

Programmed cell death in nodular palmar fibromatosis (*Morbus Dupuytren*)

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Summary. The regular loss of cellularity during involutional phase of nodular palmar fibromatosis (*Morbus Dupuytren*) indicates a regulated process known as programmed cell death (apoptosis).

Using the TUNEL method apoptosis-related DNA fragmentation is detected in numerous cells as a characteristic feature of fibromatosis noduli of involutional phase. By means of double labelling technique, α -smooth muscle actin immunohistochemistry and TUNEL method for apoptosis, it is demonstrated that the cells which underwent apoptosis are myofibroblasts. As anticipated, the antidote to apoptosis bcl-2 is not detected in involutional phase, but neither it is evidenced in proliferative phase. Immunohistochemically, Fas/APO-1 is shown to be existent in a very small number of fibroblasts in involutional phase. However, in view of the high number of TUNEL-stained cells a significance in regulating apoptosis in nodular palmar fibromatosis seems improbable.

Taking into account that the development of the fibromatosis noduli, the expression of myofibroblast phenotype, basement membrane formation and growth factor expression including TGF β culminates in involutional phase the initiation of apoptotic cell death can be discussed in relation to these growth factors and matrix protein action and the programmed cell death may be considered as the final step of myofibroblast phenotype evolution.

Key words: Nodular palmar fibromatosis, TUNEL method, Apoptosis, Myofibroblast

Introduction

The nodular palmar fibromatosis (*Morbus Dupuytren*) is a pseudo-tumorous self limited proliferation of aponeurotic fibroblasts (Ushijima et al., 1984; Enzinger and Weiss, 1988). Typically, successive stages of the disease are associated with changing from

fibroblast to myofibroblast phenotype with fundamental modulation of proliferative activity, growth factor synthesis and a shift in extracellular matrix composition (Tomasek et al., 1986; Shum and McFarlane, 1988; Pasquali-Ronchetti et al., 1993). According to Luck (1959), cell dense nodules of polygonal fibroblasts appear in the proliferative phase followed by an axial orientation of fibroblasts in the involutional phase and ending in a less cellular scar nodulus (residual phase). The modulation of the fibromatosis nodule from involutional to residual phase runs parallel with a fundamental decrease of cell number (Schürch et al., 1992). Taking into account the defined course of Dupuytren's disease with distinguished phases, a regulated process of cell diminution (programmed cell death) may be assumed. The regulating factors of diminution of cell number are not known as yet.

The programmed cell death (apoptosis) is accompanied by fragmentation of nuclear DNA by endogenous endonucleases at molecular level and occurs in an early stage of apoptosis (Lorenzen et al., 1995). These DNA double strand breaks can be specifically labelled by the TUNEL method (terminal deoxy-nucleotidyl transferase mediated X-dUTP nick end labelling) and used for indication of apoptotic cell death (Gavrieli et al., 1992).

This study was created to investigate the nature of the diminution of cell number in the late involutional phase of the nodular palmar fibromatosis and to correlate the loss of cellularity with cellular differentiation, extracellular matrix composition and the immunohistochemical expression of proteins involved in regulation of apoptosis.

Materials and methods

Tissue material

Formalin-fixed-paraffin embedded tissue as well as native shock frozen samples of fibromatosis nodules of 8 surgical Dupuytren's disease specimens were available. The phases of fibromatosis development were histologically settled according to the criteria by Luck (1959).

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Table 1. Correlation of fibromatosis phase (according to Luck, 1959) to the number of apoptotic cells marked with TUNEL method.

CASE No.	PHASE OF DUPUYTREN'S DISEASE	TUNEL STAINING
1	Proliferative phase	No apoptotic staining in the cells
2	Proliferative phase	Sporadic apoptotic staining
3	Proliferative nodules surrounded by tissue of involutinal phase	Apoptotic staining is exhibited in transition areas of proliferative nodules
4	Proliferative phase, transition into involutinal phase	Apoptosis exclusively stained situated in transition areas
5	Early involutinal phase	Weak apoptotic activity which is confined to areas of involutinal phase
6	Involutinal phase	All cells of fibromatous nodules are readily labelled
7	Involutinal phase	All cells of fibromatous nodules are readily labelled
8	Late involutinal phase	Focal staining of myofibroblast nuclei

