Expression of Bone Morphogenetic Proteins by Dupuytren's Fibroblasts

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Purpose: Dupuytren's fibroblasts, or myofibroblasts, are the primary cell type in Dupuytren's disease. Growth factors play a role in the differentiation of fibroblasts to myofibroblasts. Myofibroblasts are specialized fibroblasts that display morphologic and biochemical features similar to smooth muscle cells. Cytokines, adhesion molecules, and extracellular matrix components are all thought to play a role in myofibroblast transdifferentiation. Recent research has shown that specific cytokines, such as transforming growth factor β_1 (TGF-β_1), can modulate myofibroblast expression. We hypothesize that bone morphogenetic proteins (BMPs) play a role in the modulation of Dupuytren's fibroblasts.

Methods: Dupuytren's fibroblasts and normal palmar fascia fibroblasts (control) were analyzed for messenger RNA expression of BMPs (BMP-1, -2, -3, -4, -5, -6, -7, -8, -9, -10 and -11), their receptors (BMPR-IA, BMPR-IB, and BMPR-II), and their antagonists (follistatin and noggin) by reverse-transcription polymerase chain reaction (PCR). Western blot analysis and immunostaining also were used to confirm the differential expression of BMP-4.

Results: With reverse-transcription PCR the expression profile for normal palmar fascia fibroblasts versus Dupuytren's fibroblasts was found to show similar expression of BMP-1 and -11; qualitatively decreased expression of BMP-6, BMP-8, BMPR-IA, BMPR-IB, and BMPR-II in Dupuytren's fibroblasts; and no expression of BMP-4 in Dupuytren's fibroblasts. There was no expression of BMP-2, -3, -5, -7, -9, and -10 in both the control fibroblasts and Dupuytren's fibroblasts. In line with the messenger RNA expression pattern BMP-4 was detected in only the control fibroblasts and not in the Dupuytren's fibroblasts, whereas BMP-8 (chosen for comparison purposes) was detectable in both cell populations. Immunostaining for BMP-8 and BMP-4 confirmed our findings with reverse-transcription PCR and Western blot analysis.

Conclusions: This study reports on the expression of BMPs in Dupuytren's fibroblasts. We characterized the expression of BMPs in both normal palmar fascia fibroblasts and in Dupuytren's fibroblasts through reverse-transcription PCR, Western blot analysis, and immunostaining. The most significant difference in expression profiles was in the expression of BMP-4; that is, BMP-4 was expressed in the normal fibroblasts but not in the Dupuytren's fibroblasts. Whether BMP-4 is necessary and/or sufficient for maintaining a normal palmar fascia fibroblast phenotype is not yet known. Further studies are needed to elucidate the exact role of BMPs, and especially BMP-4, in Dupuytren's fibroblasts. (J Hand Surg 2004;29A:809–814. Copyright © 2004 by the American Society for Surgery of the Hand.)

Key words: Bone morphogenetic proteins, Dupuytren's, hand, immunostaining, myofibroblasts.

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The myofibroblast is the primary cell type in Dupuytren's disease, a fibroproliferative disorder of the palmar fascia that is characterized clinically by progressive flexion deformities of the digits.[1] Cords and nodules are formed within the diseased palmar fascia, and it is the nodules that are rich in myofibroblasts. Myofibroblasts are specialized fibroblasts that display morphologic and biochemical features similar to smooth muscle cells. In particular they express α-smooth muscle (α-SM) actin, a major component of the microfilaments that traverse the cell along its long axis and are related to the myofibroblast's ability to generate contractile force.[2–5]

Cytokines, adhesion molecules, and extracellular matrix components are all thought to play a role in myofibroblast transdifferentiation. Platelet-derived growth factor, transforming growth factor-α (TGF-α), tumor necrosis factor-α, interleukin-1, basic fibroblast growth factor, granulocyte-macrophage colony-stimulating factor, endothelin, interferon-γ, and TGF-β_1 all have been implicated as modulators of the conversion of fibroblasts to myofibroblasts.[6] Among the cytokines, TGF-β_1 has received the most attention as an inducer of myofibroblast transdifferentiation because it is capable of up-regulating fibroblast α-SM actin and collagen in fibroblasts both *in vivo* and *in vitro*.[7] Evans et al[8] showed in an *in vitro* model that the phenotypic and functional changes associated with TGF-β_1–induced pulmonary fibroblast-myofibroblast differentiation are regulated differentially by Smad proteins, especially Smad2, Smad3, and Smad4.

