Zoledronic acid affects over-angiogenic phenotype of endothelial cells in patients with multiple myeloma

Claudio Scavelli,1 Giulia Di Pietro,1 Teresa Cirulli,1 Mauro Coluccia,2 Angela Boccarelli,2 Teresa Giannini,1 Giuseppe Mangialardi,1 Raffaello Bertieri,4 Addolorata Maria Luce Coluccia,1 Domenico Ribatti,3 Franco Dammacco,1 and Angelo Vacca1

1Department of Biomedical Sciences and Human Oncology, Section of Internal Medicine and Clinical Oncology, 2Section of General Pathology, and 3Department of Human Anatomy and Histology, University of Bari Medical School, Bari, Italy; and 4Novartis Pharma, Origgio, Italy

Abstract

Therapeutic doses of zoledronic acid markedly inhibit in vitro proliferation, chemotaxis, and capillarogenesis of bone marrow endothelial cells of patients with multiple myeloma. Zoledronic acid also induces a sizeable reduction of angiogenesis in the in vivo chorioallantoic membrane assay. These effects are partly sustained by gene and protein inhibition of vascular endothelial growth factor and vascular endothelial growth factor receptor 2 in an autocrine loop. Mevastatin, a specific inhibitor of the mevalonate pathway, reverts the zoledronic acid antiangiogenic effect, indicating that the drug halts this pathway. Our results provide evidence of a direct antiangiogenic activity of zoledronic acid on multiple myeloma patient-derived endothelial cells due to at least four different mechanisms identified either in vitro or in vivo. Tentatively, we suggest that the zoledronic acid antitumoral activity in multiple myeloma is also sustained by angiogenesis, which would partly account for its therapeutic efficacy in multiple myeloma. [Mol Cancer Ther 2007;6(12):3256–62]

Introduction

Pathologic angiogenesis is a constant hallmark of bone marrow microenvironment in multiple myeloma (1), but mechanisms of its induction are not well established. Plasma cells are primary inducers because they secrete major angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF; refs. 1–3). Stromal cells and endothelial cells [multiple myeloma patient-derived endothelial cells (MMECs)] behave as secondary inducers following recruitment and activation by plasma cells (4, 5), being a huge source of these growth factors too (6). The VEGF/VEGF receptor-2 (VEGFR2) pathway greatly contributes to multiple myeloma angiogenesis and growth (7) and mediates proliferation and capillarogenesis in MMECs through an autocrine loop (8), supporting the view that effective antiangiogenesis could be achieved via VEGF-VEGFR2 inhibition (9). Zoledronic acid is a bisphosphonate used for multiple myeloma bone disease and hypercalcemia. Recent findings indicate that it has a direct cytotoxic activity on tumor cells and suppresses angiogenesis (10, 11), but the associated molecular events have not been fully characterized yet. Zoledronic acid inhibits bFGF-dependent proliferation and capillary-like tube formation on Matrigel of human umbilical vascular endothelial cells (11, 12). Here we show that its antiangiogenic activity in MMECs at diagnosis is mediated by abrogation of their VEGF/VEGFR2 autocrine loop. Data suggest that zoledronic acid has an antiangiogenic effect in multiple myeloma, which may be involved in an indirect antitumor activity.

Materials and Methods

Patients and Endothelial Cell Cultures

Twenty-four patients fulfilling the International Myeloma Working Group diagnostic criteria (13) for multiple myeloma were studied at diagnosis. They were 14 male and 10 female, of ages 44 to 75 years (median, 61.5 years), and staged (14) as II A (n = 9), III A (n = 13), and III B (n = 2); the M-component was IgG (n = 17), IgA (n = 5), κ or λ (n = 2). The study was approved by the local Ethics Committee and all patients gave their informed consent in accordance with the Declaration of Helsinki.

