forms of collagen.

Materials and Methods

Materials

A total of twenty-five surgically excised specimens from patients with Dupuytren's disease have been examined. The age of the patients was 40–65 years and

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0014-2972/80/0200-0009 $02.00

the duration of the disease 3–10 years. Some of the samples were dissected into 'nodules', 'contractures' and apparently normal parts of the aponeurosis; others were taken as whole excised specimens.

Age-matched control tissue was obtained from four specimens of aponeurosis taken immediately after death from 45–55-year-old normal subjects, and carefully separated from the adjacent fat tissue.

Pepsin was obtained from Worthington Biochemical Corp., Freehold, N.J., USA (3250 units/mg); KB^3H4 (650 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks, U.K. Chemicals for Bray's solution [6] (scintillation fluid) were supplied by Nuclear Enterprises (GB) Ltd, Edinburgh, Scotland, U.K.

Fluorescein conjugated anti-rabbit serum was obtained from Gibco.

Water content and analysis of collagen

The dissected normal and pathological tissues were minced into pieces of about 1 mm^3, washed in Tris-HCl buffer (0·005 mol/l Tris adjusted to pH 7·4 with HCl) containing NaCl (0·02 mol/l) and tetrasodium EDTA (0·01 mol/l) and then used for the following analyses:

(1) *Water content*. The excess water was removed by blotting with filter paper, the tissue weighed (about 500 mg) and then dried to constant weight at 100°C.

(2) *Total collagen content* was determined from the hydroxyproline content of at 90°C trichloracetic acid extract [7].

(3) *Collagen solubility:* (a) *Neutral salt-soluble collagen*. The minced tissue was homogenized (Ultraturax homogenizer, full speed, 1 min, several times) in 10 volumes (weight by volume of Tris-HCl buffer (0·05 mol/l Tris adjusted to pH 7·4 with HCl) containing NaCl (1 mol/l) at 4°C. The homogenate was stirred during 24 h at 4°C and then centrifuged (40,000 *g*; 1 h; 4°C). The extraction procedure was repeated three times and the collagen precipitated from the pooled extracts by bringing the NaCl concentration to 4·4 mol/l. (b) *Acid-soluble collagen*. The residue from the above salt extraction was then extracted three times with citrate buffer [10 ml of trisodium citrate (0·1 mol/l) + 50 ml of demineralized H_2O, adjusted to pH 3·5 with citric acid (0·1 mol/l] at 4°C for 6 h. The collagen was precipitated from the pooled extracts by adding NaCl to a final concentration of 0·85 mol/l.

(4) *Pepsin-solubilized collagen* (Scheme 1). Pepsin digestion was performed directly on the minced tissue or after neutral salt and acidic extractions as shown on Fig. 1 [30 parts of substrate for 1 part enzyme (weight by weight), in 10 volumes (weight by volume) of 0·5 mol/l acetic acid at 15°C for 6 h]. The digest was centrifuged (30 min; 40,000 *g*; 4°C) and the supernatant neutralized with NaOH (0·5 mol/l) to pH 7. The sediment was homogenized in 0·5 mol/l acetic acid and digested again with pepsin for 6 h. The same procedure was repeated several times to dissolve the maximal amount of collagen (checked from the hydroxyproline content of the insoluble residue): e.g. five times for nodules, six times for contractures and normal Dupuytren's aponeurosis, and eight times for control normal aponeurosis. During neutralization, a precipitate formed which was partly redissolved in 0·1 mol/l acetic acid; after centrifugation (40,000 *g*; 4°C; 30 min) supernatant I was obtained.

The neutralized supernatants of the pepsin-digested homogenates were precipitated by bringing the solution to 3·4 mol/l NaCl at 4°C for 24 h and were then centrifuged (40,000 *g* for 1 h at 4°C). The precipitate was dissolved in 0·1 mol/l acetic acid and dialysed against Tris-HCl buffer (0·05 mol/l Tris adjusted to pH 7·4 with HCl) containing NaCl (1 mol/l). A precipitate formed during dialysis. Centrifugation (40,000 *g*; 4°C; 30 min) separated supernatant II and a pellet which was dissolved in acetate buffer (0·06 mol/l Na-acetate adjusted to pH 4·4 with 0·6 mol/l acetic acid) containing urea (4 mol/l). This supernatant was used for collagen analysis, i.e. hydroxyproline content and genetic type determination through SDS-polyacrylamide gel electrophoresis.

