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Differential effect of zoledronic acid on human vascular smooth muscle cells

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ABSTRACT

Introduction: The activation of human vascular smooth muscle cell proliferation, adhesion and migration is essential for intimal hyperplasia formation. These experiments were designed to test whether zoledronic acid (ZA) would modulate indices of human smooth muscle cell activation, exert differential effects on proliferating *versus* quiescent cells, and determine whether these effects were dependent on GTPase binding proteins prenylation. ZA was chosen for testing in these experiments because it is clinically used in humans with cancer, and has been shown to modulate rat smooth muscle cell proliferation and migration.

Methods: Human aortic smooth muscle cells (HASMC) were cultured under either proliferating or growth arrest (quiescent) conditions in the presence or absence of ZA for 48 hours, whereupon the effect of ZA on HASMC proliferation, cellular viability, metabolic activity, and membrane integrity were compared. In addition, the effect of ZA on adhesion and migration were assessed in proliferating cells. The effect of increased concentration of ZA on the mevalonate pathway and genomic/cellular stress related poly-adenosine diphosphate ribose polymerase enzyme activity were assessed using the relative prenylation of Rap-1A/B protein and the formation of poly adenosine diphosphate-ribosylated protein, respectively.

Results: There was a dose dependent inhibition of cellular proliferation, adhesion and migration following ZA treatment. ZA treatment decreased indices of cellular viability and significantly increased membrane injury in proliferating *versus* quiescent cells. This was correlated with the appearance of unprenylated Rap-1A protein and dose dependent down regulation of activity.

Conclusions: These data suggest that ZA is effective in inhibiting HASMC proliferation, adhesion, and migration, which coincide with the appearance of unprenylated RAP-1A/B protein, thereby suggesting that the mevalonate pathway may play a role in the inhibition of HASMC activation.

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1. Introduction

The incidence of peripheral vascular disease continues to increase among our aging population as the risk factors such as diabetes, obesity, and hyperlipidemia continue to rise [1]. The development of surgical- and endovascular-based therapies for peripheral vascular disease has been life-saving, with increased limb-salvage and decreased disability, and represents an important achievement in medicine [2,3]. Despite massive global research efforts, including the development of adjunctive therapies and mechanical techniques, 30%–40% of patients develop restenosis within 3 to 24 mo of intervention [4]. The major processes involved in the development of restenosis are complex and include responses to injury and inflammation [5].

Animal models have shown that bisphosphonates (BP), which are typically used to treat conditions associated with excessive bone resorption, may play an inhibitory role in the development of atherosclerosis and neointimal hyperplasia [6–9]. There are also reports of marked BP accumulation in both the healthy aorta and atherosclerotic aorta in rabbits [10,11]. zoledronic acid (ZA), which is the most potent member of the nitrogen containing BP [12], is currently used in the treatment of osteoporosis and it is being tested in the treatment of bone metastasis in clinical trials [13–15]. Recent *in vitro* studies have demonstrated ZA to inhibit proliferation, adhesion and migration of vascular smooth muscle cells derived from rats [16]. However, a similar role in human cells has not been shown. These experiments were performed because the effects of drugs on animal tissue do not always correlate with similar effects on human tissue [17–19].

The aim of the present study was to verify whether ZA would sustain an inhibitory effect on activated human vascular smooth muscle cell proliferation, adhesion, and migration, which are essential components in the pathogenesis of atherosclerosis and intimal hyperplasia following vascular injury in humans. Experiments were also designed to determine whether ZA exerts distinct effects on growth induced proliferating human aortic smooth muscle cells (HASMC) viability, metabolic, and stress related activities compared with non-induced quiescent cells. BPs are known to modulate the prenylation of GTPase binding proteins of the Ras superfamily, which play a role in several cellular activities including adhesion, growth and survival [20,21]. Therefore, we investigated whether ZA treatment would alter the post-translational modification of selected members of the Ras superfamily GTPase binding proteins. Additionally, we tested the effect of ZA on PARP enzyme activity, which is an important modulator of cellular stress and smooth muscle cell cellular phenotypic alteration, proliferation, and inflammation [5,22–24].

2. Materials and methods

2.1. Cell culture

Human aortic smooth muscle cells (HASMC; Invitrogen Co., Carlsbad, CA, passage 6-7) were serially grown in medium-231, smooth muscle growth supplement (Invitrogen Co.) containing 100 units/mL penicillin, 0.01 mg/mL streptomycin, 0.25 μg/mL amphothericin-B, 5% fetal bovine serum, recombinant human basic fibroblast growth factor, recombinant human epidermal growth factor and insulin [growth medium, (GM)]. For experiments performed under growth arrest conditions, cells with were exposed to quiescent medium (QM) consisting of medium-231 containing 0.5% fetal bovine serum for 48 h. ZA (Novartis, Basel, Switzerland) was diluted in the either GM or quiescent medium prior to each experiment to concentrations of 1, 10, and 100 μM and added to cells in wells or tissue culture flasks.

2.2. Cellular proliferation

HASMC cultured in 6-well plates (1×10^5 cells per well) were subjected to growth arrest using QM. After an initial 48 h period, some of the wells were supplemented with 1, 10, or 100 μM of ZA. In separate wells, medium was exchanged to GM, for proliferating cells, and similar concentrations of ZA were added. The control group was identical to the experimental group except that ZA was not added. After 48 h incubation, cells were suspended using trypsin/EDTA mixture wherein viable cells were counted with hemocytometer using trypan blue exclusion. Data was expressed as average cells per well $\times 10^6 \pm$ SEM. Each analysis was repeated six times.

2.3. Cellular adhesion

To determine the effect of ZA on cellular adhesion, subconfluent HASMC were divided into 1.0×10^4 HASMC aliquots and incubated in GM containing 1, 10, or 100 μM ZA for 30 min. Cells were subsequently seeded on to type I collagen coated 24 well plates (BD Biosciences, Bedford, MA) containing the same type of media and incubated for 30 min at 37°C in a 5% CO_2 tissue culture incubator. Wells without ZA served as controls for the experiment. Following the 30-min incubation period, the wells were rinsed vigorously three times with phosphate buffered saline (PBS), and the remaining cells in the well were stained using 0.1% crystal violet dye dissolved in ethyl alcohol and counted in six fields in each eight independent wells, as previously described [16]. Data were expressed as adherent cells per high power field (hpf).

2.4. Cellular migration

Cellular migration was measured using a 24 transwell tissue culture plates (Corning Corporation, Corning, NY). HASMC were suspended by 0.05% Trypsin-EDTA in serum-free medium supplemented with 0.2% bovine serum albumin containing 0, 1, 10, or 100 μM ZA. Cell suspensions were placed into the upper well at a concentration of 104 cells/100 μL. The designated lower compartments were filled with 600 μL total volume of GM containing 0, 1, 10, or 100 μM ZA each. Following 4 h incubation, the non-migrating cells on the upper surface were scraped and washed out three times with PBS. Migrating cells that made it through the membrane on the lower surface were fixed and stained with 0.1% crystal violet dye in ethanol