Regulation of Sclerostin Expression in Multiple Myeloma by Dkk-1: A Potential Therapeutic Strategy for Myeloma Bone Disease

Homare Eda,1 Loredana Santo,1 Marc N Wein,2 Dorothy Z Hu,2 Diana D Cirstea,1 Neelharika Nemani,1 Yu-Tzu Tai,3 Sarah E Raines,4 Stuart Allen Kuhstoss,4 Nikhil C Munshi,3 Henry M Kronenberg,2 and Noopur S Raje1

1Massachusetts General Hospital Cancer Center, Harvard Medical School, Boston, MA, USA
2Endocrine Unit, Massachusetts General Hospital, Boston, MA, USA
3LeBow Institute for Myeloma Therapeutics and Jerome Lipper Center for Multiple Myeloma Research, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA
4Regenerative Biology, Lilly Research Laboratories, Indianapolis, IN, USA

ABSTRACT
Sclerostin is a potent inhibitor of osteoblastogenesis. Interestingly, newly diagnosed multiple myeloma (MM) patients have high levels of circulating sclerostin that correlate with disease stage and fractures. However, the source and impact of sclerostin in MM remains to be defined. Our goal was to determine the role of sclerostin in the biology of MM and its bone microenvironment as well as investigate the effect of targeting sclerostin with a neutralizing antibody (scl-Ab) in MM bone disease. Here we confirm increased sclerostin levels in MM compared with precursor disease states like monoclonal gammopathy of undetermined significance (MGUS) and smoldering MM. Furthermore, we found that a humanized MM xenograft mouse model bearing human MM cells (NOD-SCID.CB17 male mice injected intravenously with 2.5 million of MM1.S-Luc-GFP cells) demonstrated significantly higher concentrations of mouse-derived sclerostin, suggesting a microenvironmental source of sclerostin. Associated with the increased sclerostin levels, activated β-catenin expression levels were lower than normal in MM mouse bone marrow. Importantly, a high-affinity grade scl-Ab reversed osteolytic bone disease in this animal model. Because scl-Ab did not demonstrate significant in vitro anti-MM activity, we combined it with the proteasome inhibitor carfilzomib. Our data demonstrated that this combination therapy significantly inhibited tumor burden and improved bone disease in our in vivo MM mouse model. In agreement with our in vivo data, sclerostin expression was noted in marrow stromal cells and osteoblasts of MM patient bone marrow samples. Moreover, MM cells stimulated sclerostin expression in immature osteoblasts while inhibiting osteoblast differentiation in vitro. This was in part regulated by Dkk-1 secreted by MM cells and is a potential mechanism contributing to the osteoblast dysfunction noted in MM. Our data confirm the role of sclerostin as a potential therapeutic target in MM bone disease and provides the rationale for studying scl-Ab combined with proteasome inhibitors in MM. © 2016 American Society for Bone and Mineral Research.

KEY WORDS: TUMOR-INDUCED BONE DISEASE; CYTOKINES; OSTEOBLASTS; STROMAL/STEM CELLS; THERAPEUTICS

Introduction

Multiple myeloma (MM), defined by a clonal expansion of plasma cells within the bone marrow (BM) microenvironment, remains incurable despite recent advances with novel therapies.1 More than 80% of MM patients develop bone disease characterized by osteolytic bone lesions resulting in skeletal-related events (SREs). These SREs are associated with increased morbidity and mortality,2–5 negatively impacting both patients’ quality of life and survival.