Along with TGF-β isoforms and activins, BMPs constitute 1 of the 3 major groups of proteins in the TGF-β superfamily.[9] BMPs are present in several tissues, notably cartilage and bone but also others. They regulate biologic processes as diverse as cell proliferation, cell differentiation, cell determination, and apoptosis. They are involved in the development of nearly all organs and tissues including somites, lung, kidney, and the skeletal system. At the cellular level BMP cell signaling involves a complex cascade. BMPs bind to a cell surface receptor complex (BMP-R) that possesses serine-threonine kinase activity.[10–12] On binding of BMPs to the type I receptor (BMP-RI), the type II receptor (BMP-RII) associates with the BMP-RI, which it phosphorylates. This phosphorylation triggers the activation of downstream signaling cascades that result in the phosphorylation of a class of DNA-binding proteins called Smads. BMP-R phosphorylates only Smad-1 and Smad-5, whereas TGF-β receptor activates Smad-2 and Smad-3. Activation of Smad-1 or Smad-5 allows their heterodimerization with Smad-4. This complex translocates from the cytoplasm into the nucleus where it interacts with various transcription factors to regulate gene expression.

The purpose of this study was to characterize the expression of BMPs in both normal palmar fascia fibroblasts and Dupuytren's fibroblasts.

Materials and Methods

Cells

Dupuytren's fibroblast and normal palmar fascia fibroblast cell lines were generously provided by Dr. James J. Tomasek of the University of Oklahoma Health Sciences Center. Dupuytren's fibroblasts were obtained from nodules and cords harvested at the time of partial fasciectomy for Dupuytren's disease. Normal palmar fascia was harvested from patients without Dupuytren's disease who had an open carpal tunnel release.

Reverse-Transcription PCR

Total RNAs were isolated from Dupuytren's fibroblast and normal palmar fascia fibroblast cell lines (RNAeasy kit; Qiagen, Valencia, CA). One microgram of total RNA per sample was reverse-transcribed in a 20-μL sample volume consisting of 500 ng oligonucleotide (deoxythymidine [dT]), 15,500 μmol/L deoxyribonucleoside triphosphate (dNTP), 25 mmol/L Tris-HCl (pH 8.3), 37.5 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 10 mmol/L dithiothreitol, and 200 U Superscript II (Superscript II; Life Technologies, Grand Island, NY) for 50 minutes at 42°C and then 70°C for 15 minutes.

The PCR primers used are outlined in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as a control for the reverse-transcriptase reaction. For each set of primers the PCR amplification was performed using 5 μL of the reverse-transcription product in a final reaction volume of 25 μL containing 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 mmol/L each dNTPs, 200 nmol/L each primer, and 0.5 U Taq polymerase (Life Technologies). Positive controls in each case consisted of the respective DNA plasmid sequence to ensure integrity of the system. After initial denaturation at 94°C for 4 minutes, 38 cycles of PCR were performed in a thermal cycler (Perkin Elmer GeneAmp 2400; Perkin Elmer, Foster City, CA). Each cycle included denaturation at 94°C for 1 minute, annealing at either 59°C (BMP-1, -2, -4, -6, -7, -9, -10, -11) or 63°C (BMP-3, -5, -8) for 90

