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The Increased Expression of Receptor Activator of Nuclear-κB Ligand (RANKL) of Multiple Myeloma Bone Marrow Stromal Cells Is Inhibited by the Bisphosphonate Ibandronate

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ABSTRACT

The receptor activator of nuclear factor-kappaB ligand (RANKL) and interleukin-1beta are osteoclast activating factors which are abnormally expressed in bone marrow stromal cells and plasma cells of multiple myeloma patients. In this work we analyzed RANKL expression in human bone marrow mesenchymal stromal cells and the effect of the bisphosphonate ibandronate on RANKL expression after IL-1beta activation of ERK pathway. Mesenchymal stromal cells were obtained from bone marrow iliac aspirates from multiple myeloma patients at stages II/III and non-osteoporotics control donors; these cells were maintained under long-term culture conditions. Cells were cultured in the presence or the absence of 5 ng/ml IL-1beta and/or 5 μM ibandronate, during selected periods. mRNA for RANKL and protein levels were assayed by RT-PCR and Western blot, respectively. Human bone marrow stromal cell line HS-5 was used for assessing IL 1beta- and ibandronate-ERK phosphorylation responses. Multiple myeloma mesenchymal stromal cells differentiate from control cells by increased basal RANKL expression. IL-1beta up regulated RANKL expression showed dependent on activated MEK/ERK pathway. Finally, the bisphosphonate ibandronate, that hindered activation of the MEK/ERK pathway significantly inhibited both basal and IL-1beta dependent RANKL expression by cells. Results indicate that RANKL expression involves the MEK/ERK pathway in multiple myeloma mesenchymal stromal cells, and that early obstruction of this path, such as that achieved with ibandronate, significantly deters RANKL protein expression. J. Cell. Biochem. 111: 130–137, 2010.

KEY WORDS: MULTIPLE MYELOMA; HUMAN MESENCHYMAL STROMAL CELLS; RANKL EXPRESSION; IBANDRONATE; BISPHOSPHONATES

Multiple myeloma (MM) is a B-cell malignancy characterized by the accumulation of a clonal population of plasma cells in the bone marrow secreting a monoclonal immunoglobulin protein found in plasma and/or urine. MM cells have a high capacity to induce osteolytic bone lesions which are not followed by reactive bone formation, resulting in extensive lytic lesions [Bataille et al., 1991]. Bone disease manifests itself in the majority of MM patients associated with bone pain, fractures, hypercalcemia and reduced quality of life [Oyajobi, 2007].

Cellular interactions between osteoblast/stromal lineage cells and hematopoietic osteoclastic progenitor cells are crucial for maintaining osteoclastogenesis [Boyle et al., 2003]. Bone marrow stromal cells (BMSCs) and osteoblasts are responsible for the synthesis of compounds such as receptor activator of nuclear factor-kappa B ligand (RANKL), osteoprotegerin (OPG), macrophage colony-stimulating factor (MCSF), interleukin-1 beta (IL-1β), tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), integrating a network of cytokines involved in initiating and maintaining osteoclast differentiation and activity. RANKL plays a pivotal role in transducing an essential differentiation signal to osteoclast lineage cells through binding to its receptor, RANK, expressed on the latter cell population [Matsuzaki et al., 1998; Burgess et al., 1999]. Moreover, OPG, a soluble decoy receptor for RANKL, inhibits osteoclast development by blocking RANKL/RANK interaction [Jilka, 1998; Kearns et al., 2008].

In MM, alteration of the marrow microenvironment collaborates in maintaining and promoting bone disease. For the colonization and expansion of tumor cells in bone a reciprocal interaction between tumor cells and the bone microenvironment is necessary and bone resorption, promoted by imbalanced RANKL/OPG ratio, plays a critical role in this process [Giuliani et al., 2001; Pearse et al., 2001; Roux and Mariette, 2004]. The responsibility of plasma myeloma cells in RANKL overproduction appears to be both direct [Heider et al., 2003] and indirect by inducing RANKL expression by the bone marrow stromal cells and osteoblasts [Giuliani et al., 2001; Pearse et al., 2001]. On the other hand, myeloma cells bind,

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internalize and degrade OPG, and inhibit the activity of osteoblasts [Giuliani et al., 2001; Pearse et al., 2001; Standal et al., 2002].

As osteoclasts resorb bone, a variety of growth factors are activated and released into the bone microenvironment [Clézardin et al., 2005]; thus, deregulated production of several cytokines by bone marrow stromal and myeloma cells plays an important role in allowing the establishment and progression of the tumor within the skeleton. IL-1β among others, increases the expression of adhesion molecules, induces paracrine IL-6 production and increases RANKL production by stromal/osteoblasts cells [Pacifici, 1998; Boyle et al., 2003].

