The differential expression of osteoprotegerin (OPG) and receptor activator of nuclear factor κB ligand (RANKL) in human osteoarthritic subchondral bone osteoblasts is an indicator of the metabolic state of these disease cells

S. Kwan Tat1, J.-P. Pelletier1, D. Lajeunesse1, H. Fahmi1, M. Lavigne2, J. Martel-Pelletier1

1Osteoarthritis Research Unit, University of Montreal Hospital Centre, Notre-Dame Hospital, Montreal, Quebec; 2Department of Orthopaedics, Maisonneuve-Rosemont Hospital, Montreal, Quebec, Canada.

Abstract
Objective
We previously reported that human OA subchondral bone osteoblasts could be discriminated into two subpopulations identified by their levels of endogenous production (low [L] or high [H]) of PGE2. Here, we investigated the OPG and RANKL expression levels, the histologic analysis of the subchondral bone as well as the osteoclast differentiation effect of osteoblasts on normal and both OA subpopulations (L and H), and further examined on the L OA osteoblasts the modulation of bone remodelling factors on the OPG and RANKL levels, as well as on the resorption activity.

Methods
Gene expression was determined using real-time PCR, PGE2, and OPG levels by specific ELISA, and membranous RANKL by flow cytometry. Histological observation of the subchondral bone was performed on human knee specimens. Osteoclast differentiation and formation was assayed by using the pre-osteoclastic cell line RAW 264.7. OPG and RANKL modulation on L OA osteoblasts was monitored following treatment with osteotropic factors, and the resorption activity was studied by the co-culture of differentiated PBMC/osteoblasts.

Results
Human OA subchondral bone osteoblasts expressed less OPG than normal. Compared to normal, RANKL gene expression levels were increased in L OA and decreased in H OA cells. The OPG/RANKL mRNA ratio was significantly diminished in L OA compared to normal or H OA (p<0.02, p<0.03), and markedly increased in H OA compared to normal. Inhibition of endogenous PGE2 levels by indomethacin markedly decreased the ratio of OPG/RANKL on the H OA. In contrast to H OA osteoblasts, L OA cells induced a significantly higher level of osteoclast differentiation and formation (p<0.05). Histological analysis showed a reduced subchondral bone on the L OA and an increased bone mass on the H OA compared to normal. Treatment of L OA osteoblasts with osteotropic factors revealed that the OPG/RANKL mRNA expression ratio was significantly reduced by vitamin D3 and significantly increased by TNF-α, P1H and PGE2, while IL-1β demonstrated no effect. OPG protein levels showed similar profiles. No true effect was noted on membranous RANKL upon treatment with IL-1β, PGE2, and PTH, but a significant increase was observed with vitamin D3 and TNF-α. The resorption activity of the L OA cells was significantly inhibited by all treatments except IL-1β, with maximum effect observed with vitamin D3 and PGE2.

Conclusion
OPG and RANKL levels, and consequently the OPG/RANKL ratio, differed according to human OA subchondral bone osteoblast classification; it is decreased in L and increased in H OA. These findings, in addition to those showing that L OA osteoblasts have a reduced subchondral bone mass and induce a higher level of osteoclast differentiation, strongly suggest that the metabolic state of the L OA osteoblasts favours bone resorption.

Key words
Osteoarthritis, osteoblasts, subchondral bone, osteoprotegerin, RANK ligand.
OPG and RANKL in human osteoarthritic osteoblasts / S. Kwan Tat et al.

Steeve Kwan Tat, PhD; Jean-Pierre Pelletier, MD; Daniel Lajeunesse, PhD; Hassan Fahmi, PhD; Martin Lavigne, MD; Johanne Martel-Pelletier, PhD.

