Bone Morphogenetic Proteins 2 and 6, Expressed in Arthritic Synovium, Are Regulated by Proinflammatory Cytokines and Differentially Modulate Fibroblast-Like Synoviocyte Apoptosis

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Objective. To examine the expression, regulation, and potential roles of bone morphogenetic proteins (BMPs) in arthritic synovium.

Methods. Expression of BMPs in arthritic synovium from patients with rheumatoid arthritis (RA) or spondylarthropathy (SpA) and in noninflamed synovium from patients undergoing diagnostic or therapeutic arthroscopies was studied by reverse transcription–polymerase chain reaction (RT-PCR), Western blot, immunohistochemistry, and 2-color immunofluorescence. In vitro regulation of gene expression in fibroblast-like synoviocytes (FLS) was determined by real-time quantitative RT-PCR and immunohistochemistry. We used 3H-thymidine incorporation after serum deprivation–induced growth arrest to examine effects on FLS proliferation. FLS apoptosis was evaluated by flow cytometry cell cycle analysis. Apoptotic cells in synovial tissue were detected by TUNEL staining.

Results. Transcripts of different BMPs, most strikingly BMP-2 and BMP-6, were detected in synovial tissues. By Western blot, BMP-2 and BMP-6 precursor protein was found in RA and SpA synovial tissue extracts, but not in extracts of noninflamed synovial tissue. By immunohistochemistry, BMP-2 and BMP-6 were detected in the hyperplastic lining and the sublining layer of synovium from RA and SpA patients, both in CD90+ cells (FLS) and in some CD68+ cells (macrophages). Proinflammatory cytokines, such as interleukin-1β and tumor necrosis factor α, but not interferon-γ, enhanced the expression of BMP-2 and BMP-6 transcripts in FLS in vitro. Neither BMP-2 nor BMP-6 affected FLS proliferation. BMP-2 promoted FLS apoptosis, whereas BMP-6 protected against nitric oxide–induced FLS apoptosis. BMP-2–positive apoptotic cells were found in arthritic synovium.

Conclusion. BMP-2 and BMP-6 are expressed in arthritic synovium and are strongly up-regulated by proinflammatory cytokines. Although BMP signaling has been proposed to be involved in cartilage and bone repair in arthritis, this pathway may be equally important in modulating FLS cell populations in inflamed synovium.

Chronic inflammatory arthritides, including rheumatoid arthritis (RA) and the spondylarthropathies (SpA), are an important cause of morbidity and disability. Research efforts have mainly focused on inflammatory and immune pathways, leading to the identification of specific cytokines, chemokines, and tissue-destructive enzymes as key players in the pathologic process. Using this approach, successful targeted therapies, such as tumor necrosis factor (TNF) blockade, have been developed for both RA and SpA patients (1,2).

Which cells play the leading role in the development of chronic arthritis is still a subject of debate. The role of immune cells and, more particularly, T cells and autoantigens has been extensively studied (for review, see ref. 3). In RA, interactions between B cells and T cells in the synovium are critical (4). In recent years, it has become clear that other cell populations, including nonimmune cells, also contribute to the pathology and probably to the pathogenesis of chronic arthritis (5). The role of fibroblast-like synoviocytes (FLS) in particular has gained attention in both RA (5,6) and SpA (7). Activated and partially transformed FLS may escape current antiinflammatory and immune-mediated thera-
pies. In addition, multipotential mesenchymal stem cells have been identified in the synovium and in FLS cultures after in vitro expansion (8,9). These cells are suggested to contribute to tissue remodeling and repair (9) as well as disease pathogenesis (8).

Modulation of the intrinsic tissue repair capacity is an emerging therapeutic option for the treatment of both degenerative and inflammatory joint diseases (10). In arthritis, such strategies are of critical importance in restoring the damaged tissue that causes morbidity and disability, even after control of the inflammation has been achieved. Therefore, there is an emerging interest in embryonic signaling, more specifically in those growth factors and morphogens known to be involved in the embryologic formation of the skeletal tissues and the joint. Members of the transforming growth factor β (TGFβ) superfamily, including TGFβ1–3, bone morphogenetic proteins (BMPs), and activins, are among those key players (11). The TGFβ superfamily is a large group of structurally related polypeptide cytokines with pleiotropic functions in regulating development, homeostasis, and repair of different tissues (12). Originally identified as polypeptides responsible for the in vivo bone-inductive properties of demineralized bone matrix (13,14), BMPs have been recognized as key molecules both in early developmental patterning (15) and in late morphogenesis in different organs including the skeleton and the joints (11,16). A role for BMPs in skeletal repair (e.g., fracture healing) has been demonstrated (17). An important role for both TGFβ and BMPs has been suggested in homeostatic and remodeling mechanisms in osteoarthritis (OA) (18–20).

Little is known about the potential role of BMPs in chronic inflammatory joint diseases. BMPs are currently considered to be pleiotropic cytokines that influence proliferation, growth, differentiation, and apoptosis of many different cell types. Therefore, any role of BMPs in arthritis may extend beyond those in bone and cartilage. BMPs mainly affect cells of mesenchymal origin (e.g., chondrocytes and osteoblasts). Therefore, FLS may be a specific target of BMP signaling.

