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Expression of matrix metalloproteinases and their inhibitors in cords and nodules of patients with Dupuytren's disease

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Abstract
Introduction Dupuytren's disease is a fibroproliferative disorder characterized by thickening of the palmar fascia. Several studies indicate that MMPs and TIMPs may play a key role in the onset or progression of Dupuytren's disease and related disorders. In this study, we used a quantitative reverse-transcription PCR methodology to profile the expression of TIMP1, TIMP2, MMP2, and MMP9 in nodule and cord tissue from patients with Dupuytren's disease and compared this with normal palmar fascia taken at carpal tunnel release.
Materials and methods Tissue from patients with Dupuytren's disease was taken at fasciectomy ($n = 30$; 23 men and 7 women; average age 61.3 ± 9.5 years). Samples were divided into regions of nodule and cord according to gross morphology. Normal fascia was taken from patients without Dupuytren's contracture who had carpal tunnel release ($n = 30$; 14 men and 16 women; average age 63 ± 11 years). Expression of mRNA was calculated using a relative quantification method (Pfaffl). Statistical analysis was performed using the Mann–Whitney test. The level of significance was considered to be $P < 0.05$.
Results In comparison to normal fascia, the cords and nodules from patients with Dupuytren's disease showed significant upregulation for TIMP1 and TIMP2 ($P < 0.05$). The expression of TIMP1 was significantly higher in nodules in comparison to cord tissue ($P < 0.05$). The expression of MMP2 was significantly upregulated in tissue of patients with Dupuytren's contracture in comparison to normal tissue ($P < 0.05$). The expression of MMP2 was significantly higher in nodules in comparison to cord tissue ($P < 0.05$). There was no significant difference in the relative expression of MMP9 in nodules and cord tissue of patients with Dupuytren's contracture in comparison to normal fascia from patients with carpal tunnel syndrome.
Conclusions The balance between MMPs and their natural inhibitors is disturbed in patients with Dupuytren's disease. The decrease in MMP-to-TIMP expression can cause increased synthesis and deposition of collagen, leading to palmar fibromatosis. The high expression of MMP2 may represent an unsuccessful attempt to reduce collagen deposition. In the future, a treatment that downregulates TIMPs but increases the activity of MMPs may be an appropriate therapy for Dupuytren's disease.

Keywords Dupuytren's disease · Matrix metalloproteinases · Tissue inhibitors of metalloproteinases · Fibrosis

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Introduction

Dupuytren's disease is a fibroproliferative disorder characterized by thickening of the palmar fascia and often complicated by cord-like structures that extend from the palm into the affected fingers. In this latter situation, there is a frequent contracture of the metacarpophalangeal and

the proximal interphalangeal joints. The contracture is named after Baron Guillaume Dupuytren, a French physician and surgeon who popularized the recognition of this hand disease through a series of lectures in 1831. The disease usually occurs bilaterally, with one hand more involved than the other [8]. Several epidemiological studies have suggested correlations with diabetes, epilepsy, and alcoholism [11, 19, 24]. Also, a genetic predisposition has been discussed [1]. Nevertheless, the etiopathogenesis of Dupuytren's disease is still unknown [4, 12, 33, 35, 37, 39].

Three distinct histologic phases have been described by Luck [20], with a proliferative phase leading to the development of a nodular lesion, an involutional phase in which cells align themselves to lines of stress, and a residual phase leaving a scar-like cord tissue. The nodules are thought to represent an active phase of the disease with myofibroblast proliferation, whereas the cords represent late-stage disease with large, hypocellular bands of contracted, collagen-rich cords and the absence of myofibroblasts [1, 4, 32]. The growing nodules and the arrangement of newly formatted fibers entail tissue reorganization coupled with degradation of the surrounding ECM [23].