MM bone disease results from the disruption of the delicate balance between the activity of osteoblasts (OBs), osteoclasts (OCs), and marrow stromal cells (MSCs).6,7 MM cells inhibit OB differentiation and stimulate OC function, resulting in bone resorption and development of osteolytic lesions.8,9 Moreover, the BM microenvironment provides a permissive niche for MM cell growth, and targeting the interactions within the bone milieu represents an important strategy to suppress MM progression.10–13 Typically, the bone niche in MM has increased osteolytic activity with impaired/suppressed osteoblastic

Received in original form October 29, 2015; revised form January 7, 2016; accepted January 8, 2016. Accepted manuscript online January 13, 2016. Address correspondence to: Noopur S Raje, MD, POB 216, MGH Cancer Center, Massachusetts General Hospital, 55 Fruit Street, Boston, MA 02114, USA. E-mail: nraje@partners.org
Additional Supporting Information may be found in the online version of this article.
DOI: 10.1002/jbmr.2789
© 2016 American Society for Bone and Mineral Research
activity. This niche serves as a sanctuary site for the growth and proliferation of MM cells, resulting in homing of tumor, progression of disease, and development of drug resistance. Stimulating OB function within the BM niche will balance bone resorption and has the potential to not only reverse bone disease but also mitigate MM cell growth. This underscores the importance of understanding the OB axis and targeting it with therapeutic intent. [10,13–17]

Sclerostin, encoded by the SOST gene, is a small glycoprotein secreted by osteocytes. [18–20] Sclerostin blocks canonical Wnt signaling by binding to LRPS and LRP6, Wnt coreceptors on the surface of OBs. [21] Consequently, sclerostin may inhibit OB proliferation and differentiation, resulting in suppression of bone formation. [19] Recent studies have addressed the importance of sclerostin in osteoporosis, [22,23] making sclerostin an important tool in the treatment of bone conditions with high catabolism. [24]

Higher than normal circulating levels of sclerostin have been noted in newly diagnosed MM patients, and sclerostin concentration correlated with MM disease stage and fractures at diagnosis, [25] suggesting a role for sclerostin in MM and associated bone disease. Although previous studies have reported that MM cells produce sclerostin, [26–28] the main source and exact mechanism by which sclerostin plays a role in the context of MM and related bone disease remain to be elucidated.

Our data confirm increased sclerostin levels in MM patients compared with precursor disease states such as monoclonal gammopathy of undetermined significance (MGUS) and smoldering MM. Moreover, we show significantly higher concentration of sclerostin expressed by mouse cells in our human MM xenograft mouse model, suggesting a microenvironmental source as opposed to only tumor cells. To ensure the interaction between MM cells and the microenvironment we used our previously described mouse model that guarantees the homing of the MM cells to the bone marrow as demonstrated by bioluminescence imaging and immunohistochemistry. [29,30]

Using a high-affinity grade scl-Ab resulted in restoring bone anabolic effects with reversal of osteolytic bone disease in this MM mouse model. Because of lack of direct anti-MM activity in vitro, we combined the scl-Ab with carfilzomib, a selective proteasome inhibitor approved for the treatment of relapsed and refractory MM. [31–36] Our data demonstrate that this combination therapy significantly lowered tumor burden and improved bone disease. We confirmed that the high concentration of sclerostin in MM patients’ plasma is secreted by MSCs and OBs in addition to osteocytes, and this was in part mediated by Dkk-1. These findings underscore the significance of sclerostin as a potential therapeutic target in MM bone disease and provide a strong rationale for investigating scl-Ab in combination with carfilzomib as a novel combination in MM treatment.

Materials and Methods

Reagents and cell lines

A novel high-affinity grade scl-Ab was supplied by Lilly Research Laboratories (Indianapolis, IN, USA). The other scl-Ab, Dkk-1 neutralizing antibody, goat IgG control, human recombinant sclerostin, and Dkk-1 were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Carfilzomib was supplied by Onyx Pharmaceuticals Inc. (South San Francisco, CA, USA) MM cell line, MM.1S, was obtained from American Type Culture Collection (Manassas, VA, USA). MM cell lines (H929, DOX40, and INA-6) were provided by the Jerome Lipper Center and LeBow Institute for Myeloma Therapeutics at Dana Farber Cancer Institute (Boston, MA, USA). All MM cell lines were cultured as previously described. [37]

