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

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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 antiangiogenesis, 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 IIA ($n = 9$), IIIA ($n = 13$), and IIIB ($n = 2$); 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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Table 1. RT-PCR: primers, amplification, and products

Gene sequence of primers (5′–3′)	Amplification conditions	Product length (bp)
$VEGF_{165}$		
Forward: GCTGCACCCATGGCAGAAGG	30 cycles at 95°C 1 min and 30 s, 95°C	367
Reverse: GAGCAAGGCCCACAGGGATTT	1 min, 65°C 30 s, 72°C 30 s	
VEGFR2		
Forward: CCGTCAAGGGAAAGACTACG	35 cycles at 95°C 1 min and 30 s,	496
Reverse: CTTTACCCCAGGATATGGAG	95°C 30 s, 58°C 30 s, 72°C 1 min	
bFGF		
Forward: CTCACGTGGCACCAGTGGAT	35 cycles at 95°C 1 min and 30 s, 95°C	918
Reverse: CACAGAGGATGAATAGTAGC	1 min, 62°C 45 s, 72°C 1 min	
FGFR1		
Forward: TACCACCGACAAAGAGATGG	30 cycles at 94°C 3 min, 94°C 25 s, 58°C 25 s,	287
Reverse: CTGGCTGTGGAAGTCACTCT	72°C 25 s and 1 cycle at 72°C 5 min	
FGFR2		
Forward: TGGAGCGATCGCCTCACCG	28 cycles at 94°C 3 min, 94°C 25 s, 58°C 25 s,	352
Reverse: CTTCCAGGCGCTGGCAGAACTGT	72°C 25 s and 1 cycle at 72°C 5 min	
FGFR3		
Forward: CACCACCGACAAGGAGCTA	30 cycles at 94°C 3 min, 94°C 25 s, 58°C 25 s,	433
Reverse: GCTCGAGCTCGGAGACATT	72°C 25 s and 1 cycle at 72°C 5 min	
FGFR4		
Forward: GGGTCCTGCTGAGTGTGC	35 cycles at 94°C 3 min, 94°C 25 s, 58°C 25 s,	406
Reverse: GGGGTAACTGTGCCTATTCG	72°C 25 s and 1 cycle at 72°C 5 min	
HGF		
Forward: CTCAGATCAGTATCTAATTG	38 cycles at 95°C 1 min and 30 s, 95°C	1,375
Reverse: GACATGACTCTACCCTGTTC	1 min, 55°C 45 s, 72°C 1 min	
c-MET		
Forward: CTAGACACATTTCAATTGGT	30 cycles at 95°C 5 min, 94°C 30 s, 58°C 20 s, 72°C 45 s	364
Reverse: TGTTGCAGGGAAGGAGTGGT	and 1 cycle at 94°C 30 s, 58°C 20 s, 72°C 5 min	
GAPDH		
Forward: CCCTCCAAAATCAAGTGGGG	22 cycles at 95°C 1 min and 30 s, 95°C	347
Reverse: CGCCACAGTTTCCCGGAGGG	45 s, 60°C 45 s, 72°C 30 s	

europaeus-1 lectin (the receptor of which is highly and specifically expressed by endothelial cells), and transfer of beads with bound MMECs to plates in complete medium (RPMI 1640 supplemented with 10% heat-inactivated FCS and 1% glutamine) to allow cells to spread to the plate surface and grow.

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.

[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 3×10^3 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 microchamber 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 5 oil-immersion field/membrane and given as mean ± SD.

Matrigel Angiogenesis Assay. MMECs (2×10^5 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 ×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).

