

# Biomechanical properties of normal tendons, normal palmar aponeuroses, and tissues from patients with Dupuytren's disease subjected to elastase and chondroitinase treatment

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## Summary

Normal tendons, normal palmar aponeuroses and palmar aponeuroses from patients with Dupuytren's disease were subjected to elastase or chondroitinase treatment. Young's modulus was derived from the linear portion of stress–strain graph. It showed the lowest value for the apparently normal palmar aponeuroses and the highest value for tendon samples. Elastase treatment caused an increase of extensibility and a reduction of Young's modulus of normal palmar aponeuroses and tendons, but not of contracture bands. In normal tendons, normal palmar aponeuroses and apparently normal palmar aponeuroses residual strain and hysteresis loop increased significantly as a linear function of the amount of digested elastin. In contrast these biomechanical parameters were not affected significantly in contracture bands. In normal and apparently normal areas incubation with chondroitinase ABC resulted in a significant increase of residual strain and, as opposed to elastase, a decrease of normalized hysteresis loop. In contracture bands, however, these biomechanical parameters remained unchanged.

## Relevance

The increasing evidence of a correlation between morphological changes of palmar elastin and ground substance with the progress of Dupuytren's disease emphasizes the need to determine the relative importance of these connective tissue components for the pathogenesis of Dupuytren's disease.

Key words: Elastase, chondroitinase ABC, Dupuytren's disease, palmar aponeurosis, biomechanics

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## Introduction

Dupuytren's disease is a connective tissue disorder classified as a fibromatosis and characterized by a progressive irreversible contraction of one or more fingers<sup>1</sup>. The pathogenesis of these changes has not been determined despite many excellent histological and clinical studies. Typical changes are the shrinkage

of parts of the palmar aponeurosis and the development of nodules. The early stage of the condition is characterized by the thickening of collagen fibres and changes in thickness and distribution of elastin fibres<sup>2</sup>. At a later stage nodular proliferation of the fibrous tissue of the palmar fascia, accompanied by fibroblast proliferation originating from perivascular areas, leads to flexion contracture beginning at the fourth and fifth fingers. The disease process infiltrates the distal palm to involve the proximal parts of the fingers forming a continuum of fibroplasia from the palmar aponeurosis to the proximal or intermediate segments of the fingers.

In a previous study we observed that human normal

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palmar aponeuroses have a low viscous stress component, i.e. the hysteresis loop is small and the residual strain after complete removal of load is negligible. Palmar aponeuroses consist of collagen fibres, which have great tensile strength and represent the main load bearing element, extreme flexible elastic fibres and proteoglycans, contributing to the viscous stress component. Elastase treatment caused a significant increase both of hysteresis loop and residual strain, i.e. an increase of the viscous fraction<sup>3</sup>.

Changes of the elastic fibres can be observed in early stages of Dupuytren's disease, i.e. in apparently unaffected aponeurotic areas<sup>2</sup>. In thickened fibres and contracted areas, there is a reduction or complete loss of elastic fibres<sup>4,5</sup>. Collagen synthesis increases and the collagen fibres finally fuse to major bands. Electron-microscopic analysis, however, shows a desaggregation into thinner collagen fibrils<sup>6</sup>. The contracture bands themselves do not contain elastic fibres, but there are masses of elastic material between the fibrous cords. As to the ground substance changes, significant differences in the type and amount of glycosaminoglycans were found between the various manifestations of the disease process and the normal palmar connective tissue<sup>7</sup>.

The purpose of the present study was to examine the mechanical changes resulting from of an exposure of tissues from patients with Dupuytren's disease to elastase or chondroitinase ABC.