Table 1. Oligonucleotide Primers Used for Reversed-Transcription PCR

Target Template	PCR Primers	Product Size (bp)
BMP-1	5′-AGGTACAGCAGGCTGTGGAT	563
	5′-AACTTCCTGAAGATGGAGCC	
BMP-2	5′-TTGCGGCTGCTCAGCATGTT	315
	5′-TTCCGAGAACAGATGCAAGATG	
BMP-3	5′-AGGTCTCTGAACACATGCTG	604
	5′-ATCAAGCTTACAGGGACACC	
BMP-4	5′-AGCCATGCTAGTTTGATACC	382
	5′-TCAGGGATGCTGCTGAGGTT	
BMP-5	5′-AGACAATCATGTTCACTCCAGTT	722
	5′-AGCTGTAAGCCCAAATTATTCTGG	
BMP-6	5′-ACATGGTCATGAGCTTTGTGA	528
	5′-GTAGAGCGATTACGACTCTGT	
BMP-7	5′-CAGCCTGCAAGATAGCCATT	276
	5′-AATCGGATCTCTTCCTGCTC	
BMP-8	5′-CGTGCAGCGCGAGATCCTGG	539
	5′-GCCTCTATGTGGAGACTGAG	
BMP-9	5′-GAAGATGTTTCTGGAGAACG	914
	5′-GCTTCTTCCCCTTGGCTGAC	
BMP-10	5′-CAGCTTACTTGGTTTCTGGC	649
	5′-CGGCTAGAAATAGATACCAG	
BMP-11	5′-TGCAGCAGATCCTGGACC	545
	5′-GGAGCTTCGAGTCCTAGAGA	
BMPR-IA	5′-TGTTCAAGGACAGAATCTGG	399
	5′-TTGATGGCAGCATTCGATGG	
BMPR-IB	5′-AAGAAAGAGGATGGTGAGAG	791
	5′-CCTGGACCCAGTTGTACCTA	
BMPR-II	5′-AGATCCGTATCAGCAAGACC	983
	5′-GGCTGACTGGAAATAGACTG	
Noggin	5′-GCACCCAGCGACAACCTGCCC	425
	5′-GCTGCCCACCTTCACGTAGCG	
Follistatin	5′-GAACTGAGCAAGGAGGAGTG	571
	5′-CACTTTCCCTCATAGGCTAATCC	
	5′-ATATGTGGAGGTGCCATCAAT	452
GAPDH	5′-ACCACAGTCCATGCCATCAC	

seconds, and extension at 72°C for 90 seconds, with a final extension of 7 minutes at 72°C. Five microliters of PCR-amplified sequences then were electrophoresed and analyzed on 1.0% agarose-ethidium bromide gels. The intensity of each band was compared with the expression of GAPDH (serving as an internal control).

Western Blot

Both Dupuytren's fibroblast and normal palmar fascia fibroblast cells were cultured in Dulbecco's modified Eagle medium with 10% fetal bovine serum. Confluent dishes were washed with phosphate-buffered saline (PBS) and then lysed in cell lysate buffer (PBS, pH 7.4, 1% Triton X-100, and 5 mmol/L ethylenediaminetetraacetic acid). Lysates were sonicated briefly and the protein concentrations were determined by using a protein assay kit (BioRad, Hercules, CA). Twenty micrograms of proteins were subjected to 4% to 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrotransferred onto a polyvinylidene difluoride membrane. The membrane was rinsed in a PBS solution containing 0.1% Tween 20 (PBS-T) and blocked by incubation in 5% weight/volume dried milk dissolved in 0.1% PBS-T solution. Membranes were washed in 0.1% PBS-T and incubated at room temperature with either primary antibody to mouse monoclonal antibodies BMP-4 or rabbit polyclonal antiserum to BMP-8 (1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA). After the PBS-T washes, membranes were incubated with appropriate secondary goat anti-rabbit or anti-mouse antibodies conjugated by horseradish peroxidase. Membranes were washed in 0.1% PBS-T and then subjected to enhanced chemiluminescence detection.

