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ORIGINAL ARTICLE

Dysregulation of the Receptor Activator of NF-κB Ligand and Osteoprotegerin Production Influence the Apoptosis of Multiple Myeloma Patients' Bone Marrow Stromal Cells Co-Cultured with Myeloma Cells

Barbara Zdzisińska · Agnieszka Bojarska-Junak · Adam Walter-Croneck · Martyna Kandefer-Szerszeń

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Abstract The interaction of multiple myeloma (MM) cells and bone marrow stromal cells (BMSCs) induces profound changes in the bone marrow environment, influencing osteoclastogenesis and MM cell survival. Differences in receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG) production in BMSCs derived from MM patients and control subjects and the apoptosis of BMSCs and MM cells in co-cultures of both cell types were examined. RANKL and OPG expressions were examined by ELISA and semiquantitative RT-PCR. Apoptosis of BMSCs after contact with RPMI8226 and U266 cells was measured by flow cytometry and the level of ALP activity by the spectrophotometric method. OPG production by BMSCs was significantly inhibited after direct contact with RPMI8226 cells. Production of soluble RANKL was enhanced and the increase was more significant in the BMSCs of the MM patients than in those of the controls. In co-cultures of BMSCs and MM cells, significant apoptosis was detected with a concomitant decrease in ALP activity. This apoptosis decreased significantly in the presence of RANK-Fc, an antagonist of RANKL. Disturbances in the RANKL/OPG system are more profound in the BMSCs of MM patients than in those of control subjects after direct contact with RPMI8226 cells. Moreover, direct contact with RPMI8226 and U266 cells induces apoptosis of BMSCs which is mediated by an overproduction of RANKL.

Keywords Multiple myeloma · Bone marrow stromal cells · RANKL · OPG · Apoptosis

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Introduction

Multiple myeloma (MM) is a plasma cell malignancy characterized by an accumulation of monoclonal plasma cells in the bone marrow (BM) and the capacity to induce osteolytic bone lesions. Increased bone resorption, a major characteristic of MM, is due to interactions of myeloma cells with the BM microenvironment and osteoclast stimulation. An essential cytokine system for osteoclast biology has already been identified (Heider et al. 2006; Hofbauer et al. 1998; Hofbauer and Heufelder 1998; Sezer et al. 2003). It consists of the receptor activator of NF-κB ligand (RANKL), its specific receptor (receptor activator of NF-κB, RANK), and its decoy receptor antagonist osteoprotegerin (OPG). RANKL is a member of the tumor necrosis factor (TNF) family and is produced mainly by osteoblastic lineage cells and stromal cells (Nakashima et al. 2000). It exists both as a cell membrane-bound isoform, which is a secondary soluble variant cleaved from the cellular form by metalloproteinases and TNF-α converting enzyme, and as a primary secreted isoform (Nagai et al. 2000). The cellular receptor for RANKL, RANK, is expressed by osteoclast precursors and mature osteoclasts. The osteoclast receptor RANK

has also been detected on bone marrow stromal cells (BMSCs), but not on myeloma cells (Giuliani et al. 2004). Farrugia et al. (2003) in contrast, found that myeloma cells expressed RANK mRNA. OPG acts as a decoy receptor antagonist for RANKL. It is secreted mainly by osteoblastic lineage and stromal cells (Hofbauer and Heufelder 1998). A balanced RANKL/OPG ratio is essential for normal bone turnover.

Several studies have investigated the role of the RANKL/RANK/OPG system in myeloma bone disease. It has been shown that myeloma cells induce RANKL expression by stromal cells and endothelial cells within the BM through direct cell-to-cell contact (Okada et al. 2003). However, there is controversy as to whether myeloma cells express RANKL directly. While some authors found that RANKL was not expressed in the plasma cells of myeloma patients (Roux et al. 2002) or in different myeloma cell lines (Giuliani et al. 2005a), others detected RANKL in both types of these cells (Farrugia et al. 2003; Fiumara et al. 2001; Lai et al. 2004) on the protein level and by reverse transcriptase-polymerase chain reaction (RT-PCR). In addition to their effect on RANKL, myeloma cells decrease OPG availability within the BM microenvironment. They lead to reduced OPG secretion by osteoblasts and stromal cells (Giuliani et al. 2001). Moreover, myeloma cells produce and shed syndecan-1 (CD138), which binds OPG and mediates its internalization and lysosomal degradation (Standal et al. 2002).

Several studies have demonstrated that co-culture of myeloma cells and BMSCs leads to a marked induction of RANKL expression by both cell types and a concomitant downregulation of OPG expression by BMSCs (Giuliani et al. 2001; Roux et al. 2002; Sezer et al. 2003). These changes were abrogated by a neutralizing antibody to the α_4 integrin receptor subunit, indicating that cell-to-cell interactions are necessary for the upregulation of RANKL and the downregulation of OPG (Oyajobi et al. 1998).

As the differences in soluble RANKL (sRANKL) and spontaneous OPG production in BMSCs derived from patients with MM and from healthy subjects have not yet been examined, we decided to study these differences after direct cell-to-cell contact of the BMSCs with RPMI8226 myeloma cells and after indirect contact in which the two types of cells were separated by a transwell. The expressions of RANKL and OPG in BMSCs and RPMI8226 cells were examined by ELISA and semiquantitative RT-PCR. Apoptosis of BMSCs after contact with two different myeloma cell lines, RPMI8226 and U266, in the presence or absence of RANK-Fc chimeric antibodies against RANKL, was measured by flow cytometry. The level of ALP (a marker of osteoblast activity) was measured by the spectrophotometric method.

Materials and Methods

Patients

Bone marrow samples were obtained from 17 patients (49–76 years of age, mean 62.7 years, 8 men and 9 women) with newly diagnosed MM and 11 control subjects (41–67 years of age, mean 57.2 years, 6 men and 5 women; healthy volunteers and patients from whom BM samples were taken for diagnostic reasons and in whom no tumor cells were detected) with their written informed consent. The MM patients had not received any type of therapy before the experiments. The disease stages of the patients according to Durie and Salmon were four with stage I, six with stage II, and seven with stage III of the disease (Durie and Salmon 1975). The study was approved by the local ethics committee.