## Methods

### Specimens

Normal tendons from the palmaris longus muscle ( $n = 14$ ) and normal palmar aponeuroses ( $n = 23$ ) were obtained during surgery of carpal tunnel syndrome (18 patients, 2 male and 16 female; age range: 41–78 years). Apparently normal palmar aponeuroses ( $n = 9$ ) and contracture bands ( $n = 20$ ) were obtained at surgery of 16 male patients (age range: 42–70 years) with Dupuytren's disease. Generally, specimens of the apparently normal areas were excised from the 2nd or

3rd finger, whereas the contracture bands originated from the area of the 4th or 5th finger. A sketch of the palmar aponeurosis and the location of the fibre bundles excised for the investigations is presented in Figure 1. The specimens had a length of approximately 20 mm and a diameter of 2 mm. Rat-tail tendons ( $n = 12$ ) were obtained from adult Sprague–Dawley rats. The tail tendons were dissected free and single tendon fibres were pulled from the proximal end of the tail avoiding any straining. The diameter of rat tail tendon fibres was approximately 0.2 mm.

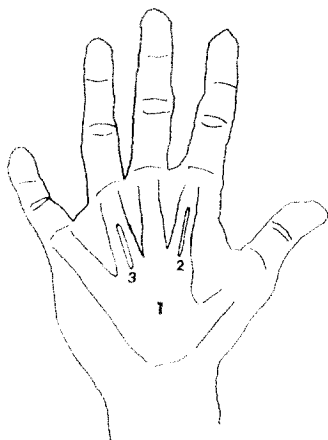
### Elastase treatment of tissue samples

Ten milligrams of saline-washed tissues were suspended in 1.0 ml phosphate-buffered saline (PBS, pH 7.2) containing 10 units of pancreatic elastase purified by affinity-chromatography to reduce non-specific protease contamination (Sigma E 0258, St Louis, MO, USA). According to the manufacturer the elastase activity of the enzyme was 86 U/mg protein, 1.7 U/mg trypsin. Digestion of elastin was performed at 37°C for 3 h at an enzyme:substrate ratio of 1:100 (w/w) in the presence of 0.1 mg soybean trypsin inhibitor per millilitre (Sigma, type I-S). This addition was necessary, since a significant tryptic activity was observed if 0.001 M benzoylarginine ethyl ester was used as substrate in the absence of soybean trypsin inhibitor. After the incubation interval the samples were centrifuged. Solubilized elastin peptides were determined<sup>8</sup> using the Lowry method<sup>9</sup> (Folin reagent). Soluble elastin from ligamentum nuchae was used as standard. In addition, we determined the hydroxyproline content of solubilized peptides using the chloramine-T method described by Stegemann and Stalder<sup>10</sup>. The activity of elastase was examined using elastin-congo red as substrate (Sigma; E-0502). To determine the degree of elastin digestion elastase-treated and control specimens were fixed in formalin, embedded in paraffin, and cut. Elastic fibres were demonstrated using the 'elastic stain' from Sigma (HT 25-A). In addition, the elastin content of the samples was determined before and after the elastin digestion<sup>11</sup>.

We determined the degree of enzymatic fibronectin degradation by immunofluorescence using a polyclonal rabbit antifibronectin antibody as primary antibody and a fluorescein isothiocyanate-labelled antibody from swine as second antibody (Dako, Copenhagen, Denmark).

### Chondroitinase treatment of tissue samples

Tissue specimens were washed in PBS and transferred to 1 ml PBS containing 1 U of chondroitinase ABC from *Proteus vulgaris* (C-2905, Sigma). The samples were incubated at 37°C for 24 h. The glycosaminoglycan content of enzyme-treated samples was compared to that of controls incubated in the absence of chondroitinase ABC. Small tissue samples were cut into pieces ( $<1 \text{ mm}^3$ ) and incubated in 0.5 ml 4 M guanidinium hydrochloride at room temperature for 18 h under continuous agitation. After centrifugation the resulting



**Figure 1.** Palmar aponeurosis 1, including 2, a specimen of an apparently normal tissue from the 2nd finger, and 3, a sample of a contracture band from the 4th finger.

supernatants were dialysed against PBS in order to remove the guanidinium hydrochloride. The dialysed fractions were treated with immobilized papain for 30 min at 37°C (P-4406; Sigma) and the digested supernatants recovered after centrifugation of the agarose beads. After dialysis against distilled water the glycosaminoglycans were determined via their uronic acid content by the carbazole method<sup>12</sup>.

