OP-1 (BMP-7) stimulates osteoprogenitor cell differentiation in the presence of polymethylmethacrylate particles

Shawn Kann, Richard Chiu, Ting Ma, Stuart B. Goodman
Department of Orthopaedic Surgery, Stanford University Medical Center, Stanford, California

Received 31 January 2009; revised 14 July 2009; accepted 21 July 2009
Published online 22 February 2010 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.32712

Abstract: Polymethylmethacrylate (PMMA) particles have been shown to inhibit the differentiation, proliferation, and mineralization of osteoprogenitor cells in vitro. In this study, we investigated the effects of OP-1 (BMP-7) on the osteogenesis of MC3T3-E1 osteoprogenitor cells exposed to PMMA particles in vitro. MC3T3-E1 cells challenged with PMMA particles on the 1st day of differentiation in osteogenic culture showed a significant dose-dependent decrease in mineralization and alkaline phosphatase expression over a 20-day culture period. Exposure of these cells to OP-1 (200 ng/mL) during days 1–4, 1–20, and 4–20 in the presence of PMMA particles resulted in significant increases in mineralization and alkaline phosphatase expression at all particle doses. Addition of OP-1 to MC3T3-E1 cultures challenged with PMMA particles on the 4th day of differentiation in osteogenic media also resulted in significant increases in mineralization and alkaline phosphatase expression. This study has shown that OP-1 stimulates osteogenesis in MC3T3-E1 osteoprogenitor cells that have been inhibited by PMMA particles. Local administration of OP-1 to the site of osteolysis may be a potential adjunctive therapy to reverse the bone destruction due to wear particles. © 2010 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 94A:485–488, 2010

Key Words: osteogenic protein-1, bone morphogenetic protein-7, osteoprogenitor differentiation, MC3T3-E1 cells, polymethylmethacrylate particles

INTRODUCTION
Periprosthetic osteolysis in total joint replacements is mediated by bone destruction inflammatory cells and osteoclasts in the presence of orthopaedic wear debris.1 Recent studies have shown that wear debris also inhibits the osteogenesis of murine osteoprogenitors and human mesenchymal stem cells (MSCs) in vitro.2–4 Given that osteoprogenitors and MSCs are the precursors to bone cells, their differentiation is essential to bone regeneration and the longevity of total joint replacements.

Bone morphogenetic protein-7 (BMP-7), also known as osteogenic protein-1 (OP-1), is a member of the transforming growth factor superfamily of proteins that induces bone formation. The binding of OP-1 to surface cell receptors leads to phosphorylation of Smads 1 and 5, which complex with Smad4 to activate Runx2, the primary transcription factor that regulates osteoblast differentiation, maturation, and matrix mineralization.5 Because of its osteoinductive properties, OP-1 has been used as a therapeutic agent for treating fractures, promoting spine fusion, and enhancing osteogenesis in bone grafts.

Previous studies have shown that polymethylmethacrylate (PMMA) particles inhibit the osteogenic differentiation of MC3T3-E1 osteoprogenitor cells with respect to mineralization and alkaline phosphatase expression in vitro.6 We hypothesized that the addition of OP-1 to osteoprogenitor cultures could stimulate osteogenesis by increasing mineralization and alkaline phosphatase expression. In this study, we exposed MC3T3-E1 osteoprogenitor cells challenged with PMMA particles to OP-1 at different time points and analyzed the time-dependent effects of this growth factor at various stages of cellular differentiation.

MATERIALS AND METHODS
MC3T3-E1 cultures
MC3T3-E1 subclone 14 osteoprogenitor cells (Cat# CRL-2594; American Type Culture Collection, Manassas, VA) were grown in 12-well plates in nonosteogenic medium composed of ascorbic acid-free alpha minimum essential medium (α-MEM; Cat# 11,075-044; Invitrogen-Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin (Cat# 15,140-122; Invitrogen-Gibco), 100 μg/mL penicillin, and 100 μg/mL streptomycin (Cat# 15,140-122; Invitrogen-Gibco) for 4 days. Cells were then grown in osteogenic medium consisting of α-MEM (Cat# 12,571-053; Invitrogen-Gibco) containing 50 μg/mL ascorbic acid (Cat# A4544; Sigma, St. Louis, MO) and 10 mM β-glycerophosphate (Cat# G9891; Sigma, St. Louis, MO) for 20 days to promote osteogenic differentiation.