Bone marrow endothelial cells isolated from each of the 24 multiple myeloma patients as described (6, 15) were distinctly used in all experiments. Briefly, centrifugation on Ficoll gradient of heparinized aspirates was followed by polystyrene flask adherence to isolate stromal cells, removal of suspended plasma cells, detachment of adherent cells with a trypsin-EDTA solution, immunodepletion of macrophages and possible residual plasma cells with CD14 and CD38 (macrophage and plasma cell markers, respectively) monoclonal antibody–coated flasks (both monoclonal antibodies were from Immunotech-Coulter), absorption to magnetic microbeads coated with Ulex

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Requests for reprints: Angelo Vacca, Department of Internal Medicine and Clinical Oncology, University of Bari Medical School, Policlinico, Piazza Giulio Cesare, 11, I-70124 Bari, Italy. Phone: 39-80-559-34-44; Fax: 39-80-559-21-89. E-mail: a.vacca@dimo.uniba.it

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The purity and viability of MMEC cultures grown at least one passage (>97% viable cells) were assessed by fluorescence-activated cell sorting (FACScan, Becton Dickinson) with double positivity for factor VIII–related antigen (a highly specific endothelial cell marker) and CD105 (or endoglin, a molecule strongly expressed by MMECs) and negativity for CD14, CD38 (6, 15), and MCA1399G (AbD Serotec; a fibroblast marker; Supplementary Fig. S1)5 monoclonal antibodies; followed by reverse transcriptase-PCR (RT-PCR) for mRNA of factor VIII–related antigen, CD38, CD105, and IgH VDJ region; and by trypan blue viable staining (15).

The human endothelial cell–like immortalized cell line EA.hy926, derived from the fusion of human umbilical vascular endothelial cells with the lung carcinoma cell line A549 (16), was used as control because its over-angiogenic phenotype, in terms of enhanced proliferative, chemotactic, and capillarogenic activities, overlaps that of MMECs (6, 9). It was maintained in DMEM containing 10% FCS and 1% glutamine.

Treatment with Zoledronic Acid and Mevastatin. Preparation of Conditioned Media, and Total RNA

Zoledronic acid (Zometa, Novartis Pharma) was solubilized in distilled water and stepwise diluted from 1 to 3, 10, 30, and 50 μmol/L in the medium. The intermediate doses 3 to 30 μmol/L correspond to concentrations achieved in sites of active bone resorption after long-term use of zoledronic acid (4 or 8 mg i.v. infusion) in an adult (70 kg) patient due to its selective uptake and long persistence in the bone (17, 18). In selected experiments, zoledronic acid was used in combination with mevastatin (Sigma-Aldrich), a specific inhibitor of hydroxymethylglutaryl CoA reductase of the mevalonate pathway, to assess whether some effects of zoledronic acid were mediated by this pathway.

