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# Differential Expression of Fibronectin Splice Variants, Oncofetal Glycosylated Fibronectin and Laminin Isoforms in Nodular Palmar Fibromatosis

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## SUMMARY

*The tissue formation process in nodular palmar fibromatosis (Morbus Dupuytren) was investigated by the demonstration of fibronectin splice variants (ED-A and ED-B fibronectin), de novo glycosylated fibronectin and laminin isoforms (A, M, B1, B2, s chains) in association to the proliferative activity (Ki-67 antigen) and the occurrence of myofibroblast phenotype (α-smooth muscle actin, desmin). The proliferative noduli of the fibromatosis were characterized by a diffuse immunostaining for alpha-smooth muscle actin, and single cells positive for desmin and the Ki-67 antigen. In contrast to the surrounding aponeurosis as extracellular matrix, components of the whole proliferative noduli were defined: ED-A, ED-B and de novo glycosylated fibronectin, B1 and B2 laminin chain, tenascin and collagen type IV. The demonstration of the A and M laminin chain was restricted to a few cells of the proliferative noduli. S laminin could be visualized in the majority of palmar aponeurotic fibroblasts. As revealed by mRNA, in situ hybridization a de novo synthesis of fibronectin could only be detected within proliferative noduli.*

*There is a positive correlation between the myofibroblast phenotype formation, cellular proliferation and the occurrence of ED-A and ED-B containing fibronectin, as well as de novo glycosylated fibronectin in Dupuytren's disease. The ultrastructural irregularities of myofibroblastic basal lamina and the heterogeneity of the myofibroblast phenotype are equivalent to the variability of laminin isoform immunostaining.*

## Introduction

Nodular palmar fibromatosis (Morbus Dupuytren) is a self-limited tumorous proliferation of fibroblasts[18,65]. At the onset of the disease proliferative noduli with predominant myofibroblasts are formed. A second phase is characterized by a loss of proliferative activity, a decrease of myofibroblasts and an abundant collagen matrix formation. The process results in aponeurotic scar noduli with an irreversible contracture[28,46,55,56].

In fully developed proliferative noduli an extensive fibronectin deposition in the extracellular space[62] and specialized myofibroblast-matrix contacts (so-called fibronexus) can be found[19,64]. In the immediate vicinity of myofibroblasts the extracellular matrix (ECM) is mostly organized in the form of a discontinuous basal lamina[23]. According to our own findings[15] and in contrast to earlier reports[15,62], it corresponds biochemically and immunohistochemically to a complete basal lamina containing laminin and collagen



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type IV. Interactions between myofibroblasts and ECM during proliferation and/or differentiation of these cells have relevance for regulating the process of tumorous tissue formation in palmar fibromatosis[1]. In this context attention may be focused to fibronectin deposited abundantly during cell proliferation, and to laminin which appears as basal lamina component of the typical myofibroblast. Reports on relations between the alternative splicing and glycosylation of fibronectin[7,36], as well as the expression of laminin isoforms[17] and processes of cellular maturation and differentiation, have prompted us to examine these ECM components in dependence on the proliferative activity and myofibroblast differentiation in nodular palmar fibromatosis; the study has been aimed at deepening the knowledge of myofibroblasts and to describe the tumorous tissue formation process in this disease in relation to fibronectin and laminin variants.

## Results

The proliferative nodules of palmar fibromatosis exhibited activated fibroblast phenotypes, myofibroblast phenotypes substantiated by alpha-smooth muscle actin (Fig. 1a) and/or desmin demonstration, a high cellular density and a distinct proliferative activity visualized by Ki-67 immunostaining. The extracellular matrix of the proliferative noduli contained tenascin and somewhat lesser collagen type IV.

Using the antibody IST 4 which reacts with all variants of fibronectin an abundant extracellular fibronectin matrix was found both in the pre-existing palmar aponeurosis and in the proliferation nodules of the fibromatosis (Fig. 1b). In contrast to this immunohistochemical staining pattern, the presence of the fibronectin splice variants containing the ED-A and ED-B domains and of the de novo glycosylated fibronectin was exclusively restricted to the proliferative nodules (Fig. 1c, d, e). As a rule, the immunoreaction of ED-A specific antibody IST 9 and the antibody to the de novo glycosylated fibronectin 5C10 in the proliferative nodules was more intensive and extensive than the reaction with the ED-B specific antibody BC1. A difference between the staining pattern of IST 9 and 5C10 was not observed.

