

MYOFIBROBLASTS OF PALMAR FIBROMATOSIS CO-EXPRESS TRANSFORMING GROWTH FACTOR-ALPHA AND EPIDERMAL GROWTH FACTOR RECEPTOR

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SUMMARY

Several studies have shown that different growth factors are involved in the pathogenesis of palmar fibromatosis. The aim of the present study was to investigate whether transforming growth factor alpha (TGF- α) and its cellular receptor, epidermal growth factor receptor (EGF-R), are expressed in palmar fibromatosis. Nodules from 20 patients with palmar fibromatosis and control normal palmar fascias were studied by the reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry. RT-PCR followed by Southern blotting demonstrated that palmar fibromatosis nodules contained high levels of TGF- α and EGF-R messenger RNA (mRNA) transcripts, while normal fascias showed only low levels. Depending on the degree of cellularity and fibrosis, the three following histological phases were recognized in palmar fibromatosis nodules: proliferative, involutinal, and residual. Immunohistochemistry, using α -smooth muscle actin as a cellular marker for myofibroblasts, revealed that TGF- α and EGF-R are co-expressed by myofibroblasts in the highly cellular areas of both proliferative and involutinal phases, while they are absent or only focally detectable in the fibroblasts of normal fascia and in hypocellular and fibrotic areas of both involutinal and residual phases. The restricted co-expression of TGF- α and EGF-R to myofibroblasts, the proliferating cellular component of nodules, suggests that an autocrine and/or juxtacrine growth stimulation by TGF- α via the EGF-R may be involved in the pathogenesis of palmar fibromatosis.

KEY WORDS—myofibroblasts; TGF- α ; EGF-R; palmar fibromatosis

INTRODUCTION

Palmar fibromatosis (Dupuytren's contracture) is a fibro-proliferative disease characterized by proliferation of myofibroblasts and variable deposition of mature collagen fibres,¹ leading to a single or an ill-defined conglomerate of multiple aponeurotic scar nodules with an irreversible contracture.² The aetiopathogenesis of palmar fibromatosis is still unknown. Previous studies have suggested that abnormal local release of polypeptide regulatory factors may be involved in this disease.³ It has been shown that basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and transforming growth factor- β (TGF- β) are expressed at the mRNA^{3,4} and protein^{4–6} levels in nodules of Dupuytren's contracture. Since these growth factors are mitogens for cultured cells from normal palmar fascia and Dupuytren's fascia,^{7,8} their possible role has been postulated in the pathophysiology of palmar fibromatosis.

To our knowledge, there have been no studies on the expression and distribution of transforming growth factor- α (TGF- α) and its cellular receptor, epidermal growth factor receptor (EGF-R), in palmar fibromatosis. The present study was undertaken to investigate whether TGF- α and EGF-R are expressed at the mRNA and protein levels in palmar fibromatosis.

MATERIALS AND METHODS

Samples

Surgical samples were obtained from 20 patients undergoing surgical palmar fasciectomy for treatment of palmar fibromatosis. Tissues consisted of single or multiple macroscopic nodules of variable size, associated with portions of palmar fascia. Samples of palmar fascia from five patients, who had hand surgery for carpal tunnel syndrome, were used as control tissues. In all cases, the tissues were snap-frozen in liquid nitrogen immediately after surgical removal.

RNA extraction

Ten nodules from ten different patients and samples of control palmar fascias were treated to extract RNA for expression studies. Tissues were extensively homogenized and total RNA was extracted by the guanidinium isothiocyanate/phenol/chloroform method.⁹ RNA concentrations were determined using spectrophotometric readings at absorbance 260 nm.

RT-PCR and Southern blot analysis of amplification products

1 μ g of total RNA was reverse-transcribed in 20 μ l of reaction mixture (50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM MgCl₂; 10 mM DTT) using Moloney Murine Leukemia Virus RT (200 U/reaction) (Amersham,

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Table I—Sequence and cDNA position of primers and probes used for TGF- α and EGF-R detection

Primer	Sequence	nt position
TGF- α 5'	5'-CTGGCTGTCCTTATCATCAC-3'	365–384
TGF- α 3'	5'-GACGGAGTTCTTGACAGAGT-3'	688–669c
TGF- α probe	5'-TGTGCCACAGACCTTCTACTTGGCCTGTA-3'	607–636
EGF-R 5'	5'-ATACCATGCAGAAGGAGG-3'	2790–2807
EGF-R 3'	5'-ATCATCCAGCACTTGACC-3'	3044–3027c
EGF-R probe	5'-CACAGAATCTATACCCACCAGAGTGATGTC-3'	2847–2876

c=complementary strand.

