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Antiangiogenic activity of zoledronic acid: inhibition of the VEGF-VEGFR-2 autocrine loop in the endothelial cells of patients with multiple myeloma

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Blood vessels are an important component of bone marrow microenvironment in multiple myeloma (MM). Their formation (angiogenesis) parallels the transition from monoclonal gammopathy unassociated/unattributable (MG[u]) to MM, or from remission MM to relapse and the leukemic phase.[1,2] The new vessels convey oxygen and metabolites, while endothelial cells (EC) at their tips secrete growth and invasive factors for plasma cells.[3]

The mechanisms that induce formation and sprouting of new vessels, however, are not well established yet. Plasma cells are seen as 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),[4-6] and their growth precedes the sprouting.[7] Stromal cells behave as secondary inducers following recruitment and activation by plasma cells. EC are themselves a vivid source of growth factors too.[8-12]

We have previously shown that bone marrow EC from patients with MM (MMEC) display a growth advantage over healthy human umbilical vein endothelial cells (HUVEC):[3] they secrete about 40 times larger amounts of VEGF into their culture medium, and express about 5 times higher levels of the cognate tyrosine kinase receptor VEGFR-2 (or kinase insert domain-containing gene KDR) suggesting the existence of a VEGF-dependent autocrine loop.

We have also shown the operativeness of this loop:[13] it mediates proliferation and capillarogenesis which are mandatory for the MM-associated angiogenesis. This loop exists in MMEC, but not MG(u)EC or HUVEC, and provides an amplification mechanism for the VEGF-driven angiogenesis in MM. Overall data support the view that efficacious antiangiogenesis could be achieved through VEGF-VEGFR-2 inhibition.

Zoledronic acid (ZA) is a bisphosphonate efficaciously used in MM for metastatic bone disease and hypercalcemia. Recent evidences indicate that it has a direct cytotoxic activity on tumor cells and suppresses angiogenesis,[14,15] but the associated molecular events have not been fully characterized. ZA inhibits the FCS-induced proliferation of HUVEC in a dose dependent manner (range 1-30 μM) and their capillary-like tubule formation on Matrigel *in vitro* (100 μM). Here, we have studied the antiangiogenic activity of ZA in MMEC of patients at diagnosis and compared it to that exerted on EA.hy926, used as control EC. We wondered to test the hypothesis that ZA directly targets the VEGF-dependent autocrine loop in MMEC.

MMEC from 8 patients were exposed on days 0, 2, 4, and 6 to both complete medium (10% fetal calf serum – FCS) and 1.5% FCS alone (positive controls) or added with ZA at different doses (1, 3, 10, 30, 50 μM), or to starvation serum-free medium (SFM - negative control). The EC proliferation rate was measured on day 8 by a colorimetric method.

ZA significantly inhibited proliferation at 3, 10, and 30 μM in a dose-dependent fashion: -25%, -45% and -52% of the positive control ($p<0.02$; Wilcoxon rank test), whereas 50 μM gave a plateau. ZA inhibited MMEC migration in a chemotaxis assay at 10 and 30 μM: -45%, and -61% of the positive control respectively ($p<0.01$; Wilcoxon rank test). The effect of ZA on capillarogenesis on the Matrigel surface was also investigated. After an 8-h incubation, unexposed MMEC gave a closely knit network whose filled areas were 27.5 ± 5.1, length 6314±708 μm, and branching points 48±6. In contrast, MMEC exposed to ZA 30 μM were less organized, and showed a lowering of all planimetric parameters, ranging from –38% to -58%. More evident inhibition was seen on mesh areas and vessel length (10±3 and 3110±290 μm respectively; $p<0.01$ or better; Student t-test for paired data).

By using RT-PCR and Real-Time RT-PCR we found that ZA down-regulates VEGF and VEGFR-2 expression in MMEC and EA.hy926, with maximum inhibition at 30

μM (-27% and –42%; $p< 0.05$ or better). The effect on MMEC seems to be specific addressed towards the VEGF- VEGFR-2 loop, since ZA does not produce any effect on the expression of both bFGF and its receptors FGFR-1/-2/-3/-4, and of HGF compared to EA.hy926, where it induces down-regulation of bFGF and HGF (-60% and –25%; $p<0.01$, respectively). Overall data provide a rationale for clinical employment of ZA in the antiangiogenic therapy of MM.

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