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TGFβ and bFGF synthesis and localization in Dupuytren's disease (nodular palmar fibromatosis) relative to cellular activity, myofibroblast phenotype and oncofetal variants of fibronectin

A. BERNDT[1]*, H. KOSMEHL[1], U. MANDEL[2], U. GABLER[1], XINMEI LUO[3], D. CELEDA[4], L. ZARDI[5] and D. KATENKAMP[1]

[1]*Institute of Pathology, Friedrich Schiller University, Ziegelmühlenweg 1, D-07740 Jena, Germany;* [2]*Department of Oral Diagnostics, School of Dentistry, University of Copenhagen, Denmark;* [3]*Max Planck Society, Research Group 'Growth Factor Signal Transduction', Jena, Germany;* [4]*Institute of Molecular Biotechnology e.V., Jena, Germany; and* [5]*Laboratory of Cell Biology, Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy*

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Summary

Nodular palmar fibromatosis is a self-limited proliferation of fibro-/myofibroblasts associated with growth factor synthesis and abundant fibronectin extracellular matrix deposition. bFGF and TGFβ are potent modulators of fibro-/myofibroblast proliferation and differentiation. Moreover, *in vitro* investigations evidenced a TGFβ1-dependent regulation of alternative splicing of fibronectin mRNA. To investigate a possible implication of these growth factors in the tissue formation process of palmar fibromatosis, TGFβ1/2 and bFGF synthesis, as well as TGFβ1/3 and bFGF tissue distribution, is demonstrated by RNA *in situ* hybridization and/or immunohistochemistry in relation to myofibroblast phenotype development (α-smooth muscle actin, desmin immunohistochemistry), expression of different fibronectin isoforms (ED-A$^+$, ED-B$^+$ and oncofetal glycosylated fibronectin immunohistochemistry, fibronectin RNA *in situ* hybridization) and cellular activity (cyclin RNA *in situ* hybridization, Ki-67 immunolabelling). The myofibroblast phenotype (α-smooth muscle actin, desmin), the growth factor synthesis (TGFβ1 and 2, bFGF), fibronectin matrix synthesis (RNA *in situ* hybridization with cDNA) and ED-A$^+$, ED-B$^+$ and oncofetal glycosylated fibronectin immunostaining are exclusively localized in the active proliferative nodules (Ki-67 immunolabelling and cyclin mRNA demonstration). Whereas the growth factor synthesis is restricted to the proliferative areas of the fibromatosis only, TGFβ1, TGFβ3 and bFGF proteins can also be detected immunohistochemically with a lower intensity in the surrounding aponeurotic tissue. The spatial correlation of myofibroblast phenotype, TGFβ and bFGF synthesis and the occurrence of the oncofetal molecular fibronectin variants (ED-B$^+$ and oncofetal glycosylated fibronectin) in the active proliferative fibromatosis nodules suggests a pathogentic role of these growth factors and matrix components in the tumorous tissue formation process. The presence of the bFGF and TGFβ1/3 proteins in fibroblasts neighbouring the proliferative nodules may point to a recruitment of quiescent aponeurotic fibroblasts in the fibromatous tissue formation process.

Introduction

Nodular palmar fibromatosis (Dupuytren's disease) is a proliferation of fibro-/myofibroblasts in the human palmar aponeurosis. The growth of the proliferatively active nodules is self-limiting and results in shortening of the palmar fascia and flexion contracture of digits. The nodules pass through three histologically definable phases: the proliferative, the involutional and the residual phase (Luck, 1959). The fibromatous nodules in the proliferative or early involutional phase are characterized by the occurrence of a myofibroblast phenotype associated with basal lamina formation and an extensive non-collagenous

*To whom correspondence should be addressed.

extracellular matrix deposition containing fibronectin (Tomasek *et al.*, 1986; Eddy *et al.*, 1988; Schürch *et al*, 1992) and tenascin (Berndt *et al.*, 1993, 1994). Fibronectin is organized in specialized cell matrix contacts of myofibroblasts called fibronexus and is involved in transmitting contractile forces generated by microfilaments to the fibrillary collagen matrix (Singer, 1979). Apart from this structural aspect, fibronectin is also of regulatory importance in tissue formation by cell–matrix interaction (Bissell *et al.*, 1982; Adams & Watt, 1993).

