A Complete Expression Profile of Matrix-Degrading Metalloproteinases in Dupuytren's Disease

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Purpose: Dupuytren's disease (DD) is a common fibrotic condition of the palmar fascia, leading to deposition of collagen-rich cords and finger contractions. The metzincin superfamily contains key enzymes in the turnover of collagen and other extracellular matrix macromolecules. A number of broad-spectrum matrix metalloproteinase inhibitors, used in cancer clinical trials, caused side effects of DD-like contractures. We tested the hypothesis that changes in the expression of specific metalloproteinases underlie or contribute to the fibrosis and contracture seen in DD.

Methods: We collected tissue from patients with DD and used normal palmar fascia as a control. We profiled the expression of the entire matrix metalloproteinase (*MMP*), tissue inhibitor of metalloproteinases (*TIMP*), and a disintegrin and metalloproteinase domain with thrombospondin motif (*ADAMTS*) gene families in these tissues using real-time reverse-transcription polymerase chain reaction.

Results: A number of metalloproteinases and inhibitors are regulated in DD. The expression of 3 key collagenases, *MMP1*, *MMP13*, and *MMP14* is increased significantly in the DD nodule, as is the expression of the collagen biosynthetic enzyme *ADAMTS14*. The expression of *MMP7*, an enzyme with broad substrate specificity, is increased in the DD nodule and remains equally expressed in the DD cord. *TIMP1* expression is increased significantly in DD nodule compared with normal palmar fascia.

Conclusions: This study measured the expression of all *MMP*, *ADAMTS*, and *TIMP* genes in DD. Contraction and fibrosis may result from: (1) increased collagen biosynthesis mediated by increased ADAMTS-14; (2) an increased level of TIMP-1 blocking MMP-1– and MMP-13–mediated collagenolysis; and (3) contraction enabled by MMP-14–mediated pericellular collagenolysis (and potentially MMP-7), which may escape inhibition by TIMP-1. The complete expression profile will provide a knowledge-based approach to novel therapeutics targeting these genes. (J Hand Surg 2007;32A:343–351. Copyright © 2007 by the American Society for Surgery of the Hand.)

Key words: ADAMTS, Dupuytren's disease, gene expression, MMP, TIMP.

Dupuytren's disease (DD) is a common condition with 4% to 6% of Caucasian populations having evidence of disease,[1] and affecting up to 20% of men older than 65 years of age.[2] Clinically it is characterized by fibrosis of the palmar fascia; progression and potentially onset of the disease involves the formation and proliferation of myofibroblasts. Three distinct histologic phases have been described,[3] 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. Thus, the nodules are thought to represent an active phase of disease with myofibroblast proliferation, whereas the cords represent late-stage disease and the absence of myofibroblasts.

The matrix metalloproteinases (MMPs) are a family of 23 enzymes in human beings that include the only mammalian enzymes able to degrade the collagen triple helix in a specific manner under physiologic conditions.[4] The classic collagenases are MMP-1, -8, and -13 of the human enzymes. More recently, MMP-2 (gelatinase A) and MMP-14 (MT1-MMP) also have been shown to make this specific cleavage, although with less catalytic efficiency.[5–7] Other MMPs also have been implicated in collagen turnover (eg, MMP-3, by virtue of its ability to activate the pro-enzyme form of MMP-1).[8] A related family of metalloproteinases, a disintegrin and metalloproteinase domain with thrombospondin motif (ADAMTSs), have 19 members in human beings and also are implicated in extracellular matrix (ECM) metabolism. These include enzymes capable of degrading the proteoglycan, aggrecan (at least ADAMTS-1, -4, -5, -8, -9, and -15), and 3 procollagen N-propeptidases (ADAMTS-2, -3, and -14). Many other members of this family are of unknown function.[9] A family of 4 specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), have been described.[10] Although the ability of the 4 TIMPs to inhibit MMPs is largely promiscuous, a number of functional differences have been noted; for example, TIMP-2, -3, and -4, but not TIMP-1, are effective inhibitors of the membrane-type metalloproteinase (MT-MMP) subclass. Specificity among the TIMPs for inhibition of the ADAMTS family of metalloproteinases also has been described, with TIMP-3 being a potent inhibitor of ADAMTS-4 (aggrecanase-1) and ADAMTS-5 (aggrecanase-2).[11] In many fibrotic diseases such as those affecting the liver, lung, and skin, MMPs (and related metalloproteinases) and TIMPs play an important role. Normal ECM turnover can be viewed as a balance between proteinase and inhibitor activities, with fibrosis coming from an imbalance away from proteolysis.[4]