Cells

The RPMI8226 (ECACC 87012702) and U266 (ATCC, Rockville, MD, USA) myeloma cell lines were grown in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, BRL, UK). BM nuclear cells separated by 0.2% methylcellulose (Sigma,) were cultured in Iscove's Modified Dulbecco's medium (IMDM; Gibco) supplemented with 10% FBS, 10% horse serum (Gibco), 1 μM hydrocortisone (Sigma), and 1% antibiotic–antimycotic solution (Gibco) for 3–4 weeks to generate BMSCs, as described previously (Zdzisińska et al. 2008). BMSCs were detached by trypsinization and frozen at liquid nitrogen temperature until use.

Cytokine Induction

After thawing, BMSCs were propagated in T75 flasks in IMDM medium supplemented with 10% FBS. Then the cells were suspended in IMDM medium with 10% FBS and seeded at a density of 2×10^5 cells/ml into six-well plastic plates (Nunc; 3 ml/well). After 24 h of incubation, the supernatants were removed and new IMDM medium supplemented with 2% FBS was added. The cultures were divided into three batches. The first batch consisted of BMSCs seeded on six-well plastic plates (6×10^5 BMSCs/well) and served as a control of spontaneous cytokine production by BMSCs. To the second batch of BMSCs, myeloma RPMI8226 cells were added at a density of 1×10^6 cells/ml in 3 ml of IMDM medium supplemented with 2% FBS (direct cell-to-cell contact) to a final density of 3×10^6 cells/well. In the third batch of wells with a monolayer of BMSCs, RPMI8226 cells were seeded onto tissue culture inserts (Transwell; Costar, Cambridge,

MA, USA) of 0.4 μm pore diameter. Supernatants of the cultures and co-cultures were collected after 18 and 72 h, centrifuged, and stored at –80°C until cytokine measurement by ELISA.

Assay for Cytokines

The concentrations of OPG and sRANKL were measured by ELISA using kits from Biomedica GmbH, Vienna, Austria (detection limits: 0.14 pmol/l for OPG and 0.08 pmol/l for sRANKL) and from BioVendor, Modrice, Czech Republic (detection limits: 0.13 pmol/l for OPG and 0.2 pmol/l for total sRANKL).

PCR-Analysis

After 18 h of monoculture or co-culture incubation, the BMSCs and myeloma cells were separated by gentle washing or, in the case of the transwell co-culture, the RPMI8226 cells were aspirated from the transwells and adherent cells were detached by trypsinization.

For the RT-PCR, total RNA was isolated from the cells using TRIZOL® LS Reagent (Invitrogen; Germany Life Technologies, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The amount of RNA was measured using a spectrophotometer. RNA was transcribed into cDNA using the Advantage® RT-for-PCR Kit (Clontech Laboratories, Inc. USA) according to the manufacturer's protocol. The Advantage 2 PCR Enzyme Kit (Clontech, USA) was used for PCR analyses performed on a DNA Engine PTC-200 thermocycler (MJ Research, USA). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was amplified as a control for the amount of template used in the RT-PCR (Clontech). Distilled water was used as a negative control. Primer Express 3.0 software was used to designate the primers for RANKL and OPG.

DNA was amplified under the following cycling conditions: denaturation at 94°C for 45 s, annealing at 60°C (for RANKL and OPG) for 45 s, and extension at 68°C for 2 min for 30 cycles (for G3PDH) or 40 cycles (for the other primer pairs). This was followed by a final extension step at 68°C for 7 min. The primer sequences used to amplify and detect RANKL and OPG are shown in Table 1. PCR fragments were separated on 2% agarose gels and visualized under UV light after ethidium bromide staining. The predicted size of each fragment was 400 bp for RANKL, 300 bp for OPG, and 983 bp for G3PDH. The primers were synthesized by Proligo (UK). The relative amounts of the PCR product were determined by quantifying the intensity of bands using the FluorImager and ImageQuant software (Molecular Dynamics, USA).

Determination of ALP-ase Activity

Alkaline phosphatase activity was measured after 3 and 11 days in the monocultures of BMSCs and in the co-cultures with RPMI8226 cells. The cell layers were lysed in a buffer consisting of 50 mmol/l Tris, pH 7.5, containing 300 mmol/l NaCl, 10 ml/l Igepal CA-630 detergent, 1 mmol/l phenylmethylsulfonyl fluoride, and 1 mg/l each of aprotinin and leupeptin. ALP-ase activity in the cell lysates was determined by a rapid colorimetric microplate assay. One hundred fifty microliters of an enzyme solution (8 mM *p*-nitrophenyl phosphate, 12 mM $MgCl_2$ in 0.1 M glycine–NaOH buffer, pH 10.5) was added to 50 μl of the cell lysate and the plate was incubated for 15 min at 37°C. The enzyme reaction was terminated by addition of 50 μl of 0.5 M NaOH. The amount of *p*-nitrophenol released by the enzyme reaction was determined by measuring the absorbance at 405 nm using a microplate reader. The *p*-nitrophenol curve was prepared separately and the molar concentrations of the resulting products were read from the curve.

An Apoptosis Assay in BMSCs after Contact with Myeloma Cells

BMSCs were plated on six-well tissue culture plates at a density of 6×10^5 cells/well/3 ml and cultured in IMDM with 10% FBS. After 24 h of incubation, supernatants were removed and fresh IMDM medium supplemented with 2% FBS was added. Then RPMI8226 or U266 cells (3×10^6 cells/well) were added directly into the wells with a monolayer of BMSCs or seeded into tissue culture

Table 1 RT-PCR primers and conditions for the amplifications

Name	Primer sequence (5′ to 3′)	Annealing temp. (°C)	Cycle no.
RANKL	**F:** CAGCATCAAAATCCCAAGTTCTC	60	40
	R: ATCTCCCACTGGCTGTAAATACG		
OPG	**F:** GCAACACAGCTCACAAGAACAGA	60	40
	R: AAGGTGTCTTGGTCGCCATT		

F forward, *R* reverse

inserts. RANK-Fc chimeric antibodies (Sigma) at a concentration 200 ng/ml were added to the wells with the direct co-culture. BMSCs in monoculture served as a control for spontaneous apoptosis. After 72 h of monoculture and co-culture incubation, supernatants were removed, the cells were detached by trypsinization, and apoptosis was measured.

