Upregulated expression of RANKL on bone marrow stromal cells can stimulate osteoclast precursors to mature into functional osteoclasts and promote survival of myeloma cells

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Abstract

Introduction: Increased expression of receptor activator of NF-kB ligand (RANKL) and/or RANK may be involved in the excessive bone resorption observed in Multiple myeloma (MM). This report describes effects of myeloma cells on the differentiation of osteoclast precursors (pOCs) into OCs in different culture systems in vitro and the interaction between OCs and myeloma cells.

Material and methods: We provide evidence that the pOCs matured in medium containing recombinant human macrophage colony-stimulating factor (rhM-CSF) and RANKL. Co-culture of RPMI 8266 and XG1 in a direct contact manner, MM cells promoted the pOCs to differentiate into mature OCs in medium with rhM-CSF and in turn the MM cells proliferated and grew well in present of mature OCs.

Results: In vitro assays showed that MM cells induced bone marrow derived monoclear cells to differentiate into adherent tartrat-resistant acid phosphatase positive multinucleated cells, indicative of the formation of functional OCs. The OCs supported the survival and proliferation of those MM cells in culture without serum by the way to protect them from apoptosis and dying, but they can't reverse the apoptosis induced by dexamethasone in vitro. The RT-PCR assays revealed that the RPMI8266, XG1 and XG7 cells didn't express mRNA transcripts for a membrane-bound and a secreted form of RANKL. MM cells RPMI8266 and XG1 triggered increased RANKL and decreased OPG expression of BMSCs in co-culture system.

Conclusions: Our results indicate that OCs and MM cells were dependent each other for their growth and survival and support the data of role of the microenvironment in tumor sustenance and progression.

Keywords: multiple myeloma, osteoclast, osteoblast, receptor activator of NF-κB ligand (RANKL), osteoprotegerin (OPG).

Introduction

Multiple myeloma (MM) is a B-cell malignancy characterized by the accumulation of malignant plasma cells in the bone marrow leading to impaired haematopoiesis and bone disease, which includes mainly lytic lesions, pathological fractures, hypercalcemia and osteoporosis [1-3]. Myeloma bone disease is the result of the increased activity of osteoclasts, which is not accompanied by a comparable increase of osteoblast function, thus leading to enhanced bone resorption [4, 5]. The excessive bone resorption observed in myeloma lesions may be explained by the production of several
interleukin 6 (IL-6), IL-1β, tumour necrosis factor α (TNF-α) and macrophage colony-stimulating factor (M-CSF) [6]. Although IL-6 seems to be primarily involved in myeloma osteolysis as well as in the growth and survival of myeloma cells, the mechanisms leading to bone destruction and effects of bone marrow stromal cells on myeloma cells in this disease are not fully understood [4].

The histomorphometric studies performed in MM patients have demonstrated that the increase of osteoclastogenesis and osteoclast activity is an early event that occurs in close contact with myeloma cells [7, 8], suggesting that local factors rather than systemic mechanisms are involved in the pathogenesis of osteolytic bone lesions. Moreover, MM patients with more extensive plasma cell infiltrates or active disease are characterized by a lower number of osteoblasts and decreased bone formation that contribute, together with the increased osteoclast activity, to the development of bone lesions. A close relationship between MAC growth, OC hyperactivity and osteolysis has been postulated clinically and demonstrated in the severe combined immunodeficient-human (SCID-hu) and the ST33 murine models (Vanderkerken et al., 2003) of MM [9-11].