Supernatants I and II were then dialysed sequentially against Tris-HCl buffer (0·05 mol/l Tris adjusted to pH 7·4 with HCl) containing NaCl 1·2, 1·5, 1·8, 2·1, 2·4, 4 mol/l. The precipitates were formed at 1·5, 1·8 and 2·4 mol/l and were recovered by centrifugation (40,000 *g*; 30 min; 4°C), dissolved in 0·1 mol/l acetic acid and used for collagen analysis; resistant insoluble residues were dissolved in acetate buffer (0·1 mol/l sodium acetate adjusted to pH 4·4 with 0·1 mol/l acetic acid) containing urea (4 mol/l), centrifuged (40,000 *g*; 30 min; 4°C) and were then subjected to collagen analysis. The fractions dissolved in 0·1 mol/l acetic acid were resubmitted to fractional salt precipitation by NaCl as above.

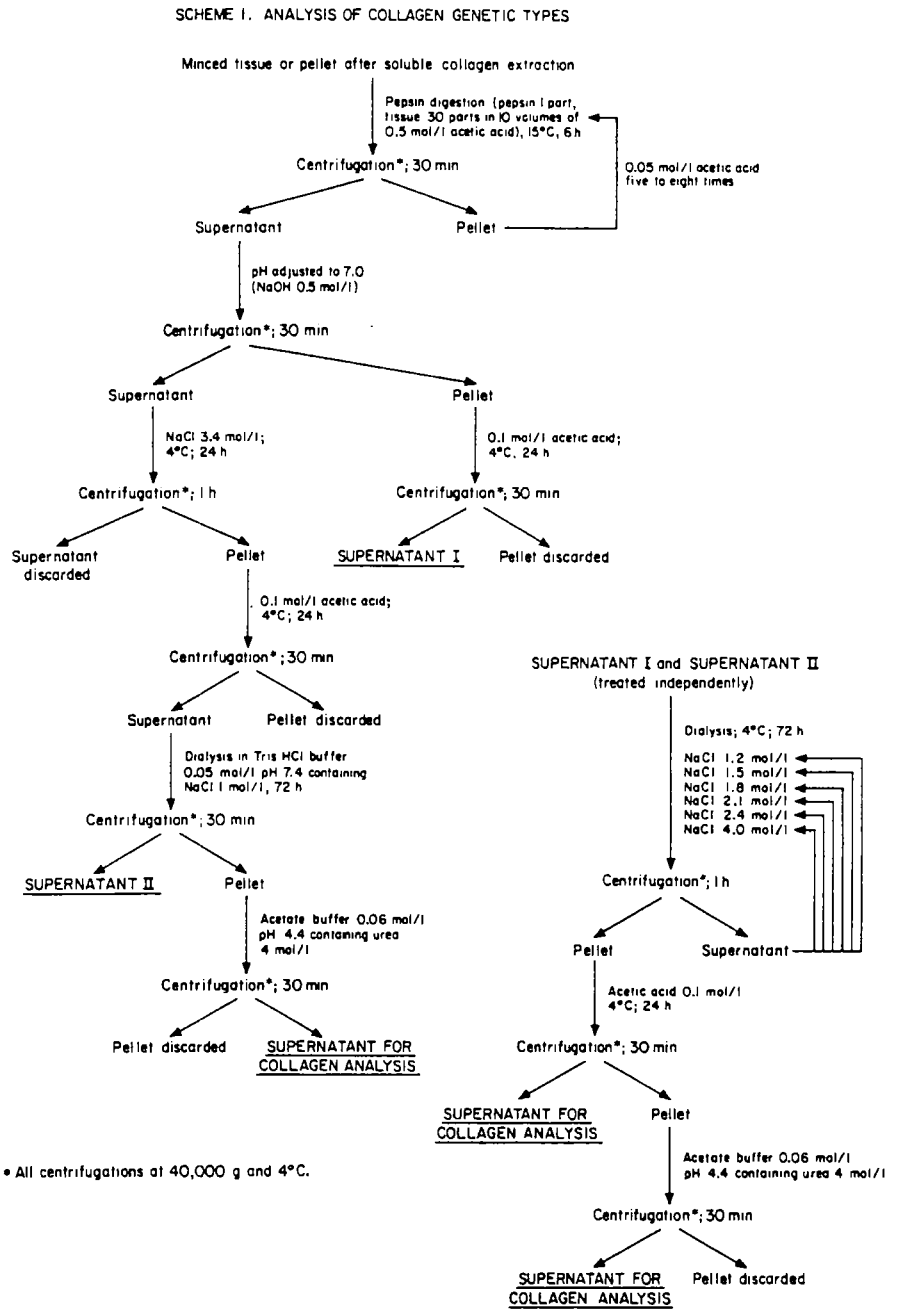
The amount of collagen in each fraction was determined by hydroxyproline analysis after hydrolysis in HCl (6 mol/l) for 24 h at 105°C [8].