Cleveland, OH, U.S.A.) and random hexamers (1 μ g/reaction) as first-strand cDNA primers (Pharmacia, Uppsala, Sweden), in the presence of 10 units of Ribonuclease Inhibitor (Amersham) and deoxyribonucleotides. Reverse transcriptase reactions were carried out at 25°C for 15 min, followed by a 45 min step at 37°C.

One tenth of each resulting cDNA product was then amplified by PCR in 100 μ l of reaction mixture containing 2.5 U of Taq DNA polymerase (AmpliQ, Perkin Elmer Cetus Co., Emeryville, CA, U.S.A.), and 12.5 pmol each of the specific primer pair.

Oligonucleotide primers were designed to amplify a 324 bp fragment of TGF- α cDNA¹⁰ and a 255 bp fragment of EGF-R cDNA.¹¹ The sequence of the primers are shown in Table I.

The reactions were carried out for 35 cycles (94°C for 1 min; 58°C for 30 s; 72°C for 45 s) for TGF- α and for 30 cycles (94°C for 1 min; 59°C for 30 s; 72°C for 45 s) for EGF-R, using a Perkin-Elmer 480 thermocycler (Perkin-Elmer Cetus Co., Emeryville, CA, U.S.A.). At the end of the PCR, samples were kept at 72°C for 10 min for final extension and were stored at 4°C until analysed.

Control reactions without M-MLV RT were also performed to exclude genomic DNA contamination as the source of any amplified signal.

Amplification products arising from RT-PCR were separated by electrophoresis on 2 per cent Nusieve: Agarose, 3:1 (FMC BioProducts, Rockland, ME, U.S.A.) in 0.045 M Tris-borate/1 mM EDTA buffer and visualized by ethidium bromide staining, using a ϕ X174 DNA *Hinf*I digested (Promega Co., Madison, WI, U.S.A.) as the size marker. Amplified products were then transferred, after denaturation and neutralization, to 0.2 μ m nylon membranes (Qiagen Inc., Chatsworth, CA, U.S.A.) as described by Southern.¹² Membranes were hybridized with an internal 30-base oligomer (Table I), end-labelled with γ -³²P-ATP and T4 polynucleotide kinase (New England Biolabs, Inc., Beverly, MA, U.S.A.). Filters were washed at high stringency and exposed on Amersham HyperScreen films for 6 h at -70°C.

Light microscopy

Four-micrometre sections of frozen tissue samples were cut on a cryostat at -20°C and stained with haematoxylin and eosin for standard histological examination. Serial sections were cut, air-dried for 20 min,

fixed in cold acetone for 10 min at room temperature, and then air-dried for 10 min for immunohistochemistry. Subsequently, the remaining tissues were fixed in 10 per cent buffered formaldehyde for 12 h, paraffin-embedded, and stained with haematoxylin and eosin.

Immunohistochemistry

Immunohistochemical studies were performed on both frozen and paraffin-embedded sections, using the avidin-biotin peroxidase (ABC) complex (Vector Laboratories, Burlingame, CA, U.S.A.).

Paraffin-embedded sections were deparaffinized in xylene and treated with 0.3 per cent hydrogen peroxide in 40 per cent (v/v) methanol in phosphate-buffered saline (PBS) for 20 min at room temperature. Enzymatic predigestion was performed with 0.05 per cent trypsin (Sigma Chemical Co., St. Louis, MO, U.S.A.) in 0.01 M PBS (pH 7.4) for 10 min at 37°C.

Incubation with primary antibodies was performed for 1 h at room temperature, followed by incubation with biotinylated anti-mouse immunoglobulins and then with the ABC complex, allowing 1 h at room temperature for each step. After each incubation, sections were washed with 0.01 M PBS (pH 7.4). Bound peroxidase was visualized using 0.05 per cent 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., U.S.A.) as the chromogenic substrate for 10 min at room temperature. Sections were counterstained with haematoxylin, dehydrated, and mounted. Controls included omission of the primary and secondary antibodies from the staining sequence.

Antibodies

The following monoclonal antibodies were used: α -smooth muscle actin (Dakopatts, dilution 1:200), TGF- α (Ab-2) (Oncogene Science, Manhasset, NY, U.S.A.; dilution 1:80) and EGF-R (Ab-4) (Oncogene Science, Manhasset, NY, U.S.A.; dilution 1:80).

