

Myeloma Cell-Osteoclast Interaction Enhances Angiogenesis Together with Bone Resorption: A Role for Vascular Endothelial Cell Growth Factor and Osteopontin

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Abstract Purpose: Similar to osteoclastogenesis, angiogenesis is enhanced in the bone marrow in myeloma in parallel with tumor progression. We showed previously that myeloma cells and osteoclasts are mutually stimulated to form a vicious cycle to lead to enhance both osteoclastogenesis and tumor growth. The present study was undertaken to clarify whether myeloma cell-osteoclast interaction enhances angiogenesis and whether there is any mutual stimulation between osteoclastogenesis and angiogenesis.

Experimental Design: Myeloma cells and monocyte-derived osteoclasts were cocultured, and angiogenic activity produced by the cocultures was assessed with *in vitro* vascular tubule formation assays and human umbilical vascular endothelial cell (HUVEC) migration and survival. Osteoclastogenic activity was determined with rabbit bone cell cultures on dentine slices.

Results: Myeloma cells and osteoclasts constitutively secrete proangiogenic factors, vascular endothelial growth factor (VEGF) and osteopontin, respectively. A cell-to-cell interaction between myeloma cells and osteoclasts potently enhanced vascular tubule formation. Blockade of both VEGF and osteopontin actions almost completely abrogated such vascular tubule formation as well as migration and survival of HUVECs enhanced by conditioned medium from cocultures of myeloma cells and osteoclasts. Furthermore, these factors in combination triggered the production of osteoclastogenic activity by HUVEC.

Conclusions: Osteoclast-derived osteopontin and VEGF from myeloma cells cooperatively enhance angiogenesis and also induce osteoclastogenic activity by vascular endothelial cells. These observations suggest the presence of a close link between myeloma cells, osteoclasts, and vascular endothelial cells to form a vicious cycle between bone destruction, angiogenesis, and myeloma expansion.

Multiple myeloma develops and expands in the bone marrow and generates devastating bone destruction by inducing bone-resorbing osteoclasts. We and others have shown that CC chemokines macrophage inflammatory protein-1 α and 1 β are

secreted from most of multiple myeloma cells and play a major role in osteoclastogenesis by multiple myeloma (1–3). Osteoclasts in turn enhance multiple myeloma cell growth and survival via a cell-to-cell interaction between multiple myeloma cells and osteoclasts. Furthermore, osteoclasts protect multiple myeloma cells from apoptosis induced by doxorubicin, suggesting that increased osteoclast number and/or activity contributes to aggressiveness or drug resistance of multiple myeloma cells. Thus, multiple myeloma cells and osteoclasts interact directly to augment their growth and activity, thereby forming a vicious cycle that leads to extensive bone destruction and multiple myeloma expansion (4).

Similar to osteoclast formation and activation, angiogenesis is enhanced in the bone marrow of multiple myeloma patients in parallel with disease progression (5–10). Such similarities of the two processes during multiple myeloma progression suggested to us that there is a link between osteoclastogenesis and angiogenesis and that enhanced osteoclastogenesis by multiple myeloma cells may play a role in stimulating angiogenesis. Multiple myeloma cells produce multiple angiogenic factors, including vascular endothelial growth factor (VEGF), hepatocyte growth factor, and angiopoietin-1 (10–12). Among them,

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VEGF is considered as the most important factor responsible for enhancement of angiogenesis by various tumors due to its potency and specificity for vascular endothelial cells. However, factors constitutively produced by multiple myeloma cells are not enough to explain enhanced angiogenesis along with the stimulation of osteoclastogenesis. We have reported previously that osteoclasts abundantly secrete an angiogenic noncollagenous matrix protein, osteopontin (4), which has been shown recently to play a role in tumor angiogenesis in addition to VEGF (13–18). Osteopontin is a ligand for $\alpha_v\beta_3$ integrin expressed on vascular endothelial cell surface (19) and enhances angiogenesis and vascular endothelial cell migration (20). In addition, VEGF is reported to enhance osteopontin actions by up-regulating $\alpha_v\beta_3$ integrin in vascular endothelial cells (21). Thus, there is a possibility that osteoclast-derived osteopontin along with multiple myeloma cell-derived VEGF act together to enhance angiogenesis around bone lesions by multiple myeloma.

The present study was undertaken to clarify whether a cell-to-cell interaction between multiple myeloma cells and osteoclasts enhances angiogenesis, whether osteopontin and VEGF play a role in the enhancement of angiogenesis mediated by multiple myeloma cell-osteoclast interaction, and whether there is any mutual stimulation between osteoclastogenesis and angiogenesis. The results show that osteopontin and VEGF play an important role in forming a vicious cycle between osteoclastogenesis and angiogenesis and that there is a mutually stimulating link between angiogenesis, osteoclastogenesis, and multiple myeloma progression.

Materials and Methods

Reagents. The following reagents were purchased from the indicated manufacturers: recombinant human macrophage colony-stimulating factor, VEGF, interleukin (IL)-6, mouse IgG₁, goat IgG, goat anti-human osteopontin antibody, and mouse anti-human VEGF monoclonal antibody were from R&D Systems (Minneapolis, MN); recombinant human soluble receptor activator of nuclear factor- κ B ligand was from PeproTech EC (London, United Kingdom); type I collagen was from Nitta Gelatin (Osaka, Japan); and purified human and recombinant human osteopontin were from Sangi (Otaru, Japan) and Biogenesis (Poole, England), respectively. Neutralizing mouse anti-human osteopontin monoclonal antibody was a kind gift from Dr. Masaki Noda (Tokyo Medical and Dental University, Tokyo, Japan).

