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

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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 PGE_2. 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, PGE_2 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 PGE_2 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 D_3 and significantly increased by TNF-α, PTH and PGE_2, 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, PGE_2 and PTH, but a significant increase was observed with vitamin D_3 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 D_3 and PGE_2.

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.

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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 E_2 (PGE_2)/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 acetaminophen, 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

(1, 2, 5, 6). Briefly, bone samples were cut into small pieces and digested for 4 hours with collagenase type I in BGJb medium (both from Sigma-Aldrich Canada, Oakville, ON) without serum at 37˚C in a humidified atmosphere of 5% CO_2/95% air. After this period the bone pieces were cultured in BGJb medium containing 20% heat-inactivated fetal calf serum (FCS; Gibco-BRL, Burlington, ON, Canada) and an antibiotic mixture (100 units/ml penicillin base and 100 µg/ml streptomycin base) (Gibco-BRL) at 37˚C in the humidified atmosphere. When cells were observed in the petri dishes, the culture medium was replaced with fresh medium containing 10% FCS until confluence. Osteoblasts passaged once were used for this study.

The effects of factors on OPG and RANKL were assessed by pre-incubating cells in DMEM (Gibco-BRL)/0.5% FCS for 24 hours followed by 18 hours (for mRNA determination) and 48 hours (for protein determination) incubation with fresh DMEM/0.5% FCS containing the factors under study. The concentration used was 50 nM for vitamin D_3 (Sigma-Aldrich Canada), 100 pg/ml for IL-1β (Genzyme, Cambridge, MA, USA), 5 ng/ml for TNF-α (R&D Systems, Minneapolis, MN, USA), 500 nM for PGE_2 (R&D Systems), 100 nM for PTH (Peninsula, Belmont, CA, USA) and 30 µg/ml indomethacin (Sigma-Aldrich Canada). These concentrations were chosen from previous works.

Identification of the OA osteoblast subpopulations was performed according to previous publications (5,6); OA osteoblasts producing low levels of PGE_2 <2000 pg/mg protein were classified as L OA osteoblasts, and those producing high levels of PGE_2 >2000 pg/mg protein as H OA osteoblasts.

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) used according to the manufacturer's specifications. The primer sequences were 5'- GTTTACTTTGGTGCCAGG (antisense), 5'- GCTTGAAACATAGGAGCTG (sense) (OPG) and 5'-GGGTATGAGAACTTGGGATT (antisense) and 5'- CACTATTAATGCCACCGAC (sense) (RANKL), and 5'- CAGAACATCATCCCTGCCTCT (antisense) and 5'- GCTTGACAAAGTGGTCGTTGAG (sense) (GAPDH). The data were given as a threshold cycle (C_T). 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 Biomedica (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 Cayman 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 10^3 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 TissuFix and stained for tartrate-resistant acid phosphatase (TRAP) according to the manufacturer's recommendation (Sigma-Aldrich Canada). Osteoclast 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, human peripheral blood mononuclear cells (PBMC; 100,000 cells/well) were inoculated 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-osteoclastic 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 the incubation period, cells were bleached (6% NaOCl, 5.2% NaCl) and extensively washed in sterilized water. Von Kossa stain was used for contrast stain for resorption as described by BD Biosciences. The quantitation was performed with a light microscope using the Bioquant software (Bioquant Osteo II, v 8.00.20; Nashville, TN, USA). Results were calculated as the percentage of the resorbed surface and expressed as the percentage of control, where control is attributed 100%.

Statistical analysis

Data are expressed as the mean ± SEM. Statistical significance was assessed by the 2-tailed Student's *t*-test, and *p*-values <0.05 were considered significant.

Results

PGE_2 levels

We first evaluated the OA osteoblast levels of PGE_2. As expected, the data revealed two subpopulations of osteoblasts: L OA osteoblasts, in which the PGE_2 levels were 1,200±214 pg/mg protein, and H OA osteoblasts having 5,393±900 pg/mg protein. The proportion of patients showing L OA osteoblasts is generally higher (60%) compared with the H OA osteoblast profile.

Modulation of OPG and RANKL expression

The data first showed (Fig. 1) that human subchondral bone osteoblasts had a much higher level of OPG expression compared to RANKL. In fact, the level of RANKL gene expression was a few thousand times less elevated than the OPG gene expression. Differences between normal (n=5 patients) and the OA subpopulations (L OA, n=7 patients; H OA, n=5 patients) were found for OPG and RANKL. OA osteoblasts were found to express less OPG than normal ($p<0.05$ *vs*. L OA osteoblasts). Moreover, L OA osteoblasts expressed significantly less OPG ($p<0.03$) than the H OA osteoblasts. For RANKL, although not statistically significant, differences were found between groups; the L OA expressed a higher mRNA level of RANKL than normal and H OA. The OPG/RANKL ratio (Fig. 1) was significantly lower in L OA osteoblasts than the normal and H OA osteoblasts ($p<0.02$ and $p<0.03$, respectively). A higher OPG/RANKL ratio was found in the H OA compared to normal, but due to the variation in the results between specimens, it did not reach statistical significance.