We examined the expression of BMP-2 and BMP-6 in arthritic synovium. These BMPs belong to different subgroups of the BMP family. In addition, we investigated the role of proinflammatory cytokines in the regulation of these morphogens and their potential role in FLS proliferation and apoptosis. Our data suggest that BMP signaling may prove to be a therapeutic target for controlling FLS behavior, thereby directing and modulating the processes of repair and remodeling in arthritis.

### PATIENTS AND METHODS

**Reagents.** Recombinant proteins were obtained from the following sources: recombinant human BMP-2 from Genetics Institute (Cambridge, MA), recombinant human TGFβ1 and recombinant human BMP-6 from R&D Systems (Abingdon, UK), recombinant human interleukin-1β (IL-1β) and recombinant human TNFα from BioSource International (Nivelles, Belgium), and recombinant human interferon-γ (IFNγ) from Boehringer (Mannheim, Germany). Goat polyclonal anti-human, anti-mouse, and anti-rat BMP-6 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Affinity-purified polyclonal chicken anti-human BMP-2 antibody was a gift from Pfizer Central Research (Groton, CT). Monoclonal mouse anti-human CD68 was purchased from Dako (Glostrup, Denmark). Monoclonal mouse anti-human CD90 was purchased from Becton Dickinson (San Jose, CA).

**Patient inclusion, needle arthroscopy, and cell isolation.** Patients were treated at the Department of Rheumatology or the Department of Orthopedic Surgery, University Hospitals Leuven, and gave written informed consent. The local Ethics Committee approved the procedure. All patients with inflammatory arthritis who were included in the study fulfilled either the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) 1987 revised criteria for RA (21) or the European Spondylarthropathy Study Group criteria for SpA (22). For a diagnosis of knee OA, the ACR criteria were used (23). Other diagnoses for the control samples were made by combining clinical, radiographic, and arthroscopy data.

Needle arthroscopy of the knee, with random synovial biopsies, was performed under sterile and standardized conditions. During needle arthroscopy, synovial hyperemia was scored macroscopically using a semiquantitative scoring system (scarce, mild, moderate, strong) as reported previously (24). Similarly, synovial hypertrophy was described as mild or manifest hypertrophy or pannus formation (defined as hypertrophic synovial tissue overlying the cartilage) by the arthroscopist (24). Biopsy samples of noninflamed synovium were obtained from patients undergoing diagnostic arthroscopies.

For histologic assessments, 6 biopsy samples were snap-frozen in TissueTek (Sakura, Zoeterwoude, The Netherlands) and stored at −70°C for later use. Two biopsy samples were immediately used for RNA isolation (see below) or protein extraction (see below). Two biopsy samples were washed twice in Hanks’ balanced salt solution (Life Technologies, Merelbeke, Belgium) supplemented with 2× antibiotic–antimyocytic solution (100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B; Life Technologies). Cells were isolated using a previously described protocol (9). Briefly, the biopsy samples were digested overnight with 0.2% type IV collagenase (Life Technologies) in high-glucose Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) containing 10% fetal bovine serum (FBS; Bio-Whittaker, Verviers, Belgium) and 2× antibiotics (as above). Passage 0 cells from both biopsy samples were pooled and washed twice in DMEM–10% FBS and antibiotics (growth medium) and then resuspended in 3 ml of growth medium and plated in 3 different wells of a 6-well tissue culture plate. Nonadherent cells were removed after 72 hours by changing
the growth medium. All tissue culture experiments were performed at 37°C in a humidified atmosphere of 5% CO₂.

**Cell expansion and stimulation.** After reaching confluence, cells were washed twice with Dulbecco's calcium- and magnesium-free phosphate buffered saline (DPBS; BioWhitaker), harvested with trypsin–EDTA (0.25% trypsin, 1 mM EDTA; Life Technologies), and replated in a T75 flask. Further passaging was done with 1:3 dilutions. Growth medium was changed twice a week. All further experiments were performed between passages 4 and 9. For cytokine stimulation experiments, harvested cells were diluted 1:3 in growth medium and plated in 6-well tissue culture plates. At confluence, cells were treated either with ligands (IL-1β, TNFα, or IFNγ) or with carrier controls. In additional experiments, cells were plated on tissue culture slides and incubated at confluence with ligands as described above.

**Total RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) analysis.** Total RNA from stimulated cells or from fresh synovial biopsy specimens was isolated using a commercially available kit (SNAP; Invitrogen, Merelbeke, Belgium) according to the manufacturer's instructions. Complementary DNA (cDNA) was obtained by RT of 1–2 μg of total RNA (Thermoscript; Invitrogen) with oligo(dT)₂₀ as primer. Conventional PCR was performed as previously described (9). Complementary DNA was mixed with 0.5 units of Taq polymerase (Eurogentec, Seraing, Belgium), 0.2 mM dNTP, 0.5 μM specific primers, and 1.5 mM MgCl₂. Primer pairs were designed using Vector NTI software (InforMax, North Bethesda, MD). The sequences of the primers, the sizes of the PCR products, and the optimum annealing temperatures are shown in Table 1.