In the 1980s and 1990s, several small molecule inhibitors of MMPs were involved in clinical trials in a variety of cancers.[12] The major side effect of these drugs was a so-called *musculoskeletal syndrome*, often referred to as musculoskeletal pain accompanied by tendonitis.[13,14] This was dose- and time-dependent and reversible on treatment discontinuation, but did not respond well to nonsteroidal anti-inflammatory drugs or low-dose steroid treatment. The clinical presentation, when reported in detail, is described as frozen shoulder or a condition resembling DD.[15] Both of these conditions involve similar fibrotic mechanisms (of the shoulder joint capsule in the case of frozen shoulder), the laying down of a collagen-rich ECM, and the involvement of myofibroblast-mediated contraction.[16,17] Although DD and frozen shoulder have different natural histories (the former a progressive disease, the latter usually self-limiting and resolving in time), they may well share common pathways leading to contracture.[16] The MMP inhibitors that cause the musculoskeletal syndrome are pan-MMP inhibitors, showing an approximately nanomolar (or lower) inhibition constant against many of the MMPs tested. Moreover, there is good evidence that they also may inhibit related metalloproteinases (eg, ADAMTSs). Indeed, the musculoskeletal syndrome usually is ascribed to the inhibition of nontarget metalloproteinases.

The measurement of a small subset of MMPs and TIMPs (MMP-1, -2, -9, TIMP-1 and -2) in the sera of patients with DD, compared with patients who had carpal tunnel release, lends some support to the tenet that expression of these enzymes is altered in DD.[18] Patients with DD had significantly higher serum TIMP-1 levels and the investigators[18] suggested that there is a systemic change in the MMP-to-TIMP ratio that may lead to increased collagen deposition. A microarray analysis of gene expression in DD and Peyronie's disease, compared with normal palmar fascia and tunica albuginea, respectively, showed upregulation of *MMP2* and *MMP9* in diseased tissue.[19] A reverse-transcription polymerase chain reaction (PCR)–based comparison of Dupuytren's tissue with frozen shoulder or control shoulder tissues showed expression of *MMP1*, *MMP2*, *MMP9*, and *TIMP1* in all tissues, with *MMP3* absent from DD tissue and *MMP14* increased compared with shoulder tissue.[20] An earlier report also showed an increased expression of MMP-2 and MMP-9 in Dupuytren's tissue in response to mechanical load.[21] None of these studies were able to look at all members of the MMP, TIMP, and related metalloproteinase gene families.

From these data we proposed the hypothesis that MMPs, ADAMTSs, and TIMPs may play a key role in the onset or progression of DD and related disorders. Hence, we used a quantitative reverse-transcription PCR methodology to profile the expression of all members of the MMP, ADAMTS, and TIMP families in nodule and cord tissue from DD patients and compared this with normal palmar fascia taken at carpal tunnel release.

Materials and Methods

Tissue Samples

All surgery was performed at the Norfolk and Norwich University Hospital under approval from the local research ethics committee and all patients gave informed consent. Tissue from patients with DD was taken at fasciectomy (n = 20; age range, 42–83 y; 3 women, 20 men). Samples were divided into regions of nodule and cord according to gross morphology. Normal palmar fascia was taken from patients without DD who had carpal tunnel release (n = 20; age range, 25–84 y; 17 women, 3 men). Tissue was dissected into approximately 5-mm pieces and snap frozen in liquid nitrogen within 15 to 30 minutes of surgery.

RNA Extraction

Tissue was weighed and homogenized in reagent (TRIzol; Invitrogen Paisley, UK) (1 mL/0.1 g tissue) using an homogenizer (UltraTurrax, IKA, Staufen, Germany). Particulates were pelleted at 10,000 *g* for 10 minutes at 4°C and the supernatant was recovered. A total of 600 μL chloroform was added per 1 mL TRIzol, vortexed for 15 seconds, and incubated at room temperature for 10 minutes. The TRIzol/chloroform solution was centrifuged at 10,000 *g* for 15 minutes at 4°C and the aqueous layer was placed into a fresh tube. A total of 0.5 × volume of 100% ethanol was added and mixed. Samples were applied to spin columns (RNeasy Mini Kit; Qiagen, Crawley, West Sussex, UK), centrifuged at full speed for 15 seconds, and the flow through was discarded as described by Price et al.[22] Columns then were washed and eluted according to the manufacturer's instructions. RNA samples were quantified using a spectrophotometer (NanoDrop; NanoDrop Technologies, Wilmington, DE) and stored at −80°C.

