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THE CONTINUOUS ELONGATION TECHNIQUE FOR SEVERE DUPUYTREN'S DISEASE

A biochemical mechanism

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Continuous extension of Dupuytren's contracture prior to fasciectomy results in a softening of the tissue, allowing straightening of the fingers. The observed change in cross-link profile indicates an increase in newly synthesised collagen due to increased turnover. This was confirmed by demonstration of the increases in levels of the degradative enzymes, the neutral metalloproteinases, collagenase and gelatinase and the acidic cathepsins B and L. Both types of enzyme effectively depolymerize the collagen fibres, albeit by different mechanisms, leading initially to loss of tensile strength and ultimately to solubilization. We suggest that the increase in enzyme activity is generated by tension on the fibroblasts of this metabolically active tissue produced during the continuous extension of the retracted fingers. The weakening of the fibres by degradation and the increase in newly synthezised collagen provide an explanation for the extension of the tissue without trauma.

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The continuous elongation technique (or Technica di Estensiona Continua, TEC) has been developed by Messina (1989) and Messina and Messina (1991) for patients with such serious contracture of the fingers that amputation is often the only alternative. The fingers are slowly extended on an adjustable frame over a period of 2 to 3 weeks until the fingers are straight, at a rate of 2 mm per day prior to fasciectomy. Initially resistance to stretching is high, but is gradually reduced until at the end of the 2 to 3 week period the tissue is noticeably softer and more pliable. There is no visible evidence of tissue rupture or haemorhages at operation. The ability to stretch these long-term tough contractures, and the gradual reduction in resistance to the applied tension, suggests that it must be due to a change in the properties of the collagen, the major mechanical component in the tissue.

Collagen fibres are generally considered to be virtually inelastic, and this mechanical strength of collagen is dependent on the formation of a series of intermolecular cross-links between the collagen molecules in the fibre (Bailey, 1991). Newly synthesized collagen is stabilized by the divalent cross-links hydroxylysino-keto-norleucine and dehydro-hydroxylysinonorleucine which are readily detectable after borohydride reduction as dihydroxylysinonorleucine (DHLNL) and hydroxylysinonorleucine (HLNL) respectively. These reducible cross-links are intermediates in the formation of the trivalent non-reducible cross-links present in mature tissue, i.e. hydroxylysyl-pyridinoline (Hyl-Pyr) and lysyl-pyridinoline (Lys-Pyr) from the keto-imine, and histidinohydroxylysinonorleucine (HHL) from dehydro-HLNL.

We have previously shown an increase in these intermediate cross-links in Dupuytren's nodules and bands (Bailey et al, 1977; Bazin et al, 1985) as would be expected in any newly synthesized collagen formed during fibrosis. We have also shown that a low but significant level of these cross-links was maintained in the long-term Dupuytren's patients (15–20 years) indicating a higher turnover of collagen than the unaffected aponeurosis even after many years. This localized high turnover is reminiscent of hypertrophic scar tissue, which, in contrast to normally healed scar, does not mature (Bailey et al, 1975), that is, achieve a low turnover with a consequent maturation of the cross-links, comparable with that of the surrounding dermis. The role of collagen in Dupuytren's disease has recently been reviewed (Bailey, 1990).

It has long been known that Dupuytren's tissue possesses a higher proportion of fibroblasts than normal surrounding tissue, and several groups have shown that under tension *in vitro* fibroblasts can be stimulated. For example, Miekle et al (1980) have shown that tensile mechanical stress on collagenous tissue also results in the release of increased amounts of collagenase. We have also shown that pressure exerted on chondrocytes releases increased amounts of cathepsins B and L (Maciewicz et al, 1991) both of which are capable of depolymerizing the extracellular collagen fibre (Woessner, 1991). We reasoned that the tension exerted on the fibroblasts in the Dupuytren's contracture when undergoing TEC could similarly increase the level of these proteolytic enzymes *in vivo*. The catheptic enzymes are known to cleave the collagen molecules within the fibre in the non-helical cross-link regions; collagenase cleaves across the collagen triple helix, whilst the gelatinases cleave type IV collagen and completes the solubilization of the fragments of the fibrous collagens. Such actions result in the depolymerization of the collagen fibres resulting in a weaker tissue which would then be capable of being stretched. In this paper we describe evidence to support this proposed mechanism for the effect of TEC on Dupuytren's tissue.