All conventional PCR reactions were carried out in a Perkin Elmer Thermal Cycler 9600 (Applied Biosystems, Leiden, Belgium). After denaturation at 95°C for 2 minutes, cycles were 10 seconds at 94°C, 10 seconds at the optimum annealing temperature, and 30 seconds at 72°C. Cycling was followed by a 10-minute elongation at 72°C. For BMP-7/osteogenic protein 1 (OP-1) PCR, a 2-step procedure with an annealing and elongation temperature of 68°C for 45 seconds was used. PCR products were electrophoresed in 1% agarose gels in Tris–borate–EDTA electrophoresis buffer, stained with ethidium bromide, and visualized by ultraviolet transillumination.

For real-time quantitative analysis, PCR was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems). PCRs were run in a 12.5-μl mixture consisting of 3.5 mM MgCl₂, 200 μM of each dNTP, 0.5 μM specific primers, 0.025 units/μl HotGoldstar enzyme, 1 μl of SYBR Green I dilution, and 1× reaction buffer (all from Eurogentec). After initial activation of the HotGoldstar enzyme (10 minutes at 95°C), 40 cycles of denaturation at 95°C for 10 seconds and elongation at 60°C for 2 minutes were run. Nonspecific amplification was detected by melting curve analysis. The identity of the PCR products was checked by DNA sequencing.

For quantitative analysis, the fluorescent signal was plotted against the cycle number, and the threshold cycle (Ct; the cycle number at which an increase above background fluorescence could be reliably detected) was determined. Serial dilutions of positive control cDNA templates were included in each PCR run, and standard curves for target gene and β-actin were generated by linear regression using log(Ct) versus log(cDNA relative dilution). Each PCR run also included no-template controls containing all reagents except cDNA. In preliminary experiments, the validity of β-actin as a housekeeping gene was tested by comparing its expression levels with those of other housekeeping genes (data not shown). The use of an active reference gene (β-actin) allows us to normalize for differences in the amount of total RNA added to a reaction and to compensate for different RT efficiencies. All PCR efficiencies were >0.87.

**Protein extraction and Western blot analysis.** For synovial tissue extraction, 2 biopsy samples (combined wet weight of tissue between 11 mg and 39 mg) were extracted in 0.5 ml of guanidine HCl buffer (1M guanidine HCl, 50 mM sodium acetate buffered at pH 7.2) overnight at 4°C. The extract was desalted and concentrated using Microcon YM-10 centrifugal spin devices (MicroPore, Lancaster, PA) and lyophilized. Proteins were normalized to wet weight by resuspension in an appropriate volume of sample buffer (50–176 μl) (NuPAGE LDS sample buffer; Invitrogen) containing 8 M urea.

Aliquots of samples (10 μl) were analyzed under reduced conditions (2% mercaptoethanol and 0.1M dithiothreitol). Samples were boiled for 5 minutes at 95°C, cooled instantly, and loaded onto a 4–12% Bis-Tris gel (Invitrogen). Electrophoresis was carried out in a commercially available running buffer (NuPAGE MES SDS running buffer [20×]; Invitrogen) at 200V for 40 minutes. Proteins were transferred from the gel onto a polyvinylidene difluoride membrane for 90 minutes at 30V in a buffer containing 0.4M glycine, 0.5M Tris base, 0.01M sodium dodecyl sulfate, and 200 μl/liter methanol. Nonspecific binding sites were blocked overnight with 0.5% milk. Blots were probed with the above-described polyclonal antibodies against BMP-2 and BMP-6 and then incubated with a horseradish peroxidase (HRP)–conjugated rabbit anti-

**Table 1.** Sequences of the primers used for polymerase chain reaction, sizes of the products, and annealing temperatures*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product size, bp</th>
<th>Annealing temperature, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP-2</td>
<td>5'-CAGAGACCCACCCACGAC-3'</td>
<td>5'-CTGTTTTGTTTGGCTTGAC-3'</td>
<td>672</td>
<td>60</td>
</tr>
<tr>
<td>BMP-4</td>
<td>5'-TTCCTGGAACGCAATGCT-3'</td>
<td>5'-GGGGCTTCATAACCTCTCAA-3'</td>
<td>555</td>
<td>60</td>
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<tr>
<td>BMP-6</td>
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<td>5'-ATGTTGCGTTGAGGTGGGAAG-3'</td>
<td>518</td>
<td>60</td>
</tr>
<tr>
<td>BMP-7/OP-1</td>
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<td>5'-GAAGTAGAGGACGGAGATGCG-3'</td>
<td>549</td>
<td>68</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>5'-CAGAAATTACAGCAAAATCTCGG-3'</td>
<td>5'-TTGCAGTTGTTATCCCTCTGTC-3'</td>
<td>186</td>
<td>60</td>
</tr>
<tr>
<td>TGFβ2</td>
<td>5'-TCCAAAGATTTAACATCTCAAC-3'</td>
<td>5'-TCCACTGTITTTTTTCTCTAGTC-3'</td>
<td>310</td>
<td>60</td>
</tr>
<tr>
<td>TGFβ3</td>
<td>5'-GCAAACGCTGGAGATGAGG-3'</td>
<td>5'-GGAGGCGTCACACAAAGTG-3'</td>
<td>650</td>
<td>60</td>
</tr>
</tbody>
</table>