Synthesis of Complementary DNA

Complementary DNA was synthesized from 1 μg of total RNA using reverse transcriptase (Superscript II, Invitrogen) and random hexamers in a total volume of 20 μL according to the manufacturer's instructions. Complementary DNA was stored at −20°C until used in downstream PCR.

Quantitative Real-Time Polymerase Chain Reaction

Oligonucleotide primers and fluorescent-labeled probes were designed (Primer Express 1.0 software; Applied Biosystems, Warrington, UK). Sequences for *MMP* and *TIMP* primers and probes were as described by Nuttall et al[23] and *ADAMTS* primers and probes were as described by Porter et al.[24] To control against amplification of genomic DNA, primers were placed within different exons close to an intron/exon boundary, with the probe spanning 2 neighboring exons where possible. Basic Local Alignment Search Tool (BLAST) searches for all the primer and probe sequences also were conducted to ensure gene specificity. The 18S ribosomal RNA (rRNA) gene was used as an endogenous control to normalize for differences in the amount of total RNA present in each sample; 18S rRNA primers and probe were purchased from Applied Biosystems.

The relative quantification of genes was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems) in accordance with the manufacturer's protocol. Polymerase chain reactions contained 5 ng of reverse-transcribed RNA (1 ng for 18S analyses), 50% 2X Master Mix (Tagman 2X Master Mix, Applied Biosystems), 100 nmol/L of each primer, and 200 nmol/L of probe in a total volume of 25 μL. Conditions for the PCR reaction were 2 minutes at 50°C, 10 minutes at 95°C, then 40 cycles each consisting of 15 seconds at 95°C and 1 minute at 60°C.

The threshold cycle (C_T), the cycle number at which the signal is detectable above the baseline, was transformed in 2 ways. To gain an approximate comparison across all genes measured, the amplification efficiency was assumed to be identical across all primer sets and normalized to 18S expression using a transformation proportional to normalized copy number ($2^{-\Delta C_T}$), where ΔC_T is C_T (target gene) - C_T (18S). When comparing the expression of a single gene across sample groups, standard curves for each gene were generated using the complementary DNA (cDNA) from one sample and making 2-fold serial dilutions across an appropriate range. Relative input amounts of template cDNA then were calculated from C_T using the standard curves; data are presented as relative levels of messenger RNA (mRNA) normalized to 18S rRNA. As a final quality control for the purified RNA samples, only those cDNAs within ±1.5 C_T of the median value for 18S for all samples were used in the downstream study.

To ascertain that the amplification product was indeed that of the desired target gene, products were subcloned and sequenced. All primer and probe sets have been shown to amplify specific products from appropriate human tissue samples.[23,24]