Notwithstanding the essential role assigned to RANKL expression by osteoblasts and stromal cells for osteoclast formation and function and also that various osteotropic factors exert their effect by modulating RANKL gene expression in osteoblasts/stromal cells [Nakashima et al., 2000; Lee et al., 2002], the molecular mechanisms by which RANKL gene expression is regulated are to a large extent unknown. The regulation of RANKL basal transcription has been recently related to the binding of the transcription factors Sp1 and Sp3 to two sequences in the proximal RANKL promoter region in both osteoblasts and stromal cells [Liu et al., 2005]. Sp1 and Sp3 have been shown to play important roles in the differentiation and function of osteoblasts [Srivastava et al., 1998; Feng et al., 2000; Mann et al., 2001]. Of note, Liu et al. [2005] hypothesized that Sp1 and Sp3, by interacting with other transcription factors such as nuclear factor-kappa B or others, could also be involved in the regulation of RANKL gene expression by osteotropic factors such as IL-1, TNF-α, 1α,25 dihydroxyvitamin D_3, dexamethasone, or TGF-β.

On the other hand, triggering of the RAS/MEK/ERK signaling pathway appears to contribute in promoting RANKL expression upstream from the specific activating transcription factors, at least in ST2 cells [Takahashi-Tezuka et al., 1997; Fan et al., 2004; Nishida et al., 2005]. Therefore, effective inhibition of RANKL expression has been observed after treatment with specific MEK1/2 inhibitors either in vitro or in vivo [Nishida et al., 2005; Breitkreuz et al., 2008].

Bisphosphonates (BPs) are used for the treatment of both benign and malignant bone disease; these compounds are synthetic, stable analogues of naturally occurring inorganic pyrophosphate. Nitrogen containing BPs (NBP) (such as pamidronate, ibandronate, risedronate, zoledronate and minodronate), inhibit the activity of farnesyl diphosphate synthase, a key enzyme in the mevalonate pathway. This leads to a reduction in the levels of geranylgeranyl diphosphate, which is required for the prenylation of small GTPases (such as Ras, Rho, and Rab) that are essential for osteoclast activity and survival [Fleisch, 1998; Russell et al., 2008].

The highly selective effect of BPs on osteoclasts does not exclude the possibility that these drugs are internalized by neighboring cells, thereby in the bone marrow microenvironment their effect extend to other cells such as osteoblasts, stromal and tumor cells. Several in vitro studies have shown direct cytostatic and pro apoptotic effects of BPs on human tumor cell lines (myeloma, breast, prostate, and pancreas) in a concentration and time-dependent manner [Shipman et al., 1997; Derenne et al., 1999; Senaratne et al., 2000; Lee et al., 2001]. The BPs' potential actions beyond their anti-osteoclasts activity are of considerable interest, particularly concerning their action on cells regulating the RANKL/OPG relationship in the bone marrow microenvironment.

Several in vitro models using primary cultures of MSCs alone or in co-culture with myeloma cells have shown the contribution of these cells to imbalanced RANKL/OPG relationship in myeloma bone disease [Oyajobi et al., 1998; Giuliani et al., 2001; Pearse et al., 2001]. However, the reciprocal interaction between MSCs and tumor cells/tumor microenvironment could, in the long-term, modify some functional properties in MM-MSCs, for instance basal and regulated RANKL/OPG expression, the activity of the MEK/ERK pathway, or the differentiation capacity of cells. The aim of this study was to further analyze RANKL/OPG expression in bone marrow stromal cells obtained from samples of control and multiple myeloma marrow donors; these MSCs were maintained under long-term culture conditions. We demonstrate that control- and MM-MSCs differ in their basal and stimulated RANKL/OPG expression, and that RANKL expression by MM-MSCs is inhibited by the bisphosphonate ibandronate.

MATERIALS AND METHODS

REAGENTS

Tissue culture media and reagents were purchased from Sigma Chemical Co. (St. Louis, MO), Gibco/BRL (Gaithersburg, MD) or US Biologicals (Swamscott, MA). Tissue culture plasticware was obtained from Nunc (Naperville, IL). Recombinant human interleukin-1β was from R&D Systems (Minneapolis, MI), and ibandronic acid (Bondronat®) from Roche (New Jersey, NJ).

Taq Polymerase, dNTPs, primers, $MgCl_2$, Trizol reagent and PCR reaction buffer, were purchased from Invitrogen Corporation (Carlsbad, CA), reverse transcriptase MMLV-RT was from New England Biolabs (Ipswich, MA). PVDF membranes were purchased from Perkin Elmer Life Sciences (Boston, MA) and ECL kit from Pierce (Rockford, IL).

Antibodies: Monoclonal mouse anti-RANKL and anti-OPG were purchased from R&D Systems (Minneapolis, MI), rabbit polyclonal anti-phospho-ERK1/2, rabbit polyclonal anti-ERK1/2 and peroxidase conjugated goat anti-rabbit antibody were from Cell Signaling Technology, Inc (Danvers, MA), monoclonal mouse anti-beta-actin was from Sigma and peroxidase conjugated goat anti-mouse IgG was from Rockland (Gilbertsville, PA).

