Bisphosphonates Enhance Osteogenic Differentiation of Human Bone Marrow Stromal Cells in vitro.

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INTRODUCTION

Bisphosphonates are well-recognized inhibitors of osteoclast activity and are widely used in the treatment of various metabolic bone diseases. Current indications include Paget's disease, post-menopausal osteoporosis and hypercalcemia of malignancy.[1] Bisphosphonates are also considered for fibrous dysplasia [2] and other disorders affecting bone metabolism such as osteogenesis imperfecta.[3]

Bisphosphonates are being investigated for their ability to prevent bony erosions in rheumatoid arthritis, osteoarthritis and peri-implant bone resorption around joint replacement prostheses.[4,5] Newer generation bisphosphonates such as zoledronate are now available, [6] and with their once-a-year dosing, might be considered for numerous clinical indications, including enhanced bone ingrowth into porous-coated orthopaedic implants.

It is widely recognized that the primary action of bisphosphonates is by the inhibition of osteoclastic bone resorption. [1] Ongoing investigations suggest that bisphosphonates may also affect osteoblastic activity. Increasing evidence from *in vitro* and *in vivo* studies support the hypothesis that bisphosphonates additionally promote osteoblastic bone formation.[4,7-8] However, little is known about the potential impact of bisphosphonates on early osteoblastic differentiation. Bone marrow stromal cells represent an important pool of osteoblastic precursors. These pluripotential cells can differentiate into osteoblasts, adipoctyes, fibroblasts and myocytes, and demonstrate remarkable elasticity between the various differentiation pathways. [9]

The purpose of this study was to determine the effects of bisphosphonates (alendronate, risedronate and zoledronate) on differentiation of human bone marrow stromal cells (hBMSC) in a clinically relevant *in vitro* cell culture model.

HUMAN BONE MARROW STROMAL CELL CULTURE MODEL

Human bone marrow was obtained from the femora of three human patients (age 69 to 76) undergoing primary total hip arthroplasty for osteoarthritis. hBMSC were separated by density centrifugation on Percoll (1.077 g/cc) and cultured at a density of 400,000 cells/cm^2 in DMEM/F-12 medium supplemented with 10% fetal bovine serum, 1% antibiotics/antimycotics, L-glutamine (2mM), 10 mM ß-glycero-phosphate and 0.1 mM L-ascorbic 2-phosphate at 37°C with 95% humidity and 5% CO_2. Cells were treated with three different bisphosphonates including 10^{-8}M alendronate (Fosamax, Merck, Rahway, NY), 10^{-8}M risedronate (Actonel, Proctor & Gamble, Cincinnati, OH), 10^{-8}M zoledronate (Zometa, Novartis, Basel, Switzerland), positive controls (addition of 10^{-8}M Dexamethasone or 10^{-8}M Vitamin D) and negative control (medium alone). Culture media was replaced with fresh media and drugs twice a week and cultures were terminated at 7, 14 and 21 days after initiation.

ANALYTICAL METHODS

Total RNA was extracted from the cell layers using TRIzol® reagent (Gibco-BRL, Grand Island, NY) according to the single step acid-phenol guanidinium method. [10] Gene expression for crucial markers of osteogenic differentiation, such as bone morphogenetic protein (BMP)-2, core binding factor alpha subunit 1 (CBFA-1), and Type 1 collagen, was analyzed using semiquantitative RT-PCR as well as quantitative real-time RT-PCR.

Semiquantitative RT-PCR

Aliquots of the extracted RNA were reverse transcribed for 1st strand cDNA synthesis (Invitrogen™, Carlsbad, CA). Template DNA was then used in PCR (MasterMix, Eppendorf, Westbury, NY) for the specified genes. GAPDH served as a housekeeping gene. All RT-PCR products were visualized on 1.5% agarose gel with 0.5g/ml ethidium bromide. Photographs were taken under ultra-violet illumination (Gel Documentation System, UVP, Upland, CA) and qualitative assessments were made of relative gene expression.