PMMA particles
PMMA particles (Cat# 19,130; Polysciences, Warrington, PA) 1–10 μm in diameter (mean 6 μm, SD 1.8 μm) were washed 3 × 10 min in 70% ethanol followed by incubation in 70% ethanol overnight. Thereafter, the particles were washed 4 × 10 min with PBS and concentrated to form a final stock

Correspondence to: S. B. Goodman; e-mail: goodbone@stanford.edu
Contract grant sponsors: Ellenberg Chair in Surgery (Stanford University), The Khosla/Santosh Foundation

© 2010 WILEY PERIODICALS, INC.
suspension in PBS. Endotoxin presence of particles were tested using the final PBS washout and the Limulus Amoebocyte Lysate kit (Cat# QCL-1000; BioWhittaker, Walkersville, MD). Endotoxin was undetectable for all the particle samples.

**Von Kossa staining and alkaline phosphatase activity assay**

The amount of mineralization in cultures was determined by the von Kossa method. Cells were washed with PBS and fixed with 10% buffered formaldehyde for 30 min. Cells were then rinsed with distilled water and subsequently incubated in 5% silver nitrate under UV light for 1 h. Digital photos of the stained culture plates were taken and the area of stained matrix in each well was measured using NIH 1.62f Imaging software and expressed as a percentage of the total area. Alkaline phosphatase was extracted by lysing each well of adherent cells in 1.5 mL of 0.5% Triton-X (Cat# X-100; Sigma) for 1 h at room temperature with shaking. Alkaline phosphatase activity levels in lysate supernatant were then measured using the QuantiChrome™ Alkaline Phosphatase Assay Kit (Cat# DALP-250; BioAssay Systems, Hayward, CA).

**Effects of OP-1 on MC3T3-E1 osteogenesis**

MC3T3-E1 cells were challenged with PMMA particles at doses of 0.000, 0.075, 0.150, and 0.300% v/v on the 1st day (day 1) of differentiation in osteogenic medium, with mineralization and alkaline phosphatase expression measured after 20 days of osteogenic culture. Cells were treated with OP-1 (Stryker, Kalamazoo, MI) at a dose of 200 ng/mL during the following days of particle treatment: (1) days 1–20, (2) days 4–20, and (3) days 1–4. Control cells were challenged with particles throughout this 20-day period but not exposed to OP-1. In a separate experiment, MC3T3-E1 cells were exposed to OP-1 (200 ng/mL) from days 1–20 of osteogenic culture but were treated with PMMA particles on the 4th day (rather than the 1st day) of differentiation. Control cells were treated with particles on the 4th day of differentiation but not exposed to OP-1. Mineralization and alkaline phosphatase expression were measured at the end of the 20-day culture period.

**Statistical analysis**

ANOVA and Fisher’s post hoc tests were performed for statistical analysis. *p*-values less than 0.05 were considered significant.

**RESULTS**

**Addition of OP-1 to MC3T3-E1 cells resulted in increased mineralization and alkaline phosphatase expression at all particle doses**