Please address correspondence and reprint requests to: Johanne Martel-Pelletier, Osteoarthritis Research Unit, University of Montreal Hospital Centre, Notre-Dame Hospital, 1560 Sherbrooke Street East, Montreal, Quebec H2L 4M1, Canada. E-mail: jm@martelpelletier.ca

Received on January 15, 2007; accepted in revised form on September 26, 2007. © Copyright CLINICAL AND EXPERIMENTAL RHEUMATOLOGY 2008.

Introduction

Osteoarthritis (OA) is the most common joint pathology and recent studies suggest that, in this disease, the subchondral bone is intimately involved in cartilage degradation and loss. In fact, abnormal subchondral bone metabolism is suggested as being the driving force behind cartilage degradation and loss. Indeed, human OA subchondral bone osteoblasts were reported to show abnormal phenotypes (1), and altered metabolism (2-4). Studies have also demonstrated that human OA subchondral bone osteoblasts can be discriminated into two subpopulations, based on their production level of prostaglandin E2 (PGE2)/interleukin-6 (IL-6) (Low or High), that otherwise demonstrate no different phenotypic features (5, 6).

It has been shown that some members of the tumour necrosis factor (TNF) family play a major role in regulating bone metabolism. In this context, a molecular triad composed of OPG/RANK/RANKL has been recently described as being the essential cytokine system for controlling the osteoclast biology (7). RANKL (receptor activator of nuclear factor κB ligand) (8) is synthesized mainly by the osteoblastic lineage cells, and is essential for mediating bone resorption through the enhancement of osteoclast differentiation and proliferation. RANKL stimulates osteoclastogenesis and osteoclast action after binding to the cell surface RANK, located on osteoclast precursors and osteoclasts. The third protagonist, OPG (osteoprotegerin), acts as a soluble decoy receptor for RANKL. OPG, by interacting with RANKL, inhibits the binding of RANKL to RANK, thereby preventing RANK activation and subsequent osteoclastogenesis and, as a result, inhibiting bone resorption (9). Although these two factors, OPG and RANKL, are of key importance in regulating bone metabolism, the ratio of OPG/RANKL is considered to better reflect environmental signals during joint pathologies (10) than the levels of each of these factors individually, in which a high ratio level is indicative of promoting bone formation while a low level favours bone resorption.

In this study, we examined the expression levels of OPG and RANKL in both human OA subchondral bone osteoblast subpopulations (low [L] and high [H]) compared to normal osteoblasts, as well as their capacity for osteoclast differentiation and formation. In vivo histological examination of the subchondral bone was also performed for each category. Further, we analysed on the L OA osteoblasts the effects of some known osteotropic factors on the modulation of the OPG and RANKL levels as well as their effect on bone resorption.

Materials and methods

Specimen selection

Normal human subchondral bones were obtained from femoral condyles within 12 hours of death (mean age±SD: 65±16). The tissues were examined macroscopically and microscopically to ensure that only normal tissue was used. Human OA specimens were obtained from femoral condyles of patients undergoing total knee arthroplasty (mean age±SD: 72±8). All patients were evaluated as having OA according to American College of Rheumatology clinical criteria (11). At the time of surgery the patients had symptomatic disease requiring medical treatment in the form of acetylsalicylic acid, NSAIDs, or selective COX-2 inhibitors. None had received intra-articular steroid injections within 3 months prior to surgery, and none had received medication that would interfere with bone metabolism. The institutional Ethics Committee Board of the University of Montreal Hospital Centre approved the use of the human articular tissues.

Subchondral bone histology

Explants from subchondral bone were fixed in TissuFix (Chaptec, Montreal, QC, Canada) and decalcified in Rapid Bone Decalcifier RDO (Apex Engineering, Aurora, IL, USA) for 4 hours. The specimens were embedded in paraffin and subjected to histological observation. Sections (5 μm) of each specimen underwent hematoxylin and eosin staining and were examined under a light microscope.

Subchondral bone osteoblast culture

The subchondral bone osteoblast culture was prepared as previously described.