It could be shown that fibronectin molecular variants exist, produced by changes in glycosylation of the IIICS region and/or by alternative splicing of mRNA. Three areas of the fibronectin monomer show sequence variations. Extra domains A and B (ED-A, ED-B) are skipped or included in a cell-type-specific manner, while the IIICS region of human fibronectin can vary in length, if present at all. ED-A and ED-B are only found in cellular fibronectin, which implies a possible role in cell–matrix communication (Schwarzbauer, 1991). Particularly the ED-B$^+$ fibronectin and the *de novo* glycosylated fibronectin are expressed in immature as well as in tumour tissues (oncofetal fibronectins) (Carnemolla *et al.*, 1989; Ffrench-Constant *et al.*, 1989; Mandel *et al.*, 1992; David *et al.*, 1993). Various cytokines, e.g. insulin-like growth factor, platelet-derived growth factor α, basic fibroblast growth factor (bFGF) and transforming growth factor β (TGFβ), are strongly expressed in proliferative nodules of palmar fibromatosis (Baird *et al.*, 1993).

The bFGF (FGF-2) induces mitogenic activity in a large number of different cell types *in vitro*, including endothelial cells and fibroblasts (Baird & Walicke, 1989; Burgess & Maciag, 1989) and is a potent regulator of differential expression of several matrix components (Tan *et al.*, 1993) as well as an efficient modulator of the expression of integrins on endothelial cells *in vitro* (Enenstein *et al.*, 1992).

TGTβ1 treatment of fibroblasts *in vitro* results in upregulation of proliferation (Okragly *et al.*, 1994) and transcription of collagen and fibronectin genes (Herlyn & Malkowicz, 1991; Grande *et al.*, 1993). Other recently published reports concerning the activities of TGFβ1 in fibroblastic cells dealt with the induction of α-smooth muscle actin, indicating a myofibroblast phenotype development, in quiescent and growing cultured fibroblast of different origins (Desmouliere *et al.*, 1993; Rønnov-Jessen & Petersen, 1993). Additionally, TGFβ1 seems to be involved in the control of alternative splicing of fibronectin pre-mRNA in fibroblasts, resulting in the re-establishment of early developmental (oncofetal) protein variants *in vitro* (Balza *et al.*, 1988; Borsi *et al.*, 1990).

The interrelations between growth factors, fibroblast proliferation and differentiation, as well as extracellular matrix mentioned above, prompted us to study TGFβ/bFGF synthesis and TGFβ/bFGF protein tissue distribution in relation to proliferative activity (cyclin mRNA *in situ* hybridization), occurrence of myofibroblasts (α-smooth muscle actin, desmin immunocytochemistry), fibronectin synthesis (fibronectin mRNA *in situ* hybridization) and deposition of ED-A$^+$ fibronectin as well as oncofetal fibronectin isoforms (ED-B$^+$ and oncofetal glycosylated fibronectin immunohistochemistry) in extracellular matrix of nodular palmar fibromatosis.

Materials and methods

Tissue material

Ten surgical specimens of proliferative active Dupuytren's disease (proliferative and early involutional phase) were available and evaluated by conventional histology according to the criteria set forward by Luck (1959). Furthermore, samples of the fresh surgical specimens, up to 5 mm in diameter, were snap frozen in fluid propane cooled by liquid nitrogen and stored at −70°C until processed.

