Expression of a Novel Gene, MafB, in Dupuytren’s Disease

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Purpose: Dupuytren’s disease (DD) is characterized by fibroblastic proliferation of the palmar fascia, often leading to flexion contracture in the hand. Although there is a strong genetic component the genome-wide expression of novel genes is not known. The purpose of this study was to use DNA microarray technology to identify upregulated genes in DD.

Methods: Human tissue samples were harvested from 3 patient sources: DD cord tissue (n = 20), normal-appearing adjacent control fascia (n = 15), and palmar fascia from patients having carpal tunnel release (n = 15). DNA microarray analysis was performed on amplified sample RNA. Novel genes were compared with known gene functions. A candidate gene of interest was studied further by using immunohistochemistry on DD tissue samples and controls.

Results: Several novel genes not described previously in the study of DD were upregulated significantly, including MafB, collagen type V, α-2 (COL5A2), collagen type VIII, α-1 (COL8A1), contactin I (CNTN1), and leucine-rich repeat containing 17 (LRRC17). These upregulated genes were compared with their known gene-expression profiles in other tissues and their purported functions. MafB was found to be of particular interest because of its prominent role in tissue development and cellular differentiation. MafB immunohistochemistry showed positive staining in 50% of the DD specimens but complete absence of MafB in all control tissues (adjacent control fascia, carpal tunnel fascia). Co-localization experiments with MafB and α-smooth muscle actin showed staining properties in similar regions but these 2 proteins were not confined solely to the same cells.

Conclusions: Microarray analysis of DD tissue has identified significant upregulated gene expression of MafB. MafB protein also is found in Dupuytren’s cords but not in control fascia. Co-localization data suggest that the association of MafB with DD is not related exclusively to myofibroblast proliferation. Because of its role in fibroblastic transformation in other models MafB and its relationship to the pathogenesis of DD deserves further study. (J Hand Surg 2006;31A: 211–218. Copyright © 2006 by the American Society for Surgery of the Hand.)

Key words: Dupuytren’s, MafB, myofibroblast.

Dupuytren’s disease (DD) is characterized by nodular fibroblastic proliferation of the palmar fascia often leading to contracture of the hand, most frequently affecting the ring and small fingers. Progression of the disease and recurrence of palmar contracture and scar formation after treatment lead to notable disability. Typically DD affects men of Northern European heritage, with a peak incidence at around 50 years of age. Prevalence is highest in elderly men from Scotland, Norway, and Iceland and can be as high as 40%. There is a higher prevalence in men, with a male-to-female ratio of approximately 6 to 1. Dupuytren’s disease is a familial disorder with a strong genetic disposition and has been associated with several risk factors including alcoholism, diabetes, epilepsy, and smoking. The effects of these risk factors and the pathogenesis of DD remain unclear, however, with variable autosomal dominance being the most likely pattern of inheritance.

Currently standard therapy consists of surgical excision of abnormal fascia and joint contracture re-
lease as necessary. The risk for disease recurrence after surgery is great; thus, surgery is not a panacea. Therefore a better understanding of the molecular pathogenesis of DD may lead to the development of new therapeutic alternatives to surgery.

In 1972 Gabbiani and Majno identified a unique type of fibroblast, named myofibroblast, as the contractile force behind the formation of the disease nodules. Histologically the nodules have a high cell density with numerous myofibroblasts. These myofibroblasts express α-smooth muscle actin (α-SMA) and have phenotypic characteristics between fibroblasts and smooth muscle cells. Additional studies verified that a greater proportion of DD nodule cells tested positive for α-SMA compared with control cells. α-Smooth muscle actin is a component of a cellular apparatus through which intracellular force is generated and transmitted to the extracellular matrix, leading to contracture formation. The source of these myofibroblasts is not known.