SDS-polyacrylamide gel electrophoresis

To check the purity and identify the molecular types of collagen present, samples of the total digest and of each of the separated collagen fractions were analysed by SDS-polyacrylamide gel electrophoresis before and after reduction with β-mercaptoethanol [9]. In order to separate the αl (III) and αl (I) collagen chains the reduction of the γ (III) to αl (III) with β-mercaptoethanol was delayed until 20 min after the start of the electrophoresis [10].

Analysis of stabilizing crosslinks

The reducible crosslinks present in the fresh tissue were analysed by reduction with tritiated potassium



borohydride and separation of the tritium-labelled compounds by ion-exchange chromatography [11].

Glycosaminoglycans

Homogenates of the fresh tissue in acetate buffer (0·1 mol/l sodium acetate adjusted to pH 5·5 with 0·1 mol/l acetic acid) were digested exhaustively with papain at 60°C for 24 h. The solubilized glycosaminoglycans were precipitated from the digest by cetyl pyridinium chloride (CPC) [12] and, after purification, characterized by electrophoresis on cellulose acetate membranes [13].

Preparation of antibodies

The antigens (type I, III and AB collagen) were extracted from human placenta by digestion with pepsin as already described [14]. The solubilized proteins were fractionated by precipitation with NaCl to give type III collagen at 1·5 mol/l NaCl, type I collagen at 2·5 mol/l NaCl and type AB collagen at 4·0 mol/l NaCl. The procedure was repeated three times on each fraction and the purity of the collagen determined by SDS–polyacrylamide gel electrophoresis. Antibodies to these collagens were raised in New Zealand white rabbits following fortnightly injections, initially in

Freund's complete adjuvant and subsequently in Freund's incomplete adjuvant. The antibodies were assayed for activity by a passive haemagglutination test [15]. Immunoabsorbent columns of collagen types I, III, and AB were used to remove non-specific antibodies. The absence of cross-reactivity was confirmed by passive haemagglutination tests.

Tissue staining

Transverse sections were stained with specific rabbit anti-human type I, III or AB, washed extensively with phosphate buffer (19 ml of 0·067 mol/l KH_2PO_4 mixed with 81 ml of 0·067 mol/l Na_2HOP_4) containing NaCl (0·15 mol/l) and then stained with fluorescein-conjugated anti-rabbit IgG as previously described [14]. For controls non-immune rabbit serum and/or rabbit IgG (Fluka AG, Buchs, Switzerland) were used in place of the specific anti-collagen antibody. The sections were viewed with a Zeiss fluorescent microscope equipped with epi-illumination and specific filter for fluorescein.

Results

Water content

In the Dupuytren's lesion there was a progressive increase in water content from the unaffected parts, to the contractures and finally the nodules. The normal aponeurosis possessed a lower water content than the apparently unaffected parts of the aponeurosis from subjects with Dupuytren's disease (Table 1).

