Effect of Cell Ratio on Osteoclast and Osteoblast Differentiation in a Ceramic Bone Substitute System

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Introduction

Patients with bone diseases are highly susceptible to fractures of the wrist, hip, and vertebrae, with high risk of non-healing due to pathological bone metabolism. Traditional fixation implants and bone constructs designed for normal physiology have limitations in stimulating healing reactions, in the face of abnormal anabolic and catabolic repair processes. Researchers employing co-cultures to evaluate efficiency of implant and material carrier system and cellular responses at the fracture site often ignore the importance of the osteoclast:osteoblast (OC:OB) cell ratio in regulating bone remodeling. A standard co-culture system that can simulate physiological balance in OC:OB ratio for normal and pathological conditions would provide consistency in methodology for pathological fracture implant evaluation. Hence, the aims of this study were to (i) evaluate the influence of RAW monocyte to D1 stromal cell ratios on OC and OB *in vitro* differentiation and (ii) determine the necessity of soluble RANKL-supplemented osteogenic co-culture conditions in a 3D ceramic system.

Methods and Materials

Cell Culture: D1 stromal and RAW monocytes were seeded at varying densities (1:1, 1:10, 1:100, 1=7.6x10^4 cells) on 150mg of heat sterilized ChronOS granules. Osteogenic (OS) and RANKL-supplemented (R) osteogenic conditions were maintained for 36 days with collection times at Day 8, 15, 22, 29, and 36.

Fluorescence Imaging: Samples were stained for tartrate-resistant acidic phosphatase (TRAP; ELF97 fluorescence kit) to demonstrate activated OCs. Nuclei and actin filaments were counterstained with blue Hoechst and red AlexaFluor 546, respectively. Images were obtained on Day 22 for RAW:D1 ratios of 1:1 and 1:10 in RANKL-supplemented medium.

Reverse Transcription Real-Time Polymerase Chain Reaction (PCR): Isolated ribonucleic acid from Days 8, 15, 22, 29, and 36 was reverse transcribed using RETROscript for PCR analysis and a Qiagen Quantitect kit and Rotor Gene 3000. Primers for Runx2, alkaline phosphatase (ALP), nuclear factor of activated T-cells (NFATc1), and cathepsin K were purchased from Integrated DNA Technologies.

Results

Fluorescence staining demonstrated that TRAP was present in all cell ratio conditions for both differentiation conditions, as seen in Figure 1 for the 1:10 ratio. Key markers of OC and OB differentiation, RANK and ALP, were expressed in similar levels for both medium conditions, as shown in Figure 2. Statistical analysis indicated no difference in gene expressions between 1:1 and 1:10, and between 1:10 and 1:100; however, there was a significant difference between 1:1 and 1:100. Gene expression levels of both RANK and ALP in the 1:10 cell ratio/osteogenic medium samples had Day 22 and Day 29 peaks characteristic of the native environment.

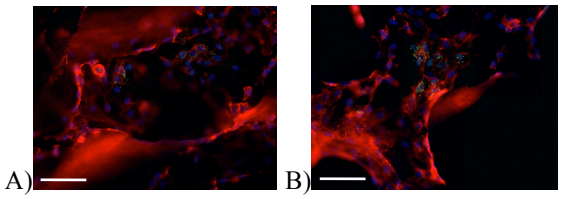

Figure 1 Fluorescent images of 1:10 osteoclast and osteoblast differentiation in osteogenic (A) and RANKL-supplemented (B) osteogenic medium at Day 22 (320x magnification). TRAP granules of activated osteoclasts stained green while nuclei and actin were counterstained with blue Hoechst and red AlexaFluor 546, respectively. Scale bar = 50μm.

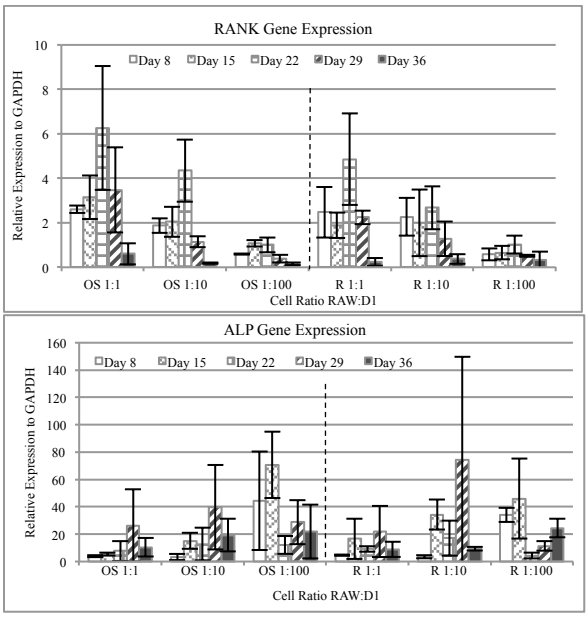

Figure 2. RANK and ALP expression levels during 36 days of OS-supplemented and R-supplemented OS conditions.

Conclusions

Osteoclast and osteoblast synchronization enables bone repair *in vivo*; hence, co-culture evaluations of implants and bone substitute materials designed for pathological fractures should be performed accordingly. The results of this study suggest that the RAW:D1 ratio of 1:10 in OS medium was more similar, in key aspects, to native conditions than the other culture conditions studied. Most importantly, the results demonstrated that the RAW:D1 ratio selection will influence information gleaned from an *in vitro* system. Future studies will focus on simulating pathological conditions with varying cell ratios.

Acknowledgements

National Science Foundation Graduate Research Fellowship, Synthes, Clemson University Hunter Endowment