Various types of proteinases are implicated in ECM degradation, but the major enzymes are considered to be matrix metalloproteinases (MMPs), also called matrixins [22, 47]. The activities of most matrixins are very low or negligible in the normal steady-state tissues, but expression is transcriptionally controlled by inflammatory cytokines, growth factors, hormones, cell–cell and cell–matrix interaction and activation of precursor zymogens [25]. Collagenases (MMP-1, MMP-8, MMP-13) cleave interstitial collagens I, II, and III into characteristic three-fourths and one-fourth fragments [10]. They also can digest other ECM molecules and soluble proteins [26]. Two other matrixins, MMP-2 and MMP-14 (MT1-MMP), have collagenolytic activity, but they are classified into other subgroups because of their domain compositions [10]. Gelatinases (MMP-2 and MMP-9) readily digest gelatine with the help of their three fibronectin type II repeats that binds to gelatine/collagen [21]. They also digest a number of ECM molecules including types IV, V, and XI collagens, laminin, aggrecan core protein, etc. MMP-2, but not MMP-9, digests collagens I, II and III in a similar manner to the collagenases [28].

MMP activities are regulated by two major types of endogenous inhibitors: α_2-macroglobulin and tissue inhibition of metalloproteinases (TIMPs) [26]. In the tissue, TIMPs are considered to be key inhibitors of MMPs [9]. Four TIMPs are currently identified in humans [9]. They are homologous proteins of 21–29 kDa consisting of two domains, an N-terminal inhibitory domain and a C-terminal domain. Each domain contains three conserved disulfide bonds. The N-terminal domain folds as an independent unit with MMP inhibitory activity. TIMPs inhibit all MMPs tested so far, but TIMP-1 is a poor inhibitor for MT1-MMP, MT3-MMP, MT5-MMP and MMP-19 [22, 31]. TIMP gene expression is regulated by growth factors and cytokines but their levels of modulation are less than those of MMPs [47].

The balance between MMPs and TIMPs is critical for the ECM remodeling in the tissue [33]. Elevated levels of TIMPs over those of MMPs are observed in diseases associated with fibrosis [42, 43]. Several studies indicate that MMPs and TIMPs may play a key role in the onset or progression of Dupuytren's disease and related disorders [2, 16, 18, 27, 29, 41]. The results concerning the upregulation of some MMPs in an active disease and the behavior of TIMP2 remain controversial. In previous studies, only tissue samples of small patient groups were analyzed. In this study, we used a quantitative reverse-transcription PCR methodology to profile the expression of TIMP1, TIMP2, MMP2, and MMP9 in nodule and cord tissue from a larger group of patients with Dupuytren's disease and compared this with normal palmar fascia taken at carpal tunnel release.

Materials and methods

Patients

The study included 30 patients (23 men and seven women; average age 61.3 ± 9.5 years) with Dupuytren's disease. The duration of the disease ranged from 9 months to 24 years. Treatment consisted of regional fasciectomy with complete excision of the diseased fascia in the palm and digits ($n = 17$) or radical fasciectomy for patients with extensive disease ($n = 13$). All surgery was performed at the Aachen University Hospital under approval from the local research ethics committee. Informed consent was obtained from all subjects. Tissue from patients with Dupuytren's disease was taken at fasciectomy. Samples were divided into regions of nodule and cord according to gross morphology. Normal fascia was taken from patients without Dupuytren's disease who had carpal tunnel release ($n = 30$; 14 men and 16 women; average age 63 ± 11 years). Tissue samples were dissected into approximately 5-mm pieces and snap frozen in liquid nitrogen immediately after surgical removal.

RNA extraction

For RNA extraction, samples were minced with a Polytron®PT 1200 (Kinematica AG, Swiss) in peqGold RNA-Pure™ (peqlab, Germany). Lysates were incubated at room temperature and then centrifuged at 8,000*g*. After

addition of two times chloroform to the supernatants followed by centrifugation at 8,000*g*, the upper phases were incubated over night with isopropanol at −20°C. The lysates were washed three times with 80% ethanol and eluated in RNAse-free water. RNAs were quantified by spectrophotometer at OD of 260 nm. A 260/280 ratio ≥1.8 was considered high purity.

Reverse transcription

Reverse transcription was performed using Gibco BRL Reverse Transcription kit (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Reactions were carried out in 20 µL volumes consisting of 1× buffer, 200 U of Moloney-murine leukemia virus reverse transcriptase, 40 U/µL RNAse inhibitor (Roche Molecular Biochemicals, Indianapolis, IN), 5 µmol/L random hexamers (Life Technologies) and 5 µL total RNA. Reaction were incubated in a PCR thermocycler at 25°C for 10 min, 37°C for 50 min, and 70°C for 15 min and then cooled to 4°C. After reverse transcriptase, samples were diluted by adding purified water.