Cells and cultures. Human multiple myeloma cell lines, U266 and RPMI8226, were obtained from the American Type Culture Collection (Rockville, MD). Human myeloma cell lines, TSPC-1 and OPC, were established in our laboratory (3). Human umbilical vascular endothelial cells (HUVEC) were purchased and maintained in the angiogenic medium EBM2 plus EGM2 from Cambrex BioScience (Walkersville, MD) on type I collagen-coated culture plates (Becton Dickinson Labware, Franklin Lakes, NJ). Peripheral blood mononuclear cells (PBMC) and bone marrow mononuclear cells were isolated from healthy volunteers or patients with multiple myeloma as described previously (3, 4). Multiple myeloma cells were further purified from bone marrow mononuclear cells with positive selection using CD138 (Syndecan-1) microbeads and Miltenyi magnetic cell sorting system (Miltenyi Biotec, Auburn, CA). The cells were cultured in MEM Eagle-Alpha Modification (α -MEM; Sigma, St. Louis, MO) with 10% heat-inactivated fetal bovine serum (FBS; Whittaker Bioproducts, Walkersville, MA), 100 units/mL penicillin (Sigma), and 100 μ g/mL streptomycin (Sigma). All procedures involving human specimens were

done according to the protocol approved by the Institutional Review Board for human protection.

Generation of human osteoclast-like multinucleated cells and cocultures. Osteoclasts were generated from PBMCs by previously described procedures (22, 23). Adherent cells were prepared from PBMCs and cultured in α -MEM supplemented with 10% FBS, 50 ng/mL recombinant human soluble receptor activator of nuclear factor- κ B ligand, and 500 units/mL recombinant human macrophage colony-stimulating factor. After culturing for 2 weeks, adherent cells were harvested with 0.05% trypsin/0.53 mmol/L EDTA (Kohjin Bio, Saitama, Japan), replated onto 24-well tissue culture plates at 1×10^5 cells/mL, and further cultured with soluble receptor activator of nuclear factor- κ B ligand and macrophage colony-stimulating factor. After washing, PBMC-derived osteoclasts were cultured alone or cocultured with multiple myeloma cells (5×10^5 cells/mL). Multiple myeloma cells were also cultured alone (5×10^5 cells/mL). Their conditioned media were collected after 2 days, filtered with 0.22- μ m filter systems (MILLEX-GV, Millipore, Bedford, MA), and stocked at -80°C until use.

Cytokine measurements. Human VEGF levels were measured by using Human VEGF Assay kit (R&D Systems). Human osteopontin levels were measured by using Human Osteopontin Assay kit-IBL (ImmunoBiological Laboratories, Gunma, Japan).

In vitro vascular tubule formation assay. Conditioned media were harvested from cultures of PBMC-derived osteoclasts and multiple myeloma cell lines, U266 and RPMI8226, and from cocultures of both types of cells. *In vitro* angiogenic activity of conditioned medium was determined using the angiogenesis assay kit that is commercially available from Kurabo (Osaka, Japan). Conditioned media were added at 10%. After 11 days of culture, vascular tubules were stained with anti-CD31 antibody and alkaline phosphatase-conjugated secondary goat anti-mouse IgG antibody, and vascular tubule scores were counted with 12 different fields under a phase-contrast microscopy (Olympus IX71, Olympus, Tokyo, Japan), equipped with a UPlanFL N $\times 10/0.30$ objective lens (Olympus) to achieve an original magnification of $\times 100$ according to the manufacturer's instruction. Images were recorded with an Olympus CCD camera (TH4-100) and Olympus DP controller software and digitally processed using Photoshop software (Adobe, San Jose, CA). Tubule score was estimated with the Chalkley count method (24).

Migration assays. Cell migration assays were done using membrane filters with 8- μ m pore-sized transmigration chambers (Chemotaxicell, Kurabo), which were preincubated for 1 h in a 0.03% solution of type I collagen and set on each well of 24-well culture plates (Becton Dickinson Labware). HUVECs were suspended in α -MEM with 1% FBS. The cells (3×10^4 cells in 300 μ L) were placed on upper chambers. Lower chambers were filled with 800 μ L of the same medium containing conditioned medium or a control medium at 20%. Cytokines and antibodies were added to lower chambers. After incubation for 8 h at 37°C in 5% CO_2 , the cells were fixed with 10% formalin (Wako Pure Chemical Industries, Osaka, Japan) and stained with 10% Giemsa solution (Merk, Tokyo, Japan). Nonmigrated cells on the upper side of the membrane filter were wiped off with a cotton swab and the filters were dried, cut out, and mounted on glass slides. Migrated cells were viewed under an Olympus BX50 microscope (Olympus) equipped with a UPlanFL N $\times 10/0.30$ objective lens to achieve an original magnification of $\times 100$ and cell numbers were counted with five different fields.

Cell viability assays. HUVECs were seeded onto wells in 24-well culture plates at a density of 5×10^4 cells per well in EBM2 plus EGM2 angiogenic medium. After 24 h, media were exchanged to α -MEM containing 1% FBS with conditioned medium (20%) or a control medium. After adding control IgG or antibodies against osteopontin or VEGF, the plates were incubated for 24 h at 37°C in 5% CO_2 . Alive cells were counted by colorimetric assays with 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt (Cell Counting Kit-8, Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. The absorbance of each well was

measured at 450 nm with a microtiter plate reader (model 450 microplate reader, Bio-Rad Laboratories, Tokyo, Japan), and the number of alive cells was calculated from the standard curve.

Collection of HUVEC conditioned medium and in vitro osteoclastogenesis. Culture plates were coated by incubating 24-well plates with recombinant human osteopontin at 1 $\mu\text{g}/\text{mL}$ in Ca/Mg-free PBS or 0.03% solution of type I collagen at room temperature for 1 h. Culture media were then removed and the plates were dried. HUVECs were seeded in wells in the 24-well culture plates coated with recombinant human osteopontin or type I collagen as above at a density of 5×10^4 cells per well in EBM2 plus EGM2 angiogenic medium. After 24 h, media were exchanged to α -MEM with 10% FBS. Cells in wells coated with recombinant human osteopontin or type I collagen were further cultured in the presence or absence of recombinant human VEGF at 10 ng/mL. After culturing 2 days, conditioned media were harvested from each well.