MC3T3-E1 cells treated with PMMA particles on the 1st day of differentiation in osteogenic media showed a dose-dependent decrease in mineralization and alkaline phosphatase expression [Fig. 1(A,B,C)]. With exposure to OP-1, these cells showed significant increases in mineralization [Fig. 1(C)] and alkaline phosphatase expression at all particle doses [Fig. 1(A,B)]. Exposure of cells to OP-1 from days 1–20 of culture resulted in a 70.8, 87.1, 118.9, and 151.9% increase in mineralization and a 42.7, 40.8, 34.2, and 184.7% increase in alkaline phosphatase expression at particle doses 0.000, 0.075, 0.150, and 0.300% v/v, respectively [Fig. 1(A,B)]. Exposure of cells to OP-1 from days 4–20 of culture resulted in a 61.5, 71.1, 102.9, and 175.9% increase in mineralization [Fig. 1(C)] and a 26.8, 30.3, –20.6, and 38.2% increase in alkaline phosphatase expression, respectively, at the particle doses listed earlier [Fig. 1(A,B)]. Exposure of cells to OP-1 from days 1–4 of culture resulted in a 65.5, 71.1, 67.4, and 125.7% increase in mineralization [Fig. 1(C)] and a 13.5, 23.3, –13.8, and 38.2% increase in alkaline phosphatase expression, respectively, at the particle doses listed earlier [Fig. 1(A,B)]. At each particle dose, the increases in mineralization and alkaline phosphatase were similar across cell groups treated with OP-1 at different time periods [Fig. 1(A,B)].

MC3T3-E1 cells treated with PMMA particles on the 4th day of differentiation in osteogenic media showed a dose-dependent decrease in mineralization (Fig. 2) and alkaline phosphatase expression, with significant reductions in both parameters observed at particle doses 0.150 and 0.300% v/v [Fig. 2(A,B)]. Exposure of these cells to OP-1 starting from the 1st day of differentiation in osteogenic media (days 1–20 of culture) resulted in a significant 71.7, 69.8, 61.5, and 125.2% increase in mineralization [Fig. 2(C)] and a 23.7, 21.6, 23.4, and 21.3% increase in alkaline phosphatase expression at particle doses 0.000, 0.075, 0.150, and 0.300% v/v, respectively (Fig. 2).

**DISCUSSION**

This study has demonstrated that OP-1 stimulates osteogenesis in MC3T3-E1 osteoprogenitor cells that have been inhibited by PMMA particles in vitro. The addition of OP-1 to the cultures throughout the entire osteogenic period resulted in significant increases in mineralization and alkaline phosphatase expression at all particle doses (Fig. 1). MC3T3-E1 cells exposed to OP-1 only during the first 4 days of culture showed similar levels of increased mineralization and alkaline phosphatase expression as cells exposed to OP-1 during the entire 20-day culture period or during days 4–20 of culture (Fig. 1). This suggests that the first 4 days of osteogenic differentiation is a sufficient time window for osteoprogenitor cells to respond to the stimulatory effects of OP-1. OP-1 also significantly increased mineralization and alkaline phosphatase expression in MC3T3-E1 cells that were challenged with PMMA particles on the 4th day of differentiation (Fig. 2), which indicates that particle exposure at later stages of differentiation does not affect the response of osteoprogenitor cells to OP-1.

Initial bone formation is a three-step process. First, within the bone marrow, MSCs are exposed to growth factors that initiate the commitment process of these cells to the osteoblast lineage. The mesenchymal cells differentiate into proliferating preosteoblasts. Thereafter, these cells mature into osteoblasts that produce bone matrix. Finally, the matrix mineralizes, encasing the osteoblasts. BMP-7/OP-
FIGURE 1. Stimulatory effects of OP-1 on MC3T3-E1 osteoprogenitor cells with respect to (A) mineralization and (B) alkaline phosphatase expression. (C) Representative pictures of the Von Kossa stained cell culture plates. MC3T3-E1 cells were challenged with PMMA particles at concentrations of 0.000, 0.075, 0.150, and 0.300% v/v for 20 days, starting from the 1st day of differentiation in osteogenic medium. Cells were exposed to OP-1 during days 1–20 (light gray bar), days 4–20 (dark gray bar), and days 1–4 (black bar) of differentiation in osteogenic medium. Control cells (white bar) were not exposed to OP-1. $N = 3$, $*p < 0.05$ compared to control (white bar) not exposed to OP-1 within the same particle dosage group. $**p < 0.05$ compared to control (white bar) of the 0% group not challenged with particles. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

