DARU Vol. 15, No. 1 2007

Benidipine hydrochloride increases calcified nodule formation in the bovine aortic smooth muscle cell cultures

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Received 21 Feb 2006; Revised 22 Aug 2006; Accepted 24 Aug 2006

ABSTRACT

Arterial calcification, a regulated process similar to ossification in bone, is common in atherosclerosis. A subpopulation of bovine aortic media cells, have osteoblastic characteristics and form spontaneously mineralized nodules in vitro. To assess whether Benidipine Hydrochloride as a potent calcium-channel blocker modulates arterial calcification, the effect of this drug on bovine vascular aortic smooth muscle cells for formation of calcified nodules and alkaline phosphatase activity in the culture medium was determined. These cells were obtained from bovine thoracic aorta, treated with different concentrations (0, 0.01, 0.1, 1, 10 nmol/L) of Benidipine Hydrochloride. Twenty-one days of treatment in comparison with control cells resulted in a significant increase in number of calcified nodules visualized by von Kossa staining, as well as by increase in alkaline phosphatase activity, a marker for osteoblastic differentiation and decrease in cell number in a dose dependent manner, compared with control cells. These results indicate that treatment with Benidipine Hydrochloride treatment in long term may contributes to vascular calcification.

Keywords: Atherosclerosis**,** Benidipine hydrochloride, Vascular calcification

INTRODUCTION

Calcification of vascular tissue is a common finding in atherosclerosis. It may increases vessel wall rigidity (1), augments plaque brittleness(2) and leads to increased plaque rupture or vessel wall dissection following balloon angioplasty and/or stent placement(3). Calcified arterial walls are unable to dilate or contract in response to physiological stimuli, resulting in regulatory disturbance of blood flow (4). Arterial calcification was once thought to be a passive process that occurs in response to tissue injury. Evidences suggest that it is an active, cell-controlled, progressive, organized and regulated process similar to ossification in bone(5) which begins quite early in life and increases with age at a rate that is roughly commensurate with the rate that atherosclerosis develops and precedes to arterial narrowing (6). Systemic hypertension is a major risk factor for cardiovascular morbidity in the general population (7). Recent studies have demonstrated an association between hypertension and atherosclerosis (8) with abnormalities in calcium metabolism. (9). One of drugs used for treatment of hypertension is Benidipine Hydrochloride (BD). Which is a potent calcium-channel blocking agent that exhibits strong antihypertensive effects (10). Recently, researchers have found that BD, increases alkaline phosphatase (ALP) activity and stimulates mineral deposition of osteoblastic cells which were isolated from neonatal mouse calvaria (11). It has been shown that BD in vitro as well as in vivo exerts antiproliferative effects on vascular smooth muscle cells (VSMCs) (12,13).

Additionally, it has been reported that benidipine reduced neointimal formation in a rat balloon-injury model (12).Considering these findings and the fact that vascular smooth muscle cells (VSMCs) and osteoblasts share a common embryonic mesenchymal derivation(14) and a subpopulation of VSMCs are capable of expressing osteoblastic specific genes to form in vitro mineralized nodules spontaneously (15). The potential of BD to increase in vitro osteoblast differentiation of bovine vascular aortic smooth muscle cells (BASMCs) to form more in vitro calcified nodules was examined in this study. Alkaline phosphatase (ALP), which is critical for calcification (16,17) was used as a marker for osteoblastic differentiation.

MATERIALS AND METHODS

Reagents

BD was purchased from Chemexper.com. DMEM, ice-cold PBS, type 1 collagenase were purchased from Gibco. Unless otherwise mentioned, all other reagents were obtained from Sigma.

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Tissue Culture

Bovine thoracic aortic media cells were cultured from explants sectioned from luminal face of aortic media (13,14). Small sections were obtained from bovine thoracic aorta. Tissue was rinsed in serum-free medium, dissected free of adventitia under the loop microscope and placed in 0.2% type 1 collagenase in a 1:1 mix of medium and PBS for 30 min at 37° C. Under the loop microscope, endothelium was gently removed and medial layer was isolated in ice-cold PBS. BASMCs were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL), sodium pyruvate (1 mmol/L), streptomycin (100 U/mL), fungizone (0.25 µg/mL), L-glutamine (2 mmol/L), sodium beta-glycerophosphate (10 mM) and ascorbic acid (50µg/ml) and by HEPES buffer (25 mmol/L) adjusted to pH 7.25. Cell clones were trypsinized before formation of nodules, plated in 12-well tissue culture dishes at a density of 16000 cells/cm^2, and were grown for 21 days. Twenty-four hours after seeding, different concentrations of BD (0, 0.01, 0.1, 1, 10 nmol/L) were added to the cultures (18). The plates with cells were incubated at 37 °C, and 5% CO_2 for 21 days. Growth medium was changed every other day. Each experiment was repeated six times in each group.