The samples were analyzed by flow cytometry directly after preparation. A FACS Calibur instrument (Becton-Dickinson) and CellQuest software were used. For each analysis, 10,000 events were acquired and analyzed. The percentage of positive cells was measured from a cut-off set using isotype-matched nonspecific antibodies.

The number of apoptotic cells was measured by the Annexin V technique (TACS Annexin V-FITC; R&D Systems, MN, USA), which enables one to investigate plasma membrane alterations at two different steps: phosphatidylserine (PS) redistribution from the inner plasma membrane leaflet to the outer leaflet is detected by Annexin V (FITC-conjugated) binding to PS and the loss of plasma membrane integrity is revealed by propidium iodide (PI), which permeates into necrotic cells. The cells were incubated with Annexin V-FITC and PI for 15 min at room temperature and for a further 15 min with anti-CD166 (a marker for human BM stromal fibroblasts) or anti-CD138 (a marker for myeloma cells) PE antibodies at room temperature.

The degree of apoptosis was also measured by chloromethyl-X-rosamine staining (Miro Tracker Red CMXRos; Molecular Probes, USA). CMXRos is a cationic lipophilic fluorochrome that does not accumulate in depolarized mitochondria and can be used to detect disruptions in the mitochondrial membrane potential ($\Delta\Psi m$). CMXRos was used in combination with monoclonal anti-CD166 FITC antibodies. The cells were incubated with CMXRos for 30 min at 37°C and, after 15 min of incubation with anti-CD166 monoclonal antibodies, were analyzed in FACSCalibur. Cells considered to be apoptotic displayed a decreased staining of the mitochondrial membrane ($\Delta\Psi m^{low}$) with CMXRos.

Data Analysis

The results were reported as the mean ± SD. The significance of differences was determined by analysis of variance (STATISTICA computer package). A number of statistical tests were performed, including two-way ANOVA with the post-hoc Tukey test to measure differences within a group and the Mann–Whitney *U* test to measure differences between groups. The concentrations of cytokines below the detection level were considered to be 0 for the purpose of the analysis. *p* Values of 0.05 or less were considered significant.

Results

Decreased Production of OPG in Co-Cultures of BMSCs of MM Patients and Controls after Direct Contact with RPMI8226 Cells

RPMI8226 cells were chosen for the experiments because they were reported to have slight adherent properties to BMSCs and to be free of Epstein-Barr virus (Lokhorst et al. 1994). The OPG level was examined by ELISA in the supernatants of monocultures of BMSCs of MM patients and healthy controls and in co-cultures of BMSCs with RPMI8226 cells under cell-to-cell contact conditions and when the two types of cells were separated by a porous membrane (transwell). As can be seen from Fig. 1a, the level of OPG released by the BMSCs of the MM patients was comparable to that of the controls. The RPMI8226 cells did not produce any detectable levels of OPG. In co-cultures of BMSCs with RPMI8226 cells, the level of OPG decreased under cell-to-cell contact conditions. The differences were, however, not statistically significant because of the high standard deviations resulting from the large differences in OPG production (both spontaneous and in response to RPMI8226 cells) among the patients. Nevertheless, when OPG release was examined in two patients, the decreased OPG production was significantly dependent on the ratio of BMSCs to RPMI8226 cells. An increased number of RPMI8226 cells caused a more pronounced decrease in the OPG level (Fig. 1b). Semiquantitative RT-PCR revealed lowered expression of OPG in direct co-cultures of BMSCs with RPMI8226 cells compared with the control. PCR analysis also confirmed that RPMI8226 cells did not express OPG (Fig. 2). In this experiment, the two types of cells were separated by gentle washing. A preliminary cytometric analysis showed that after washing, BMSCs were only 1–3% contaminated with RPMI8226 cells.

Increased Production of RANKL in BMSCs of MM Patients after Direct Contact with RPMI8226 Cells

As can be seen from Fig. 3a and b, the level of sRANKL released into the culture medium of the co-cultures of BMSCs with RPMI8226 cells was significantly higher than that released by the monocultures of BMSCs. Moreover, the increase was detected only when the BMSCs of the MM patients were in close contact with the myeloma cells. The level of sRANKL released spontaneously by the BMSCs of the MM patients was comparable to that of the control subjects; however, the BMSCs of the MM patients produced significantly more sRANKL after direct contact with the MM cells than those of the control subjects. RPMI8226 cells in monocultures also released sRANKL

