REAL-TIME-RT-PCR ANALYSIS OF OPG AND RANKL EXPRESSION IN CULTURED FIBROBLAST AND MACROPHAGE CELLS: IMPLICATIONS FOR PERIPROSTHETIC OSTEOLYSIS

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Introduction
Osteolysis is a radiographic term that describes the local loss of bone adjacent to prosthetic implants marked by periprosthetic radiolucencies. Within these radiolucencies is a membrane containing macrophages and fibroblasts which have been shown to produce cytokines implicated in bone resorption (1). These cytokines are felt to indirectly stimulate osteoclastogenesis via stimulation of 2 regulatory molecules, the receptor activator of NF-κB ligand (RANK-L), and Osteoprotererin (OPG). RANK-L is one of the key regulatory molecules in osteoclast formation that is thought to be expressed by osteoblasts/stromal cell lines as a membrane-bound protein that binds to osteoclast precursor cells. Osteoprotegerin (OPG) is a novel secreted member of the tumor necrosis factor (TNF) receptor superfamily that acts as a decoy receptor of RANKL and negatively regulates osteoclastogenesis. In vitro studies have demonstrated OPG and RANK-L protein expression in the membrane using immunohistochemistry (2). Consequently, in this study, macrophage and fibroblast cultures were stimulated with IL-1β and TNF-α, and the resulting gene expression of OPG and RANK-L was quantitatively assessed.

Materials and Methods
Cells and culture systems: Human macrophages (U-937) and fibroblasts (HS-5) cell lines were obtained from ATCC. The HS-5 cells were cultured by DMEM and U-937 by RPMI 1640 medium from Gibco BRL®. The cells were incubated in Corning T-75 flasks and observed by the Nikon microscope for confluence. Calculate cell numbers: Cell number was assessed using bright-line hemacytometry to calculate the cells after confluence. Trizol and MessageClean: Using 6-well pates, 1.0x10⁶ cells of U-937 or HS-5 were pipetted into each well, where 50 ng/ml (2) IL-1β or TNF-a (purchased from R&D) was added to duplicate wells at various time points for 0, 1, 3, 6, and 18 hours. Trizol was used to isolate the total RNA, and a MessageClean kit to remove DNA contamination from RNA. A periprosthetic membrane sample from a patient with linear osteolysis was used as a tissue standard. Realtime-RT-PCR: All samples were quantified using a BIO-RAD SmartSpecTM3000. Experimental samples were prepared at a concentration of 20 ng/µl and standard was prepared at a concentration of 100 ng/µl. Then the samples and standard were submitted for Realtime-RT-PCR. 18S rRNA was used as a control for normalization. And the Ct number (Cycle threshold number) is used as a quantitative index of the mRNA content after conversion.

Results
In the unchallenged cultures, RANK-L and OPG mRNA expression was not detected in HS-5 or U-937 cells. IL-1β and TNF-α failed to induce mRNA expression of RANK-L in U-937, and only very slightly in HS-5 cells. In fact, little if any RANK-L transcription occurred in either cell type under the conditions used. In contrast, IL-1β and TNF-α both induced OPG mRNA expression in HS-5 cells, but in U-937 cells the expression was much lower. In HS-5 cells, peak expression in response to cytokine treatment was at 6 hours. The control RNA sample (a membrane from a patient with linear osteolysis) did show the expression of both RANK-Ligand and OPG mRNA with a ratio of 1.0999 (Figure 2). The Ct number in each experiment is around 28-31. The high Ct for OPG and RANK-L in each sample pertained to the low concentration of these gene products in the respective samples. The standard curves for 18S rRNA in HS-5 and U-937 cells and in the linear osteolysis sample indicated the mRNA preparation was of high quality. When the data was normalized to 18S rRNA as a control, HS-5 cells had a higher gene expression for OPG than U-937 cells. Interestingly, RANK-L gene expression was not significantly demonstrated in HS-5 fibroblasts, i.e., mesenchymal-like cells, either by IL-1β or TNF-α.

Discussion
This study confirms other published work stating that RANK-L gene expression does not occur in cells of myeloid lineage, such as macrophages, in response to cytokine challenge. Fibroblast cells were expected to produce expression of RANK-L in response to cytokine challenge, but did not. On the other hand, gene expression of OPG was demonstrated in both the fibroblast cultures and within the osteolytic membrane. OPG expression in macrophage cells was markedly lower than in fibroblasts. Fibroblasts numbers are markedly decreased in the more aggressive erosive osteolytic lesions, and it is likely that the consequent decreased OPG production is responsible for the increased bone resorption seen in these lesions.

Figure 1. Cytokine challenge to RANK-L and OPG gene expression.

Figure 2. Assessment of RANK-L/OPG ratio in the actual osteolysis patient.

References