Competing interests: none declared.
RNA extraction, reverse transcription (RT), and real-time polymerase chain reaction (PCR)

Total cellular RNA from human osteoblasts was extracted with the TRIzol™ reagent (Invitrogen, Burlington, ON, Canada) according to the manufacturer’s specifications. The RNA was quantitated using the RiboGreen RNA quantitation kit. The RT reactions were primed with random hexamers as described previously (12).

Real-time quantitation of mRNA was performed as previously described (12) in the GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with the 2X Quantitect SYBR Green PCR Master Mix (Qiagen, Mississauga, ON, Canada) according to the manufacturer’s specifications. The primer sequences were 5’- TTGACTTTGGTGCCAGG (antisense), 5’- GCCGAAAACATAGGAGCTG (sense) (OPG) and 5’-GGGTTATGAGAACCTTTGGGAATT(antisense) and 5’- CACTATTAATGCCCACCAGAC (sense) (RANKL), and 5’- CAGAAACATCCCTGGCCT (antisense) and 5’- GCCGAGAAATGGTGCAGATCG (sense) (GAPDH). The data were given as a threshold cycle (Ct).

Data were calculated as the ratio of the number of molecules of the target gene/number of molecules of GAPDH. The primer efficiencies for the test genes were the same as for the GAPDH gene. The standard curves were generated with the same plasmids as the target sequences.

Protein determination

OPG, RANKL and PGE_{2} levels were determined in the culture media: for OPG by a specific ELISA from Bio medica (Medicorp, Montreal, QC, Canada) with a sensitivity of 2.8 pg/ml; soluble RANKL (sRANKL) by an EIA (ALPCO Diagnostics, Salem, NH, USA) with a sensitivity of 30 pg/ml; and PGE_{2} by an EIA assay from Cay man Chemicals (Ann Arbor, MI, USA) with a sensitivity of 7.8 pg/ml. All determinations were performed in duplicate for each cell culture.

Membranous RANKL determination

At the end of the incubation period, cells were washed once in 1% BSA/PBS, detached with the cell dissociation buffer enzyme-free (Invitrogen) at 37°C, and centrifuged at 500 g for 5 minutes at 4°C. The cells were resuspended in 1% BSA/PBS and a 500 μl suspension was made, having a concentration of 1 x 10^{6} cells/ml. The suspension was incubated for 30 minutes at room temperature and divided into tubes. One served as a negative control in which mouse IgG (15 μg/ml; Chemicon International, Billerica, MA, USA) was added, and the other was incubated with the anti-human RANKL antibody (15 μg/ml; R&D Systems) for 30 minutes at 4°C. After washing, a goat anti-mouse FITC-conjugated secondary antibody (7.5 μg/ml; R&D Systems) was added for another 30 minutes at 4°C. Cells were then washed in PBS, resuspended in PBS, and analysed using flow cytometry (FACSCalibur, BD Bioscience, Mississauga, ON, Canada). The control sample was used to determine background fluorescence and compared to that of the sample incubated with the specific antibody. The level of fluorescence was measured by a FACScan using the CellQuest program (BD Bioscience), calculated as the mean fluorescent intensity of positive cells, and data expressed over the control, which was assigned a value of 1.