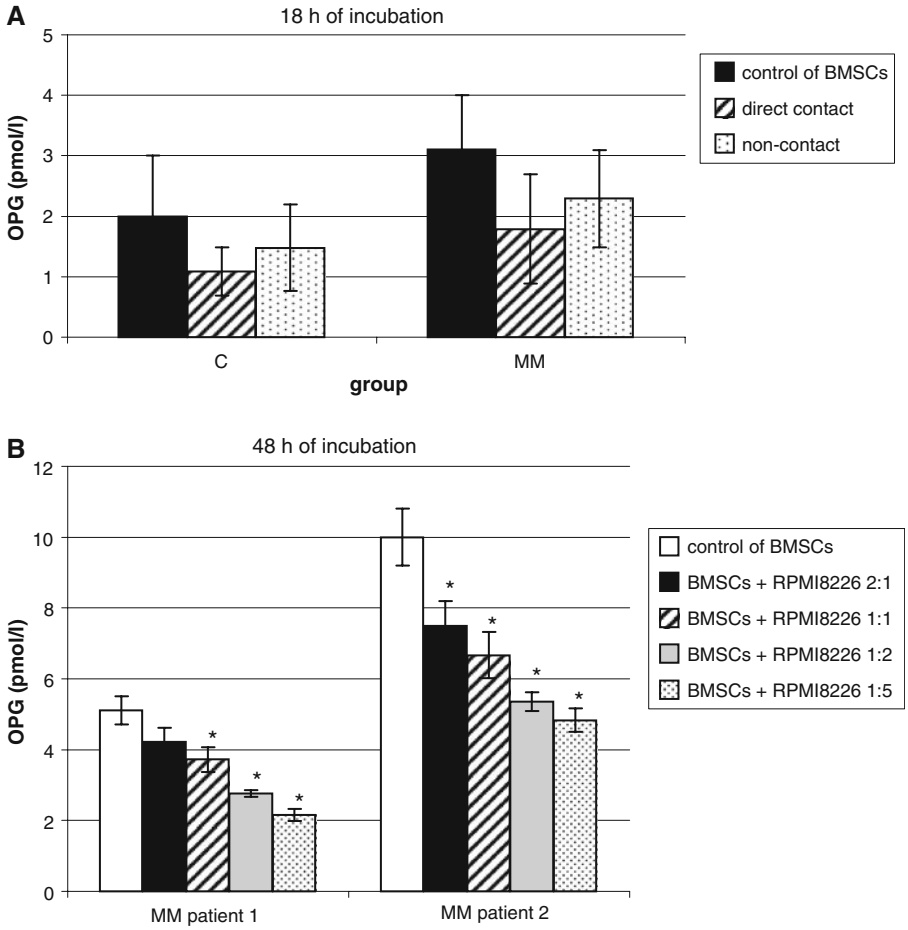

Fig. 1 RPMI8226 cells down-regulate OPG release from BMSCs. **a** BMSCs of 8 MM patients and 7 controls (6×10^5 per well) were incubated for 18 h in monocultures or with RPMI8226 (3×10^6 per well) cells under direct cell-to-cell contact or non-contact (transwell) conditions. The addition of RPMI8226 cells down-regulated the release of OPG from both MM patients and controls under direct cell-to-cell contact conditions. The OPG level was measured by ELISA. The differences were not statistically significant because of great differences among patients. **b** Down-regulation of OPG expression in the BMSCs of two MM patients depended on the BMSC/RPMI8226 cell ratio (at the same volume of cell culture) and became more pronounced with an increased number of RPMI8226 cells. *Statistically significant at $p < 0.05$ compared with monoculture of BMSCs

into the culture medium. Treatment of RPMI8226 and U266 cells with LPS from *E. coli* (1 μg/ml) significantly increased sRANKL production by these myeloma cells (Fig. 3c). The increase in sRANKL production was detected by two different kits. Semiquantitative RT-PCR revealed increased expression of RANKL in the co-cultures of the BMSCs of the MM patients and controls with RPMI8226 cells (Fig. 4). Because the primers were selected to detect all the isoforms of RANKL, it seems likely that not only the expression of sRANKL, but also of its membrane-bound forms was enhanced. RANKL was also expressed in the monocultures of RPMI8226 cells and in the cells which were removed from the co-cultures with BMSCs.

Increased Number of Apoptotic Cells and Decreased ALP Activity in BMSCs after Co-Culture with Myeloma Cells

After 3 days of co-culture of BMSCs with RPMI8226 cells, flow cytometry was used to measure the number of Annexin V-binding and CD166-positive cells; disruption of the mitochondrial membrane potential (ROS low) was also determined. The number of BMSCs which underwent apoptosis was significantly higher in the direct co-cultures than in the monocultures of BMSCs and in the co-cultures in which the cells were separated by a transwell. RPMI8226 cells did not undergo apoptosis (Fig. 5a, b). When RANK-Fc chimera antibodies were added to the co-cultures of the BMSCs of the MM patients and RPMI8226 cells or the other myeloma cell line, U266, their presence canceled the inhibition of myeloma cell-induced apoptosis of BMSCs. This inhibition was statistically significant and independent of the myeloma cell line used (Fig. 6a, b). When ALP activity was measured after 3 and 11 days of co-culture, a significant decrease in ALP activity as a marker for osteoblasts was detected. It seems likely that osteoblast precursors were among the cells which underwent apoptosis after direct contact of BMSCs with RPMI8226 cells (Fig. 7).

Discussion

The interaction of myeloma cells with BM stroma is thought to influence both osteoclastogenesis and malignant