HUMAN BONE MARROW

Human bone marrow was obtained by iliac crest aspiration from 10 newly multiple myeloma patients (6 women and 4 men) and from 7 postmenopausal women who served as controls. The former were at stage II/III, according to Durie-Salmon classification [Salmon and Durie, 1978], and the latter considered themselves healthy, except for requiring bone surgery because of arthroplasty. Control subjects were non-osteoporotics, according to their bone mineral density (BMD) at the lumbar spine (L2–L4), and they were not under glucocorticoid or hormone replacement therapy [Raisz, 1997]. All subjects were 60–75 years old; written, informed consent was obtained from all subjects and ethical approval was obtained from the Ethical Committees of both the Hospital Sótero del Río and the Instituto de Nutrición y Tecnología de los Alimentos (INTA).

HUMAN BONE MARROW MESENCHYMAL STROMAL CELLS PREPARATION AND CULTURE METHODS

MSCs were classified as control (C-MSCs) or multiple myeloma (MM-MSCs) according to whether they derived from menopausal or multiple myeloma donors. Bone marrow mononucleated cells were separated by Ficoll-Hypaque density gradient centrifugation, suspended in Minimum Essential Medium Alpha (α-MEM) containing 20% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin sulfate, and incubated at 37°C in the presence of 5% CO_2. After 3–4 weeks a monolayer of adherent cells was established, at confluence the cells were detached by a mild treatment with trypsin–EDTA (0.05% trypsin, 0.53 mM sodium EDTA) for 5 min at 37°C and plated at 1/3 the original density in α-MEM supplemented with 10% FBS (growing medium). Cells were maintained and expanded in this medium; half culture medium volume was replaced with fresh medium twice weekly. Experiments were performed with cells in passages 3–5.

Most contaminating hematopoietic stem cells were progressively lost and after the second passage, cultures contained a morphologically homogeneous cell population. This was confirmed by fluorescence-activated cell-sorting analysis showing a lack of expression of the typical hematopoietic cell surface markers, including CD45, CD34, and CD14, and positivity for CD105, CD73, and CD44 [Sotiropoulou et al., 2006; Patel et al., 2008].

For measurements, MSCs were seeded at 1×10^3 cells/cm^2 and grown until confluence in growing medium, then cells were maintained and treated in α-MEM containing 1% FBS and antibiotics (basal medium) in the presence or absence of 5 ng/ml IL-1β, or 5 μM ibandronate during specified periods. After incubations, cell viability was ascertained by the trypan blue exclusion method.

Because establishing MM-MSCs cultures is time-consuming, some experiments were carried out using the human bone marrow stromal cell line HS-5 (ATCC number CRL-11882TM). Cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS and antibiotics. All experiments were done incubating cells in basal medium.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Cells cultured for specified periods were released from the culture dishes by a mild treatment with trypsin/EDTA, collected and suspended with 1 ml of TRIzol reagent. The extracted RNA was quantified in a spectrophotometer (MBE2000, Perkin Elmer, Boston, MA) and stored at −80°C. RNA integrity was verified by electrophoresis under denaturating conditions.

The complementary DNA was synthesized from 1 μg of total RNA using reverse transcriptase MMLV-RT (New England Biolabs), incubating for 10 min at 70°C, 5 min at 4°C on ice, 90 min at 42°C and finally 15 min at 70°C. For PCR reactions, 300 ng of cDNA was amplified with 30 μL of reaction mix (3 μl of 10× PCR reaction buffer, 1.2 mM $MgCl_2$, 10 pmol of each primer, 0.125 mM dNTPs, 22.3 μl of nucleases-free water, and 2.5 U of Taq Polymerase). The PCR program for RANKL, OPG and GAPDH consisted in initial denaturation at 94°C for 5 min, followed by 39, 33, and 29 cycles, respectively. The cycling conditions for denaturation, annealing and extension were as follows: RANKL, 94°C for 30 s, 59.2°C for 30 s, and 72°C for 40s; OPG, 94°C for 30 s, 53°C for 30 s, and 72°C for 40s; GAPDH, 94°C for 30 s, 53°C for 30 s, and 72°C for 40 s. The programs finished with a final extension of 10 min at 72°C. RT-PCR was performed with a Maxi Thermal Cycler ESCO, Hatboro, PA. The primers used were: RANKL, 5′AGC GTC GCC CTG TTC TTC-3′ (sense); 5′-GGG ATG TCG GTG GCA TTA-3′ (antisense); OPG, 5′-GTG TCT TTG GTC TCC TGC TAA-3′ (sense); 5′-GGG CTT TGT TTT GAT GTT TC-3′ (antisense); GAPDH, 5′-GGA TTT GGT CGT ATT GGG-3′ (sense); 5′-GGA AGA TGG TGAT GGG ATT-3′ (antisense). RT-PCR products were separated by electrophoresis on 2% agarose gel and stained with ethidium bromide. A program Kodak image Zone was used to detect the signal intensity; mRNA expression was calculated as the intensity of the band of the target respect to house keeping (GAPDH) gene.

