Influence of Tumor Necrosis Factor α, Parathyroid Hormone, and Vitamin D₃ on Modulation of the RANKL2 Isoform: A Pilot Study

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Abstract
RANKL exists as three isoforms: RANKL1, 2, and 3. RANKL1 and 3 were reported to be differently expressed upon treatment with some osteotropic factors, but RANKL2 expression could not be reliably determined. Here, we investigated through a mechanistic model, human 293 cells stably transfected with the RANKL2cDNA, the production and modulation of RANKL2 protein stability upon treatment with TNF-α, vitamin D3, and PTH. Data showed that TNF-α significantly increased (p<0.03) RANKL2 production and its half-life/stability (p<0.005). Vitamin D3 and PTH had no effect. This information will help to better define and differentiate the pathological mechanisms operating during osteolytic diseases.

Keywords
osteoarthritis disease modification, bone, preclinical research, cytokines and growth factors, RANKL2 isoform, TNF-α, vitamin D3, PTH

Introduction
Although significant progress has been made in understanding the pathological process of osteoarthritic (OA) joint tissues, much remains to be done as far as developing a specific therapy aimed at the repair process. Recent literature suggests that both cartilage and subchondral bone should be targeted. In this context, the molecular triad osteoprotegerin (OPG)/receptor activator of nuclear factor κB (RANK)/RANK ligand (RANKL) is gaining importance in OA subchondral bone and cartilage, in which increased levels of RANKL lead to a catabolic cascade of events affecting both tissues. Targeting the inhibition of RANKL could improve or protect the patient’s joint structure by preventing its destruction.

RANKL exists as 3 isoforms: RANKL1, RANKL2, and RANKL3. In bone, these isoforms were shown to differentially regulate osteoclastogenesis: RANKL1 and RANKL2 were demonstrated to be proresorptive, whereas RANKL3 acts as an inhibitor of osteoclastogenesis by preventing the membranous localization of RANKL. RANKL isoforms form homotrimer or heterotrimer structures between themselves, and the trimeric combination of RANKL1 and RANKL2 induces osteoclast formation, whereas RANKL1 or RANKL2 with RANKL3 induces a reduced level of osteoclastogenesis.

Although very little is known about the regulation and modulation of these isoforms, it could be speculated that a tight equilibrium exists between them to maintain bone homeostasis and that dysregulation occurs during pathological processes, resulting in increased RANKL activity. In that regard, a study on RANKL isoforms was performed by our group on a human OA subchondral bone osteoblast subpopulation, demonstrating abnormal resorptive properties as well as an increased level of membranous RANKL. The latter study showed that a normal and an OA subchondral bone osteoblast subpopulation differentially express...
membranous RANKL as well as RANKL1 and RANKL3 isoforms and that treatment with the osteotropic factors tumor necrosis factor α (TNF-α) and vitamin D₃, but not parathyroid hormone (PTH), increased the levels of membranous RANKL protein. In this study, the RANKL2 gene expression level could not be evaluated because the complementary DNA (cDNA) sequence of human RANKL2 is similar to human RANKL1, except at the deletion region, and designed primers in this region inadequately amplify RANKL2. We hypothesized from our previous study that RANKL2 expression levels are highly upregulated upon TNF-α treatment and could be increased upon vitamin D₃ treatment.

We thus examined, using a cellular mechanistic model, the modulation of the RANKL2 isoform upon treatment with TNF-α and vitamin D₃, comparing data with those of PTH. This pilot study brings to light that the RANKL2 isoform can be modulated by TNF-α by increasing the protein production and its stability/half-life but not by vitamin D₃ or PTH. This information will help to better define and differentiate between the pathological mechanisms operating during arthritic diseases.

Methods and Results

Expression and Production of the RANKL2 Isoform by 293RANKL2 Cells

The mechanistic cell model consists of the human embryonic kidney 293 cell (293) that does not express RANKL but is stably transfected by the cDNA encoding the transmembranous and extracellular domains of murine RANKL2 (293RANKL2) (INSERM U643 and U957, Nantes, France). For the cloning of RANKL2, the transmembranous and extracellular domains of mouse RANKL were amplified from 293RANKL2 cells, and the amplification product (cDNA RANKL2) was cloned into the plasmid pcDNA3 (Invitrogen, Cergy-Pontoise, France). The 293 cells were then stably transfected with the plasmid pcDNA3-RANKL2, and positive cells were selected upon treatment with Geneticin (1,200 µg/mL, Invitrogen).

In this study, we first verified the RANKL2 isoform gene expression by reverse transcriptase polymerase chain reaction (RT-PCR). In brief, total cellular RNA was extracted with the TRizol reagent (Invitrogen), and determination of the RANKL2 expression level was performed using semiquantitative PCR as previously described using 18S as the housekeeping gene. The primer sequences for RANKL2 were 5′-TCCCACACGAGGGTCCGCTG (antisense) and 5′-TCAGTCTATGTCCTGAACTTTGAAAGCCCC (sense) and for the 18S were 5′-TCAAGAACGAAAAGTCCGGAGGTGGCTG (antisense) and 5′-TTATTGCTCAACTCCTGGTGCTG (sense). Further, the RANKL2 protein production from the 293RANKL2 was analyzed by Western blot in the cell lysates, as previously described with the rat anti-mouse RANKL (2 µg/mL, R&D Systems, Minneapolis, MN) and the rabbit anti-human GAPDH (1:50,000 dilution, Abcam, Cambridge, MA) as the control gene. The second antibodies were the immunoglobulin horseradish peroxidase–conjugated anti-rat (1:20,000 dilution) or anti-rabbit (1:50,000 dilution, Pierce, Rockford, IL). Data confirmed that 293RANKL2 cells strongly expressed and produced RANKL2 (Fig. 1A and 1B).