### Mechanical tests

Strain-controlled tests were performed at a rate of 1% per min. The materials testing device is shown in Figure 2. It consists of a spindle driven by a gearbox motor, a load cell (maximum load 20 N, resolution 10 mN), a potentiometer used for displacement measurement (maximum open length 100 mm, resolution 0.01 mm), a bath containing PBS, and the clamps. As padding material we used waterproof abrasive paper glued to the clamps, a method proposed by Viidik<sup>13</sup>. Strain (relative increase in length) is defined as change in length divided by original length, i.e. the maximum length of a specimen when zero load is applied. This length differs from the *in-vivo* situation by 2–3%. Strains and loads were plotted with an X–Y graph recorder and then digitized. Load was converted into stress by forming the ratio load by collagen content per unit length<sup>14</sup>. For comparison of enzyme-treated and control specimens we calculated parameters which are independent of the individual geometry of a sample: (a) normalized hysteresis loop, i.e. the ratio of dissipated energy to strain energy, and (b) the residual elongation after complete removal of load. Both quantities were measured after an unloading phase starting from a strain level of 10%.

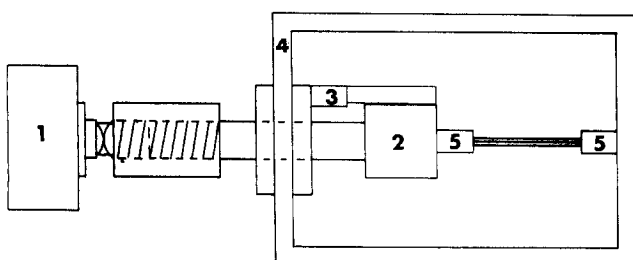
### Statistical analysis

The Kruskal–Wallis test was used to detect overall effects, followed by U-test for comparison of enzyme-treated and control specimens.  $P < 0.05$  was considered statistically significant.

## Results

### Elastase digestion

Incubation of Congo-red elastin with elastase resulted in the complete solubilization of the substrate (1 mg



**Figure 2.** Elements of the materials testing machine: 1, spindle driven by a gearbox motor; 2, load cell; 3, potentiometer for displacement measurement; 4, bath containing phosphate-buffered saline, and 5, the clamps.

incubated at 37°C for 3 h). When comparing the degree of elastin solubilization obtained by elastase treatment of different tissues, we observed the highest extraction in normal palmar aponeuroses (62.0  $\mu\text{g}/\text{mg}$  SD, 14.2), the lowest in normal tendons (26.0  $\mu\text{g}/\text{mg}$  SD, 4.6). Apparently normal palmar aponeuroses (42.3  $\mu\text{g}/\text{mg}$  SD, 5.3) and contracture bands (35.9  $\mu\text{g}/\text{mg}$  SD, 11.4) from patients with Dupuytren's disease showed intermediate solubilities. Elastin extractability shows significant differences between samples representing different manifestations of the disease process, with the exception of apparently normal areas compared to contracture bands (Table 1). Elastase treatment reduced the elastin content by 60–70% depending on sample geometry and origin. Analysis of elastase-digested tissue samples from palmar aponeuroses and human tendons by a histological stain for elastin revealed the elimination of large amounts of elastin indicating deep penetration of the enzyme. Electron-microscopic investigation showed that in most tissue areas elastin was completely digested<sup>3</sup>. Elastase digestion of rat-tail tendons did not result in the solubilization of significant amounts of collagen or other proteins, as verified by the determination of the hydroxyproline concentration and protein content of the supernatants. In contrast, the enzyme solubilized significant amounts of protein from human tendons and palmar aponeuroses. The hydroxyproline content of these solubilized peptides was 1.60% SEM, 0.4 (w/w). No significant digestion of fibronectin was observed by immunofluorescence analysis.