Culture Examination

Cultures were examined daily using phase contrast microscopy. Some cultures were fixed in 10% neutral buffered formalin and stained with cresyl violet.

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Mineralization assay

The specimens after 21 days of growth were stained for mineral deposition by Von Kossa method (19). Briefly Cell layers were fixed with 10% neutral buffered formalin for 1h, incubated with 2% silver nitrate solution for 10 min in dark, washed thoroughly with deionized water and exposed to bright light for 15 min. Calcium mineral was stained as black. Calcified nodules in each well were counted twice by the use of phase-contrast microscopy (20).

ALP activity assay

The cultures were fixed in cold (4˚C), 10% neutral buffered formalin after 14 days of growth, and incubated for 10 min with sodium-alpha-naphthyl phophate in "Tris" buffer, pH 10 in the presence of Fast Red Violet LB salt. During incubation, the cultures were protected from drying and direct light. The stained cultures were rinsed with deionized water, air-dried, and were then examined by phase contrast optics (20).For quantification of ALP, the cells at day of 14 were washed three times with PBS, lysed with 1% Triton X-100, and then 2 µl of the cell lysate incubated with 200 µl of ALP reagent. Quantitative kinetic determination of cell associated ALP activity was then determined at 30°C by monitoring absorbance at 405 nm on a microplate reader. One unit was defined as the activity producing 1 nmol of *p*-nitrophenol for 30 minutes (21).

Statistical analysis

Data are expressed as mean ± Standard Deviation (SD). Significance of treatment- mediated differences was determined by Student's *t*-test and *P* value of <0.05 was considered significant.

RESULTS

Morphology of BVSMCs

These cells, which most of them were polygonal, attached to the culture dishes at the day of 3 (Fig1) and formed cell colonies at the day of 6.

Effects of BD on the cell growth

The cells at the day of 14 were dispersed with trypsin 0.1% in 2 mmol/L EDTA in PBS for 10 minutes at 37°C. The cells were counted by hemocytometer. BD (0.01- 10 nM) dose-dependently decreased cell proliferation of BVSMCs per well at the day of 14 compared with untreated wells. The number of cells in control cultures was 1.5-fold greater than that of BD treated cultures at 10 nM (Fig 2).

Effects of BD on the ALP activity

ALP activity was observed in the cell colonies at the day of 14 (Fig3). BD was effective in a concentration-dependent manner (0.01- 10 nM) in the way that at the day of 14 the ALP activity at 10nM was about 4-fold greater than that of control cultures. (Fig 4).

Effects of BD on the number of calcified nodules

At the day of 21, cell colonies were stained with Von Kossa stain which represented mineralized nodules(Fig5).BD (0.01-10 nM) dose-dependently increased the number of calcified nodules per well at the day of 21 compared with untreated wells (Fig 6). BASMCs, as control cells, did not form any or formed only rare calcified nodules in this time period.

DISCUSSION

In the present study it is shown that BD dose-dependently increases ALP activity and the number of calcified nodules per well after 21 days of cell culture compared with untreated wells. Conversely it dose-dependently decreased cell

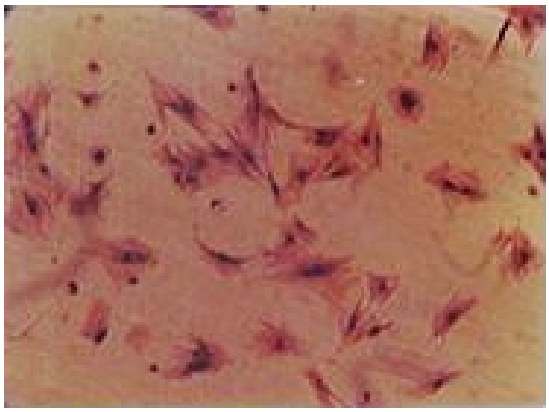

Figure1. Cresyl violet staining of BVSMCs before forming the colonies(day 3, ×400).

