Expression of RANKL and OPG in primary osteoblasts

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Summary

Objective: Osteoblasts are specialized cells responsible for bone formation. Furthermore, these cells modulate osteoclast formation and maturation, mainly by the production of RANKL and OPG. We previously reported that the bone tissue of osteoporotic patients showed increased RANKL expression and RANKL/OPG ratio when compared to osteoarthritic patients. Thus we decided to explore whether this aberrant expression may be related to an abnormal expression of these genes by osteoblasts. The aim of this study was to explore the transcriptional levels of these factors in primary osteoblasts.

Methods: Primary human osteoblasts (hOBs) were obtained by the primary explant technique from bone tissue of patients undergoing hip replacement surgery for hip fractures (n=28) or osteoarthritis (n=26). Patients with secondary osteoporosis, fractures due to high-energy trauma or secondary osteoarthritis were excluded. RANKL and OPG gene expression was explored by real time quantitative PCR.

Results: No statistical differences in RANKL and OPG gene expression were found along the in vitro mineralization of hOBs. Interestingly, OPG transcriptional levels were markedly higher than RANKL levels. However, no differences in the transcriptional levels of RANKL and OPG were observed between both groups.

Conclusions: Overall, our data confirm that osteoblasts produce RANKL and OPG. However, our results suggest that the gene expression differences found in the osteoporotic and osteoarthritic bone tissue are not explained by the intrinsic characteristics of osteoblasts.

Key words: osteoclastogenesis, bone remodeling, osteoporosis, osteoarthritis.
Introduction
Bone is a specialised form of supporting tissue formed by different cell types and mineralised materials on which they confer marked rigidity and resistance, at the same time as allowing a certain degree of elasticity. Due to this peculiar composition, bone tissue is also an important reservoir of calcium and other inorganic ions, and participates actively in the maintenance of calcium homeostasis. Each bone is sculpted by means of a complicated process called modelling. In addition, bone tissue is renewed constantly by a process known as remodelling. Remodelling plays an essential role in the homeostasis of calcium, as well as preserving the solidity of the bone by substituting old bone for new.

Differently from modelling, bone remodelling requires the coordinated action of the osteoclasts, cells which are in charge of reabsorbing bone, and osteoblasts, bone-forming cells. Basically, the process of remodelling may be divided into four phases. The first phase, known as activation, involves the process of starting remodelling by the activation of the osteoclasts in a specific region of the bone by means of the attraction of osteoclast precursors and the induction of their differentiation into mature osteoclasts. In the second stage, resorption, the osteoclasts are fixed in a bone matrix creating a resorption cavity. The resorption phase ends with the apoptosis of the osteoclasts. Together with the osteoclast apoptosis signals responsible for the recruitment of the osteoblasts to the resorption cavities are produced (the proliferation phase). This coupling between the action of the osteoclasts and the osteoblasts is sequential in such a way that the processes of bone resorption and formation do not coexist either in time or space. Indeed, this is critical in order for there to be a proper process of remodelling. Finally, during the last stage, known as the formation stage, the osteoblasts synthesise the non-mineralised organic matrix, and are subsequently responsible for their mineralisation.

In spite of the fact that all not all the details of the molecular mechanisms of these processes are known, some of them have been successfully identified. The RANKL-RANK signalling system, critical for the initiation of osteoclastogenesis, is perhaps the most important molecular mechanism for the differentiation of the osteoclasts. The receptor activator of nuclear factor κB ligand (RANKL) is a protein produced by various types of cells, among which the osteoblasts are one of the principal sources in bone tissue. This protein bonds to the receptor activator of nuclear factor κB (RANK), present in the membranes of the osteoclast precursors, inducing their differentiation through the ultimate activation of transcription factor NFATc-1, which is responsible for the modulation of the expression of the genes necessary for the formation of an osteoclast which is mature and prepared for bone resorption. On the other hand, the osteoblasts are also capable of producing osteoprotegerin (OPG), another protein which acts as a soluble receptor for RANKL, thus blocking the possible interaction between RANKL and its receptor RANK. In fact the RANKL/OPG quotient determines in great measure the rate of osteoclastogenesis. Directly related to this, in an earlier work we observed that the gene expression of RANKL and the RANKL/OPG quotient in bone tissue are significantly higher in patients with osteoporotic fractures than in patients with arthrosis, suggesting that the patients with osteoporosis may have a greater activation of osteoclastogenesis, and, as a consequence, an imbalance in bone remodelling, which provokes a loss of bone mass and a greater probability of suffering fractures. In spite of the fact that data from other authors confirm these results, for the moment what it is that provokes these differences in the production of RANKL and OPG is not known.