### Chondroitinase ABC digestion

The efficiency of glycosaminoglycan degradation by chondroitinase ABC was determined by comparing the glycosaminoglycan content of the specimens before and after enzymatic digestion. The degree of digested glycosaminoglycans from total ranged from 60 to 80%.

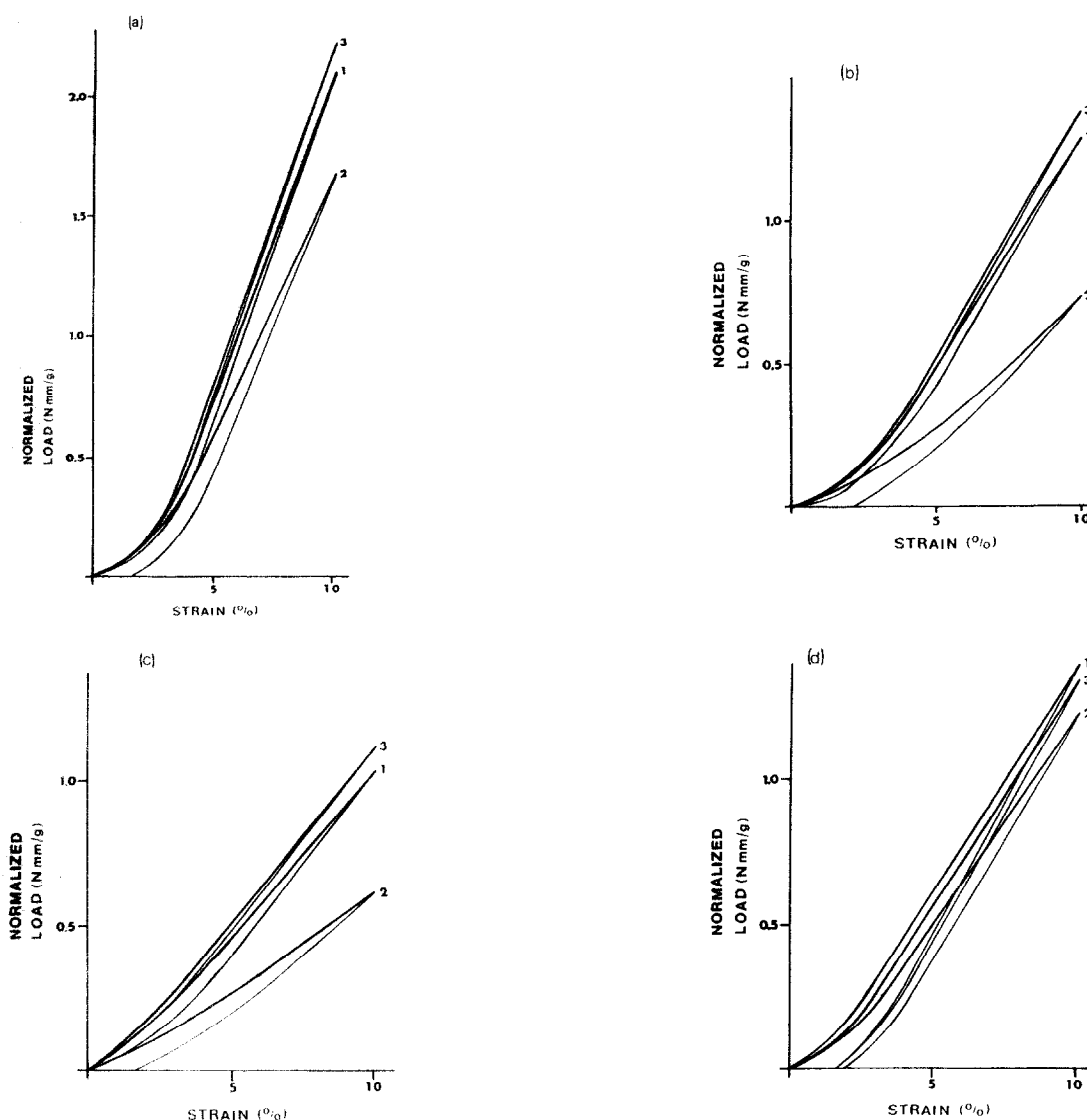
### Biomechanical results

The non-linear stress–strain relationships are presented in Figure 3. The stress–strain curves of elastase-treated samples showed increased extensibility for normal tendons, normal and apparently normal palmar aponeuroses but not for contracture bands and rat-tail tendons. Young's modulus (Figure 4) from the linear portion of the stress–strain graphs of native samples displays a maximum for normal tendons, intermediate

**Table 1.** Statistical evaluation of elastin digestion

	NT(P)	NPA(P)	ANPA(P)	CB(P)
NT		<0.01	<0.01	<0.05
NPA	<0.01		<0.01	<0.01
ANPA	<0.01	<0.01		NS
CB	<0.05	<0.01	NS	

NT, normal tendon; NPA, normal palmar aponeurosis; ANPA, apparently normal palmar aponeurosis; CB, contracture bands.



**Figure 3.** Stress–strain relationship of **a**, normal tendons; **b**, normal palmar aponeuroses; **c**, apparently normal palmar aponeuroses; and **d**, contracture bands. 1, controls; 2, after elastase digestion; and 3, after treatment with chondroitinase ABC.

values for normal palmar aponeuroses and contracture bands from patients with Dupuytren's disease, and a minimum for the apparently normal areas.

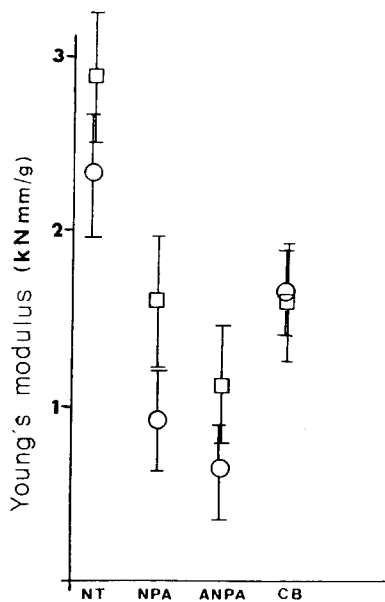
As for the viscoelastic behaviour, normal human tendon, normal palmar aponeurosis, apparently normal palmar aponeurosis, and rat-tail tendons reveal low values of residual strain and normalized hysteresis loop. In contracture bands, however, these parameters are significantly increased. Elastase digestion resulted in a significant augmentation of both biomechanical parameters in normal tendons, normal and apparently normal palmar