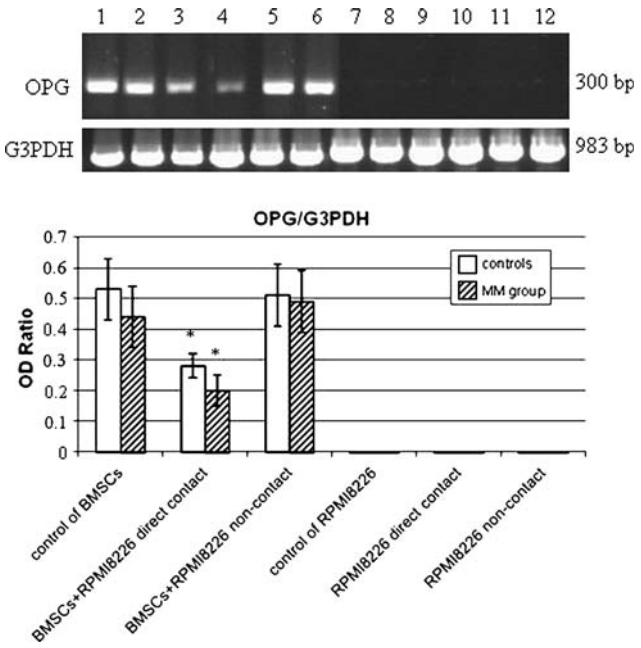

Fig. 2 RPMI8226 cells decrease OPG mRNA expression. The effect of RPMI8226 cells on the level of OPG mRNA was examined by semiquantitative RT-PCR in monocultures of cells and in a co-culture system under cell-to-cell contact and non-contact conditions. The *graphs* represent the mean optical density (OD) of OPG normalized to G3PDH (=OD ratio) ± SD of seven experiments. *Lane 1* control BMSCs of a healthy subject, *lane 2* control BMSCs of an MM patient, *lane 3* co-culture of BMSCs of a healthy subject with RPMI8226 cells in direct cell-to-cell contact, *lane 4* co-culture of BMSCs of an MM patient with RPMI8226 cells in direct cell-to-cell contact, *lane 5* co-culture of BMSCs of a healthy subject with RPMI8226 cells separated by a transwell, *lane 6* co-culture of BMSCs of an MM patient with RPMI8226 cells separated by transwell, *lanes 7 and 8* control of RPMI8226 cells, *lane 9* RPMI8226 cells after cell-to-cell contact with BMSCs of a healthy subject, *lane 10* RPMI8226 cells after cell-to-cell contact with BMSCs of an MM patient, *lane 11* RPMI8226 cells after contact with BMSCs of a healthy subject under non-contact conditions, *lane 12* RPMI8226 cells after contact with BMSCs of an MM patient under non-contact conditions. *Statistically significant at $p < 0.05$ compared with monoculture of BMSCs

cell survival (Heider et al. 2006). In this article we presented evidence that the interaction of RPMI8226 cells with the BMSCs of MM patients resulted in a more profound dysregulation of the RANKL/OPG cytokine axis than in the control subjects, which means that in co-cultures of BMSCs of MM patients with RPMI8226 cells, RANKL production was more significantly enhanced than in controls. Moreover, we found that RPMI8226 cells expressed RANKL and participated in the dysregulation of the RANKL/OPG axis. We also found that BMSCs in co-cultures with myeloma cells underwent accelerated apoptosis and that in such co-cultures there was a significant decrease in ALP activity. Moreover, we detected that BMSC apoptosis may be reversed by the addition of RANK-Fc, which is known to bind to RANKL.

The literature presents conflicting results as to whether myeloma cells express RANKL. Heider et al. (2003) utilizing flow cytometric analysis of BM-derived plasma cells, found RANKL expression by myeloma cells, the level of which correlated with osteolytic bone disease. Similarly, Sezer et al. (2002) utilizing immunohistochemical analysis, detected RANKL production in MM cell lines. In contrast, Giuliani et al. (2001) failed to detect RANKL in any of 10 human myeloma cell lines or in MM cells isolated from 26 patients with MM. Roux et al. (2002) reported similar negative findings in MM cells from the BM of 15 MM patients. However, Lai et al. (2004) using specific primers for the membrane-bound RANKL isoforms 1 and 2, detected mRNA for RANKL in several human myeloma cell lines, among others in RPMI8226 cells and in 17 out of 19 samples prepared from $CD138^+$ cells from the BM of MM patients. They also confirmed these findings using indirect immunofluorescence. In the present study, RANKL was produced in RPMI8226 cells on the protein and mRNA levels. The results obtained by Heider et al. (2003) also provide evidence of correlations between RANKL expression on BM myeloma cells and osteolytic bone disease in patients with MM. Our observations, on the other hand, strongly support the argument that RANKL produced by MM cells contributes to osteolytic lesions in MM patients.

These discrepancies in the results can be explained by the findings reported in a paper by Calvani et al., who reported that myeloma cell lines (U266 and MCC-2), when adhering to plastic surfaces, underwent a transformation to osteoclast-like cells which exhibited a multinuclear morphology with deregulated expression of RANK and RANKL (Calvani et al. 2005). The transformation was dependent on RANKL stimulation. In the present experiments, RANKL expression was found rather late, after 72 h of incubation on plastic plates (at the protein level, by ELISA) or after co-culture with BMSCs (at the mRNA level, by PCR). An additional signal of increased RANKL production was generated by the treatment of myeloma cells with LPS, which can mimic bacterial infection in vivo. It seems likely that in the organisms of MM patients, myeloma cells may initiate bone resorption by acting as functional osteoclasts, as observed in mouse plasmacytoma (McDonald et al. 1983).

Accumulating data suggest that a disruption of the balance between RANKL and OPG is of major importance in MM. Histological examinations of BM biopsies from patients with MM have shown enhanced RANKL expression as well as reduced OPG expression (Pearse et al. 2001). Serum OPG levels are also lower in MM patients than in healthy controls (Seidel et al. 2001; Terpos et al. 2003). Independent research groups (Giuliani et al. 2001; Pearse et al. 2001) have reported reduced expression of

