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RESEARCH PAPER

Zoledronate and pamidronate depress neutrophil functions and survival in mice

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BACKGROUND AND PURPOSE

Bisphosphonate-related osteonecrosis of the jaw (BRONJ) has been identified as a severe complication of patients previously treated with i.v. bisphosphonates. It has been noted that necrotic bone from BRONJ sites display signs of bacterial infection suggesting that an immune defect may play a role in the pathophysiology of BRONJ. Here, we have examined the effect of two potent bisphosphonates, zoledronate and pamidronate, on neutrophil function, differentiation and survival.

EXPERIMENTAL APPROACH

The effect of bisphosphonates on chemotaxis, NADPH oxidase activity and neutrophil survival were assessed *in vitro* using bone marrow-derived primary neutrophils or *in vitro* differentiated haematopoetic progenitors from mice. The same parameters and the number of circulating neutrophils were quantified in neutrophils isolated from mice treated *in vivo* with zoledronate. *In vivo* recruitment of neutrophils was assessed by sodium periodate-induced peritonitis.

KEY RESULTS

Zoledronate and pamidronate inhibited *in vitro* neutrophil chemotaxis and NADPH oxidase activity in a dose-dependent manner. *In vivo* recruitment of neutrophils was also suppressed. Zoledronate did not affect *in vitro* differentiation of neutrophils but shortened their life span in a granulocyte-colony stimulating factor-dependent manner. fMLP-induced activation of RhoA activity was decreased by zoledronate treatment.

CONCLUSIONS AND IMPLICATIONS

Our results show that bisphosphonate exposure leads to impaired neutrophil chemotaxis, neutrophil NADPH oxidase activity and reduced circulating neutrophil counts. This work suggests that bisphosphonates have the potential to depress the innate immune system for a prolonged time, possibly contributing to the pathogenesis of BRONJ.

Abbreviations

BRONJ, bisphosphonate-related osteonecrosis of the jaw; DHR dihydrorhodamine 123; FFP, farnesyl diphosphate; fMLP, formyl-methionyl-leucyl-phenylalanine; G-CSF, granulocyte-colony stimulating factor; GGPP, geranylgeranyl diphosphate; GM-CSF, granulocyte-macrophage colony-stimulating factor; PMA phorbol myristate acetate; PMN, polymorphonuclear leukocytes;

Introduction

Bisphosphonates (BPs) have become the primary treatment for the prevention of osteoporosis and to reduce skeletal-related symptoms and events in patients with multiple myeloma and metastatic bone lesions of solid tumour cancers (Saad *et al.*, 2008). Bisphosphonates are potent inhibitors of osteoclast-mediated bone resorption and are used to reduce

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bone turnover, increase bone mass and decrease the risk of pathological fractures (Russell and Rogers, 1999). The mechanism of action of bisphosphonates involves the perturbation of the mevalonate biosynthetic pathway, responsible for the synthesis of cholesterol and isoprenoid lipids such as isopentyl diphosphate, farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) (Amin *et al.*, 1992). FPP and GGPP are involved in post-translational modification (prenylation) of small GTPases such as Rho and Rac, which allows proper membrane localization and activation of these GTPases (Zhang and Casey, 1996). The small Rho GTPases are essential in several cells types including osteoclasts (Sato *et al.*, 1991) where they act as 'molecular switches' that can regulate several processes such as cell proliferation, apoptosis and turnover of the actin cytoskeleton (Ridley, 2001).