Not clear which MMPs and TIMPs this data describes.

MATERIALS AND METHODS

11 specimens were surgically removed from patients with Dupuytren's disease after having been subjected to the continuous elongation technique (TEC). The average age of the patients was 58 years, and the duration of the disease from 2 to 22 years. Control fibrotic specimens, i.e. non-stretched Dupuytren's tissue (ten) were obtained from patients undergoing the standard surgical procedure.

Preparation of samples for electrophoresis

The samples were frozen in liquid nitrogen, pulverized in a freezer mill (Mikro-dismembrator: B Braun Intern.) and then extracted in distilled water containing 0.1% Brij 35 (2 ml per 100 mg sample) by repeatedly (4×) freezing and thawing the samples. After centrifugation (10,000 g, 4 C), the protein content of each supernatant was determined using a Lowry based method or BCA assay (Pierce Chemicals Ltd.). Aliquots (20 μg) of each protein extract were freeze-dried, reconstituted in non-reducing sample buffer with twice normal amounts of SDS, and heated at 60°C for 1 hour prior to loading onto the polyacrylamide gels.

Preparation of the pure enzymes

Cathepsins B and L were purified from human liver essentially as described by Maciewicz et al (1989). The molar concentration of the active enzyme was determined by active site-titration using the synthetic inhibitor Ep-475 (Taisho Pharmaceuticals, Japan; Barrett and Kirschke 1981).

Partially purified collagenase was obtained from human fibroblast cell cultures stimulated for 24 hour with PMA (phorbol myristate acetate in serum free medium).

Analysis of cross-links

The reducible and mature cross-links were analyzed by reduction of the tissue with sodium borohydride, and separation by ion-exchange chromatography on an LKB amino acid analyzer. The conditions for the borohydride reduction and the subsequent concentration of the cross-links on a mini CF1 cellulose column prior to analysis on the modified amino acid analyzer, have been previously described in detail (Sims and Bailey, 1992).

The reduced cross-links were identified as DHLNL and HLNL. The mature cross-links are the pyridinolines, Hyl-Pyr and Lys-Pyr and HHL. The location of the cross-links on the analyzer has previously been confirmed with samples of the authentic compounds. The quantification was based on ninhydrin colour reaction and their known leucine equivalents.

Analysis of degradative enzymes

Cysteineproteinases

Cathepsins B and L. The samples were weighed, SDS PAGE buffer containing twice the normal amount of SDS added (5 ml) and then heated to 60°C for 1 hour. An aliquot of each was loaded into the well of a 12.5% polyacrylamide gel and the samples then electrophoresed.

The samples in the gel were electrophoretically transfered to Immobilon-P (pore size 0.45 μm, Millepore, Bedford, MA), blocked with 5% dried milk in TBS (pH 7.4) plus 0.2% Tween for 24 hours. The catheptic enzymes were detected by monoclonal antibody to human cathepsin B (19 μl in 10 ml), and a polyclonal to cathepsin L, (Maciewicz et al, 1989) and then the appropriate anti-species IgG peroxidase conjugated antibody. Visualization of the enzymes was achieved using ECL Western blotting detection system (Amersham International) and exposed X-ray film (Kodak XRP 5) for a minimum of 24 hours.

Metalloproteinases

Collagenase

A 10% polyacrylamide gel was used for separating collagenase and the protein probed with a sheep anti-collagenase following the protocol outlined in detail for the cathepsins.