Real-time polymerase chain reaction

For real-time quantitative RT-PCR, the ABI 7700 Prism (PE Biosystems) was used. Quantitative TaqMan PCR analysis was performed for MMP2, MMP9, TIMP1, TIMP2, and GAPDH. Primers and probes were synthesized by assay on demand products (850 Lincoln Centre Drive Foster City, CA 94404 USA): MMP2 (HS 00234422), MMP9 (HS 00234579), TIMP1 (HS 00171558), TIMP2 (HS 00234278), GAPDH (HS 99999905).

RT-PCR was performed in triplicate 20 µL reaction volumes consisting 10 µL TaqMan Universal PCR Master Mix (PE Biosystems), 1 µL 20× Assays-on Demand Gene Expression Assay Mix, and 9 µL 25 ng cDNA sample diluted in RNAse free water. Two steps PCR cycling was carried out as follows: 95°C for 12 min (one cycle), 95°C for 15 s, and 60°C for 1 min (40 cycles). At the end of the PCR, baselines and threshold values were established using ABI 7700 Prism software, and the Ct values were exported to Microsoft Excel (Microsoft Corp., Redmond, WA) for analysis. Expression of mRNA was calculated using a relative quantification method (Pfaffl), which determines the relative quantification of a target gene in comparison to a reference gene. Analysis was carried out using the sequence detection software supplied with the ABI 7700 (PE Biosystems). This software calculates the Ct for each reaction and uses it to quantify the amount of starting template in the reaction. The relative expression ratio (R) of a target gene is calculated based on E and the Ct deviation of an unknown sample versus a control, and expressed in comparison to a reference gene. PCR efficiencies were calculated from the given slopes in TaqMan software. The corresponding real-time PCR efficiency (E) of one cycle in the exponential phase was calculated according to the equation: $E = 10(-1/\text{slope})$.

E_{target} is the real-time PCR efficiency of target gene transcript; E_{ref} is the real-time PCR efficiency of a reference gene transcript; $\Delta\text{Ct}_{\text{target}}$ is the Ct deviation of control-sample of the target gene transcript; $\Delta\text{Ct}_{\text{ref}}$ is the Ct deviation of control-sample of reference gene transcript. For the calculation of R, the individual real-time PCR efficiencies and the Ct deviation (ΔCt) of the investigated transcript must be known.

$$\text{Ratio}(R) = \frac{(E_{\text{target}})^{\Delta\text{ct}_{\text{target}}(\text{control}-\text{sample})}}{(E_{\text{ref}})^{\Delta\text{ct}_{\text{ref}}(\text{control}-\text{sample})}}$$

Statistical analysis

Data were expressed as mean value and SD. Statistical analysis was performed using the Mann–Whitney test. The level of significance was considered to be $P < 0.05$.

Results

Relative expression of MMP2

Figure 1 shows the results for the relative expression of MMP2 in nodules and cord tissue from patients with Dupuytren's disease and from patients with normal palmar fascia. The expression was significantly upregulated in tissue of patients with Dupuytren's contracture in comparison to normal tissue ($P < 0.05$). The expression of MMP2 was significantly higher in nodules in comparison to cord tissue ($P < 0.05$).

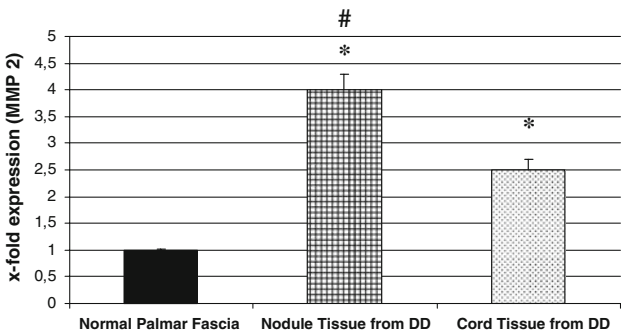

Fig. 1 Relative expression of MMP2 in nodule and cord tissue of patients with Dupuytren's disease in comparison to normal palmar fascia. * $P < 0.05$ versus normal palmar fascia; # $P < 0.05$ versus cord tissue

Relative expression of MMP9

There was no significant difference in the relative expression of MMP9 in nodules and cord tissue of patients with Dupuytren's contracture in comparison to normal fascia from patients with carpal tunnel syndrome (Figs. 2, 3).