* BMP = bone morphogenetic protein; OP-1 = osteogenic protein 1; TGFβ1 = transforming growth factor β1.
chicken or rabbit anti-goat secondary antibody (Jackson Immu-
noResearch, West Grove, PA). All washes were performed
with Tris buffered saline (TBS)–Tween/0.2% milk except in
the last washing step, when milk was omitted from the washing
buffer. For detection enhancement, 1 ml of chemiluminescent
substrate (Super signal Pico; Pierce, Rockford, IL) was applied
in the dark. Sections were washed in distilled water and counter-
stained with hematoxylin. For tissue culture slides, the same
procedure was applied.

**Immunofluorescence.** For 2-color immunofluores-
cence, sections were quenched with 0.025 mg/ml NHCl.
Blocking of nonspecific binding was done with Blocking Re-
agent (Roche Molecular Biochemicals, Brussels, Belgium).
For primary antibodies, we used the above-described anti–BMP-2 antibody and a monoclonal mouse anti-human CD68
antibody or a monoclonal mouse anti-human CD90 antibody.
For secondary antibodies, we used a Cy3-conjugated rabbit
antibody or a monoclonal mouse anti-human CD90 antibody.

**Immunohistochemistry.** Frozen sections were cut from
Tissue-Tek blocks. All 6 biopsy samples obtained at arthros-
copy were analyzed on one slide. Silanated slides were air-
dried and fixed with acetone for 10 minutes. Sections were
extensively quenched with 0.3% H2O2 in H2O. After washing 3
times in TBS for 5 minutes, blocking donkey serum (1:5
dilution in TBS) was added for 30 minutes at room tempera-
ture. Sections were subsequently incubated with primary
chicken anti-human BMP-2 antibody (5 μg/ml) or chicken IgG
(Jackson ImmunoResearch) at the same concentration (all
diluted in TBS) at 4°C overnight. After washing 3 times in TBS,
the second blocking step was added, and thereafter sections were
incubated for 30 minutes at room temperature with an HRP-
conjugated rabbit anti-chicken antibody (1:100 dilution) (Jack-
on ImmunoResearch). BMP-6 staining, a polyclonal goat
anti-BMP-6 antibody or goat IgG (Santa Cruz Biotechnology)
was used (1:100 dilution). For a secondary antibody, we used
an HRP-conjugated rabbit anti-goat antibody (1:100 dilution)
(Jackson ImmunoResearch). Diaminobenzidine (Sigma,
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**FLS proliferation assay.** FLS proliferation rates were
analyzed using a slightly modified protocol previously de-
scribed (25). After trypsin release, FLS were resuspended in
growth medium at a concentration of 10^5/ml. Two hundred
microliters of the cell suspension was plated per well in a
96-well flat-bottomed tissue culture plate, and cells were
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**Table 2.** Patient characteristics

<table>
<thead>
<tr>
<th>Patient/age/sex</th>
<th>Disease</th>
<th>Disease duration</th>
<th>ESR, mm/hour</th>
<th>CRP level, mg/liter</th>
<th>Synovial fluid cells/ml</th>
<th>DMARD</th>
<th>Synovial vascularity</th>
<th>Synovial hypertrophy</th>
</tr>
</thead>
<tbody>
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<td>MD</td>
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<td>MD</td>
<td>MD</td>
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<tr>
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<td>87</td>
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<td>Gold</td>
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<tr>
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<td>2</td>
<td>3</td>
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<td>SSZ</td>
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<tr>
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<td>MD</td>
<td>MTX</td>
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<tr>
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<td>152</td>
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<td>SSZ</td>
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<tr>
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<td>60</td>
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<td>19/31/F</td>
<td>SpA</td>
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<td>Mild</td>
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<td>Mild</td>
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<tr>
<td>21/46/M</td>
<td>SpA</td>
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<td>41</td>
<td>43</td>
<td>25,200</td>
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<td>Strong</td>
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<tr>
<td>22/72/M</td>
<td>SpA</td>
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<td>156</td>
<td>12,500</td>
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<td>Moderate</td>
<td>Manifest</td>
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<td>23/31/F</td>
<td>SpA</td>
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<td>19.7</td>
<td>4,200</td>
<td>No</td>
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<td>Mild</td>
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<tr>
<td>24/35/F</td>
<td>SpA</td>
<td>8 years</td>
<td>12</td>
<td>3</td>
<td>4,800</td>
<td>No</td>
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<td>2 months</td>
<td>14</td>
<td>20</td>
<td>100</td>
<td>No</td>
<td>Mild</td>
<td>Mild</td>
</tr>
<tr>
<td>26/36/M</td>
<td>SpA</td>
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<td>22</td>
<td>19</td>
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<tr>
<td>27/25/F</td>
<td>SpA</td>
<td>2 years</td>
<td>20</td>
<td>3</td>
<td>3,600</td>
<td>SSZ</td>
<td>Moderate</td>
<td>Mild</td>
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<tr>
<td>28/55/M</td>
<td>SpA</td>
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<td>64</td>
<td>81</td>
<td>8,200</td>
<td>SSZ</td>
<td>Moderate</td>
<td>Absent</td>
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</table>

* ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DMARD = disease-modifying antirheumatic drug; MD = missing data; RA = rheumatoid arthritis; SSZ = sulfasalazine; MTX = methotrexate; SpA = spondylarthropathy.
DMEM–1% FBS–1× antibiotics for 72 hours. The cultures were supplemented with ligands for 24 hours or 48 hours. One microcurie of 3H-thymidine (ICN Biomedicals, Costa Mesa, CA) was added immediately or after 24 hours. Cells were washed with DPBS, trypsinized, and resuspended in 200 μl of serum-free DMEM. The DNA-incorporated radioactivity was determined in a liquid scintillation counter. Alternatively, cells were lysed after 24 hours or 48 hours of ligand stimulation. DNA content was measured using SYBR Green I fluorescence in a 96-well plate.

**FLS apoptosis assay–flow cytometric cell cycle analysis.** After trypsin release, FLS were plated in 24-well tissue culture plates, grown to confluence in growth medium, and then kept overnight in DMEM–1% FBS–1× antibiotics. Cells were subsequently incubated with ligands or carrier controls for 4 hours, after which sodium nitroprusside (SNP; Sigma) was added at different concentrations in the appropriate experimental conditions. Sixteen hours later, both adherent and nonadherent cells were collected and washed 3 times with PBS. Subsequently, 1% Triton X-100 (Sigma) and 100 μg/ml RNase A (Serva, Heidelberg, Germany) were added for 10 minutes on ice. Cell DNA was stained with 50 ng/ml propidium iodide (Sigma). Nuclear DNA content was analyzed on a FACSscan (Becton Dickinson). The number of sub-G1 cells in the DNA histogram was counted. Experiments were performed in duplicate or triplicate.

**Staining of apoptotic cells.** Apoptotic cells in synovial tissue were detected using TUNEL staining. Frozen sections were processed for TUNEL assays according to the manufacturer's instructions (In Situ Cell Death Detection kit; Roche Diagnostics, Mannheim, Germany). After TUNEL staining, sections were further processed for BMP staining as mentioned above. Cells were counterstained with DAPI.

**Statistical analysis.** Comparisons among three or more groups were made by a nonparametric Friedman analysis of variance (ANOVA), and those between two groups were made by a nonparametric Wilcoxon test. P values less than 0.05 were considered significant.

**RESULTS**

BMP gene expression in arthritic synovium. We set out to study the expression of TGFβ superfamily members from different subfamilies in arthritic synovium from patients with RA or SpA and in noninflamed synovium from patients undergoing diagnostic or therapeutic arthroscopies. Patient characteristics are shown in Table 2. In preliminary experiments, we found transcripts from different BMPs, including BMP-2, BMP-4, BMP-6, and BMP-7/OP-1, to be expressed in synovial tissues. Since BMP-2 and BMP-6 messenger RNA (mRNA) appeared to be most consistently expressed, we further studied levels of these BMPs by real-time quantitative PCR on 15 samples (5 normal synovial tissues...
[from patients 1–5], 5 RA tissues [from patients 9–13], and 5 SpA tissues [from patients 19–23]). These BMPs are part of distinct subfamilies with different receptor affinities (the BMP-2 and BMP-4 subfamily preferentially binding activin receptor–like kinase 3 [ALK-3] and the subfamily that includes BMPs 5, 6, and 7, which preferentially bind ALK-2 and/or ALK-6) (10). No differences in mRNA levels of these BMPs were seen between the normal and arthritic synovium after normalization to the housekeeping gene β-actin (Figure 1).

**Figure 3.** BMP-2 expression in arthritic synovium. **A** and **B,** Immunohistochemical staining showing many BMP-2–positive cells in the lining layer and the sublining layer in synovium from an SpA patient (**A**) and a few weakly BMP-2–positive cells in synovium from a patient with cartilage injury (**B**). Bars = 200 μm. **C–H,** Two-color immunofluorescence staining of the synovium from an RA patient. Shown are BMP-2 staining in the hyperplastic lining layer (**C**) and sublining zone (**F**), CD68 staining (**D**) and superimposed image (**E**) showing nuclear 4′,6-diamidino-2-phenylindole (DAPI) staining and CD68+ cells positive for BMP-2, and CD90 staining (**G**) and superimposed image (**H**) showing nuclear DAPI staining and CD90+ cells positive for BMP-2. Bars = 25 μm. See Figure 1 for other definitions.
BMP protein expression in arthritic synovium.
To study whether BMP proteins were also expressed in the synovium, we used Western blot. Patient characteristics are shown in Table 2. BMP-2 and BMP-6 precursor proteins were found in synovium from RA patients (patients 14–18) and SpA patients (patients 24–28), but not in noninflamed synovium from controls (patients 6–8) (Figure 2). By extending the time of exposure of the chemiluminescent substrate to the film, small amounts of processed protein could also be detected (results not shown).