WESTERN BLOTTING

Cells under basal or specified treatment conditions were washed twice with ice-cold PBS. Cells were then treated with RIPA buffer (50 mM Tris–HCl, pH 7.5; 150 mM NaCl; 1% Nonidet P-40; 0.5% deoxicholate; 0.1% SDS), in the presence of protease inhibitors (100 μg/ml PMSF; 1 mM sodium orthovanadate; 1 μg/ml leupeptine; 0.7 μg/ml pepstatine; 1 μg/ml aprotinine and 2 mM *N*-ethyl maleimide). Proteins (20 μg) from cell lysates were denatured with sample buffer (10% SDS, 10% glycerol, 0.5 M Tris–HCl pH 6.8, 0.05%, bromophenol blue, 20% β-mercaptoethanol) during 5 min at 70°C and separated by 10% SDS–PAGE. Samples were then transferred to PVDF membranes, for 90 min at 100 V. Membranes were treated with blocking solution (5% non-fat dry milk in TBS-0.1% Tween-20) for 1 h at room temperature and exposed to the corresponding primary antibody (anti-RANKL or anti-OPG at a 1:1000 dilution, anti-phospho ERK1/2 at a 1:2,000 dilution, anti-ERK1/2 at 1:1,000 and anti-β actin at a 1:5,000 dilution, in blocking solution) during 16 h at 4°C. Membranes were washed three times with TBS–0.1% Tween-20, and incubated with the corresponding HRP-conjugated secondary antibody at a 1:5,000 dilution during 1h at room temperature. Immunoreactive bands were visualized using the ECL-plus detection system. Quantification was performed using Kodak Digital 1D software, β actin was used as load control.

STATISTICAL ANALYSIS

Unless otherwise stated all experiments were reproduced at least three times. Values are expressed as the mean ± SEM. All statistical analysis was done using GraphPad Prism 4 (GraphPad software, Inc., San Diego, CA). For nonparametric data sets, statistical analysis was done using the Mann–Whitney test. Differences among groups were tested by one or two way ANOVA; $P < 0.05$ was considered statistically significant.

RESULTS

PRODUCTION OF RANKL AND OPG BY HUMAN BONE MARROW MESENCHYMAL STROMAL CELLS

It has been proposed that in multiple myeloma bone disease the relationship among RANKL and OPG levels is disrupted. In this study we evaluated initially the expression of these cytokines by MSCs comparing their levels in cells derived from MM patients and control

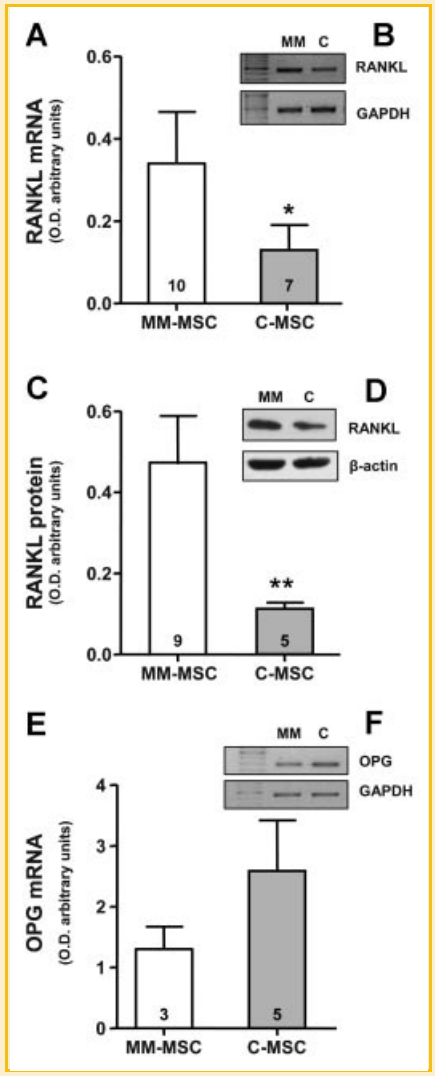

Fig. 1. RANKL mRNA (A,B) and protein (C,D), and OPG mRNA (E,F) expression in MM-mesenchymal (open bars) and control-mesenchymal (gray bars) stromal cells. Cells were incubated in α-MEM 1% fetal calf serum for 48 h. A: The mean optical density (OD) of RT-PCR analysis of RANKL values normalized to the OD of GAPDH mRNA expression. B: Photographs of RANKL RT-PCR. C: The mean OD of RANKL immunoblot analysis, values are normalized to the OD of β-actin expression. D: Photographs of RANKL Western blot. E: The mean OD of RT-PCR analysis of OPG values normalized to the OD of GAPDH mRNA expression. F: Photographs of OPG RT-PCR. The bars represent the mean ± SEM; inserted numbers in columns indicate total number of samples analyzed. $^{*}P < 0.05$; $^{**}P < 0.01$.

donors. The level of RANKL in MM- and control-MSCs was assessed measuring the expression of both mRNA and protein levels in cells incubated in basal medium for 48 h. MM-MSCs expressed significantly higher levels of both mRNA (Fig. 1A,B) and protein (Fig. 1C,D) for RANKL, than control cells. In contrast, the content of mRNA for OPG in MM-MSCs (Fig. 1E,F) was not statistically different from the values measured in control cells.