TUNEL-method

Apoptotic cells were detected on dewaxed paraffin sections using the TUNEL (terminal deoxynucleotidyl transferase mediated dUTP-fluorescein nick end labelling) method according to Gavrieli and co-workers (1992) applying the In Situ Cell Death Detection Kit/AP (Boehringer Mannheim, Germany). Briefly, tissue sections were predigested by incubation with proteinase K (20 µg/ml in 10mM Tris/HCl, pH 7.4-8.0) for 20 minutes at 37 °C. The samples were incubated with terminal deoxynucleotidyl transferase and fluorescein labelled dUTP containing nucleotide mixture (TUNEL reaction mixture) in a humid atmosphere at 37 °C for 60 minutes. The converter-AP solution (alkaline phosphatase-conjugated anti fluorescein antibody) was applied at 37 °C for 30 minutes. After rinsing in PBS, bound alkaline phosphatase was detected using the components of the Nucleic Acid Detection Kit (NBT/BCIP system, Boehringer Mannheim, Germany) yielding a purple-blue coloured precipitate. Slides were incubated with substrate solution for 10 min, 20 min and 30min, rinsed in PBS, mounted under glass coverslips and analysed light microscopically. A negative control was included in each experimental setup by omitting the terminal transferase from the TUNEL reaction mixture.

Immunohistochemistry

Paraffin as well as cryostat sections of the respective tissue samples were available for immunohistochemical staining using the APAAP (alkaline phosphatase monoclonal anti-alkaline phosphatase) method (Gustmann et al., 1991).

Briefly, the primary antibodies to α -smooth muscle actin (clone 1A4, diluted 1:40, Dako, Denmark), collagen type IV (clone CIV22, diluted 1:50, Dako, Denmark), laminin (β 1 chain, clone 4E10, diluted 1:20000, Telios, USA), bcl-2 (clone 124, diluted 1:40, Dako, Denmark) and Apo-1/Fas (clone SMI/17, diluted

1:100, Serva, FRG), TGF β (pan specific, polyclonal, diluted 1:20, R&D systems, UK) were incubated for 30 min at room temperature. After washing with Tris buffer, sections were treated with rabbit anti mouse antibodies (polyclonal, diluted 1:70, Dako, Denmark), and then with the mouse APAAP-complex (Dako, Denmark). In the case of polyclonal TGF β antibody an additional mouse anti rabbit antibody (polyclonal, diluted 1:400, Dako, Denmark) was introduced. Both incubations were done for 30 min at room temperature. 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 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.