It was also observed that when these tissues had been homogenized and their neutral-and-acid-soluble collagen extracted, the washed residue, centrifuged for 1 h at 40,000 *g*, retained much more water in the case of Dupuytren's disease than in the case of normal aponeurosis: 93% in nodules and 91% in apparently unaffected parts compared with 59% in the control aponeurosis. This indicates that the extent and nature of the cross-linking in diseased tissues allowed extensive swelling.

Collagen content

The collagen content progressively increased from the apparently unaffected tissue to the contractures and to the nodules, and in all cases was greater than the normal control specimens (Table 1).

The proportions of the neutral salt-soluble and acid-soluble fraction were small but significantly higher in Dupuytren's lesions than in control aponeuroses (Table 1).

The proportion of pepsin-solubilized collagen was higher in Dupuytren's lesions than in control aponeuroses, and increased from the normal part through the contractures to the nodules. However, the solubilization process was not identical in all these tissues, indicating differences in the nature of the cross-links and possibly associated glycoproteins. In the case of nodules, the first three digestions contained more than 60% of the solubilized collagen. In the case of contractures and of apparently normal parts of Dupuytren's disease, the first four extractions contained only 30–35% of the solubilized collagen. In contrast, the first three extractions of control aponeurosis contained only traces of collagen. However, up to 80% of the total collagen could be solubilized through the five subsequent extractions, but this extensive pepsin treatment led to some degradation of the collagen.

Types of collagen

A high proportion of the tissue was solubilized by the pepsin digestion (Table 1) and hence analysis of this soluble collagen can be considered as being representative of the total collagen.

(1) *Total pepsin digest*. Electrophoresis of the total pepsin digest revealed that the normal aponeurosis comprised almost pure type I collagen (Fig. 1). The tissue of the nodules and contactures had a higher proportion of type III collagen compared to normal aponeurosis and, interestingly, the apparently unaffected aponeurosis of the patients with Dupuytren's disease also had higher amounts of type III collagen compared to normal aponeurosis.

Table 1. Collagen analysis in normal palmar aponeurosis and Dupuytren's lesions

	Water content (%) in fresh tissue	Total collagen (μg hydroxyproline/ mg dry tissue)	Collagen fractions (% of total collagen)			Type III collagen (% of pepsin-solubilized collagen)
			Neutral salt soluble	Acid soluble	Pepsin solubilized	
Dupuytren's nodules	62·1 (±2)	109·9 (±2·4)	0·17 (±0·02)	0·26 (±0·10)	97·4 (±5)	20–30
Dupuytren's contractures	60·7 (±1·8)	89·5 (±1·5)	0·17 (±0·02)	0·28 (±0·10)	95·2 (±6)	30–40
Apparently unaffected aponeurosis from Dupuytren's patients	59·0 (±·15)	71·1 (±3)	0	0	91·5 (±4)	10–15
Aponeurosis from normal patients	54·3 (±1·4)	58·1 (±3·2)	0	0·02	83	Not determined

± = maximum deviation observed.

