Macrophage Cell Lines Produce Osteoinductive Signals That Include Bone Morphogenetic Protein-2

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Bone wound healing requires osteoinductive signals that are attributed to (the) bone morphogenetic proteins (BMPs). The cellular origin of such osteoinductive signals has only been partially elucidated. Because of the central role of the macrophage in cutaneous wound healing, we hypothesized that the macrophage could play a similar role in osseous wound healing. It was the aim of the present investigation to examine the possible expression of BMP by the macrophage, and to evaluate the contribution of macrophage products to an early step of bone formation modeled in an in vitro culture system. The synthesis of BMP-2 and BMP-6 by cultured human and murine macrophages was evaluated by reverse transcription-polymerase chain reaction (RT-PCR). When human mesenchymal stem cells (hMSCs) were grown in conditioned media from J774A.1 cells, alkaline phosphatase expression increased. This induction was blocked by anti-BMP-2 antibody and by anti-transforming growth factor-β1 (TGF-β1) antibody. Modeling of the macrophage expression of osteoinductive signals by potential physiological situations was evaluated by treatments with lipopolysaccharide (LPS) or macrophage chemotactic peptide-1 (MCP-1). Macrophage BMP-2 expression was reduced by proinflammatory LPS stimulation (which was confirmed to induce release of the proinflammatory cytokine, TNF-α), and conditioned media from LPS-treated macrophages had no ability to increase alkaline phosphatase activity in hMSCs. This first study of macrophage BMP-2 expression indicates that the macrophage is capable of physiological regulation consistent with a key role in osteoinduction for osseous wound healing. (Bone 30:26–31; 2002) © 2002 by Elsevier Science Inc. All rights reserved.

Key Words: Macrophage; Bone morphogenetic protein (BMP); Osteoinduction; Osseous wound healing.

Introduction

The initial stages of wound healing involve fibrin clot formation and population by hematopoietic cells including peripheral monocytes that give rise to macrophages. The macrophage plays a pivotal role in wound healing, principally by contributing to wound sterilization and debridement, and by secreting reparative growth factors. Many of these growth factors regulate mesenchymal stem cell recruitment, proliferation, differentiation, and synthesis of extracellular matrix proteins. For example, macrophage transforming growth factor (TGF)-β1 expression regulates fibrosis during cutaneous wound healing. A similar role for the macrophage in osseous wound healing has not been considered.

Osseous wound healing requires the induction of mesenchymal stromal cells to differentiate along the osteoblastic lineage for new bone formation. The central role of bone morphogenetic proteins (BMPs) in osteoinduction is well defined. BMP expression by osteoblastic cells has been demonstrated in vivo and in vitro. In development, BMP expression by nonosteoblastic cells results in osteoinduction and bone morphogenesis. Mesenchymal cells in developing tissues are influenced by BMP prior to any innate ability to produce BMP themselves.

In bone healing, BMP expression has been attributed to the osteoblastic cell. Alternative cellular origins of bone-inducing signals have not been considered. However, immunolocalization of BMP-2 during fracture healing revealed its limited expression in a minimum number of primitive cells of the fracture callus. Osseous wound healing may involve cells other than those of mesenchymal origin in the initial osteoinductive signaling by BMP.

It was the aim of this initial study to determine the macrophage’s ability to produce BMP and to determine if the macrophage could provide osteoinductive signals to promote mesenchymal stem cell differentiation along the osteoblastic lineage. Human and murine macrophage cell lines were used as a model of active macrophages to evaluate BMP expression. The expression of BMP-2 mRNA was associated with BMP-2 specific activity in conditioned media. Anti-BMP-2 antibody inhibited the osteoinductive effects of J774A.1 cell-conditioned media on human mesenchymal stem cell differentiation along the osteoblastic lineage. These findings have many implications for the control of osteogenesis in healing bone.

Materials and Methods

Cell Culture

The human THP-1 and the mouse J774A.1 and RAW264.1 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Inc., Rockville, MD), in a 5% CO2 atmosphere at 37°C. Cells were passaged every third day. For preparation of conditioned media, 2 × 10^6 cells were plated into 20-mm-diameter dishes (J774A.1 and RAW264.1) or T25 culture flasks (THP-1).
containing 10 mL of medium. After overnight growth in complete media, cells were rinsed with phosphate-buffered saline (PBS), and grown in 1% FBS containing media for 24 h. Cells were cultured for an additional 24 h in either 1% FBS medium alone, 1% FBS medium supplemented with 100 ng/mL Escherichia coli 055:B5 lipopolysaccharide (LPS; Sigma Co., St. Louis, MO), or 1% FBS medium supplemented with 10 ng/mL recombinant murine JE/MCP-1 (Monocyte Chemotactic Peptide-1, R&D Systems, Inc., Minneapolis, MN). Culture supernatants were collected and used directly for conditioned media experiments, or stored at −70°C for further enzyme-linked immunoassay (ELISA) analyses. Cells were processed for RNA extraction as indicated in what follows.

