

RECOMBINANT HUMAN BONE MORPHOGENETIC PROTEIN-2 RESCUES DEXAMETHASONE-INHIBITED MINERALIZATION IN MC3T3-E1 OSTEOBLAST CULTURES

*Luppen, C A; *Smith, E; **Spevak, M; **Boskey, A L; +*Frenkel, B
+*University of Southern California Keck School of Medicine, Los Angeles, CA

INTRODUCTION: Glucocorticoids (GC) are frequently prescribed for the treatment of autoimmune and inflammatory diseases and are known to induce bone loss. GC have both direct and indirect adverse effects on bone metabolism, especially on osteoblasts. Previous studies have shown that GC inhibit osteoblast cell cycle, collagen synthesis, alkaline phosphatase activity, expression of bone phenotypic genes and mineral deposition. Bone morphogenetic protein 2 (BMP-2), a member of the TGF β superfamily, is known to induce osteoblast differentiation and bone formation. To gain insight into the potential anti-GC actions of BMP-2 in osteoblasts, we studied which of the inhibitory effects of GC are reversed by rhBMP-2.

METHODS: A robustly mineralizing subclone of the MC3T3-E1 cell line was used in this study. Cells were cultured in 12 well plates for histological and biochemical assays and in 100 mm plates for cell cycle analysis as well as for the preparation of protein and RNA extracts. The cells were maintained in α -MEM medium with 10% fetal bovine serum and 1% penicillin/streptomycin. At 80% confluency (day 3), the culture medium was supplemented with 50 μ g/ml ascorbic acid and 10 mM β -glycerophosphate to support differentiation. Treatment with dexamethasone (DEX; 1 μ M) and/or rhBMP-2 (10ng/ml or 100ng/ml) was initiated at 80% confluency and continued for the duration of the experiment (14 days). Calcium deposition was demonstrated histologically by Alizarin Red staining of ethanol-fixed cultures. Calcium ions in the cell layer were quantitated by Sigma procedure no. 587 and corrected for total DNA. Phosphate deposition was determined by von Kossa staining. The mineral was characterized using x-ray diffraction and Fourier Transform Infrared Spectroscopy (FTIR). Cell cycle progression was estimated by flow cytometry analysis of ethanol-fixed propidium iodide-stained cells. Collagen accumulation was evaluated by Sirius Red staining. Alkaline phosphatase (AP) activity was measured by Sigma procedure no. 104 and corrected for total cellular DNA. mRNA levels for α 1(I) collagen, osteocalcin and Cbfa1 were measured as the incorporation of radionucleotides during reverse transcription PCR (RT-PCR) reactions using gene-specific primers. The RT-PCR products were resolved by gel electrophoresis, quantitated by exposure to a phosphor screen, and normalized to 18S RNA expression. Cbfa1 DNA-binding activity was determined by electromobility shift assay using the OSE2 binding site from the mouse osteocalcin promoter as a probe. All the experiments were performed in triplicate.

RESULTS: We have optimized an osteoblast culture system in which GC at pharmacological levels inhibit calcium and phosphate deposition by more than 90%. rhBMP-2 alone also inhibited mineral deposition in these highly differentiated cultures, especially at the 100 ng/ml dose. rhBMP-2 at both 10 ng/ml and 100 ng/ml, rescued mineralization in DEX-treated cultures to levels close to those observed in the non-treated cultures (Figure 1). Furthermore, both the control and rhBMP-2-rescued mineral were characterized as bone-like apatite by x-ray diffraction and FTIR. DEX only minimally decreased AP activity in MC3T3-E1 cultures. rhBMP-2 stimulated AP activity in both DEX-treated and non-treated cultures by 2-4 fold. DEX strongly attenuated cell cycle progression in differentiating MC3T3-E1 cultures and this was reflected by the inhibition of multilayering and the lack of nodule formation. Cell cycle progression was also attenuated in the rhBMP-2-treated cultures. In contrast to its anti-GC action on mineralization, rhBMP-2 did not rescue the attenuated cell cycle in the DEX-treated cultures. As expected, both collagen and osteocalcin gene expression were strongly inhibited in the DEX-treated cultures (Figure 2). The effect of DEX on collagen was apparent both by RT-PCR and by Sirius Red staining of the extracellular matrix. rhBMP-2 alone increased both collagen and osteocalcin mRNA levels but decreased extracellular collagen accumulation. rhBMP-2 rescued the DEX-inhibited collagen and osteocalcin mRNA levels. However, extracellular collagen accumulation in the DEX-treated cultures was still inhibited in the presence of BMP-2 co-treatment. The DNA-binding activity of the osteoblast-specific transcription factor Cbfa1 was maximal on day 8 of culture. At this developmental stage, DEX strongly inhibited Cbfa1

DNA-binding activity and rhBMP-2 restored this activity to levels observed in the non-treated cultures. Similar effects were also observed for Cbfa1 mRNA levels on day 6 (Figure 2).

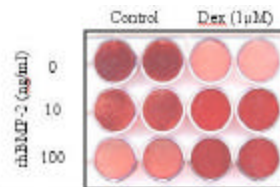


Figure 1. Alizarin Red staining of MC3T3-E1 osteoblasts after 14 days of culture.

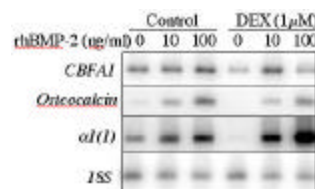


Figure 2. Effects of DEX and rhBMP-2 on bone-phenotypic gene expression after 6 days of culture.

DISCUSSION: Our MC3T3-E1 culture system provides a model in which the adverse effects of GC on osteoblast differentiation can be studied in isolation. In this study, BMP-2 rescued some but not all of the inhibitory effects of GC. rhBMP-2 counteracted the DEX-inhibition of Cbfa1 DNA-binding activity, osteocalcin and collagen mRNA levels and mineral deposition. No difference was observed between the DEX-inhibited/rhBMP-2-rescued and the non-treated cultures in terms of the inorganic components of the extracellular matrix. Thus, rhBMP-2 seems to have fully restored the mechanisms responsible for the formation of the apatite crystals. However, rhBMP-2 did not fully restore normal bone tissue formation in the presence of DEX. Specifically, collagen accumulation and multilayering/nodule formation were not rescued in the DEX/rhBMP-2 cultures. The lack of multilayering/nodule formation may have been due to the inhibition of collagen accumulation and/or the attenuation of cell cycle progression, both of which were persistent in GC-treated cultures regardless of the presence of rhBMP-2. In summary, this study demonstrates two separable types of inhibitory effects exerted by GC on osteoblast differentiation. The first type consists of GC-mediated effects that are rescuable by rhBMP-2: inhibition of Cbfa1 DNA binding activity, down regulation of mRNA of Cbfa1 target genes and deposition of apatite crystals. The second type consists of effects that cannot be rescued by rhBMP-2: inhibition of collagen accumulation and attenuation of cell cycle progression. Both of these types of GC effects on osteoblast differentiation should be addressed in the future design of bone-sparing immunosuppressive therapies for autoimmune and inflammatory diseases.

ACKNOWLEDGEMENT: The authors would like to thank Wyeth Research for the generous supply of rhBMP-2. This work was funded by NIAMS (NIH) and by the Arthritis Foundation.

**Hospital for Special Surgery, New York, NY