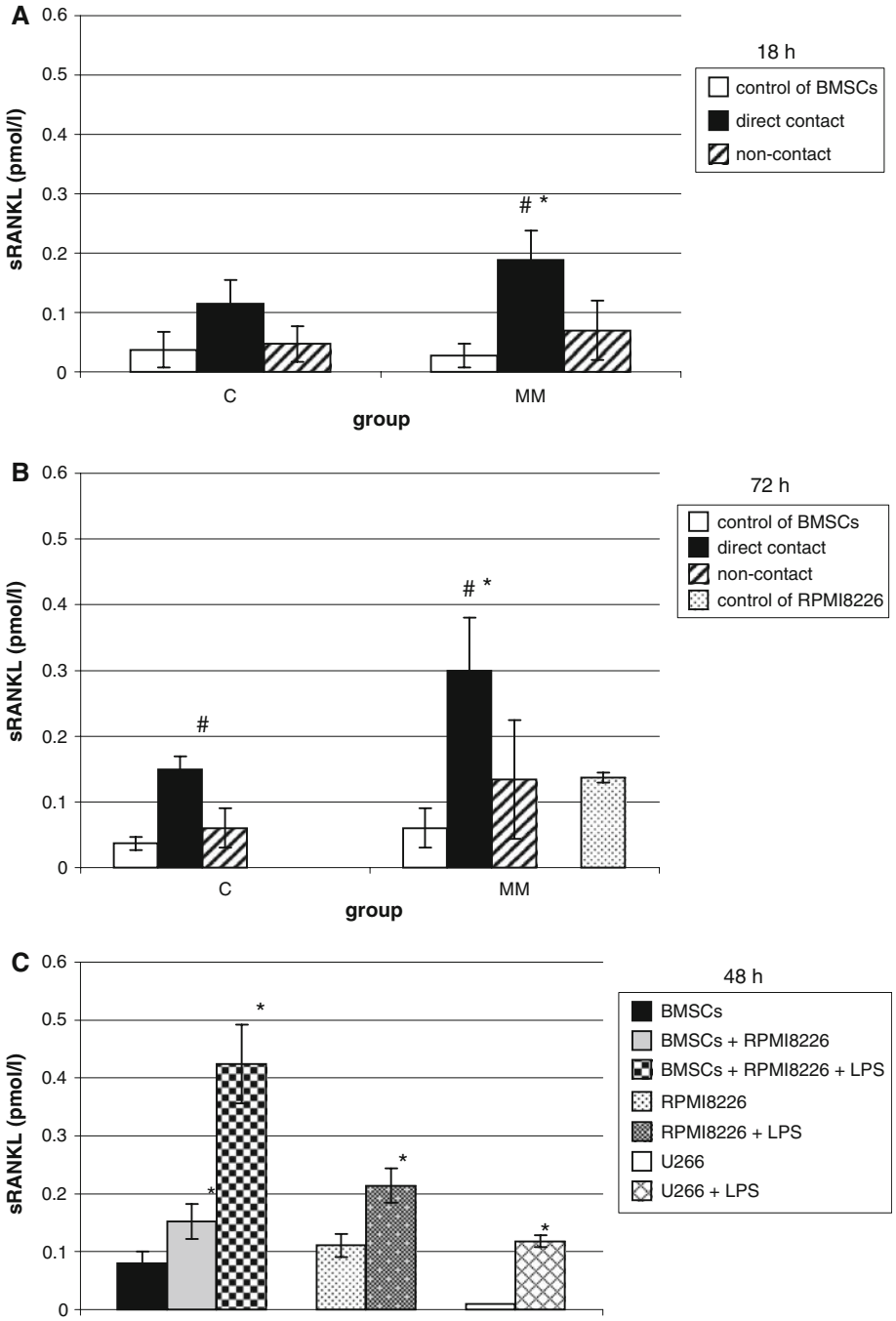

Fig. 3 The induction of sRANKL expression in co-cultures of BMSCs with RPMI8226 cells. Supernatants from monocultures of BMSCs and RPMI8226 cells and from co-cultures under cell-to-cell contact and non-contact conditions (transwell) (BMSCs/RPMI8226 1:5) were assessed by ELISA. RPMI8226 cells significantly increased the level of RANKL in cell-to-cell contact co-cultures with BMSCs of MM patients compared with the co-cultures with BMSCs of control subjects after 18 h (**a**) and 72 h (**b**) of incubation. (**c**) RPMI8226 cells in monoculture after 48 h of incubation produced sRANKL. The level of sRANKL increased in culture of RPMI8226 and U266 cells after LPS stimulation. *Statistically significant compared with BMSCs of the control group at $p < 0.05$. #Statistically significant within the group compared with the appropriate control (monoculture of BMSCs)

OPG by stromal cells in the BM of myeloma patients compared with non-myeloma patients. Furthermore, it has been shown that myeloma cells reduce stromal cell OPG production in vitro. In a co-culture system, the presence of a tissue insert completely blunted the effect of MM cells on OPG and RANKL production, indicating that this effect was mediated by cell-to-cell contact between MM cells and BMSCs rather than by the release of a soluble factor (Giuliani et al. 2001).

In the present experiments we found decreased production of OPG in the co-cultures of BMSCs with RPMI8226 cells compared with the monoculture of BMSCs and decreased OPG expression measured at the mRNA level. These results confirm observations of other authors (Giuliani et al. 2001; Pearse et al. 2001). Moreover, our experiments seem to imply that in vivo suppression of OPG expression may depend on the tumor burden. In the present study the reduction in OPG level was dependent on

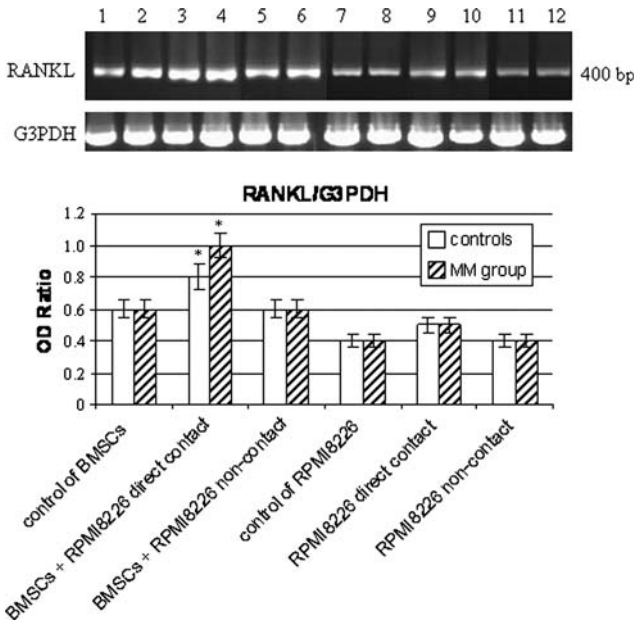

Fig. 4 The induction of RANKL mRNA expression in co-culture of BMSCs with RPMI8226 cells. Semiquantitative RT-PCR analysis of RANKL and G3PDH mRNA in monocultures of BMSCs and RPMI8226 cells and in co-cultures under cell-to-cell contact and non-contact conditions (transwell). The *graphs* represent the mean optical density (OD) of RANKL normalized to OD of G3PDH (=OD ratio) of seven experiments. *Lane 1* control BMSCs of a healthy subject, *lane 2* control BMSCs of an MM patient, *lane 3* co-culture of BMSCs of a healthy subject with RPMI8226 cells in direct cell-to-cell contact, *lane 4* co-culture of BMSCs of an MM patient with RPMI8226 cells in direct cell-to-cell contact, *lane 5* co-culture of BMSCs of a healthy subject with RPMI8226 cells separated by a transwell, *lane 6* co-culture of BMSCs of an MM patient with RPMI8226 cells separated by transwell, *lanes 7 and 8* control of RPMI8226 cells, *lane 9* RPMI8226 cells after cell-to-cell contact with BMSCs of a healthy subject, *lane 10* RPMI8226 cells after cell-to-cell contact with BMSCs of an MM patient, *lane 11* RPMI8226 cells after contact with BMSCs of a healthy subject under non-contact conditions, *lane 12* RPMI8226 cells after contact with BMSCs of an MM patient under non-contact conditions. *Statistically significant at $p < 0.05$ in comparison to monoculture of BMSCs

the number of RPMI8226 cells in direct contact with BMSCs.