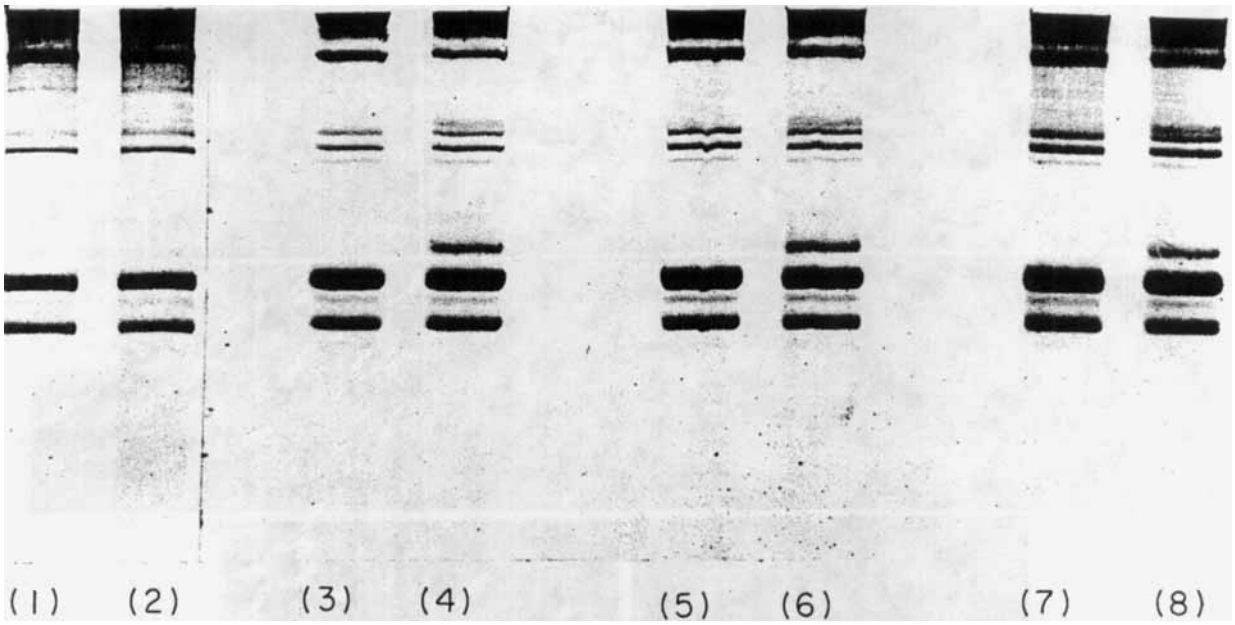


Figure 1. Sodium dodecyl sulphate/polyacrylamide gel electrophoresis of the total pepsin digests of tissue dissected from various sites in the palmar fascia: (1) and (2) aponeurosis from a normal subject without and with reduction by β-mercaptoethanol respectively; (3) and (4) Dupuytren's nodules without and with β-mercaptoethanol respectively; (5) and (6) Dupuytren's contractures without and with β-mercaptoethanol respectively; (7) and (8) unaffected aponeurosis from Dupuytren's patient without and with β-mercaptoethanol respectively. Electrophoresis interrupted and β-mercaptoethanol added 20 min after start to resolve αI (III) and αI (I).

The presence of the two bands of type AB collagen were barely discernible on electrophoresis of the total digest, but analysis of the precipitate formed at 4·0 mol/l NaCl clearly demonstrated the presence of this collagen in all normal aponeurosis. It has previously been shown to be present in bovine tendon [14].

A second basement membrane type of collagen shown to precipitate at 2 mol/l NaCl from digests of human placenta [16], could not be detected in intramuscular collagen [14] and similarly appeared to be absent from the aponeurosis (although it may be present in quantities undetectable by the present techniques).

(2) *Neutral salt soluble and acid soluble fractions.* Both these fractions showed only types α1 and α2 chains, type III collagen was not detected.

(3) *Fractional salt precipitation of pepsin digests.* In those fractions which would only dissolve in acetate buffer containing urea (4 mol/l) as well as those formed at 1·5 mol/l NaCl, only type III collagen could be detected by SDS-polyacrylamide gel electrophoresis. These were not detected in normal control aponeurosis.

SDS–polyacrylamide-gel electrophoresis showed that fractions precipitated at 1·8 mol/l NaCl contained both type I and type III collagens if they had been prepared from diseased samples but were devoid of type III if they had come from control aponeurosis.

Only type I collagen could be identified in the fractions precipitating at 2·4 mol/l NaCl.

Immunofluorescent localization of collagen types

Using the indirect immunofluorescent technique with antibodies to type I collagen, staining occurred throughout the whole aponeurosis. However, when type III and types AB antibodies were used the staining of the cross-sections was restricted to the fine sheath around the fibre bundles (Fig. 2a–f). A similar analysis of the nodules from subjects with Dupuytren's disease revealed an intensive staining for collagen types III and AB, within the major bundles; these appeared broken-up into small fibrils (Fig. 2d, f). In the apparently unaffected aponeurosis of patients with Dupuytren's disease, the pattern of staining for collagen types I, III or AB was in many areas similar to that seen in the aponeurosis of normal patients (Fig. 2g). and in other areas similar to that seen in Dupuytren's nodules (Fig. 2h).