Patient samples and primary cells

Peripheral blood (PB) and BM samples from MM patients were obtained after providing written informed consent per the Declaration of Helsinki and approved by the institutional review board of the Massachusetts General Hospital. After plasma separation, mononuclear cells from BM were separated by Ficoll-Hypaque density gradient centrifugation, and CD138+ cells, CD14+ cells, and OBs and MSCs were separated using CD138 and CD14 MicroBeads (Miltenyi Biotec Inc., Cologne, Germany) according to the manufacturer’s instructions. Additionally, 14 BM biopsy samples from MM patients and 3 BM samples from osteoarthritides (OA) patients were obtained to perform immunohistochemistry and double immunofluorescence analysis.

Osteoblast differentiation

MSCs were grown to confluence and cultured with osteoblasto-genic media containing α-minimum essential medium (α-MEM) with 20% fetal bovine serum (FBS), 2% penicillin streptomycin (Corning Cellgro, Corning, NY, USA), b-glycerophosphate at 1.08 mg/mL, ascorbic acid at 0.05 mg/mL, and dexamethasone at 80 nmol/L (Sigma-Aldrich, St. Louis, MO, USA). OB differentiation was confirmed by detection of OB differentiation markers by qPCR and calcification as previously described. [38] For murine osteoblastic cell MC3T3-E1, murine OB differentiation medium (α-MEM with 10% FBS, 2% penicillin streptomycin, b-glycerophosphate at 2.16 mg/mL, ascorbic acid at 0.05 mg/mL) was used.

Enzyme-linked immunosorbent assay (ELISA)

ELISAs for human sclerostin or mouse sclerostin (American Laboratory Products Company, Salem, NH, USA) were performed with PB and BM plasma from MM patients, plasma from mice, and supernatant from OB cocultured with MM cells previously collected and stored at –80°C according to manufacturer’s instructions. Human Dkk-1 was detected using human Dkk-1 Quantikine ELISA (R&D Systems) in mouse samples. Osteocalcin and P1NP were similarly measured by Mouse Osteocalcin EIA Kit (Biomedical Technologies, Inc., Stoughton, MA, USA), Rat/Mouse P1NP EIA (Immunodiagnostic Systems Ltd., Boldon, UK) and RatLaps EIA (Immunodiagnostic Systems) according to the manufacturer’s instructions.

MTT assays

The viability of MM primary cells or cell lines were assessed by measuring MTT (Chemicon International, Temecula, CA, USA) dye absorbance, as described previously. [29]

Coculture assay

For coculture experiments, cell culture inserts (BD Biosciences, San Jose, CA, USA) were used. Human MSCs or immature human OBs were cocultured in OB differentiation medium (α-MEM with 20% FBS, 2% penicillin streptomycin, b-glycerophosphate at 2.16 mg/mL, ascorbic acid at 0.05 mg/mL, and dexamethasone at 10 nmol/L) with MM cells with or without 1 µg/mL sclerostin/0.4 µg/mL Dkk-1 neutralizing antibody (Ab) or goat IgG control.
antibody (R&D Systems) as indicated by using cell culture inserts (BD Biosciences) to avoid cell-cell contact. For MC3T3-E1 coculture, murine OB differentiation medium was used.

Quantitative PCR analysis

mRNA was purified from MSCs, OBs, monocytes, primary MM cells, and MM cell lines, and cDNA was obtained as previously described. The details for primers are shown in Supplemental Table S1. qPCR analysis was performed using RT2 SYBR Green ROX qPCR Master Mix according to the manufacturer’s specifications (Qiagen Inc., Valencia, CA, USA).

