

EPIDERMAL GROWTH FACTOR RECEPTOR (EGF-R) IN DUPUYTREN'S DISEASE

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The aim of this paper was to examine participation of the epidermal growth factor receptor (EGF-R) signal pathway in the pathogenesis of Dupuytren's disease. The study showed changes in the ratio of membrane EGF-R to its intracellular level during the different clinical stages of Dupuytren's contracture progression. Our observations of a high ratio of surface to intracellular EGF-R in the palmar aponeurosis of patients with second degree of Dupuytren's disease (Iselin's classification), which was significantly higher than this ratio in control palmar fascia ($P = 0.022$), would suggest that EGF-R has a role in the involutional phase of the disease.

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INTRODUCTION

The most characteristic feature of Dupuytren's disease is growth of nodular masses, which demonstrate a remarkable increase in the cellularity, primarily due to the proliferation of fibroblasts and myofibroblasts. These cells are also believed to be responsible for deposition of abnormally large amounts of extracellular matrix components (Berndt et al., 1995; Gabbiani and Majno, 1972; Magro et al., 1995, 1997; Tomasek et al., 1999). Depending on the relative degree of cellularity and fibrosis, different morphologic phases and equivalent clinical stages of disease progression are observed during the course of contracture. The histological changes are closely associated with abnormal expression of various structural and signal molecules such as collagen, fibronectin, $\alpha 5\beta 1$ integrins and growth factors, including these activating the epidermal growth factor receptor (Augoff et al., 2005; Baird et al., 1993; Chansky et al., 1999; Magro et al., 1995, 1997; Murrell et al., 1991; Tomasek et al., 1999).

Epidermal growth factor receptor (EGF-R) (erbB-1) is a member of erbB family of cell surface receptor tyrosine kinases. It is a large transmembrane glycoprotein ($M_r \cong 175,000$) which is found in three major regions: the extracellular domain which contains the growth factor binding site, the hydrophobic transmembrane domain and the cytoplasmic domain which contains the tyrosine-specific protein kinase (Bennett and Schultz, 1993; Waterfield, 1989). EGF-R can bind several different epidermal growth factor family hormones (EGFs), such as epidermal growth factor (EGF), transforming growth factor- α (TGF- α), amphiregulin or heparin-binding epidermal growth factor (HB-EGF) (Cheng et al., 2002; Damjanov et al., 1986; Kim et al., 1998; Kömüves et al., 2000; Madtes et al., 1999; Riese et al., 1996). Binding of a ligand to the extracellular domain induces EGF receptor dimerization, which activates the catalytic domain and leads to tyrosine autophosphorylation. Activated receptors, by association with the specific group of signal transducers and

activators of transcription, initiate a cascade of biological processes which lead to DNA synthesis and result in proliferation, differentiation, migration and adhesion of many connective tissue cells, including fibroblasts (Bennett et al., 1993; Cheng et al., 2002; Kim et al., 1998; Laato et al., 1987; Rumalla and Borah, 2001; Shiraha et al., 2000; Wakita and Takigawa, 1999). In addition, EGF-R activation stimulates collagen and glycosaminoglycans synthesis and expression of matrix metalloproteinases, or tissue inhibitors of metalloproteinase, and, thus, may be important in extracellular matrix remodelling (Bennett et al., 1993; Laato et al., 1987; Madtes et al., 1999; Rumalla and Borah, 2001).

Several in vitro and in vivo studies have shown that deregulation of signalling networks composed of the erbB family receptors and epidermal growth factor family of peptide hormones, by alterations in expression or activity of cell surface EGF receptors, may initiate changes in cell physiology, leading to morphologic defects or tumoral transformation (Riese et al., 1996; Rumalla and Borah, 2001; Wakita and Takigawa, 1999; Waterfield, 1989).

Since analysis of the cellular sites of receptor localization can provide information regarding their activity, the purpose of the present study was to analyse immunochemically, by use of enzyme-linked immunosorbent assay (ELISA) test, changes in activity of EGF-R, defined by the ratio of surface (high-affinity membrane-localized) to intracellular (latent internalised or newly biosynthesized) EGF-R, in palmar aponeurosis through the different clinical stages of Dupuytren's disease.

MATERIALS AND METHODS

Fragments of pathological palmar aponeurosis, taken intraoperatively from 63 patients (53 male and 10 female), aged 47 to 73 years, treated surgically for Dupuytren's contracture between 2000 and 2003 at the

Department of Traumatic Surgery and Hand Surgery of Wrocław University of Medicine, were the object of the study. Iselin's classification was used to identify disease progression (Degree I—palmar nodules and small cords without signs of contracture, Degree II—contracture of the metacarpophalangeal (MCP) joint, Degree III—contracture of the proximal interphalangeal (PIP) joint and Degree IV—severe contracture of the MCP and the PIP joints with hyperextension of the distal interphalangeal (DIP) joint, together with advanced lesions in the osseous system (Nagay, 1985). According to this classification, we examined palmar fascia tissue from 7 cases with first degree disease, 20 with second degree, 20 with third degree and 16 cases with fourth degree disease. For comparative purposes, we analysed fragments of normal palmar fascia obtained from 8 patients (7 female and 1 male) during surgery for carpal tunnel syndrome.