 NIH3T3 cells were also cultured in a 5% CO2 atmosphere at 37°C in DMEM containing 10% FBS, passed 1:3 every third day using 0.5% trypsin/ethylene-diamine tetraacetic acid (EDTA), and used to prepare untreated cell-conditioned media as described earlier.

 Human mesenchymal stem cells (hMSCs) were generously provided by Osiris Therapeutics, Inc. (Baltimore, MD). They were cultured in DMEM containing 10% FBS and passed 1:3 upon attaining confluence using 0.05% trypsin. The hMSCs (10,000/well) were then plated in 96 well culture plates. After overnight growth in complete media, cell layers were rinsed three times in cold PBS and subsequently grown for 0–72 h in conditioned media from untreated, LPS-, or MCP-1-treated NIH3T3 cells. Additional cultures were grown in unconditioned media and conditioned media from untreated NIH3T3 cells.

 For blocking experiments monoclonal anti-human BMP-2 antibody (1:100 dilution, R&D Systems) or monoclonal anti-human/mouse/pig TGF-β1 antibody (1:500 dilution, BD Pharmingen, San Diego, CA) were added to the hMSCs cultures at the same time as the conditioned media.

### Alkaline Phosphatase Assay

Following culture of hMSCs in unconditioned or conditioned media, cell layers were rinsed three times with 200 μL of ice-cold PBS and then incubated for 4 h in 100 μL Sigma 104 reagent. The conversion of substrate by alkaline phosphatase was measured at 405 nm using a microtiter plate reader (Model 550, Bio-Rad Laboratories, Inc., Hercules, CA). All samples were blanked against reaction wells previously containing conditioned media alone. Five wells were measured for each experimental condition and timepoint in four separate experiments.

### Table 1. Polymerase chain reaction (PCR) oligonucleotide primer sequences and amplification product sizes

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin forward</td>
<td>5'-ATCTGGCCACCCACTCTCTCTAAATGACTGGG</td>
<td>838 bp</td>
</tr>
<tr>
<td>β-actin reverse</td>
<td>5'-CACACCCTACTGCTGGCTGATCCACATCTGC</td>
<td></td>
</tr>
<tr>
<td>Mouse BMP-2 forward</td>
<td>5'-AACCTGGCAGGATCTTGCCGGCCTG</td>
<td>479 bp</td>
</tr>
<tr>
<td>Mouse BMP-2 reverse</td>
<td>5'-TCTGGCAGATGTTGAAACTCGTCA</td>
<td></td>
</tr>
<tr>
<td>Human BMP-2 forward</td>
<td>5'-AGATTTCGATGGCTGTCGTC</td>
<td>442 bp</td>
</tr>
<tr>
<td>Human BMP-2 reverse</td>
<td>5'-TGAGTCACTAACTCCTGGTGTC</td>
<td></td>
</tr>
<tr>
<td>Mouse BMP-6 forward</td>
<td>5'-AGACTCTGGATGCGAGGCGGG</td>
<td>383 bp</td>
</tr>
<tr>
<td>Mouse BMP-6 reverse</td>
<td>5'-ACCATCCTGCTGTGGCCGCGC</td>
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<tr>
<td>Human BMP-6 forward</td>
<td>5'-GCGAGGAAGGATCGTTCGGCGG</td>
<td>624 bp</td>
</tr>
<tr>
<td>Human BMP-6 reverse</td>
<td>5'-GTCCTCGTGTCTGTCGTCCTCCT</td>
<td></td>
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<tr>
<td>TGF-β1 forward</td>
<td>5'-GACCGTGAGACACCAACTATTGCT</td>
<td>161 bp</td>
</tr>
<tr>
<td>TGF-β1 reverse</td>
<td>5'-AACGCTCCTTGAATGACCCCGGG</td>
<td></td>
</tr>
</tbody>
</table>

**KEY:** BMP, bone morphogenetic protein; TGF, transforming growth factor.

### Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Evaluation of Gene Expression in THP-1, J774A.1, RAW264.1, and NIH3T3 Cells