Immunostaining

Paraffin sections of Dupuytren's tissue were immunostained for BMP-4 and BMP-8. Sections were deparaffinized and rehydrated, followed by an enzymatic pretreatment of hyaluronidase at 1 mg/mL in Tris buffer, pH 7.4, for 20 minutes at room temperature. A protein block (Dako Serum-Free Protein Block; Dakocytomation, Fort Collins, CO) was applied for 10 minutes at room temperature before incubation of the primary antibodies. Mouse anti-mouse BMP-4 (Santa Cruz Biotechnology) was diluted at 1:50, incubated at 4°C overnight, and detected using anti-mouse link and streptavidin label (Super Sensitive Alkaline Phosphatase kit; Biogenex, San Ramon, CA). Rabbit antihuman BMP-8 (Santa Cruz Biotechnology) was diluted 1:50, incubated at 4°C overnight, and detected using the anti-rabbit link and streptavidin label from an alkaline phosphatase kit. Alkaline phosphatase substrate (Vector Red; Vector Laboratories, Burlingame, CA) was used for visualization, and sections then were counterstained with Mayer's hematoxylin. The primary antibody was substituted (Negative Control Serum for Super Sensitive Antibodies; Biogenex) with mouse and rabbit control serum, respectively, for negative control sections.

Results

To examine the expression profile of BMPs in Dupuytren's fibroblasts a reverse-transcription PCR assay was performed. BMP-1 remained unchanged in both control (normal palmar fascia) fibroblasts and Dupuytren's fibroblasts, whereas Dupuytren's fibroblasts exhibited decreased expression of BMP-6, -8, -11, BMPR-IB, and BMPR-II, and no expression of BMP-4 (Fig. 1). Levels of BMP-2, -3, -5, -7, -9, and -10 were undetectable in both the control fibroblasts and the Dupuytren's fibroblasts (not shown). Noggin, a BMP antagonist, was not expressed in either cell population, whereas follistatin, another BMP antagonist, was expressed in both. GAPDH, expressed in all metabolically active cell populations, was used as a control for this part of the study and was expressed strongly in both cell populations.

Given that BMP-4 and BMP-8 messenger RNA are highly expressed in the normal palmar fascia fibroblasts while not expressed or poorly expressed in Dupuytren's fibroblasts, a Western blot analysis was performed to test the protein levels of these components. In line with the messenger RNA expression pattern, BMP-4 was detected in only the control