We further studied synovial BMP-2 (Figure 3) and BMP-6 expression by immunohistochemistry. BMP-2–positive cells were demonstrated in both RA and SpA synovium (Figures 3A, C, and F). BMP-2–positive cells were found in the hyperplastic lining layer and in infiltrating sublining and perivascular cells. In biopsy samples from the control patients with noninflamed synovium, only a few scattered BMP-2–positive cells were found (Figure 3B). Morphologic characteristics of the BMP-2–positive cells suggested expression in FLS (type B synoviocytes) as well as in macrophages (type A synoviocytes). To further characterize BMP-2–expressing cells, we used 2-color immunofluorescence staining, which showed that both CD90+ cells (FLS) and CD68+ cells (macrophages) stained for BMP-2 (Figures 3C–H). Areas with large lymphoid infiltrates were mostly BMP-2 negative. Similarly, BMP-6 expression was found both in lining and in sublining layer cells (results not shown). Chicken, goat, or mouse IgG was used as a negative control for all stainings.

Up-regulation of BMP gene expression in FLS cultures by TNFα and IL-1β, but not by IFNγ. We further examined the influence of critical proinflammatory cytokines on gene expression of BMPs and TGFβs in FLS cultures. FLS from both nonarthritic and arthritic joints were cultured in 6-well tissue culture plates in growth medium until confluence and were then stimulated for 24 hours with TNFα (10 ng/ml), IL-1β (10 ng/ml), or IFNγ (100 units/ml).

As shown by real-time quantitative RT-PCR analysis, both BMP-2 and BMP-6 expression were up-regulated in the presence of TNFα or IL-1β stimulation, but not with IFNγ (Figure 4). We consistently found BMP-2 and BMP-6 up-regulation by IL-1β or TNFα in all FLS cultures, regardless of whether they originated from nonarthritic, RA, or SpA synovium (Figure 4). (Data are from 12 different experiments in which FLS from 4 different donors were used.) Treatment with 10 ng/ml IL-1β resulted in mean 35.87-fold and 14.50-fold increases, respectively, in BMP-2 and BMP-6 expression levels (both \( P < 0.01 \)), and incubation with 10 ng/ml...
TNFα resulted in mean 17.24-fold and 5.21-fold increases, respectively, in BMP-2 and BMP-6 expression levels \((P < 0.01\) and \(P < 0.05\), respectively) (Figure 4). BMP up-regulation by IL-1β and TNFα was dose-dependent (data not shown). We also studied BMP-4 and BMP-7 regulation by TNFα and IL-1β. No up-regulation of BMP-4 or BMP-7 was seen (data not shown). Overall expression levels of BMP-7 were very low. No significant effects on expression levels of TGFβ1 (Figure 4) or on expression levels of TGFβ2 or TGFβ3 (data not shown) were seen.

To extend these findings, BMP-2 and BMP-6 proteins were examined in FLS cultured on tissue culture slides. FLS were treated with IL-1β (10 ng/ml) for 24 hours. BMP-2 and BMP-6 proteins were clearly detected using immunohistochemistry (Figure 5).

**FLS proliferation stimulated by TGFβ, but not by BMP.** We studied the influence of BMPs and TGFβ on FLS proliferation in vitro after serum deprivation–induced growth arrest. FLS from 5 different patients (1 with OA, 2 with RA, and 2 with SpA) were studied, all

\[\text{Figure 5. Up-regulation of BMP-2 and BMP-6 protein expression by IL-1β in fibroblast-like synoviocytes (FLS). Normal FLS were cultured on tissue culture slides. At confluence, FLS were cultured in growth medium alone (A and C) or were stimulated for 24 hours with 10 ng/ml IL-1β (B and D). BMP-2 (A and B) and BMP-6 (C and D) were visualized by immunohistochemistry. Bars = 25 μm. See Figure 4 for other definitions.}\]

\[\text{Figure 6. Effects of TGFβ1 and BMPs 2 and 6 on fibroblast-like synoviocyte (FLS) proliferation. FLS proliferation was studied by }^{3}\text{H-thymidine incorporation after serum deprivation–induced growth arrest. After 24 hours of serum deprivation (1% serum), cells were stimulated with BMP-2 (100 ng/ml), BMP-6 (100 ng/ml), or TGFβ1 (10 ng/ml) for 48 hours. During the last 24 hours, }^{1}\text{Ci }^{3}\text{H-thymidine was added to the cultures. Values are the mean and SD. FLS from 5 different patients were studied. * = } P < 0.05 \text{ versus unstimulated controls. OA = osteoarthritis; RA = rheumatoid arthritis; SpA = spondylarthropathy (see Figure 4 for other definitions).}\]
between passages 4 and 7. We studied both early (first 24 hours) and late (second 24 hours) effects on proliferation. In contrast to TGFβ1 (10 ng/ml), which consistently increased FLS proliferation after 48 hours (Figure 6), but not after 24 hours (data not shown), no effect of BMP-2 (100 ng/ml) or BMP-6 (100 ng/ml) was seen at 24 hours (data not shown) or 48 hours (Figure 6). No differences were seen with either dosage variation of BMPs or with combinations of BMPs and TGFβ1 (data not shown). We confirmed these data obtained by ³H-thymidine incorporation by measuring DNA content after 24 hours and 48 hours (data not shown).