Gelatinase

Analysis of gelatinolytic metalloproteinases (MMPs) was performed on aliquots of tissue extracts. Unreduced samples were run on 10% polyacrylamide SDS gels co-polymerized with 0.5 mg/ml bovine gelatin (Sigma Ltd) After electrophoresis, gels were washed in 2.5% Triton X-100 to displace SDS and renature the enzymes, and then incubated for 18 hours at 37°C in 50 mM Tris/HCl (pH 7.8) supplemented with 50 mM calcium chloride, 0.5 M sodium chloride, and 1 mM APMA to activate the pro-enzymes. Gels were stained with 0.1% Coomassie Blue in 10% acetic acid-methanol mix. Zones of MMP gelatinolytic activity were characterized by clear bands in the gel, and compared with molecular weight standards and control gelatinase-A pro-enzyme prepared from conditioned medium of WERI-1 tumour cells treated with 0.2 μM retinoic acid.

Zones of proteolysis in the gels were quantitated by scanning densitometer (LKB Gelscan XL), each sample lane being scanned along three different axes to ensure representative readings. Both width and intensity of the zones were estimated by calculating the area over the deflection for each band.

RESULTS

Analysis of collagen cross-links

Reducible cross-links

The reducible cross-links were barely detectable in the normal mature aponeurosis but increased five to ten-fold in the unstretched Dupuytren's fibrotic tissue as anticipated from previous results on this type of tissue (Bazin et al, 1985; Bailey, 1990). Examination of the tissue after subjection to the TEC revealed a further significant increase in both reducible cross-links indicating the presence of a higher proportion of newly synthesized collagen, i.e. increased synthetic activity of the fibroblasts. The effect of TEC on these immature cross-links is shown in Figure 1a.

Mature cross-links

HHL. The normal aponeurosis from age-matched controls revealed a high level of HHL as expected from a mature tissue. In the Dupuytren's tissue the level was significantly reduced due to the increased proportion of newly synthesized collagen. The level of the HHL in the Dupuytren's tissue subjected to the TEC was found to be reduced to an even lower level. This decrease in the major mature cross-link is consistent with the increase in the proportion of newly synthesized collagen, i.e. an increased turnover rate, over and above the turnover rate of the unstretched tissue.

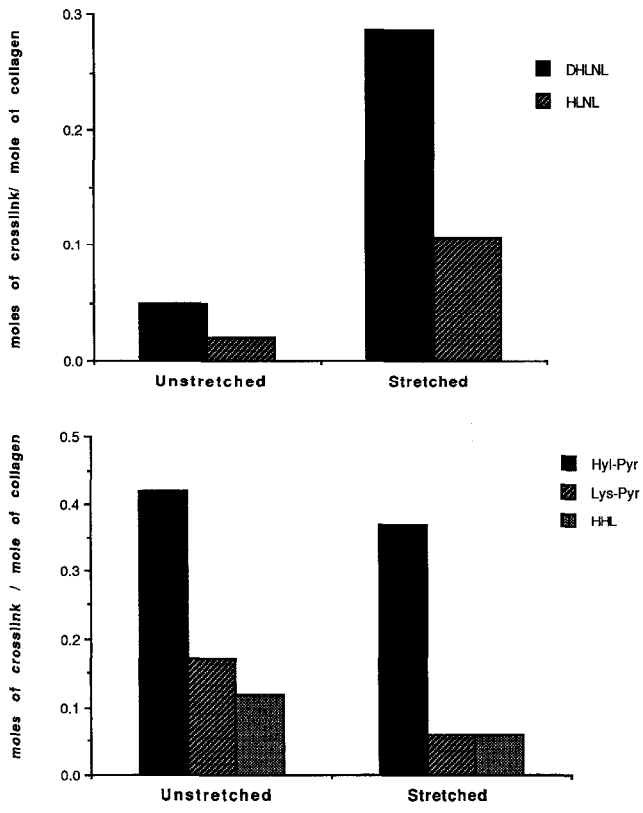

Fig 1 Change in cross-link levels following TEC on Dupuytren's tissue. (a) The effect of TEC on the levels of the immature cross-links HLNL and DHLNL in Dupuytren's tissue compared to unstretched tissue. (b) The effect of TEC on the levels of the mature cross-links, Hyl-Pyr, Lys-Pyr and HHL, in Dupuytren's tissue compared to unstretched tissue.