In order to investigate whether endogenous PGE_2 acts on the OPG and RANKL mRNA, experiments were carried out in which osteoblasts from L (n=3) and H (n=3) OA were incubated in the absence/presence of a PGE_2 inhibitor, indomethacin, at a concentration of 30 μg/ml, and OPG and RANKL expression determined. Data revealed a significant inhibition of PGE_2 levels by 55% ($p<0.04$) and 64% ($p<0.05$) for the L and H 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 modulated on the L OA osteoblasts. On H OA osteoblasts, however, OPG was decreased and RANKL increased, leading to a marked decrease in OPG/RANKL ratio ($p<0.06$) upon treatment with indomethacin (Fig. 2B).

Modulation of osteoclast differentiation and formation

Since the data revealed a decrease in the OPG/RANKL ratio in the L OA osteoblasts, 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 subchondral bone osteoblast category, normal (n=3), L OA (n=3) and H OA (n=4), to orient the differentiation and for-

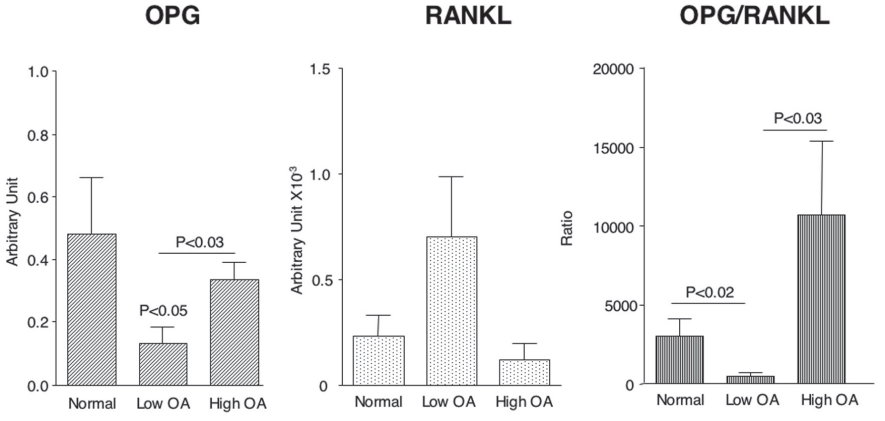

Fig. 1. Expression of OPG and RANKL, and the ratio of OPG/RANKL in human normal, Low or High OA subchondral bone osteoblasts. Total RNA was extracted and processed for real-time PCR, and the data are expressed as the mean ± SEM of arbitrary unit as described in *Materials and methods*. Of note, the RANKL arbitrary unit is represented as $X10^{-3}$. Statistical significance was assessed by the Student's t-test versus normal or as indicated (underlined).