Mouse xenograft human MM model

Animal protocols were approved by the Institutional Animal Care and Use Committees of Massachusetts General Hospital and performed in accordance with the regulations and guidelines. Housing and husbandry were in the Animal Research Facility at the Massachusetts General Hospital. The xenograft MM mouse model was generated as previously described. Briefly, mice were irradiated at day 0 followed by injection of MM.1S-LucNeo GFP + cells a day after. Mice were then assigned into four groups (n = 10) at random: treatment with scl-Ab (10 mg/kg intraperitoneally for 2 days per week for 4 weeks); treatment with carfilzomib (2 mg/kg intravenously for 2 days per week for 4 weeks); treatment with combinations of both agents; or vehicle control. These were started at day 6. Bioluminescence imaging (BLI) was performed at the end of the experiment. Animals were euthanized at the end of treatment, and samples were collected as previously described. Experimental outcomes were assessed by bone mass and BLI.

Micro-computed tomography and bone volume fraction quantification

Five (randomly chosen) L3 and L4 vertebrae in each group were scanned using micro-computed tomography for three-dimensional reconstruction and for bone volume fraction quantification at the Center for Advanced Orthopedic Studies, Beth Israel Deaconess Medical Center, Harvard Medical School. Image acquisition and analysis protocols adhered to the JBMR guidelines.

Immunohistochemistry and double immunofluorescence staining analysis

Formalin-fixed paraffin-embedded tissue sections for hematoxylin and eosin staining and immunohistochemistry were prepared as previously described. Detailed experimental procedures employed for immunohistochemical staining or double immunofluorescence staining are provided in Supplemental Table S1. Images were obtained with a microscope (Eclipse TS100; Nikon Instruments Inc., Melville, NY, USA) with SPOT Insight Camera for immunohistochemistry. The immunohistochemical staining samples were counterstained with hematoxylin. Negative controls were only incubated in blocking buffer.

Statistical analysis

All in vitro experiments were repeated at least three times. All in vivo experiments were performed with more than five samples. The statistical significance of differences was determined using Student’s t test or Mann-Whitney U test with a minimal level of significance of p = 0.05. For quantitative morphometric analyses, one-way ANOVAs were run followed
by Tukey’s multiple comparisons test. One-way ANOVAs were determined using the GraphPad Prism (GraphPad, Inc., La Jolla, CA, USA).

Results
A high-affinity grade sclerostin neutralizing antibody reverses osteolytic bone disease in the xenograft MM model

We confirmed increased sclerostin levels in patient samples (Fig. 1A) compared with healthy controls, other hematopoietic cancers (leukemia), and precursor states such as MGUS. We next measured sclerostin concentration in our xenograft MM model, in which human MM.1S cells were injected intravenously in NOD SCID mice as previously described. In agreement with MM patients’ data, significantly higher concentration of mouse sclerostin was detected in MM-bearing mice compared with non-tumor-bearing controls (Fig. 1B). Interestingly, human sclerostin was not detected in either non-tumor-bearing controls or MM-bearing mice (data not shown), confirming a microenvironmental source of sclerostin. High sclerostin levels in this model were associated with inhibition of activated β-catenin protein expression in femoral bones of tumor-bearing animals (Fig. 1C). To ascertain whether sclerostin is a potential therapeutic target in MM bone disease, we used a high-affinity grade scl-Ab in our xenograft MM model. Mice were treated with scl-Ab and/or carfilzomib, a proteasome inhibitor approved for the treatment of MM, because scl-Ab alone did not show direct effect on MM cells (Supplemental Fig. S1A, B). Scl-Ab-treated bones showed higher levels of activated β-catenin expression, suggesting that the inhibition of β-catenin activity in MM mouse bone was caused by sclerostin, confirming that this model is appropriate for sclerostin research in MM bone disease (Fig. 1C).