The tissue fragments were washed in 0.9% NaCl, dried on blotting paper and homogenized in 5 volumes of lysis 50mM buffer Tris-HCl pH 7.4 with 1mM EDTA, 0.25M saccharose, 1mM DTT and 20mg/ml PMSF in a glass Potter's homogenizer. Homogenates were centrifuged for 30 minutes at 13,500*g*. The extracts were stored in -20 °C and used for intracellular EGF-R estimation. Pellets were suspended in 3 volumes of 1% Tween 20 in PBS, incubated for 30 minutes at room temperature and then centrifuged for 30 minutes at 13,500*g*. These extracts were also stored in -20 °C and used for surface EGF-R estimation. EGF receptors were studied by the indirect ELISA system.

Proteins (50 µg/ml) were passively absorbed to microtiter plates in 96-well format (Nunc) for 2 × 30 minutes at 37 °C. We used bovine serum albumin in PBS (1% w/v) to block non-specific binding. Attached antigens were incubated for 3 h at 37 °C with primary mouse monoclonal antibodies against human EGF-R (Santa Cruz Biotechnology, cat. No sc-120, diluted 1:200) and, then, for 1.5 h at room temperature with secondary goat antibodies anti-mouse-IgG HRP conjugate (BIO-RAD, cat. No 170-6516, diluted 1:3000). Bound enzyme was exposed to detection solution of 0.04% *o*-phenylenediamine dihydrochloride (Sigma) dissolved in 0.05M buffer sodium citrate with 0.15M sodium phosphate, pH 6.0 containing 0.01% H₂O₂ for 30 minutes at room temperature. The extinction values were read with a reader for ELISA (DHN, EL - 01) using a 492 nm filter.

The results, expressed as the ratio of surface EGF-R to intracellular EGF-R in the tested tissues, were analysed statistically using the non-parametric Mann-Whitney U-test (<http://eatworms.swmed.edu/~leon/stats/utest.html>). *P* values of less than 0.05 were considered statistically significant.

RESULTS

Changes of membrane EGF-R to intracellular EGF-R ratio in the tissues with Dupuytren's contracture for

each degree of disease are presented in Table 1. Low EGF-R ratio was found in the early phase of the disease in the group of patients with first degree disease (median 0.15). The range of these values did not differ from the control. With progression of the disease, this ratio increases, achieving a maximum median (0.45) in the group of patients with second degree disease. In comparison with the control group, the ratio of surface to intracellular EGF-R in second degree disease was significantly different (*P* = 0.022). Decreasing EGF-R ratio was observed in the tissues taken from patients with third degree disease (median 0.39). Although the ratio was still higher than in the control, the difference was not statistically significant (*P* = 0.059). Tissue from patients with fourth degree disease, the most advanced stage of the disease, showed a range of EGF-R ratio most similar to that of the control group.

DISCUSSION

Dupuytren's disease is a fibroproliferative disorder which is characterized by shortening of the palmar fascia leading to progressive digital flexion deformity (Tomasek et al., 1999). It is a result of a complex biological process which is well documented at a microscopic level, but its biochemical/molecular regulation is still an intriguing puzzle for scientists. From a histological point of view, there are three phases in the course of contracture: proliferative, involutional and residual (Luck, 1959). Each of them is characterized by domination of a different type of cells, starting with fibroblasts through myofibroblasts to fibrocytes (Fitzgerald et al., 1999).

The proliferative phase could be referred to as the first clinical degree of disease progression. It is associated with formation of nodular thickening, usually at the base of the 4th and 5th finger, without a noticeable contracture. It has been demonstrated that specialized fibroblasts, myofibroblasts, appear during the proliferative phase of the disease and come to comprise almost all of the cells present in highly cellular nodules (Tomasek et al., 1999). The mechanism which activates the normal quiescent cells of connective tissue is unknown although accumulating data suggests that cytokines such as interleukin-1 α (IL-1 α), interleukin-1 β (IL-1 β), transforming growth factor- β (TGF- β), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF) play a central role in initiating and/or sustaining the early growth response (Baird et al., 1993; Berndt et al., 1995; Chansky et al., 1999; Fitzgerald et al., 1999; Kömüves et al., 2000).