In recent years, it has been noted that a small subset of cancer patients (1–10%) treated with i.v. bisphosphonates are at an increased risk of developing a debilitating, non-healing, necrotic area in the oral cavity known as bisphosphonate-related osteonecrosis of the jaw (BRONJ) (Walter *et al.*, 2008). In addition, a very low risk for developing BRONJ also exists for patients treated with oral bisphosphonates (Hong *et al.* 2010; Sedghizadeh *et al.*, 2009). The majority of BRONJ cases in patients receiving bisphosphonates develop after dental surgery. There are several mechanisms by which bisphosphonates are thought to cause BRONJ, which include anti-angiogenesis and bisphosphonate toxic effects that inhibit wound healing (Reid *et al.*, 2007). Another emerging theory is the idea that infection may play a primary role in the pathogenesis of BRONJ (Reid, 2009). The presence of infection appears to be a consistent finding among histological reports of BRONJ (Marx *et al.*, 2007) with *Actinomyces* being a common, if not universal, finding (Hansen *et al.*, 2006). In addition, there are reports that some bisphosphonates affect the innate immune system, including inhibition of neutrophil enzymes that affect wound healing, such as matrix metalloproteinase 8 (Teronen *et al.*, 1997), increased risk of neutropenia (Coukell and Markham, 1998) and decreased generation of reactive oxygen species formation (Serretti *et al.*, 1993). This is not surprising in view of the mechanism of action of bisphosphonates, which target small GTPases that are signalling proteins integral to neutrophil differentiation and function (Dinauer, 2003). Despite this fact, the idea that bisphosphonates may negatively affect the innate immune system has not received much focus in the literature.

Our *in vitro* and *in vivo* studies are the first to present clear evidence that two potent nitrogen bisphosphonates, zoledronate and pamidronate, can depress neutrophil functions including chemotaxis and NADPH oxidase activity as well as neutrophil survival in mice.

Methods

Animals

All animal care and experimental procedures were carried out in accordance with the Guide for the Humane Use and Care of Laboratory Animals and were approved by the University of Toronto Animal Care Committee. We used 3 month old CD-1 and C57BL/6J mice, at the University of Toronto Animal Care Facility.

Dosing of zoledronate and pamidronate in vivo *and* in vitro

Zoledronate disodium (1-hydroxy-2-imidazol-1-yl-1-phosphono-ethyl)phosphonic acid; CAS no: 165800-07-7) was purchased from Sequoia Research, Pangbourne, UK. Pamidronate disodium (3-amino-1-hydroxypropylidene-bisphosphonate pentahydrate, CAS no: 109552-15-0) was purchased from Tecoland Corporation, NJ. Using a modification of a previously described rat protocol (Pfister *et al.*, 2003), 3 month old mice were given (i.v.) 2 μg of zoledronate, 20 μg pamidronate or carrier alone. The *in vivo* doses were based on the recommended human therapeutic dose of 4 mg every 4 weeks. Assuming an average patient weight of 60 kg, for a 30 g mouse the equivalent dose would be 2 μg·per mouse. The concentration of zoledronate used *in vitro* was based on data from a previous study in rats that used radiolabelled zoledronate to quantify its concentration in bone, soft tissue and blood from 1 to 280 days after a single infusion (Green and Rogers, 2002). Based on this and more recent data (Weiss *et al.*, 2008), we estimate that the concentrations of zoledronate in bone marrow are between 20 and 100 μM. For *in vitro* experiments, we used concentrations within this range. At the doses and incubation times used here, there were no obvious signs of toxicity with normal circulating cell counts, except for neutrophils (see Results).

Isolation of murine bone marrow neutrophils

Mouse neutrophils were isolated from the bone marrow of mice, as described previously (Glogauer *et al.*, 2003). The murine neutrophil isolation protocol routinely yields cell suspensions that are >90% neutrophils with >98% viability, as judged by Wright stain and Trypan blue exclusion respectively. All of the neutrophil studies were carried out at 37°C.

Measurement of NADPH oxidase activity

Neutrophil-associated oxidant content was measured using dihydrorhodamine 123 (DHR; Molecular Probes, Eugene, OR). Isolated peripheral blood neutrophils were incubated in the presence of 1 mM DHR for 15 min, followed by incubation with 10μM phorbol myristate acetate (PMA) and 1μM formyl-methionyl-leucyl-phenylalanine (fMLP). Cell-associated fluorescence was quantified after 10 min incubation at 37°C using a Guava flowcytometer (Siddiqi *et al.*, 2001).