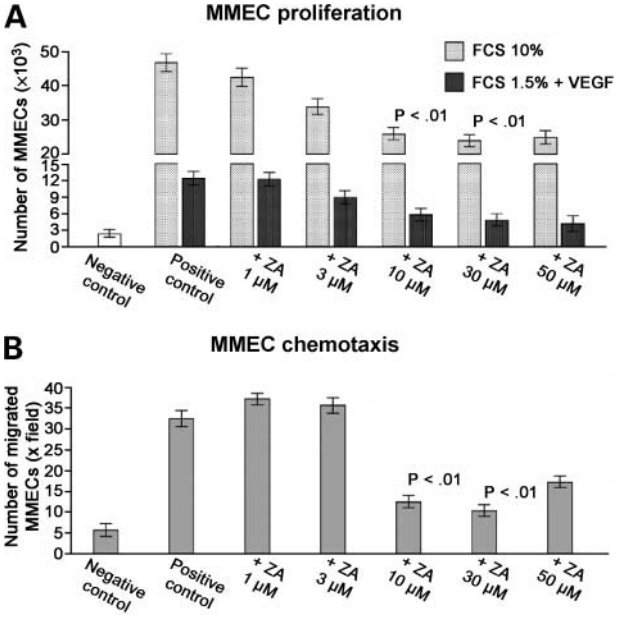

Figure 1. **A,** effect of different zoledronic acid (*ZA*) doses on 10% FCS– and VEGF-induced proliferation of MMECs. *Columns,* mean cell counts; *bars,* SD. **B,** effect of the same doses on VEGF-induced chemotaxis of MMECs. *Columns,* mean number of migrated cells per five high-power fields in triplicate wells; *bars,* SD. Significance of changes by the Wilcoxon-Wilcox test.

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 ΔC_t. The ΔC_t of the basal (medium) endothelial cells was then subtracted from the ΔC_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, FGF 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 $VEGF_{165}$ 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).[5]

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 $VEGF_{165}$ (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 $VEGF_{165}$ (9) as opposed to the selective activity on bFGF-dependent norm-angiogenic human umbilical vascular endothelial cells (11).

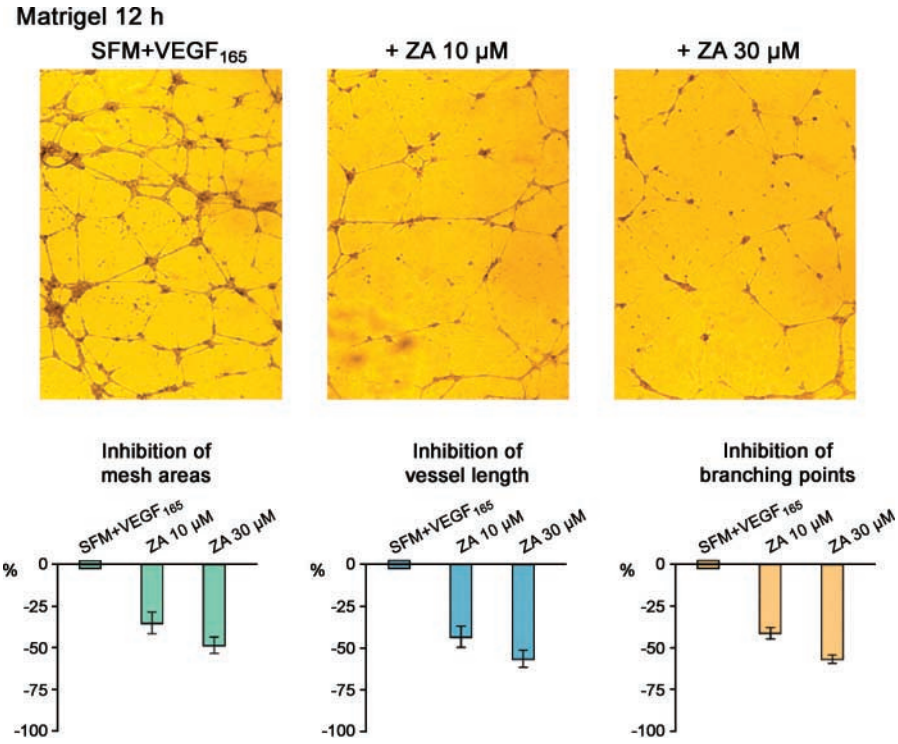

Figure 2. Capillarogenesis on Matrigel. MMECs of a representative patient were seeded on Matrigel in serum-free medium (*SFM*) supplemented with $VEGF_{165}$ alone and with the zoledronic acid doses. After a 12-h incubation, their three-dimensional organization was examined planimetrically by a computed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