Trabecular bone thickness and trabecular bone volume in vertebrae were also increased in mice treated with scl-Ab (Supplemental Fig. S2). Micro-CT reconstructions and quantification showed that scl-Ab increased trabecular bone volume in lumbar vertebrae (Fig. 2A, B). We also measured markers of bone turnover and, interestingly, the level of the bone-formation marker osteocalcin was significantly increased in scl-Ab-treated mice in comparison with control mice after 1 week of treatment (Fig. 2C, left panel). Furthermore, 1-week administration of scl-Ab resulted in significantly increased levels of the bone-formation marker procollagen type 1 amino-terminal propeptide (P1NP) compared with the control group (Fig. 2C, right panel) (the percentage change in P1NP concentration: control –66.64%, scl-Ab 38.29%, carfilzomib 1228 EDA ET AL. Journal of Bone and Mineral Research

Fig. 2. Scl-Ab alone and in combination with carfilzomib increases bone formation. NOD-SCID CB17 mice (n = 40) were inoculated intravenously with MM.1S-LucNeo GFP cell and then treated with saline (n = 10), sclerostin neutralizing antibody (scl-Ab) (n = 10), carfilzomib (CFZ) (n = 10), or the combination of sclerostin neutralizing antibody plus carfilzomib (combo) (n = 10). (A, B) Lumbar vertebrae (L5 and L6) were scanned using micro-CT after completion of treatment. (C, left) Osteocalcin concentration was detected after 1 week of treatment. (C, right) The percentage change in P1NP concentration between pretreatment and 1 week post-treatment samples in each mouse were normalized. (D, left) Bioluminescence imaging (BLI) was performed at the end of treatment. (D, right) After treatment, femurs were collected and GFP cells were detected by immunohistochemistry. *p < 0.05, **p < 0.01, ***p < 0.001.
–34.01%, combination 26.54%, \( p < 0.05 \). Tumor burden and GFP-positive MM cells in mice femur bones were not, however, significantly inhibited by scl-Ab (Fig. 2D). Treatment with the combination of carfilzomib resulted in increased trabecular bone thickness compared with the carfilzomib group (Supplemental Fig. S2). Moreover, micro-CT reconstructions and quantification showed that the combination treatment increased cancellous bone volume in lumbar vertebrae more than with carfilzomib alone (Fig. 2A, B). Osteocalcin and P1NP concentration in mouse plasma were also significantly increased in scl-Ab in combination with carfilzomib treatment group in comparison with control mice or carfilzomib-treated mice (Fig. 2C). Importantly, scl-Ab in combination with carfilzomib inhibited tumor burden and GFP-positive cells compared with the control group (Fig. 2D). No adverse event related to drug treatment was observed.

Marrow stromal cells and osteoblasts in myeloma bone marrow secrete sclerostin

Because our in vivo data confirmed a microenvironmental source of sclerostin in MM bone disease, we sought to identify the source of sclerostin in MM. Sclerostin is known to be secreted by osteocytes,\(^{(40,41)}\) however, viable osteocytes and mature OBs are significantly decreased in number in MM patients.\(^{(8,9,42)}\) Moreover, no significant difference in the expression of sclerostin between MM patient and healthy control osteocytes has been observed.\(^{(8,9,42)}\) Therefore, osteocytes alone would be an unlikely source of increased sclerostin found in myeloma patients. Although increased sclerostin in MM patients is thought to be produced by MM cells\(^{(26–28)}\) there is no direct evidence that this is the case. Because we could detect higher concentration of sclerostin in MM patients’ BM, SOST mRNA expression levels corrected for GAPDH mRNA in primary MM cells, BM monocytes, and OBs and MSCs derived from MM patients’ BM were evaluated. Interestingly, the OB and MSC fraction showed higher SOST expression than the MM cell or BM monocyte fraction in 8 of 12 patients’ BM (Fig. 3A). Moreover, the 8 patients who showed higher SOST mRNA expression in the OB and MSC fractions (Fig. 3B, left bar) demonstrated a tendency for higher concentrations of sclerostin in BM than the other 4 patients (Fig. 3B, middle bar) or leukemia patients (Fig. 3B, right bar, \( p = 0.057 \)). To confirm the source of sclerostin, we next evaluated sclerostin expression in BM biopsy samples from MM patients by immunohistochemistry (IHC). Osteocytes in MM patient bone showed sclerostin expression (Fig. 4A) in agreement with previous reports of sclerostin serving as an osteocyte marker.\(^{(40,41)}\) Interestingly, in addition to osteocytes, spindle-shaped cells in BM stroma and on trabecular bone surfaces showed sclerostin expression (Fig. 4A). These sclerostin-positive spindle-shaped BM stromal cells stained for the mesenchymal stem cell marker Mel-CAM,\(^{(43,44)}\) and spindle-shaped cells on trabecular bone surfaces stained for the OB marker osteocalcin.\(^{(45)}\) These sclerostin-positive spindle-shaped cells were negative for CD138, an MM cell marker (Fig. 4A). As a negative control, we stained sclerostin in bone specimens from osteoarthritis (OA) patients and noted that sclerostin-positive cells in OA patient biopsies were only osteocytes (Fig. 4B).