Given their capacity to produce RANKL and OPG, the osteoclasts are not only crucial for bone formation, but they are also essential for the initiation of osteoclastogenesis and a correct coupling of resorption-formation. Therefore, the aim of this work was to study the changes in expression of RANKL and OPG in primary osteoblasts during the process of mineralisation in vitro and to compare the levels of transcription in osteoblasts taken from osteoporotic and arthritic patients.

Materials and methods
Cell cultures
Bone tissue samples were obtained from the femoral head during substitutive arthroplasties. The study was approved by the regional committee for ethics in clinical research and all the samples were taken after obtaining informed consent. The primary osteoblast samples were obtained using the explants technique, using trabecular bone tissue from the central part of the femoral head, avoiding the fractured areas and subchondral regions. The osteoblasts were cultivated in DMEM, complemented with 10% foetal bovine serum and antibiotics, and kept in an incubator at 37° C under 5% CO2 and with a relative humidity of above 90%.

For the mineralisation experiments the cells were plated at a density of 40,000 cells/CM2. Once the cells had arrived at a state of confluence osteogenic medium (DMEM, complemented with 10% foetal bovine serum, antibiotics, 50 μM ascorbic acid and 10mM of β-glycerophosphate) was added. The progress of calcification was studied by staining with Alizarin Red S. For the analysis of the expression of RANKL and OPG in arthritic and osteoporotic patients cultures of primary osteoblasts with a confluence of 80% (passes 1-2) from patients subject to the insertion of a hip prosthesis due to fracture or arthrosis were used. The patients were selected using the determining criteria for the diagnosis of osteoporosis and arthrosis, in the former, the presence on fractures and in the latter the presence of serious arthrosis. Excluded from the study were those patients with secondary osteoporosis, with fractures due to high energy trauma or with secondary arthrosis.
Gene expression
RNA was extracted from semiconfluent primary osteoblasts using TRIzol, following the instructions of the manufacturer (Invitrogen). For the synthesis of cDNA 1 μg of RNA was used and the instructions of the maker of the Superscript III First Strand kit (Invitrogen) were followed. During the culture of the cells in osteogenic medium RNA was extracted consecutively every 7 days.

The gene expression for RANKL and OPG was quantified using quantitative PCR (RT-qPCR) with TaqMan probes following the manufacturer’s instruction (Applied Biosystems). To avoid interexperimental variability samples from the both groups were included in each of the analyses carried out. The TaqMan probes used were: TNFSF11 (isoform I, RANKL; Hs00243519_m1 and TNFRSF11B (OPG): Hs00900360_m1. For each gene the relative expression was calculated using as a reference the gene of the TATA box binding protein (TBP), expressed consecutively in the cells. The expression was calculated using the 2\(^{-\Delta\Delta Ct}\) method, \(\Delta Ct\) being the difference between the umbral cycle (C) of the gene of interest and TBP.

Statistical analysis
For the comparison of the expression of RANKL and OPG during the process of osteogenic expression the Student’s t-test (two tails), was used. The statistical significance of the differences between the patients with osteoporosis and arthrosis were studied with the Mann-Whitney test (two tails). The value \(p=0.05\) was fixed as the limit of statistical significance. The dat were analysed with the GraphPad Prism statistical programme, version 5.01.

Results
Study of the expression of RANKL and OPG during the process of mineralisation in vitro
The primary osteoblast cultures in osteogenic conditions showed a progressive mineralisation, evidenced by staining with Alizarin Red S (Figure 1A). In parallel we studied the expression of RANKL and OPG during the process (Figure 1B). The expression of RANKL showed a tendency to increase with the advance in calcification, becoming notable after three weeks in culture, although it did not become statistically significant. On the other hand, we detected no differences in transcriptional levels of OPG (Figure 1C). It should be noted that the gene expression of OPG was much higher than that of RANKL during the whole process. As a consequence, the values of the RANKL/OPG quotient were noticeably low, although again we observed a non-significant tendency to increase with the time in culture (Figure 1D).

Study of the expression of RANKL and OPG in primary osteoblasts from patients with fracture and with arthrosis
Table 1 summarises the characteristics of those patients from whom the samples were obtained. We found no significant differences in the gene expression of RANKL, OPG or in the RANKL/OPG quotient, between the two groups of osteoblasts analysed (Table 2). As with the mineralization medium cultures in both groups OPG expression was notably higher than that of RANKL. On the other hand we analysed the results by stratifying the samples by age. In spite of the fact that no significant differences were found, the osteoblasts obtained from the arthrosic patients aged between 81 and 90 years showed a clear tendency to express more OPG. Similarly, the RANKL/OPG quotient was lower in the osteoblast cultures from patients with arthrosis (Table 3).