By double immunostaining it could be shown that the myofibroblastic phenotype demonstrated by alpha-smooth muscle actin was associated with fibronectin isoforms containing the ED-B domain and the de novo glycosylated fibronectin (Fig. 1f). Worth mentioning in this context, all of the alpha-smooth muscle actin positive cells were accompanied with these fibronectin isoforms but there were also additional fibroblastic cells without any muscle-specific actin in ED-B domain rich and de novo glycosylated fibronectin positive areas.

By means of non radioactive in situ hybridization for whole fibronectin mRNA, a reaction could only be found within cells of proliferation nodules (Fig. 2). The no probe control, as well as the RNAse pre-digestion control, were completely negative.

An extracellular laminin matrix could regularly be demonstrated within fully developed proliferation nodules, whereas the adjacent aponeurotic tissue was negative (Fig. 3a).

The monoclonal antibody 4C7 to the A chain of laminin only produced a delicate positivity particularly within the cytoplasm of some single cells or cell groups of the proliferation nodules (Fig. 3b). The B1 and B2 chains of laminin were regularly demonstrable in active nodules (monoclonal antibodies 4E10 and 4E8) and the immunostaining pattern was identical to that of the polyclonal laminin antisera (Fig. 3c, d). The staining intensity for B1 was stronger than for B2.

The laminin M chain (monoclonal antibody 5H2) was expressed like the A chain in only a few cells or cell groups within the proliferation nodules (Fig. 3e). In contrast to the localization of laminin chains B1, B2, A and M exclusively in proliferation nodules, the s chain visualized by the monoclonal antibody C4 could not only be recognized within cells of the proliferative nodules, but also with a weak immunoreaction in the cytoplasm of fibroblasts in the surrounding preexisting aponeurosis (Fig. 3f).

## Discussion

### *Fibronectin Variants*

Fibronectin is a multifunctional glycoprotein demonstrable in the extracellular matrix as well as in body fluids. The fibronectin molecule consists of two subunits with a molecular weight of 250,000 daltons made up of three types of sequence homologies (type I, II, III) joined by two disulphide bonds at the carboxy-terminal end of the molecule. Fibronectin is expressed and secreted by many cell types and is involved in the fundamental processes of cell-cell adhesion, cell-matrix adhesion and cell migration, as well as in the regulation of proliferation and differentiation[1,31,32,66]. The proliferation noduli in palmar fibromatosis (Dupuytren's disease) display a copious formation of non-collagenous extracellular matrix proteins and collagen type IV[4]. Fibronectin may have special relevance because of its extensive deposition and its association to specialized cytoskeleton-extracellular matrix connections, the so-called fibronexus[60,63,64].

The functional significance, the occurrence and tissue distribution of fibronectin splice and de novo glycosylated variants in fibro/myofibroblastic proliferating tissue is largely unknown[43]. The different fibronectin isoforms are derived from a single gene by an alternative processing of the primary RNA transcript or by post-translational modifications in the C-terminal region of fibronectin with a specific O-linked glycosylation of a single threonine residue[40,41]. The fibronectin molecule has three sites of alternative splicing, the domains ED-A, ED-B and the variable (V) region. ED-A and

ED-B are alternatively type III repeats, encoded by a single exon and skipped or included in a specific manner[5,58].

Whereas by immunohistochemical methods in Dupuytren's disease a fibronectin matrix could be seen everywhere in the aponeurotic tissue (although with an accentuation in the proliferative noduli[62]), non radioactive mRNA in situ hybridization has shown that the site of fibronectin de novo-synthesis was restricted to the proliferation nodules. By immunohistochemistry, the fibronectin splicing variants containing the ED-A and ED-B domain, as well as the de novo glycosylated form, were also demonstrable only in the proliferation nodules. From these findings it may be concluded that the de novo-synthesized fibronectin in proliferating nodules of Dupuytren's disease is classified by ED-A and ED-B domains and the de novo glycosylation. These fibronectin variants could mainly be found in embryonal tissues and neoplasms up to now (so called oncofetal fibronectins[6,10,14,20,21,44,45]). Their emergence in proliferation nodules of Dupuytren's disease may be explained by a proliferation-associated cellular dedifferentiation with a resultant tissue immaturity.