![Fig. 3.](image)

**Fig. 3.** MSCs and OBs express sclerostin in MM BM. (A) SOST mRNA levels, corrected for GAPDH mRNA, was detected in primary MM cells, monocytes or MSCs, and OBs derived from 12 MM patients’ BM aspirations. Black bars indicate SOST/GAPDH mRNA expression in MSCs/OBs fraction, white bars indicate SOST/GAPDH mRNA expression in monocytes fraction, and hatched bars indicate SOST/GAPDH mRNA expression in MM cells fraction. Asterisks indicate significance between MSCs/OBs versus MM. (B) Sclerostin concentration was evaluated in the BM plasma of patients who had high SOST mRNA expression in MSCs/OBs fraction (as showed in Fig. 3A) and in patients who did not have high SOST mRNA expression in MSCs/OBs fraction. Sclerostin plasma levels were then compared with concentration in leukemia patients’ BM. Sclerostin in MM showed higher SOST in MSCs versus leukemia; \( p = 0.057 \). * \( p < 0.05 \), ** \( p < 0.01 \).
agreement with previous reports. For further confirmation of these results, we performed double immunofluorescence staining for the presence of sclerostin and Mel-CAM or sclerostin and CD138 in BM cells. In accordance with the IHC data, sclerostin+ cells in MM BM were Mel-CAM+ in patients’ BM (Fig. 4C). CD138+ MM cells were sclerostin- in the same patients (Fig. 4C). In contrast, Mel-CAM+ MSCs in OA BM were both sclerostin- and CD138- (Supplemental Fig. S3). Because we could detect higher concentration of mouse sclerostin in the MM mouse model, we next performed IHC for mouse sclerostin in the femurs from these mice. In agreement with MM patients’ BM IHC, mouse sclerostin+ cells were detected on the bone surface and BM stroma in addition to osteocytes (Supplemental Fig. S4). In contrast, only osteocytes were sclerostin+ in non-tumor-bearing control mouse bones (Supplemental Fig. S4). These sclerostin+ areas were devoid of GFP+ MM cells (Supplemental Fig. S4, lower middle panel), although many GFP+ cells were noted in other areas of the same femur (Supplemental Fig. S4, lower right panel).

MM induces inhibition of OB differentiation via stimulating sclerostin expression in immature OBs by Dkk-1

We next investigated the effect of MM cells on inhibition of OB differentiation. We first cocultured OBs derived from MM patients with MM cells by using cell culture inserts to avoid cell-cell contact. INA-6 MM cells inhibited expression of OB