**Figure 7.** Different effects of bone morphogenetic protein 2 (BMP-2) and BMP-6 on fibroblast-like synoviocyte (FLS) apoptosis. FLS apoptosis was studied by flow cytometry with propidium iodide cell cycle analysis. A, Cells were stimulated with or without BMP-2 (100 ng/ml) or BMP-6 (100 ng/ml) for 20 hours. * = P < 0.05 versus unstimulated controls. B, Cells were pretreated with BMP-2 (100 ng/ml), BMP-6 (100 ng/ml), or carrier control. After 4 hours, sodium nitroprusside (SNP) was added to the cultures for 16 hours. In each experiment, the concentration of SNP used for analysis was chosen in such a way that a 15% increase in apoptotic cells was seen 16 hours after adding SNP. ** = P < 0.05 versus SNP-stimulated cells. Data from 7 different experiments are shown. Horizontal bars are medians.

**Figure 8.** Expression of bone morphogenetic protein 2 (BMP-2) in synoviocytes undergoing apoptosis. A, Double staining showing BMP-2–positive (brown) and TUNEL-positive (blue) cells. Several double-positive cells are seen. B, Superimposed image showing nuclear 4',6-diamidino-2-phenylindole staining, cytoplasmic BMP-2 positivity, and nuclear TUNEL positivity. Bars = 25 μm.
stimulation had no direct effect on FLS apoptosis. However, BMP-6 pretreatment for 4 hours protected FLS from NO-induced apoptosis \( (P < 0.004 \) by Friedman ANOVA; \( P < 0.02 \) by nonparametric Wilcoxon test) (Figure 7B). Appropriate concentrations of SNP (the NO liberator) were chosen, resulting in an increase of \( \sim 15\% \) in apoptotic cells compared with controls. To show specificity of the effect on FLS and to exclude a nonspecific cytotoxic effect of our BMP-2 preparation, we also treated myogenic cell line C2C12 with BMP-2. As recently described, BMP-2 had no direct effect on C2C12 apoptosis, but protected against TNF\( \alpha \)-induced cell death (data not shown) (26).

Expression of BMP-2 in synoviocytes undergoing apoptosis. To examine whether the in vitro effect of BMP-2 on synoviocyte apoptosis may be relevant in vivo, we used immunohistochemistry to study BMP-2 expression in cells undergoing apoptosis (Figure 8). We found BMP-2- and TUNEL-positive cells in both the lining and sublining layer of arthritic synovium. We also found BMP-2-positive, TUNEL-negative cells and BMP-2-negative, TUNEL-positive cells. Our data suggest that BMP-2 expression may precede the process of apoptosis. In the double-positive cells, TUNEL positivity was not covering the whole nucleus. In later stages of apoptosis, the whole cell nucleus was TUNEL positive, and little or no BMP-2 staining was seen, probably due to progression of apoptosis and shrinkage of the cell cytoplasm (Figure 8). Normal IgG were used as negative controls for the double staining.

DISCUSSION

In this study, we demonstrated the expression and presence of specific BMPs in arthritic synovium by RT-PCR and immunohistochemistry. Real-time quantitative RT-PCR for BMP-2 and BMP-6 did not show significant differences between normal and arthritic synovium. Although PCR techniques are extremely sensitive tools for detecting small amounts of mRNA or DNA, the need to normalize gene expression levels to those of a housekeeping gene limits their value in the analysis of complex tissues such as the synovium. Cellularity and cell types may be different from patient to patient due to differences in disease status, disease type, and random sampling. Housekeeping gene expression levels show a large variability between different cell types and therefore often do not allow for a simple comparison. Similarly, alternative normalization to total RNA transcribed in the RT reaction is also influenced by cell types and cellularity as well as by the potential presence of inhibitors of the RT-PCR reaction. In Western blot, however, both BMP-2 and BMP-6 precursor protein was present in extracts from arthritic synovium, but not in extracts obtained from the synovium of control patients. Our immunohistochemistry studies corroborate these findings, showing more BMP-2- and BMP-6-positive cells in arthritic synovium than in nonarthritic control synovium.

Two-color immunofluorescence demonstrated BMP-2 and BMP-6 expression both in type A synoviocytes (macrophages) and in type B synoviocytes (FLS). The expression of BMPs in cells of mesenchymal origin (FLS) as well as in those of hematopoietic origin was not surprising, since BMPs have been reported to play a role in mesenchyme differentiation (15) and hematopoiesis (27). The spatial distribution of BMP-2- and BMP-6-producing cells in the inflamed synovium does not provide specific clues about potential targets. In addition, BMPs are secreted proteins and are thus expected to work both in an autocrine and in a paracrine manner.