The expression of BMP-2, BMP-6, TGF-β1, and β-actin were evaluated by RT-PCR using total RNA isolated from THP-1, J774A.1, RAW264.1, or NIH3T3 cells. The sequence of the primers used for these reactions is represented in Table 1. Total RNA was isolated from cell layers or pellets using Tri-reagent (Sigma), based on the single-step method described by Chomczynski and Sacchi.4 RNA was quantified by ultraviolet (UV) spectrophotometry and 3.5 μg total RNA was used for first-strand synthesis reactions using Superscript reverse transcriptase (Life Technologies, Inc., Rockville, MD). One tenth of the resulting cDNA was used for PCRs. Standard 100 μL reactions were prepared and 20–30 cycles of amplification were performed (initial denaturing step for 1 min at 95°C, increasing cycle numbers of 45 sec at 95°C, 45 sec at 60°C, and 2 min at 72°C). At 20, 22, 24, 26, 28, and 30 cycles, 10 μL aliquots were removed and evaluated by UV transillumination of the electrophoretic pattern in 1.5% agarose gels stained with ethidium bromide. The images were digitally captured and evaluated using a LUMI-IMAGER F1 workstation (Roche Molecular Biochemicals, Indianapolis, IN). The relative intensity of each band was determined and plotted as the relative abundance of BMP-2, BMP-6, or TGF-β1 amplification product/β-actin amplification product abundance vs. cycle number. Each set of RT-PCR evaluations was repeated for each target mRNA using three separate cultures.

### Measure of TNF-α Expression in J774A.1 Cell Culture Supernatants

The secretion of TNF-α into J774A.1 cell-conditioned media was measured using an ELISA assay (mouse TNF-α Cyto- screen from BioSource International, Inc., Camarillo, CA). Cells were untreated or treated with 100 ng/mL E. coli 055:B5 LPS or 10 ng/mL mouse MCP-1 for 24 h. Conditioned media from five separate experiments were tested in duplicate wells undiluted (medium alone and untreated cultures), diluted 1:2 to 1:20 (MCP-1-treated cells), or diluted 1:100 to 1:500 (for LPS-treated cultures), following the manufacturer’s standard procedures.
Results are expressed as mean \pm standard deviation. Values obtained from untreated cells were compared with values obtained from LPS- or MCP-1-treated cells using the t-test with one-way analysis of variance (ANOVA). \( p < 0.05 \) was considered statistically significant.

**Results**

**Pro-osteogenic Cytokine Expression in Macrophages**

The expression of BMP-2 mRNA was revealed by the RT-PCR evaluation of total RNA isolated from untreated human and murine macrophage cell lines grown in the presence of 1% FBS (Figure 1, top panels, lanes 2). The murine macrophage cell lines J774A.1 and RAW264.1 also expressed BMP-6, although at a lower level than BMP-2 (Figure 1B,C, middle panels). No BMP-6 expression was detected in the human macrophage cell line THP-1 (Figure 1A, middle panel). Moreover, BMP-7 expression was not observed in any of these macrophage cell lines (data not shown).

LPS treatment (100 ng/mL) resulted in a reduction of BMP-2 expression in all three macrophage cell lines (Figure 1, upper panels, lanes 4 compared with 2). Similarly, LPS treatment abolished BMP-6 expression in the two murine macrophage cell lines (Figure 1, middle panel, lanes 4 compared with lanes 2). In the murine J774A.1 cells, LPS treatment led to an 80% reduction in BMP-2 expression (Figure 2A and Figure 3A). When the cells were treated with 10 ng/mL MCP-1, BMP-2 expression was slightly reduced in the murine macrophage cell lines (Figure 1, upper panels, lanes 3 compared with lanes 2). NIH3T3 cells grown under similar conditions failed to express BMP-2 mRNA (Figures 2B and 3A). MCP-1 treatment had little effect on BMP-6 expression (Figure 1, middle panels). A 20% reduction was noted when MCP-1 was included in the culture media of J774A.1 cells (Figures 2A and 3A).

Treatment of J774A.1 cells with LPS or MCP-1 resulted in different TGF-\( \beta \)-mRNA expression. When compared with NIH3T3 cell results, RT-PCR resulted in similar levels of the TGF-\( \beta \)-specific amplicon after 30 cycles (Figures 2A and 3B). Treatment of J774A.1 cells with MCP-1 increased the amount of TGF-\( \beta \)-specific amplicon present after 26 PCR cycles (Figures 2A and 3B). LPS treatment reduced by one-half the abundance of TGF-\( \beta \)-specific amplicon (Figures 2A and 3B).