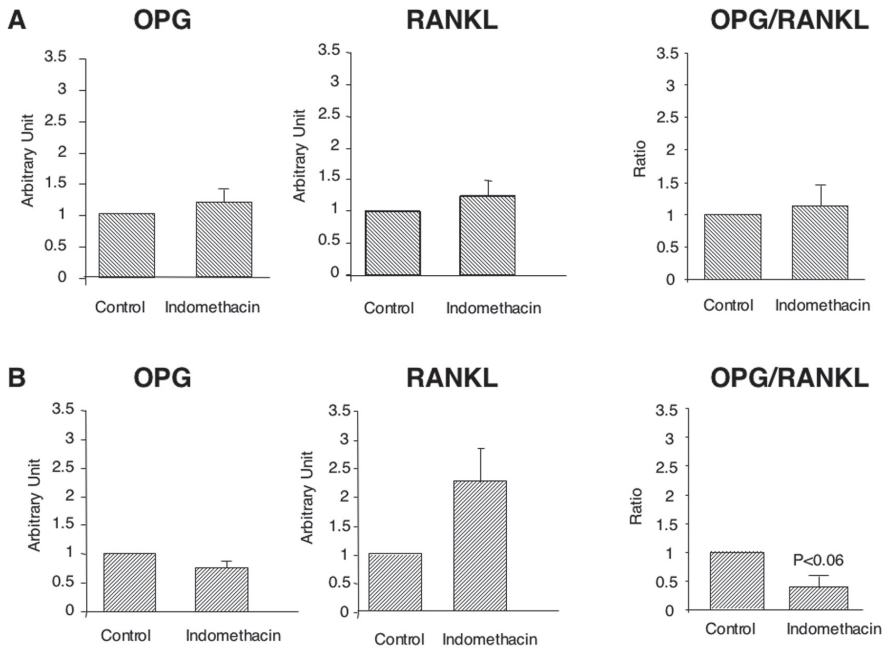

Fig. 2. Expression of OPG and RANKL, and the ratio of OPG/RANKL in human **A)** Low or **B)** High OA subchondral bone osteoblasts incubated in the absence or presence of indomethacin (30 μg/ml). Data are expressed over the control, in which a value of 1 was assigned to the control. Statistical significance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

mation of osteoclast cells. 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

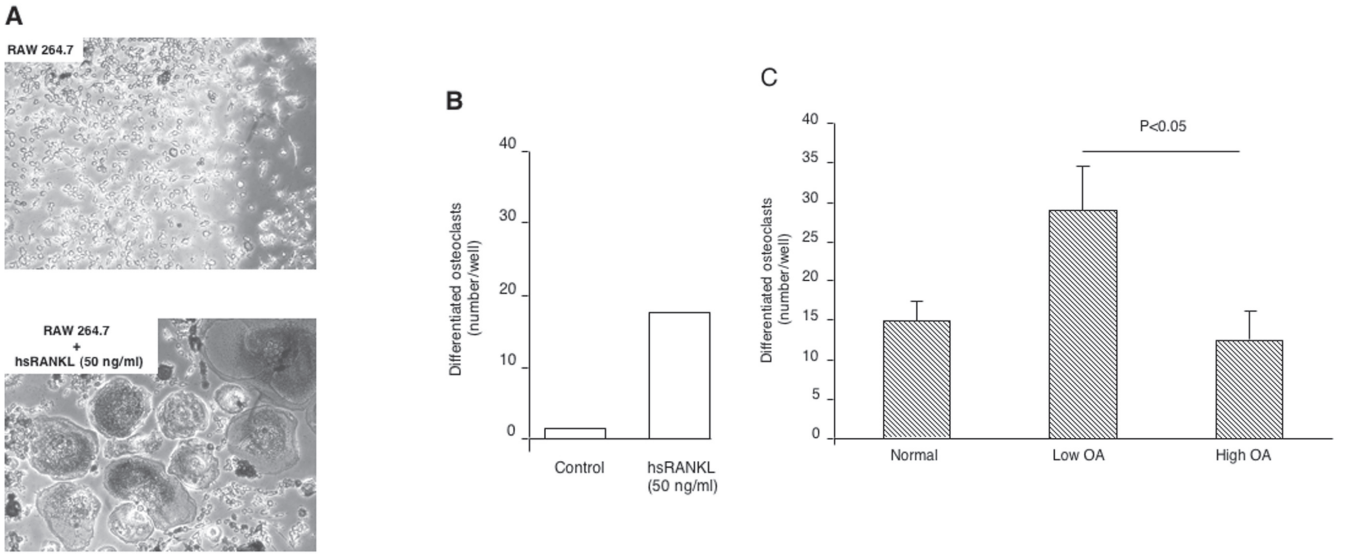

Fig. 3. Osteoclast differentiation and formation performed with the RAW 264.7 cells incubated in the absence or presence of 50 ng/ml of human soluble (hs)RANKL or conditioned culture medium from normal, Low or High OA human subchondral bone osteoblasts. **A**) Pictures of the TRAP staining of the RAW 264.7 cells in the absence or presence of human soluble RANKL (original magnification X100). Of note is the formation of multinucleated cells which stained for TRAP activity when cells were incubated in the presence of hsRANKL. **B)** Histogram of the TRAP positive RAW 264.7 cells incubated in the absence (control) or presence of hsRANKL. **C**) The number of TRAP positive cells when the RAW 264.7 cells were incubated in the presence of the human normal, Low or High OA subcondral bone osteoblasts conditioned culture medium as described in *Materials and methods*. The number of positive TRAP multinucleated cells with at least 3 nuclei are counted and data are expressed as the mean ± SEM of the number of positive-cells/well. Statistical significance was assessed by Student's *t*-test.

