[haematologica reports]
2006;2(3):39-41

Zoledronic acid inhibit angiogenesis and impairs tumorigenesis in a mouse model of cervical carcinogenesis

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A B S T R A C T

The angiogenic phenotype has been characterized in a mouse model involving the human papillomavirus type-16 (HPV-16) oncogenes that develops cervical cancers via lesional stages analogous to those in humans. These studies revealed intense angiogenesis in high-grade cervical intraepithelial neoplasias (CIN-3) and carcinomas. Matrix metalloprotease-9 (MMP-9), a pro-angiogenic protease implicated in mobilization of VEGF, appeared in the stroma concomitant with the angiogenic switch, expressed by infiltrating macrophages, as occur in humans. Preclinical trials sought to target MMP-9 and angiogenesis with a prototypical MMP inhibitor and with a bisphosphonate, zoledronic acid (ZA), revealing both to be anti-angiogenic, producing comparable effects to a MMP-9 gene knockout in impairing angiogenic switching, progression of pre-malignant lesions, and tumor growth. ZA treatment increased neoplastic epithelial and endothelial cell apoptosis without affecting hyperproliferation, indicating ZA was not anti-mitotic. The analyses revealed cellular and molecular targets of ZA's actions: ZA suppressed MMP-9 expression by infiltrating macrophages, and inhibited metalloprotease activity, reducing association of VEGF with its receptor on angiogenic endothelial cells. Given its track record in clinical use with limited toxicity, ZA holds promise as an *unconventional* and safe MMP-9 inhibitor for antiangiogenic therapy of cervical cancer, and potentially for additional tumors and other diseases where MMP-9 expression by infiltrating macrophages is evident.

Main results and aims

In this report, we have used a mouse model of human cervical carcinogenesis, K14-HPV16/E_2, to study the mechanisms regulating angiogenesis and malignant progression, and to begin exploring possible anti-angiogenic therapies with the potential to prevent and/or regress cervical carcinoma.[1-3] Herein we have characterized the angiogenic phenotype in this mouse model of human cervical carcinogenesis, and evaluate anti-angiogenic therapy with an amino-bisphosphonate previously approved for use in treating bone metastases, to determine its possible utility for prevention and therapy of cervical cancer.[2]

Molecular and histological evaluation of cervical cancer progression in humans and in HPV/E2 mice presents striking parallels between the two pathways.[2] The HPV/E2 mouse model thus presents a platform for preclinical testing of drugs designed to interrupt critical pathways implicated in angiogenesis and lesional progression of cervical carcinoma.[2] Given the above data implicating MMP-9 in angiogenesis and progression, we became interested in assessing the effects of MMP inhibitors (MMP-I) on tumor growth and angiogenesis. Therapeutic trials in a transgenic mouse model of neuroendocrine cancer had previously demonstrated the capability of a prototypical MMP-I, BB-94, to inhibit angiogenesis and tumor growth when treatment was initiated during premalignant stages. Therefore we conducted analogous early stage trials targeting CIN-2/3 lesions with BB94, which produced reductions in the density of the angiogenic neovasculature (data not shown) and in tumor incidence and tumor growth/burden. While encouraged, we were aware that human clinical trials in various disease indications using BB94 and other active-site targeted MMP-I had failed, due to side effects and toxicity as well as poor efficacy against end stage tumors.[4] We were thus led to consider a much different agent, Zoledronic Acid (ZA, or Zometa), a nitrogen-containing bisphosphonate (N-BP) that is FDA-approved to reduce skeletal complications of bone metastasis in patients with multiple myeloma and several solid tumor types, with minimal side effects.[5,6] Recent studies in cell culture and xenotransplant tumor models have variously suggested that ZA and oth-

