Bone Morphogenetic Proteins Induce Osteoblast Apoptosis in a Maturation State-Dependent Manner

INTRODUCTION
Bone morphogenetic proteins (BMPs) are a family of proteins known to induce bone and cartilage formation and enhance osteogenesis. BMP2 is used clinically in orthopaedic applications to induce bone formation in challenging non-union fractures and large bony defects, as well as for spinal fusion. Clinically, a large (on the order of milligrams), bolus dose of BMP2 must be delivered to the desired site. However, recent incidences of complications after BMP2 treatment have been reported, including soft tissue swelling, ectopic bone formation, and even resorption of the adjacent vertebral bodies. Because of the effectiveness of the treatment, it is important to attempt to characterize mechanism of the deleterious effects to optimize treatment with these proteins.

In both mesenchymal and osteoblastic cell lines, it has been demonstrated that BMPs are able to induce expression of BMP antagonists, including Cerberus, Noggin, DAN, and Gremlin. Noggin is the most well studied of these inhibitors, and is known to regulate the action of both BMP2 and BMP4 by sequestering the BMP, blocking its attachment to its specific receptors, thus inhibiting the BMP signaling cascade. The aim of this study was to determine the effect of BMP2 and BMP4 on survival of cells in different stages of maturation: mesenchymal stem cells, pre-osteoblasts, and mature osteoblasts.

METHODS
Human mesenchymal stem cells (HMSCs), human osteosarcoma MG63 cells (which represent an immature osteoblast phenotype), and normal human osteoblasts (NHOst) (mature osteoblasts) were cultured to confluence. At confluence, cells were treated with 50, 100, or 200ng/ml rhBMP2 or rhBMP4. After 12h of treatment, RNA was harvested and expression of BMPs (BMP2, BMP4), BMP inhibitors (NOG, NBL1, GREM1), and apoptotic pathway molecules (BAX, BCL2, p53) were measured by real-time qPCR and normalized to GAPDH. After 24h of treatment, cells were harvested for quantitative colorimetric in situ TUNEL assay or caspase-3 activity assay. Finally, a model system of increased endogenous BMPs was created by generating MG63 cells with knocked-down NOG expression (shNOG). For these experiments, TUNEL, MTT, total DNA content, caspase-3 activity, expression of BAX and BCL2, and BMP2 and BMP4 production were measured over seven days. Data presented are the mean±SEM of n=6 independent cultures per variable. Statistical significance was determined by ANOVA followed by Bonferroni’s modification of Student’s t-test.

RESULTS
BMP treatment of HMSCs did not affect TUNEL; caspase-3 activity was increased only at 200ng/ml BMP2. The ratio of BAX/BCL2 expression decreased in HMSCs treated with 50 and 100ng/ml BMP2, but was not affected at 200ng/ml. BMP4 increased BAX/BCL2 at 50ng/ml but had no effect at higher doses. Expression of p53 decreased in a dose-dependent manner after BMP2 treatment; 50ng/ml BMP4 decreased p53 expression, but there was no effect at either of the higher doses.

Treatment with BMP2 increased the BAX/BCL2 ratio in MG63 at the highest treatment dose (200ng/ml). However, BMP4 increased BAX/BCL2 at both 100 and 200ng/ml doses.

In NHOst, BMP2 increased caspase-3 activity and TUNEL at all doses, and BMP4 increased caspase-3 activity at all doses. Both BMP2 and BMP4 decreased p53 expression in a dose-dependent manner. The ratio of BAX/BCL2 increased in NHOst treated with BMP4 in a biphasic manner, with the highest ratio at 100ng/ml. However, treatment with BMP2 dose-dependently increased BAX/BCL2, with an 8-fold increase in expression in cells treated with 200ng/ml when compared to control.

Expression of BMPs (BMP2 and BMP4) and BMP inhibitors GREM1, NBL1, and NOG were regulated by BMP treatment in HMSCs, MG63, and HOBs, but the effect was dependent on the dose and cell type.

shNOG-MG63 cells, a cell line with reduced levels of noggin and therefore continuous exposure to high levels of BMP2 and BMP4, increased apoptosis two days after plating, as demonstrated by an increase in TUNEL and caspase-3 activity over WT MG63 cells. Apoptosis in shNOG-MG63 reached a peak at four days. This was confirmed by MTT, total DNA content, and BAX/BCL2.

DISCUSSION
This study demonstrates that BMP2 and BMP4 modulate cell survival in a cell-dependent manner. In HMSCs, treatment with these growth factors has only small effects on apoptotic signaling, but has a strong osteogenic effect as was shown before. MG63 cells are sensitive to treatment with BMP2 and BMP4, but BMP4 is more potent in inducing apoptosis. While we hypothesize this is due to their immature osteoblast phenotype, it is important to consider the osteosarcoma origins of this cell line. Interestingly, treatment with BMP2 induces expression of BMP4, which may then induce cell apoptosis in immature osteoblasts. This hypothesis is further supported by the high levels of BMP4 in shNOG-MG63 cells in comparison to WT cells throughout the culture period. However, in NHOst, these growth factors induce apoptosis depending on their dose, an important fact given the exceedingly high doses used clinically.

Taken together, the results suggest that the effect of BMP treatment on osteoblast and progenitor apoptosis depends on the maturation state of the cell. While in progenitor cells BMP2 and BMP4 have minimal effects on cell apoptosis, mature osteoblasts these potent factors induce apoptosis, especially at higher doses.

Figure 1. Ratio of expression of BAX/BCL2 measured by Real-time PCR in HMSCs (A), MG63 (B), and NHOst (C) treated with doses of BMP-2 (left bar) or BMP4 (right bar). *p<0.05 vs. control.

Figure 2. TUNEL of MG63 and shNoggin-MG63 cells throughout six days in culture. shNoggin-MG63 cells are a model for high endogenous BMP levels in the presence of low levels of Noggin. *p<0.05 vs. MG63; #p<0.05 vs day 1.

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