We evaluated then whether IL-1β affected the production of RANKL by this MSCs system. Results showed that cell treatment with 5 ng/ml IL-1β for 48 h, significantly increased the content of RANKL

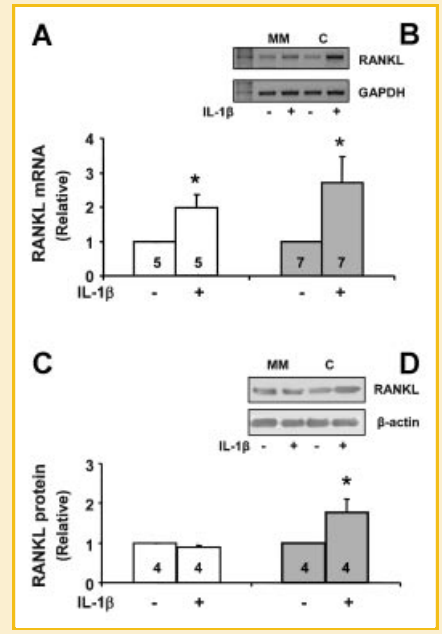

Fig. 2. Interleukin 1-β (IL-1β) stimulated RANKL mRNA (A,B) and protein expression (C,D) in MM-mesenchymal (open bars) and control-mesenchymal (gray bars) stromal cells. Cells were incubated in α-MEM 1% fetal calf serum for 48 h, in the presence or the absence of 5 ng/ml IL-1β. A: Mean OD of RT-PCR analysis of RANKL normalized to the OD of GAPDH mRNA expression, relative to the corresponding value in the absence of the cytokine. B: Photographs of RANKL RT-PCR. C: The mean OD of RANKL immunoblot analysis, values normalized to the OD of β-actin expression are shown relative to the corresponding value in the absence of the cytokine. D: Photographs of RANKL Western blot. The bars represent the mean ± SEM; inserted numbers in columns indicate total number of samples analyzed. $^{*}P < 0.05$.

mRNA in both the control- and MM-MSCs, compared to untreated cells (relative OD values = 0.13 ± 0.061 and 0.34 ± 0.12, respectively; Fig. 2A,B); but the RANKL protein level increased significantly only in control cells (Fig. 2C,D).

On the other hand, IL-1β treatment for 48 h had no effect on the expression of OPG mRNA in both, control and MM-BMSCs (data not shown).

THE EXPRESSION OF RANKL mRNA BY MM-MSCS IS REPRESSED BY IBANDRONATE

The direct effect of ibandronate on MM-MSCs in culture has been scarcely analyzed; in our MM-MSCs system 1–10 μM ibandronate did not modify cell viability, which was 95–98% after 5 days in culture. However, a time dependent inhibitory effect of 5 μM ibandronate on the expression RANKL mRNA was observed in these cells, thus after 4 days of treatment the level of RANKL mRNA reduced by 45% (Fig. 3A). This effect was reflected in the RANKL protein level found in cells, after 4 days in the presence of the bisphosphonate (Fig. 3B,C). Moreover, 5 μM ibandronate restrained also the 5 ng/ml IL-1β stimulatory effect on RANKL mRNA, but inhibition by ibandronate required that cells be exposed to the bisphosphonate prior to IL-1β treatment. Thus, the cytokine positive effect on RANKL mRNA expression (incubation condition 1, Fig. 4A,B) was significantly restrained by pre-treating cells with

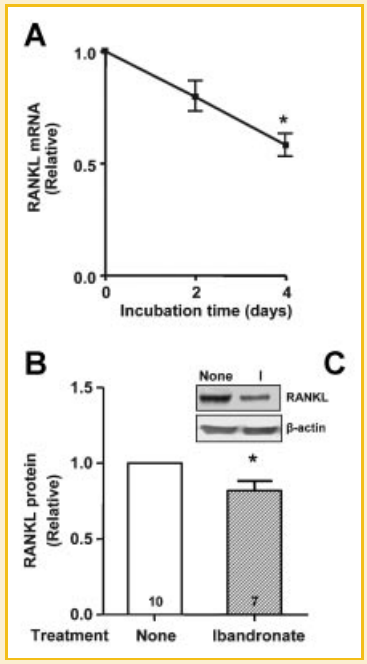

Fig. 3. Effect of ibandronate on basal RANKL mRNA (A) and protein (B,C) expression. Cells were incubated in α-MEM 1% fetal calf serum in the presence or absence of 5 μM ibandronate up to 4 days. A: Mean OD of RT-PCR analysis of RANKL normalized to the OD of GAPDH mRNA expression; values are relative to the corresponding value in the absence of the bisphosphonate. B: The mean OD of RANKL immunoblot analysis, values normalized to the OD of β-actin expression obtained after 4 days of treatment. Values are the mean ± SEM; inserted numbers indicate total number of samples analyzed. $^{*}P < 0.05$. Photographs of RANKL Western blot.