Table 1. RT-PCR: primers, amplification, and products

<table>
<thead>
<tr>
<th>Gene sequence of primers (5‘–3’)</th>
<th>Amplification conditions</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VEGF</strong>&lt;sub&gt;165&lt;/sub&gt;</td>
<td>Forward: GCTGCACCATGGCCAGGAAGG</td>
<td>30 cycles at 95°C 1 min and 30 s, 95°C 1 min, 65°C 30 s, 72°C 30 s</td>
</tr>
<tr>
<td></td>
<td>Reverse: GACGCGCTCGCAAGCTG</td>
<td></td>
</tr>
<tr>
<td><strong>VEGFR2</strong></td>
<td>Forward: GCTGCACCATGGCCAGGAAGG</td>
<td>35 cycles at 95°C 1 min and 30 s, 95°C 30 s, 58°C 30 s, 72°C 1 min</td>
</tr>
<tr>
<td></td>
<td>Reverse: GACGCGCTCGCAAGCTG</td>
<td></td>
</tr>
<tr>
<td><strong>bFGF</strong></td>
<td>Forward: TACCACCGACAAGAGATGG</td>
<td>30 cycles at 94°C 3 min, 94°C 25 s, 58°C 25 s, 72°C 25 s and 1 cycle at 72°C 5 min</td>
</tr>
<tr>
<td></td>
<td>Reverse: GACGCGCTCGCAAGCTG</td>
<td></td>
</tr>
<tr>
<td><strong>FGFR1</strong></td>
<td>Forward: GCTGCACCATGGCCAGGAAGG</td>
<td>32 cycles at 94°C 3 min, 94°C 25 s, 58°C 25 s, 72°C 25 s and 1 cycle at 72°C 5 min</td>
</tr>
<tr>
<td></td>
<td>Reverse: GACGCGCTCGCAAGCTG</td>
<td></td>
</tr>
<tr>
<td><strong>FGFR2</strong></td>
<td>Forward: GCTGCACCATGGCCAGGAAGG</td>
<td>32 cycles at 94°C 3 min, 94°C 25 s, 58°C 25 s, 72°C 25 s and 1 cycle at 72°C 5 min</td>
</tr>
<tr>
<td></td>
<td>Reverse: GACGCGCTCGCAAGCTG</td>
<td></td>
</tr>
<tr>
<td><strong>FGFR3</strong></td>
<td>Forward: GCTGCACCATGGCCAGGAAGG</td>
<td>32 cycles at 94°C 3 min, 94°C 25 s, 58°C 25 s, 72°C 25 s and 1 cycle at 72°C 5 min</td>
</tr>
<tr>
<td></td>
<td>Reverse: GACGCGCTCGCAAGCTG</td>
<td></td>
</tr>
<tr>
<td><strong>FGFR4</strong></td>
<td>Forward: GCTGCACCATGGCCAGGAAGG</td>
<td>32 cycles at 94°C 3 min, 94°C 25 s, 58°C 25 s, 72°C 25 s and 1 cycle at 72°C 5 min</td>
</tr>
<tr>
<td></td>
<td>Reverse: GACGCGCTCGCAAGCTG</td>
<td></td>
</tr>
<tr>
<td><strong>HGF</strong></td>
<td>Forward: GCTGCACCATGGCCAGGAAGG</td>
<td>30 cycles at 94°C 3 min, 94°C 25 s, 58°C 25 s, 72°C 25 s and 1 cycle at 72°C 5 min</td>
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<tr>
<td></td>
<td>Reverse: GACGCGCTCGCAAGCTG</td>
<td></td>
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<tr>
<td><strong>c-MET</strong></td>
<td>Forward: GCTGCACCATGGCCAGGAAGG</td>
<td>30 cycles at 94°C 3 min, 94°C 25 s, 58°C 25 s, 72°C 25 s and 1 cycle at 72°C 5 min</td>
</tr>
<tr>
<td></td>
<td>Reverse: GACGCGCTCGCAAGCTG</td>
<td></td>
</tr>
<tr>
<td><strong>GAPDH</strong></td>
<td>Forward: GCTGCACCATGGCCAGGAAGG</td>
<td>30 cycles at 94°C 3 min, 94°C 25 s, 58°C 25 s, 72°C 25 s and 1 cycle at 72°C 5 min</td>
</tr>
<tr>
<td></td>
<td>Reverse: GACGCGCTCGCAAGCTG</td>
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5 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
MMECs or EA.hy926 at 90% confluence were cultured in duplicate in serum-free medium alone or supplemented with zoledronic acid (10 and 30 μmol/L) for 24 h. Conditioned media were collected and stored as described (6).

Total RNA was extracted with the Trizol reagent (Invitrogen, Life Technologies), purified using the RNeasy total RNA Isolation Kit (Qiagen), and verified for integrity with an Agilent Bioanalyzer (Agilent Technologies).

**Functional Studies**

**Proliferation Assay.** MMEC proliferation was evaluated as described (9). Triplicate $3 \times 10^5$ seeded cells per well in 96-well plates in starvation serum-free medium (negative control) or supplemented with 10% FCS or with VEGF$_{165}$ or bFGF (both 10 ng/mL; Sigma Chemical Co.) in 1.5% FCS alone (positive controls) or added with the zoledronic acid doses were counted on day 8 by a colorimetric method, and data were expressed as mean ± SD.

**Chemotaxis Assay.** This was done in triplicate according to the Boyden microchemotactic technique toward serum-free medium alone (negative control) or admixed with 10 ng/mL VEGF$_{165}$ or bFGF alone (positive controls) or supplemented with the zoledronic acid doses as described (9). Cells were counted on $1,000 \times$ oil-immersion field/membrane and given as mean ± SD.