Osteoclast differentiation and formation

Osteoclast differentiation and formation was carried out by using the pre-osteoclastic RAW 264.7 cell line (generously provided by Dr. Svetlana Komarova, McGill University, Montreal, QC, Canada). These cells in the presence of RANKL differentiate into multinucleated TRAP positive osteoclasts (13). In brief, the RAW 264.7 were seeded at a density of 5 x 10^{5} cells/well in a 24 well plate with the culture medium containing DMEM/10% FCS/1% sodium pyruvate and antibiotics, for 6 days and incubated at 37°C in a humidified atmosphere, in the absence or presence of human sRANKL (50 ng/ml; Cell Sciences, Canton, MA, USA) or in the presence of the subchondral bone osteoblast conditioned culture medium. The culture medium was changed every 2 days. The conditioned culture medium was obtained from confluent osteoblasts incubated in a T80 flask for 72 hours in DMEM/0.5% FCS and antibiotics. As the medium of the osteoclasts had to be changed every 2 days, one third of the osteoblast conditioned culture medium was used each time at a 1:1 dilution with the osteoclast culture medium. At the end of the 6th day, the osteoclasts were
fixed with TissueFix and stained for tar-
trate-resistant acid phosphatase (TRAP) according to the manufacturer’s recom-
mendation (Sigma-Aldrich Canada). Os-
teoclast formation was quantified using a
light microscope by counting the newly
differentiated multinucleated TRAP-
positive cells containing at least 3 nuclei.
Results were calculated as the number of
differentiated osteoclasts/well.

Resorption activity determination
Resorption activity was measured using
the BD BioCoat Osteologic Bone Cell Culture System (BD Biosciences,
Oakville, ON, Canada). In brief, hu-
man peripheral blood mononuclear cells (PBMC; 100,000 cells/well) were
incubated into the wells with culture
medium containing DMEM/10% FCS, antibiotics, and 25 ng/ml M-CSF (R&D
Systems) and incubated for 3 days at
37°C in order to induce pre-osteoclas-
tic differentiation (14). Human OA
subchondral bone osteoblasts (10,000
cells/well) were then inoculated with the
differentiated PBMC (pre-osteoclast)
and incubated for another 3 days. At the
end of this period, cells were incubated
at 37°C with fresh DMEM containing
M-CSF, 10% FCS and antibiotics for
3 weeks with the factors under testing.
Media was changed every 3 days. At the
end of this period, cells were incubated
with 40% and 50% inhibition on the L
OA osteoblasts respectively (data not shown). A similar inhibition profile was obtained in a previous study
with 40% and 50% inhibition on the L
and H OA (5). As illustrated in Figure
2A, OPG and RANKL were not mod-
ulated on the L OA osteoblasts. On H
OA osteoblasts, however, OPG was de-
creased and RANKL increased, leading
to a marked decrease in OPG/RANKL
ratio (p<0.06) upon treatment with in-
domethacin (Fig. 2B).

Modulation of osteoclast
differentiation and formation
Since the data revealed a decrease in
the OPG/RANKL ratio in the L OA os-
teoblasts, while it was increased in the
H OA osteoblasts, suggesting that L OA
osteoblasts are enriched with factors
promoting bone resorption, we further
explored the capacity of each subchon-
dral bone osteoblast category, normal
(n=3), L OA (n=3) and H OA (n=4),
to orient the differentiation and for-
OPG and RANKL in human osteoarthritic osteoblasts

S. Kwan Tat et al.

To this end, experiments were performed with the pre-osteoclastic cell line, RAW 264.7. Although RAW 264.7 is a murine cell line, human soluble RANKL was able to markedly induce the formation of multinucleated TRAP-positive cells (Figs. 3A and B), as previously reported (15). Of note, osteoclast formation was minimal in the absence of RANKL.

Interestingly, Figure 3C shows that the conditioned medium from the L OA osteoblasts induced a statistically significant increase (p<0.05) in differentiated osteoclasts when compared to the H OA osteoblasts. This data further supports the hypothesis of the presence in the L OA osteoblasts of higher bone resorptive factors.

Subchondral bone histology

In order to verify if these data reflect the in vivo situation, experiments were performed in which we looked at the histology of human subchondral bone in normal (n=3), L OA (n=5) and H OA (n=5). Interestingly, and as illustrated in Figure 4, differences were found between each population. Hence, the subchondral bone from normal was thick and dense, showing only a limited number of small remodelling units. In contrast, the L OA specimens showed a marked reduction in the subchondral bone surface, whereas an increase in bone surface was found in H OA subchondral bone.