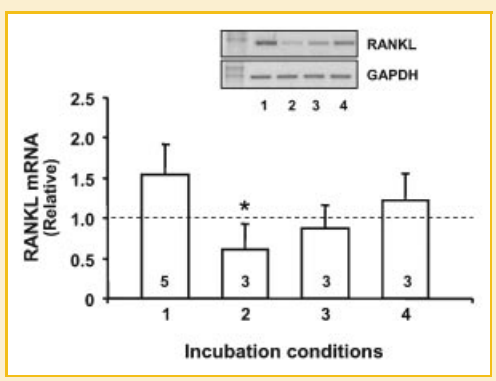

Fig. 4. Effect of ibandronate on IL-1β stimulated RANKL mRNA expression (A,B). Cells were incubated in α-MEM 1% fetal calf serum under the following conditions: (1) basal medium for 72 h, then 48 h with 5 ng/ml IL-1β; (2) 5 μM ibandronate for 72 h, then 5 ng/ml IL-1β in the presence of the bisphosphonate for further 48 h; (3) 5 μM ibandronate for the initial 72 h, and then 5 ng/ml IL-1β for 48 h, in the absence of the bisphosphonate; (4) basal medium for 72 h and further 48 h with 5 μM ibandronate and 5 ng/ml IL-1-β. Results in (A) represent mean OD of RT-PCR analysis of RANKL normalized to the OD of GAPDH mRNA expression; values are relative to the corresponding value in the absence of the bisphosphonate. Discontinue line represents value in the absence of IL-1β and ibandronate. Values are the mean ± SEM; inserted numbers indicate total number of samples analyzed. $^{*}P < 0.05$. B: Photographs of RANKL Western blot.

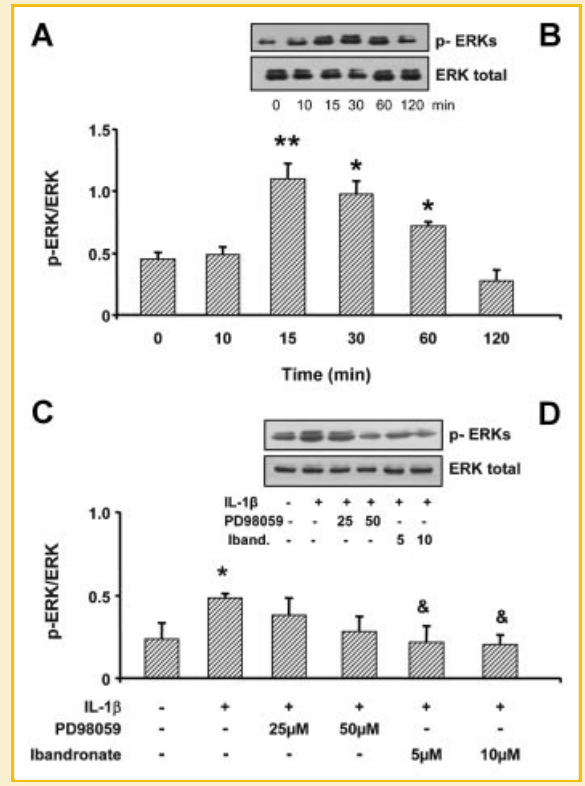

Fig. 5. Time-dependent level of phospho ERKs after 5 ng/ml IL-1β treatment of HS-5 cells (A,B), and inhibition of the level of phospho ERKs by the presence of PD98059 (25 or 50 μM) or ibandronate (5 or 10 μM) (C,D). Cells were incubated in DMEM 1% fetal calf serum for the indicated times in the absence or the presence of 5 ng/ml IL-1β (A,B). Cells were incubated during 30 min with IL-1β with or without the indicated concentrations of ibandronate or PD98059 (C,D). Results represent the mean OD of phospho-ERK immunoblot analysis normalized to the OD of total ERKs expression. B,D: Photographs of phospho-ERK Western blots. The bars represent the mean ± SEM of three independent experiments. In (A), $^{*}P < 0.05$; $^{**}P < 0.01$. In (C), $^{*}P < 0.05$ as compared with control values; $^{\&}P < 0.05$ as compared with IL-1β treated value.

5 μM ibandronate for 72 h, and maintaining this compound during the further 48 h under IL-1β treatment (incubation condition 2, Fig. 4A,B). Moreover, partial reduction in the level of RANKL mRNA was observed when cells were incubated with 5 μM ibandronate for the initial 72 h and then adding IL-1β for 48 h, in the absence of ibandronate (incubation condition 3, Fig. 4A,B), or with basal medium for the initial 72 h and further 48 h with 5 μM ibandronate and IL-1β (incubation condition 4, Fig. 4A,B), although the inhibitory effect did not reach statistical significance.