Using double immunostainings a correlation between myofibroblasts and the oncofetal fibronectin isoforms could be established in palmar fibromatosis. This codistribution of myofibroblast and oncofetal fibronectins was not only limited to tumorous mesenchymal proliferations as the Morbus Dupuytren; Brouty-Boyé and Magnien[8] reported on myofibroblasts and associated ED-B fibronectin in cultures produced from human stromal cells of breast carcinomas and non-neoplastic breast tissue. Moreover, ED-B and de novo glycosylated fibronectin could be detected in the stroma of other carcinomas[36,37,42] including, as well known, abundant myofibroblasts[32,59].

The myofibroblast dominated proliferative noduli in Dupuytren's disease expressed numerous growth factors and their receptors[3,25,49]. Both the occurrence of myofibroblasts and the fibronectin splicing were growth factor modulated[2,51,53,68]. Therefore, the concurrent accumulation of the myofibroblast and oncofetal fibronectins in the palmar fibromatosis might be interpreted as the result of a co-ordinated growth factor mediated connective tissue reorganization. Despite the unclear functional role of the oncofetal fibronectins[58], a modulation of cellular adhesion seems to be present supported by the codistribution of tenascin in the palmar fibromatosis known as an adhesion modulating factor[12,48]. Referring to a biological significance it should be pointed out that tumorigenic fibroblasts, in contrast to non-tumorigenic fibroblasts in culture, preferably incorporated ED-B fibronectin in the extracellular matrix[38].

*Laminin Variants*

At the ultrastructural level, myofibroblast can at times have a fairly well developed basal lamina, but more often than not, this is interrupted and occurs only in patches as a basal lamina-like material or is absent[23]. We have demonstrated the major proteins of basal lamina (collagen type IV and laminin)[47,70] in the proliferation nodules of palmar fibromatosis by showing their close relation to myofibroblastic cells[5]. Laminin is a heterotrimeric cross-shaped molecule containing one large A chain of 400 000 daltons and two smaller B1 and B2 chains of about 200,000 daltons[13,61]. Recent studies have revealed that several genetically distinct subunit chains and consequently several laminin isoforms exist[69]. In addition to the A, B1 and B2 chains, merosin (M chain), a homologue of the A chain[16,34,67] and s-laminin (s chain), a homologue of the B1 chain[27] has been characterized. In the proliferation nodules of Dupuytren's disease, the B1 and B2 chain of laminin, which could be visualized by the antibodies 4E10 and 4E8, were found in a similar diffuse distribution as the fibronectin variants with the ED-A and ED-B domain and with de novo glycosylation. But only single cells or small cell groups stained for laminin M and/or A. This heterogeneous distribution of the A and M chain in proliferation nodules could be based on the well-known variability of the myofibroblast phenotype[54] which also includes irregularities in basal lamina formation. The de novo expression of M chain in the proliferative noduli may be interpreted as a proliferation associated dedifferentiation of fibro/myofibroblastic cells[67].

At present, most authors believe that laminin assembly follows the rule that one of the heavy chains combines with two light chains[9,52]. Accordingly, a congruent immunohistochemical pattern of light chains and at least one heavy chain should be expected. In proliferative nodules of Dupuytren's disease, such a congruent immunostaining of light and heavy chains could not be observed. Provided that the immunohistochemical pattern was not caused by a deficient affinity of the applied antibodies to the heavy chains, these unexpected results might indicate the possibility of an independent expression of laminin chains or could speak for the existence of further laminin heavy chains (e.g. K-laminin[39,50,67]). The finding of only s-laminin outside the proliferating nodules could be a further hint at the possibility of an independent expression of laminin chains in this disease.

Summarizing our results, there was an obvious correlation between the myofibroblast phenotype formation (alpha-smooth muscle actin and/or desmin immunostaining), cellular proliferation (Ki-67 labelling) and the occurrence of ED-A, ED-B containing fibronectin, as well as de novo glycosylated fibronectin in Dupuytren's disease. The ultrastructural irregularities of myofibroblastic basal lamina and the heterogeneity of the myofibroblast phenotype are equivalent to the variability of laminin isoform immunostaining.