RESULTS

RNA expression

RT-PCR demonstrated that palmar fibromatosis nodules contained both TGF- α and EGF-R mRNA transcripts. TGF- α and EGF-R were expressed at high



Fig. 1—Composed Southern blot showing high expression levels of both TGF- α and EGF-R mRNA in two different nodules (N) of palmar fibromatosis, in contrast to the low levels in two control palmar fascias (F)

Table II—Immunohistochemical expression of α -smooth muscle actin, TGF- α , and EGF-R in the different phases of palmar fibromatosis and in normal palmar fascia

	α -Actin	TGF- α	EGF-R
Normal fascia	— — —	+ — —	+ — —
Proliferative phase	+++	+++	+++
Involucional phase	++ —	++ —	++ —
Residual phase	+ — —	+ — —	+ — —

+++ = diffuse immunoreactivity; ++ — = immunoreactivity restricted to highly cellular areas; + — — = focal immunoreactivity; — — — = no immunoreactivity.

levels, as assessed by Southern blot following RT-PCR, in all nodules when compared with control palmar fascia (Fig. 1). The levels of expression were variable, probably dependent of the different degrees of cellularity in the samples.

Light microscopy

Depending on the degree of cellularity and fibrosis, the following three histological phases were recognized in palmar fibromatosis nodules: proliferative, involucional, and residual. The proliferative phase was characterized by high stromal cellular density and marked vascularization, while the extracellular matrix contained few mature collagen fibres. The involucional phase also showed high cellularity, but spindle-shaped fibroblasts tended to be aligned in the same direction and an increased amount of collagen fibres was observed between cells. In some areas, a fibrotic transformation of the extracellular matrix was also noted, enveloping individual fibroblasts. The residual phase was hypocellular and the aligned fibroblasts were surrounded by thick bands of mature collagen fibres, giving them a tendon-like appearance.

Immunohistochemistry

Immunohistochemical results were similar both on frozen and on formaldehyde-fixed tissues. A summary of the results is shown in Table II.

Immunostaining for α -smooth muscle actin was performed to reveal the presence and distribution of myofibroblasts within palmar fibromatosis nodules. A strong cytoplasmic reaction for α -smooth muscle actin was diffusely detected in the spindle-shaped cells of the

highly cellular areas of both the proliferative and the involucional phases (Figs 2A and 3A). These cells were then identified as myofibroblasts. A strong reaction also found in smooth muscle cells of small blood vessels served as an internal control (Figs 2A and 3A). The spindle-shaped cells present in the control palmar fascia and in the hypocellular and fibrotic areas of both involucional and residual phases (Fig. 3A) were unstained by α -smooth muscle actin antibody and were considered fibroblasts.

Immunostaining for TGF- α and EGF-R revealed cytoplasmic positivity in the myofibroblasts of the highly cellular areas of both the proliferative and the involucional phases (Figs 2B, 2C, and 3B). The fibroblasts of the hypocellular and fibrotic areas of both involucional and residual phases showed focal or absent immunoreactivity to TGF- α (Fig. 3B) and EGF-R. Focal and weak staining was also detected in the fibroblasts of the control palmar fascia (not shown). Endothelial cells of blood vessels of both normal palmar fascia and palmar fibromatosis nodules were stained by TGF- α and EGF-R (Figs 2B, 2C, and 3B).

DISCUSSION

Dupuytren's contracture is a fibro-proliferative disease progressing through three histological stages: proliferative, involucional, and residual.^{1,13} Several studies have shown that myofibroblasts are the principal cellular component of palmar fibromatosis and that antibodies against α -smooth muscle actin are reliable markers to identify these cells.¹ In agreement with several studies,^{1,14,15} we found that it is only the stromal cells of the highly cellular areas of the proliferative and involucional phases that are myofibroblasts, as demonstrated by their cytoplasmic immunoreactivity to α -smooth muscle actin. These cells, showing contractile properties and a capacity to produce several extracellular matrix components^{1,14-18} and some of their cellular ligands,¹⁹ are believed to play a critical role in the mechanisms leading to tissue retraction in palmar fibromatosis.^{1,17,19} Although the inflammatory component is minimal or absent in palmar fibromatosis, this pathological condition is considered biologically similar to normal wound repair.¹⁻⁵ Since it is known that several growth factors regulate cellular events during wound repair,^{20,21} some authors have postulated and then demonstrated the presence and the involvement of growth factors also in palmar fibromatosis,³⁻⁸ suggesting the possibility that contracture may be the result of a complex and abnormal local regulation of cellular proliferation.^{3,7}

