Thermal Stability and Fibrillogenesis of Collagen from Tissue of Patients with Dupuytren's Disease

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Introduction

Dupuytren's disease (DD) is a spontaneously occurring tissue rearrangement with a number of biochemical and cellular variations compared to normal fibroblast activity (McFarlaine 1983; Gabbiani and Majno 1972). Fibroblasts are influenced by a number of environmental factors, e.g., cytokines, drugs, or biomechanical stress, which modulate gene expression and therefore the rate of collagen production and extracellular matrix formation. The extracellular matrix of connective tissue is basically constructed of collagen fibrils and various noncollagenous extracellular matrix proteins (Piez 1984). Specific collagen types and proteoglycans modulate the biomechanical character of tissues including skin, bone, cornea, or palmar aponeurosis (Romanic et al. 1991; Birk et al. 1990; Scott 1988; Lapiere et al. 1977). In pathological situations such as fibrosis, the pattern of collagen expression is changed (Bailey et al. 1977).

In Dupuytren's contracture, mainly quantitative changes in the amount of extracellular matrix components are observed. In addition, beyond the formation of nodules and bands, the ultrastructure of the collagen fibers is altered. Areas of active proliferation are characterized by their cellularity and by a meshwork of fibrils (polymeric collagen) which only focally coalesce into discrete fibers but which then separate again. An important feature is that both the constituent fibrils and the fibers themselves tend to be oriented in one direction in contrast to what occurs in mature tissue (Hunter et al. 1975).

The events leading to altered production of collagen fibrils and contraction of the tissue are presently not clear. It has been suggested that contraction is due to the transformation of fibroblasts into myofibroblasts, since myofibroblasts have been found in diseased palmar aponeurosis (Gabbiani and Majno 1972). Hereditary factors, sex, metabolic diseases, and drugs such as alcohol may be initiating factors in DD. (Hueston 1990).

The biochemical changes of DD tissue include the following:

1. A change in the relative content of the different collagen types, especially an increase in collagen III (Bailey et al. 1977)
2. Increased proteoglycan content relative to collagen (Tunn et al. 1988)
3. A lysyl overmodification of collagen I (Bailey et al. 1977)

4. Increase fibronectin content in the tissue (Brokaw et al. 1985; Menzel 1984)
5. Fewer cross-links in the tissue than found in normal aponeurosis (Bailey, this volume)

Organization of the extracellular matrix is a self-regulated process involving the various collagen types and proteoglycans. Posttranslational modifications of collagen, such as hydroxylation of prolyl and lysyl residues and glycosylation of hydroxylysine, may also have a regulatory effect on extracellular matrix formation.

It was our aim to acquire biochemical data, with respect to the changes listed above, from collagen obtained from Dupuytren's nodules. These data were compared to the special ultrastructural features found in DD tissue and to the unbalanced biochemical turnover which leads to a net overproduction of collagen.

Materials and Methods

Biochemical Analysis of Skin, Palmar Aponeurosis, and Tissue from DD Patients

Tissue samples were manually homogenized under liquid nitrogen using a stainless steel homogenizer followed by dialysis against 0.05% acetic acid for 3 days. All dialysis steps were carried out in the presence of phenylmethansulfonylfluoride (3 mg/l) to inhibit proteases. After centrifugation at 100 000 × g (1 h, 4°C), an aliquot (5 ml) of the supernatant was lyophilized and analyzed on SDS-PAGE (Bätge et al. 1990). The remainder was used for the extraction procedures described below. Skin, palmar aponeurosis, and tissue from DD patients was defatted and then homogenized prior to extraction.

Extraction of Collagens

Limited pepsin digestion was used to solubilize bulk collagen. Samples (2–10 g dry weight) were stirred in a pepsin (0.1 mg/ml) (Boehringer Mannheim, FRG) solution at 4°C for 24 h. After centrifugation at 65 000 × g (1 h, 4°C), supernatants were neutralized and stored at −20°C. This digestion procedure was repeated six times; for determination of the total collagen composition all neutralized supernatants were pooled.