Up-regulation of BMP-2 mRNA by IL-1β in OA FLS has previously been reported (28). We extended these findings and added novel information by demonstrating a striking up-regulation of BMP-2 and BMP-6 (but not of BMP-4) by the proinflammatory cytokines TNF\( \alpha \) and IL-1β (but not by IFN\( \gamma \)). This selective modulation suggests a relevant function for these BMPs as autocrine or paracrine peptides in the pathologic microenvironment of chronic arthritis. It is clear that BMPs and proinflammatory cytokines such as IL-1 and TNF\( \alpha \) are part of a tightly controlled network, suggesting that local balances and autocrine or paracrine effects, rather than the overall amount of protein, are the determinants of cell fate and function.

It remains unclear whether BMPs are part of the synovial defense and repair mechanisms, or whether they play a role in disease pathogenesis. Specific BMPs are strongly up-regulated in response to proinflammatory cytokines in vitro. We hypothesize that any tissue damage elicits a response from the body's inherent defense and repair capacity. Reactivation of embryonic signaling pathways essential for tissue growth and differentiation during morphogenesis is likely to be such a mechanism (11). Such phenomena, involving BMPs, have been clearly demonstrated in fracture healing (29).

Cartilage-derived morphogenetic proteins, which are also members of the BMP family, are expressed in the superficial layers of the articular cartilage in normal individuals (30). In OA, however, their expression extends into the deeper cartilage tissue layers of the damaged areas. Their expression is also up-regulated after trypsin treatment, resulting in matrix depletion (30). Local overexpression of BMPs may provide a
metabolic stimulus for chondrocytes and osteoblasts or osteocytes at the site of damage, increasing local resistance. Osteophyte formation in OA may be a partially BMP-driven repair/remodeling effort to increase joint stability after cartilage damage has occurred. Injection of TGFβ1 or BMP-2 into murine joints results in osteophyte formation (19).

BMP-2 has also been shown to up-regulate osteoprotegerin in osteoblastic cells, thereby providing a negative signal for osteoclast differentiation and activation (31,32). However, up-regulation of both receptor activator of NF-κB ligand and cyclooxygenase 2 in osteoblast-like cells has been reported to contribute to osteoclastogenesis in coculture experiments of osteoblast-like cells and hematopoietic cells (33). It is noteworthy that BMP-4 has been reported to act as a strong chemotactic agent for mononuclear cells (34). Local BMP production within the synovium may therefore also contribute to disease pathogenesis and progression.

Although there is striking structural similarity between all members of the superfamily, biologic effects are diverse. Different ligand–receptor affinities, target cell receptor expression, specific intracellular pathway activation, and interactions of the DNA transcription complex with activators and inhibitors define specificity and diversity in signaling (35). In view of this, the contrasting effects of BMP-2, BMP-6, and TGFβ1 on FLS apoptosis and proliferation are not surprising.

Different BMPs have been associated with apoptosis in development (36,37). BMP-2 and the strongly related BMP-4 have been described as proapoptotic molecules in different in vitro settings (38–40). However, in contrast to its effects on osteoblasts (38), BMP-2 has also been reported to inhibit apoptosis in cardiac myocytes (41). BMP-6 protects cortical neural cells in vitro against H2O2-induced cell death and reduces brain ischemia/reperfusion injury in rats (42). In arthritis, BMP-2–induced apoptosis may be a protective mechanism for controlling extensive and aggressive pannus proliferation. The antiapoptotic effect of BMP-6 is not inconsistent with this protective mechanism, since it may protect the synovium from excessive cell death due to accumulation of NO or reactive oxygen molecules. In this respect, the identification of BMP receptor type IA (BMPRIA)–positive cells in arthritic synovium (8) and their potential role in RA pathogenesis may define a specific target cell for BMP-2 signaling in arthritis. It is noteworthy that such cells were not found in OA synovium (6). In the absence of BMPRIB (ALK-6) expression in synovium, as demonstrated by RT-PCR (data not shown), BMP-6 signaling would preferably proceed through activin receptor type Ia (ALK-2) (43). We have not yet identified a specific target cell in the inflamed synovium for BMP-6 signaling, although we are continuing research in this area.

In conclusion, we believe that this is the first report to describe synovial expression of BMP and some of its potential roles in the arthritic synovium. Further research is now warranted to assess the protective and/or pathogenetic role of distinct BMPs in specific forms of arthritis. We propose that BMPs may further tilt the balance between destructive and reparative mechanisms in the joint in favor of tissue repair, not only by promoting cartilage and bone formation, but also by modulating FLS cell populations in the inflamed synovium.

ACKNOWLEDGMENTS

The authors wish to thank all collaborators at the Laboratory for Skeletal Development and Joint Disorders for support and useful discussions. We also thank Drs. René Westhovens, Kurt De Vlam, Patrick Verschueren, and Johan Vanlaeve for providing biopsy materials.

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