In the present paper, we give the first demonstration of the expression of TGF- α and its cellular receptor, EGF-R, at the mRNA and protein levels in palmar fibromatosis nodules, as shown by RT-PCR and immunohistochemistry, respectively. Immunohistochemical study showed a differential expression of both TGF- α and EGF-R in the different phases of palmar fibromatosis. TGF- α and EGF-R were localized in myofibroblasts of the highly cellular areas of the

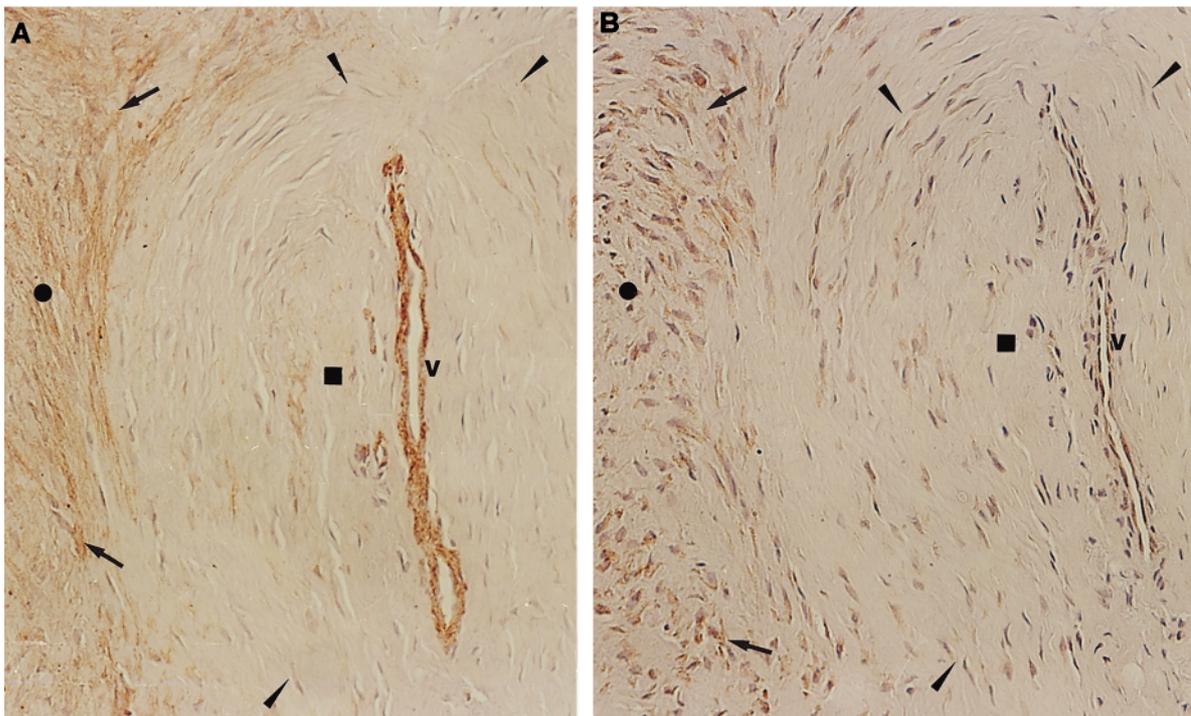
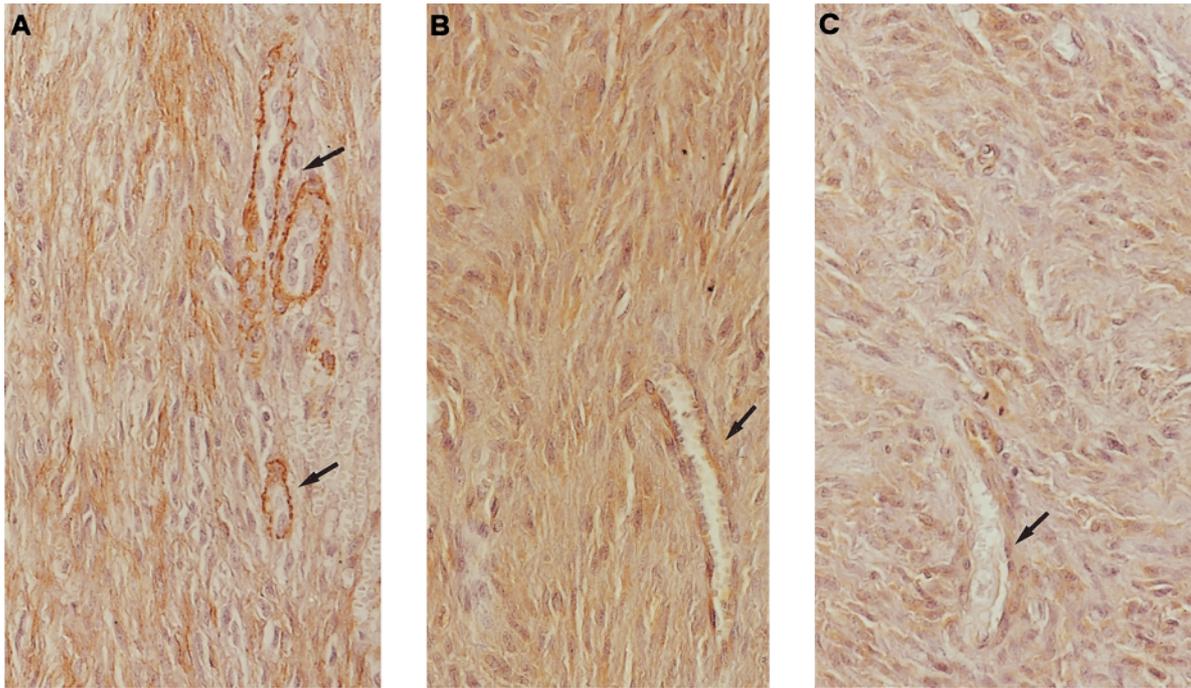