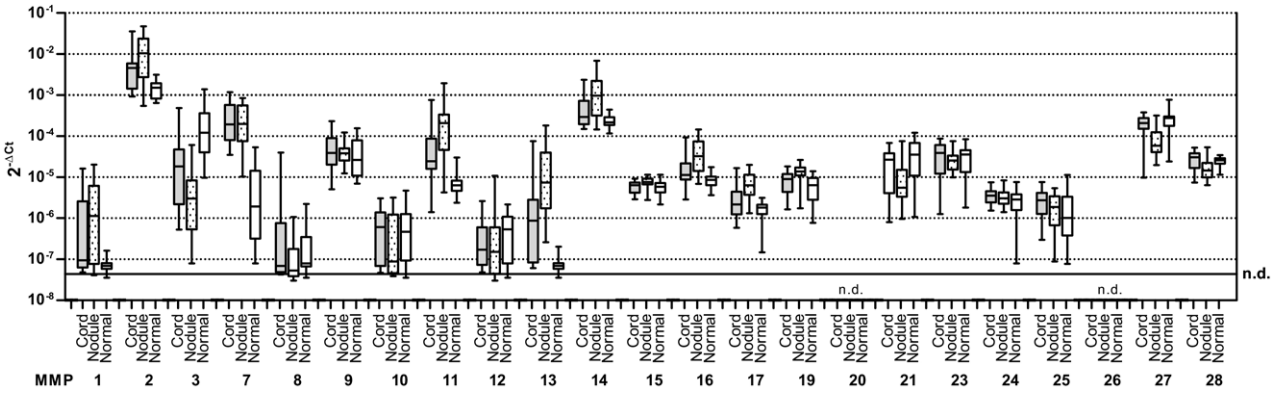

Figure 1. Comparative expression of *MMP* genes in normal palmar fascia versus cord and nodule tissue from DD. The expression level of each gene was determined as described in the Materials and Methods section and normalized to the level of *18S rRNA* gene expression using ΔC_T (C_T [target gene] - C_T [18S]). Open boxes, normal; filled boxes, cord; dotted boxes, nodule. The box-and-whisker plot shows the median, and each section represents a quartile of the data. N.D., not detected.

Statistical Analysis

Analyses were performed on 19 samples from each group because 1 sample in each group failed quality control as detailed previously. Differences between the 3 groups were defined using a Kruskal-Wallis test or pair-wise comparison using a 2-sided Mann-Whitney U test (in GraphPad Prism 4; GraphPad Software Inc, San Diego, CA); these nonparametric tests make no prior assumption on the data distribution. To account for differences in age and gender, standardized expression data using the standard curve method were subjected to a logarithmic transformation to approximate normalization and tested by analysis of covariance (ANCOVA; SPSS for Windows; SPSS Inc, Chicago, IL). Further analysis of correlation was performed using the Spearman rank correlation (SPSS for Windows).

Results

Examining the overall pattern of gene expression shown in Figures 1 to 3, a striking feature is that the level of *TIMP* gene expression is higher than that of the proteinase genes. Broadly, the level of steady-state mRNA across the 3 gene families assayed is *TIMP* > *ADAMTS* > *MMP*. Table 1 shows the differences in gene expression between the DD nodule and cord tissue compared with normal palmar fascia.

Although DD obviously involves the laying down of excess collagenous matrix, this matrix is presumably also remodeled and shortened during disease

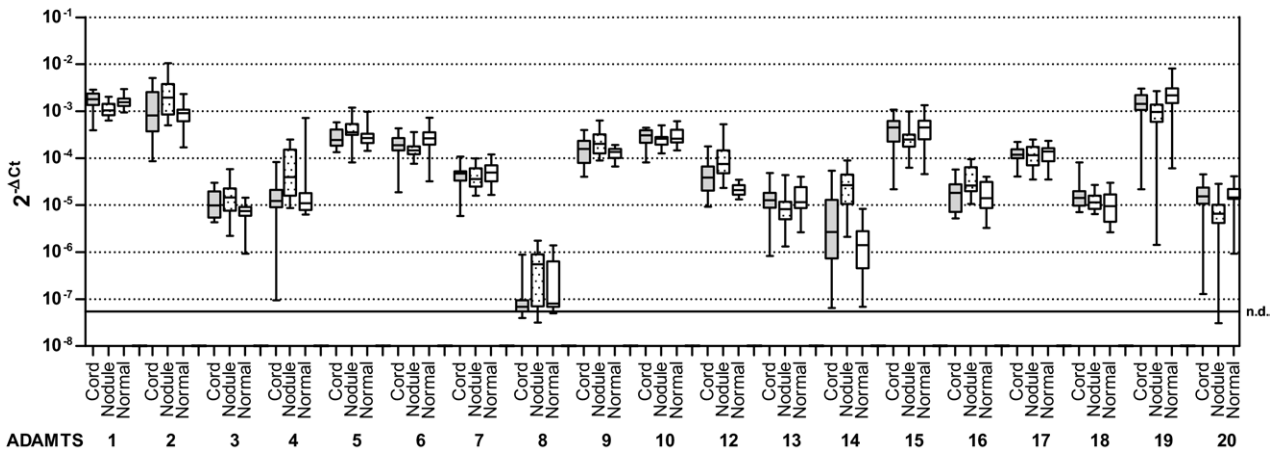

Figure 2. Comparative expression of *ADAMTS* genes in normal palmar fascia versus cord and nodule tissue from DD. The expression level of each gene was determined as described in the Materials and Methods section and normalized to the level of *18S rRNA* gene expression using ΔC_T (C_T [target gene] - C_T [18S]). Open boxes, normal; filled boxes, cord; dotted boxes, nodule. The box-and-whisker plot shows the median, and each section represents a quartile of the data. N.D., not detected.