---

**Figure 4.** Sclerostin expression in osteocytes, spindle-shaped cells in BM stroma, and on trabecular bone surfaces. (A) Detection of sclerostin, Mel-CAM, osteocalcin, and CD138 by immunohistochemistry in serial sections of the same region of MM patients’ BM biopsy was performed. Original magnification is ×400. Evidence of lack of staining with secondary antibody is shown on the right panel. (B) Detection of sclerostin by IHC in osteoarthritis patients’ BM was performed. Original magnification is shown in the figure. (C) Detection of sclerostin and Mel-CAM or CD138 by double immunofluorescence staining of MM patients’ BM biopsy was performed. Original magnification is ×400. Evidence of lack of staining with secondary antibody is shown on the right panel.
dissociation markers such as Runt-related transcription factor 2 (runx2) and alkaline phosphatase (ALP) mRNA in OBs (Fig. 5A), and also inhibited OB matrix mineralization (Fig. 5B), in agreement with previous reports confirming that MM cells inhibit OB differentiation. Importantly, OB viability was maintained as shown by immunofluorescence staining (Fig. 5C), and MM cell-induced suppression of OB differentiation was reversed by scl-Ab (Fig. 5A, B). We next examined sclerostin expression in this coculture system by evaluating SOST mRNA expression in OBs without cell-cell contact, suggesting a cytokine-mediated mechanism. Because Dickkopf-related protein 1 (Dkk-1) stimulates SOST mRNA and sclerostin expression in mature OBs (8,9) and MM cells stimulate SOST mRNA expression in OBs without cell-cell contact, we investigated the effect of Dkk-1 on OBs. SOST mRNA expression was significantly stimulated in OBs when cocultured with INA-6 or H929 MM cells (Fig. 6A). These data highlight the fact that MM cells stimulate SOST mRNA expression in OBs without cell-cell contact, suggesting a cytokine-mediated mechanism. Because Dickkopf-related protein 1 (Dkk-1) stimulates SOST mRNA and sclerostin protein expression in mature OBs (8,9) and given that Dkk-1 is secreted by MM cells (16,47), we investigated the effect of Dkk-1 neutralizing antibody in this coculture system. Interestingly, Dkk-1 neutralizing antibody inhibited the effect of MM cells on SOST mRNA expression in OBs (Fig. 6A). Furthermore, recombinant human Dkk-1 significantly stimulated SOST mRNA expression in immature OBs (Fig. 6B). To confirm these data, we cocultured MC3T3-E1 cells with H929 human MM cells in OB differentiation medium using cell culture inserts. Mouse sclerostin was detected in the supernatant of MC3T3-E1 cells cocultured with H929 cells, whereas it was absent in the supernatant of MC3T3-E1 alone (Fig. 6C). Human sclerostin was not detected in any of these cultures, as expected (data not shown), confirming that sclerostin is secreted by MC3T3-E1 cells upon stimulation by MM cells. Furthermore, Dkk-1 neutralizing antibody inhibited the stimulation of mouse sclerostin expression by H929 MM cells (Fig. 6C). Finally, we measured human Dkk-1 in our xenograft MM model, which had been injected with human-derived MM.1S MM cells, as these mice exhibited higher levels of murine sclerostin (Fig. 1B). Because MM.1S MM cells are human-derived, we would expect to detect human Dkk-1 if Dkk-1 is indeed secreted by MM cells. In agreement with our in vitro data, we detected significantly higher concentration of human Dkk-1 in the plasma of mice injected with MM.1S cells compared with levels in healthy mice plasma (Fig. 6D).

Discussion

Recent studies have addressed the importance of sclerostin in osteoporosis (22,23); however, the exact role of sclerostin and that of targeting it in the treatment of osteolytic bone disease in oncology and specifically in MM remains unclear. We first confirmed increased sclerostin levels in MM patients. We next defined the role of sclerostin in MM patients and demonstrate the impact of targeting sclerostin in the context of MM and associated bone disease. Our data clearly show that scl-Ab improves MM bone disease in a MM xenograft mouse model, suggesting that sclerostin is a promising target for MM bone disease. We also show that MM cells have the ability to stimulate sclerostin expression in OBs via secretion of Dkk-1, a likely mechanism of inhibition of OB differentiation. These findings suggest that the high sclerostin blood levels detected in MM patients result from secretion not only by osteocytes but also by MSCs and OBs, and that MM cells play a central role in stimulating these cells to secrete sclerostin. The Dkk-1 effect on sclerostin expression in OBs may be caused by positive feedback; however, further studies are necessary to confirm this hypothesis. These data point to a new mechanism of OB inhibition noted in MM and emphasize the importance of targeting sclerostin for treatment of MM bone disease.