Modulation of L OA osteoblast OPG and RANKL by osteotropic factors

We then further investigated the factors

![Figure 2](image1.png)

**Fig. 2.** Expression of OPG and RANKL, and the ratio of OPG/RANKL in human A) Low or B) High OA subchondral bone osteoblasts incubated in the absence or presence of indomethacin (30 μg/ml). Data are expressed over the control, in which a value of 1 was assigned to the control. Statistical significance was assessed by the Student’s t-test versus control.

![Figure 3](image2.png)

**Fig. 3.** Osteoclast differentiation and formation performed with the RAW 264.7 cells incubated in the absence or presence of 50 ng/ml of human soluble (hs)RANKL or conditioned culture medium from normal, Low or High OA human subchondral bone osteoblasts. A) Pictures of the TRAP staining of the RAW 264.7 cells in the absence or presence of human soluble RANKL (original magnification X100). Of note is the formation of multinucleated TRAP-positive cells (Figs. 3A and B), as previously reported (15). Of note, osteoclast formation was minimal in the absence of RANKL. Interestingly, Figure 3C shows that the conditioned medium from the L OA osteoblasts induced a statistically significant increase (p<0.05) in differentiated osteoclasts when compared to the H OA osteoblasts. This data further supports the hypothesis of the presence in the L OA osteoblasts of higher bone resorptive factors.

Subchondral bone histology

In order to verify if these data reflect the in vivo situation, experiments were performed in which we looked at the histology of human subchondral bone in normal (n=3), L OA (n=5) and H OA (n=5). Interestingly, and as illustrated in Figure 4, differences were found between each population. Hence, the subchondral bone from normal was thick and dense, showing only a limited number of small remodelling units. In contrast, the L OA specimens showed a marked reduction in the subchondral bone surface, whereas an increase in bone surface was found in H OA subchondral bone.

Modulation of L OA osteoblast OPG and RANKL by osteotropic factors

We then further investigated the factors
that could possibly contribute to a change in the synthetic profile of these cells from L to H OA osteoblasts. To this end, L OA osteoblasts were treated with osteotropic factors known to modulate the metabolism of osteoblasts. These included inflammatory factors such as IL-1\(\beta\), TNF-\(\alpha\), PGE\(_2\), as well as vitamin D\(_3\) and PTH. As illustrated in Figure 5A, the expressions of OPG and RANKL (n=5) were differentially regulated by vitamin D\(_3\) and TNF-\(\alpha\); vitamin D\(_3\) had no true effect on OPG but upregulated RANKL expression, whereas the opposite was found for TNF-\(\alpha\), which increased OPG and almost abrogated RANKL. PGE\(_2\) and PTH, while having no true effect on OPG, significantly reduced RANKL expression. On the OPG/RANKL ratio (Fig. 5B) data showed that vitamin D\(_3\) significantly reduced it (\(p<0.05\)), and IL-1\(\beta\) had no effect. In contrast, the ratio increased markedly in response to TNF-\(\alpha\) (335-fold; \(p<0.0001\)) and to a lesser extent to PTH and PGE\(_2\) (30- and 7-fold respectively; \(p<0.02\), \(p<0.0005\)).

The protein levels of OPG and the soluble RANKL released in the culture medium were also measured by ELISA/EIA. As illustrated in Figure 6, the level of OPG protein (n=5) showed a similar profile to that of the mRNA expression for all factors. IL-1\(\beta\) and TNF-\(\alpha\) significantly increased its level, and PGE\(_2\) and PTH had similar OPG levels to that of the control. For soluble RANKL, the levels detected with the EIA were at the limit of the assay and did not permit reliable measurement.

We then measured the level of membranous RANKL using the flow cytometry methodology. Data (n = 6) showed (Fig. 6) that vitamin D\(_3\) and TNF-\(\alpha\) significantly increased membranous RANKL whereas none of the other factors truly modulated its level. Because of the different techniques used for OPG and RANKL protein measurement, the OPG/RANKL protein ratio could not be calculated.