**Matrigel Angiogenesis Assay.** MMECs ($2 \times 10^5$ per well) were plated in duplicate in 24-well plates precoated with Matrigel (300 μL/well; Becton Dickinson) in 1 mL/well serum-free medium alone supplemented with VEGF$_{165}$ (10 ng/mL) alone or with the zoledronic acid doses. After a 12-h incubation and skeletonization of the mesh, its topological variables, (a) “areas,” (b) “vessel length,” and (c) “branching points,” were measured by computed image analysis as described (19), and given as mean ± SD of percent inhibition versus the positive control.

**Chorioallantoic Membrane Assay.** This was done on fertilized White Leghorn chicken eggs (20 per group) incubated at 37°C at constant humidity as described (20). Briefly, a square window was opened in the shell at day 3, and 2 to 3 mL of albumen were removed to allow detachment of the chorioallantoic membrane. At day 8, the chorioallantoic membranes were implanted with 1-mm$^3$ sterilized gelatin sponges (Gelfoam Upjohn) loaded with 1 μL of PBS (negative control), 1 μL of PBS containing 250 ng of VEGF$_{165}$ (positive control), or 1 μL of culture conditioned media from MMECs alone or added with 10 or 30 μmol/L zoledronic acid. The angiogenic response was evaluated at day 12 as the number of vessels converging toward the sponge recognized at $\times 50$ and photographed in ovo under an Olympus stereomicroscope (Olympus).

**RT-PCR and Real-time RT-PCR**

Two micrograms of total RNA were reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Invitrogen) and 1 μg of cDNA was subjected to PCR with primers (Invitrogen) shown in Table 1. The PCR products were separated and stained, and bands were measured as numbers of pixels as described (9).

Real-time RT-PCR was done in a Smart Cycler (Cepheid) using the OmniMix HS (TaKaRa Bio, Inc.) added with SYBR Green I (Sigma Chemical). To compensate for differences in RNA quality or reverse transcription efficacy, each sample was processed with parallel assays for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene; the absolute levels of each mRNA were thus normalized to the GAPDH mRNA content (21). The same primers used for RT-PCR were applied on duplicate RNA samples. Measurements were taken at the end of the 72°C extension step in each cycle, and the second-derivative method was used to calculate threshold cycle ($C_t$). Melt curve analysis showed a single sharp peak for all samples. The average $C_t$ of GAPDH gene was subtracted from the average $C_t$ of each gene to yield the $\Delta C_t$. The $\Delta C_t$ of the basal (medium) endothelial cells was then subtracted from the $\Delta C_t$ of endothelial cells exposed to each zoledronic acid dose to obtain the $\Delta \Delta C_t$ (22).

**Western Blot and ELISA**

Western blot was done as described (9) to evaluate the content of VEGFR2, phospho-VEGFR2, phospho-extracellular signal–regulated kinase 1/2, FGFR receptor (FGFR)-1/FGFR2/FGFR3/FGFR4, and c-MET in cell extracts without (medium) and with exposure to zoledronic acid alone and plus mevastatin. Briefly, proteins (40 μg) were subjected to 8% SDS-PAGE, electrotransferred to a polyvinylidene difluoride membrane, incubated with primary and secondary antibodies (Santa Cruz Biotechnology, Inc.), analyzed by
enhanced chemiluminescence, and were revealed by Kodak Biomax film (Eastman Kodak Co); their band intensity was expressed as fold expression of the medium value by arbitrary absorbance taken as unit.

Fifty microliters (VEGF and HGF) and 100 μL (bFGF) of conditioned media were tested with a sandwich ELISA (Quantikine, R&D Systems). The interassay coefficients of variations were 6.7% (VEGF), 8% (bFGF), and 6.9% (HGF); the intraassay coefficients were 4.7%, 5.2%, and 5.8%, respectively.