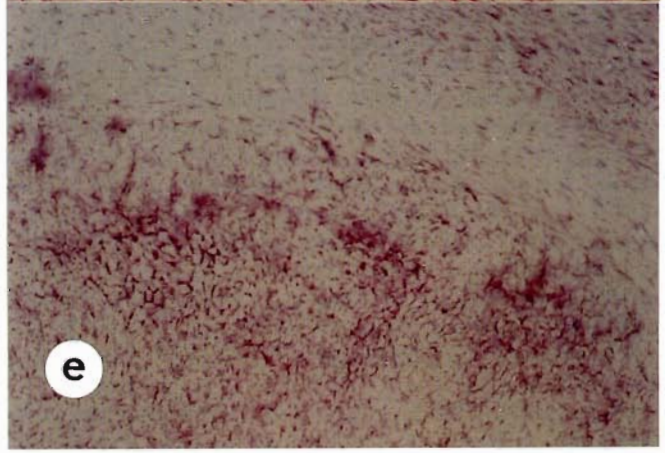
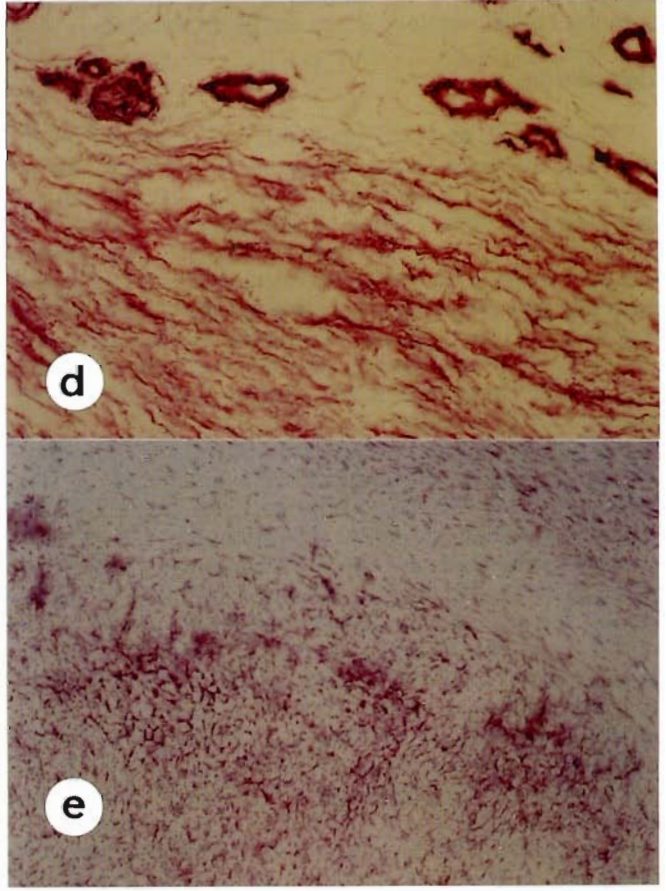
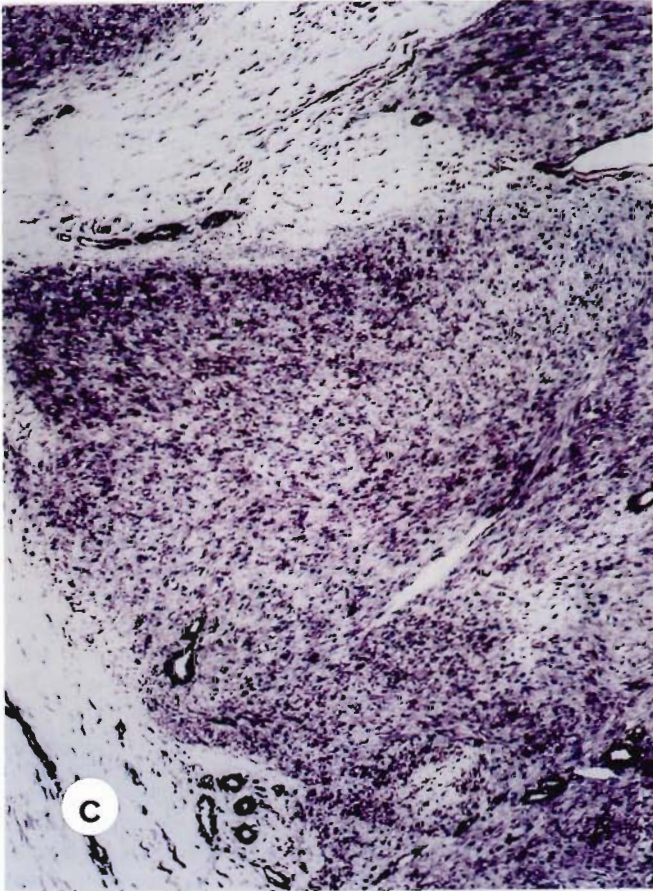
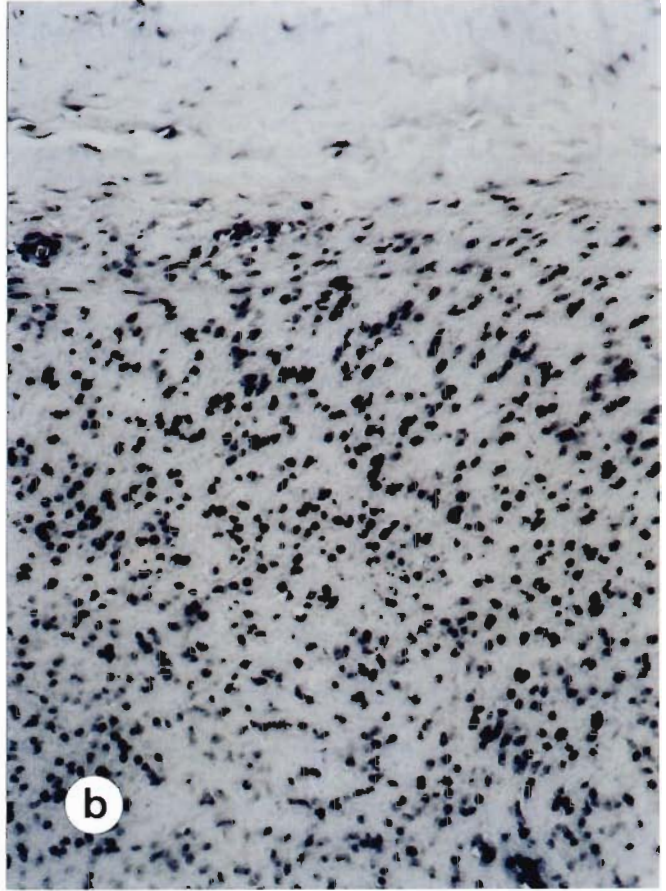
Double staining procedure for α -smooth muscle actin and apoptosis