Sequential Salt Precipitations

In order to separate collagen I from the other collagen types, sequential neutral salt precipitations were performed by consecutive dialysis against 1.0, 1.8, and 2.5 *M* NaCl solutions (0.05 *M* Tris, pH 7.4) (Miller and Rhodes 1982). The 2.5 *M* precipitate consisted of highly purified collagen I. After

centrifugation (65000 × g, 1 h, 4°C), the pellet was redissolved in 0.05% acetic acid and dialyzed against the same solvent to remove residual salt. For all further studies only the fraction precipitating at 2.5 *M* NaCl was used.

Electrophoretic Separation

Small aliquots of each tissue sample were lyophilized and redissolved in SDS sample buffer at a concentration of 1 mg/ml. Each sample was heated to 95°C for 2 min and quenched on ice prior to sample loading (Laemli 1970). After separation of collagen the polyacrylamide gels were stained with Coomassie blue and the relative amounts of the different components were measured by densitometric scanning using a video scanner (Computer and Vision, Lübeck, FRG). Relative amounts of collagen types were determined by measuring the integrated absorption of the bands in a lane.

Amino Acid Analysis

Lyophilized collagen I was hydrolyzed with 6 *M* HCl and 0.1% mercaptoethanol under nitrogen. Analysis was performed on a Beckman 6300 amino acid analyzer (Beckman, Munich, FRG). The relative content of hydroxylysine and hydroxyproline per mole of fractionated collagen was expressed as Hyl/(Hyl + Lys) and Hypro/(Pro + Hypro), respectively.

In Vitro Fibril Formation and Fibril Melting

Fibrillogenesis. Self-assembly conditions followed a modification of the method described by Williams et al. (1978): 100 µg/ml collagen I, 30 m*M* Tris, 30 m*M* K_2HPO_4, 150 m*M* NaCl. In order to reach 90% saturation of fibril formation within 2 h for all samples, the incubation was performed at 37.0°C and pH 7.4 in a thermocontrolled Gilford quartz cuvette, unless otherwise stated.

Fibril Melting. After 18 h the sample was exposed to a linear temperature gradient of 0.5°C/min. Assembly and disassembly was monitored by continuous recording of the absorbance at 313 nm in a Gilford spectrophotometer.

Definition of the Characteristic Values

Fibrillogenesis and fibril melting is a process which can be described with an unsymmetric sigmoid function. We have differentiated this curve and determined the following parameters, which mainly characterize the process of collagen assembly: (1) turning point (maximum of the differentiated curve), (2) maximal slope (height of the differentiated curve), and (3) maximal turbidity

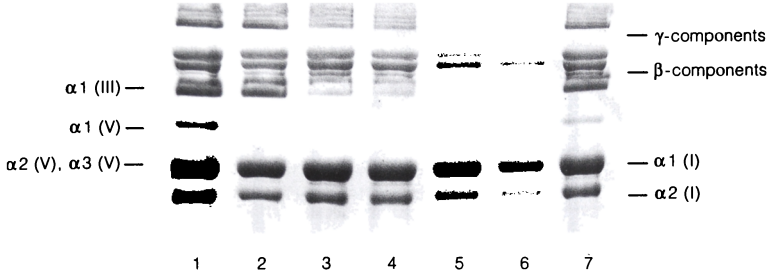

Fig. 1. SDS gel electrophoresis of pepsin extracts of Dupuytren's nodules. *Lanes 1–6* are successively extracted collagens; *lane 7* displays the overall composition of collagens

(area under the differentiated curve). For the melting curve, the value of the maximal transition rate (the maximum of the differentiated curve) is defined here as the fibril melting temperature, T_m.

Results

Unaffected tissue from palmar aponeurosis was taken during hand surgery from patients suffering from carpal tunnel syndrome. Tissue from five DD patients came from nodules of the midhand.

Composition of Collagens

The tissue material from the DD patients was totally digested after five rounds of pepsin digestion. Densitometric evaluation of the electrophoretically separated proteins of the digests showed only collagen types, I, III, and V. Samples taken from every digest and from the collagen composition of the total pool were analyzed by gel electrophoresis (Fig. 1).