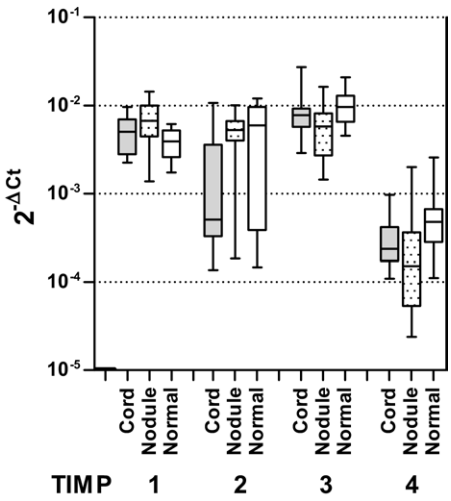

Figure 3. Comparative expression of *TIMP* genes in normal palmar fascia versus cord and nodule tissue from DD. The expression level of each gene was determined as described in the Materials and Methods section and normalized to the level of *18S rRNA* gene expression using ΔC_T (C_T [target gene] - C_T [18S]). Open boxes, normal; filled boxes, cord; dotted boxes, nodule. The box-and-whisker plot shows the median, and each section represents a quartile of the data.

progression either as a consequence of tension or contributing to increased tension. Thus, the expression of 3 procollagen N-propeptidases, *ADAMTS2*, *ADAMTS3*, and particularly *ADAMTS14*, is increased in the DD nodule, consistent with an increase in collagen synthesis. Conversely, the expression of several enzymes capable of mediating collagen breakdown, *MMP1*, *MMP2*, *MMP13*, and *MMP14*, also is increased in the DD nodule compared with normal palmar fascia, consistent with increased collagen turnover. The increase of all of the earlier-described enzymes is to some extent attenuated in the DD cord.

Comparing the DD nodule with the cord, it is clear that many genes that are induced in the nodule are at least less induced in the cord, with many not significantly different in the cord compared with normal palmar fascia. The only gene whose expression is increased in the DD nodule and remains increased to a similar level in the DD cord is *MMP7*; MMP-7 has a broad substrate specificity,[25] and this may represent a continued matrix remodeling process in the DD cord (see later).

The most significantly downregulated gene in the DD nodule is *MMP3*. This appears to be a recurring theme in diseases involving matrix destruction because similar findings have been reported in osteoarthritic cartilage[26] and degenerative tendinopathy.[27]

Six ADAMTS enzymes have been shown to be aggrecanases, at least *in vitro*, ADAMTS-1, ADAMTS-4, ADAMTS-5, ADAMTS-8, ADAMTS-9, and ADAMTS-15. *ADAMTS15* is expressed in normal palmar fascia, but not regulated in DD tissue. *ADAMTS4*, *ADAMTS5*, and *ADAMTS9* all are expressed in normal palmar fascia and increased in the DD nodule, but not the cord. *ADAMTS1* is expressed robustly in normal palmar fascia and expression is lower in the DD nodule. *ADAMTS8* is undetectable in most tissue samples.

Of the *TIMPs*, *TIMP1* expression is increased in the DD nodule, but *TIMP2*, *TIMP3*, and *TIMP4* all are expressed at lower levels in the DD nodule than in normal palmar fascia.

Analysis of covariance showed that there is a relationship between the patient's age and the level of gene expression for *MMP3*, *MMP16*, *MMP17*, *MMP25*, *TIMP1*, *TIMP2*, *ADAMTS1*, *ADAMTS5*, and *ADAMTS18*. Analysis of covariance also showed a weak relationship between gender and expression of *MMP16*, *TIMP2*, *ADAMTS3*, and *ADAMTS5*. The statistically significant differences in gene expression for these genes between groups (detailed previously), however, are paralleled in this analysis in all cases apart from *ADAMTS18*. The expression of *ADAMTS18* shows no statistical difference between groups under ANCOVA analysis but shows weak significance when analyzed by either Kruskal-Wallis or in pair-wise analysis using the Mann-Whitney U test. For the expression of *MMP3*, *TIMP1*, and *TIMP2*, there is a strong correlation with age in normal palmar fascia (the former 2 decreasing with age, the latter increasing) that is lost in DD tissues. This shows that regulatory mechanisms for these genes are aberrant in disease. Table 2 shows a Spearman rank correlation analysis for the 9 genes showing an effect of age in ANCOVA. Figure 4 shows the correlation of *MMP3* expression with age in the normal palmar fascia.