aponeuroses, but not in contracture bands (Tables 2 and 3) and rat-tail tendons (Young's modulus: 8400 Nmm/g SEM, 540, residual elongation: 0.14% SEM, 0.03 and normalized hysteresis loop: 10.2% SEM, 0.6). Figures 5 and 6 indicate that after the extraction of elastin residual strain and normalized hysteresis loop represent an approximately linear function of the amount of elastin digested.

The alterations of biomechanical properties induced by chondroitinase ABC were not as pronounced as the

effects of elastase. The stress–strain curves were not affected significantly by chondroitinase ABC, although there was a slight decrease of extensibility in normal and apparently normal palmar aponeuroses. The residual strain was significantly increased for normal and apparently normal palmar aponeuroses, whereas the normalized hysteresis loops were reduced. Again, changes in biomechanical parameters of contracture bands were not significant (Tables 2 and 3).

### Discussion

Normal palmar aponeuroses are composed of 'wavy' collagen fibres and thin, scattered elastin fibres forming highly branching networks between bundles of collagen. For skin and tendon it has been shown that collagen fibrils wind around elastin cores<sup>15</sup>. It was proposed by Minns et al.<sup>16</sup> and Oxlund et al.<sup>17</sup> that the elastin fibres are responsible for the recoiling of extended collagen fibrils. During the course of Dupuytren's disease the number of elastic fibres decreases within the 'collagen bands',



**Figure 4.** Young's modulus of the untreated tissues compared with elastase-digested tissues. The different sample classes were normal tendons (NT), normal palmar aponeuroses (NPA), apparently normal areas (ANPA), and contracture bands (CB) of patients with Dupuytren's disease. □ Controls; ○ elastase-digested tissues.

so that finally, in the contracture bands, elastic fibres are only found within the perifascicular tissue<sup>5,6</sup>.