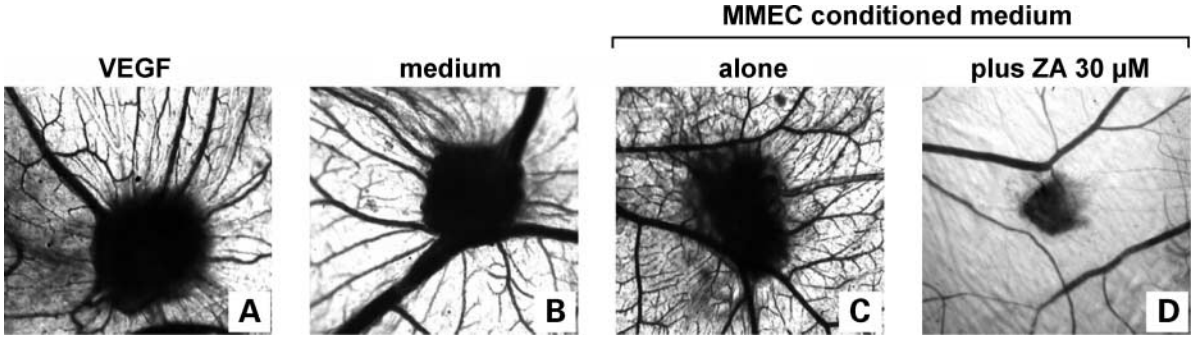

Figure 3. Chorioallantoic membranes treated with sponges loaded with $VEGF_{165}$ or MMEC conditioned media were surrounded by allantoic vessels developing radially toward the implant in a spoked-wheel pattern (**A** and **C**). No vascular response was detectable around the sponges loaded with vehicle (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**).

Inhibition of $VEGF_{165}$/VEGFR2 Autocrine Loop, But Not of bFGF/FGFR and HGF/c-MET Loops, in MMECs

The high sensitivity to zoledronic acid of $VEGF_{165}$-induced angiogenic functions prompted us to investigate whether the inhibition of $VEGF_{165}$/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