Quantitative Real-time RT-PCR

RNA was treated with DNAse I using the DNA-free kit (AMS Biotechnology Ltd, CH, Abingdon Oxon, UK). cDNA synthesis was performed by incubating the RNA with random hexamers, using Stratrascript reverse transcriptase (Stratagene, NL, La Jolla, CA). Real-time quantitative RT-PCR reactions were performed and monitored using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA). In the same reaction, cDNA samples were analysed both for the gene of interest and the reference gene (18-S rRNA), using a multiplex approach (Perkin Elmer User Bulletin N. 2). Technical settings, primers and probes sequences were as previously described. [11]

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Statistical Analysis

Statistical analysis of real-time RT-PCR data was assessed using one-way analysis of variance (ANOVA) and post-hoc paired, double-sided *t*-tests generated from 2 independent hBMSC cultures, with $p < 0.05$ considered to be significant.

RESULTS

All three bisphosphonates enhanced osteoblastic differentiation of hBMSC *in vitro* (Fig. 1). Semiquantitative RT-PCR and quantitative real-time RT-PCR analysis demonstrated upregulated mRNA expression for CBFA-1, BMP-2, and type I collagen in hBMSC after administration of alendronate, risedronate, and zoledronate (Fig. 2). These effects were most pronounced after 14 days of culture, particularly under treatment with zoledronate ($p < 0.05$ versus control for Collagen type I), risedronate ($p < 0.05$ versus control for Collagen type I) and alendronate (Fig. 3).

DISCUSSION

This study provides further evidence that bisphosphonates have anabolic effects on osteoblasts. Different bisphosphonate treatments induced an upregulated gene expression pattern of hBMSC *in vitro* and triggered differentiation of omnipotential hBMSC along the osteoblastic differentiation pathway. These findings are consistent with reports of osteogenic differentiation, by Frank, et al. [11] Interestingly, these effects followed a time- and type-dependent pattern. Of note, the highly potent new bisphosphonate, zolendronate, tended to have the strongest effects on osteogenic differentiation of hBMSC reflecting the higher biological potency of this drug as demonstrated in recent clinical trials. [6]

The mechanism of action behind the anabolic effects of bisphosphonates on osteoblastic differentiation of hBMSC *in vitro* is not known. Our data suggests that bisphosphonates might initially promote expression of key genes like BMP-2 or CBFA-1, which secondarily causes a pronounced osteogenic differentiation of pluripotential hBMSC.

Further investigation is needed to determine how our *in vitro* results translate to bone quality and bone turnover *in vivo*. In summary, our findings suggest that the *in vivo* use of bisphosphonates could lead to enhanced recruitment of bone forming cells, and ultimately show pronounced bone formation and net gain of bone mass. An enhanced understanding of the complex interactions of bisphosphonates with bone metabolism, on both the osteoblastic and osteoclastic side, might open up a broad application of these drugs to critically improve the biological fixation and durability of implants in orthopaedic surgery.

ACKNOWLEDGEMENTS

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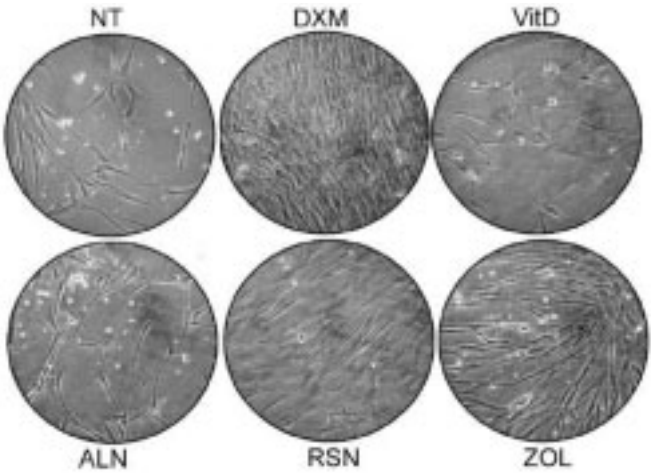

Figure. 1: Enhanced osteoblastic differentiation under bisphosphonate treatment after 14 days of hBMSC culture.

