Myeloma cells suppress osteoblasts through sclerostin secretion

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Introduction

Multiple myeloma (MM) is a hematological B-cell malignancy that results from the clonal expansion of plasma cells within bone marrow (BM), mostly with overproduction of monoclonal immunoglobulins.1–3 Symptomatic MM is defined by the evidence of end-organ or tissue damage attributable to plasma cell proliferation according to the CRAB criteria, that consist of C: hypercalcemia (>11.5 mg/dl); R: renal failure (serum creatinine >173 mmol/l); A: anemia (hemoglobin <11.5 mg/dl); and B: bone disease (lytic lesions, severe osteopenia or pathological fractures).2,3 Symptomatic MM needs appropriate therapy, and it is differentiated from monoclonal gammapathy of undetermined significance (MGUS) and asymptomatic (smoldering) MM based on the presence or absence of end-organ damage. The staging of symptomatic MM according to the International Staging System (ISS) is based on two laboratory tests, serum levels of β2-microglobulin and albumin, and it divides patients into three distinct stages with different outcomes.4 MM bone disease has a major impact on patient morbidity and mortality. Myeloma cell has a pivotal role in the pathogenetic mechanism of MM bone disease, which is characterized by an increased osteoclastic bone resorption accompanied by a suppressed osteoblastic function.5 Osteoclastogenesis is regulated by a signaling pathway, involving both receptor activator of nuclear factor-kB (RANK) expressed on the surface of mature osteoclasts and their precursors, and RANK ligand (RANKL) expressed on BM stromal cells (BMSCs).5 In addition, osteoprotegerin (OPG), a soluble decoy receptor secreted by osteoblasts (OBs) and BMSCs, competes with RANK for binding to RANKL, resulting in an anti-osteoclastogenic effect. Myeloma cell stimulates osteoclastogenesis by triggering both RANKL increase and OPG reduction.6–8

Osteoblastogenesis involves the differentiation of BMSCs into functional OBs, implicating the activation of different transcription factors such as Cbfα1/Runx2, which directly regulates the expression of the major OB markers such as collagen type I (COLL I), osteopontin, bone sialoprotein II (BSP II) and osteoprotegerin (OSTC).9,10 Another relevant transcription factor is the activator protein 1 (AP-1), which consists of multiple dimers of proteins belonging to Fos (c-Fos, FosB, Fra-1 and Fra-2) and Jun families (c-Jun, JunB and JunD).11 Mice with conditional deletion of fra-1 develop osteopenia, that seems to be due to the reduced expression of bone matrix genes encoding for OSTC, COLL I and matrix Gla protein.12 Recently a direct transcriptional regulation of genes encoding for COLL I and OSTC byfra-2 in human has been demonstrated.13 One of the most relevant signaling regulating OB differentiation is represented by the canonical Wingless-type (Wnt) pathway. Wnts are secreted cysteine-rich glycoproteins known as regulators of hematopoietic and mesenchymal cell differentiation as well as of embryonic development.14–16 The activation of canonical Wnt signaling, induced by binding of Wnt proteins to both Frizzled receptor and low-density lipoprotein receptor-related protein (LRP-5/6) co-receptor, is followed by β-catenin translocation into the nucleus,17,18 resulting in the activation of major OB transcription factors. Wnt pathway is tightly regulated by several secreted antagonists, that is, soluble frizzled-related proteins (sFRPs), which interfere with Wnt/Frizzled receptor binding, or Dickkopf (DKK) proteins and sclerostin, which bind the co-receptor LRP5/6.19 The OB suppression occurring in MM bone disease has been related to the inhibition of the canonical Wnt signaling, through DKK1 (ref. 20) and sFRP-2 and -3 (refs 21–23) secreted from the myeloma cells; otherwise, there are no
literature data on a possible involvement of sclerostin, except for those showing high serum level of sclerostin in MM patients. Sclerostin, the product of SOST gene, is prominently produced by osteocytes embedded in newly formed bone. In humans, sclerostin has been reported to inhibit the differentiation and mineralization of preosteoblastic cells. In humans, mutations in the SOST gene cause sclerosing bone dysplasia, such as sclerosteosis and Van Buchem disease, related to increased bone formation. Recently, studies on these two rare bone disorders led to the identification of sclerostin as an important negative regulator of bone formation. Further, the ongoing development of new therapeutic approaches for reduced bone mass diseases spares antibodies against Wnt antagonists, including sclerostin. Here we studied sclerostin involvement in the impaired bone formation of MM bone disease.