Results and Discussion

Functional Studies

Proliferative response of MMECs to both complete medium and VEGF<sub>165</sub> was significantly reduced by zoledronic acid in a dose-dependent fashion, being maximal at 30 μmol/L (~52% of untreated cells; P < 0.01, Wilcoxon-Wilcox test), whereas 50 μmol/L gave a plateau (Fig. 1A). Zoledronic acid at 10 and 30 μmol/L exerted an inhibitory effect on the MMEC migration in a chemotaxis assay: ~55% and ~68% of the positive control, respectively (P < 0.01, Wilcoxon-Wilcox test; Fig. 1B). Zoledronic acid did not produce inhibitory effects on bFGF-induced proliferation and chemotaxis (Supplementary Fig. S2).<sup>5</sup>

Zoledronic acid markedly inhibited the capillarogenesis on Matrigel (Fig. 2A). After a 12-h incubation, unexposed MMECs produced a closely knit capillary network with thin, branching, and anastomosing tubes linked through numerous junctions: mesh areas were 43.2 ± 8; length, 7,914 ± 708 μm; and branching points, 48 ± 6. Exposure to zoledronic acid at 10 μmol/L and even more at 30 μmol/L gave rise to a poorly organized plexus with few strait and disorganized tubes with scarce junctions: mesh areas, 29.6 ± 4.2 and 18.4 ± 2.8, respectively; vessel length, 4,712 ± 413 and 3,145 ± 326 μm; and branching points, 27 ± 4 and 16 ± 3 (P < 0.01 or better).

Chorioallantoic membranes treated with sponges loaded with VEGF<sub>165</sub> (positive control) or with the MMEC conditioned media were surrounded by allantoic vessels converging radially toward the sponge in a “spoked-wheel” pattern (number of vessels, 28 ± 5 and 26 ± 4, respectively; Fig. 3A and C). No vascular response was detectable around the sponges loaded with vehicle alone (number of vessels, 6 ± 2; Fig. 3B). When zoledronic acid at 10 and 30 μmol/L was added to the MMEC conditioned media, a significant reduction of the angiogenic response was found (number of vessels, 15 ± 3 and 8 ± 2, respectively; P < 0.001; Fig. 3D).

Because zoledronic acid is a calcium chelator, parallel control experiments with EDTA at equimolar doses, assessing a possible contribute of cation chelation to zoledronic acid effect, gave no significant results (data not shown).

These results provide direct evidence that zoledronic acid exerts antiangiogenic activity on MMECs by blocking almost specifically the over-angiogenic response to VEGF<sub>165</sub> (9) as opposed to the selective activity on bFGF-dependent norm-angiogenic human umbilical vascular endothelial cells (11).

Figure 2. Capillarogenesis on Matrigel. MMECs of a representative patient were seeded on Matrigel in serum-free medium (SFM) supplemented with VEGF<sub>165</sub> alone and with the zoledronic acid doses. After a 12-h incubation, their three-dimensional organization was examined planimetrically by a computed image analysis. Columns, mean percentage of inhibition of the indicated topological variables; bars, SD.

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Inhibition of VEGF₁₆₅/VEGFR2 Autocrine Loop, But Not of bFGF/FGFR and HGF/c-MET Loops, in MMECs

The high sensitivity to zoledronic acid of VEGF₁₆₅-induced angiogenic functions prompted us to investigate whether the inhibition of VEGF₁₆₅/VEGFR2 autocrine loop in MMECs (8) may account for the functional results, and whether other main regulators of angiogenesis (i.e., bFGF and HGF-dependent loops; ref. 23), which are expressed at high levels in the multiple myeloma bone marrow (6), were affected by zoledronic acid. By using semiquantitative

Figure 3. Chorioallantoic membranes treated with sponges loaded with VEGF₁₆₅ or MMEC conditioned media were surrounded by allantoic vessels developing radially toward the implant in a spoked-wheel pattern (A and C). No vascular response was detectable around the sponges loaded with vehicle (medium) alone (B). Zoledronic acid added to the conditioned media at 30 μmol/L significantly inhibits the angiogenic response (D). Original magnification, ×50 (A–D).