To study the role of elastin in Dupuytren's disease, normal tendons, normal palmar aponeuroses, and tissues from various disease manifestations were incubated with highly purified elastase plus soybean trypsin inhibitor. Control studies were performed ascertaining that collagen fibres were not degraded by our elastase preparation: no collagen was solubilized from rat-tail tendons, and the mechanical properties of this tissue, which contains no elastin<sup>18</sup>, remained unchanged after the enzymatic digestion. As to the digestion of elastin from palmar aponeuroses, we find a decrease in this parameter from normal via apparently normal palmar aponeuroses to contracture bands. These results correlate with the elastin content of the

respective tissues, as verified by the histological studies of Millesi<sup>4</sup>. The degree of elastin extraction (60–70%) was similar in all tissue samples, irrespective of the clinical involvement. The hydroxyproline content of the solubilized peptides was low and independent of the relative amount of type III collagen, indicating that neither type I nor the more susceptible type III collagen was attacked by the enzyme. Immunohistochemical analysis with anti-type-III antibodies demonstrated that the fine type III collagen fibres were not significantly degraded. The addition of soybean trypsin inhibitor, which abolished the small original tryptic activity of our elastase preparation, cannot prevent the degradation of fibronectin, since this glycoprotein is readily digested by elastase itself. Fibronectin was found to be even more sensitive to proteolysis than the majority of other extracellular matrix proteins<sup>19</sup>. Fibronectin may be viewed as a 'link' protein forming mechanical bonds (a) between cells and their immediate extracellular environment, and (b) between the extracellular matrix components themselves. According to our study, however, immunofluorescence analysis did not reveal the disappearance of fibronectin after elastase digestion. This can be explained by the fact that collagen-binding fibronectin fragments, generated by incubation with elastase, stick to the collagen matrix and are detected by the polyclonal antifibronectin antibodies. This does not exclude the possibility that fibronectin bridges between proteoglycans and collagen may be destroyed by the enzyme.

During the loading procedure the initially 'wavy' collagen fibres become more and more parallel to the axis of applied load resulting in a non-linear stress-strain relationship (Figures 3a-d). The following nearly linear portion of the stress-strain behaviour indicates that the collagen fibres themselves are strained and represent the 'reserve strength' of collagenous tissues<sup>20</sup>. Normal tendons show significantly higher values of Young's modulus as compared to specimens from normal as well as diseased palmar aponeurosis, among which the apparently normal aponeurotic areas display the lowest value of Young's modulus, a finding which

**Table 2.** Residual strain of different tissues compared to normal tendons and its change after enzyme treatment

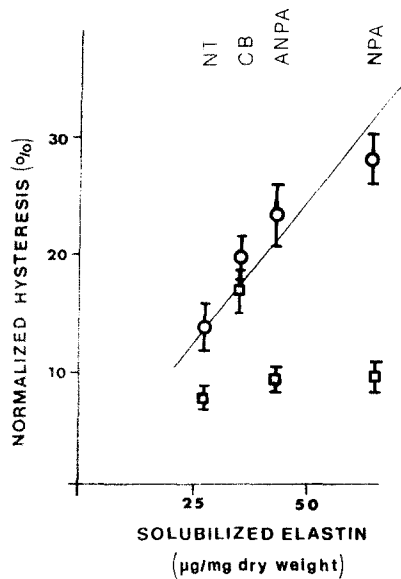
Tissue	Residual strain of untreated samples ( $\times 10^{-3}$ )	Relative change after enzymatic treatment (%)	
		Elastase	Chondroitinase
Normal tendon	2.3 (0.5)	+465 (176) <sup>b</sup>	+74 (9.1) <sup>n</sup>
Normal palmar aponeurose	1.6 (0.3) <sup>n</sup>	+1275 (225) <sup>b</sup>	+119 (15) <sup>a</sup>
Apparently normal palmar aponeurose	2.2 (0.4) <sup>n</sup>	+673 (154) <sup>b</sup>	+141 (29) <sup>b</sup>
Contracture bands	12.0 (2.0) <sup>d</sup>	+41.7 (4.9) <sup>n</sup>	+7.5 (2.2) <sup>n</sup>