Recently, two molecules belonging to the TNF receptor ligand superfamily, osteoprotegerin (OPG) and its ligand OPG-L, namely the receptor activator of NF-κB ligand (RANKL), also known as TNF-related activation-induced cytokine (TRANCE), have been identified as critical in the regulation of osteoclast activity, leading to a new paradigm in bone biology [12, 13]. RANKL is encoded by a single gene at human chromosome 13q14. Alternative splicing of RANKL mRNA allows expression of a type II transmembrane glycoprotein or a soluble ligand. RANKL is expressed by activated T cells, marrow stromal cells and osteoblasts and binds to its receptor, RANK, which is expressed by osteoclast precursors, chondrocytes and mature osteoclasts. OPG is encoded by a single gene on chromosome 8q24 and is mainly secreted by marrow stromal cells. Extensive studies have shown that OPG and RANKL exert a coupled control of bone resorption [14-19]. Therefore, it is the balance between the expression of RANKL and OPG that determines the extent of osteoclast activity and subsequent bone resorption.

The microenvironment is now recognized as a principal facilitator of the malignant disease process. Tumours have been shown to induce changes in their microenvironment and in turn require these changes for their continued survival. The bone marrow stromal cells and OC comprise the bone marrow microenvironment and are thus important not only in normal haematopoiesis, but also in the pathogenesis of MM. The mechanisms that enhance OC function in malignant plasma cells have not been completely elucidated. The present study was designed to observe the in vitro expansion of osteoclast precursors from bone marrow using rhM-CSF and the ability of these cells to mature as osteoclasts in the presence of RANKL and/or multiple myeloma in different culture systems in vitro. Moreover, we examined the effects of osteoclast precursors on proliferation and survival of MM cells, especially in sera starvation or in medium containing dexamethasone. The effects of MM cells on expression of RANKL and OPG of bone marrow stromal cells were investigated using a co-culture technique.

Material and methods

Culture of primary osteoclast precursors and mature osteoclasts

Because osteoclast precursors are mainly presented in bone marrow (BM) as adherent cells when cultured in vitro, we used BM as a source of osteoclast precursors (pOCs) and mature OCs. Primary pOCs were isolated using a modification of Yamamoto’s methods [20]. Briefly, Human BM cells were obtained from 10 healthy volunteers after informed consent was obtained, in accordance with the guidelines of the ethics committee of Soochow University Hospital. The bone was penetrate at the posterior superior iliac spine after local anaesthesia with 1% lidocaine. A total volume of 10 ml of bone marrow was aspirated. The bone marrow mononuclear cells (BMMNCs) were separated by Ficoll-Hypaque density gradient centrifugation. After washing twice with Hank’s solution supplemented with 5% FBS, the cells were cultured at 37°C in a humidified atmosphere of 5% CO2 in air at 2 x 10⁵ cells/ml in 50 ml flasks in α-MEM supplemented with 20% FBS. The non-adherent cells were removed after 72 hours of incubation and the adherent cells collected as a source of pOCs. To generate OCs, OC medium was added which contained RANKL (50 ng/ml), human M-CSF (25 ng/ml), and 10 nM dexamethasone, and the cultures were continued for an additional 6-10 days, at which time they contained large numbers of multinucleated OCs with bone-resorbing activity.

Culture of primary bone marrow stromal cells (BMSCs)

Mononuclear cells (MNCs) were separated from bone marrow as mentioned above by Ficoll-Hypaque density gradient centrifugation. To remove haematopoietic stem cells and prevent overgrowth of the cultures with macrophages, CD45+ cells were depleted by negative immuno-magnetic cell selection using the Mini MACs device (Miltenyi Biotec, Germany) according to the manufacturer’s instructions. Samples obtained before and after depletion of the CD45+ cells were analyzed by flow cytometry.
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(FCM). The resulting CD45-depleted MNCs were cultured as described previously [21]. When adherent cells reached 80% confluence, cells were isolated by treatment with 0.25% trypsin/EDTA and replated at 104 cells/ml. After a 10-day incubation, the number of colony-forming-unit fibroblasts (CFU-F) was determined by fixing the cultures with methanol for 5 min, staining with Wright’s stain for 20 min, and then counting the number of colonies.