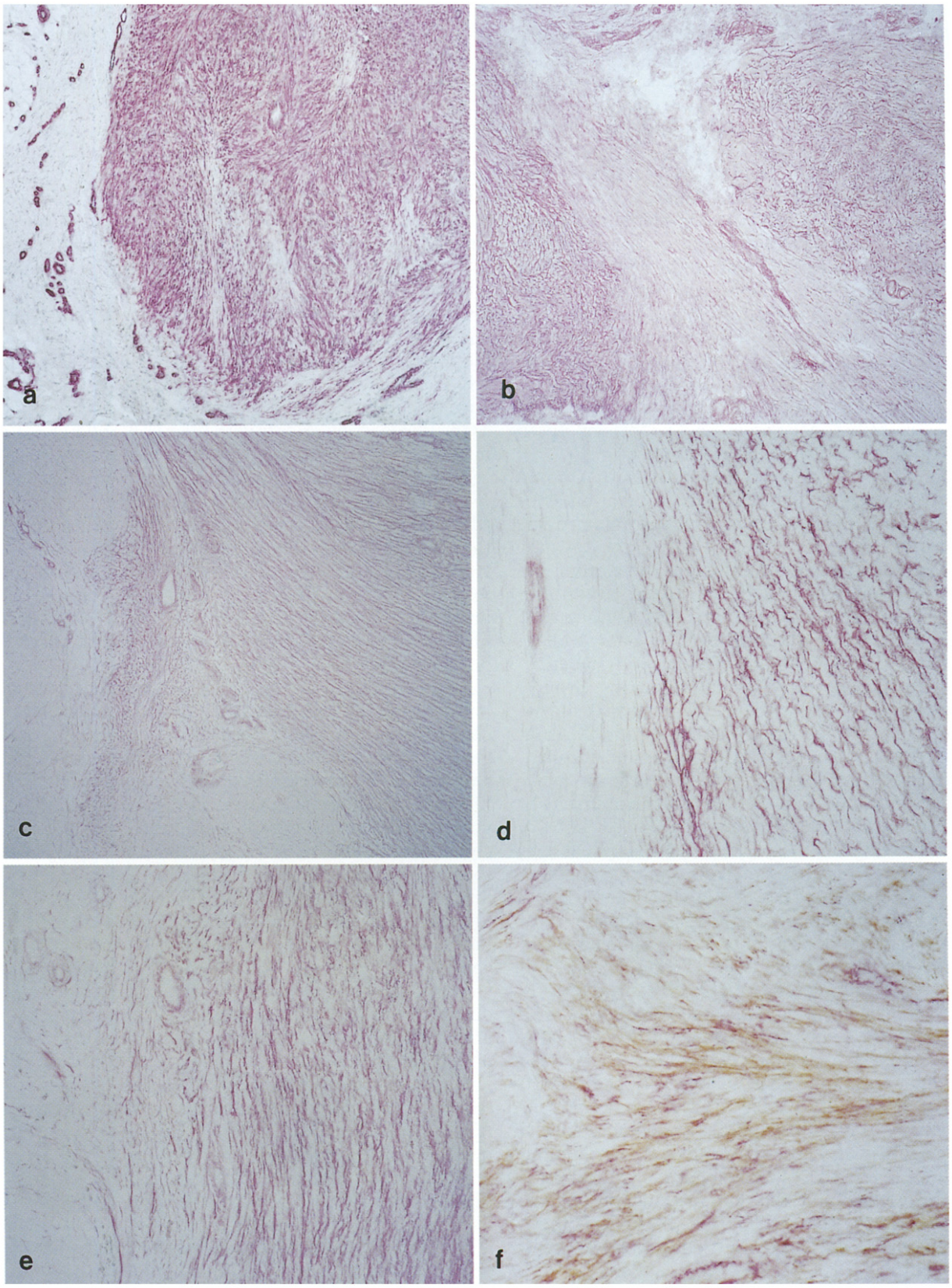



Fig. 1. Immunohistochemical demonstration of the myofibroblastic phenotype within a proliferative nodulus of Dupuytren's disease applicating a monoclonal antibody against α-smooth muscle actin (a, × 40). Immunostaining using the antibody IST 4 which reacts with all variants of fibronectin. The fibronectin matrix is distributed within the whole palmaraponeurosis with a slightly accentuated staining of proliferative nodules (b, × 75). The ED-A-fibronectin could be visualized within the proliferative nodulus and in some fibro/myofibroblastic cells neighbouring the nodulus (c, × 75), whereas the ED-Fibronectin (d, × 150) and the de novo glycosylated fibronectin (e, × 150) is exclusively restricted to the proliferative nodulus. Colocalization of α-smooth muscle actin (red, APAAP technique) and de novo glycosylated fibronectin (brown, ABC-peroxidase technique) in a proliferative nodulus (f, × 150).