For *in vitro* osteoclastogenesis, osteoclast precursors were first prepared from unfractionated bone cells according to the previously described method (3). In brief, long bones of 5-day-old white rabbits were minced, and bone particles were removed. The rabbit bone cells were seeded on bone slices from calf femur in 96-well plates at 5×10^4 cells per well and cultured for 4 days in α -MEM containing 3% FBS in the absence or presence of 20% conditioned medium collected from HUVEC cultures as described above. After 4 days, cells were stained for tartrate-resistant acid phosphatase using leukocyte acid phosphatase kit (Sigma) and tartrate-resistant acid phosphatase-positive multinucleated cells were viewed under an Olympus BX50 microscope equipped with a UPlanFL N $\times 10/0.30$ objective lens to achieve an original magnification of $\times 100$. Cell numbers were counted with three different bone slices with a mesh glass installed in the ocular lens.

Statistical analysis. Statistical significance was determined by one-way ANOVA with Scheffe post hoc tests. The minimal level of significance was $P = 0.05$.

Results

Osteoclasts enhance angiogenesis in concert with multiple myeloma cells. Although multiple myeloma cells enhance not only osteoclastogenesis but also angiogenesis in the bone marrow (9, 11, 12, 25–28), little is known about the involvement of osteoclasts in the enhanced angiogenesis of multiple myeloma bone marrow. Therefore, we first investigated the role of osteoclasts as well as multiple myeloma cell-osteoclast interaction in angiogenesis using an *in vitro* angiogenesis assay. Microscopic images of newly formed vascular tubules are shown in Fig. 1A. Vascular tubule scores were measured to estimate angiogenic activity quantitatively (Fig. 1B and C). Conditioned medium from PBMC-derived osteoclasts enhanced vascular tubule formation to a level similar to those from multiple myeloma cell lines, U266 and RPMI8226, as well as primary multiple myeloma cells (~ 1.5 -fold increase compared with control medium). Conditioned medium from cocultures of osteoclasts with these multiple myeloma cells enhanced vascular tubule scores more potently (>2 -fold). Thus, osteoclasts produce angiogenic activity as potently as multiple myeloma cells and cooperate with multiple myeloma cells to stimulate angiogenesis.

Osteopontin is a major angiogenic factor produced by osteoclasts. Osteopontin has been shown recently as a factor responsible for tumor angiogenesis (13–18). As we showed previously (4), PBMC-derived osteoclasts abundantly produced osteopontin ($13,000 \pm 2,570 \text{ ng}/10^5 \text{ cells}/2 \text{ days}$; $n = 5$). In contrast to osteoclasts, none of multiple myeloma cells