Discussion

In screens of gene expression the need to account for multiple testing in the statistical analyses is problematic. The Bonferroni correction is often conservative, removing type I error (false positive) at the expense of type II error (false negative). This would limit the utility of gene expression studies in which the validity of any multiple testing procedure has yet to be ascertained.[28,29] In Table 1, we have not applied any correction for multiple testing, although at the 5% significance level ($p = .05$) a false-positive rate of 1 in 20 would be expected.

Of prime importance to the phenotypic outcome of these changes in gene expression is the final balance

Table 1. Comparison of Gene Expression Between Normal Palmar Fascia, Cord, and Nodule Tissue From DD

	± p value					
Gene	Normal versus nodule (+, higher in nodule)		Normal versus cord (+, higher in cord)		Nodule versus cord (+, higher in cord)	
MMP1	+	.0009		NS		NS
MMP2	+	<.0001	+	.0043	-	.0043
MMP3	-	<.0001	-	.0002	+	.0091
MMP7	+	<.0001	+	<.0001		NS
MMP8		NS		NS	+	.044
MMP9		NS		NS		NS
MMP10		NS		NS		NS
MMP11	+	<.0001	+	<.0001	-	.004
MMP12		NS		NS		NS
MMP13	+	<.0001	+	.0003	-	.0012
MMP14	+	<.0001		NS	–	.0061
MMP15	+	.0144		NS	-	.0098
MMP16	+	<.0001	+	.036	–	.004
MMP17	+	<.0001		NS	-	<.0001
MMP19	+	<.0001		NS	-	.0028
MMP20		ND		ND		ND
MMP21	+	.0071		NS		NS
MMP23		NS		NS		NS
MMP24		NS		NS		NS
MMP25		NS		NS		NS
MMP26		ND		ND		ND
MMP27	-	.0003		NS	+	.002
MMP28		NS		NS	-	.04
ADAMTS1	-	.011		NS	+	.0043
ADAMTS2	+	.0012		NS	-	.012
ADAMTS3	+	.0034	+	.018		NS
ADAMTS4	+	.0024		NS	-	.0005
ADAMTS5	+	.0066		NS	-	.017
ADAMTS6	-	.0043		NS	+	.04
ADAMTS7		NS		NS		NS
ADAMTS8	-	.0056		NS	+	.0043
ADAMTS9	+	.0037		NS		NS
ADAMTS10		NS		NS		NS
ADAMTS12	+	<.0001	+	.0028	-	.002
ADAMTS13		NS		NS		NS
ADAMTS14	+	<.0001		NS	-	.0002
ADAMTS15		NS		NS	+	.036
ADAMTS16	+	.0223		NS	-	.0024
ADAMTS17		NS		NS		NS
ADAMTS18	+	.036	+	.0144		NS
ADAMTS19	-	.0056		NS	+	.021
ADAMTS20	-	.0034		NS	+	.0031
TIMP1	+	.0008		NS	-	.028
TIMP2	-	.0115		NS	+	.0005
TIMP3	-	.004		NS		NS
TIMP4	-	.004		NS	+	.03

Statistical analysis was performed pair-wise using the Mann-Whitney U test.
NS, not significant; ND, not detected.

of proteinase versus inhibitor. Although this is impossible to ascertain from a study at the steady-state mRNA level such as this, it is clear that *TIMP1* is the only *TIMP* whose expression is increased significantly in the DD nodule compared with normal palmar fascia. Indeed, *TIMP2*, *TIMP3*, and *TIMP4* all are reduced in the DD nodule. Thus, in the DD nodule, the situation may be similar to that described

in liver fibrosis, in which the potential for matrix degradation is present, even in advanced disease, but is prevented by TIMP expression.[30] Of potential importance here is the fact that TIMP-1 is a poor inhibitor of MMP-14, MMP-15, MMP-16, and MMP-24.[10] Because expression of *MMP14* and *MMP16* is significantly increased in the DD nodule, these proteinases might escape inhibition from TIMP-1. This also may provide a mechanism for collagen remodeling, particularly in the pericellular environment (by MMP-14, a membrane-bound enzyme) during contraction in the presence of TIMP-1. Similarly, MMP-7 (whose expression is increased equally in both the DD nodule and the cord), lacks a C-terminal hemopexin-like domain that is common to most MMPs, decreasing its affinity for at least TIMP-1,[31] and potentially resisting inhibition in pathology.