Effects of MM cells on the pOgs

The pOgs were cultured at a density of 2 × 105/ml in 24-well plates alone or with MM cell line RPMI8266 (ATCC, USA), XG1 (kind gifts of Prof. Zhang Xueguang, Institute of Biotech, Soochow University) in 1 ml of α-MEM with or without 25 ng/ml rhM-CSF (Peprotech, USA) and 50 ng/ml RANKL (Cytolab, USA). The MM cells, pOgs and cytokine combinations used in these studies were 1) direct contact groups; α)pOgs alone; β)pOgs + RPMI8266 (XG1); γ)pOgs + RPMI8266 (XG1) + rhM-CSF; δ)pOgs + RPMI8266 (XG1) + RANKL; 2) indirect contact groups. A Transwell (0.45-μm pore size, Corning Co, USA) culture system was used in this group according to the manufacturer’s instructions. α)pOgs in the lower chamber and RPMI8266(XG1) in the upper chamber; β)pOgs and rhM-CSF in the lower chamber + RPMI8266(XG1) in the upper chamber, and γ)pOgs and RANKL in the lower chamber + RPMI8266(XG1) in the upper chamber. The cultures were fed every 48 hours with the same factor-supplemented media. Cultures were fixed with methanol for 5 min in triplicate on day 6, staining with TRAP (Sigma, USA) according to the manufacturer’s instructions.

MM cell and bone marrow stromal cell co-cultures

The cultured primary bone marrow stromal cells (pBMSCs) were plated to 24-well plates at a density of 5 × 104/ml and grew to 80% confluence, after which time the MM cells were added to it in direct contact. The RPMI8266 and XG1 MM cells were plated in triplicate at a density of 3 × 106 cells/ml. After incubation for 24 hours, the cultured cells were washed three times with PBS to detach and remove the non-adherent MM cells with PBS. The residual pBMSCs were collected for further analysis of RANKL and OPG expression.

Reverse transcription polymerase chain reaction (RT-PCR)

To determine the RANKL and OPG expression in pBMSCs before and after co-culture with MM cell lines RPMI8266, XG1 and XG7, we therefore analyzed expression of RANKL and OPG by RT-PCR. Briefly, total RNA was isolated, reverse transcribed and amplified using the ‘Thermo-Script RT-PCR system’ according to the manufacturer’s instructions (Invitrogen, USA). PCR was optimized by 1.5 mmol/l MgCl2 and 0.5 U of Platinum Taq DNA polymerase in a 20-μl reaction. Amplification conditions included 35 cycles at 94°C for 50 s, 62°C for 20 s, 72°C for 20 s, for a total 35 cycles with a final extension of 10 min at 72°C in a thermocycler (Eppendorf, Hamburg, Germany). β-actin served as an internal control. Primers were designed as follows: RANKL 5’-GGTCCGGCATTTGGAATTAAAGATGAC ACCAG-3’ (antisense); OPG 5’-AACCCCAGAGGCAAACACAC-3’ (sense) and 5’-AAAGAGGC CTCTTCCACAC-3’ (antisense); β-actin 5’-GTTGGGCAGCTGGGCA CGAGGC-3’ (sense) and 5’-CCTTTTCAAGAGCGATTCC-3’ (antisense). PCR products were visualised on 1.5% agarose gels by ethidium bromide staining and ultraviolet (UV) transillumination using the Quantity One software in a Fluor-S Multimager (BioRad, Hercules, CA, USA). The PCR products of RANKL, OPG and β-actin were 814bp, 219bp and 538bp respectively.

MM cells and OC co-cultures

Pre-cultured OCs were washed three times with PBS to detach and remove the non-adherent cells. RPMI8266, XG1 and XG7 MM cells (5 × 105 cells/ml α-MEM medium supplemented with M-CSF and RANKL but lacking dexamethasone) were cultured alone or added to OCs in duplicate in 24-well plates (1 ml/well) for 14 days. In additional experiments, RPMI8266, XG1 and XG7 MM cells were cultured with media conditioned by OCs or by MM cells and OC co-cultures. At the end of day 3, 7, and 10 MM cells were counted. To test the effects of OCs on survival of MM cells, RPMI8266, XG1 and XG7 MM cells were plated to 6-well plates at a concentration of 5 × 104/well alone or containing freshly prepared OCs or by MM cells and OC co-cultures. The cultures were continued for 48 hours and cell numbers, viability (trypan blue assay) and annexin V/PI binding were determined by flow cytometry.