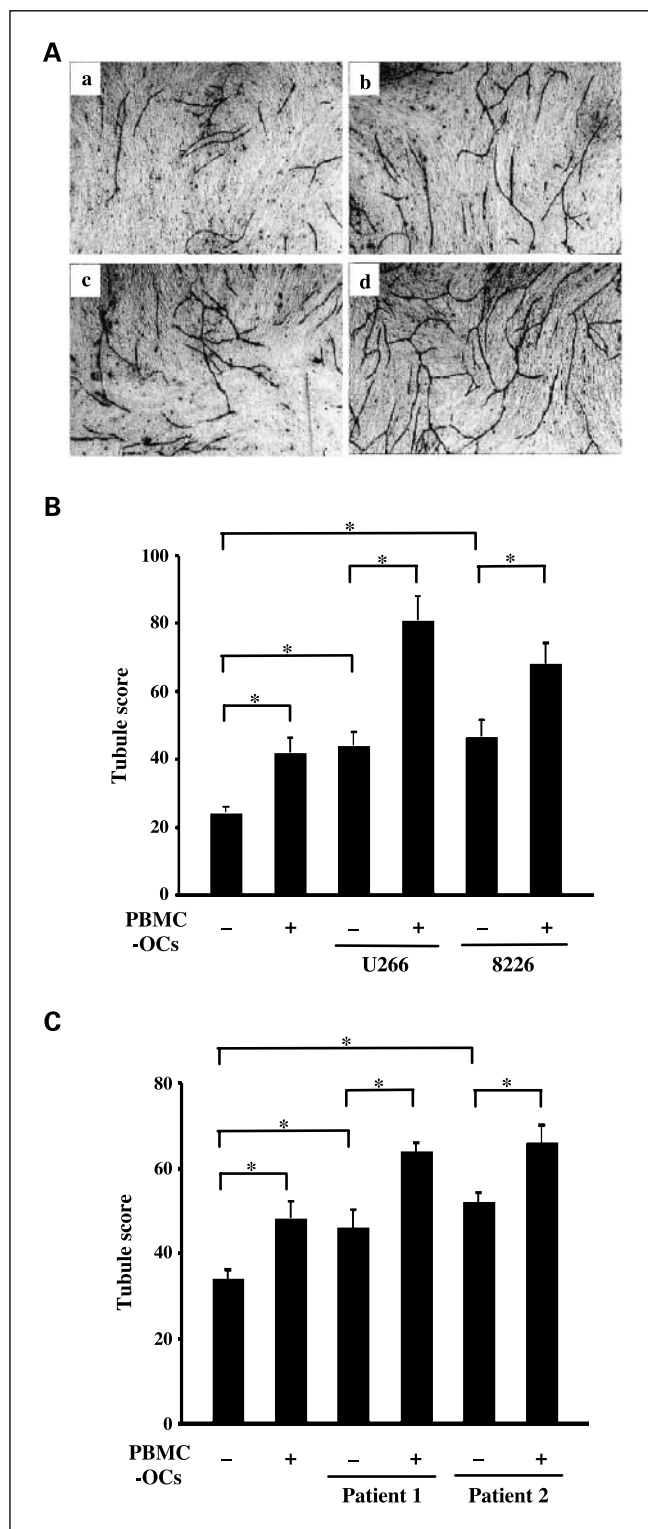


Fig. 1. Conditioned media from cultures of osteoclasts and/or multiple myeloma cells enhance angiogenesis. *In vitro* angiogenic activity of conditioned medium was determined using a commercially available angiogenesis assay kit. Vascular tubules were visualized by staining with anti-CD31 antibody. **A**, microscopic images of newly formed vascular tubules in the absence (**a**) or presence of conditioned medium from U266 cells (**b**) or PBMC-derived osteoclasts (OCs; **c**) or from cocultures of both cells (**d**). Vascular tubule scores for cultures with conditioned medium from PBMC-derived osteoclasts or multiple myeloma cells, the multiple myeloma cell lines U266 and RPMI8226 (**B**), or primary multiple myeloma cells isolated from patients with multiple myeloma (**C**) or from cocultures of both types of cells. Columns, mean of results in quadruplicate; bars, SD. *, $P < 0.05$.

obtained from five patients produced detectable levels of osteopontin, although a portion of primary multiple myeloma cells were reported to produce osteopontin (29, 30). Among multiple myeloma cell lines, only RPMI8226 cells produced osteopontin, whereas most of the other multiple myeloma cell lines, including U266, TSPC-1, and OPC, did not produce detectable levels of osteopontin (<5 ng/mL). Together with our previous observation that the levels of osteopontin produced by osteoclasts is about 140-fold higher than those by bone marrow stromal cells (4), these results indicate that osteoclasts are a major source of osteopontin in the bone marrow microenvironment surrounding multiple myeloma.

Similar to the recent observation by Colla et al. (30), osteopontin alone enhanced vascular tubule formation at a concentration (1 μ g/mL) that is comparable with the final concentrations of osteopontin in osteoclast-conditioned medium used in the angiogenesis assays (Fig. 2A). Addition of a blocking antibody against $\alpha_V\beta_3$ integrin abrogated the effect of osteopontin on angiogenesis, indicating $\alpha_V\beta_3$ integrin as a major receptor for the angiogenic actions of osteopontin (Fig. 2A). We next examined whether osteopontin plays any

role in osteoclast-mediated angiogenesis. The enhancement of angiogenesis by osteoclast-conditioned medium was again completely abrogated by a blockade of osteopontin actions using a neutralizing antibody against osteopontin (Fig. 2B). These results show that osteoclast-derived osteopontin is responsible for osteoclast-mediated angiogenesis.

Osteopontin and VEGF are responsible for angiogenesis enhanced by cocultures of osteoclasts and multiple myeloma cells. VEGF is one of the most potent angiogenic factors produced by tumor cells, including multiple myeloma cells (11, 31). In contrast to osteopontin, all multiple myeloma cell lines examined thus far constitutively secrete VEGF (12, 32). However, osteoclasts do not produce detectable levels of VEGF. A neutralizing antibody against VEGF blocked angiogenesis enhanced by conditioned medium from multiple myeloma cell lines, RPMI8226 and U266, as well as primary multiple myeloma cells (Fig. 3A), suggesting that VEGF is a predominant angiogenic factor derived from these multiple myeloma cells.

In addition to the respective angiogenic actions, osteopontin and VEGF are reported to have cooperative effects on vascular endothelial cells. Osteopontin enhances VEGF-mediated HUVEC migration, and VEGF up-regulates $\alpha_V\beta_3$ integrin expression and the activity of osteopontin in vascular endothelial cells (21). Thus, there is a possibility that osteopontin and VEGF cooperatively enhance angiogenesis under a cell-to-cell interaction between multiple myeloma cells and osteoclasts. As expected, osteopontin and VEGF in combination more potently enhanced vascular tubule formation compared with osteopontin or VEGF alone by *in vitro* angiogenesis assays (Fig. 3B and C).

We next investigated a role for osteopontin and VEGF in angiogenesis enhanced by cocultures of osteoclasts and multiple myeloma cells. Anti-osteopontin-neutralizing antibody or anti-VEGF-neutralizing antibody alone partially suppressed vascular tubule formation enhanced by the conditioned medium from the cocultures of osteoclasts and U266 cells, whereas both antibodies in combination completely abrogated vascular tubule formation to control levels (Fig. 4A and B). Similar results were obtained in cocultures of primary multiple myeloma cells and osteoclasts (Fig. 4C). These results are consistent with the notion that osteoclast-derived osteopontin and multiple myeloma cell-derived VEGF cooperatively enhance angiogenesis and that enhanced angiogenesis in the bone marrow microenvironment surrounding multiple myeloma cells is largely mediated by coordinated actions of these two factors under cell-to-cell interaction between multiple myeloma cells and osteoclasts.

Multiple myeloma cells and osteoclasts cooperatively promote migration and survival of HUVEC by elaborating osteopontin and VEGF. Angiogenesis is a multistep process, and migration and survival of vascular endothelial cells in response to angiogenic stimuli are important initial steps of angiogenesis leading to sprouting of vascular endothelial cells. To further clarify the mechanism of enhancement of angiogenic process by cell-to-cell interaction between osteoclasts and multiple myeloma cells, we investigated the effects of osteopontin and VEGF in conditioned medium from cocultures of osteoclasts and multiple myeloma cells on migration and survival of HUVEC. Conditioned medium from cocultures of osteoclasts and multiple myeloma cells enhanced migration of HUVEC >2-fold (Fig. 5A). Antibodies against VEGF or osteopontin alone