Figure 4. A, expression levels of VEGF₁₆₅, VEGFR2, bFGF, FGFR2, HGF, and c-MET genes in MMECs after exposure to zoledronic acid (10 and 30 μmol/L) alone or with mevastatin (Mev) at equimolar doses, as evaluated by RT-PCR. B, real-time RT-PCR analyses on the same genes in MMECs and EA.hy926. Columns, mean percentage of inhibition or stimulation compared with the baseline value; bars, SD. Significance of changes by the Wilcoxon-Wilcox test. C, effect of zoledronic acid alone or added with mevastatin on VEGF, bFGF, and HGF concentrations in MMEC conditioned media as measured by ELISA. Columns, mean; bars, SD. Significance of changes by the Wilcoxon-Wilcox test. D, protein levels of VEGFR2, FGFR2, and c-MET in MMEC lysates by Western blot analysis. The band intensity was expressed as fold expression of the medium value (untreated cells) by arbitrary absorbance (optical density).
RT-PCR, we found that zoledronic acid down-regulates VEGF165 and VEGFR2 expression maximally at 30 μmol/L (Fig. 4A). Real-time RT-PCR quantification showed a significant dose-dependent down-regulation of VEGF165 (−22% and −37% at 10 and 30 μmol/L, respectively; P < 0.005) and VEGFR2 (−32% and −45%; P < 0.01); no effect on bFGF, FGFR2 (Fig. 4B), and FGFR1/FGFR3/FGFR4 (Supplementary Fig. S3); and mild up-regulation of HGF (+13% and +25%; P < 0.05) but not of c-MET (Fig. 4B).

Parallel to the mRNA expression, Western blot showed that VEGFR2 protein content was reduced in MMEC lysates by zoledronic acid at 10 and 30 μmol/L, whereas FGFR2 and c-MET remained unchanged (Fig. 4D). ELISA of MMEC conditioned media displayed a significant dose-dependent reduction of VEGF by zoledronic acid, whereas bFGF and HGF were substantially unvaried (Fig. 4C). In addition, phosphoactivation of VEGFR2 and extracellular signal–regulated kinase 1/2 (downstream signaling transducers) was also dose-dependently inhibited by zoledronic acid at 10 and 30 μmol/L (Fig. 5), confirming at molecular level a specific inhibition of the VEGF165/VEGFR2 autocrine loop in MMECs.

Contrary to MMECs, in EA.hy926 cells a relevant down-regulation was observed for all angiogenic factors and VEGFR2 with 10 and 30 μmol/L (Fig. 4B): VEGF, −27% and −42%; bFGF, −42% and −64%; HGF, −19% and −27%; and VEGFR2, −36% and −48% (P < 0.03 or better). The divergent response to zoledronic acid between MMECs and EA.hy926 cells may be attributable to activation and/or acquisition of specific pathways due to a different origin of these endothelial cell types: EA.hy926 cells are hybrids between human umbilical vascular endothelial cells and a tumor cell line and may harbor different pathways from MMECs that are under the oncogene control.

Zoledronic Acid – Induced Inhibition of VEGF/VEGFR2 Autocrine Loop in MMECs Is Mediated by the Mevalonate Pathway

To examine whether the effect on VEGF/VEGFR2 expression was dependent on inhibition of farnesyl pyrophosphate synthase through the mevalonate pathway, a target of zoledronic acid (24), MMECs and EA.hy926 cells were exposed to zoledronic acid in the presence of mevastatin, which inhibits hydroxy-methylglutaryl CoA reductase, thereby indirectly preventing protein posttranslational modification (i.e., prenylation) required for the regulation of cell proliferation, cell survival, and cytoskeletal organization (25).

The inhibitory effect was almost completely abrogated by mevastatin at equimolar doses both at gene and protein levels (Fig. 4A–B and C–D, respectively). It is thus plausible that accumulation of unprenylated proteins may disrupt various downstream signaling pathways, including those implicated in the regulation of the VEGF/VEGFR2 expression.

Conclusion

This study adds new information on the zoledronic acid antiangiogenic activity. It inhibits bFGF-induced prolifera-

### Acknowledgments

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