Phagocytosis by OCs

RPMI8266 myeloma cell lines were incubated with 10-4 M dexamethasone for 5 days to induce cell death. The cells were then stained with trypan blue (diluted 1:1 with PBS) for 10 min and washed five times with 30 ml of PBS, resuspended in OC media, and added to OC cultures in 8-well chamber slides (105 cells/well). Phagocytosis of dead myeloma cells by OCs were observed under a light microscope 4–24 hours later.

Statistical analysis

Unless otherwise indicated, all values are expressed as mean ± SE. Statistical analysis was performed using SPSS software (Chicago, IL). Statistical significance was determined using the nonparametric Mann-Whitney U test or Student t test. A P value of < 0.05 was considered significant.
Results

Proliferative characterization of bone marrow pOCs and mature OCs in vitro

Cultures of pOCs and mature multinucleated OCs were prepared from BMMNCs from healthy adults. The non-adherent cells were removed after 72 hours, leaving adherent cells of the myelomonocytic lineage. Most of them expressed tartrate-resistant acid phosphatase (TRAP) (Figures 1A-1D). All OC cultures were devoid of fibroblast-like cells and the OCs remained viable for more than 30 days.

MM cells promote pOCs to differentiate into mature OCs

We initially tested whether RPMI8266 and/or XG1 MM cell lines can affect differentiation of pOCs in vitro. Pre-cultured pOCs were cultured alone or incubated with RPMI8266 and XG1 MM cells for 7–14 days in medium containing M-CSF, RANKL respectively or a combination of those two factors. The results indicated that pOCs matured in medium containing rhM-CSF and RANKL. In co-culture of RPMI8266 and XG1 in direct contact, MM cells promoted the pOCs to differentiate into mature OCs in medium with rh-M-CSF and in turn the MM cells proliferated and grew well in the presence of mature OCs. But the MM cells would gradually undergo apoptosis as in controls and in an indirect contact culture manner with or without rh-M-CSF. Our data also show that MM cells or RANKL alone or in combination did not induce pOCs to differentiate into mature OCs and MM cells and pOCs dissolved as time passed (Figures 2A-2D).

MM cells up-regulate RANKL expression and disrupt the balance of RANKL/OPG of BMSCs

The RT-PCR assays on the human myeloma cell lines revealed that the RPMI8266, XG1 and XG7 cells did not express mRNA transcripts for the membrane-bound and a secreted form of RANKL (Figures 3A, 3B). To determine whether the RPMI8266 and XG1 cell lines had effects on stromal cells from normal human BM, the stromal cells were cultured with RPMI8266 and XG1 for 24 hours; RT-PCR assay found that MM cells could up-regulate the RANKL expression of stromal cells (Figure 3C). In the meantime, the expression of OPG of bone marrow stromal cells was down-regulated in co-culture with MM cells (Figure 3D).

OCs inhibit MM cell apoptosis induced by sera starvation

To test the role of OCs on supporting the survival of MM cells cultured in medium without serum, MM...
cell lines RPMI8266, GX1 and XG7 were co-cultured with pre-cultured OCs in DMEM without serum for 48 hours. The total number of myeloma cells recovered from cultures of myeloma cells alone was significantly lower than that from co-cultures (34.5±6.87 vs. 56.6±11.5%), and their viability was also significantly lower (38 vs. 74.2%, P<0.001), while their apoptotic rate was significantly higher (27.66 vs. 57.71%, 36.40 vs. 82.18%, and 26.24 vs. 90.92% respectively, by annexin V) than those of myeloma

![Figure 2](image-url)