Pyridinolines. The proportion of the major pyridinoline, Hyl-Pyr was high in the mature controls but surprisingly remained high in the Dupuytren's tissue, both stretched and unstretched. The Lys-Pyr appeared to be lower in the stretched tissue, but the difference was not significant. Although pyridinoline is generally considered a mature cross-link, values are often high in tissues with a rapid turnover, e.g. periodontal ligament. The effect of TEC on the level of these mature cross-links is shown in Figure 1b.

Degradative enzymes

Metalloproteinases

Gelatinases. Zymographic analysis of samples of TEC treated as compared with untreated tissue of similar disease duration, about 15 years, show a clear increase in the levels of gelatinase-A (Fig 2). Similar high levels of gelatinase were evident in tissue from short duration disease, i.e. about 2 years. Specimens from unstimulated fibrotic tissue of long duration (>10 years) revealed low measurable quantities of gelatinase-A, but predominantly in the form of inactive pro-enzyme. As well as an overall increase in enzyme levels, there was seen to be a clear shift towards activated enzyme in tissue stimulated by TEC, and in the case of short duration disease (Fig 3). The mechanism by which gelatinase-A is activated is not well characterized, but is believed to be cell membrane associated (Murphy and Docherty, 1992), and in this instance is probably a result of fibroblast stimulation.

Collagenase. Neutral collagenase showed an increase in the levels of the active enzyme in the TEC treated tissues. Comparisons of non-stretched and stretched tissue were made on patients with a similar duration of Dupuytren's disease. Initial studies were carried out on tissue from patients with a disease duration of 10 to 20 years. Analysis of tissue from short duration Dupuytren's disease revealed high levels of enzyme prior to TEC indicating a high turnover of the tissue in early disease (Fig 4b).

Cysteineproteinases

Cathepsins B and L. Examination of samples from unstretched Dupuytren's tissue revealed low levels of cathepsin B and L. In contrast, tissue from Dupuytren's