On the other hand, it was discovered that recombinant OPG can, in a dose-dependent manner, inhibit TRAIL/Apo2L-induced apoptosis of RPMI8226 cells, which suggests that OPG may function as a survival factor for myeloma cells (Shipman and Croucher 2003). It is intriguing that myeloma cells can down-regulate the production of a tumor-survival factor. However, the nature of the role of OPG as a survival factor in vivo will depend on the relative concentrations, timing, and location of the expressions of OPG, TRAIL/Apo2L, and their ligands in the local BM environment.

In this study we detected accelerated apoptosis of BMSCs after direct contact with RPMI8226 cells together with a strong inhibition of ALP activity, which can be considered as myeloma cell-induced apoptosis of the osteoblastic lineage. It has already been established that myeloma cells suppress the formation of osteoblast progenitors. Moreover, an inhibitory effect on osteocalcin, alkaline phosphatase, collagen I mRNA, and RUNX2/CBFA1 activity by human preosteoblastic cells in co-cultures with myeloma cells has been observed (Giuliani et al. 2005b). Several studies suggest that in addition to blocking osteoblast formation, MM cells may directly act on mature osteoblastic cells. MM cells have been reported to up-regulate osteoblast apoptosis when co-cultured with osteoblasts (Silvestris et al. 2003). Recently, Tinhofer et al. (2006) reported that MM cells induced apoptosis of osteoblasts and sensitized osteoblastic cells to cell death mediated by recombinant TRAIL. In turn, osteoblastic cells protected MM cells from TRAIL-mediated apoptosis by secretion of OPG (Tinhofer et al. 2006). The results of the present study confirm that at least part of BMSCs undergo apoptosis after direct contact with myeloma cells. Moreover, in our experiments this accelerated apoptosis of

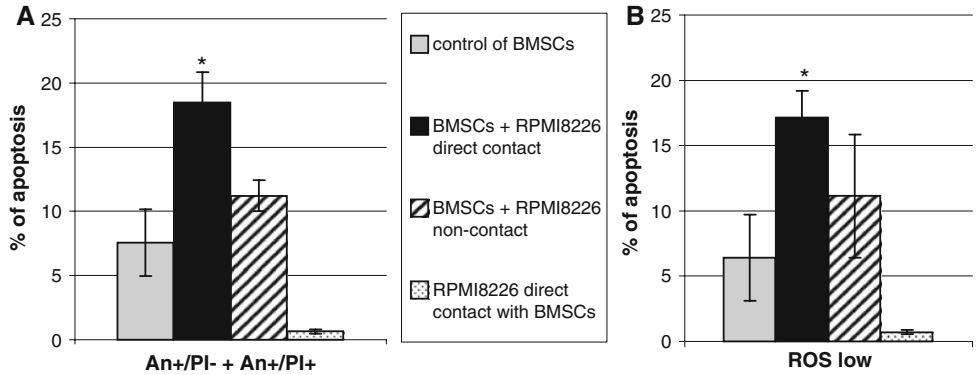

Fig. 5 RPMI8226 and U266 cells induce apoptosis of BMSCs. BMSCs ($CD166^+$) of MM patients were cultivated alone or together with RPMI8226 cells for 72 h. Apoptosis of BMSCs ($CD166^+$) was determined by flow cytometry using the Annexin V/PI binding assay (**a**) or by CMX Ros, detecting a decreased mitochondrial membrane potential ($\Delta\Psi m$) (**b**). Results from eight independent experiments are presented. *Statistically significant at $p < 0.05$ compared with the control of BMSCs

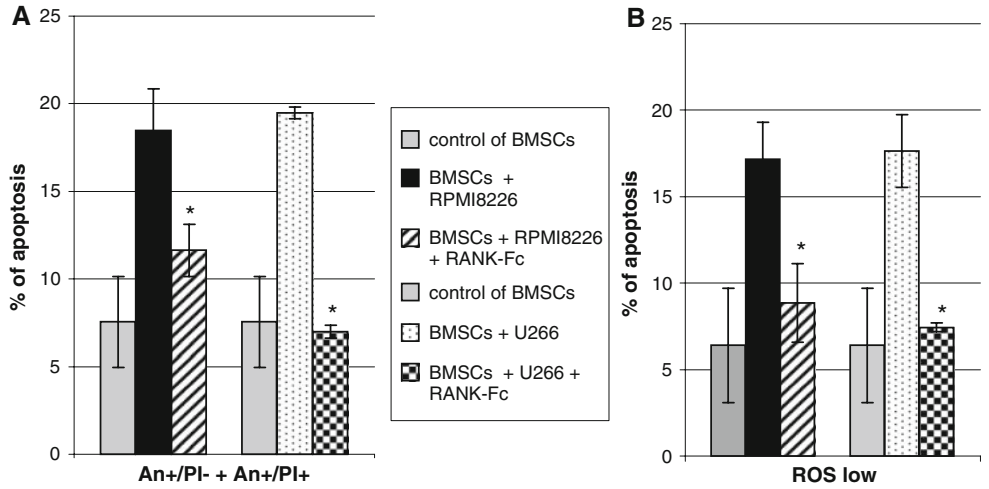

Fig. 6 RANK-Fc inhibits myeloma cell-induced apoptosis of BMSCs ($CD166^+$ cells). BMSCs of MM patients were cultivated with RPMI8226 or U266 cells in the absence or presence of RANK-Fc chimera antibodies at a concentration of 200 ng/ml. After 3 days of co-culture, the myeloma cells were removed, BMSCs were trypsinized, and apoptosis of the $CD166^+$ cells was measured by Annexin V-binding with propidium iodide (**a**) or by chloromethyl-X-rosamine staining (**b**). Apoptotic cells displayed a decreased mitochondrial membrane staining (ROS low). *Statistically significant at $p < 0.05$ compared with direct co-culture of BMSCs with RPMI8226 or U266 myeloma cells