Finally, we analysed the data separating the samples by sex. No differences were found between women and men when the two groups of osteoblasts were compared. Similarly, no differences were found between women and men in the same group (Table 4).

Discussion
Since the discovery of the RANKL-OPG-RANK signalling system there have been many studies designed to understand the mechanisms which regulate it. Studies with “knock out” mice have shown that the absence of these genes is directly related to changes in bone mass8. Therefore, this system has become a valuable therapeutic target for the treatment of prevalent skeletal diseases such as osteoporosis. In fact, the antibodies against RANKL have been shown to reduce the number of fractures in postmenopausal osteoporotic patients5. Therefore, it seems clear that this is one of the signalling mechanisms critical for bone homeostasis, capable of tipping the balance towards greater or lesser resorption as a function of the relationship between the production of RANKL and OPG.

At the moment it is not known precisely which are the mechanisms which trigger the commencement of bone remodelling, but it is thought that it is the osteocytes which are responsible for signalling the location and for recruiting the osteoclast precursors to the region, supposedly through a process of chemoattraction10,11. The production of RANKL by the osteoblasts, and, as has recently been published, by the osteocytes12, induces the maturation of the osteoclast precursors and starts the resorption. However, osteoblast and osteoclasts do not coexist in time or space. On the one hand, it is known that the osteoclasts undergo apoptosis and disappear. On the other, our data from in vitro mineralisation using primary osteoblasts suggest that during bone formation there is a greater gene expression of OPG than RANKL. This, theoretically, would reduce the possibility of an interaction between RANKL and RANK, thus preventing a possible activation of osteoclastogenesis during bone formation. Curiously, the expression of RANKL and OPG does not appear to be modified significantly during the process of mineralisation. However, there is observed a slight tendency to an increase in the expression of RANKL in later days, in those cases in which the
mineralisation was more patent. This slight increase in the expression of RANKL is accompanied by a slight increase in the RANKL/OPG quotient.

Osteoporosis and arthrosis are two skeletal diseases characterised by having opposing bone mass values. While the osteoporotic patients showed a reduction in bone mass compared with healthy subjects, those with arthrosis tended to have a higher bone mass. Given the impact that RANKL and OPG have on bone homeostasis, it may be expected that the difference in the production of these proteins may explain, at least in part, the differences in bone mass between these patients. Thus, our group has recently shown that the production of RANKL, and as a consequence, the RANKL/OPG quotient, is increased in the bone tissue of osteoporotic patients. Other authors have reported similar results, which suggests that this really is a pattern of expression characteristic of osteoporotic patients. In an attempt to find an explanation for this deregulation of RANKL expression, we studied whether there were differences in the methylation of the promoter regions for this gene in bone tissue of osteoporotic and arthritic patients. However, it should be taken into account that bone tissue is formed by different cell types, a number of which are capable of producing RANKL and OPG. Therefore, we decided to study the expression of these genes selectively in primary osteoblasts from osteoporotic and arthritic patients. As observed in primary osteoblast mineralisation, in both osteoporotic and arthritic patients the values of OPG expression were higher than those of RANKL. In the overall analysis of the results, we found no significant differences in the expression of RANKL or OPG between the two groups studied. These results are in line with earlier observations carried out by other groups. Nor did we find any differences when we analysed expression by sex or age. However, we did find a clear tendency to a higher expression of OPG in arthritic patients aged between 81 and 90 years, and as a consequence, those same patients showed a lower RANKL/OPG quotient. Together, these results suggest that the differences observed in bone tissue were not due to the osteoblasts of the osteoporotic or arthritic patients being intrinsically different in terms of the expression of these genes. It is possible that the differences at the level of tissue may be explained by differential production on the part of other types of cell, such as the osteocytes or cells of the immune system. In concordance with this, the RANKL/OPG quotient found in the primary osteoblast cultures was much lower than that found in the bone tissue, which means that other cells different from the osteoblasts contribute to the production of RANKL in the bone tissue. Similarly, it has recently been suggested that the osteocytes are the principal source of RANKL in the bone, at least in mice.

It is therefore possible to speculate that the differences found may be due to a differential production of RANKL specifically in this type of cell.