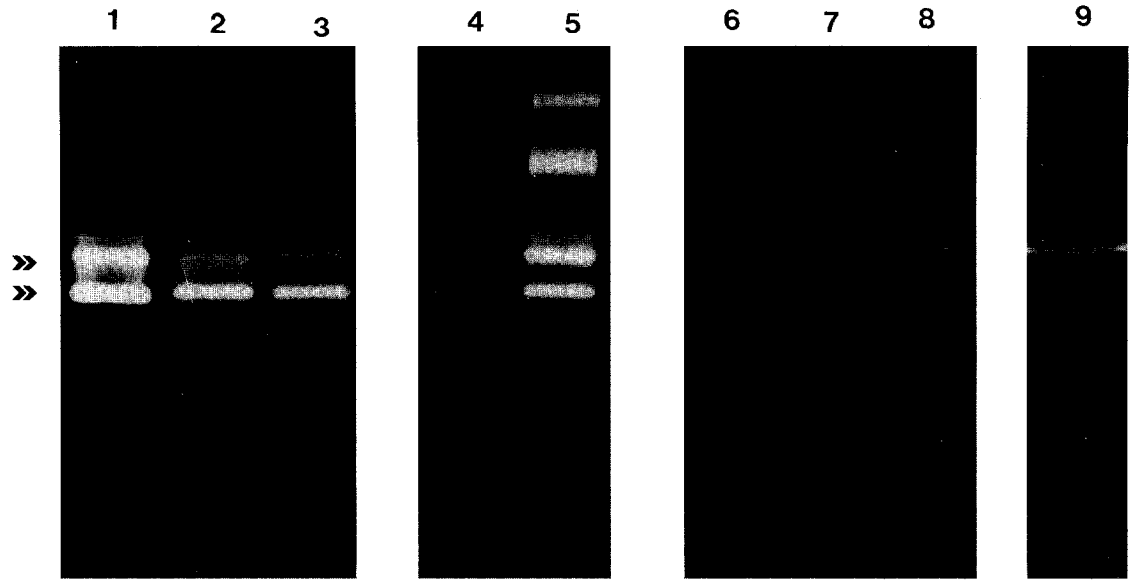

Fig 2 Zymogram showing gelatinase A activity in TEC treated Dupuytren's tissue. (a) Tracks 1 to 3 TEC treated tissues of long duration (>10 years); tracks 4 and 5, untreated short duration disease tissue (at 2 years); tracks 6 to 8 untreated long duration tissue (>10 years); track 9 pro-gelatinase A marker. Arrow heads indicate inactive gelatinase A (upper) and active gelatinase A (lower). (Note in tracks 1–3 and 4–5 greater overall levels of gelatinase A, and a greater proportion of the active enzyme than is seen in the long duration untreated controls, tracks 6–8. Gelatinolytic activity at higher molecular weight in track 5 of the zymogram suggests the presence of gelatinase B but this has not been confirmed.)

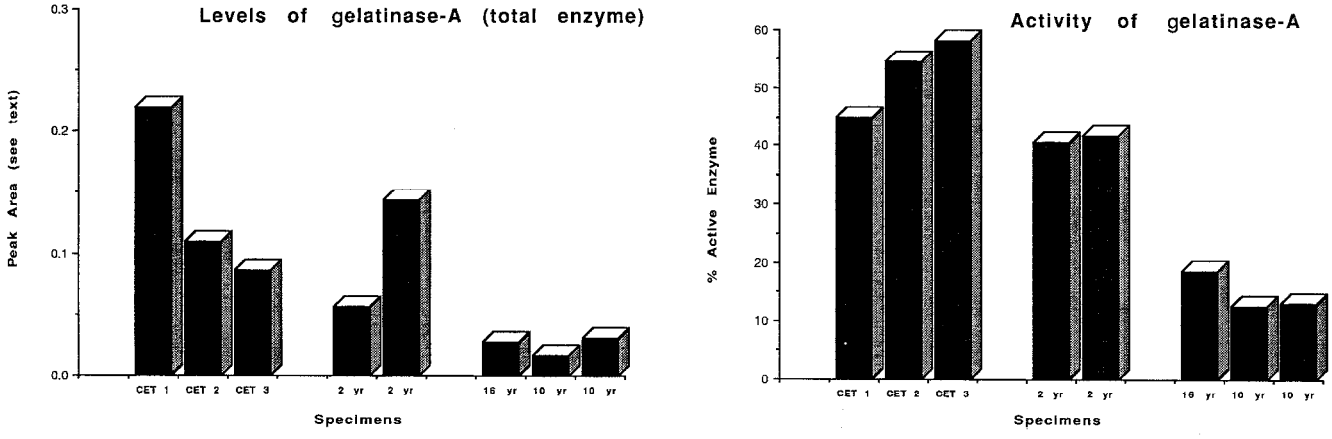

Fig 3 Comparison of the levels of gelatinase A determined from the zymogram as (a) total enzyme, and (b) % of active enzyme, for the TEC treated and untreated samples at about 2 year and >10 year duration of the disease. The levels were determined by scanning densitometer and calculation of the area of the zones of proteolysis.

subjects after subjection to the TEC revealed substantial quantities of both cathepsin B and L as seen on the immunoblots. The tension on the fibroblasts induced by the TEC clearly results in an increase in the amount of the degradative cathepsins present in the tissue. Similar results were obtained for cathepsins B and L; typical results are shown for cathepsin B in Figure 4a.

DISCUSSION

The increase in the intermediate cross-links and concomitant decrease in the mature cross-links, together with the increased presence of the metalloproteinases and the catheptic enzymes, clearly indicate an increase in both the synthesis and degradation of the collagen in Dupuytren's tissue following subjection to the TEC. The hydroxylation and type III collagen levels were found to be unchanged after TEC, suggesting that the rate of new collagen synthesis is low compared to the rate of degradation.