Although it is known that cytokines from the EGF family stimulate proliferation of fibroblasts in vitro and that actively proliferating cells express the EGF-R mainly on the membrane, our results showed no changes in the ratio of surface to intracellular EGF-R in tissues

Table 1—Median and ranges of membrane to intracellular EGF-R ratio in the normal palmar fascia tissue (control group) and in four groups of tissues of palmar aponeurosis with Dupuytren's contracture with increasing clinical degrees of disease progression

Tested groups		EGF-R membrane/ intracellular
Control group <i>n</i> = 8	Median	0.23
	Range	0.08–0.56
Group I First degree of contracture <i>n</i> = 7	Median	0.15
	Range	0.1–0.42
Group II Second degree of contracture <i>n</i> = 20	<i>P</i>	0.816
	Median	0.45
Group III Third degree of contracture <i>n</i> = 20	Range	0.11–2.5
	<i>P</i>	0.022*
Group IV Fourth degree of contracture <i>n</i> = 16	Median	0.39
	Range	0.07–2.83
	<i>P</i>	0.059
	Median	0.30
	Range	0.09–0.57
	<i>P</i>	0.358

P- probability value in relation to the control group;

*difference significant.

taken from patients with first degree disease, in comparison to the control. Hence, we conclude that the EGF-R signalling system is not involved in mediation of fibroblast division in the early stage of Dupuytren's disease. Magro et al. (1997), in immunohistochemical studies, have observed strong immunostaining for EGF-R in highly cellular areas of Dupuytren's aponeurosis in the proliferative phase. However, this study includes no information about the cellular localization of the EGF-receptors. It remains possible that the EGF-R/ligand complex is rapidly internalized and then degraded in lysosomes. The low amount of EGF-R on the cell surface may be due to the "down-regulation" phenomenon (Damjanov et al., 1986; Waterfield, 1989). Moreover, it is known that the transient EGF-R internalization may also be induced by several cytoplasmic kinases, which affect the state of phosphorylation of the EGF receptor (Damjanov et al., 1986; Wakita and Takigawa, 1999).

High levels of surface EGF-R relative to intracellular EGF-R characterize pathological fascia taken from patients with second and third degree of disease progression. The second and early third degree include the beginning of fibrosis and is called the "involutional phase". It is associated with reorganization of extracellular matrix protein network and with significant changes in cellular density and activity. Areas with intense cellular proliferation ajoin areas of relatively inactive tissue. The nodules begin to disappear. The morphological features of the myofibroblasts also change. The cells are smaller, show a tendency to

penetrate highly collagenized areas and align themselves with the lines of stress (Tomasek et al., 1999). Contracture gradually intensifies as a result of structural matrix components remodelling. EGF-R may have an essential role as a signal messenger, stimulating migration and differentiation of myofibroblasts at this stage of progression of the disease. Using a model of cell culture, Barrandon and Green (1987) have suggested that the effect of the two main ligands for EGF-R (EGF and TGF- α) in, promoting multiplication of the keratinocyte colony, depends rather on their ability to increase the rate of cell migration, than on stimulation of proliferation. It is known that EGF, and/or TGF- α , also enhance connective tissue maturation (Laato et al., 1987). Whether it is possible that these growth factors promote terminal differentiation of the myofibroblasts and/or fibroblasts, as well as keratinocytes, gives an opportunity for future studies.

The final stage, the "residual" phase, including advanced third and fourth clinical degrees of the disease, is characterized by increasing contracture and digital flexion by obvious fibrotic structures of connective tissue joined by homogenous hyaline bodies, dislodging subcutaneous fatty tissue (Nagay, 1985). Myofibroblasts are not observed in the residual phase. The cellular structure of the fascia returns to the state which is observed in the normal palmar aponeurosis. Fibroblasts, which constitute a small number of the cells, lose their proliferative phenotype and develop into a more mature cell phenotype-fibrocytes (Tomasek et al., 1999). In the specimens taken from the patients with fourth degree disease, we observed regression to the normal/control levels of EGF-R ratio. This is in agreement with the findings of Roholl et al. (1991) and Magro et al. (1997). These authors also showed that reactivity for EGF-R antibody in advanced palmar fibromatosis is similar to the EGF-R reactivity in normal fascia. This fact is not a surprise because it has been observed that cells with a low replication capacity have a reduced level of EGF-R production (Damjanov et al., 1986). Moreover, in the residual phase of Dupuytren's disease, a low concentration of TGF- α , as well as of EGF, has also been found and it is known that ligands for EGF-R normally autoregulate the synthesis of its own receptor (Augoff et al., 2005; Kömüves et al., 2000; Magro et al., 1997).

In the present study, we showed changes in the ratio of surface epidermal growth factor receptor (EGF-R) to its intracellular levels during the different clinical stages of Dupuytren's contracture progression. From these observations, we postulate that changes in the ratio of surface to intracellular EGF-R, observed during the progression of Dupuytren's disease, indicate that the EGF-R signalling pathway may contribute to the development of this disorder. Our observations would suggest that EGF-R has a role in the involutional phase of the disease. However, this supposition remains to be investigated further.

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