Fig. 2. The fibronectin synthesis is exclusively restricted to the proliferative nodulus (mRNA in situ hybridization for whole fibronectin, × 150). ►

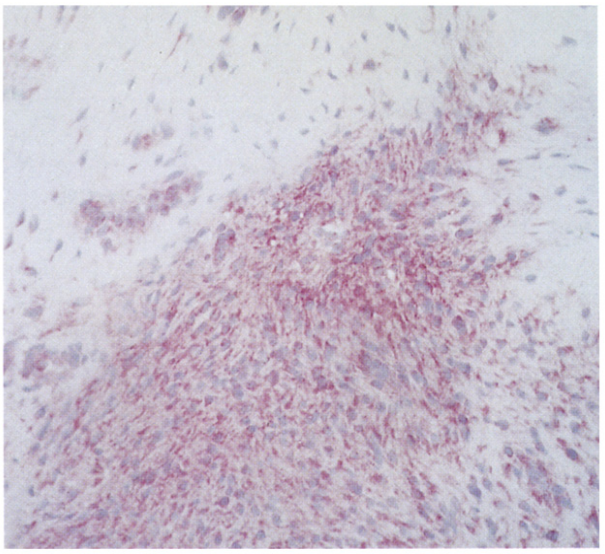
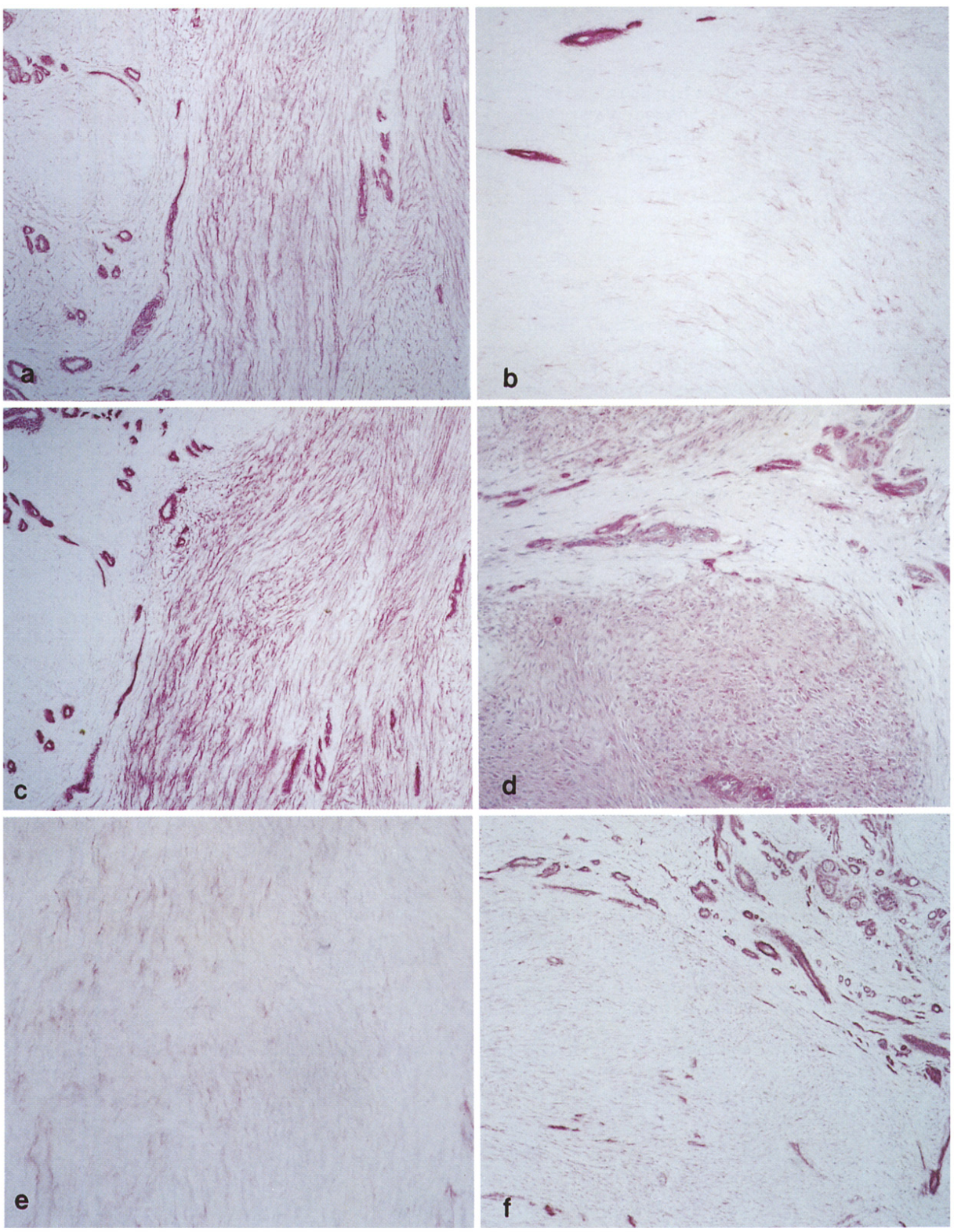