Immunohistochemistry

Cryostat sections of the respective frozen tissue samples were fixed in ice-cooled acetone for 15 min and subjected to immunohistochemistry. The following primary antibodies were employed: antibodies to vimentin (clone V9, 2.2 μg ml^{-1}, Dako, Denmark), desmin (clone D33, 6.2 μg ml^{-1}, Dako, Denmark), α-smooth muscle actin (clone 1A4, 2.4 μg ml^{-1}, Dako, Denmark), ED-A containing fibronectin (clone IST 9, culture supernatant, diluted 1:500 (Borsi *et al.*, 1987)), ED-B containing fibronectin (clone BC1, culture supernatant, diluted 1:20 (Carnemolla *et al.*, 1992)), oncofetal glycosylated fibronectin (clone 5C10, culture supernatant, diluted 1:20 (Mandel *et al.*, 1992)), proliferation marker Ki-67 antigen (rabbit anti-Ki-67 antigen polyclonal antibodies, 2.8 μg ml^{-1}, Dako, Denmark), bFGF (polyclonal, protein concentration 28 μg ml^{-1}, Sigma, USA), TGFβ1 (LAP/TGFβ1 precursor, polyclonal, 2 μg ml^{-1}, R&D Systems, USA) and TGFβ3 (clone 236-5.2, 5 μg ml^{-1}, Dianova, FRG). Immunohistochemical staining was performed using the APAAP (alkaline phosphatase monoclonal anti-alkaline phosphatase) method (Gustmann *et al.*, 1991).

The primary antibody was incubated for 30 min at room temperature. After washing with Tris buffer, sections were treated with rabbit anti-mouse immunoglobulins (IgG, Z-259, diluted 1:70, Dako, Denmark), and then with the mouse APAAP-complex (Dako, Denmark). Both incubations were done for 30 min at room temperature. In 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. Naphthol-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^{-1} levamisole (Sigma, L-9756, USA).

For evaluation of immunostaining, the primary antibody was replaced by non-immune serum as negative control. Blood vessels of the aponeurotic tissue were used as inherent positive control for smooth muscle actin, desmin and fibronectin.

mRNA in situ *hybridization*

Slide preparation and pretreatment. Non-radioactive mRNA *in situ* hybridization was performed on cryostat sections of Dupuytren's tissue containing histologically definable proliferative nodules. Tissue sections (7 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 min at 4°C. Then slides were briefly rinsed in 70% ethanol and air-dried. Sections were stored at −70°C until processed.

mRNA in situ *hybridization with oligonucleotide probes for bFGF, TGFβ1, TGFβ2 and cyclin (PCNA, proliferating cellular nuclear antigen)*

Before hybridization procedures the slides were handled as follows: 15 min at room temperature in 0.2 M HCl, rinsed in PBS, and 15 min at 37°C in 50 mM Tris–HCl, pH 7.6/proteinase K (0–5 μg ml^{-1}). Slides were washed twice in distilled water, dehydrated in graded ethanol (70% and 96%) and air-dried.

Preparation of labelled oligonucleotide probes. The oligonucleotide probes for human TGFβ2 and cyclin were purchased from Oncogene Science/Dianova (Germany) and biotin labelled usmg the labelling system Bio-ULS (Kreatech/Dianova, Germany). The oligonucleotide probes for human TGFβ1 and bFGF (R&D systems) were biotin-labelled probe cocktails containing probes for three different exons of about 30 bases in lengths.

Prehybridization and hybridization. The hybridization solution contained 4 × SSC (0.6 M NaCl and 0.06 M sodium citrate), 25% (v/v) deionized formamide, 1 × Denhardt's solution, 1 mM EDTA (ethylene diaminetetraacetic acid), 5% (w/v) dextran sulphate, 100 μg ml^{-1} heat-denatured calf thymus DNA, 100 μg ml^{-1} heat-denatured transfer RNA and 200–400 ng ml^{-1} biotin-labelled probes. Prehybridization was carried out with hybridization medium without oligonucleotides, 60 min at 37°C in a moist chamber. Then 50 μl hybridization solution were applied to each slide, ribonuclease (RNAse)-free coverslips were mounted and hybridization was allowed to proceed at 37°C overnight. Slides were then washed for 30 min in 2 × SSC and 30 mm in 1 × SSC at room temperature.

mRNA in situ *hybridization with cDNA probe for fibronectin*

Pretreatment of slides. 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^{-1}). 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 (Kornblihtt *et al.*, 1983, 1985) was purchased from GIBCO BRL, UK (1.4 kb, nucleic acids: 1993–3364), amplified in sufficient amounts according to a new polymerase chain reaction technique which does not require specific primers (manuscript in preparation) 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^{-1} heat-denatured calf thymus DNA, 100 μg ml^{-1} heat-denatured transfer RNA and 200 ng ml^{-1} biotin-labelled fibronectin cDNA. Prehybridization was carried out with hybridization medium without cDNA for 60 min at 37°C in a moist chamber. Then 50 μl hybridization solution containing 200 ng ml^{-1} 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 h in 10 mM Tris–HCl, pH 7.6 / 2 × SSC / 50% formamide / 1 mM EDTA followed by 1 h at 55°C in OmniBuff (JenaBioTech and WAK Chemie Medical GmbH, Germany) and rinsed in Omnibuff at room temperature.