SEM in parentheses; n, not significant; a,  $P < 0.05$ ; b,  $P < 0.01$  versus control; d,  $P < 0.01$  versus normal tendon.

**Table 3.** Normalized hysteresis loop of different tissues compared to normal tendons and its change after enzyme treatment

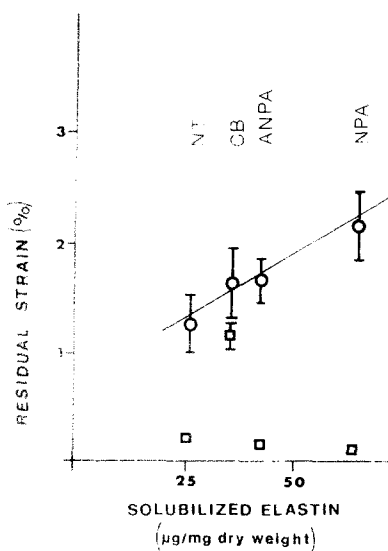
Tissue	Hysteresis loop of untreated samples ( $\times 10^{-3}$ )	Relative change after enzymatic treatment (%)	
		Elastase	Chondroitinase
Normal tendon	79.0 (5.0)	+80 (11.5) <sup>b</sup>	-64.6 (7.5) <sup>b</sup>
Normal palmar aponeurose	86.0 (8.0) <sup>n</sup>	+231 (25.0) <sup>b</sup>	-48.8 (5.2) <sup>b</sup>
Apparently normal palmar aponeurose	87.0 (9.0) <sup>n</sup>	+175 (18.0) <sup>b</sup>	-57.5 (11) <sup>b</sup>
Contracture bands	191 (28.0) <sup>c</sup>	+4.2 (3.7) <sup>n</sup>	-16.8 (5.0) <sup>n</sup>

SEM in parentheses; n, not significant; a,  $P < 0.05$ ; b,  $P < 0.01$  versus controls; c,  $P < 0.05$  versus normal tendon.



**Figure 5.** Residual strain of normal tendons (NT), normal palmar aponeuroses (NPA), apparently normal areas (ANPA), and contracture bands (CB) after removal of elastin as a function of the amount of digested elastin. □ Controls; ○ elastase-digested tissues.

could be explained by progressive fibre fragmentation as described for this stage by Nemetschek<sup>6</sup> et al. After straining the samples into the linear range, the unloading curves differ from the loading curves resulting in a hysteresis loop, which is a measure for the energy loss during the loading–unloading cycle. We observed an increase of both normalized hysteresis loop and residual strain from normal via apparently normal palmar aponeuroses to contracture bands. The increase of residual strain of apparently normal as compared to normal palmar aponeuroses may be due to the fact that



**Figure 6.** Normalized hysteresis loop of normal tendons (NT), normal palmar aponeuroses (NPA), apparently normal areas (ANPA), and contracture bands (CB) after removal of elastin as a function of the amount of digested elastin. □ Controls; ○ elastase-digested tissues.

in apparently normal tissues collagen bundles are always surrounded by thin strands of collagen not aligned in the direction of the palmar aponeurosis<sup>2</sup>. Biochemically, apparently normal palmar aponeuroses differ significantly from the normal controls<sup>2,21</sup>. The much higher viscous stress component of contracture bands as compared to normal palmar aponeuroses may be explained by the formation of non-covalent chemical interactions among adjacent molecules resulting in the stabilization of collagen<sup>22</sup>, and by the strong increase of type III collagen relative to type I collagen<sup>21</sup>. In addition the morphology of contracture bands is completely different from normal and apparently normal palmar aponeuroses: collagen fibres are fused into compact huge bundles oriented in the direction of the aponeurotic fascia.