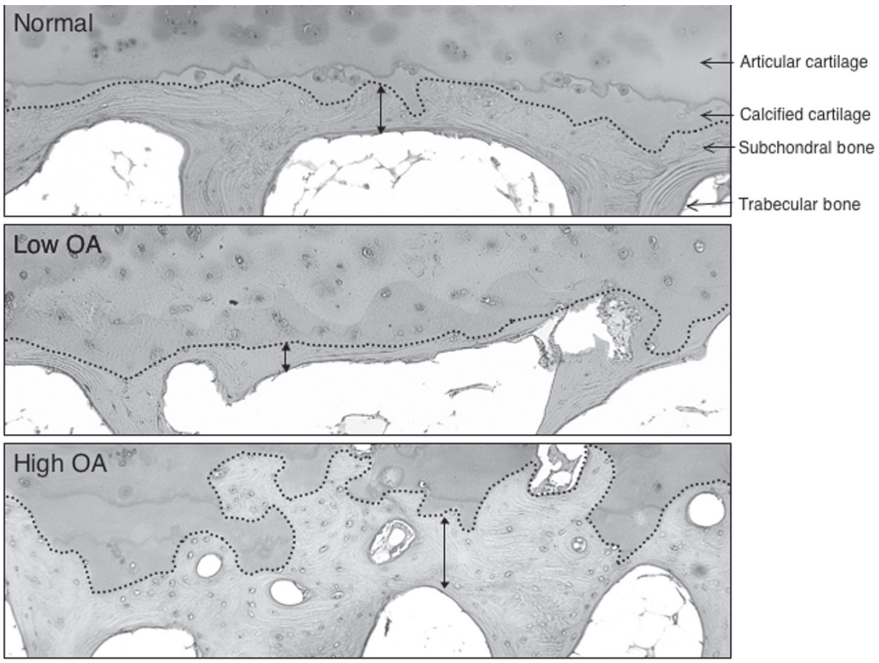

Fig. 4. Representative histologic sections of femoral condyles of human subchondral bone (↔) in normal, Low and High OA. Localization of the articular cartilage, calcified cartilage, subchondral as well as trabecular bone are indicated. Original magnification X63.

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-1β, TNF-α and 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-α; vitamin D_3 had no true effect on OPG but upregulated RANKL expression, whereas the opposite was found for TNF-α, 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-1β had no effect. In contrast, the ratio increased markedly in response to TNF-α (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-1β and TNF-α 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-α 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-α 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.

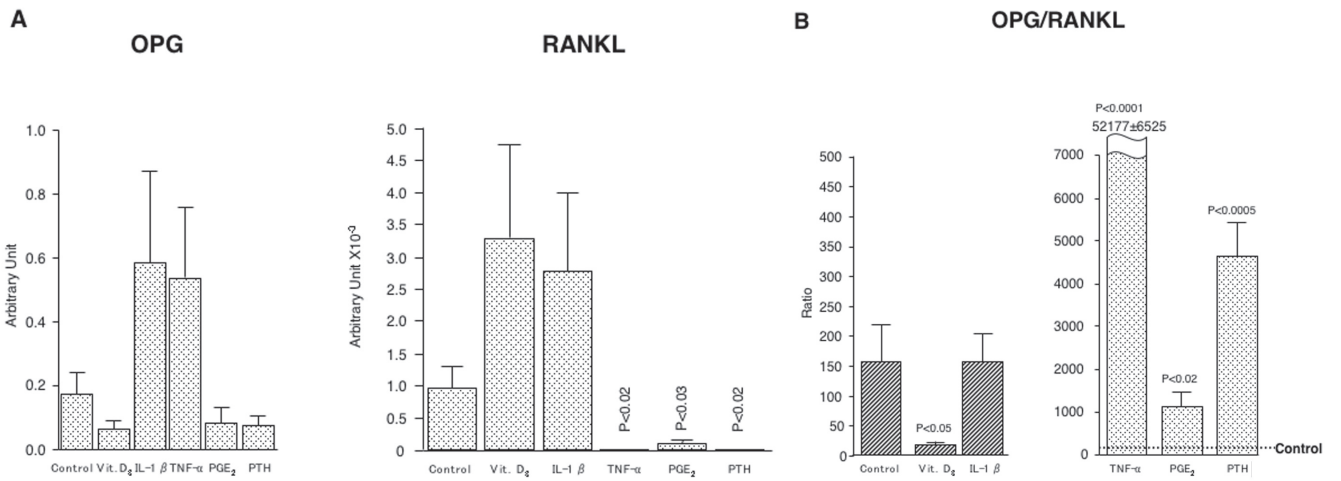

Fig. 5. **A**) OPG and RANKL mRNA and **B**) OPG/RANKL mRNA ratio in human Low OA subchondral bone osteoblasts incubated in the absence (Control) or presence of osteotropic factors including vitamin D_3 (Vit.D_3), 50 nM; IL-1β, 100 pg/ml, TNF-α, 5 ng/ml; PGE_2, 500 nM; and PTH, 100 nM. Data are expressed as the mean ± SEM of arbitrary unit as described in *Materials and methods*. Of note, the RANKL arbitrary unit is represented as $X10^{-3}$.