Patients and methods

Patients

The samples consisted of BM aspirates from the iliac crest from 60 patients (mean age, 68 years; range, 55–87 years) newly diagnosed as having active symptomatic MM, requiring therapy in accordance with the International Myeloma Working Group criteria and classified according to ISS. A total of 43 of such patients showed radiological evidence of bone involvement, including osteolysis, osteoporosis, pathological fractures, spinal cord compression and plasmacytoma. Some patients deserved magnetic resonance imaging or computerized tomography to assess the symptomatic bony sites with negative skeletal survey, suspected cord compression or size of tumor mass. The controls included BM aspirates from 38 subjects with MGUS without evidence of bone disease, matched for age and sex with the patients diagnosed as having MM. Informed consent to the study was given according to the tenets of the Declaration of Helsinki. Approval was obtained from the Institutional Review Board of the Department of Internal Medicine and Public Medicine of University of Bari.

Cells

Human myeloma cell lines (HMCLs) and CD138+ cells. HMCLs (H929, RPMI 8226, U266 and Karpas 929) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco Invitrogen, Milan, Italy). Malignant plasma cells from BM aspirates of patients (MM plasma cells) and controls, identified as CD138+ cells, were cultured with a magnetic cell-sorting separator (Miltenyi Biotec, Bergisch-Gladbach, Germany) using magnetic microbeads (Miltenyi Biotec) coupled to anti-CD138 monoclonal antibody (mAb). Only samples with a purity of more than 97%, checked by flow cytometry, were considered. Fresh CD138+ cells were used for RNA or protein extraction, or co-culture experiments.

Human BM cells. BM aspirates of MGUS controls were subjected to Histopaque 1077 density gradient (Sigma Aldrich, St Louis, MO, USA). The buffy coat cell fraction was entirely cultured to obtain the BM mononuclear cells (BMNCs) used in colony-forming unit-fibroblast (CFU-F) and colony-forming unit-OB (CFU-OB) assays. BMSCs were obtained from adherent fraction of BMNCs and used in co-culture experiments.

Cell culture conditions and co-cultures

BMNCs were plated at the density of 4 × 10^5/cm² in an OB differentiating medium, consisting of z-minimum essential medium supplemented with 10% fetal bovine serum, 50 µg/ml ascorbic acid and 10⁻⁶ M dexamethasone (Sigma Aldrich). These cells were co-cultured with 1 × 10^7/cm² HMCLs or CD138+ cells from symptomatic MM patients, or purified B lymphocytes from normal donors. All experiments of co-culture were performed in the absence or in the presence of 50 and 500 ng/ml neutralizing anti-sclerostin mAb (R&D Systems, Minneapolis, MN, USA), or anti-immunoglobulin G (IgG) control Ab. These co-cultures were maintained in OB differentiating medium for 21 days to evaluate the formation of CFU-O, identified as alkaline phosphatase-positive colonies. In parallel, we maintained the co-cultures in osteogenic medium, consisting of OB-differentiating medium supplemented with 10 mM β-glycerophosphate, for 30 days to evaluate the formation of CFU-OB, identified with Von Kossa staining. Moreover, we also performed in OB-differentiating medium short-term (48 h) co-cultures and in osteogenic medium long-term (30 days) between semiconfluent BMSCs and HMCLs, or CD138+ cells from symptomatic MM patients, or B lymphocytes. We analyzed in the former co-cultures the expression of COLL I, RANKL, OPG, Runx2, Osterix, Fra-1, Fra-2, JunD, β-catenin and sclerostin–LRP-5/6 immunocomplex, and in the latter co-cultures the expression of BSP II and OSTC. In these experiments, HMCLs or CD138+ cells were removed using magnetic cell-sorting separator, whereas B lymphocytes were removed using a positive immunoselection with anti-CD19 mAb (Dynal, Lake Success, NY, USA), and the BMSCs were subjected to RNA or protein extraction. BMSC viability was performed as previously described to exclude any toxic or apoptotic effect induced by HMCLs in the co-culture system.