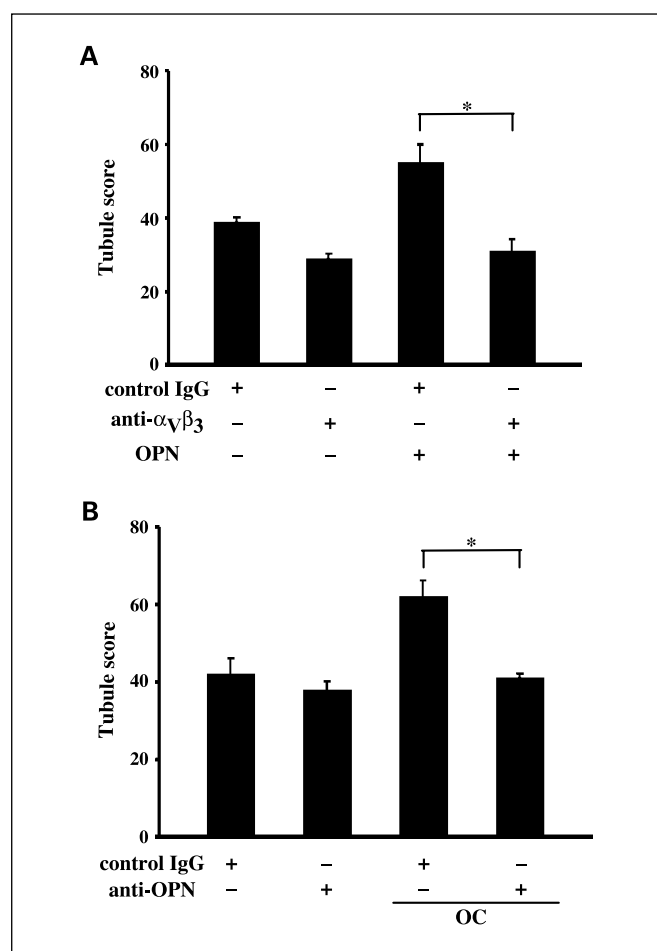


Fig. 2. Osteopontin elaborated by osteoclasts is responsible for angiogenesis. **A**, angiogenic activity of osteopontin (OPN) was determined in the presence of control IgG or anti- $\alpha_V\beta_3$ integrin blocking antibody using *in vitro* angiogenesis assays. Osteopontin was added at 1 μ g/mL. **B**, angiogenic activity of conditioned medium from PBMC-derived osteoclasts at 10% was determined in the presence of control IgG or anti-osteopontin-neutralizing antibody. Columns, mean of results in quadruplicate; bars, SD. *, $P < 0.05$.

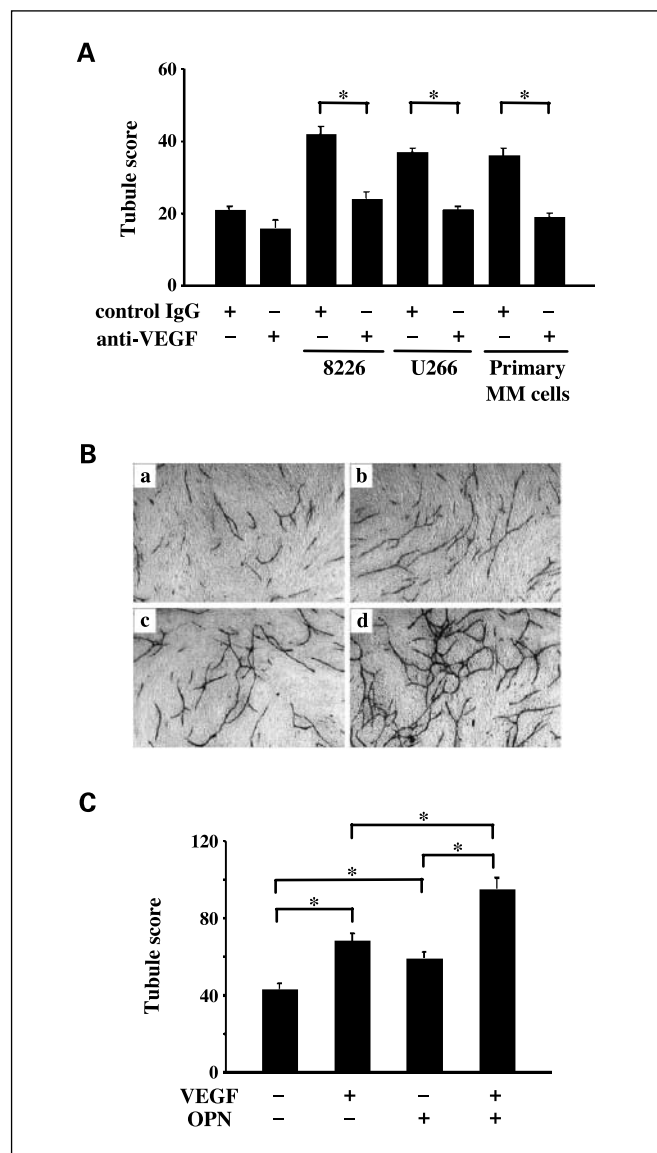


Fig. 3. Angiogenic activity of multiple myeloma cell – derived VEGF and cooperative angiogenic actions of VEGF and osteopontin. *A*, angiogenic activity of conditioned medium from the multiple myeloma (*MM*) cell lines RPM18226 and U266 or primary multiple myeloma cells at 10% was determined in the presence of control IgG or anti – VEGF-neutralizing antibody. *B*, microscopic images of vascular tubules were shown in the absence (*a*) or presence of osteopontin (1 µg/mL; *b*) or VEGF (10 ng/mL; *c*) or both in combination (*d*). *C*, vascular tubule scores were calculated in the absence or presence of VEGF (10 ng/mL) or osteopontin (1 µg/mL) or both in combination. Columns, mean of results in quadruplicate; bars, SD. *, *P* < 0.05.

partially inhibited, and both antibodies in combination mostly abrogated the migratory effects of conditioned medium from cocultures of osteoclasts and multiple myeloma cells (Fig. 5A). HUVEC is highly dependent on nutrition and growth factor and soon dies under factor-deprived conditions. Conditioned medium from cocultures promoted survival of HUVEC by ~ 2.5-fold under factor-deprived conditions compared with control culture medium (Fig. 5B). Antibodies against VEGF and osteopontin in combination substantially reduced supporting effects of the conditioned medium on HUVEC survival. Each antibody alone suppressed HUVEC survival less potently