A combination of APAAP technique and TUNEL method was used for double staining of α -smooth muscle actin positive cells and apoptotic nuclei. At first, the APAAP technique for α -smooth muscle actin visualization was performed as described. Secondly, after thoroughly rinsing in Tris buffer the TUNEL procedure was carried out.

Results

Using the TUNEL method for demonstration of apoptosis-related DNA fragmentation dark blue-labelled nuclei could be detected within nodular palmar

Fig. 1. a. Nodulus of nodular palmar fibromatosis in proliferative phase exhibiting few dark-blue labelled nuclei (positive staining by TUNEL method. Without counter staining. x 150. b. Involutinal phase of nodular palmar fibromatosis. Numerous cells within the fibromatosis nodulus are labeled by TUNEL method. Without counter staining. x 150. c. Nodulus of nodular palmar fibromatosis in involutinal phase. The double labelling technique for α -smooth muscle actin immunohistochemistry and TUNEL method for apoptosis shows a predominant correlation of apoptosis to myofibroblasts. Without counter staining. x 75. d. Immunohistochemical demonstration of laminin β 1 chain in a fibromatosis nodulus of *Morbus Dupuytren*. Without counter staining. x 150. e. Immunohistochemical demonstration of TGF β 1 in a fibromatosis nodulus of *Morbus Dupuytren*. Without counter staining. x150



fibromatosis. There is a clear correlation of the number of stained nuclei to the disease phase of the fibromatosis. Whereas in the pre-existing aponeurosis as well as in the residual nodules no or only single marked nuclei were observed, in the proliferative phase few (Fig. 1a) and in the involutinal phase numerous stained nuclei were recognized (Table 1, Fig. 1b). In fibromatosis nodules of the involutinal phase the majority of the nuclei were regularly labelled. Within these fibromatosis nodules of the involutinal phase were blood vessels, their endothelial cells showing an apoptosis related DNA fragmentation, as well (Fig. 1c). As to the cellular differentiation of the apoptotic cells double labelling with α -smooth muscle actin immunohistochemistry for myofibroblast phenotype and TUNEL method for apoptosis revealed the majority of apoptotic cells as myofibroblasts (Fig. 1c).

In involutinal phase a strong immunolabelling of laminin and collagen type IV could be demonstrated restricted to the myofibroblast-containing fibromatosis nodules.

Within the same areas the growth factor TGF β could be visualized (Fig. 1d,e).

A true bcl-2 immunostaining of the cells involved in fibromatosis process was not achieved. With the application of monoclonal antibodies to Fas/APO-1 only some single fibroblastic cells could be positively labelled, whereas a correlation to the staining pattern with the TUNEL method could not be established.

Discussion

Despite the fact that apoptosis has attracted much scientific attention in the recent years and may have a distinct light microscopic feature consisting of cytoplasmic shrinkage, loss of cell contacts, distinctive alterations of the nucleus, such as chromatin condensation and nuclear fragmentation, cellular budding and fragmentation, and phagocytosis of the fragments by macrophages or adjacent cells (Kerr et al., 1972), to our knowledge no data are available about the nature of the reduction of cell number during the course of Dupuytren's disease.

At present, for visualization of apoptotic cell death in histological slides two methods are available: the in situ nick translation and the in situ end labelling (TUNEL method; Gold et al., 1994). Whereas the in situ nick translation demonstrates DNA single strand breaks, the TUNEL method indicates both single and double strand breaks. Because single strand breaks preferentially occur in necrosis, the TUNEL method is considered to be slightly more sensitive for assessment of programmed cell death by morphological criteria (Gold et al., 1994). In literature, the TUNEL method is widely accepted for specific in situ demonstration of apoptosis (Hockenbery, 1995; Ikeda et al., 1995; Money et al., 1995; Moreira et al., 1995).