Badalamente et al showed transforming growth factor–β1 staining in fibroblasts, myofibroblasts, and capillary endothelial cells and transforming growth factor–β2 in myofibroblasts in DD samples. In addition both isoforms had positive effects on DD myofibroblast proliferation. Other studies have identified fibronectin, α-SMA, and α-5 β-1 integrin as components of the cellular apparatus through which intracellular force is generated and transmitted to the extracellular matrix, leading to contracture formation. Laminin and tenascin C also were identified by immunohistochemistry as present in the proliferative DD nodules. Finally, other extracellular matrix proteins such as matrix metalloproteinases and tissue inhibitors of metalloproteinases have been identified as possible components of the disease process.

Beyond these studies little is known about the etiology and pathogenesis of DD. Expression patterns of genes important in DD are as yet unknown and the downstream targets of the growth factors and the cellular mechanisms that regulate disease progression are unexplained.

DNA microarray technology allows observation of upregulation or downregulation of up to 40,000 genes. This screening method is useful in finding novel genes that may be expressed differentially in disease. The main objective of our study was to identify novel genes upregulated in DD. These upregulated genes would be correlated with their known functions to focus on a novel gene candidate. Finally, this novel gene candidate would undergo tissue analysis by immunohistochemistry to confirm expression.

### Materials and Methods

#### Microarray Analysis: Patient Demographics and Tissue Collection

Men between the ages of 36 and 72 years with clinically diagnosed DD having elective surgical excision were enrolled in the study. The excised tissue was obtained under a protocol approved according to standard guidelines set by the institutional review boards of Stanford University and the Veterans Affairs Palo Alto Health Care System. Informed consent from each patient was obtained before enrollment. Additional men between the ages of 50 and 89 years having carpal tunnel release were enrolled as age-matched controls.

Palmar fasciectomy for the treatment of DD contracture involved removal of the diseased cords from the palmar fascia. Normally the excised tissue is discarded. The tissue consists of single or multiple cords of variable size localized in palmodigital and digital areas. As a control, normal-appearing palmar fascia adjacent to the diseased cord was excised.

As a second control normal palmar fascia was obtained from age- and gender-matched patients having elective carpal tunnel release. During carpal tunnel release a small amount of overlying normal palmar fascia was removed. Previous studies have established and validated the use of palmar fascial tissue from patients having carpal tunnel release as controls for studies of DD. For microarray analysis the tissue samples were processed from 3 different specimen groups: (1) DD tissue obtained from patients having elective DD fasciectomy (n = 4), (2) normal-appearing DD-adjacent control fascia from the same DD patients (n = 4), and (3) normal palmar fascia obtained from patients having carpal tunnel release (n = 3). After excision each tissue sample was divided, flash-frozen in liquid nitrogen, and stored at −80°C before RNA isolation.

#### Microarray Gene Profiling

Extraction of total RNA from tissue samples was accomplished with a reagent (Trizol; Life Technologies, Gaithersburg, MD) and further purified (RNeasy mini kit; Qiagen, Valencia, CA) according to the manufacturer’s protocols. Total RNA isolated from all tissue samples was amplified (MessageAmp aRNA kit; Ambion, Austin, TX) to obtain sufficient amounts of RNA for microarray experiments. RNA quality and quantity were measured by spectrophotometry. Each sample used in the microarray experiments achieved an absorbance ratio (A260/A280) in the range of 1.7 to 2.3. Amplified RNA was reverse-transcribed into complementary DNA (cDNA) (Superscript II reverse transcriptase; Life Technologies) and random hexamer (Amersham, Piscataway, NJ)
and microarray analysis was performed on cDNA chips (Stanford Functional Genomics Facility, Stanford, CA; www.microarray.org). Each array chip contained approximately 42,000 human cDNA elements, representing more than 30,000 unique genes. A type II experimental design was applied in which a universal human reference RNA (Stratagene, La Jolla, CA) served as the common reference probe in all hybridizations. In addition the universal human RNA was amplified and used to make cDNA probes labeled with Cy5 (Cyanine 5) (Amersham) and amplified RNA from tissue samples was used to make cDNA probes labeled with Cy3 (Cyanine 3). Probe hybridization was performed by adding 20 μg of yeast transfer RNA, 20 μg of polydeoxyadenylic acid, and 20 μg of human CoT1 DNA (Gibco-BRL, Gaithersburg, MD) to a solution containing 3-times-standard saline citrate, 0.3% sodium dodecyl sulfate, and labeled probes. This mixture was aliquoted onto the microarray chip and incubated for 18 hours at 65°C. Washes and scans were performed as described previously.17,18 After gridding, array data were uploaded to the Stanford Microarray Database (SMD) (http://genome-www5.stanford.edu/MicroArray/SMD) and analyzed.