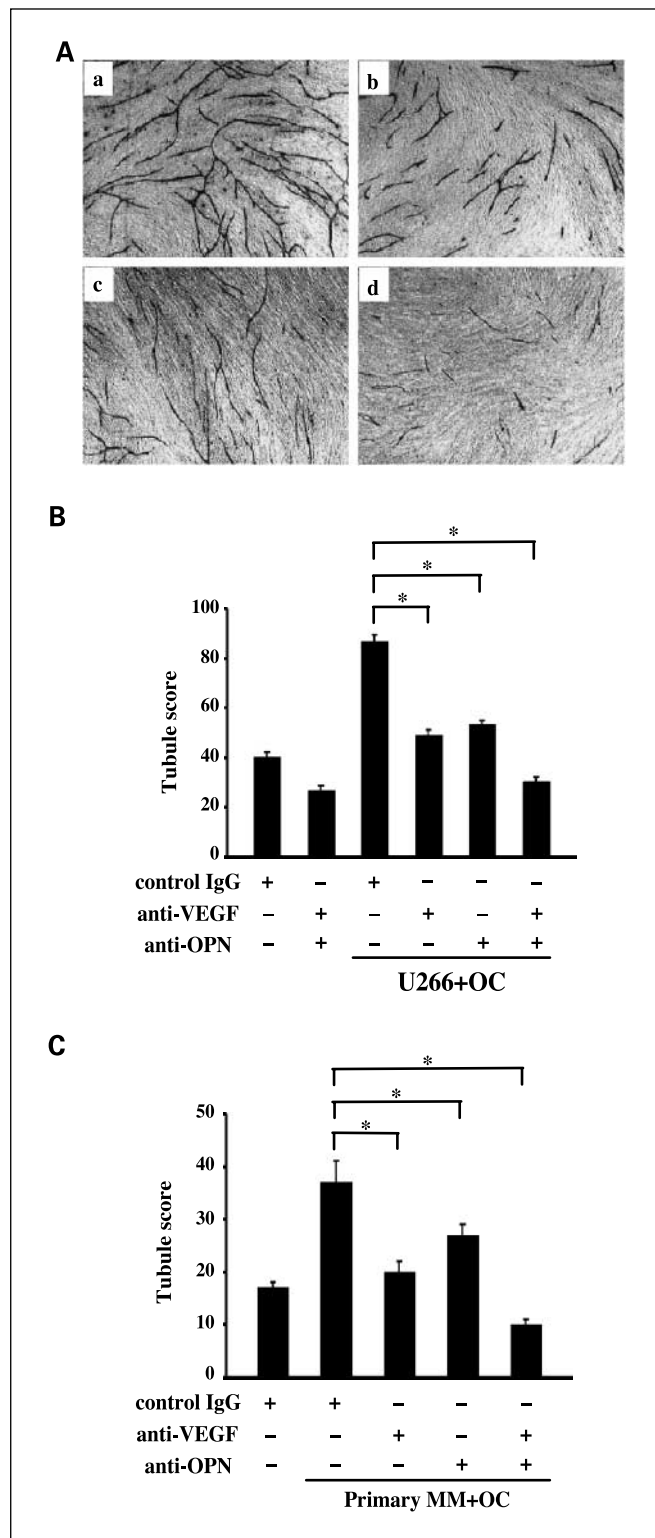


Fig. 4. Osteopontin and VEGF elaborated by osteoclasts and multiple myeloma cells largely mediate angiogenesis. Conditioned media from cocultures of osteoclasts and U266 cells were added at 10%, and their angiogenic activity was determined using *in vitro* angiogenesis assays (*A*). Microscopic images of vascular tubules were shown in the absence (*a*) or presence of anti – VEGF-neutralizing antibody (*b*) or anti – osteopontin-neutralizing antibody (*c*) at 10 µg/mL or both in combination (*d*). Vascular tubule scores were measured in the same assays using 10% conditioned medium from cocultures of osteoclasts and U266 cells (*B*) or primary multiple myeloma cells (*C*). Columns, mean of results in quadruplicate; bars, SD. *, *P* < 0.05.

(Fig. 5B). Thus, osteoclast-multiple myeloma cell interaction facilitates major steps of the angiogenic process, including migration and survival of vascular endothelial cells largely via the actions of elaborated osteopontin and VEGF.

Osteopontin and VEGF trigger the induction of osteoclastogenic activity by HUVEC. Given that osteoclasts and multiple myeloma cells create a microenvironment rich in osteopontin