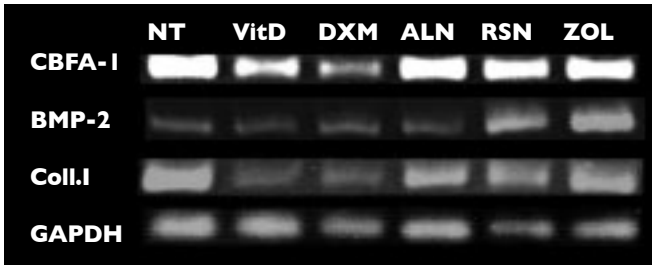

Figure 2: Gene expression after 14 days of hMBSC culture determined using semi-quantitative RT-PCR. GAPDH served as housekeeping gene.

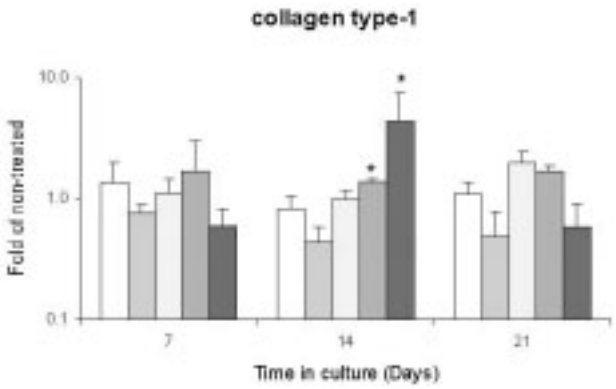

Figure 3: mRNA levels of collagen type I determined using real-time PCR. Data is presented as fold difference and measured in cells from 2 independent donors under all treatment conditions. * $p<0.05$ over negative control.

References

1. **Rodan GA, Martin TJ.** Therapeutic approaches to bone diseases. *Science* 2000; 289:1508-1514
2. **Lane JM, Khan SN, O'Connor WJ, Nydick M, Hommen JP, Schneider R, Tomin E, Brand J, Curtin J.** Bisphosphonate therapy in fibrous dysplasia. *Clin Orthop* 2001; 382:6-12.
3. **Devogelaer JP.** New uses of bisphosphonates: osteogenesis imperfecta. *Curr Opin Pharmacol* 2002; 2:748-53.
4. **Venesmaa PK, Kroger HP, Miettinen HJ, Jurvelin JS, Suomalainen OT, Alhav EM.** Alendronate reduces periprosthetic bone loss after uncemented primary total hip arthroplasty: a prospective randomized study.
   J Bone Miner Res 2001; 16:2126-2131.
5. **Shanbhag AS, Hasselman CT, Rubash HE**. The John Charnley Award. Inhibition of wear debris mediated osteolysis in a canine total hip arthroplasty model. Clin Orthop 1997; 344:33-43.
6. **Reid IR, Brown JP, Burckhardt P, et al.** Intravenous zoledronic acid in postmenopausal women with low bone mineral density. *N Engl J Med* 2002; 346:653-661
7. **Mundy G, Garrett R, Harris S, Chan J, Chen D, Rossini G, Boyce B, Zhao M, Gutierrez G.** Stimulation of bone formation in vitro and in rodents by statins. *Science* 1999; 286:1946-9.
8. **Im G, Puskas B, Rubash H, Shanbhag AS.** Bisphosphonates enhance osteoblast proliferation and maturation. *Trans OR* 2002; 64.
9. **Aubin J** E. Bone stem cells. *J Cell Biochem Suppl* 30-31:73-82, 1998.
10. **Chomczynski P, Sacchi N.** Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 1987; 162:156-9.
11. **Frank O, Heim M, Jakob M, Barbero A, Schafer D, Bendik I, Dick W, Heberer M, Martin I.** Real-time quantitative RT-PCR analysis of human bone marrow stromal cells during osteogenic differentiation in vitro. *J Cell Biochem* 2002; 85:737-746.

John Abraham, Jen Cook, Travis Matheney, Sean Rassman, and Chris Forthman enjoy the festivities at an the chef's open-house BBQ

Sean Rassman, Ben Bengs, and Jeff Zarins enjoy some free time at the AO course in Colorado