Reducible cross-links

Distinct differences in the cross-link patterns of the various dissected tissues could be seen (Fig. 3): the nodules exhibited at 2·5:1 molar ratio of dihydroxylysinonorleucine to hydroxylysinonorleucine and a negligible amount of the hexosyl-lysines. the contractures showed a decreased proportion of dihydroxy-

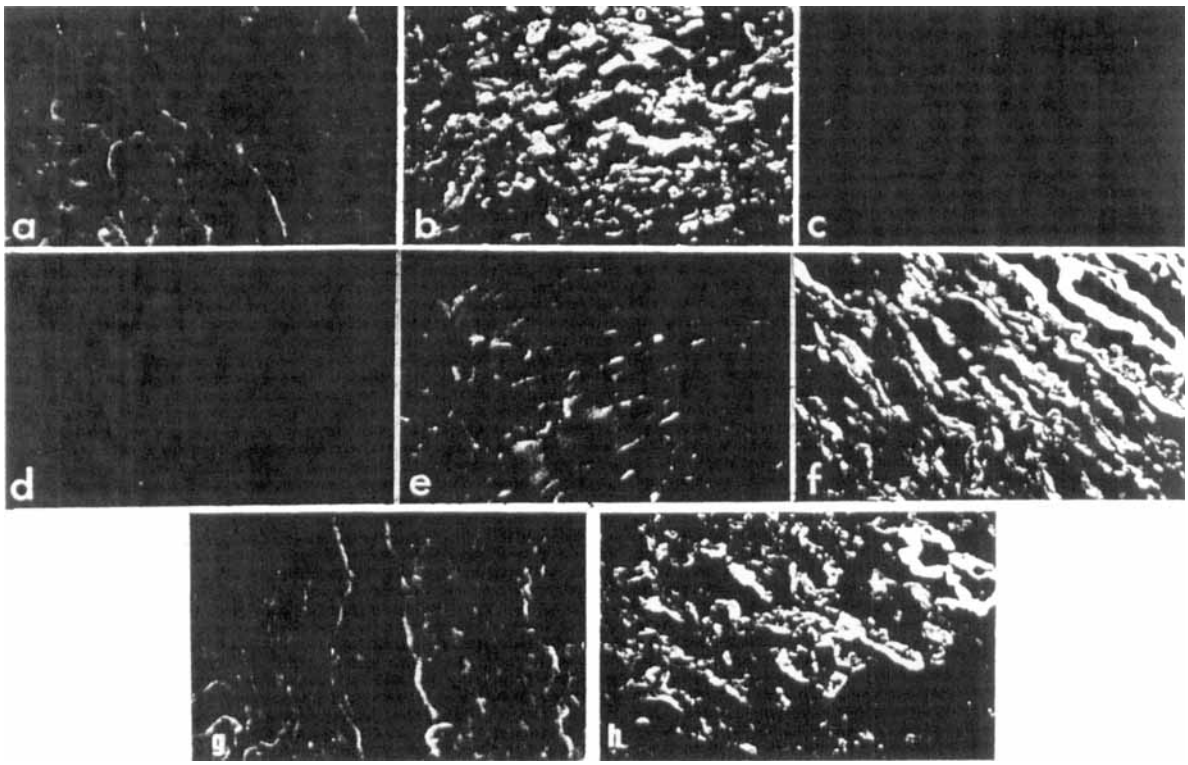


Figure 2. Immunofluorescent staining of frozen section of normal palmar aponeurosis and Dupuytren's lesions with anticollagen antibodies. (a) Normal aponeurosis incubated with rabbit serum containing anti-type III collagen antibodies followed by fluorescein-conjugated goat anti-rabbit IgG. The staining is weak and localized essentially at the periphery of the regularly arranged collagen bundles. (b) Dupuytren's nodules treated as in (a). The intensity of the staining is stronger than in (a) and the antibody is located throughout the small irregularly-distributed collagen bundles. (c) and (d) Normal aponeurosis and Dupuytren's nodules respectively treated with normal rabbit serum instead of serum containing anti-type II collagen antibodies. No staining. (e) and (f) Normal aponeurosis and Dupuytren's nodules stained with rabbit serum containing anti-type AB collagen antibodies followed by fluorescein-conjugated goat anti-rabbit IgG. The distribution of the staining is similar to that observed for anti-type III collagen antibodies. (g) and (h) Apparently normal zones in the palmar aponeurosis of Dupuytren's disease, treated as in (a) with anti-type III collagen. In many areas (g) a weak staining is present at the periphery of the collagen bundles which are arranged regularly, and in other areas (h) a more intense staining is diffused throughout the bundles. × 400.