Figure 1. Gene expression of RANKL and OPG in a process of mineralisation in vitro in primary osteoblasts. A) Every 7 days, the mineral deposition was evaluated using Alizarin Red S. B) The expression of RANKL showed a non-significant tendency to increase with time. C) The expression of OPG was constant throughout the process of mineralisation in vitro. D) The RANKL/OPG quotient increases non-significantly in the final days of the process. Presented in the figures are the mean and standard deviation of the values of relative expression obtained from the process of mineralisation with 4 independent samples of primary osteoblasts.
In fact, it would not be surprising that the osteocytes in osteoporotic patients had an abnormal production of RANKL, since we have already observed in earlier works a change in the expression of various genes involved in bone metabolism. Similarly, the osteoblastic lining cells, considered to be inactive osteoblasts, may be implicated in the differences in the expression of RANKL in bone tissue, since our studies indicate that the pattern of methylation of the promoter of the RANKL gene allows these cells to be capable of expressing both RANKL and OPG.

On the other hand, we should not forget that, when working with primary cells, these are isolated in their usual surroundings, the bone micro-environment, without their being stimulated by factors capable of modulating the production of RANKL and OPG, positively or negatively. Various molecules, such as the parathyroid hormone or the estrogens, among many others, can alter the expression of these genes and, therefore, may be responsible for the deregulation reported in osteoporotic patients. Finally, our analysis has focused exclusively on gene expression, without evaluating the quantity of protein present. Similarly, earlier works have shown that, in spite of finding no differences at a transcriptional level, the synthesis of OPG is increased in osteoblasts taken from osteoporotic patients. The causes of this de-synchronisation between transcriptional and

Table 1. Distribution of sex and age of the samples collected for the analysis of the expression of RANKL and OPG

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Women</td>
</tr>
<tr>
<td>FRX</td>
<td>28</td>
<td>23</td>
</tr>
<tr>
<td>ART</td>
<td>26</td>
<td>9</td>
</tr>
<tr>
<td>Value p</td>
<td>0.003</td>
<td>0.20</td>
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Table 2. Values for gene expression (mean and standard deviation) for the RANKL and OPG gene in primary osteoblasts

<table>
<thead>
<tr>
<th></th>
<th>OPG</th>
<th>RANKL</th>
<th>RANKL/OPG</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRX</td>
<td>25.52±4.06</td>
<td>0.024±0.013</td>
<td>0.001±0.0006</td>
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<tr>
<td>ART</td>
<td>45.86±19.67</td>
<td>0.042±0.023</td>
<td>0.04±0.039</td>
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<tr>
<td>Value p</td>
<td>0.57</td>
<td>0.50</td>
<td>0.81</td>
</tr>
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</table>

Table 3. Analysis stratified by age for the gene expression of RANKL and OPG in primary osteoblasts (mean and standard deviation)

<table>
<thead>
<tr>
<th>Age</th>
<th>OPG</th>
<th>RANKL</th>
<th>RANKL/OPG</th>
</tr>
</thead>
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<tr>
<td></td>
<td>70-80</td>
<td>81-90</td>
<td>70-80</td>
</tr>
<tr>
<td>FRX</td>
<td>75±1</td>
<td>85±3</td>
<td>24.92±4.7</td>
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<tr>
<td>ART</td>
<td>73±1</td>
<td>82±2</td>
<td>25.0±8.2</td>
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<tr>
<td>Value p</td>
<td>0.21</td>
<td>0.14</td>
<td>0.69</td>
</tr>
</tbody>
</table>

Table 4. Analysis stratified by sex for the gene expression of RANKL and OPG in primary osteoblasts (mean and standard deviation)

<table>
<thead>
<tr>
<th></th>
<th>OPG</th>
<th>RANKL</th>
<th>RANKL/OPG</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRX</td>
<td>26.73±0.01</td>
<td>23.02±7.05</td>
<td>0.028±0.012</td>
</tr>
<tr>
<td>ART</td>
<td>96.77±55.88</td>
<td>18.96±5.02</td>
<td>0.096±0.066</td>
</tr>
<tr>
<td>Value p</td>
<td>0.35</td>
<td>0.47</td>
<td>0.93</td>
</tr>
</tbody>
</table>
protein levels are currently unknown. One may speculate about a possible deregulation of the expression of certain miRNAs being capable of influencing the process of translating messenger RNA or changes in post-translation marks which affect the stability or half-life of the protein. So it would be interesting to carry out studies to confirm whether the differences observed at a transcriptional level also occur at the level of the proteins, as well as fathoming those post-transcriptional and post-translational mechanisms which influence the quantity of protein produced.

Taken together, our data suggest that the differences in the production of RANKL and OPG found in bone tissue between osteoporotic and arthritic patients are not due to the intrinsic characteristics of the osteoblasts in these patients, but must have their origin in other skeletal cells, or be the consequence of the complex cellular and molecular interactions which take place in the bone microenvironment. New studies will be needed which focus on other types of cells to identify the mechanisms which underlie the deregulation of the expression of RANKL in osteoporotic bone.

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**Bibliography**