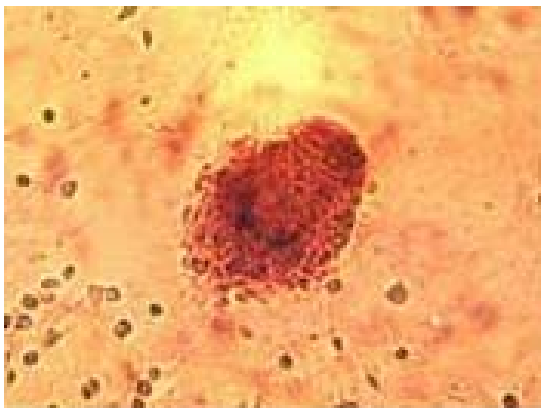

Figure 3. ALP activity determined in one cell colony (day 14, ×100).

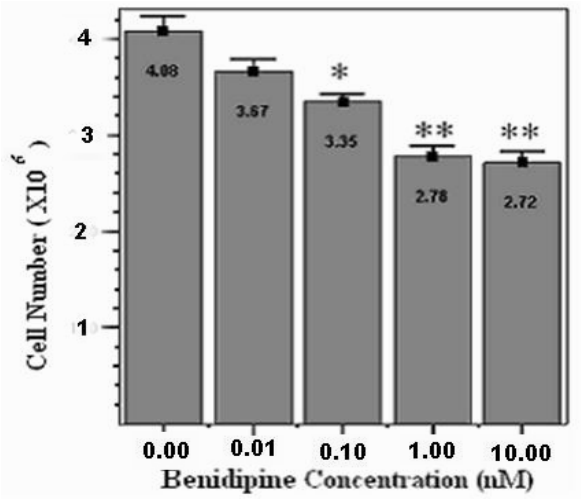

Figure 2. Effects of BD on cell growth in BVSMCs. The number of cells per culture well was followed in 14 days treatment with BD. The number of cells in control cultures was 1.5-fold greater than that of BD treated cultures at 10 nM(*P<0.5 , **P<0.1).Values represent the mean +- S.E. (n=6 wells)

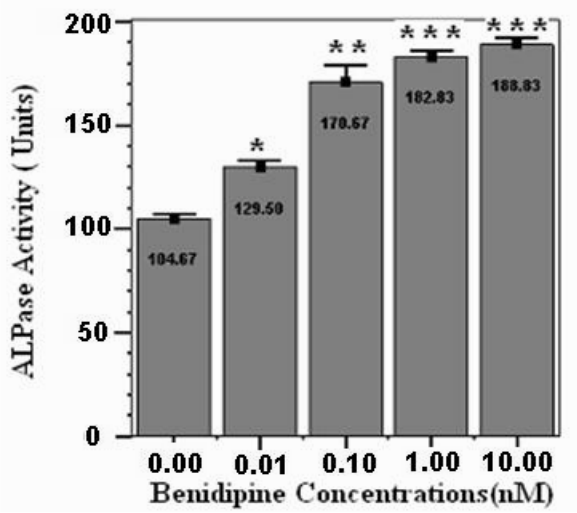

Figure 4. Effect of BD on ALPase activity in BVSMCs. Alpase activity of BASMCs per well at the day 14 after treatment with BD compaired with untreated wells. ALP activity at 10nM was about 4-fold greater than that of control cultures(*P<0.05 **P<0.01 ***P<0.001) . Values represent the mean± S.E. (n=6 wells).

proliferation of BVSMCs per well at the day of 14. Previously it has been shown that BD exerts antiproliferative effects on VSMCs in vivo as well as in vitro (12) because it increases the protein level of p21, a negative regulator of the cell cycle. The over-expression of p21 may directly inhibit the activities of cyclin D /CDK complexes and PCNA in vitro (22). Adenovirus-mediated over-expression of p21 inhibits proliferative responses to growth factors in cultured VSMCs and neointimal formation in the rat balloon-injury model (23,24). It has been reported that 1 nM of BD, increases ALP activity of osteoblastic cells derived from mouse calvaria (11).Furthermore, it is reported that cell differentiation is often accompanied with expression of p21 (25). These data indicate that BD may inhibit cell cycle progression by its anti-proliferative action, leading to differentiation of VSMCs. Identification of the molecular target of BD is an important issue for future investigation. The opposite effect of BD on ALP activity and cell number is usually exhibited between the differentiation and proliferation of various cells, including osteoblasts. Some agents inhibiting the proliferation of osteoblast-lineage cells, can