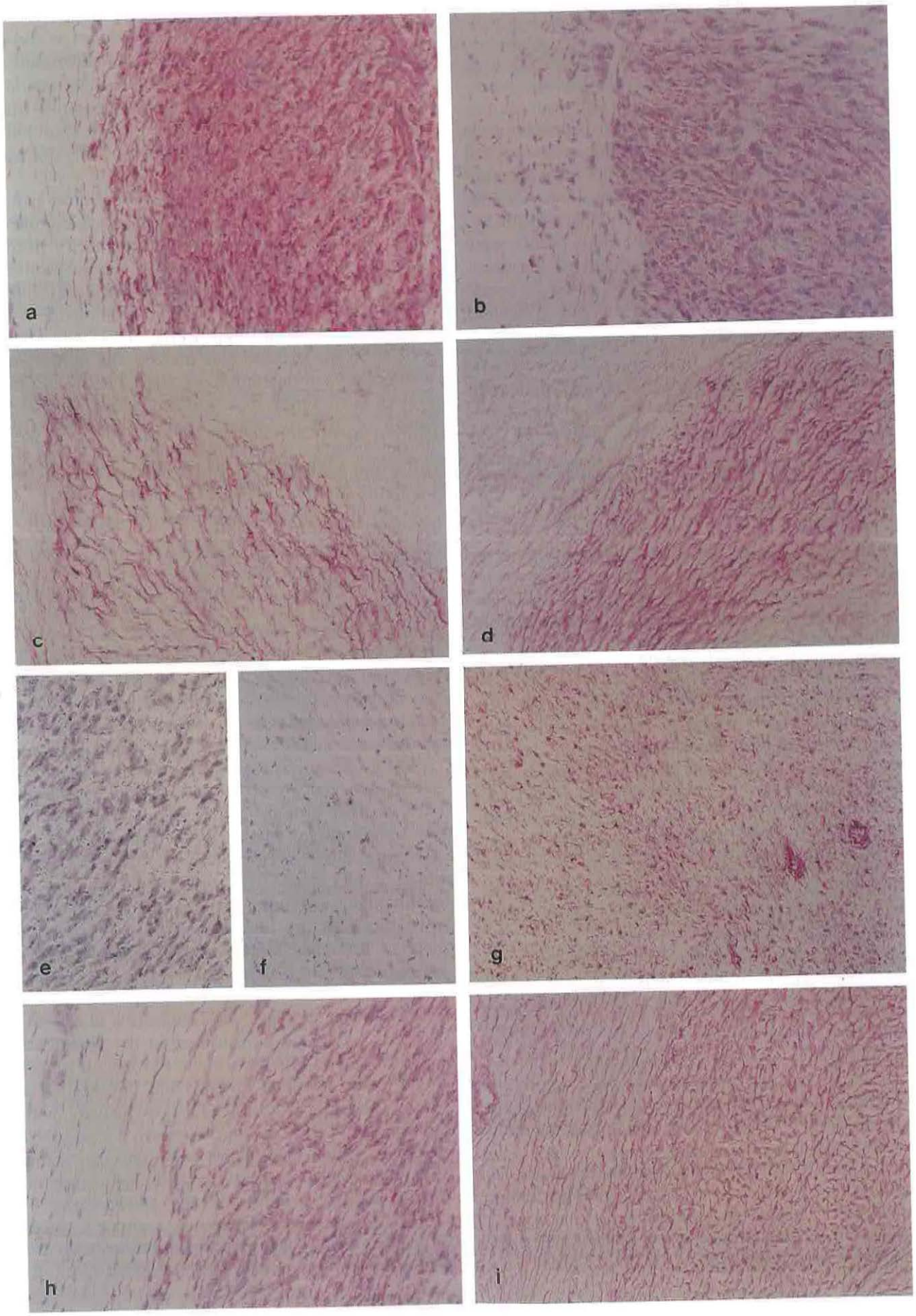
Detection

Hybridized probes were detected using an anti-biotin antibody (DAKO, Denmark) and the APAAP technique mentioned above. In the case of bFGF, *in situ* hybridization biotinylated probes were detected using alkaline phosphatase conjugated streptavidin (steptavidin AP) and 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium (BCIP/NBT) as substrate/chromogen (DAKO *In situ* Hybridization Detection System, Dako, Denmark).

Controls

No-probe hybridization and ribonuclease (RNase) predigestion were used as negative control and specificity control, respectively.

Fig. 1. (a) Cyclin RNA *in situ* hybridization indicating a high cellular activity in the proliferative phase of nodular fibromatosis (detection with APAAP technique, ×150). (b) Fibronectin synthesis revealed by RNA *in situ* hybridization is restricted to the cell-dense active fibromatosis nodule (detection with APAAP technique, ×150). (c) Immunohistochemical detection of ED-B$^+$ fibronectin and (d) oncofetal glycosylated fibronectin in fibromatosis nodules of early involutional phase (APAAP technique, ×150). (e) Detection of bFGF synthesis in the centre of a nodule (*in situ* hybridization, BCIP/NBT, ×150. (f) RNase control of bFGF *in situ* hybridization. (g) Diffuse cellular immunostaining of bFGF in a fibromatotic nodule (APAAP technique, ×150). (h) Demonstration of TGFβ1 synthesis by means of RNA *in situ* hybridization in fibromatosis nodule of early involutional phase (detection with APAAP technique, ×150). (i) Diffuse immunohistochemical distribution of TGFβ1 in a fibromatosis nodule as well as in surrounding aponeurotic tissue (APAAP technique, ×150).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Results

Myofibroblast phenotype and proliferative activity