Fig. 2—Proliferative phase. Stromal cells show cytoplasmic staining for α -smooth muscle actin (A), TGF- α (B), and EGF-R (C). Smooth muscle cells of small blood vessels (arrows) are stained by α -smooth muscle actin (A), while endothelial cells are positive for TGF- α (B) and EGF-R (C). (Formaldehyde-fixed tissues; ABC-peroxidase technique; haematoxylin counterstaining)

Fig. 3—Involutinal versus residual phase. Consecutive sections immunostained by α -smooth muscle actin (A) and TGF- α (B). Myofibroblasts (arrows) of the involutinal phase (●) are positive for both α -smooth muscle actin and TGF- α , while focal or absent staining is observed in fibroblasts (arrow-heads) of the residual phase (■). v=blood vessel. (Formaldehyde-fixed tissues; ABC-peroxidase technique; haematoxylin counterstaining)

proliferative and involutinal phases, while they disappeared from the fibroblasts of the hypocellular and fibrotic areas of the involutinal and residual phases. The immunohistochemical detection of TGF- α in the cytoplasm of myofibroblasts suggests that these cells are the source of this growth factor. Since TGF- α is a polypeptide mitogen which acts through the EGF-R,^{22,23} the co-expression of EGF-R by myofibroblasts indicates that these cells are probably the main target for TGF- α action in palmar fibromatosis. Although it is widely known that some growth factors such as PDGF, granulocyte macrophage-colony stimulating factor (GM-CSF), and TGF- β may modulate the phenotype of fibroblasts in several fibrotic pathological conditions,²⁴ to our knowledge no information is available on the expression of TGF- α and EGF-R in human superficial and deep fibromatoses.

The co-expression of TGF- α and EGF-R in myofibroblasts has been reported only in human umbilical cord.²⁵ Moreover, previous studies have shown that myofibroblasts may synthesize and release TGF- α ,²⁶ and this growth factor, through EGF-R, may modulate the cellular biology of wound repair^{27,28} and the fibroproliferative response in experimental models of lung injury.^{29,30}

Although further studies are necessary, the restriction of co-expression of the TGF- α /EGF-R complex to myofibroblasts, which are considered to be the proliferating cellular components of palmar fibromatosis nodules,¹ suggests that TGF- α and EGF-R may be the components of an autocrine/paracrine regulatory pathway involved in the stromal proliferative events occurring in palmar fibromatosis. Identification of inhibitors to TGF- α and EGF-R, as well as to other growth factors detected in palmar fibromatosis, may be clinically advantageous in the prevention of palmar nodule formation. It remains to be established whether co-expression of the TGF- α /EGF-R complex is a common finding in the myofibroblasts of other fibrotic pathological conditions.

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