*Figure 2. Myeloma cells directly induce differentiation of precursors (pOCs) to mature OCs. A – pOCs + RPMI8266 (XG1), B – pOCs + RPMI8266+RANKL, C – multinucleated OCs were increased in pOCs incubated with RPMI8266 and M-CSF. The cells are stained with TRAP (+100), D – when pOCs were cocultured with RPMI8266, M-CSF, and RANKL, most of cells showed characteristic of mature OCs (multinucleated) (+200) as stained with TRAP.*

![Figure 3](image-url)

*Figure 3. Effect of MM cells on expression of RANKL/OPG of bone marrow stromal cells (BMSCs). A and B – RPMI8266, XG1 and XG7 MM cells did not express RANKL and OPG (line 1 positive control, 1. 8266; 2. XG1 and 3. XG7), C – MM cells upregulate the RANKL expression of the stromal cells (line 1 BMSCs, control; 2. BMSCs + 8266; 2. BMSCs + XG1), D – MM cells downregulate the OPG expression of BMSCs (line 1 BMSCs control, 2. BMSCs + 8266; 3. BMSCs + XG1).*
cells cultured with OCs. Although OCs protected the MM cells from apoptosis, they had no effects on the MM cells whether the cells were cultured alone or with OCs in medium containing $2 \times 10^{-7} \mu M$ dexamethasone in vitro. The rates of apoptosis of MM cells were about 82.84 vs. 74.01%, 83.94 vs. 85.02%, and 41.36 vs. 30.71% respectively as determined by flow cytometry. There was no statistical significance between them ($P>0.05$), as shown in Table I.

**Table I. Effects of OCs on MM cells in conditions of sera starvation and dexamethasone**

<table>
<thead>
<tr>
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<th>MM cells/MM cells + Dex</th>
<th>MM cells + OCs/MM cells + Dex + OCs</th>
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<tr>
<td></td>
<td>Annexin V (%)</td>
<td>Annexin V/PPI (%)</td>
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<tr>
<td>XG1</td>
<td>36.4/9.36</td>
<td>47.53/83.94</td>
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<tr>
<td>XG7</td>
<td>35.49/16.81</td>
<td>36.76/41.36</td>
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<tr>
<td>8226</td>
<td>18.08/12.85</td>
<td>54.07/82.84</td>
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Discussion

Osteoclast-mediated bone resorption is an essential component of bone development, growth, and remodelling. However, in a variety of pathological conditions, including multiple myeloma (MM), osteoclast recruitment and activity exceed osteoblast-mediated bone formation, leading to focal bone loss throughout the axial and craniofacial skeleton. This osteolytic bone disease is responsible for the most debilitating clinical symptoms of MM, which include bone pain, pathological fractures, spinal cord compression, hypercalcemia, renal failure, and death [22].

The excessive bone resorption observed in myeloma is general in the close vicinity of myeloma cells and is related to an increased osteoclast number and activity at sites of MM cell infiltration. Under the influence of a variety of BM- and MM-derived hormones, growth factors, and cytokines, the pre-osteoclasts differentiate into multinucleated osteoclasts capable of bone resorption. A critical role of BM stromal cells in supporting the growth of malignant plasma cells as well as in myeloma-induced bone resorption has been suggested [23]. These stromal cells are able to produce a number of bone resorption cytokines such as IL-6, and their cytokine production may be modulated by malignant plasma cells either via cell-cell interactions or via secreted products. Hofbauer and his colleagues [24] demonstrated that osteotropic factors and hormones such as parathyroid

![Figure 4](https://example.com/figure4.png)
Upregulated expression of RANKL on bone marrow stromal cells can stimulate osteoclast precursors to mature into functional osteoclasts and promote survival of myeloma cells

Hormone (PTH), 1,25(OH)₂-vitamin D₃, IL-11, IL-1β, TNF-α or prostaglandin E₂ (PGE₂) up-regulate RANKL expression in osteoblast/stromal cells. RANKL, a TNF-ligand family member in its membrane-associated or soluble form, binding to a TNF receptor family member expressed by osteoclast precursors, plays an important role in osteolytic bone disease of myeloma [25-30]. A soluble TNF receptor family member, termed osteoprotegerin, acts as a decoy receptor for RANKL. Significantly, osteoclast formation is determined principally by the ratio of RANKL to OPG in the bone marrow [31]. From these data, an emerging concept is that cytokines and hormonal factors involved in bone resorption may act by a common final pathway involving RANKL and OPG.