Of note is that, although the levels of membranous RANKL were significantly higher (2.5 fold) in the TNF-\(\alpha\) treatment, the OPG protein level was 3-fold greater when compared to control, and that the level of OPG protein released was much more elevated than that of soluble RANKL which was at the limit of detection.
Modulation of L OA osteoblast resorption activity by osteotropic factors

Osteoclastogenesis was investigated following treatment of the cells with the above osteotropic factors. The experiment was performed by determining the area of the resorbed surface by the differentiated PBMC co-cultured with L OA osteoblasts (n=5). Data showed (Fig. 7) that all the factors tested, with the exception of IL-1β, significantly reduced the resorption activity when compared to control (Fig. 7). A maximum inhibition was observed with vitamin D₃ and PGE₂.

Discussion

Bone is permanently turned over by a tightly balanced and coordinated action of bone-resorbing and bone-forming elements. Imbalance in subchondral bone resorption/formation has been shown to occur in the pathogenesis of OA. However, a major area not yet entirely studied in OA is the complete characterization in the subchondral bone of specific biochemical changes that distinguish the different stages of the disease. This information is of major importance for better identifying specific strategic treatment for this disease.

One of the emerging data from this study is that L and H OA osteoblasts from human subchondral bone have reached different metabolic states, L OA being enriched with factors promoting bone resorption and H OA having reduced resorptive properties. Of note, osteoblasts from human subchondral bone have already been shown to be mature differentiated cells, as they express the bone specific markers including alkaline phosphatase and osteocalcin (1, 5).

The above hypothesis was first suggested by the OPG and RANKL expression level findings, in which these factors and consequently the OPG/RANKL mRNA ratio differ according to human OA subchondral bone osteoblast category. In the L OA osteoblasts, the OPG/RANKL ratio favours RANKL compared to the H OA osteoblasts, which favour OPG. The suggestion that the L OA osteoblasts are enriched in factors promoting bone resorption was further strengthened by the data showing a higher propensity of this cell subpopulation in orienting the osteoclast differentiation, as well as the finding of a reduced subchondral bone surface when examined histologically. Conversely, the H OA subpopulation, although previously shown to have no difference in phenotypic markers from the L OA (alkaline phosphatase and osteocalcin) (5), demonstrated a higher level of OPG and a reduced level of osteoclast differentiation and formation when compared to the L OA osteoblasts. This result also concurs with the histologic examination in which H OA subchondral bone appears to favour bone formation.

In order to sustain that OA osteoblast category discrimination by endogenous PGE₂ level reflects the metabolic state of these cells, experiments were carried out in which cells were treated with a PGE₂ inhibitor. Data are in agreement with this hypothesis suggesting that a low level of PGE₂ favours a low OPG/RANKL ratio as they showed, on one hand, that indomethacin treatment markedly decreased the OPG/RANKL expression level in H OA osteoblasts and, on the other hand, did not modulate the L OA. These findings thus point towards the local production of
PGE$_2$, being instrumental in regulating OPG/RANKL in human OA subchondral bone.

In the OA population, the L OA osteoblasts are generally found in a larger number of patients than the H OA osteoblasts. Hence, the majority of OA patients seem to have a subchondral bone metabolic state that favours bone resorption. This also agrees with the recent report of Bettica et al. (16) showing that in knee OA patients, disease progression is correlated with resorption of the subchondral bone. Since the L OA osteoblasts are involved in a remodelling process in favour of bone resorption, we therefore focused our study on investigating the effects of several factors and cytokines on the L OA osteoblasts, in order to identify the signal(s) that can trigger a switch in the activities of these cells from L to H.