RNA isolation and real-time PCR amplification

RNA from CD138+ cells, HMCLs and BMSCs was extracted and subjected to real-time PCR as previously described. Appropriate primer pairs were used for the PCR amplification.

Western blot analysis and immunoprecipitation

Western blot analysis and immunoprecipitation were performed as previously described. The following primary Abs were used: polyclonal anti-COLL I, anti-BSP-II, anti-Fra-2, anti-JunD, anti-OPG, anti-Lamin B1, anti-ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-RANKL (R&D Systems) and anti-β-catenin (Cell Signaling Technology, Danvers, MA, USA); monoclonal anti-β-actin, anti-Fra-1 (Santa Cruz Biotechnology), and anti-sclerostin (R&D Systems).

Statistical analyses

Statistical analyses were performed by Student t-test with the Statistical Package for the Social Sciences (spssx/pc) software (SPSS, Chicago, IL, USA).

Results

Expression of sclerostin by HMCLs or CD138+ cells from patients

By means of real-time PCR or western blot analysis, we determined the expression of sclerostin by four HMCLs (that is, H929, RPMI-8226, U266 and Karpas 909) or by freshly purified CD138+ cells from BM aspirates of both MM patients and controls. Owing to the low number of CD138+ cells obtained in individual BM aspirates, the expression of sclerostin from each donor could only be assessed at mRNA or at protein
level. By real-time PCR, the lowest level of sclerostin mRNA was found in controls, whereas it was several folds higher in almost all MM patients in which higher levels were detected when bone disease was evident. However, it should be noted that in some cases an overlap of sclerostin mRNA levels between symptomatic MM patients both with and without bone disease and controls was observed.

Among the HMCLs, H929 expressed significant high mRNA levels of sclerostin (Figure 1a). By western blots, we determined that sclerostin was much strongly expressed in MM bone disease patients, whereas it was undetectable in the controls. In the four HMCLs, sclerostin was well evident at protein levels with the highest amount in H929 (Figure 1b). Of note, although increased mRNA levels of sclerostin were only detected in some samples either from patients (8/20) or HMCLs (1/4), all samples from symptomatic MM with bone disease as well as all HMCLs expressed sclerostin at protein level (Figure 1b). An overview of clinical characteristics of symptomatic MM patients and sclerostin expression has been reported in Supplementary Table 1.

Expression of OB differentiation markers in BMSCs co-cultured with HMCLs

On the basis of our findings on the sclerostin expression by myeloma cells, we investigated whether this protein could impair OB differentiation. We performed long-term co-cultures (allowing cell-cell contacts) of BMNCs from MGUS controls and H929 in the presence or in the absence of 50 and 500 ng/ml anti-sclerostin mAb. The formation of CFU-F or CFU-OB was assessed as indicator of early or late phase of OB differentiation, respectively. The results were compared with those obtained by culturing BMNCs alone. We found that H929 significantly inhibited CFU-F formation after 21 days (Figure 2a), and the formation of CFU-OB after 30 days (Figure 2b). The addition of anti-sclerostin mAb at two different concentrations partially restored CFU-F and CFU-OB formation (Figures 2a and b). As reported in Supplementary Table 2, all HMCLs exhibited comparable results. Similarly, CD138+ cells from symptomatic MM patients inhibited the formation of both CFU-F and CFU-OB respect to BMNCs alone, and this inhibition was partially rescued in the presence of anti-sclerostin mAb (Figures 2a and b). These data suggest a sclerostin involvement in the inhibition of OB differentiation. Conversely, B lymphocytes did not inhibit the formation of CFU-F and CFU-OB (data not shown).