Lai et al. [32] had demonstrated that MM cells directly contributed to the pool of RANKL in bone. In the present study, we employed an in vitro assay to demonstrate that myeloma cell lines RPMI8266, XG1, and XG2 did not express RANKL mRNA transcripts for a membrane-bound and a secreted form of RANKL and tried to investigate the interaction between MM cells and RANKL/OPG expression of bone marrow stromal cells. We found that pOCs matured in a medium containing rhM-CSF and RANKL. In co-culture of RPMI8266 and XG1 in a direct contact manner, MM cells promoted the pOCs to differentiate into mature OCs in medium with rh-M-CSF and in turn the MM cells proliferated and grew well in the presence of mature OCs. But the MM cells would gradually apoptose as in controls and in an indirect contact culture manner with or without rh-M-CSF. The RT-PCR assays on the human myeloma cell lines revealed that when BMSCs were cultured with RPMI8266 and XG1 for 24 hours, the expression of RANKL BMSCs was up-regulated, suggesting that RANKL may contribute to the high rate of bone resorption observed in this disease, and may be the result of interactions between malignant plasma cells and BMSCs, via cell-cell contact or production of cytokines known to stimulate RANKL expression. It has recently been reported that three isoforms of RANKL may be produced, of which one lacks the transmembrane domain and could act as a soluble form [33-35]. However, it is not clear whether this soluble form of RANKL plays a role in vivo in normal bone homeostasis or in pathological processes characterized by increased bone resorption. Our data also show that MM cells or RANKL alone or in combination did not induce pOCs to differentiate into mature OCs and MM cells and pOCs became non-viable as time passed. Moreover, our results could suggest that the expression of OPG by stromal cells was down-regulated in co-culture with MM cells.

Previous studies reported that BM stromal cells supported survival and growth of primary human myeloma cells and that BM mesenchymal cells from patients with myeloma abnormally expressed adhesion molecules and cytokines, suggesting that these changes are required to promote myeloma [36-38]. The close association between myeloma cell growth and OCs activity has been gleaned clinically and has been demonstrated in our SCID-hu model for primary myeloma [22, 39]. Our OCs/myeloma cells co-culture experiments clearly demonstrate the ability of OCs from healthy donors to support long-term survival and proliferation of MM cells, especially when the MM cells were cultured devoid of serum. But the OCs did not support the survival of MM cells in medium containing dexamethasone. Additionally, the OCs actively phagocytized dying myeloma cells, explaining the lower number of MM recovered from co-cultures compared with control cultures and making a microenvironment suitable for MM cells. Although myeloma cells did not firmly adhere to OCs, cell-cell contact was essential, as

Figure 5. Osteoclast (OC) phagocytosis of dying tumour cells. The photos show different stages of phagocytosis (×400)
demonstrated in non-contact experiments and using media conditioned by OC or OC/MM cell co-cultures. This indicates that soluble factors released by OCs alone or after their direct interaction with MM PCs are insufficient to promote myeloma cell growth and survival. Taken together with the clinical reports and the data from the experimental models, these results underscore the critical role that OCs play in sustaining the myeloma disease process.

In conclusion, our OC/myeloma cell co-culture experiments clearly demonstrate the ability of OCs from healthy donors to support long-term survival and proliferation of MM cells, especially when the MM cells were cultured without serum. MM cells directly induce osteoclastogenesis through increasing RANKL expression of BMSCs, in turn supporting cell-cell contact. At the same time, the expression of OPG of BMSCs was down-regulated. Additionally, the OCs actively phagocytized dying myeloma cells, explaining the lower number of MM recovered from co-cultures compared with control cultures and making a microenvironment suitable for MM cells.

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