lysinonorleucine, its molar ratio to hydroxylysinonorleucine being 1·5:1. A significant amount of the hexosyl-lysines was also present. The apparently unaffected part of the aponeurosis revealed the presence of the reducible cross-links with a similar ratio to the contractures, i.e. 1·7:1, but with a greater amount of hexosyl-lysines. The aponeurosis from a normal subject of the same age gave a pattern typical of mature tissues in that the only major reducible components were the hexosyl-lysines.

The gradual change of pattern shown by different parts of the diseased aponeurosis indicates that the nodules contained a high proportion of newly-formed collagen, the contracture being mainly newly-synthesized collagen but with some mature collagen, whilst the 'unaffected' aponeurosis contained mainly mature collagen with a little newly-synthesized collagen. The normal control aponeurosis was clearly mature collagen.

Glycosaminoglycans

The glycosaminoglycans of the Dupuytren's disease specimens were hyaluronate, chondroitin sulphates and dermatan sulphate in approximately equivalent proportions.

The glycosaminoglycans of the control normal aponeurosis were present at a low level, and were mainly dermatan sulphate with traces of hyaluronate.

Discussion

When compared to normal aponeurosis, the tissue from Dupuytren's lesions appears to have biochemical features similar to those of granulation tissue which in turn is similar to embryonic tissue. Thus, increased amounts of soluble collagen as well as an increased proportion of reducible cross-links are present, both indicating synthesis of new collagen. In addition there is an increased proportion of type III collagen, increased hydroxylation and glycosylation of the reducible cross-links, and an appreciable proportion of chondroitin sulphate. All these findings are characteristic of experimental granulation tissue and human normal or hypertrophic scar tissue [17, 18].

