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The effect of nitrogen containing bisphosphonates, zoledronate and alendronate, on the production of pro-angiogenic factors by osteoblastic cells

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ABSTRACT

Bisphosphonates (BPs) have been shown to influence angiogenesis. This may contribute to BP-associated side-effects such as osteonecrosis of the jaw (ONJ) or atypical femoral fractures (AFF). The effect of BPs on the production of angiogenic factors by osteoblasts is unclear. The aims were to investigate the effect of (1) alendronate on circulating angiogenic factors; vascular endothelial growth factor (VEGF) and angiopoietin-1 (ANG-1) *in vivo* and (2) zoledronate and alendronate on the production of VEGF and ANG-1 by osteoblasts *in vitro*. We studied 18 post-menopausal women with T score $\leqslant -2$ randomized to calcium/vitamin D only (control arm, $n = 8$) or calcium/vitamin D and alendronate 70 mg weekly (treatment arm, $n = 10$). Circulating concentrations of VEGF and ANG-1 were measured at baseline, 3, 6 and 12 months. Two human osteoblastic cell lines (MG-63 and HCC1) and a murine osteocytic cell line (MLO-Y4) were treated with zoledronate or alendronate at concentrations of 10^{-12}–10^{-6} M. VEGF and ANG-1 were measured in the cell culture supernatant. We observed a trend towards a decline in VEGF and ANG-1 at 6 and 12 months following treatment with alendronate ($p = 0.08$). Production of VEGF and ANG-1 by the MG-63 and HCC1 cells decreased significantly by 34–39% ($p < 0.01$) following treatment with zoledronate (10^{-9}–10^{-6} M). Treatment of the MG-63 cells with alendronate (10^{-7} and 10^{-6}) led to a smaller decrease (25–28%) in VEGF ($p < 0.05$). Zoledronate (10^{-10}–10^{-6} M) suppressed the production of ANG-1 by MG-63 cells with a decrease of 43–49% ($p < 0.01$). Co-treatment with calcitriol (10^{-8} M) partially reversed this zoledronate-induced inhibition. BPs suppress osteoblastic production of angiogenic factors. This may explain, in part, the pathogenesis of the BP-associated side-effects.

1. Introduction

Skeletal remodelling is a physiological process which occurs throughout adult life within the basic multicellular unit (BMU) [1]. In the BMU, osteoblasts and osteoclasts are in close proximity with endothelial cells of the vasculature. Studies have indicated that bone remodelling occurs in a highly vascular closed compartment termed the bone remodelling compartment (BRC) [2,3]. Angiogenesis plays an essential part in the functioning of the BMU within the BRC as it supplies nutrients and oxygen and enables transport of osteoprogenitors and osteoclasts to the BRC [4].

The expression and production of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) and angiopoietin (ANG) which affect endothelial cell growth, migration and vessel formation in many tissues also play an important role in the regulation of vascular growth in the skeleton [4,5]. In bone, all these factors have been detected at sites of bone remodelling confirming the role of vascular growth within the remodelling unit [6]. These factors are also produced by bone cells including osteoblasts and osteocytes [6,7]. Stimuli for their release include bone-derived cytokines and hypoxia, simulating bone injury [8]. ANG-1 has also been shown to be expressed by osteoblasts and osteoclasts in remodelling bone [6]. Following VEGF-mediated production of immature blood vessels, ANG-1 recruits surrounding mesenchymal cells via tyrosine kinase Tie 2 receptor signalling, and promotes the differentiation of cells into vascular smooth muscle cells [9]. Besides its angiogenic role, VEGF may also act directly on bone

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cells. The Wnt/β-catenin signalling has been implicated in the regulation of osteoblastic VEGF-A gene transcription. The VEGF gene promoter contains 7 binding regions for β catenin/Tcf, suggesting basal VEGF secretion in mineralised tissue may be part of the osteoblast's normal physiological role [10]. *In vitro* studies suggest that VEGF may be an effective regulator of osteoblast survival via autocrine feedback [5]. VEGF has also been shown to behave as a chemoattractant for osteoclastic cells [11]. It is unclear whether ANG-1 has direct actions on bone cells, although osteoblast-specific ANG-1 overexpression has been shown to lead to increased bone mass [12].

Bisphosphonates (BPs) are widely recognised as standard treatment in disorders of bone remodelling including post-menopausal osteoporosis (PMO), Paget's disease and metastatic bone disease (MBD) [13]. Differences in potency occur among the N-containing BPs with zoledronate being the most potent, followed by alendronate [13]. BPs inhibit osteoclastogenesis and osteoclast activity and also promote osteoclast apoptosis. Their main cellular mechanism of action is through disruption of the cytoskeleton by inhibition of the mevalonate pathway [14]. Their actions is not limited to osteoclasts as there is evidence that BPs can also affect other cells in the bone micro-environment including osteoblasts and osteocytes, although data are conflicting [15]. BPs have been shown in *in vitro* studies to stimulate osteoblast proliferation, differentiation and inhibit apoptosis, although negative effects on osteoblasts have also been reported [16]. One of the explanations for the discrepancy in the findings may be due to the dose-dependent differential effects of BPs on cells from the osteoblastic lineage. It has been reported that in contrast to their anti-osteoclastic effects, lower concentrations of BPs may be sufficient for their effects on osteoblasts [16]. Lower concentrations of BPs have been shown to exert beneficial effects on osteoblasts whilst amino-BPs induce osteoblast apoptosis and inhibit cell differentiation at high concentrations, 10^{-5} M or higher, although this needs further clarification [17].