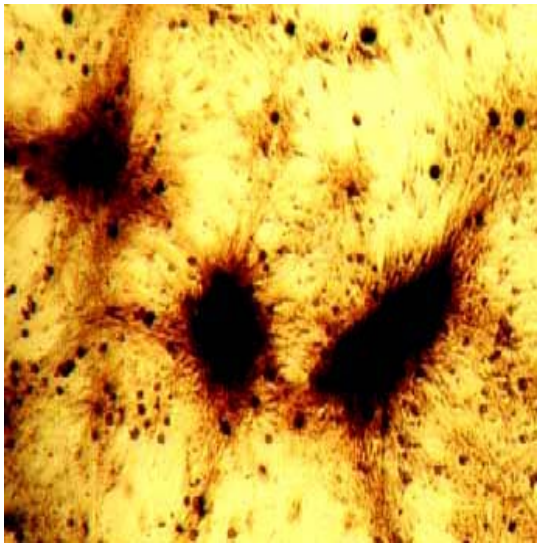

Figure 5. Von Kossa staining of BD–treated cell clonies at the day of 21 (x 50).

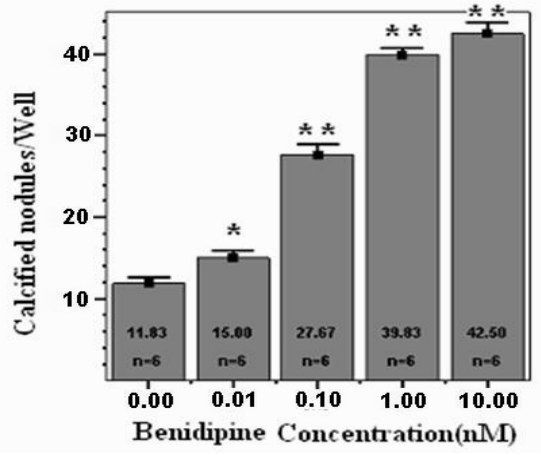

Figure 6. Effect of BD on formation of mineralized nodules in BVSMCs. The number of mineralized nodules per culture well after 21 days of treatment with BD. BD (0.01-10 nM) dose-dependently increased the number of calcified nodules per well at the day of 21 compared with untreated wells(*P<0.05 ** P< 0.001). Each bar represents the mean of six wells.

induce osteoblastic differentiation (26) . It is therefore possible that antiproliferative effect of BD on BASMCs may induce osteoblastic differentiation under certain conditions. A variety of factors, including interleukin 1-β, tumor necrosis factor-α, and bone morphogenetic proteins (BMP) have been implicated in osteoblastogenesis (27). It is possible that BD alters the expression of one or more of these cytokines. Alternatively, BD may binds to critical growth factors, such as basic-fibroblast growth factor (bFGF), which could potentially regulates both osteoblastic activity and differentiation. It is well documented that osteoblasts have L-type calcium channel (28). This channel provides an important pathway for entry of Ca^{+2} into vascular and cardiac muscles (29) and is activated by numerous factors like vitamin D3 (30). Therefore, they have a role in regulation of osteoblast function. It is also reported that level of expression of ALP was correlated with that of the L-type calcium channel (31). Therefore another possible mechanism of BD as a calcium channel blocker in enhancement of osteoblast differentiation is that the drug prevents Ca^{+2} entry induced by some factors that are inhibitory to differentiation. These factors may be produced by osteoblasts. Furthermore this drug decreases the basal concentration of intracellular Ca^{+2} (32). Because of regulation of many cellular functions by intracellular Ca^{+2} ion as a second messenger (33), intracellular Ca^{+2} levels may regulates osteoblast differentiation. It is necessary to elucidate the role of the calcium channel in regulation of osteoblast differentiation in future investigation.

CONCLUSIONS

The results of this investigation indicate that in vitro spontaneous formation of calcified nodules from the bovine aortic media is increased in response to treatment with BD.

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