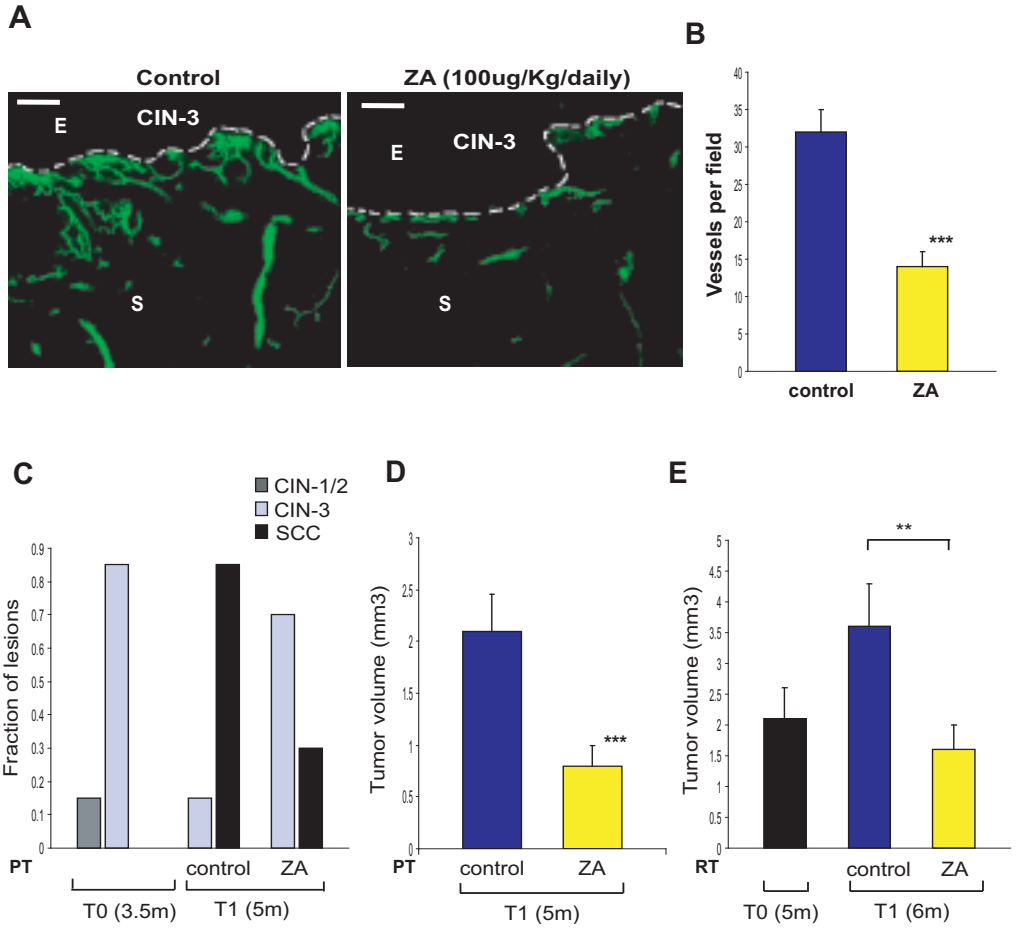

Figure 1. ZA inhibits angiogenesis and reduces tumor incidence and growth. (A) Perfusion of HPV/E2 control mice and ZA-treated mice (6 weeks of treatment, PT) with fluorescin-lectin revealed dramatic changes in the three-dimensional organization of the vasculature proximal to CIN-3 lesions of treated mice at 5 months of age. (B) Vessels density, as assessed by MECA-32 immunostaining, was significantly reduced in ZA treated mice as compared to controls (56% reduction, ***p<0.001). Results are mean ± s.e.m. of five fields per mouse from a total of 8 mice. (C) CIN-2/3 lesion-bearing mice (T0, 3.5 month old) treated with ZA or vehicle for 6 weeks (PT) showed a 55% reduction in the tumor incidence at the end of the treatment T1 (5 month old) (n=16 control, n=8 ZA treated). (D) Tumor volume of ZA treated mice was reduced by 61% compared to untreated controls (PT, ***p<0.001, n=16 control, n=8 ZA treated). (E) Mice bearing SCC (5 month old, T0) treated with ZA for 1 month (T1) in a RT showed a 57% decrease in tumor volume (p<0.01, n=15 control, n=10 ZA treated). Values are mean ± s.e.m. p values were calculated using the Wilcoxon test. Tumor volume and histological scores were determined as described in the methods. "E" denotes epithelium and "S" stroma; "PT" indicates a Prevention Trial and "RT" a Regression Trial. The scale bar corresponds to 50 μm.**

er bisphosphonates have anti-angiogenic and MMP-inhibitory activity This data led us to test ZA on cervical neoplasias in HPV/E2 mice.[7,8]