The nodules of active palmar fibromatosis are characterized by a high cellular density and immunohistochemically by α-smooth muscle actin positive cells (myofibroblasts). Additionally, single cells or cell groups are desmin positive. A distinct immunostaining pattern of Ki-67 antigen and a clear accumulation of cyclin gene transcription as shown by *in situ* hybridization (Fig. 1a) evidence the high proliferative activity of fibro-/myofibroblastic cells in the fibromatosis. Additionally, cyclin mRNA could be found in single fibroblasts in aponeurotic tissue beyond fibromatosis nodules and in vascular structures.

Immunohistochemical distribution of oncofetal fibronectin variants

Fibronectin splice variants containing the ED-A and ED-B domains and the oncofetal glycosylated fibronectin are exclusively demonstrated in proliferative nodules (compare Figs 1c and 1d). As a rule, the immunoreaction of ED-A specific antibody IST 9 and the antibody to the oncofetal glycosylated fibronectin 5C10 was stronger and more extensive than the reaction with the ED-B specific antibody BC1. A difference between the staining pattern of IST 9 and 5C10 is not observed.

Immunohistochemical distribution of growth factors

There was an accumulation of bFGF in proliferative areas, whereas in the surrounding aponeurosis, blood vessels and only a few single fibroblastic cells showed a clear reaction (Fig. 1g). TGFβ1 (Fig. 1i) and TGFβ3 can be detected in the whole examined material with a concentration in the proliferative nodules. In general, the staining pattern of TGFβ1 is more intensive than that of TGFβ3. Additionally, there is an accentuation of strongly TGFβ3-positive cells in perivascular regions.

Growth factor and fibronectin synthesis

Synthesis of TGFβ1 (Fig. 1h), TGFβ2, bFGF (Figs 1e and 1f) and fibronectin (Fig. 1b) as revealed by non-radioactive RNA *in situ* hybridization can be detected in all fibro-/myofibroblastic cells within the proliferative areas. Only some single cells out of the nodules as well as blood vessels are additionally immunostained.

The hybridization pattern of growth factors, fibronectin and cyclin, as well as the immunostaining pattern for oncofetal glycosylated and alternatively spliced fibronectin isoforms, are identical, as determined by serial sections.