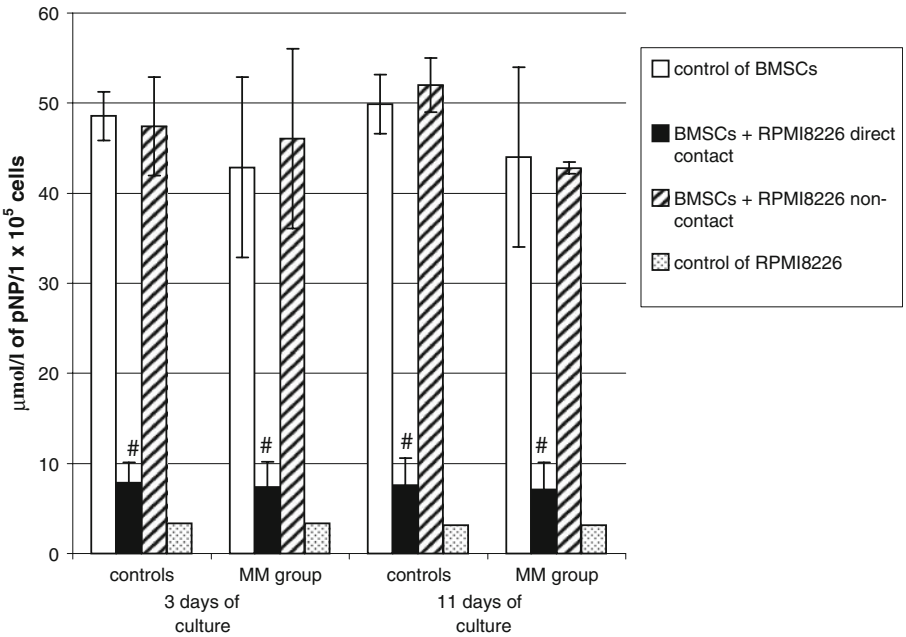

Fig. 7 Alkaline phosphatase activity is decreased in co-cultures of BMSCs with RPMI8226 cells. ALP activity in lysates of BMSCs of MM patients and controls cultivated in monocultures or in co-cultures under cell-to-cell contact and non-contact conditions was measured spectrophotometrically. #In co-cultures of BMSCs under cell-to-cell contact conditions with RPMI8226 cells, a significant decrease of ALP activity was detected at $p < 0.05$

BMSCs was connected with an overproduction of RANKL, which was demonstrated by the addition of RANK-Fc, a recombinant RANKL antagonists, to the co-cultures. RANK-Fc is a fusion protein of the extracellular domain of RANK with the constant region of human IgG1 and is prepared using the baculovirus expression system in Sf9 cells. In the presence of RANK-Fc, RPMI8226- or U266-induced apoptosis of BMSCs was completely abolished. The involvement of RANKL in BMSC apoptosis can be explained either by the direct pro-apoptotic activity of RANKL on the BMSC subpopulation, probably on the precursors of osteoblasts, as apoptosis was connected with a decrease in ALP activity, or by an indirect activity of OPG. In the absence of RANKL, OPG can bind TRAIL and in this way inhibit apoptosis of osteoblastic precursors in BMSCs. It is known that OPG possesses similar affinity to TRAIL as to RANKL (Vitovski et al. 2007). The role of TRAIL in killing osteoblasts by myeloma cells was already described by Tinhofer et al. (2006).

Summing up, we detected an intrinsic capacity of the BMSCs of MM patients to overproduce RANKL after direct cell-to-cell contact with RPMI8226 myeloma cells. In

contrast, the direct contact of the two types of cells caused decreased expression of OPG. Moreover, we found that RPMI8226 cells produced RANKL, but did not produce OPG. The cell-to-cell contact of the BMSCs with RPMI8226 and U266 cells increased the number of BMSCs which underwent apoptosis. These changes were connected with a significant decrease in ALP activity, a marker of osteoblast precursors, and were reversed by treating the co-cultures with RANK-Fc, a RANKL antagonist.

During the last decade, novel therapies against MM targeting both the tumor cells and the BM microenvironment were introduced. The proteasome inhibitor bortezomib and the immunomodulatory drugs thalidomide and its derivative lenalidomide exert their anticancer effect both directly by inducing apoptosis of MM cells and indirectly by interrupting the interactions between myeloma and stromal cells (which are crucial for myeloma cell growth and survival). Recently it was shown that bortezomib counteracts the abnormal balance of RANKL/OPG, leading to osteoclast inhibition and decreased bone destruction. Moreover, this agent stimulates osteoblast function by reducing dickopf-1 (Terpos et al. 2006, 2007). In addition, the combination of bortezomib, melphalan, dexametasone, and intermittent thalidomide reduces angiogenic cytokines, osteoclast regulators, dickopf-1, and bone resorption in patients with MM (Terpos et al. 2008). Bortezomib as well as lenalidomide decrease $\alpha V\beta 3$-integrin, TRAP-positive cells, and bone resorption on dentin discs. Both agents decrease RANKL secretion of BMSCs derived from MM patients and significantly decrease growth and survival factors, such as MIP1-α, BAFF, and a proliferation-inducing ligand. Additionally, in serum from patients treated with lenalidomide, RANKL as well as the RANKL/OPG ratio were significantly reduced, whereas OPG was increased (Breitkreutz et al. 2008). Thus, the present paper suggests that these novel therapies may be very effective in the treatment of patients with MM.

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