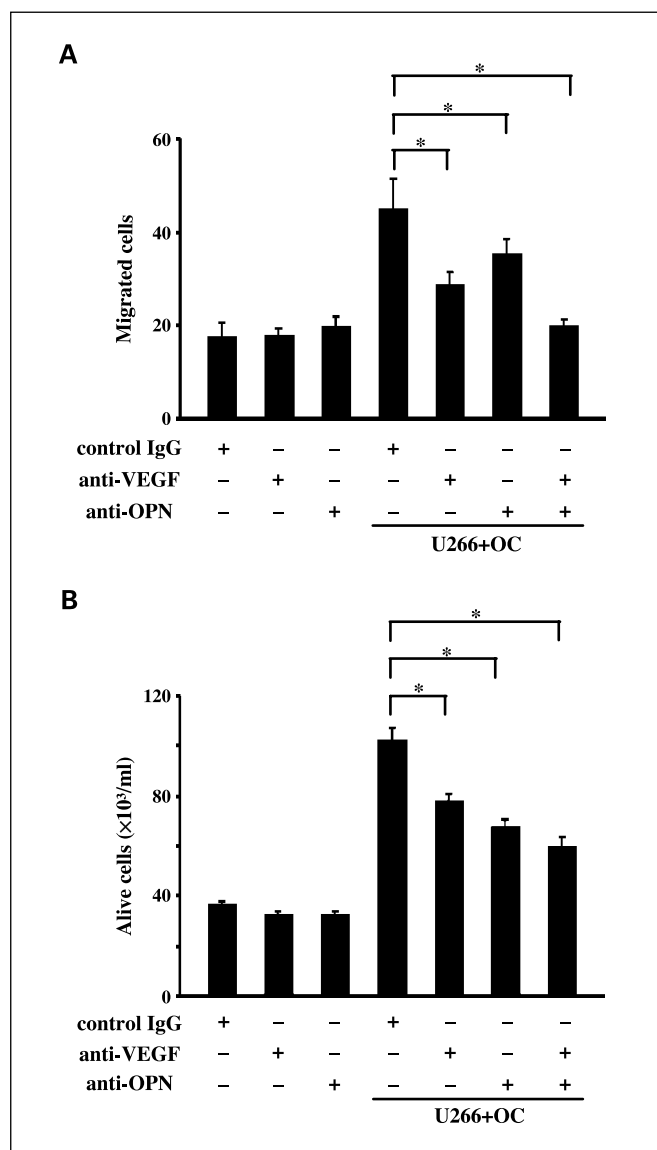


Fig. 5. Osteoclast-derived osteopontin and multiple myeloma cell – derived VEGF cooperatively promote migration and survival of HUVEC. **A**, conditioned media from cocultures of osteoclasts and U266 cells were added to lower chambers at 20%. Anti – VEGF-neutralizing antibody or anti – osteopontin-neutralizing antibody or control IgG was added at 10 µg/mL to the indicated chambers. HUVECs were placed onto the upper chambers in quadruplicate and allowed to migrate for 8 h. The numbers of the migrated cells to the lower side of pore filters were counted. Columns, mean of migrated cell numbers; bars, SD. *, *P* < 0.05. **B**, HUVECs were plated out onto type I collagen-coated 24-well culture plates and cultured in α-MEM supplemented with 1% FBS. Conditioned media from cocultures of osteoclasts and U266 cells at 20% with or without anti – VEGF-neutralizing antibody or anti – osteopontin-neutralizing antibody or control IgG at 10 µg/mL were added to the indicated wells. Cultures were done in quadruplicate. Viable cell number was counted after 24 h with 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt colorimetric assays. Columns, mean; bars, SD. *, *P* < 0.05.

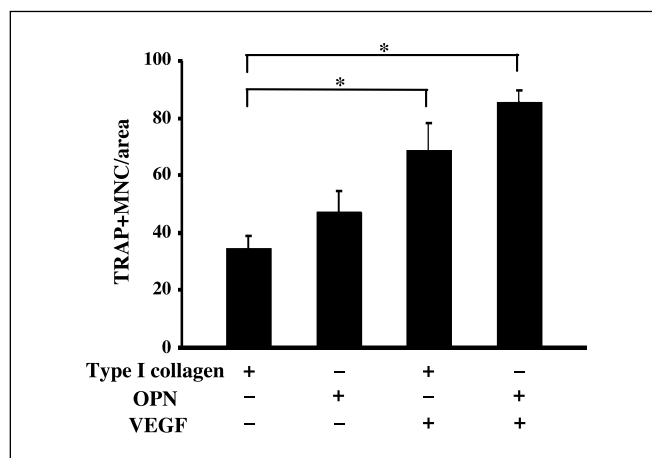


Fig. 6. Osteoclastogenic activity is produced by HUVEC on stimulation with VEGF and osteopontin. HUVECs were first cultured for 1 d on either osteopontin-coated or type I collagen-coated wells as described in Materials and Methods. After medium change, HUVECs were cultured in the presence or absence of VEGF for 2 d and conditioned media were collected. The conditioned media were added to rabbit bone cell cultures. Anti-VEGF antibody was added at 10 µg/mL to cancel out the effect of VEGF contained in the conditioned medium. Osteoclastogenesis was determined by numbers of tartrate-resistant acid phosphatase (TRAP) – positive multinucleated cells (MNC) at day 4 as described in Materials and Methods. Columns, mean of results in quadruplicate; bars, SD. *, *P* < 0.05.

and VEGF and induce angiogenesis around them in multiple myeloma bone lesions, there is a possibility that vascular endothelial cells supported by osteopontin and VEGF may in turn influence nearby cells of osteoclastic lineage. Therefore, we next examined whether VEGF and osteopontin were able to induce osteoclastogenic activity in HUVEC using mixed bone marrow cell cultures. Because HUVEC was cultured and maintained on type I collagen-coated wells at a quiescent state and because contamination of exogenously added osteopontin in conditioned medium had to be avoided for the following experiments, we analyzed osteoclastogenic potential of HUVEC cultured on osteopontin-coated wells compared with that cultured on type I collagen-coated wells. Conditioned media were collected from HUVEC cultured on either osteopontin-coated or type I collagen-coated wells in the presence or absence of VEGF. Because VEGF in itself enhances osteoclastogenesis, we added a surplus of anti-VEGF antibody to the conditioned medium to cancel out the effect of exogenously added VEGF to examine osteoclastogenic potential of HUVEC conditioned medium added to mixed bone marrow cell cultures. Conditioned medium from HUVEC cultured on osteopontin-coated wells with VEGF enhanced osteoclast formation most potently (2.6-fold) compared with conditioned medium from type I collagen-coated wells without VEGF as a control, whereas conditioned medium from type I collagen-coated wells with VEGF and osteopontin-coated wells without VEGF enhanced 2- and 1.4-fold, respectively (Fig. 6). These results indicate cooperative actions of VEGF and osteopontin on induction of osteoclastogenic activity by HUVEC. Taken together, in addition to a direct osteoclastogenic activity of VEGF and osteopontin, these factors elaborated by multiple myeloma cell-osteoclast interaction may further stimulate osteoclastogenesis by triggering the secretion of osteoclastogenic factor(s) from vascular endothelial cells in the bone marrow microenvironment around multiple myeloma.