Modulation of RANKL2 Protein

The effects of the factors on RANKL2 were assessed by incubating the cells for 72 hours with the factors in Dulbecco’s modified Eagle’s medium (DMEM, Wisent, Saint-Bruno, QC, Canada) supplemented with 1% BSA with an antibiotic mixture (100 U/mL penicillin base and 100 µg/mL streptomycin base, Wisent). The factors were TNF-α (5 ng/mL, R&D Systems), vitamin D₃ (50 nM, Sigma-Aldrich Canada, Oakville, ON, Canada), and PTH (100 nM, Peninsula, Belmont, CA). RANKL2 production was determined in the cell lysates using a specific DUOset ELISA (R&D Systems) and determinations performed in triplicate for each cell culture. Modulation of the RANKL2 protein upon treatment with vitamin D₃, TNF-α, and PTH (n = 5) showed that only TNF-α significantly increased (P < 0.03) its level (Fig. 1C).
Figure 2. RANKL2 protein stability/half-life modulation in 293RANKL2 ($n = 3$) following incubation with an increased time period with actinomycin D (5 µg/mL) and (A) vitamin D$_3$ (50 nM) or (B) TNF-α (5 ng/mL). RANKL2 protein levels were determined in the cell lysates using a specific ELISA. Data are expressed over the control, which was attributed a value of 1. Statistical analysis was assessed by the Student t test, and the $P$ value is as indicated.

RANKL2 Production Stability

The 293RANKL2 cells produce RANKL2 protein constitutively, and because the cDNA excludes the RANKL promoter, we were only able to investigate the effect of factors on the RANKL2 protein stability/half-life. For the protein stability determination, cells were pretreated for 1 hour at 37 °C with cycloheximide (2 and 10 µg/mL, R&D Systems) or actinomycin D (5 µg/mL, R&D Systems) alone and incubated for increased durations (0-24 hours) with or without the factors, and RANKL2 protein was measured in the cell lysates.

Data showed that treatment with cycloheximide at both concentrations used induced a cytotoxic effect on both the control 293 cells and on the 293RANKL2 cells. Upon treatment with actinomycin D ($n = 3$) alone, as expected, the RANKL2 protein level decreased with time, reaching a minimum at 8 hours (Fig. 2A and 2B). Treatment with vitamin D$_3$ in conjunction with actinomycin D was without effect on the stability of RANKL2 protein (Fig. 2A). PTH treatment resulted in similar observations (data not shown). On the other hand, TNF-α plus actinomycin D increased the stability of RANKL2 by increasing its half-life, and statistical difference ($P < 0.005$) between actinomycin D alone and TNF-α plus actinomycin D was reached at the 4-hour incubation period (Fig. 2B).

Discussion

The present study first showed that TNF-α, but not vitamin D$_3$ or PTH, significantly increased RANKL2 protein levels. Secondly, in order to investigate the stability/half-life of the protein and because cycloheximide induces nonspecific results in these cells, we used an RNA inhibitor. Indeed, in these cells, the RANKL promoter is excluded, but RANKL protein is constitutively produced, allowing investigation with actinomycin D (a general RNA cell transcription inhibitor) on the modulation of the remaining pool of RANKL over time.

Data showed that TNF-α, but not vitamin D$_3$ or PTH, significantly increased the RANKL2 protein level by increasing RANKL2 protein half-life. This finding suggests that upon treatment with TNF-α, RANKL2 could play an important role in potentiating membranous RANKL localization. In this context, one could speculate that the TNF-α–induced increase in membranous localization of RANKL observed on the human OA subchondral bone osteoblast subpopulation$^{10}$ resulted from the association of RANKL1 with RANKL2 proteins.

The lack of effect of vitamin D$_3$ on the stability of RANKL2 is not surprising, as this factor did not increase the RANKL2 protein level, and in the human OA subchondral bone osteoblast subpopulation, it markedly upregulated RANKL1,$^{10}$ which could account for the induced membranous RANKL.

If these data are translated in vivo, it could reflect the differential effects of these factors in bone remodeling. Indeed, although it is well known that in some diseases TNF-α induces bone loss at least in part via RANKL activity,$^{12,13}$ conflicting reports exist on the effects of the vitamin D$_3$ effect on bone resorption. Indeed, although vitamin D$_3$
was found to increase membranous RANKL, depending on the situation, this is not always followed by a corresponding increase in bone resorption activity.\textsuperscript{14}

This study has some limitations. In this mechanistic model, mouse RANKL2 was genetically modified to resemble human RANKL2 in that the mouse RANKL2 lacked its intracytoplasmic domain and, as expected, had no membranous localization. In fact, the intracellular domain of RANKL is important for such membranous localization. Hence, quantification of membranous RANKL could not be performed. Moreover, as the RANKL2 resides intracellularly, it was also impossible to perform osteoclastogenesis assays with, for example, co-culture with osteoclasts. Another limitation was that, as we had a strong viral promoter to overexpress the RANKL2 protein, we were not able to look at the mRNA half-life/stability in the presence of the factors. Finally, for a better comprehension of their pathological processes, further investigation should be carried out on the regulation of each RANKL isoform in both cartilage and bone (subchondral bone) as well as in diseases with high RANKL activity.

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