Expression of OB differentiation markers in BMSCs co-cultured with HMCLs

By using the same co-culture system, we investigated whether myeloma cells could impair, through sclerostin production, specific parameters of OB differentiation such as production of extracellular bone matrix proteins. By western blots, we found a strong inhibition of COLL I synthesis in a sclerostin-dependent manner in short-term co-cultures. Indeed, the results were partially reverted by the addition of 50 ng/ml anti-sclerostin mAb, and totally reverted by 500 ng/ml mAb (Figure 3Aa). In long-term co-cultures, we also demonstrated a significant reduction of BSP II and OSTC, which are expressed during the late phase of OB differentiation. BSP II was detected at protein levels (Figure 3Ab), and OSTC at mRNA level (Figure 3Ac). In the presence of 50 and 500 ng/ml anti-sclerostin mAb, there was a partial rescue, clearly indicating a role for sclerostin. In particular, the effect on BSP II expression appeared to be almost the same at both mAb concentrations (Figure 3Ab), whereas the effect on OSTC expression was observed in the presence of 500 ng/ml mAb (Figure 3Ac). In both the short- and long-term co-cultures, the inhibition of the OB marker synthesis exerted by CD138+ cells from symptomatic MM patients or by all the other HMCLs as well as the effect of the mAb was quite
comparable; additionally, the expression of the OB markers was not affected by anti-IgG Ab or B-lymphocytes (data not shown). Any toxic- or apoptotic-induced effect of HMCLs on OB cultures was excluded by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assay.

**Expression of OB transcription factors in BMSCs co-cultured with HMCLs**

On the basis of results obtained on bone protein expression, we evaluated whether myeloma cells could affect, through sclerostin, the expression of transcription factors tightly regulating OB differentiation, such as Runx2, Osterix and AP-1. By using the same co-culture system, we show that the presence of H929 induced a reduced expression of the dimeric AP-1 complex in BMSCs due to the decreased expression of Fra-1, Fra-2 and JunD. Fra-1 and JunD were rescued in a dose-response manner by anti-sclerostin mAb added to the system, whereas Fra-2 expression was only partially rescued, even in the presence of the highest mAb concentration (Figure 3B). The inhibition of the OB transcription factors exerted by CD138+ cells from symptomatic MM patients or by all the other HMCLs was quite comparable; conversely anti-IgG Ab or B-lymphocytes did not affect the expression of such factors (data not shown).

**Expression of RANKL and OPG by BMSCs co-cultured with HMCLs**

On the basis of the knowledge that RANKL/OPG ratio can be unbalanced by Wnt inhibitors, we investigated whether in our co-culture system myeloma cells could affect, through sclerostin production, the expression of these cytokines, respectively with pro- and anti-osteoclastogenic activity. H929 upregulated RANKL and strongly downregulated OPG expression by BMSCs in a sclerostin-dependent manner. In fact these effects were progressively reverted in the presence of anti-sclerostin mAb (Figure 3C). Both CD138+ cells from symptomatic MM patients and all the other HMCLs similarly influenced RANKL and OPG expression, conversely their levels were not affected by anti-IgG Ab or B-lymphocytes (data not shown).

**Formation of the LRP5/6–sclerostin complex and nuclear β-catenin accumulation in BMSCs co-cultured with HMCLs**

To investigate whether myeloma cells might affect the canonical Wnt signaling in BMSCs, we evaluated in our co-culture system the formation of LRP5/6–sclerostin complex and the nuclear accumulation of β-catenin. The formation of this complex is followed by the inhibition of Wnt signaling, whereas the
accumulation of nuclear β-catenin is a hallmark of Wnt pathway activation. In the presence of H929, we detected the formation of LRP5/6–sclerostin complex, which was proportionally reduced in the presence of anti-sclerostin mAb at the two different concentrations (Figure 4a). Moreover, HMCLs prevented β-catenin accumulation in BMSC nuclei and this effect was slightly influenced by anti-sclerostin mAb only at the concentration of 500 ng/ml (Figure 4b). MM plasma cells and all HMCLs induced the formation of the LRP5/6–sclerostin complex, and inhibited the accumulation of nuclear β-catenin in a comparable way. Differently, in the presence of an anti-IgG Ab or B lymphocytes, no effect was seen on both the LRP5/6–sclerostin complex formation and the nuclear β-catenin accumulation (data not shown).