FIGURE 2. Stimulatory effects of OP-1 on MC3T3-E1 osteoprogenitor cells at a more advanced stage of differentiation with respect to (A) mineralization and (B) alkaline phosphatase expression. (C) Representative pictures of the Von Kossa stained cell culture plates. MC3T3-E1 cells were challenged with PMMA particles at concentrations of 0.000, 0.075, 0.150, and 0.300% v/v on the 4th day of differentiation in osteogenic medium. Cells were either exposed to OP-1 throughout the entire osteogenic period (days 1–20, black bar) or not (control cells, light gray bar). $N = 3$, $*p < 0.05$ compared to control (light gray bar) not exposed to OP-1 within the same particle dosage group. $**p < 0.05$ compared to control (light gray bar) of the 0% group not challenged with particles. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
1 plays a key role in the transformation of uncommitted MSCs into osteoblasts by recruiting stem cells from the surrounding tissue and to initiate the bone formation process. Recent clinical trials have shown OP-1 to be an effective therapeutic treatment for fracture healing and repair of musculoskeletal tissues. The addition of OP-1 to open tibial fractures resulted in significantly fewer delayed unions and nonunions at 6 months compared with controls without OP-1. For distal tibial fractures, the addition of OP-1 decreased mean time for fracture union and was associated with less average time of absence from work. Furthermore, the addition of OP-1 has proved useful as an adjunct in lumbar fusion.

In this study, exposure of cells to OP-1 from days 1 to 20 of culture resulted in a 70.8, 87.1, 118.9, and 151.9% increase in mineralization and a 42.7, 40.8, 34.2, and 184.7% increase in alkaline phosphatase expression at particle doses 0.000, 0.075, 0.150, and 0.300% v/v, respectively (Fig. 1). Exposure of cells to OP-1 from days 4 to 20 of culture resulted in a 61.5, 71.1, 102.9, and 175.9% increase in mineralization and a 26.8, 30.3, –20.6, and 38.2% increase in alkaline phosphatase expression respectively at the particle doses listed earlier (Fig. 1). Exposure of cells to OP-1 from days 1 to 4 of culture resulted in a 65.5, 71.1, 67.4, and 125.7% increase in mineralization and a 13.5, 23.3, –13.8, and 38.2% increase in alkaline phosphatase expression respectively at the particle doses listed earlier (Fig. 1).

When challenged with PMMA particles, a dose-dependent decrease in mineralization and alkaline phosphatase is observed reflecting inhibition of MC3T3-E1 osteoprogenitor cell differentiation and maturation. The addition of OP-1 has been shown to increase mineralization and alkaline phosphatase expression in the present experiments. However, these latter effects do not follow a strict dose-response curve, as the effects of growth factors are known to be pleiotropic.

In this experiment, the addition of OP-1 was successful in mitigating the direct adverse effects of PMMA particles on osteoprogenitor cells. However, in the in vivo situation, wear particles stimulate proinflammatory cytokine release from many cell lines including macrophages, fibroblasts, and others. It is not known whether osteolysis could be prevented by the addition of growth factors such as OP-1 in the presence of pro-inflammatory cytokines released by these other cell types. In other words, it is possible that the proinflammatory cytokines released from other cell lines might downregulate osteoprogenitor cell proliferation, differentiation, and maturation such that the addition of growth factors such as OP-1 would be less efficacious than in this study. Mixed culture studies to include osteoprogenitors and other cell types such as macrophages and fibroblasts exposed to PMMA particles with and without the addition of OP-1 would help resolve this issue. Periprosthetic osteolysis is the result of both increased bone destruction and decreased bone formation secondary to wear debris. Therapeutic strategies to modulate this adverse reaction to wear particles would prove useful in maintaining and reconstituting bone stock. In this respect, this study has shown that OP-1 enhances differentiation and maturation of osteoprogenitor cells challenged with PMMA particles. The local administration of OP-1 to the site of particle-induced periprosthetic osteolysis may be a useful adjunct in mitigating the adverse effects of particles.

ACKNOWLEDGMENT

The OP-1 was a generous gift from Stryker Biotech.

REFERENCES