In keeping with data reported by others (26–28) regarding sclerostin being produced by MM cells, we detected SOST mRNA expression in cells derived from 2 of 12 primary MM patients’ BM. Moreover, we also show a correlation between sclerostin concentration and serum creatinine (Supplemental Fig. S5), suggesting that renal function can modify sclerostin levels in MM. Taken together, our data suggest that sclerostin is secreted by OBs and MSCs in addition to some from MM cells and that sclerostin concentrations may be regulated by creatinine clearance. This underscores the importance of targeting sclerostin in addition to MM cells for treatment of MM bone disease to improve patients’ quality of life and survival. Because scl-Ab showed no direct effect on tumor cells (although it

Fig. 5. MM inhibits OB differentiation by stimulating sclerostin expression from immature OBs. (A) MSCs derived from MM patients were differentiated into OBs with or without INA-6 by using cell culture inserts in the presence of 1 μg/mL goat IgG control antibody or 1 μg/mL sclerostin neutralizing antibody (Ab). At day 10, runx2 and ALP mRNA expression was detected by qPCR. (B) MSCs were differentiated in OB media with or without INA-6 by using cell culture inserts in the presence of 1 μg/mL goat IgG control antibody or 1 μg/mL sclerostin neutralizing Ab. The effect on OB differentiation was evaluated by using Alizarin red staining at day 42. (C) MSCs were differentiated into OBs with or without INA-6 by using cell culture inserts. At day 42, actin and nuclei were visualized by immunofluorescence. ***p < 0.001.
showed a significant effect on bone turnover, we combined the scl-Ab with carfilzomib. The combination of carfilzomib and scl-Ab stimulated OB calcification in vitro in an additive manner and had potent anti-MM activity. Our xenograft MM model confirmed our in vitro observations with effects on both tumor as well as BM microenvironment. The animals that received only carfilzomib alone in our studies did not show significant increase of bone formation likely because of the low concentration and shorter administration of carfilzomib. In fact, we have previously shown significant effects of carfilzomib resulting in increased bone mass in the same xenograft MM model with higher concentration and longer administration of carfilzomib. Here, combination treatment achieved inhibition of tumor burden and stimulated OB function with consequent increased cancellous bone volume and trabecular bone thickness. Our data reassuringly show an additive effect of carfilzomib and scl-Ab together. Our data warrant exploring the potential of an anti-sclerostin strategy in MM either alone in precursor disease states or in combination with other anti-MM therapies in active MM and osteolytic bone disease.

**Disclosures**

SER and SAK are employees of Lilly Research Laboratories. NSR has received research funding from Eli Lilly and Onyx. All other authors state that they have no conflicts of interest.

**Acknowledgments**

We thank the Center for Skeletal Research Histology Core (NIH P30 AR 066261) for performing IHC on MM patients and mouse samples. This work was supported by a LLS Clinical Scholar Award (to NSR), NIH NRSA DK100215 (to MNW), and NIH P01 DK011794 (to HMK).
Authors’ roles: HE and NSR designed the research. HE, LS, MMW, DZH, DDC, and NN performed research. Y-TT, SER, SAK, NCM, and HMK provided samples or compounds. HE, LS, MMW, HMK, and NSR contributed to data interpretation and discussion. HE, LS, and NSR wrote the paper. All coauthors read and revised the manuscript and approved the final version.

References