Discussion

Expansion of multiple myeloma bone disease is associated with an enhancement of angiogenesis around multiple myeloma cells. Both VEGF and osteopontin are well-known multifunctional factors involved in various aspects of cancer progression, including multiple myeloma. Both factors trigger growth, survival, and migration of multiple myeloma cells (29, 33), increase angiogenesis (10, 30, 31), as well as osteoclastic bone resorption (20, 34). Abnormalities in the expression and/or signaling pathways of VEGF and osteopontin can, therefore, play an important role in the development and progression of multiple myeloma. The present study showed that VEGF and osteopontin are abundantly produced by multiple myeloma cells and osteoclasts, respectively, and are responsible for the enhanced angiogenesis induced by multiple myeloma cell-osteoclast interactions in part through an enhancement of migration and survival of vascular endothelial cells.

Osteopontin binds to $\alpha_v\beta_3$ integrin and VEGF binds to VEGF receptor 2 (KDR/Flk-1) as major receptors that are expressed on vascular endothelial cells (35). Osteopontin binding to $\alpha_v\beta_3$ integrin up-regulates the expression (36) as well as VEGF-induced phosphorylation of VEGF-R2 (37). Conversely, VEGF binding to VEGF receptor 2 up-regulates the expression of $\alpha_v\beta_3$ integrin (21). Furthermore, ligand binding to either $\alpha_v\beta_3$ integrin or VEGF receptor 2 alone induces coassociation of these receptors (38). Those previous reports indicate that $\alpha_v\beta_3$ integrin and VEGF receptor 2 closely interact each other after binding of their respective ligands to efficiently transduce their downstream signaling. Based on these observations, it is plausible to assume that osteopontin secreted from osteoclasts and VEGF from multiple myeloma cells cooperate each other to induce angiogenesis by mutually enhancing downstream signaling in the bone marrow microenvironment around multiple myeloma.

Osteopontin is also produced by various types of cells other than osteoclasts, including bone marrow stromal cells/osteoblasts and aberrantly by some tumor cells (10, 13–18, 39, 40). A portion of multiple myeloma cells produce a significant amount of osteopontin, and elevated osteopontin levels have been detected in the bone marrow plasma of patients with multiple myeloma producing osteopontin, which may further potentiate angiogenesis (30). However, the production levels of osteopontin per cell is by far higher in osteoclasts than in bone marrow stromal cells or multiple myeloma cells (4). The importance of osteoclasts as a source of osteopontin was further corroborated by the present observation that osteopontin-negative multiple myeloma cells cooperatively enhanced *in vitro* angiogenesis with osteoclasts similar to osteopontin-producing RPMI8226 cells (Fig. 1B; refs. 17, 30). Furthermore, inoculation of osteopontin-producing human multiple myeloma cell line, ANBL-6, in nonobese diabetic/severe combined immunodeficient mice resulted in an elevation of murine osteopontin but not tumor cell-derived human osteopontin in the serum (29). Taken together, these observations are consistent with a notion that osteoclasts surrounding multiple myeloma cells rather than

multiple myeloma cells themselves are a major source of osteopontin to create osteopontin-rich environment and enhance angiogenesis around multiple myeloma cells.

Because angiogenesis and osteoclastogenesis seem to progress in parallel in the multiple myeloma bone marrow microenvironment, there is a possibility that vascular endothelial cells that are formed under stimulation with VEGF and osteopontin by an interaction between multiple myeloma cells and osteoclasts also have the ability to enhance osteoclastogenesis. In fact, HUVEC treated with VEGF and osteopontin together, but not quiescent HUVEC, produced osteoclastogenic activity (Fig. 6). In an effort to clarify osteoclastogenic factor(s) secreted from HUVEC on stimulation with VEGF and osteopontin, cytokine antibody arrays were done. Among 125 different cytokines analyzed, VEGF and osteopontin treatment substantially up-regulated the production of several cytokines and chemokines by HUVEC, and IL-8 was most prominently induced by VEGF and osteopontin treatment (data not shown). IL-8 is known as an osteoclastogenic factor and is involved in osteoclastogenesis induced by cancer metastasis to bone (41). However, blockade of IL-8 only partially suppressed osteoclastogenesis induced by conditioned medium from VEGF and osteopontin-treated HUVEC cultures. Thus, factors other than IL-8 may also be involved in the osteoclastogenic activity produced from HUVEC under stimulation with VEGF and osteopontin.

We have reported previously that multiple myeloma cell-osteoclast interaction enhances multiple myeloma cell growth and survival and that blockade of IL-6 action only partially inhibits multiple myeloma cell growth and survival, although secretion of IL-6 from osteoclasts is enhanced by multiple myeloma cell-osteoclast interaction (4). Because IL-6 plays an important role both in proliferation/survival of multiple myeloma cells and in osteoclastogenesis in multiple myeloma, we envisaged an important role for IL-6 in angiogenesis in the bone marrow in patients with multiple myeloma. Roles for IL-6 produced in the multiple myeloma bone marrow microenvironment remains to be elucidated *in vivo*. Osteoclasts produce B-cell-activating factor and a proliferation-inducing ligand, members of tumor necrosis factor family (42). Our previous results showed that binding of B-cell-activating factor and a proliferation-inducing ligand secreted from osteoclasts to their cognate receptors expressed on multiple myeloma cells plays an important role in multiple myeloma cell growth and survival in the bone marrow microenvironment and the formation of a vicious cycle between multiple myeloma cells and osteoclasts (43). In addition to the direct interaction between multiple myeloma cells and osteoclasts, the present study showed that enhanced angiogenesis induced by multiple myeloma cell-osteoclast interaction further facilitates multiple myeloma cell growth and survival as well as osteoclast formation to aggravate multiple myeloma cell expansion and bone destruction. These results also suggest that VEGF and osteopontin can be considered as a therapeutic target to disrupt the pathognomonically skewed cellular interactions in multiple myeloma bone marrow microenvironment to ameliorate bone destruction and multiple myeloma expansion.

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