Relative expression of TIMP1

Figure 3 shows the relative expression of TIMP1 in different tissues. In comparison to normal fascia, the cords and nodules from patients with Dupuytren's disease showed significant upregulation for TIMP1 ($P < 0.05$). The expression was significantly higher in nodules in comparison to cord tissue ($P < 0.05$).

Relative expression of TIMP2

The relative expression of TIMP2 was significantly higher in nodules and cord tissue of patients with Dupuytren's disease in comparison to normal palmar fascia ($P < 0.05$; Fig. 4). There was no remarkable difference in the expression of TIMP2 between cords and nodules.

Discussion

The integrity of the ECM is controlled by a simple balanced equation of synthesis and degradation of ECM components [30]. This phenomenon is tightly coupled with functioning of the extracellular proteolytic system, which includes the activity of matrix metalloproteinases and their inhibitors [48]. Normal ECM turnover can be viewed as a balance between proteinase and inhibitor activities, with fibrosis coming from an imbalance away from proteolysis [21, 22, 26]. Whereas patients with chronic wounds present a lack of TIMPs with an uncontrolled activity of MMPs [36], an elevated systemic TIMP-1 concentration can be observed in severe fibroproliferative disorders [42, 43].

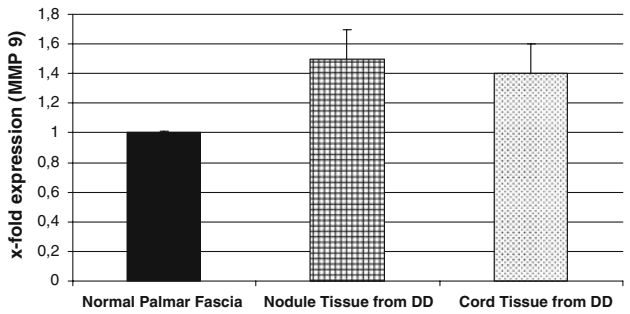

Fig. 2 Relative expression of MMP9 in nodule and cord tissue of patients with Dupuytren's disease in comparison to normal palmar fascia

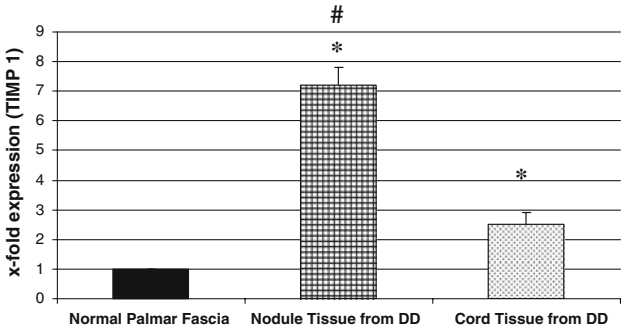

Fig. 3 Relative expression of TIMP1 in nodule and cord tissue of patients with Dupuytren's disease in comparison to normal palmar fascia. * $P < 0.05$ versus normal palmar fascia; # $P < 0.05$ versus cord tissue

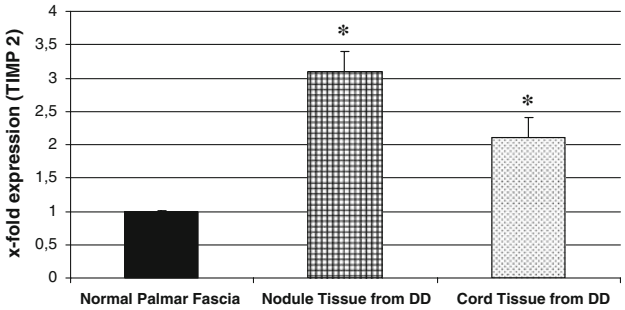

Fig. 4 Relative expression of TIMP2 in nodule and cord tissue of patients with Dupuytren's disease in comparison to normal palmar fascia. * $P < 0.05$ versus normal palmar fascia