Data showing that vitamin D$_3$ has no true effect on OPG expression and protein production while markedly increasing RANKL, thus significantly reducing the OPG/RANKL ratio, are indicative of a possible increase in bone remodelling/resorption capacity. Although these findings agree with the most recent literature in which vitamin D$_3$ is able to promote osteoclastogenesis by acting on the differentiation of osteoblast precursors (17), our data, however, showed that vitamin D$_3$ induced on these cells a marked and significant decrease in resorative activity. The inhibition of the resorption activity of OA osteoblasts with vitamin D$_3$ could relate to a direct effect of this factor on osteoclasts. Indeed, Itonaga et al. (18) showed a marked decrease in the formation of TRAP$^+$ and VNR$^+$ multinucleated cells from PBMC when treated with vitamin D$_3$ and suggest that this factor inhibits osteoclastogenesis through a direct effect on osteoclast precursors.

The addition of PGE$_2$ to L OA osteoblasts revealed a strong inhibition of the resorptive activity. Addition of PGE$_2$ increased the OPG/RANKL expression mRNA ratio, having little effect on OPG but significantly reducing RANKL gene expression. Yet these data did not perfectly correspond with the protein/membranous level of OPG and RANKL. However, Figure 6 illustrates that the fold increase in OPG protein and membranous RANKL following treatment with PGE$_2$ is similar (1.5 fold) and, as mentioned earlier, since the OPG protein level is at a much higher level than that of RANKL, this points towards a net increase in OPG production, hence an inhibition of the resorptive activity. In addition, the high inhibition of resorptive activity of PGE$_2$ on L OA osteoblasts could be explained by the data of Take et al. (19), demonstrating that PGE$_2$ could also act directly as an inhibitor of osteoclastogenesis. They demonstrated the presence of a direct PGE$_2$-induced inhibition of osteoclast precursor formation, which occurs through the interaction of PGE$_2$ on its specific receptors. These data are also well in line with the report of Raisz et al. (20), showing that low concentrations of PGE$_2$, favour bone resorption, while high concentrations favour bone deposition. In this line of thought, we performed the osteoclast differentiation and formation experiments, which revealed that the L OA cells, which expressed a lower level of PGE$_2$, enhanced the formation of osteoclasts, whereas the H OA cells, expressing a higher level of PGE$_2$, did not (Fig. 3C). Treatment with TNF-$\alpha$ and PTH did favour an increase in the OPG/RANKL ratio that correlates with a significant inhibition of the resorptive activity. Contrasting with our data, previous reports have shown that, in some types of osteoblasts, PTH can stimulate RANKL synthesis (21-24). However, our study was carried out with osteoblasts from human OA rather than from small animal species and with osteoblasts from subchondral rather than trabecular bone. In addition, it was also shown that human OA subchondral bone osteoblasts have an abnormal PTH signalling pathway (25), which might be an explanation for its effect on the RANKL modulation.

TNF-$\alpha$ induced a marked increase in the OPG/RANKL mRNA expression ratio. These data showed a good correlation with the OPG protein production, and also agree with the resorption assay results showing an inhibition upon TNF-$\alpha$ treatment. The discrepancy between the lower RANKL expression and the increased level of membranous RANKL upon TNF-$\alpha$ treatment could be explained by the TNF-$\alpha$ modulation on the different isoforms of RANKL. Indeed, it is now known that human RANKL exists as 3 isoforms, namely RANKL1, 2 and 3 (26). Although membranous RANKL (RANKL 1 and 2) has been demonstrated to induce osteoclastogenesis, RANKL3 (cytoplasmic) was recently shown to be an inhibitor of this phenomenon by preventing the membranous localisation of RANKL (26, 27). It can thus be hypothesized that the increase in the level of membranous RANKL upon treatment with TNF-$\alpha$ is mediated mainly through a decreased expression of RANKL3.

In our study, the finding showing that soluble RANKL in the culture medium could not be detected together with the flow cytometry experiment demonstrating an increased level of the membranous RANKL, appears to contradict the resorption assay data which showed a slight inhibition upon TNF-$\alpha$ treatment. The explanation could be that, although an increase of 2.5 fold is noted for membranous RANKL, the OPG production is 3 fold greater. Thus, the slight inhibition of the resorption activity can be explained by the excess of OPG.