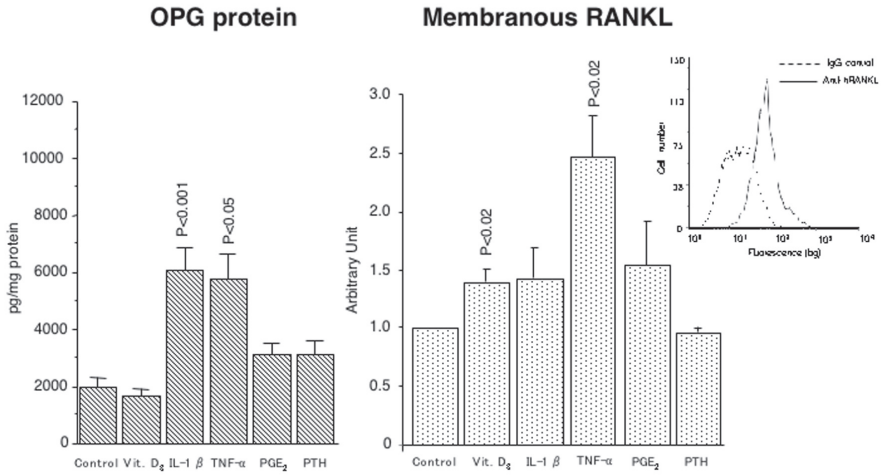

Fig. 6. OPG protein production (pg/mg protein) and RANKL membranous level (mean fluorescence intensity (MFI) over control, in which a value of 1 was attributed to the control) in human Low OA subchondral bone osteoblasts incubated in the absence (Control) or presence of the various osteotropic factors: vitamin D_3 (Vit.D_3), 50 nM; IL-1β, 100 pg/ml, TNF-α, 5 ng/ml; PGE_2, 500 nM; and PTH, 100 nM. The inset is a representative histogram of the membranous RANKL flow cytometry in which a mouse IgG served as control for background fluorescence and a specific anti-human RANKL (anti-hRANKL) antibody as described in *Materials and methods*. Statistical significance was assessed by Student's t-test versus control.

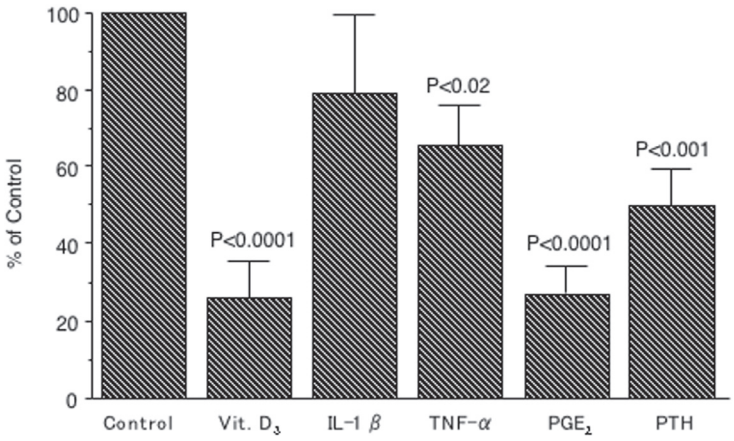

Fig. 7. Resorption activity of human Low OA subchondral bone osteoblasts co-incubated with PBMC in the presence of M-CSF and osteotropic factors including Vitamin D_3 (Vit.D_3), 50 nM; IL-1β, 100 pg/ml, TNF-α, 5 ng/ml; PGE_2, 500 nM; and PTH, 100 nM. Data are expressed as the mean percentage of the total surface resorbed upon treatment with the factors compared to control. Statistical significance was assessed by Student's *t*-test versus control.

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 D_3 and PGE_2.

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 PGE_2 level reflects the metabolic state of these cells, experiments were carried out in which cells were treated with a PGE_2 inhibitor. Data are in agreement with this hypothesis suggesting that a low level of PGE_2 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 resorptive 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-α 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-α 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-α treatment. The discrepancy between the lower RANKL expression and the increased level of membranous RANKL upon TNF-α treatment could be explained by the TNF-α 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-α 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-α 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-α is known to induce osteoclast differentiation (28) and resorption, our data reveal an anti-resorptive process upon treatment with TNF-α. 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-α 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 subpopulations 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 L 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 PGE_2.

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