Microarray Data Analysis

Online software from the SMD was used to analyze selected data points that met the following spot-quality criteria: spot regression correlation greater than 0.4 and “spot flag” and “failed” filters equal to 0. These user-selectable criteria were intended to exclude spots with nonuniform, dim, or otherwise unreliable signals. Relative changes in gene expression were evaluated by fold-change as determined from the log2 of the red-to-green normalized ratio reported by the SMD, as previously described.17 For a given gene a 4-fold change from the mean in at least 1 array was used as a cut-off level in our experiments (nonbiased analysis). Only genes and arrays with greater than 80% good data were included in the results. The gene-expression profile in terms of fold-change from the mean was compared using the Student t test. Results with a p value of less than .05 were considered significant. Genes and arrays also were clustered according to their expression patterns using SMD online software.19

Immunohistochemistry: Patient Demographics and Tissue Collection

Patients were enrolled using criteria similar to those described previously for the microarray experiments. Tissue samples were collected from 3 different specimen groups: (1) DD tissue obtained from patients having elective DD fasciectomy (n = 16), (2) nor-

mal-appearing DD-adjacent control fascia from the same DD patients (n = 10), and (3) normal palmar fascia obtained from patients having carpal tunnel release surgery (n = 9). After excision each tissue sample was flash-frozen in liquid nitrogen and stored at −80°C.

Histology

The frozen specimens were cut on a cryostat (Leica Microsystems GmbH, Germany) into 6-μm sections, adhered onto room-temperature glass slides, and fixed with acetone for 10 minutes. Hematoxylin-eosin staining was performed using standard techniques. Additional slides were stored for immunohistochemistry and co-localization staining with α-SMA as described later.

Immunohistochemistry

The slides first were rinsed with 3 changes of phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked by treatment of hydrogen peroxide (H2O2, 0.5%) in PBS for 10 minutes and then rinsed in 3 changes of PBS. To block nonspecific binding to antibody sites the sections were incubated with normal blocking serum (1.5% goat, Vectastain; Vector Laboratories, Burlingame, CA) for 1 hour. Sections then were incubated with MafB antibody and normal normal blocking serum (1:500, goat polyclonal; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4°C. Slides were rinsed in 3 changes of PBS and then incubated for 30 minutes with anti-goat immunoglobulin G (IgG) biotin-conjugated secondary antibody. After rinsing with 3 changes of PBS the slides were incubated with avidin biotin enzyme reagent (Vector) for 30 minutes and then rinsed again. The reaction product was visualized with diaminobenzidine (Vector), rinsed in distilled water, and counterstained with hematoxylin followed by an ammonia rinse and 5 changes of distilled water. Slides were dehydrated through alcohols (2 changes 70%, 2 changes 100%), allowed to dry, and then coverslipped (Permount; Fisher, Pittsburgh, PA).

Co-localization of α–Smooth Muscle Actin and MafB

By double-staining diseased cord tissue with both polyclonal MafB antibody and α-SMA we attempted to localize better the expression of MafB in DD. Our goal was to identify the cell type and location of MafB expression, possibly in myofibroblasts. Co-localization of MafB and α-SMA was performed in 2 steps. Sections first were stained for MafB and visualized with diaminobenzidine as described in the Immunohistochemistry section. Rather
than dehydrating them with alcohol we then incubated the sections in anti–human α-SMA antibody (mouse monoclonal IgG; R&D Systems, Inc., Minneapolis, MN) for 30 minutes at room temperature, then rinsed them in 3 changes of PBS followed by a 30-minute incubation with anti–goat IgG biotin-conjugated secondary antibody. After rinsing with 3 changes of PBS the slides were incubated with avidin biotin enzyme reagent (Vector) for 30 minutes and then rinsed again. Staining was visualized with VIP (Vector), producing a purple-red color that was compared with the brown diaminobenzidine MafB staining.