**J774A.1 Macrophage-conditioned Media Induces Alkaline Phosphatase Expression in hMSCs**

The induction of alkaline phosphatase activity in hMSC cultures was used as a rapid in vitro test for the presence of osteoinductive cytokines in J774A.1-conditioned media. When hMSCs were cultured in unconditioned media, a low level of endogenous alkaline phosphatase activity was measured (Figure 4). This level did not change over 4 days of culture. The addition of conditioned media from untreated J774A.1 cells increased alkaline phosphatase activity measured at days 1–4 (Figure 4). This increase was in contrast to the absence of any increased alkaline phosphatase activity in hMSCs cultured in the presence of conditioned media from LPS-treated J774A.1 cells (Figure 4). MCP-1 treatment of J774A.1 cells did not alter the effect of J774A.1 cell-conditioned media on the induction of alkaline phosphatase expression in the hMSCs (Figure 4). Treatment of
hMSCs with NIH3T3 cell-conditioned media did not increase hMSCs alkaline phosphatase expression.

**J774A.1 Cell-conditioned Media Effects on Osteoinduction Are Mediated by BMP-2**

To begin to understand which of the specific cytokines secreted by J774A.1 cells were responsible for the induction of alkaline phosphatase activity in the hMSCs, blocking experiments were performed using anti-BMP-2- and anti-TGF-β1-specific antibodies. The addition of anti-BMP-2-specific antibody effectively eliminated the induction of hMSC alkaline phosphatase expression at days 1–4 (Figure 5). The addition of anti-TGF-β1 antibody also prevented the induction of alkaline phosphatase expression (Figure 5). There was no change from the low baseline levels of alkaline phosphatase expression in hMSCs when unconditioned media were used or when antibodies to BMP-2 or TGF-β1 were added to the unconditioned media.

**Proinflammatory Response of J774A.1 Cells**

To confirm that the LPS treatment of J774A.1 cells—which reduced BMP-2 expression—also induced a classical proinflammatory response in this macrophage cell line, we measured TNF-α secretion by ELISA. As expected, the expression of TNF-α was increased nearly 150-fold by the addition of LPS to J774A.1 cultures. MCP-1 treatment resulted in a measurable, but minor (twofold), increase in TNF-α secretion (Table 2).

**Discussion**

The main finding of this preliminary investigation was that macrophage cell lines produce BMPs. BMP-2 mRNA expression was readily indicated by RT-PCR amplification from total RNA of both human and mouse macrophage cell lines, whereas BMP-6 mRNA expression was detected only in murine macrophage cell lines. Conditioned media from J774A.1 cultures increased hMSC alkaline phosphatase expression (Figure 3), an initial step during in vitro differentiation toward osteoblasts. BMP-2 is a key regulator of bone morphogenesis and several other organ systems during embryonic development. Its role in bone formation is clear and its ability to act as an osteoinductive agent when targeting progenitor cell populations has been demonstrated repeatedly in vivo and in vitro. The importance of BMP-2 in the hMSC response to culture supernatants from J774A.1 cells was indicated by the successful blocking of this induction by anti-BMP-2 antibody (Figure 4). The induced differentiation of this hMSC culture system is well documented, whereas Detmer et al. evaluated BMP expression among hematopoietic cells and have identified placent bone morphoge-
Statistically significant differences were evaluated by t-test and are indicated (*p < 0.05, **p < 0.01).![](image)

Table 2. Levels of TNF-α released from J774A.1 cultured cells

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>TNF-α levels (pg/ml)</th>
<th>p values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% FBS medium</td>
<td>&lt;10.0</td>
<td></td>
</tr>
<tr>
<td>J774A.1 conditioned medium</td>
<td>59.4 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>J774A.1 + LPS conditioned medium</td>
<td>8974.0 ± 309.1</td>
<td>0.0156</td>
</tr>
<tr>
<td>J774A.1 + MCP-1 conditioned medium</td>
<td>110.3 ± 0.3</td>
<td>0.0048</td>
</tr>
<tr>
<td>NIH3T3 conditioned medium</td>
<td>&lt;10.0</td>
<td></td>
</tr>
</tbody>
</table>

*Cells were preincubated overnight in 1% FBS-containing medium, and further incubated for an additional 24 h in either fresh 1% FBS-containing medium, or 1% FBS medium + 100 ng/mL E. coli LPS, or 1% containing medium + 10 ng/mL MCP-1.