Reports in recent years have raised concerns about two potential complications following long-term treatment with BPs; osteonecrosis of the jaw (ONJ) and atypical femoral fractures (AFF) [18,19]. Estimates of cumulative incidence suggest that ONJ occur in between 1% and 10% in oncology patients receiving high doses of BPs intravenously [20]. The estimated incidence in patients given oral BPs at doses used for osteoporosis is lower at less than 1 in 100,000 patient treatment years [18]. In contrast, the incidence of AFF is not established [21]. A dose–response relationship has not been documented but the risk appears related to duration of treatment. AFF has been described following treatment with alendronate but can also occur with zoledronate.

The pathogenesis of BP-associated ONJ or AFF is unclear. Recent reports have suggested an impairment in angiogenesis as ONJ has been described in the oncology setting in patients treated with bevacizumab, an anti-angiogenic agent that inhibits VEGF [18]. In one clinical study, zoledronate infusion administered to 30 patients with metastatic bone disease was shown to reduce circulating concentrations of VEGF, although data on other angiogenic factors such as ANG are lacking [22]. Reduced angiogenesis has been shown in the oral mucoperiosteal tissue in BP-associated ONJ [23]. Moreover, data from preclinical studies have demonstrated that BPs act directly on endothelial cells and interfere in the angiogenic process including endothelial cell adhesion, migration and survival [24]. In the oncology setting, BPs may have anti-cancer effects through their inhibition of tumour angiogenesis [25,26]. Inhibition of angiogenesis by BPs could also be implicated in the pathogenesis of AFF as isolated skeletal injury results in a local and systemic angiogenic response, mediated primarily by VEGF [27].

The potential direct effect of BPs, at non-toxic clinically relevant concentrations, on the production of angiogenic factors by osteoblasts is not fully known and in light of the possible involvement of these factors in the maintenance of skeletal homeostasis, this merits further investigations. Moreover, it is still unclear whether doses of nitrogen-containing BPs used for other disorders of skeletal remodelling such as osteoporosis still have anti-angiogenic effects. The aim of the study was therefore to investigate the (1) *in-vivo* effect of alendronate in post-menopausal women, given at the osteoporosis dose (70 mg weekly) and (2) *in vitro* effect in osteoblastic and osteocytic cells of zoledronate and alendronate at concentrations ranging from 10^{-12} to 10^{-6} M on the production of the pro-angiogenic factors VEGF-A and ANG-1.

2. Materials and methods

2.1. Patients

Eighteen ambulatory postmenopausal women aged over 50 years with osteoporosis or moderate osteopenia (T score of $\leqslant -2$) at either the lumbar spine (LS), total hip (TH) or femoral neck (FN) who had never received bisphosphonates were studied. The patients were randomized to receive calcium (600 mg daily) and vitamin D3 (400 IU daily) only (the control group) or alendronate 70 mg weekly in addition to the combined calcium/vitamin D supplements. Subjects were excluded if they had a metabolic bone disease such as Paget's disease, chronic kidney disease, history of reflux disease or gastritis or were receiving treatment which could influence bone turnover such as hormone replacement therapy with oestrogens, previous use of bisphosphonates, strontium ranelate or teriparatide. Ethical approval was obtained from the Research and Ethics Committee of Guy's and St. Thomas' NHS Trust. All study participants gave written informed consent. All subjects underwent a DXA scan to assess their bone mineral density (BMD) at baseline and at 12 months. BMD at the TH and LS were measured using dual X-ray absorptiometry (DXA) scan (Discovery A QDR Series, Hologic, Inc., USA). The CV for BMD measurement was 0.35%.

The study participants were reviewed at 3, 6 and 12 months when their adherence with study medication was determined and any adverse events reported. Blood samples were obtained at baseline, 3, 6, and 12 months. Serum samples were stored at −70 °C for analysis of the angiopoietic factors; VEGF and ANG-1. Patient demographics are summarised in Table 1.

2.2. Cell culture and in vitro experimental protocol

Two different clonal cell lines with early osteoblast characteristics were used. We cultured the human osteosarcoma cell line;

Table 1
Summary of demographics and baseline biochemical parameters of the control and treatment groups. There was no significance difference in baseline characteristics between the controls and treatment group.

Baseline characteristics mean (SD)	Control group $n = 8$	Treatment group $n = 10$
Age	59 (3.5)	58 (4.5)
Years since menopause	9 (5.4)	8 (4.3)
Weight (kg)	67.5 (9.5)	63.9 (12.9)
Body mass index (kg/m^2)	23.9 (3.7)	25.2 (0.5)
Albumin corrected calcium (mmol/L)	2.2 (0.1)	2.2 (0.1)
Phosphate (mmol/L)	1.1 (0.1)	1.1 (0.1)
eGFR (ml/min/1.73^2)	83.5 (10.0)	85.5 (0.7)
Vitamin D (nmol/L)	61.1 (12.7)	54.3 (23.4)
Lumbar spine BMD (g/cm^2)	0.782 (0.1)	0.775 (0.1)
Lumbar spine T score	−2.4 (0.6)	−2.5 (0.5)
Femoral neck BMD (g/cm^2)	0.653 (0.1)	0.631 (0.1)
Femoral neck T score	−1.8 (0.7)	−2.0 (0.8)
Total hip BMD (g/cm^2)	0.795 (0.1)	0.778 (0.1)
Total hip T score	−1.2 (0.5)	−1.4 (0.8)