The increased ability to stretch the tissue more easily within a few days of initiating the TEC, and the increased levels of the degradative enzymes, suggest that the effect must be due to the depolymerization of the collagen

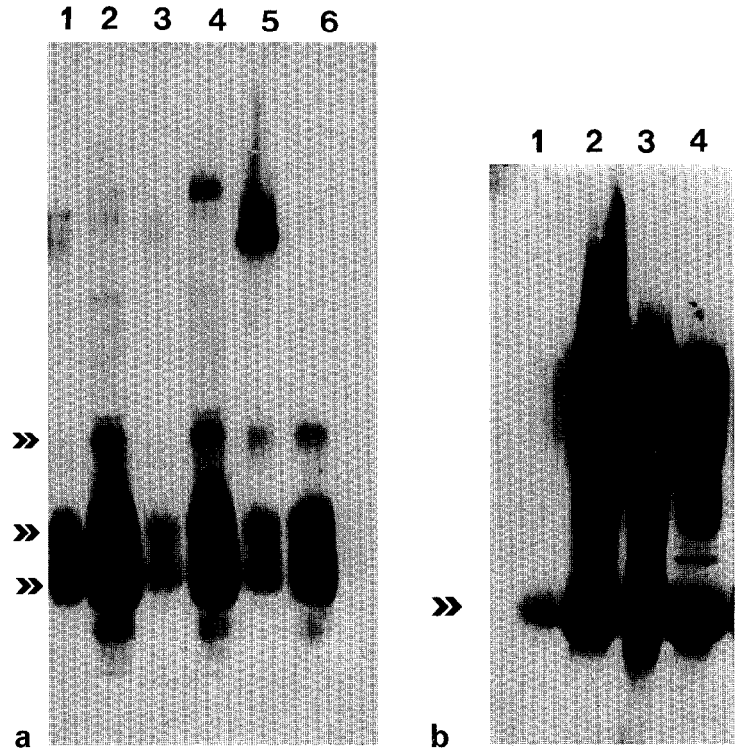

Fig 4 Detection of proteinases in Dupuytren's tissue by Western blotting using monoclonal antibodies. (a) Detection of Cathepsin B using a monoclonal antibody to cathepsin B. Track 1 cathepsin B marker; track 2 TEC treated tissue at 15 years; track 3 non-TEC tissue at 15 years; track 4 TEC treated tissue at 15 years; track 5, non-TEC tissue at 2 years; track 6 TEC treated tissue at 15 years. Arrows denote procathepsin B (upper) and the two active forms of cathepsin B of different molecular weight (two sets of lower arrows). (b) Detection of neutral collagenase using a monoclonal antibody to collagenase. Track 1 non-TEC 20 years duration; track 2 TEC treated tissue at 15 years duration; track 3 TEC treated tissue at 10 years duration; track 4, non-TEC 2 years duration. Arrows denote position of active collagenase.

fibres. The cathepsins are known to act by cleavage of the polymerized collagen molecules in the non-helical cross-link region. Only a few cleavage points along the fibres would be necessary to allow slippage and release of the contracture when subjected to tension. The collagenase cleaves the collagen within the triple helix and the gelatinases degrade collagenous fragments, similarly leading to loss of mechanical strength. This reduction in mechanical strength of the existing tissue is then further enhanced by replacement of the mature trivalent cross-links by newly synthesized collagen stabilized only by the intermediate divalent cross-links. The linking of the microfibrils through HHL and pyridinoline is lost and consequently slippage of the individual microfibrils under tension can occur. The precise role of the trivalent pyridinoline cross-link is not clear since it is retained at a high level in the stretched tissue.

We therefore suggest that the mechanism stimulating the initial depolymerization of the 'mature' collagen and the subsequent increased turnover during extension are the effects of the tension on the fibroblasts of the contracture stimulating the cells to secrete excess degradative enzymes. These enzymes cause sufficient depolymerization and degradation of the collagen fibres to account for the ability of the TEC to stretch the mature Dupuytren's fibrotic tissue until the finger is completely extended, and thus permit normal surgery.

The release of the tension should, if the fibrotic tissue is not surgically removed, lead rapidly to contraction and reversion to the original position of the finger as the activated fibroblasts revert to normal, and contraction occurs through the action of the myofibroblasts on stable collagen fibres. The mechanism by which mechanical deformation activates fibroblasts is not understood, and may involve cytoskeleton elements in mediating signals through specific integrins as membrane receptors (Wang et al, 1993).

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