Amino Acid Composition

The collagen I α-chains are lysyl-overhydroxylated in Dupuytren's nodules. The value of the relative content of hydroxylysine (Hyl/Hyl + Lys) is 0.25 compared to 0.17 in normal aponeurosis. The relative hydroxyproline content (Hyp/Hyp + Pro), 0.44, is unchanged (Table 1).

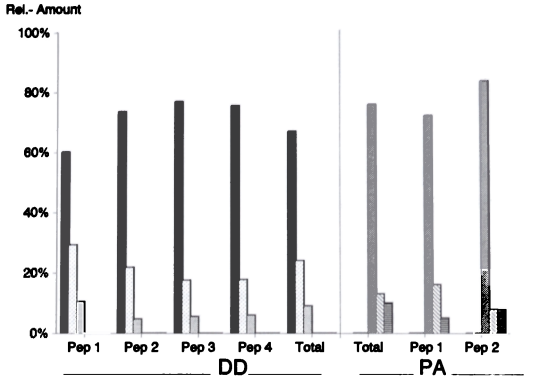

Fig. 2. Histograms of the densitometrically determined relative collagen concentrations of the α-bands of collagen types I, III and V (from left to right; see Fig. 1). *Pep*, successive pepsin digests (also shown is the total pool of pepsin-soluble collagen); *DD*, Dupuytren's disease; *PA*, palmar aponeurosis

Table 1. Biochemical and biophysical data regarding collagen type I

Human tissue	Fibril T_m (°C)	Triple helix T_m (°C)	Hyl/Hyl + Lys	Hyp/Hyp + Pro
Skin	49.9	41.5	0.12	0.41
Palmar aponeurosis	n.d.	42.2	0.17	0.46
Dupuytren's disease nodules	50.2	42.8	0.25	0.46

T_m, fibril melting temperature; Hyl, hydroxylysine; Lys, lysine; Hyp, hydroxyproline; Pro, proline.

Composition of Collagen Types

Electrophoretic separation and subsequent determination of the relative content of collagen types in the individual pepsin digests and in the total tissue are shown in Fig. 2. Most of the collagen III is present in the first digest and collagen V is nearly absent in the last digests. The overall content of collagen III is higher in tissues derived from DD patients.

Thermal Stability of Collagen

Collagen I (2.5 *M* NaCl sediment) from DD patients has as higher melting temperature than collagen from normal aponeurosis. The T_m value of collagen

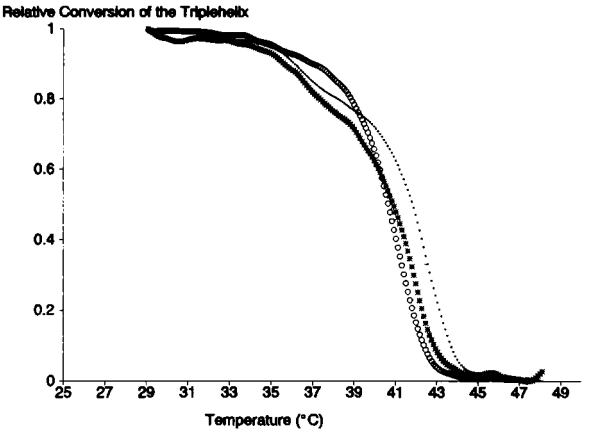

Fig. 3. Melting curve of solute collagen molecules in 0.05% acetic acid. Ellipticity at 221 nm is normalized in terms of triple helix to random coil conversion. □, collagen I from Dupuytren's disease patients; *, collagen I from normal aponeurosis; ○, collagen I from control skin

from DD patients is 42.8°C. For the unaffected aponeurosis we measured 42.2°C. Denaturation curves of skin, DD collagen and palmar aponeurosis collagen obtained from circular dichroism (CD) measurements are shown in Fig. 3.