Several studies have compared the contractile properties of Dupuytren's cord- or nodule-derived fibroblasts with control cultures.[32–35] Results varied depending on the model of contraction used, but the consensus appears to be that Dupuytren's fibroblasts can generate significantly increased contractile force compared with control cells, with nodule-derived cells showing greatest contraction. In such models, broad-spectrum synthetic MMP inhibitors have been shown to inhibit contraction,[36,37] and this is paralleled by the inhibition of wound contraction in a mouse model of skin wound healing.[38] This latter study also reported a decrease in α-smooth muscle actin expression in the presence of the MMP inhibitor. This blockade of contraction is difficult to dovetail with the reported side effects of some of these MMP inhibitors in clinical trials in which Duputyren's-like contracture and frozen shoulder were observed[13–15]; however, this may reflect the fact that culture models of collagen lattice contraction only represent a single facet of a more complex disease process. Again, drawing on the analogy with liver fibrosis, a treatment that downregulates TIMPs but increases the activity of MMPs may be an appropriate therapy for DD.[30] One such possibility is relaxin, 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.[39–42]

It also may be possible to target collagen biosynthesis via blockade of ADAMTS-14. Functional overlap between the 3 ADAMTS procollagen-N-propeptidases (ADAMTS-2, -3, and -14) provides the potential to inhibit ADAMTS-14 without disruption of global collagen synthesis,[43] but this may be sufficient to slow or halt collagen deposition in DD.

The current study examines the expression of the entire *MMP*, *ADAMTS*, and *TIMP* families in DD. These enzymes and inhibitors have critical roles in extracellular matrix homeostasis, which is clearly dysregulated in DD. These data will allow future work to focus on the function of specific metalloproteinases in the disease process.

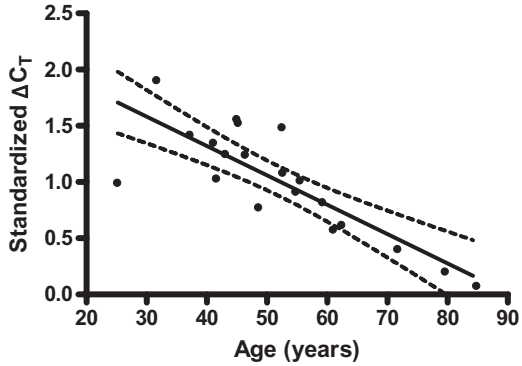

Figure 4. Correlation of *MMP3* expression with age in normal palmar fascia. The expression level of *MMP3* was determined using the standard curve method as described in the Materials and Methods section and normalized to the level of *18S rRNA* gene expression using an adjusted ΔC_T (C_T [target gene] - C_T [18S], allowing for efficiency of primer-probe sets). The solid line shows the best fit as determined by linear regression, with dotted lines defining 95% confidence limits.

Table 2. Correlation of Gene Expression With Age in Normal Palmar Fascia, Cord, or Nodule Tissue From DD

	p value (r value)		
Gene	Cord	Nodule	Normal
MMP3	.318	.273	<.001 (−.774)
MMP16	.204	.307	.063 (−.435)
MMP17	.322	.024 (−.516)	.616
MMP25	.323	.358	.115
TIMP1	.632	.557	.012 (−.565)
TIMP2	.280	.416	.013 (.558)
ADAMTS1	.051 (.454)	.263	.473
ADAMTS5	.627	.011 (−.567)	.743
ADAMTS18	.022 (−.523)	.468	.348

Genes shown are those identified by ANCOVA. Statistical analysis was performed using the Spearman rank correlation. The Spearman *r* value is shown where correlation is significant (positive value indicates an increase in expression with age, negative value indicates a decrease in expression with age).

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