Hence, although TNF-$\alpha$ is known to induce osteoclast differentiation (28) and resorption, our data reveal an anti-resorptive process upon treatment with TNF-$\alpha$. These conflicting findings may be linked to the use of different model systems (pathology), source, location of osteoblasts ( trabecular bone vs. subchondral) and culture conditions. This is well illustrated with the data on the soluble RANKL determination with the EIA (which was at the limit of detection) which appear to contrast with those obtained from human trabecular bone osteoblasts from hip periprosthetic loosening (29), in which TNF-$\alpha$ increases soluble RANKL. Such difference between the RANKL protein data could rely on the provenance of cells in which, in the Lavigne et al. study (29), due to the osteolysis pathological process involving osteoblasts but also macrophages, more cytokines and other factors could have
stimulated the osteoblasts to produce soluble RANKL. In conclusion, our data point to specific molecular mechanisms that appear to be operating during human OA progression. Our study demonstrates that the abnormal OPG and RANKL levels in human OA osteoblasts are dependent on the metabolic state of the cells. The differential abnormal expression of OPG and RANKL in the two human OA subchondral osteoblast sub-populations could indicate different stages of attempts to repair the damaged subchondral bone tissue; an increase in bone resorption followed by abnormal bone sclerosis. These concur with the in vivo findings from an OA dog model, in which at an early stage of the disease process there is an enhancement of bone loss and resorption with subchondral bone exhibiting a surface and trabecular thickness reduction, and an increased number of osteoclasts, as well as production of catabolic factors including cathepsin K and MMP-13 (30). Moreover, an in vivo experiment performed at an early stage on such an OA dog model demonstrated upon treatment with bisphosphonates (an inhibitor of osteoclast function) an inhibition of the subchondral bone loss (31). At a later stage, Brandt et al. (32) reported on the dog model 54 months after OA induction that the histomorphometric analysis of the subchondral bone was markedly increased in volume with an active bone formation. Thus, in humans, the OA osteoblasts may be in a milieu with factors that promote osteoclast differentiation and formation, thereby increasing bone resorption, a hypothesis supported by the increased TRAP positive cells from the RAW 264.7, and reduced subchondral bone volume. On the other hand, the H OA cells are under the influence of factors favouring bone deposition, thus explaining the sclerosis of the subchondral bone. The latter could be due to a flare in disease activity with circumstances favouring the upregulation of factors such as TNF-α and PGE₂.

Acknowledgments

The authors are grateful to Dr. Svetlana Komarova, Faculty of Dentistry, McGill University, Montreal, Quebec, Canada for generously providing the osteoclast cell line RAW 264.7. The authors also thank Dr. Marika Sarfati, Dr. Guy Delespesse and Manuel Rubio from the Research Unit of Immunoregulation and Allergy Research Unit at the University of Montreal Hospital Centre for utilizing their flow cytometry apparatus, and Aline Delalandre, François Mineau, and François-Cyril Jolicoeur from the Osteoarthritis Research Unit at the University of Montreal Hospital Centre for their expert technical assistance, and Virginia Wallis for the manuscript preparation.

References

23. HORWOOD NJ, ELLIOTT J, MARTIN TJ, GILLESPIE MT: Osteotropic agents regulate the expression of osteoclast differentiation factor and osteoprotegerin in osteoblasts.
OPG and RANKL in human osteoarthritic osteoblasts / S. Kwan Tat et al.


28. THOMSON BM, MUNDY GR, CHAMBERS JN: Endogenous prostaglandin E2 and insulin-like growth factor 1 can modulate the levels of parathyroid hormone receptor in human osteoarthritic osteoblasts. J Bone Miner Res 2001; 16: 713-21.