Fibrillogenesis of Heterotypic Collagen

Various mixtures of the collagen precipitated at 1.8 *M* NaCl (collagen I + collagen III) and at 2.5 *M* NaCl (pure collagen I) were incubated together in order to determine the effect of minor collagen types on fibril formation.
In Fig. 4 the maximum turbidity values, which are proportional to the mass per unit length of the collagen fibrils, are summarized. The mass per unit length of collagen fibrils decreases by a factor of five with increasing amounts of collagen III. Also, as shown in Fig. 4, the amount of collagen which is not assembled into fibrils represents only 20% of the total amount of collagen. Free collagen concentration is increased by 5% if the collagen III concentration is raised. The structure of the in vitro formed collagen fibrils was demonstrated to be the same as native collagen by negatively stained electron micrographs (not shown).

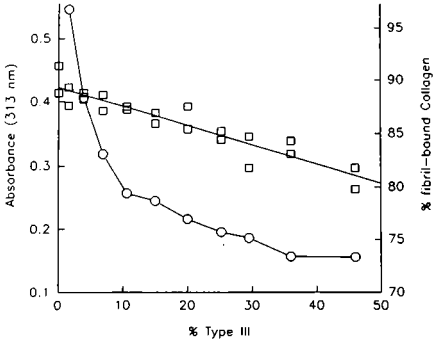

Fig. 4. Influence of collagen III on fibril structure. Amount of collagen III in a solution with collagen I (always 100 µg/ml) is plotted against maximum turbidity (○) and against relative amount of collagen in the supernatant after sedimentation of the fibrils (□)

Fibrillogenesis and Fibril Stability of Collagen I from Palmar Aponeurosis and from Dupuytren's Disease Patients

In Fig. 5a overhydroxylated collagen of DD patients is compared with normal skin collagen (highly purified collagen from normal palmar aponeurosis cannot be obtain in sufficient quantity). Lysyl-overhydroxylated collagen from DD patients did not show different dynamic patterns of fibrillogenesis as compared to collagen I from control skin, but the mass per unit length was nearly twice as high for the overhydroxylated collagen. The thermal stability of fibrils formed in vitro from these isolated collagens was also not significantly different (Fig. 5b). The melting temperature was about 50°C for both control skin collagen I and collagen I from DD patients. Presumably, because of the thicker fibrils of DD patients, melting is slightly shifted to higher temperatures.

Discussion

In comparing the collagenous extracellular matrix components of normal palmar aponeurosis and palmar aponeurosis altered by the typical symptoms of DD contracture, two changes, the results of an as yet unknown initiating biochemical mechanism, can be found: (1) quantitative changes in the collagen composition and (2) posttranslational collagen modification. Posttranslational modification of collagen stabilizes the collagen triple helix, which is a structural prerequisite for the molecule to withstand proteolytic degradation. Therefore, the degree of posttranslational modification indirectly influences the net collagen production rate. Additionally, collagen fibril structure and fibril

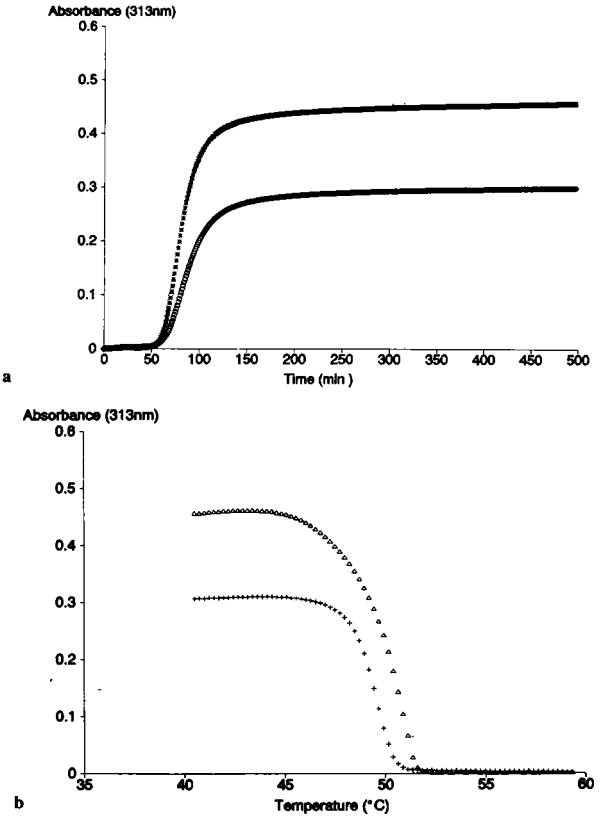

Fig. 5. **a** Turbidity-time curve of neutralized collagen at 37°C monitored at 313 nm; *, skin collagen I; O, overhydroxylated collagen I from Dupuytren's disease patients. **b** Turbidity-temperature curve of fibrils to determine the melting temperature of fibrils; +, skin collagen I; △, collagen from Dupuytren's disease patients