**TNF-α levels were determined by enzyme-linked immunoassay in cell culture supernatants diluted appropriately. Values represent means ± SD of duplicate wells, expressed in picograms per milliliter, from one experiment that was representative of five.

***TNF-α levels measured in conditioned medium from stimulated J774A.1 cells were compared with levels measured in conditioned medium from J774A.1 resting cells by t-test, and calculated p values are reported.

Figure 5. Kinetics of blocking by anti-BMP-2 and anti-TGF-β1 monoclonal antibodies of alkaline phosphatase activity in hMSCs incubated with J774A.1 cell-conditioned media. hMSCs were preincubated for 24 h in 1% FBS-containing medium, and further incubated for 24, 72, or 96 h in the presence of fresh 1% FCS medium (open bars), conditioned medium from J774A.1 cells incubated overnight in 1% FBS medium (closed bars), fresh 1% FBS medium + anti-BMP-2 antibody (vertical hashed bars), conditioned medium from J774A.1 cells incubated overnight in 1% FBS medium + anti-BMP-2 antibody (horizontal hashed bars), fresh 1% FBS medium + anti-TGF-β1 antibody (oblique ascending hashed bars), or conditioned medium from J774A.1 cells incubated overnight in 1% FBS medium + anti-TGF-β1 antibody (oblique descending hashed bars). At the indicated timepoints, culture supernatants were removed and replaced by alkaline phosphatase substrate. Alkaline phosphatase activity was evaluated after a 4 h developing time by measuring optical density at 405 nm. Plotted values represent mean ± SD of five replicate wells from one representative experiment out of four. Statistically significant differences were evaluated by t-test and are indicated (*p < 0.05, **p < 0.01).!

Figure 6. Putative pathways of macrophage phenotypic development and role in osseous wound healing (adapted from Riches17). The extravasation and subsequent differentiation of the monocyte is dependent on environmental factors that include inflammatory stimuli such as LPS. Bacterial LPS promotes an inflammatory macrophage phenotype that can direct local inflammatory processes by abundant TNF-α production. The exposure of monocytic cells to the osteoprotegerin ligand (OPGL) produced by osteoblastic cells and T lymphocytes promotes osteoclastogenesis and bone resorption. A third possible fate is a wound healing-associated phenotype in which TNF-α expression is low or even absent and morphogenetic proteins such as TGF-β1 and BMP-2 are secreted by the macrophage to promote wound healing and bone regeneration. Other activities beyond osteoinduction, including increasing cell migration and promoting angiogenesis, may be attributed to BMPs, and underscore the suggested importance of BMP expression by wound healing macrophages. The factors affecting the pivotal role of the macrophage in wound healing require elucidation.
BMP expression by macrophages may be contributory or even essential in this osseous wound healing process. During development and repair, osteoblastic cells are not the first cells to produce BMPs. In fact, nonmesenchymal cells composing the developing epithelium are a source of BMPs. The macrophage is a good candidate as an early and nonmesenchymal source of BMP because it is present in all tissues, it is recruited in the early blood clot, and it has been shown to be present at alloplastic surfaces shortly following placement into endosseous environments.

The macrophage has been previously suggested to play a negative role in osseous wound healing. Particular materials that are shed proximal to endosseous implant materials and joint prostheses result in fibrosis and bone loss. The macrophage, as part of the body’s response to foreign particles, has been implicated as a central mediator of cellular responses to particulate materials. Whereas other cells also respond negatively to particulate materials, the induction of inflammatory cytokines and fibrosis-promoting growth factors by stimulated macrophages suggests a prominent negative or osteolytic effect for the proinflammatory macrophage.

The present preliminary report suggests that the macrophage may also contribute osteoinductive and osteogenic cytokines to the osseous wound healing environment. This may be particularly important in the context of macrophage-mediated responses to alloplastic materials used for bone fixation, osseointegration, or bone regeneration. In fact, the macrophage is one member of the cell population that can adhere to endosseous implants. Its response to autogenous or alloplastic endosseous materials may contribute to the biochemical and cellular context for wound healing. Macrophage elaboration of BMP-2 would promote osteogenesis during aseptic wound healing. Macrophage elaboration of TNF-α would elicit an inflammatory response and fibrosis. Both possibilities are relevant to the clinical challenge of osseous wound healing, particularly with regard to implanted biomaterials, and warrant further investigation.

References

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