In contrast, treatment of MM-MSCs with 5 μM ibandronate did not modify OPG mRNA expression (data not shown).

IBANDRONATE INHIBITS PHOSPHORYLATION OF ERKS

From the former observations the inhibition of some key enzymatic step(s) could be inferred, and considering that IL-1β action on cells is dependent in part on ERKs activity we analyzed whether ibandronate modified cells' ERKs phosphorylation. To ascertain incubation time-conditions, the HS-5 cell line was employed. The time-dependent level of phospho ERKs was established after treating

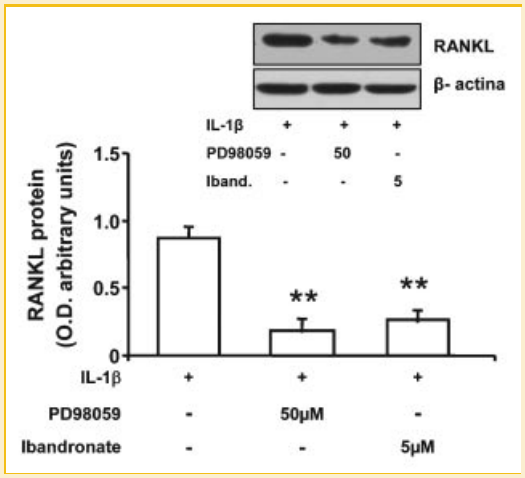

Fig. 6. Inhibition of IL-1β stimulated RANKL protein level in MM-mesenchymal stromal cells by PD98059 or ibandronate (A,B). MM-MSCs cells were incubated in α-MEM 1% fetal calf serum with or without 50 μM PD98059 or 5 μM ibandronate for 30 min, followed by 5 ng/ml IL-1β treatment for 15 min; cells were washed and maintained in basal medium for further 48 h. Results in A represent the mean OD of RANKL immunoblot analysis normalized to the OD of β-actin expression. The bars represent the mean ± SEM of four independent experiments. $^{**}P < 0.01$. B: Photographs of RANKL Western blot.

cells with 5 ng/ml IL-1β, observing maximal phosphorylation of ERKs at 15–30 min of treatment (Fig. 5A,B). Then, the level of phospho ERKs was analyzed incubating these cells for 30 min in the presence or the absence of ibandronate (5 and 10 μM), or the recognized ERKs inhibitor PD98059 (25 and 50 μM), subsequently 5 ng/ml IL-1β was added for further 15 min. Results showed that ibandronate, significantly inhibited the cell level of phospho ERKs (Fig. 5C,D).

Considering the former observations, the effect of either 5 μM ibandronate or 50 μM PD98059 on the expression of RANKL protein was studied, incubating MM-MSCs for 30 min with each reagent, followed by treatment with 5 ng/ml IL-1β for 15 min. Afterwards, ibandronate, PD98059 and the cytokine were washed out and cells were maintained in basal medium until completing 48 h. As shown in Figure 6A,B, the interleukin effect was significantly blocked when either PD98059 or ibandronate was added to the incubation medium prior to IL-1β treatment.

DISCUSSION

It was noticeable that our h-MSCs system replicated the distinctive dissimilar production of RANKL and OPG cytokines, depending on to whether cells originated from bone marrow of control- or MM patients- donors. Thus, enhanced RANKL production was observed in MM-MSCs, compared to control cells, both at the mRNA and protein level, while OPG mRNA content was higher in control-MSCs. It should be taken into consideration that these features expressed in MSCs in the absence of pro osteoclastic hormones or cytokines, and the absence of other cell types, in cells obtained after three to five passages. In the bone marrow MSCs among other cell types, are responsible for RANKL production; the increased RANKL expression observed in MM bone disease has been ascribed to the direct myeloma- to mesenchymal stromal-cell contacts [Giuliani et al., 2001; Pearse et al., 2001; Roux et al., 2002]. Our observations validate h-MSCs as one of the sources of RANKL and OPG cytokines, although the dissimilar expression of these cytokines by control- and MM-cells maintained through several subcultures, independently of the presence of other cell types. Whether MM-MSCs display increased basal RANKL expression because these cells were isolated from MM donors in advanced stages of the disease, is actually unknown. All former in vitro studies demonstrating a disrupted RANKL/OPG cytokine axis in MM employed primary human or murine MSCs cultures, requiring either osteotropic stimulation or co culture with myeloma cells [Hofbauer et al., 1999; Giuliani et al., 2001; Pearse et al., 2001].