stability is influenced by collagen type composition and posttranslational modification (Notbohm et al. 1992). It is well known that prolyl overhydroxylation can affect triple helix stability. The influence of lysyl overhydroxylation and underhydroxylation have been demonstrated recently for collagen from pathologically altered bone and skin (Fig. 6, Notbohm et al. 1992). A decrease of the melting temperature of about 1°C was observed when the hydroxylysine content increased by a factor of three. Collagen I from DD patients is lysyl- but not prolyl-overhydroxylated, as compared to collagen I

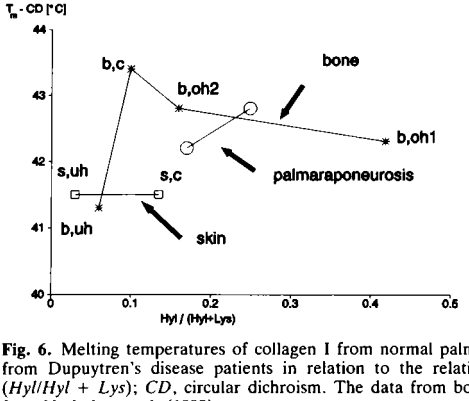

Fig. 6. Melting temperatures of collagen I from normal palmar aponeurosis and collagen I from Dupuytren's disease patients in relation to the relative hydroxylysine content (○) (*Hyl/Hyl + Lys*); *CD*, circular dichroism. The data from bone (*) and skin (□) are taken from Notbohm et al. (1992)

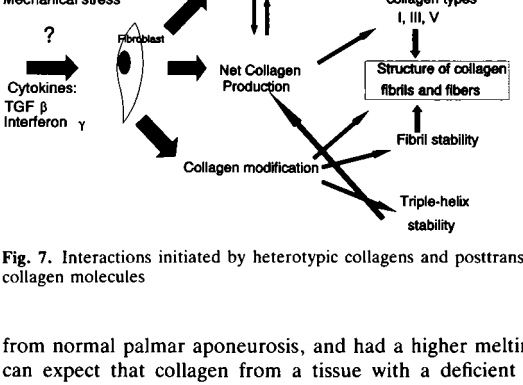

Fig. 7. Interactions initiated by heterotypic collagens and posttranslational modifications of collagen molecules

from normal palmar aponeurosis, and had a higher melting temperature. One can expect that collagen from a tissue with a deficient collagen mass (e.g., osteogenesis imperfecta; Brenner et al. 1989) would melt at a lower temperatures than collagen from tissue with a surplus of extracellular matrix (fibrosis).

Fibrils of tissue from DD patients were investigated by electron microscopy (Hunter and Ogdnon 1975). Most frequently, the tissue was described as consisting of more irregularly arranged fibrils which were also smaller in diameter. From our in vitro studies, we can conclude that overhydroxylated

collagen I from DD nodules does not produce fibrils of smaller diameter. Therefore, changes in collagen type composition may play a dominant role in the formation of smaller fibrils. Furthermore, in agreement with in vivo observations that formation of thick bundles of fibers is impaired in DD nodules, the in vitro experiments demonstrated that fibrils did not tend to form bundles when greater amounts of collagen III were present in solution. The interdependencies of collagen metabolism and extracellular matrix formation are summarized in Fig. 7.

In conclusion, our observations provide preliminary evidence that changes in extracellular matrix biochemistry and structure do not initiate Dupuytren's contracture. The characteristics of these alterations strongly argue for a mechanism which induces an acceleration of fibrogenesis.

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