The mechanisms of elastin elasticity are still unresolved and the pattern of the molecular organization of elastin molecules into fibrils may strongly influence the mechanical properties of the tissues. The relationship between fibril and matrix mechanics has been investigated only for ligament elastin<sup>23</sup>. Of all the connective-tissue components, elastin is the most inert protein and has the lowest turnover rate. It is therefore particularly susceptible to long-term modifications, as seen in Dupuytren's disease. However, the potential impact of elastin alterations on the pathogenesis of Dupuytren's disease has not been explored so far. In analogy to the results of Oxlund et al.<sup>17</sup> obtained with elastase-digested skin specimens, we found greater extensibility and loss of elastic behaviour in tendons and palmar aponeuroses after elastase treatment. In normal and apparently normal palmar aponeuroses Young's modulus was reduced to approximately 60% of the original value by elastase in contrast to the contracture bands showing no mechanical changes. Since collagen itself was not attacked by the enzyme, our interpretation of this phenomenon is that the removal of elastin and other minor components such as fibronectin<sup>2</sup> changes the whole spectrum of interactions between the remaining components, resulting in sum in a decreased collagen–collagen interaction. The dramatic increase of residual strain after elastase digestion in normal and apparently normal palmar aponeuroses indicates that elastin fibres are responsible for the recoiling mechanism after stress or deformation has been applied. After elastase treatment, however, the viscous fraction of normal tendons was still smaller than that of contracture bands, while in normal and apparently normal palmar aponeuroses this parameter even exceeded that of fibrotic cords. In contrast, the already elevated residual elongation of untreated contracture bands was not significantly affected by elastase, suggesting that the interaction between collagen fibrils and elastic cores had already been impaired before elastase digestion by marked pathologic structural alterations characteristic for the fibrotic cords. The final value of both residual elongation and hysteresis loop after elastase treatment, representing the 'pure' collagen mechanics of the different tissues,

was proportional to the amount of digested elastin. Thus only the removal of most of the elastin revealed an altered viscoelastic behaviour of apparently normal areas, corresponding to the already altered collagen structure in early disease stages. In advanced stages of the disease biomechanical alterations can no longer be remedied by the elastin component.

Like collagen, elastin is intimately associated with proteoglycans<sup>24,25</sup>. Several investigators studied the contribution of the ground substance matrix to the biomechanical properties of tendons<sup>13,26</sup> and skin<sup>17,27</sup>. In our study we observed that the overall alterations of normalized hysteresis loop and remaining elongation induced by chondroitinase ABC was not as conspicuous as that induced by elastase. Chondroitinase ABC digestion caused a decrease of the normalized hysteresis loop, which might be due to a reduced intrinsic viscosity of the ground substance and/or reduced electrostatic interaction between proteoglycans and collagen fibres after the enzymatic elimination of negatively charged glycosaminoglycan chains<sup>28</sup>. In contrast the remaining elongation was slightly increased after chondroitinase ABC treatment, indicating an impaired recoiling mechanism after the elimination of proteoglycan bridges between elastin and collagen. In this respect the effect of chondroitinase ABC resembles that of elastase.

In conclusion, in normal palmar aponeuroses the elastic fibres are responsible for the recoiling mechanism and contribute to the maintenance of an elastic state with a low viscous stress component. Since the viscous fraction of apparently normal palmar aponeuroses after elastase treatment (15.2% SEM, 4.4) is similar to the elevated value of untreated contracture bands (19.1% SEM, 2.8), the loss of the mechanical function of elastin, as simulated by elastase digestion, may be regarded as pathogenetically relevant for Dupuytren's disease.

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