We treated female mice bearing CIN-3 lesions with ZA (100 μg/kg, daily, subcutaneously) or with vehicle alone for 6 weeks, asking whether ZA could prevent tumor formation and/or affect angiogenesis (in a 'Prevention Trial', or PT). Although ZA is inoculated intravenously on a monthly regimen in humans, a number of preclinical studies suggested that it was best supplied in a daily regimen in mice, reflecting much different pharmacokinetics. Fluorescin-lectin perfusion and immunostaining with Meca-32 antibody revealed a decrease by 56% in the number of blood vessels proximal to the basement membrane in dysplastic lesions and cervical carcinomas from ZA-treated mice compared to analogous lesions in controls (Figure 1A and B). At the defined endpoint (5 months of age, 5 m), the control mice had a tumor incidence of 85% (Figure 1C). Remarkably, ZA was able to limit the tumor incidence to 30% (Figure 1C), and reduce tumor volume by 61% (Figure 1D). Next, to assess the effects of ZA on the growth of established cervical carcinomas, we performed a *Regression Trial* (RT) treating the mice with ZA for 1 month starting at 5 months of age, when 85% had malignant tumors, and the remainder CIN-3 lesions. One month later the control mice had a tumor incidence of 90% and increased tumor volume (Figure

1E). In contrast, the ZA regimen impaired growth of the invasive carcinomas, reducing tumor volume by 57% from that at the start (Figure 1E). Thus ZA was able to impact both premalignant and malignant stages of cervical carcinogenesis, reducing angiogenesis both in high-grade dysplasias and cancers, interfering with progression to and subsequent growth of invasive tumors.[2]

Discussion and Conclusions

We have analyzed the angiogenic phenotype and assessed a novel anti-angiogenic therapy in a mouse model of cervical carcinogenesis that is driven by the etiologic viral oncogenes, faithfully recapitulating the lesional stages of the human disease. Having identified MMP-9 and its macrophage source as functional contributors to angiogenesis and tumor progression, we evaluated a targeted therapy involving a clinically approved drug, the bisphosphonate Zoledronic Acid, which demonstrated promising efficacy against both premalignant lesions and cancers of the cervix. In the course of a Prevention Trial, ZA inhibited angiogenesis and limited the progression of pre-malignant precursors to invasive carcinomas of the cervix, suggesting that ZA (or better yet an orally bioavailable derivative) might be considered for adjuvant treatment following excision of CIN-3 lesions for women at high risk for recurrence and progression. We have also shown that ZA is able to interfere with the growth of established cervical carcinomas, producing 'partial responses' as a monotherapy. Perhaps ZA could be added to the standard of care for cervical cancer,[9] to assess its potential for improving efficacy by inhibiting angiogenesis. Furthermore, given that we have identified molecular and cellular targets for ZA, thereby increasing the body of evidence that MMP-9 and *tumor-enhancing macrophages* are functionally important for angiogenesis and carcinogenesis in a variety of organs,[10,11] it may be reasonable to consider incorporating ZA into conventional therapeutic regimens for additional tumor types, and indeed other diseases with an angiogenic component where these bio-markers are present. In that regard, it may be pertinent to consider more frequent dosing than the current monthly schedule, which was based on ameliorating pain associated with bone metastasis and not on dose limiting toxicity or clinical anti-neoplastic efficacy; instead, perhaps suppression of MMP-9 expression in tissue-infiltrating macrophages (or imaging of its protease activity in the target tissue) may be an appropriate biomarker for optimizing an efficacious antiangiogenic dosing regimen.

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