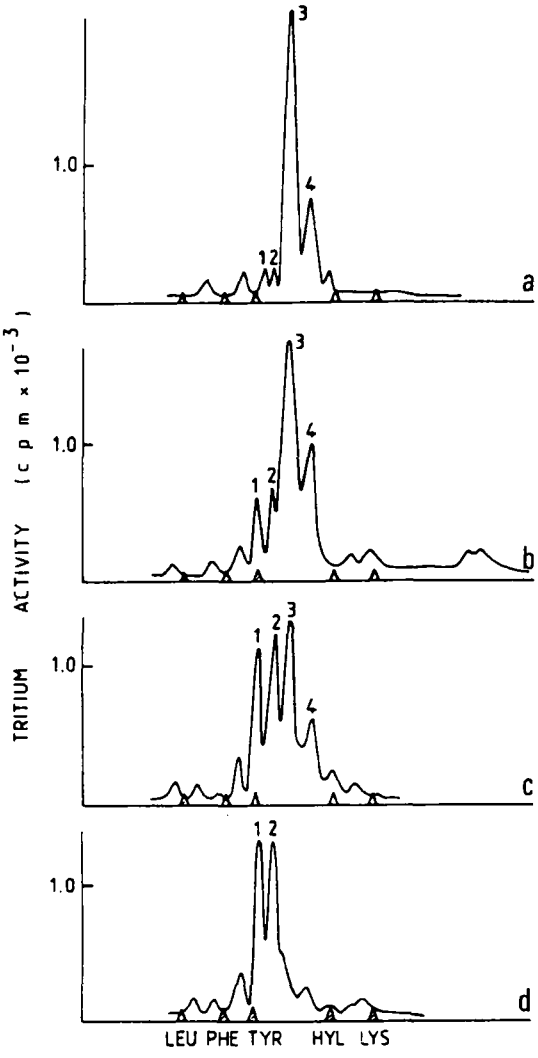


Figure 3. Ion-exchange elution pattern of an acid hydrolysate of Na B^3H4-reduced collagen. (a) Dupuytren's nodule; (b) Dupuytren's contracture; (c) Dupuytren's unaffected aponeurosis; (d) aponeurosis of normal subject. Peaks 1 and 2 represent hexitol-lysines and are not involved in cross-linking. Peak 3 is the reduced cross-link dihydroxylysinonorleucine and Peak 4 is the reduced cross-link hydroxylysinonorleucine.

The increased collagen content in the nodules which are also packed with cells rather like tumours, confirms the classification of the condition among the so-called fibromatoses.

An increase of type III collagen in nodules and contractures was anticipated from previous studies on granulation tissue [17]. Type III collagen may be related to the thin argyrophilic fibrils reported to be present in the nodules by a number of workers [19]. Our results by means of immunofluorescence show that these thin fibres in the nodules stain intensely with anti-type III, thus supporting the interpretation [20] that argyrophilic fibres (also called reticulin) contain type III collagen.

As reported in our preliminary communication [5], the clinically 'unaffected' part of the aponeurosis also revealed the presence of increased amounts of type III collagen, and reducible cross-links indicating synthesis of new collagen. Since our previous studies [14] had located type III collagen in the endotendineum of Achilles tendon we investigated its presence in the Dupuytren's lesions. The proportion of type III collagen was clearly increased in the apparently normal aponeurosis of Dupuytren's patients compared with the control aponeurosis; moreover, the type III staining was observed on several occasions within the large fibre bundles, which appeared to be dissociated into microbundles. These observations show that Dupuytren's lesions probably begin focally and support our proposal that the initiating site is within the aponeurosis [5]. The cells, mainly fibroblasts, migrate along the collagen bundles, and as in most cases of inflammation, the initial response is the synthesis of type III collagen; this is followed by multiplication of fibroblasts which transform into myofibroblasts until the mass of tissue looks rather like scar tissue.

Closer analysis of the fibroblasts in the apparently unaffected aponeurosis has shown that a certain proportion of them have several of the characteristics of myofibroblasts, such as intracellular bundles of microfilaments, and a basement lamina-like condensation just beneath the plasmalemma; however, gap junctions typically connecting myofibroblasts in granulation tissue and Dupuytren's nodules were not observed [21].

Myofibroblasts have been reported previously to be the most common cellular component of nodules and to a lesser extent of contractures [4]. They have distorted nuclei, thick bundles of cytoplasmic actomyosin filaments, gap junctions connecting one cell to another and hemidesmosomes attaching them to the basal lamina. These cells may be responsible for the contractile events of Dupuytren's disease [4]. We propose that during the initial steps of Dupuytren's disease, certain fibroblasts in the aponeurosis gradually acquire some of the morphologic features of myofibroblasts and actively synthesize collagen; they would then migrate and multiply to form typical nodules producing digital retraction.

Although the aetiology of the disease is unknown, our investigations indicate the disease is not strictly focal and limited to the nodules. This is in accordance with the well-accepted clinical observation that Dupuytren's disease can recur within the same aponeurosis and that the disease is often bilateral and frequently accompanied with other types of fibromatoses [21]. The increase of type III collagen and the presence of myofibroblasts in the 'unaffected' aponeurosis suggests that the disease is initiated and/or propagated by the cells migrating along collagen bundles.

Acknowledgments

The authors are very grateful to Marie-Pierre Gassama and Anne De Almeida for their excellent technical

assistance. This work has been supported in part by the Swiss National Science Foundation, Grant Nr 3.445-0.79.

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