Several studies indicate that the disturbance between MMPs and TIMPs in patients with Dupuytren's disease might contribute to palmar fibromatosis. Nevertheless, the involvement of TIMP-2 and MMP-2 is discussed controversially. In a series of 12 patients with advanced inoperable gastric carcinoma who had treatment with the synthetic MMP inhibitor Marimastat (British Biotech Ltd., Oxford, UK), six patients developed a frozen shoulder or a condition resembling Dupuytren's disease [16]. The authors postulated that the development of the frozen shoulder and the Dupuytren-like condition in their patients was caused by the decrease in the systemic MMP-to-TIMP ratio, which might cause increased synthesis and deposition of collagen and connective tissue.

Recently, we have determined serum concentrations of MMP-1, MMP-2, MMP-9, TIMP-1, and TIMP-2 in 22 patients with Dupuytren's disease using an enzyme-linked immunosorbent assay [41]. Tissue samples were obtained for immunohistochemistry. Patients with Dupuytren's contracture presented a significantly elevated TIMP-1 concentration in their sera in comparison to control patients. Patients with a proliferative active disease had a significantly higher TIMP-1 concentration than patients with a disease in the late involutional and residual phase.

There were no significant differences in the TIMP-2, MMP-1, MMP-2, and MMP-9 serum concentration between patients with palmar fibromatosis and the control group. Patients with Dupuytren's disease presented a significantly lower MMP-to-TIMP ratio than patients with normal palmar fascia. Patients with an active palmar fibromatosis presented a significantly reduced ratio compared with those in later phases. TIMP-1 and TIMP-2 could be detected in tissue of patients with Dupuytren's contracture, with an accumulation in nodules. MMP-2 could be detected locally in Dupuytren's tissue in a few patients, with less positive staining than that for TIMPs.

Tarlton et al. [40] were able to demonstrate an increased expression of MMP2 and MMP9 in Dupuytren's tissue in response to mechanical load.

Augoff et al. [2] determined the level of MMP-2 activation in the palmar fascia of patients with Dupuytren's disease with reference to the clinical stages of disease progression and recurrence of the contracture after surgery. They showed that regardless of the clinical stage of Dupuytren's disease, the activation ratios of MMP-2 remain significantly elevated even in the terminal phase of fibrosis when the cellular structure of the fascia returns to the state observed in the normal palmar aponeurosis. In this context, the activity of MMP-2 seems to be dependent on factors of nonmyofibroblastic origin. The authors were not able to find a correlation between high level of MMP-2 activation and the recurrence in the area of surgically treated Dupuytren's contracture. In their study, TIMPs were not analyzed.

First microarray studies of diseased palmar fascia from six patients were described by Pan et al. [27]. The authors found 23 genes with levels that differed consistently from control levels. Qian et al. [29] also performed microarray analysis and compared the gene expression profiles between Peyronie's disease (PD) and Dupuytren's contracture. In nine patients with Dupuytren's disease, 16 genes were upregulated and 3 genes were downregulated in at least 4 of the patients. Of the genes upregulated in Peyronie's and Dupuytren's disease, the ones most prominently increased were MMPs, specifically MMP-2 in all the nodules and either MMP-2 or MMP-9 in one half of the PD plaques.

Johnston et al. [18] described in a study, concerning the expression profile of matrix-degrading metalloproteinases in patients with Dupuytren's disease, a higher expression for TIMPs in comparison with proteinase genes. In their study, an upregulation of MMP2 and TIMP1 could be observed in nodules compared with normal palmar fascia. This was true in our study, too. Comparing the nodule with the cord, the expression for MMP2 was significantly higher in the nodule. There was no significant difference in the expression of MMP9 in the nodules, cords, and normal palmar fascia. TIMP2 was expressed at lower levels in the nodule than in the normal palmar fascia and the cord. This was different in our present study. We were able to demonstrate a TIMP2 upregulation in the tissue of 30 patients with Dupuytren's disease in comparison to physiological palmar fascia.