Results

Microarray Gene-Expression Profile of Novel Genes in Dupuytren’s Disease

Each microarray chip contained approximately 42,000 human cDNA elements, representing more than 30,000 unique genes. Cord tissue samples from DD (n = 4) were compared with normal-appearing adjacent control fascia in the same patient (n = 4) and control palmar fascia from patients who had carpal tunnel release (n = 3). Many genes previously described as involved in DD were confirmed to be upregulated, including α-SMA, fibronectin, β1 integrin, laminin, tenasin C, heat shock protein 47, transforming growth factor–β2, and collagen I. In addition several novel genes not described previously in the study of DD were upregulated significantly. These included MafB, collagen type V, α-2 (COL5A2), collagen type VIII, α-1 (COL8A1), contactin I (CNTN1), and leucine-rich repeat containing 17 (LRRC17) (Fig. 1).

These upregulated genes were compared with their known gene-expression profiles in other tissues and their purported functions. MafB was of particular interest because of its prominent role in tissue development and cellular differentiation, including its ability to induce cellular transformation.20,21 MafB gene expression was more than 4-fold upregulated in the diseased cord tissue when compared with diseased cord–adjacent palmar fascia (p < .05) (Fig. 2). Expression of MafB in the control normal fascia from patients having carpal tunnel release was minimal and not statistically different from DD-adjacent control fascia.

Figure 1. Several novel genes not associated previously with DD were found to be upregulated. Columns represent individual samples. Red and green colors represent increased and decreased gene expression, respectively; black indicates approximately the same level of gene expression as the mean across all samples. Color saturation is proportional to the magnitude of the difference from the mean. Cord, DD cords; Fascia, adjacent control fascia; CTR, carpal tunnel control fascia.

Figure 2. MafB-specific gene expression by microarray analysis. See Figure 1 for details.
Patient Demographics for Immunohistochemistry
To confirm upregulation of MafB, immunohistochemistry was performed to detect its presence in DD tissue versus control tissue. Dupuytren’s cord tissue from 16 additional patients was obtained. The average age of patients was 57 years (range, 36–72 y). Thirteen of the 16 patients had bilateral DD and 1 patient also had Ledderhose disease (fibrosis of the plantar aspects of the feet). None of the patients had previous surgery in the same location on the hand. Contractures involved the middle, ring, and/or small fingers. Metacarpophalangeal and/or proximal interphalangeal joints were involved and indications for surgery included contracture of more than 30° at the metacarpophalangeal joint or any contracture of the proximal interphalangeal joint. In addition 9 normal palmar fascia samples were collected from patients having carpal tunnel release to serve as controls. The average age of these control patients was 60 years (range, 50–89 y).

Histology of Dupuytren’s Disease Cord and Control Tissue
Subtle differences in histology and basic tissue architecture between DD cord tissue, adjacent control fascia, and carpal tunnel palmar fascia were observed on hematoxylin-eosin staining, particularly pertaining to cellularity. Control carpal tunnel fascia was composed mainly of collagen and was hypocellular. Adjacent control fascia was more cellular and DD cord tissue was most cellular, often containing hypacellular clusters (Fig. 3).

MafB Immunohistochemistry
For initial confirmation of MafB immunohistochemistry mouse pancreas sections were used as a positive control. MafB is localized in the nuclei of islet β cells in both insulin- and glucagon-producing cells. No MafB was detected in the surrounding acinar cells, which is consistent with published studies. Mouse pancreas sections were included each time DD tissue was processed to verify positive MafB staining. MafB-blocking peptide (Santa Cruz Biotechnology, Inc.) was used as a negative control.

The presence of MafB was examined immunohistochemically and compared among the 3 patient groups (Table 1). Observation of sections by light microscopy showed a pattern of positive MafB staining in DD tissue in 8 of 16 patients (Fig. 4A). Both controls, the adjacent control fascia and the carpal tunnel control fascia, showed complete absence of MafB staining in all specimens (Figs. 4B,C). Review of patient demographic data showed no significant differences between those patients whose DD cord tissue had positive staining and those with negative staining. The presence of staining did not correlate with disease severity.