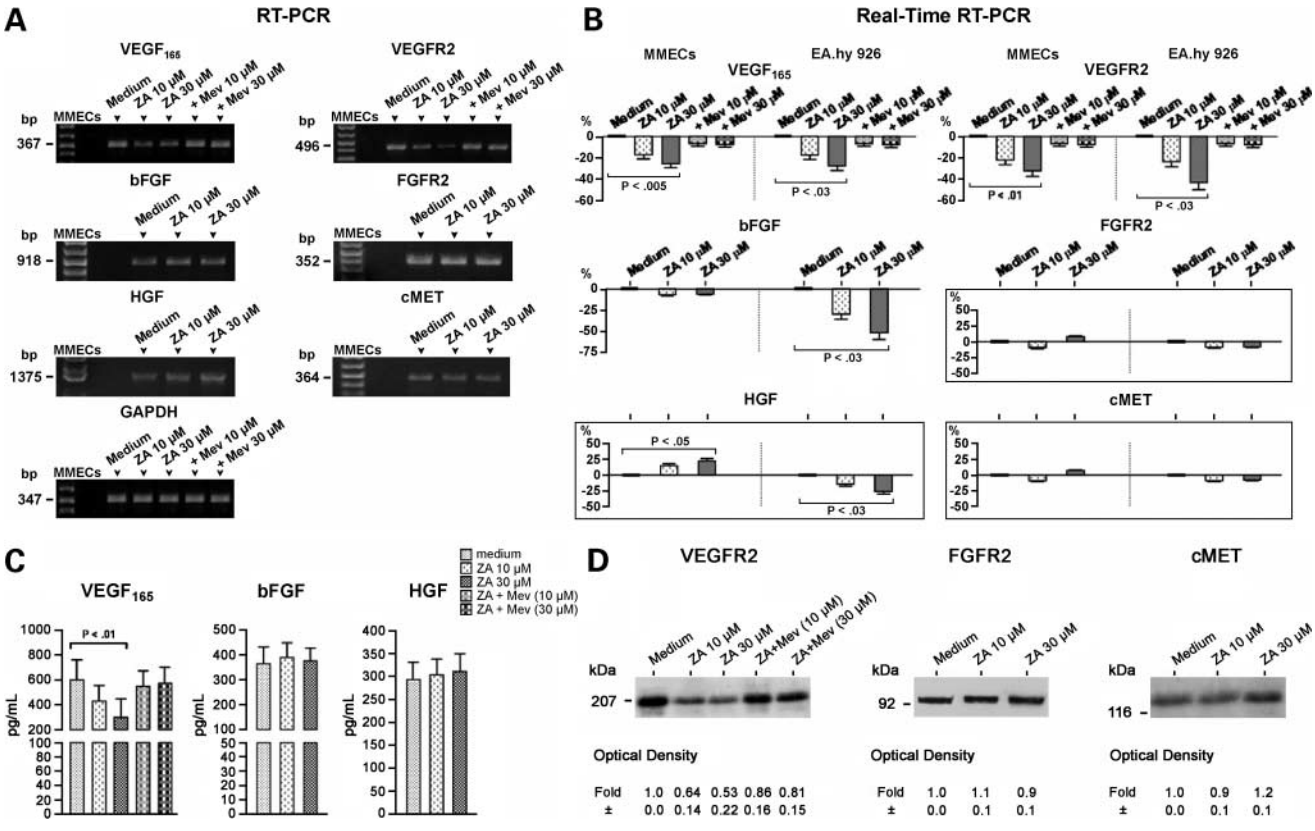

Figure 4. **A,** expression levels of *$VEGF_{165}$, VEGFR2, bFGF, FGFR2, HGF*, and *c-MET* genes in MMECs after exposure to zoledronic acid (10 and 30 μmol/L) alone or with mevastatin (*Mev*) at equimolar doses, as evaluated by RT-PCR. **B,** real-time RT-PCR analyses on the same genes in MMECs and 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 $VEGF_{165}$ and *VEGFR2* expression maximally at 30 μmol/L (Fig. 4A). Real-time RT-PCR quantification showed a significant dose-dependent down-regulation of $VEGF_{165}$ (−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);[5] 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 $VEGF_{165}$/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 proliferation (11), adhesion, migration, and survival of human umbilical vascular endothelial cells (26); decreases bone tumor–associated angiogenesis in the murine 5T2 multiple myeloma model (27); inhibits revascularization of the ventral prostate gland in castrated rats treated with testosterone (12); modulates $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (28); inhibits matrix metalloproteinase-2 and matrix metalloproteinase-9 (29); and modulates in cancer patients serum levels of proangiogenic growth factors including VEGF and bFGF (30). *In vivo*, zoledronic acid antiangiogenic activity may be also mediated via inhibition of osteoclasts, which actually produce angiogenic factors (31).

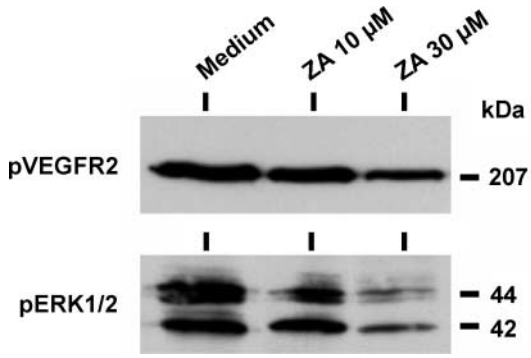

Figure 5. Inhibition of VEGFR2 and extracellular signal–regulated kinase (*ERK*)-1/2 constitutive phosphorylation by zoledronic acid in MMECs. Total cell lysates from untreated (medium) and zoledronic acid (10 and 30 μmol/L)–treated MMECs were probed with the indicated antibodies. MMECs starved overnight were treated with the zoledronic acid doses for 4 to 6 h in the presence of serum. Whole-cell lysates were analyzed by SDS-PAGE. Figure shows a dose-dependent inhibition of phosphoactivation of VEGFR2 and extracellular signal–regulated kinase 1/2.

Our results provide evidence of a direct antiangiogenic activity of zoledronic acid on endothelial cells of multiple myeloma patients due to (*a*) inhibition of $VEGF_{165}$-dependent proliferation, chemotaxis, and angiogenesis *in vitro*; (*b*) inhibition of the *in vivo* angiogenesis in the chick embryo chorioallantoic membrane-sponge assay; and (*c*) block of $VEGF_{165}$/VEGFR2 autocrine loop, mediated by the mevalonate pathway. Tentatively, we suggest that the zoledronic acid antitumoral activity in multiple myeloma is also sustained by antiangiogenesis.

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