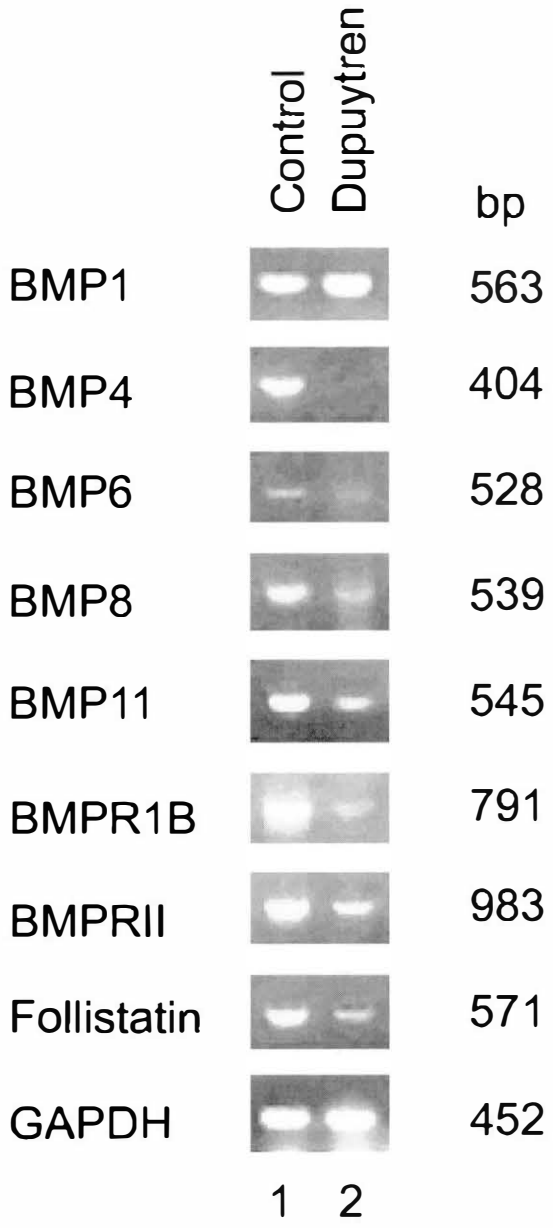

Figure 1. Expression analysis of BMPs, BMPRs, and tissue inhibitors by reverse-transcription PCR. Amplification products for BMPs, BMPRs, and inhibitors from Dupuytren's fibroblasts and normal palmar fascia fibroblasts were resolved by 1.0% agarose-ethidium bromide gel electrophoresis. Positive control GAPDH is indicated.

fibroblasts and not in Dupuytren's fibroblasts, whereas BMP-8 was detectable in both cell populations (Fig. 2).

Immunostaining for BMP-8 and BMP-4 confirmed our findings with reverse-transcription PCR and Western blot analysis (Fig. 3). Immunostaining for BMP-4 was completely negative, and the test sections were indistinguishable from the negative control section. Sections immunostained for BMP-8 ex-

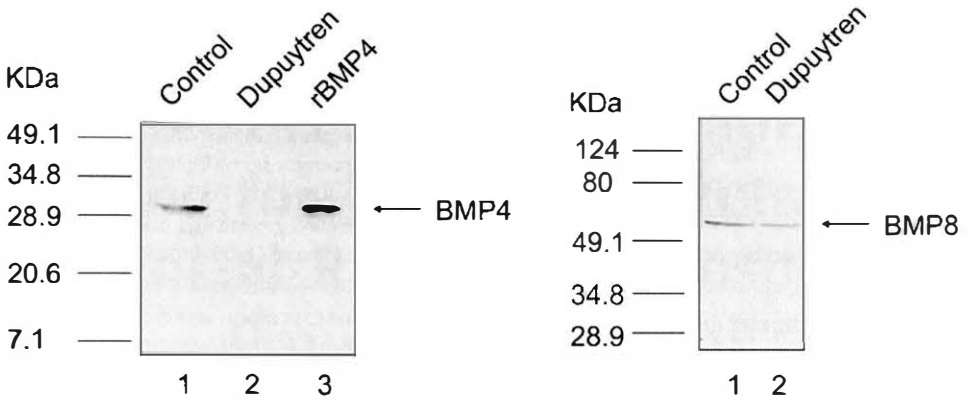

Figure 2. Western blotting assay for examining expression of BMP-4 and BMP-8. Protein samples (20 μg) extracted from the indicated tissues. Dupuytren's fibroblast and normal palmar fascia fibroblast cells (Control) were subjected to 4% to 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, electrotransferred to the membrane, and probed with specific antibodies against BMP−4, and -8. Positions of BMP-4 and BMP-8 are indicated by arrows.

hibited variable positivity depending on the area of the section examined. Positive immunostaining was restricted consistently to the cytoplasm of the proliferating myofibroblasts. No positive staining was visible in 10% to 20% of the area of the sections. In approximately 30% of the section area the staining was diffusely and weakly positive. In the remainder of the section area the staining was present in discrete, variably sized foci within the cytoplasm and strongly positive. The smooth muscle myocytes of arteries and arterioles in these sections exhibited a staining pattern that was similar to that of the proliferating myofibroblasts.