Co-localization of α-Smooth Muscle Actin, Myofibroblast Marker, and MafB
To determine whether MafB was upregulated specifically in myofibroblasts a known myofibroblast marker, α-SMA, was used to double-stain the DD

| Table 1. MafB Staining Properties of Tissues From 3 Patient Groups Used in This Study |
|-----------------------------------------------|-----------------|-----------------|-----------------|
| **Dupuytren’s Cord Tissue (n = 16)**              | **Adjacent Control Fascia (n = 10)** | **Carpal Tunnel Control (n = 9)** |
| Positive MafB staining, n (%)                  | 8 (50)           | 0 (0)           | 0 (0)           |
| Negative MafB staining, n (%)                  | 8 (50)           | 10 (100)        | 9 (100)         |

Figure 3. Hematoxylin-eosin staining of DD cord tissue and adjacent control fascia. These sections show the subtle difference in cellularity between (A) diseased cord and (B) adjacent control fascia. Note the hypercellular cluster in the cord tissue.
cord tissue along with MafB. MafB was expressed in the same general areas as the α-SMA but was not limited exclusively to the same cells. The α-SMA shared areas of overlapping staining with the nuclear MafB, showing a fibrillar pattern interspersed with nuclei. In addition nuclear MafB was seen also in areas of tissue that did not express α-SMA (Fig. 5).

**Discussion**

Dupuytren’s disease is believed to have a strong genetic component. Previous studies have shown links to several genes, particularly in relation to myofibroblastic proliferation. Our study attempted to search for additional genes that may play a major role in the pathogenesis of DD. With microarray technology several novel genes were found to be upregulated in DD cord tissue compared with control tissues. These genes included collagen type V, α-2 (COL5A2), collagen type VIII, α-1 (COL8A1), contactin I (CNTN1), and leucine-rich repeat containing 17 (LRRC17). In addition MafB gene expression was more than 4-fold upregulated in the diseased cord tissue when compared with both the adjacent control fascia and the carpal tunnel control fascia.

Attention was focused on the upregulation of MafB. MafB is a member of a basic leucine zipper transcription factor network and is a product of the Maf proto-oncogene family. Maf proteins are believed to play prominent roles in tissue development and cellular differentiation. The Maf oncogene initially was identified in an oncogenic avian retrovirus that induced musculoaponeurotic fibrosarcomas in vivo and also cellular transformation of fibroblasts when expressed in chicken embryos in vitro. The origin of the family name Maf is taken from musculoaponeurotic fibrosarcoma. Dupuytren’s disease in its early stages is histopathologically similar to fibrosarcoma. This ability to cause transformation of fibroblasts makes MafB relevant to the pathogenesis of DD. The activity of Maf also can be modulated by other proteins such as Hox, underlining the complexity
of these transcriptional activators.\textsuperscript{28} It would be useful to see how relevant these findings are in the setting of DD pathogenesis.

In this study MafB immunohistochemistry showed positive staining in 50\% of the DD specimens but complete absence of MafB in all control tissues (adjacent control fascia, carpal tunnel fascia). This intermittent positive staining in DD tissue may reflect differences in cellularity in tissue samples or differences in stages of disease progression that were not evident in the review of clinical cases. For example one sample may be derived from a region of tissue with relative cellular inactivity and therefore would have low MafB staining. In future experiments it will be interesting to determine the temporal and spatial course of MafB expression in the clinical progression of DD.

Myofibroblasts have been implicated in the formation of DD cords. \(\alpha\)-smooth muscle actin is a known myofibroblast marker that is present in greater quantities in DD.\textsuperscript{8} Possible co-localization of MafB with myofibroblasts in DD was assessed. When double immunohistochemistry was performed MafB and \(\alpha\)-SMA showed staining properties in similar regions but these 2 proteins were not confined solely to the same cells. These histologic data suggest that the association of MafB with DD is not related exclusively to myofibroblast proliferation.

References

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