**Discussion**

Bone disease is the most characteristic marker of end-organ or tissue damage in MM. It has been demonstrated that myeloma cell disrupts the balance between osteoclastic bone resorption and osteoblastic bone formation. Increased osteoclastic activity is mainly due to MM-induced RANKL and other osteoclastogenic cytokines,

1,7,8 whereas OB suppression involves a number of Wnt inhibitors, such as DKK1, sFRP-2 and -3.

Sclerostin is another molecule inhibiting Wnt signaling, whose expression is restricted to bone tissue. High serum levels of sclerostin were reported in long-term immobilized patients,

35 and in ankylosing spondylitis.

However, there are no data on its role in MM bone disease, except for data presented by Terpos et al. at the last
meeting of ASH in which they correlated the increase of sclerostin serum levels with ISS-3 disease in newly diagnosed patients with MM. The aim of our study was to understand whether sclerostin has a role in MM bone disease. We demonstrate that it is expressed by myeloma cells isolated from patients with MM bone disease and that, although its mRNA expression does not always correlate with the extent of bone damage, sclerostin protein levels seem to be associated with the severity of their disease. This was identified by the evidence of pathological fractures, cord compression or plasmacytoma, as well as by the concomitant renal impairment and/or anemia and ISS-2 or ISS-3 disease. Otherwise, low mRNA levels of sclerostin and undetectable amount of the protein were assessed in MGUS patients. Of note, no progression to MM was detected in these control subjects until the time of the writing. Accordingly, we demonstrated, by means of an in vitro model consisting of a co-culture of BMSCs isolated from MGUS controls and HMCLs or CD138+ cells, that the OB suppression observed in co-cultures in the presence of myeloma cells is largely sclerostin dependent. In fact, OB inhibition could be partially restored by the addition of a neutralizing anti-sclerostin mAb. The OB inhibition resulted in a reduced expression of OB functional differentiation markers, as ALP and COLL I in short-term co-cultures, and BSP II, OSTC and mineralized nodule formation in long-term co-cultures. The OB inhibiting effect exerted by myeloma cells on OB activity has been already described,21,23 suggesting a possible involvement of both Fra-1 and Fra-2 in all the differentiation phases of OBs. In our system, the implication of sclerostin in the inhibition of Fra-1, Fra-2 and JunD was evident from the partial rescue of their expression in the presence of neutralizing anti-sclerostin mAbs.

The role played by sclerostin in the suppression of OB function is also supported by our findings concerning the formation of LRP5/6-sclerostin complex, as well as the inhibition of β-catenin translocation into OB nuclei: the former abolished and the latter partially restored by the addition of the anti-sclerostin mAbs. Our findings are consistent with the literature data,23 demonstrating a significantly reduced translocation of β-catenin into the OB nuclei in the presence of myeloma cells. Wnt pathway also regulates osteoclast formation and resorption modulating the expression of both RANKL and OPG in OBs. Consistently, we demonstrated that sclerostin mediated the upregulation of RANKL and the downregulation of OPG in our co-culture system.

In conclusion, our findings demonstrate for the first time the expression of sclerostin by myeloma cells and its association with the advanced ISS of the disease. Furthermore, sclerostin contribution in the development of MM bone disease could be related to both a direct induction of OB suppression with reduced bone formation, and an indirect activation of osteoclast bone resorption through the unbalanced RANKL/OPG ratio. Thus, sclerostin can provide a promising potential target for the development of novel therapeutics to rebuild bone mass in MM bone disease.

Conflict of interest

The authors declare no conflict of interest.

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Sclerostin role in multiple myeloma bone disease
C. Colucci et al.

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