Fig. 3 ▼



## Material and Methods

### *Tissue Material*

10 surgical specimens of Dupuytren's disease in the proliferative phase or the beginning of involutional phase were available defined according to criteria set forward by Luck[18,24,35] as evaluated by conventional histology.

Fibromatosis noduli of these specimens with high cellularity, low content of collageneous extracellular matrix and proliferative activity (Ki-67 labelling) are designated as proliferative noduli.

Samples of the fresh surgical specimens, up to 5 mm in diameter, were shock frozen in fluid propane cooled by liquid nitrogen.

### *Immunohistochemistry*

Cryostat sections of the respective frozen tissue samples were fixed in ice cooled acetone for 15 minutes and subjected to immunohistochemistry. Primary antibodies were employed against: vimentin (clone V9, diluted 1 : 40, Dako, Denmark), desmin (clone D33, diluted 1 : 30, Dako), alpha-smooth muscle actin (clone 1A4, diluted 1 : 40, Dako), tenascin (clone TN2, diluted 1 : 5000, Telios, USA), all variants of fibronectin, including cellular and plasma fibronectin (clone IST 4, culture supernatant, diluted 1 : 20, Dr. L. Zardi[6]), ED-A containing fibronectin (clone IST 9, culture supernatant, diluted 1 : 500, Dr. L. Zardi[6]), ED-B containing fibronectin (clone BC1, culture supernatant, diluted 1 : 20, Dr. L. Zardi[11]), oncofetal de novo glycosylated fibronectin (clone 5 C 10, culture supernatant, diluted 1 : 20, Dr. Ulla Mandel[36]), laminin (rabbit antihuman laminin polyclonal antibodies, diluted 1 : 300, Telios, USA), laminin B1 chain, equivalent in new nomenclature for laminin chains β1[9] (clone 4E10, diluted 1 : 20 000, Telios, USA), laminin B2 chain, synonymous γ1 (clone 4E8, diluted 1 : 500, Telios, USA), laminin A chain, synonymous α1 (clone 4C7, diluted 1 : 10 000, Telios, USA), laminin s chain, synonymous β2 (clone C4, diluted 1 : 1000, Dr. J. Sanes, DSHB, Baltimore, USA), laminin M chain, so-called merosin, synonymous α2 (clone 5H2, diluted 1 : 5000, Telios, USA), collagen type IV (clone 8C5 5A5, diluted 1 : 40, Dianova, Germany) and proliferation marker Ki-67 antigen (rabbit anti- Ki-67 antigen polyclonal antibodies, diluted 1 : 70, Dako, Denmark).

Immunohistochemical staining was performed using the APAAP (alkaline phosphatase monoclonal anti-alkaline phosphatase) method[26].

The primary antibody was incubated for 30 minutes at room temperature. After washing with Tris buffer, sections were treated with rabbit anti-mouse gamma G immunoglobulin (IgG, Z-259, diluted 1 : 70, Dako, Denmark), and then with the mouse APAAP-complex (Dako, Denmark). Both incubations were done for 30 minutes at room temperature. In the case of primary polyclonal rabbit antibodies a second mouse anti-rabbit antibody (diluted 1 : 400, Dako, Denmark) was introduced.