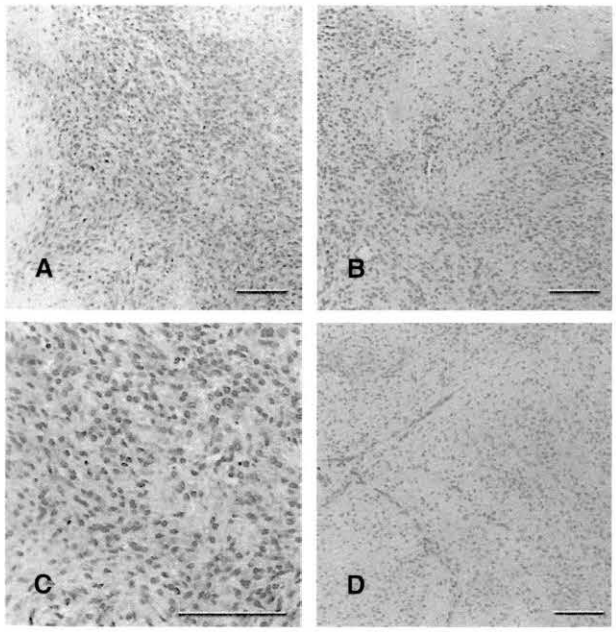

Figure 3. Immunostaining of Dupuytren's tissues for (A) BMP-8, (B) negative control for BMP-8, (C) BMP-8 ([A] at higher magnification), and (D) BMP-4. Bar = 200 μm.

Discussion

BMPs comprise at least 16 members of the TGFβ superfamily based on the presence of 7 highly conserved cysteines in the carboxyl terminus. Their role in cellular proliferation and differentiation has been studied extensively in various organ systems. Corneal fibroblasts have been shown to express BMP-2, -4, -5, and -7.[13,14] In canine keratinocytes TGF-β_1 has been shown to cause the nuclear translocation of Smad-2.[15] This process seems to depend at least in part on cell density, which can modulate myofibroblast differentiation.[15,16] In the cornea, myofibroblast differentiation plays a significant role in the induction of scar formation and the reduction of corneal transparency.[17–20] A specific BMP also may play varying roles depending on cell type, as has been shown for BMP-7. You and Kruse[9] found that BMP-7 did not increase α-SM–myosin expression in corneal fibroblasts, whereas activin A did. On the other hand, Dorai et al[21] reported that BMP-7 increased α-SM–myosin expression in vascular smooth muscle cells. In the musculoskeletal system, BMP-12 and -13 inhibit terminal differentiation of myoblasts by inhibiting the expression of myosin, but do not induce their differentiation into osteoblasts.[22] BMP-2 also downregulates myosin and simultaneously induces markers for osteoblast differentiation.[23]

BMP-4 is expressed in initial cartilage condensations and during early and late fracture healing, suggesting roles for this protein in early skeletal development and bone repair.[24,25] BMP-4 also influences, in several ways, the morphogenesis of different portions of the urogenital system.[26] In this study, we characterized the expression of BMPs in both normal palmar fascia fibroblasts and in Dupuytren's fibroblasts through reverse-transcription PCR, Western blot analysis, and im-

munostaining. The most notable difference in expression profiles was seen in the expression of BMP-4; that is, it was shown that BMP-4 is expressed in normal fibroblasts but not expressed at all in Dupuytren's fibroblasts. It is possible that the variable positivity found in our immunostaining is caused by the 2 Dupuytren's cell types, that is, cells derived from cords and cells derived from nodules. It has been reported that although nodules are myofibroblast-rich, they contain both fibroblast (non–α-SM actin–containing) and myofibroblast (α-SM actin–containing) cell types; cords, on the other hand, do not have myofibroblasts and possess only a sparse cell population of fibroblasts.[27] Whether BMP-4 is necessary and/or sufficient for maintaining a normal palmar fascia fibroblast phenotype is not yet known. It also is not known whether the lack of BMP-4 expression is necessary and/or sufficient for the development of a Dupuytren's fibroblast phenotype. Further studies are needed to clarify the exact role of BMPs, and especially BMP-4, in Dupuytren's fibroblasts.

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