IL-1β treatment increased significantly the expression of mRNA for RANKL in both MM- and control-MSCs, this effect appears increased in control cells because of their low level of RANKL expression under basal conditions. IL-1β treatment of cells significantly increased cell-associated RANKL protein only in control-MSCs, while in MM-MSCs the high RANKL protein level observed under the basal conditions remained unchanged after treatment. Since protein measurements were restricted to cell-associated RANKL levels, we cannot rule out that MM-MSCs release substantial RANKL protein into the extracellular milieu, given the high levels of both RANKL mRNA and protein present in these cells under basal condition. Several studies have demonstrated that IL 1-β stimulates the expression of RANKL mRNA in different human cells like osteoblasts/stromal, microvascular endothelial, mesenchymal stem and periodontal ligament cells [Hofbauer et al., 1999; Collin-Osdoby et al., 2001; Wei et al., 2005; Oikawa et al., 2007].

IL-1β treatment did not change the expression levels of mRNA for OPG in control and MM-MSCs; former studies on the action of IL-1β demonstrated increased expression of mRNA for OPG in the MG-63 osteosarcoma cell line [Hofbauer et al., 1999], but not in preosteoblasts [Giuliani et al., 2001].

Myeloma bone disease is characterized by an altered RANKL/OPG system favoring the increased activation of osteoclasts. Our results imply an unbalanced RANKL/OPG expression by isolated MM-MSCs; these observations highlight the contribution of this cell type in establishing the pro-osteolytic MM-bone marrow microenvironment.

Currently, the well-known antiresorptive action of bisphosphonates on pre-osteoclast/osteoclast cell types has been broadened to include their direct action on other bone cell types, including tumor cells [Guise, 2008]. The cells' response generated by these reagents appears to be dependent on the cell type and the chemical structure of the bisphosphonate [Russell et al., 2008]. There is scarce information on the effects of bisphosphonates on human bone marrow stromal cells, therefore in this study we analyzed whether the expression of RANKL by MM-MSCs could be inhibited by the bisphosphonate ibandronate. Results showed that 5 μM ibandronate directly inhibited time-dependently the basal expression of RANKL mRNA and protein by these cells, without affecting their viability. Moreover, ibandronate action hindered also IL-1β mediated up regulation of RANKL expression, but requiring that cells were exposed to bisphosphonate prior to the cytokine plus ibandronate treatment. The bisphosphonate effect on RANKL

expression could not be the consequence of a general toxic effect on MM-MSCs, because the reagent did not affect OPG expression. Therefore, the effect of ibandronate on RANKL mRNA expression appeared rather specific on RANKL regulation.

IL-1β-triggered early ERK activation was investigated in HS-5 cells to ascertain time-dependent phospho ERK's level; it was afterwards observed that this activation was clearly inhibited by the administration of ibandronate. This outcome was analogous to that exerted by the ERKs' inhibitor PD98059. These observations substantiated that in MM-MSCs both reagents PD98059 and ibandronate, significantly inhibited IL-1β mediated up-regulation of RANKL protein expression. This was accomplished under incubation conditions guaranteeing inhibition of IL-1β dependent ERK activation, such as short-term pre-treatment of cells with the inhibitors and further maintaining these reagents for the 15 min required for maximal IL-1β dependent ERK activation. These results indicate that RANKL expression involves the MEK/ERK pathway in MM-MSCs, and that early interruption of this path, such as that achieved with ibandronate or PD98059, significantly inhibits RANKL protein expression. These observations in MM-MSCs support other data implicating activation of the MEK/ERK signaling pathway in the pathogenesis of MM [Hideshima et al., 2006; Solit et al., 2006; Tai et al., 2006, 2007].

The specific step blocked by ibandronate in the MEK/ERK path was not identified in the present study, but ibandronate, like other clinically used NBPs, by limiting farnesyl- and geranyl-pyrophosphate formation [Barrett et al., 2004; Russell et al., 2008] inhibits the isoprenylation of the low-molecular G-proteins involved in signal transduction [Luckman et al., 1998; Fisher et al., 1999]. Inhibition of RANKL mRNA expression by the bisphosphonate YM529/ONO-5920 in the stromal cell line ST2 has been recently related to hindered prenylation of Ras and subsequent blocking of signal transduction in the Ras-MEK-ERK pathway [Nishida et al., 2005].

As far as we know this is the first report on a direct ibandronate effect on RANKL expression by human MM-MSCs. Further studies are needed to determine whether these in vitro observations extend to the in vivo situation; however, it may be suggested some benefit from the early use of ibandronate in MM patients, prior to the development of osteolytic bone disease. The effect of ibandronate on other bone marrow cells showed that it did not significantly inhibit the cell growth at the concentrations expected in vivo [Cruz et al., 2001], nor induced myeloma cell apoptosis in a murine model [Shipman et al., 2000].

In summary, our results show that MM-MSCs differ from control cells by an increased basal RANKL expression. The IL-1β up-regulated RANKL expression showed to be dependent on an activated MEK/ERK pathway. Finally, the bisphosphonate ibandronate, hindered the activation of the MEK/ERK pathway and significantly inhibited both basal and IL-1β dependent RANKL expression by MM-MSCs.

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