To increase the staining intensity, the incubation with the rabbit anti-mouse immunoglobulin and with the APAAP-complex was repeated twice. Naphtol-AS-biphosphate (Sigma, 2250, USA) and new fuchsin (Merck, 4040, Germany) were used as substrate and developer, respectively. To inhibit endogenous tissue enzyme activity, the developing solution was supplemented with 0.25 mmol/l levamisole (Sigma, L-9756, USA).

For the evaluation of immunostaining, the primary antibody was replaced by nonimmune serum as negative control. Blood vessels of the aponeurotic tissue were used as inherent positive control for smooth muscle actin, desmin, fibronectin, laminin, collagen type IV and tenascin.

A combination of ABC peroxidase technique and APAAP technique was used for double immunostaining. At first, the demonstration of the de novo glycosylated fibronectin or the ED-B containing fibronectin was carried out using the Vectastain ABC Peroxidase Kit (Vector Laboratories, Burlingame, CA, USA). Secondly, the APAAP technique for α-smooth muscle actin visualization was performed.

### *mRNA in Situ Hybridization*

*Slide preparations and pretreatment for mRNA in situ hybridization of fibronectin:* Non radioactive fibronectin in situ hybridization was performed on cryostat sections of immediately after surgery snap frozen specimens of Dupuytren's diseased aponeurotic material containing histological definable proliferative nodules. Tissue sections (10 µm) were mounted on slides coated with 3% (v/v) 3-aminopropyltriethoxysilane in acetone, briefly air dried and fixed in 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS) for 20 minutes at 4 °C. Then slides were shortly rinsed in 70% ethanol and air dried. Sections were stored at – 70 °C until processed. Before hybridization the slide preparations were handled as follows: 20 min at room temperature in 0.2 M HCL, 30 min at 70 °C in 2 × SSC and 15 min at 37 °C in 50 mM Tris · HCl, pH 7,6/proteinase K (1–5 µg/ml). Slides were rinsed twice in distilled water, dehydrated in graded ethanol (70% and 96%) and air dried.

*Preparation of Labelled cDNA Probe:* The cDNA for human fibronectin[29,30] was purchased from GIBCO BRL, UK (1,4 Kb, nucleic acids: 1993-3364) and biotin labelled using the Nick Translation Kit and biotin-11-dUTP from Boehringer Mannheim, Germany.

*Prehybridization and hybridization:* The hybridization solution contained 4 × SSC, 50% (v/v) deionized formamide, 1 × Denhardt's solution, 1 mM EDTA, 5% (w/v) dextran sulphate, 100 µg/ml heat denatured calf thymus DNA,100 µg/ml heat denatured transfer RNA and 200 µg/ml biotin labelled fibronectin cDNA. Prehybridization was carried out with hybridization medium without cDNA, 60 min at 37 °C in a moist chamber. Then fifty microliters hybridization solution containing 200 ng/ml probe were applied to each slide, RNAse free coverslips were mounted and hybridization was allowed to proceed at 37 °C overnight. Slides were then washed for 2 hr in 10 mM Tris · HCl, pH 7.6/2 × SSC/50% formamide/1 mM EDTA, followed by 1 hr at 55 °C in OmniBuff (JenaBioTech and WAK Chemie Medical GmbH, Germany) and rinsed in Omnibuff at room temperature. Hybridized cDNA was detected using an anti biotin antibody (DAKO, Denmark) and the APAAP technique mentioned

◀ Fig. 3. The immunodetection of laminin in nodular palmar fibromatosis is restricted to proliferative areas (a, polyclonal antibodies, × 75). The laminin A chain (b, × 75) could be demonstrated in a delicate manner, whereas the B1 chain (c, × 75) and the B2 chain (d, × 75) showed a strong reaction within the proliferative noduli. The M chain could be visualized within the proliferative noduli but only slight in some fibro/myofibroblastic cells (e, × 150). The s chain of laminin was abundantly found in vascular structures and in fibro/myofibroblastic cells all over in the palmar aponeurosis of Dupuytren's disease (f, × 40).

above. No probe hybridization and RNAse predigestion were used as negative control and specificity control, respectively.

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