Satish et al. [34] compared the gene expression profiles of fibroblasts isolated from Dupuytren's contracture patients and controls using two different microarray platforms (GE Code Link^{TM} and Illumina^{TM}). They identified 69 and 40 differentially regulated gene transcripts using the GE Code Link^{TM} and Illumina^{TM} platforms, respectively. In their study, they did not identify MMPs and TIMPs. In their opinion, this might be due to differences between tissues and cultured cells expressing a solitary phenotype. In our opinion their results might be due to the analysis of candidate genes from cord-derived and not from nodule-derived fibroblasts.

The data from our present study indicate that the balance between MMPs and their natural inhibitors is disturbed in patients with Dupuytren's disease. The decrease in MMP-to-TIMP expression can cause increased synthesis and deposition of collagen, leading to palmar fibromatosis. Remarkable changes in gene expression were the ones related to defense mechanisms against fibrosis. We were not able to demonstrate these effects in a recent study concerning the concentration of MMPs and TIMPs in the sera of patients with Dupuytren's disease [41]. Using immunohistochemistry, a positive staining for MMP-2 could be observed in proliferative active areas. Our actual observations confirm that the nodules of patients with Dupuytren's disease are in active remodeling and not in terminal involution. The high expression of MMP-2 may represent an unsuccessful attempt to reduce collagen deposition, owing to resistance either by cross-links caused by advanced glycation end products [13] or by inhibition of MMP activity through accumulation of MMP inhibitors [31]. It is also possible that MMP-2 is a potent promotor of fibrosis [18]. Its activation may have an important effect on the regulation of profibrotic transforming growth factor-β_1 (TGF-β_1), which can be found at high levels in all stages of Dupuytren's disease [3, 7]. TGF-β_1 is secreted and maintained in a latent complex with a small proteoglycan, decorin. This complex functions as a reservoir of TGF-β_1 in the extracellular matrix [17]. Decorin is susceptible to degradation by MMP-2. Thus, MMP-2 might release TGF-β_1 from the decorin–TGF-β_1 complex and might play a key role in the control of TGF-β_1 activation and fibrosis promotion [2, 18].

Currently, surgery still remains the mainstay of treatment. Operative techniques include partial or total fasciectomy. Hoegemann et al. [14] performed a retrospective study with 61 patients with Dupuytren's disease. They suggest that total aponeurectomy is a promising

alternative to partial fasciectomy with low risk for a recurrence and slightly increased risk for nerve lesion. It is a common practice that operation in patients with first degree affection and exclusive nodules in the palm are only operated if the nodules are a cause of pain [46].

One of the most encouraging results in the conservative management of Dupuytren's disease has been with collagenases enzymatic fasciotomy. Starkweather et al. [38] showed that clostridial collagenases injection into cords may lead to a decrease of their elasticity by 93%. In 1999, Hurst and Badalamente [15] described enzymatic fasciotomy using collagenases injection. One year later, they analyzed the clinical safety and efficacy of clostridial collagenases injection as a nonsurgical treatment of Dupuytren's disease in a phase II open-label trial [5]. Twenty-nine patients had collagenases injections at a dose level of 10,000 U into a cord, causing contractures of 34 MCP joints, 9 PIP joints, and 1 thumb. Ultrasonographic imaging was used to guide the injection into the cord. After the injection, the hand was manipulated, often rupturing the cord. Twenty-eight of the 34 MCP joint contractures corrected to normal extension (0°). Two of the 34 MCP joint contractures corrected to 5° of normal extension. In patients with PIP joint contractures, 4 of the 9 joints corrected to normal (0°). In 2007, the authors performed a randomized double-blind, placebo-controlled trial [6]. Sixteen of twenty-three patients with Dupuytren's disease showed clinical success with contracture correction to within 5° of normal after one injection. After three injections, clinical success could be achieved in 21 of 23 patients. No placebo-treated patients had joint correction. Altogether, the injection appeared to have merit as nonsurgical treatment of Dupuytren's disease.

In the future, a treatment that not only downregulates TIMPs but also increases the activity of MMPs may be an appropriate therapy for Dupuytren's disease. The use of relaxin would be one possibility. It is an insulin-like growth factor hormone reported to decrease collagen expression, increase MMP, and decrease TIMP expression in a variety of in vitro and in vivo models [44, 45].

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