Discussion

The growth of benign and malignant soft-tissue tumours may be associated with a strong expression of numerous growth factors and their receptors, interpreted as evidence for autocrine growth stimulation (Perosio & Brooks, 1989; Roholl *et al.*, 1991; Gonzalez *et al.*, 1992).

In palmar fibromatosis the synthesis of growth factors (TGFβ, bFGF) could be visualized by mRNA *in situ* hybridization. Whereas growth factor mRNA could be demonstrated exclusively within proliferative fibrous nodules, immunohistochemically bFGF, TGFβ1 and TGFβ3 could be seen additionally in the adjacent aponeurotic tissue, in the fibroblastic cells as well as in the extracellular matrix. The immunohistochemical demonstration of extracellular localized growth factors indicates diffusion processes. The diffusion of growth factors from synthetically active fibrous nodules to adjacent aponeurotic tissue may cause an activation of quiescent fibroblasts by a paracrine stimulation in palmar fibromatosis.

TGFβ comprises a superfamily of structurally related regulatory proteins including three mammalian isoforms, TGFβ1, -β2, and -β3, with 70–80% sequence identity, as well as activin/inhibins and bone morphogenetic proteins which have 30–40% sequence identity. TGFβ is the prototypical multifunctional growth factor (Sporn & Roberts, 1988). The biological activity of TGFβ isoforms seems to be functionally equivalent *in vitro*, although there are differences in potency on different cell types. The nature of the activity in a particular cell type depends critically on many parameters, such the state of differentiation, the growth conditions and on the presence of other growth factors (Sporn *et al.*, 1987). In the proliferative phase of nodular fibromatosis significant differences between the mRNA patterns of bFGF, TGFβ1 and TGFβ2 could not be observed. The simultaneous activation of these genes in the proliferative phase of the fibromatosis is in line with the state of fibroblast activation and known for fibroblasts of different origins.

Whereas fibronectin occurs in the matrix of the whole palmar aponeurosis (Tomasek *et al.*, 1986) fibronectin synthesis is restricted to the proliferative nodules. The so-called oncofetal fibronectin isoforms (ED-B$^+$ and oncofetal glycosylated fibronectin) and ED-A$^+$ fibronectin are localized exclusively within the fibrous nodules of the proliferative phase (Halliday *et al.*, 1994). The pattern of fibronectin synthesis and immunohistochemical oncofetal isoform demonstration indicates that only the newly formed fibronectin in palmar fibromatosis nodules is alternatively spliced and/or glycosylated (Kosmehl *et al.*, 1995). Furthermore, there is an obvious co-localiza-

tion of oncofetal fibronectin isoforms, growth factor mRNA's myofibroblasts (α-smooth muscle actin positive cells) and fibromatosis proliferation (visualized by cyclin labelling). Therefore, the activated fibroblast phenotype in palmar fibromatosis may be defined by growth factor synthesis, oncofetal matrix expression and myofibroblastic phenotype.

TGFβ1 is said to be a potent inductor of expression of extracellular matrix proteins (Wang *et al.*, 1991; Bachem *et al.*, 1993; Grande *et al.*, 1993) including the regulation of alternative splicing of fibronectin (Balza *et al.*, 1988; Borsi *et al.*, 1990) and of the myofibroblast phenotype revealed by α-smooth muscle actin (Björkerud, 1991; Desmouliere *et al.*, 1993; Rønnov-Jessen & Petersen, 1993). The present investigations support these *in vitro* findings by a co-distribution of newly formed matrix proteins and TGFβ synthesis for fibroblasts in nodular palmar fibromatosis *in vivo*.

In summary, the results substantiate a *de novo* synthesis of TGFβ1, TGFβ2 and bFGF in nodular palmar fibromatosis which should play a role in the formation of the 'quasi-neoplastic' tissue of the proliferative nodules. Secondly, there is an obvious co-distribution of growth factor synthesis, oncofetal fibronectin matrix and myofibroblast phenotype formation as well as cellular proliferation (cyclin RNA *in situ* hybridization) in active fibromatosis nodules.

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