Using the TUNEL method, exclusively in the late involutinal phase of Dupuytren's disease numerous

nuclei of the fibromatosis nodules could be intensely dark blue labelled, interpreted as a specific sign of programmed cell death (apoptosis). It can be concluded that limitation of the quasineoplastic growth of nodular palmar fibromatosis and decrease of cell number in the fibromatosis nodules is realized by apoptosis.

The dominating cellular phenotype of fibromatosis nodules in involutinal phase is the myofibroblast (Schürch et al., 1992). As demonstrated by double labelling technique with α -smooth muscle actin immunohistochemistry and TUNEL method for apoptosis, it could be confirmed that cells with the myofibroblast phenotype preferentially undergo apoptotic cell death. This finding in nodular palmar fibromatosis is in line with the observation of Desmoulière and co-workers (1995) who described that apoptosis mediates the decrease of cellularity during the transition between granulation tissue and scar. In congruence to our results yielded by double labelling they described ultrastructurally apoptotic and myofibroblast features within the same cell. Taking into account the course of nodular palmar fibromatosis in the different phases and the formation of the myofibroblast phenotype it can be concluded that myofibroblast evolution includes the programmed cell death as the final step. Moreover, in reparative processes including smooth muscle cells or myofibroblasts, e.g. atherogenesis, apoptosis seems to be a regular phenomenon of these cell types to control the tissue formation (Leszczynski et al., 1994; Bochaton-Piallat et al., 1995; Han et al., 1995).

The number of molecules with a putative role in regulation of programmed cell death is continuously increasing. Among them, bcl-2 and Fas/APO-1 are discussed in a direct relation to the apoptotic process (Itho et al., 1991; Craig 1995; Hockenbery, 1995; Korsmeyer, 1995; Higaki et al., 1996). Fas/APO-1 which releases apoptosis is only present in a few single fibroblasts in involutinal phase and bcl-2 which prevents apoptosis and should be expected in proliferative phase is not demonstrated. Therefore, these proteins do not seem to be involved in programmed cell death in nodular palmar fibromatosis.

The fibro-/myofibroblasts of proliferative and involutinal phase in nodular palmar fibromatosis show an abundant growth factor and extracellular matrix protein synthesis (Baird et al., 1993; Halliday et al., 1994). Concerning the limitation of proliferation and the initiation of programmed cell death the myofibroblast-associated expression of the transforming growth factor β family and the basal lamina development seems to be of special interest. As shown here and previously by us, the expression of TGF β 1 and 3, visualized by in situ hybridization, and the myofibroblast-associated basal membrane formation culminate in the late involutinal phase (Berndt et al., 1993, 1995; Kosmehl et al., 1995). The loss of basement membrane during the progression from the late involutinal phase to the residual phase runs parallel with increasing apoptosis in nodular palmar

fibromatosis and is comparable to the involution of mammary gland, where the loss of the ductal basement membrane results in the apoptosis of ductal epithelial cells (Wicha et al., 1980; Talhouk et al., 1992; Lochter and Bissell, 1995). Moreover, an induction of apoptosis by TGF β is evidenced for liver parenchymal cells (Gressner et al., 1996).

The course of the tissue modulation process in palmar fibromatosis, the expression of TGF β and basement membrane formation and the apoptosis induction of these proteins known from the literature let us assume they play a role in regulation of programmed cell death in fibromatosis, as well.

The presented results evidence the restriction of quasineoplastic growth in nodular palmar fibromatosis by programmed cell death. The double-labelling experiments, α -smooth muscle actin immunohistochemistry and TUNEL method for apoptosis, demonstrate apoptosis as an event associated with myofibroblast phenotype evolution. The correlation between the decrease of basement membrane and increase of apoptotic activity in epithelial cells as well as in myofibroblasts of granulation tissue and fibromatosis points to a special role of extracellular matrix in regulation of tissue cellularity.

Because the apoptotic cell death seems to be included in the physiological development of myofibroblast phenotype one may expect that the loss of control of apoptosis contributes to malignant myofibroblast growth as known for myofibrosarcoma.

Acknowledgements. The authors are grateful to Dr. J. Stoltz and Dr. Ph. Zollmann, Praxis für Chirurgie Jena, for placing the surgical specimens at our disposal and to Mrs. Christina Martin for skilful technical assistance. The work was generously supported by Boehringer Mannheim GmbH, Germany.

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Accepted June 14, 1997