Zigmond chamber chemotaxis

A neutrophil suspension (1×10^6 cells mL^{-1}) was allowed to attach to BSA-coated glass coverslips (22 × 40 mm) at 37°C for 20 min. The coverslip was inverted onto a Zigmond chamber, and 100 μL HBSS media was added to the left chamber, while 100 μL HBSS media containing 1 μM fMLP was added to the right chamber. Time-lapse video microscopy was used to examine neutrophil movements in the Zigmond chamber. The images were captured at 20 s intervals with a Nikon Eclipse E1000 Microscope. Cell-tracking software (Retrac

version 2.1.01 Freeware) was used to characterize cellular chemotaxis from the captured images.

Sodium periodate peritonitis

To induce an experimental peritonitis, 1 mL of 5 mM sodium periodate (Sigma, Oakville, ON, Canada) in PBS was injected i.p. The mice were killed 3 h later, and the peritoneal exudate was collected by lavage with chilled PBS (5 mL·per mouse). Neutrophils were counted by a haemocytometer and Coulter Counter (Becton Dickinson, Mississauga, ON, Canada).

Circulating neutrophil levels

Two and four weeks following zoledronate and vehicle treatment, 200 µL of blood was isolated from the great saphenous leg vein of the mouse before killing. A complete blood count was performed using a hemavet (Hemavet®950, Drew Scientific, Oxford, CT).

Rho GTPase pulldown/activity assay

The Pak-binding domain (PBD) and Rhotekin-binding domain (RBD) assays were carried out as described previously (Benard and Bokoch, 2002). In short, PMN were pretreated with bisphosphonate or mock carrier prior to stimulation with fMLP. Cells were then lysed, and GTP-loaded GTPases were pulled down with GST-PBD or GST-RBD beads and quantified by densitometric Western blotting. The amounts were normalized for the total amount of GTPase present in the lysate. Data represent the mean value from three separate experiments.

Apoptosis assay

The percentage of apoptotic neutrophils was determined by Annexin-V staining using the Annexin V-FITC Apoptosis Detection Kit (BioVision Inc., Mountain View, CA, USA) according to the manufacturer's protocol. Stained cells were analyzed by FACS, and cells positive for both Annexin-V and propidium iodide (PI) or Annexin-V alone were considered apoptotic.

In vitro *differentiation of neutrophils*

Haematopoetic progenitors were isolated from bone marrow using the EasyStep™ Hematopoietic Progenitor Enrichment kit (Stemcell Technologies, Vancouver, Canada). Stem cells were subsequently differentiated into neutrophils as described previously (Buitenhuis *et al.*, 2005). In brief, progenitors were cultured for 3 days in Iscove's modified Dulbecco's medium (Gibco, Burlington, ON, Canada), 10% FCS, 50 µM β-mercaptoethanol, 10 u·mL^{-1} penicillin/streptomycin, 2 mM glutamine, stem cell factor (SCF; 50 ng·mL^{-1}), FLT-3 ligand (50 ng·mL^{-1}), granulocyte-macrophage colony-stimulating factor (GM-CSF; 0.1 nM), IL-3 (0.1 nM) and granulocyte-colony stimulating factor (G-CSF) (30 ng·mL^{-1}). After this initial period, the medium was replaced every 3 days with medium containing only G-CSF (30 ng·mL^{-1}) with or without either (1, 10, 50, 200 µM) zoledronate. Cells were harvested between day 12 and 14 (as mentioned in the Results section). This culture method ensures that the majority of cells differentiate into neutrophils, which is also clear by assessing their morphology after 5–7 days *in vitro* with the presence of a typical segmented nuclear morphology.

Statistics

Normally distributed data were analysed by unpaired Student's *t*-tests. ANOVA with Bonferroni's correction was used for multiple comparisons. A *P*-value of <0.05 was considered to be significant. Data are expressed as mean ± SD unless indicated otherwise.

Results

We first assessed whether the bisphosphonate, zoledronate, affects neutrophil functions *in vitro*. Isolated murine neutrophils were exposed *in vitro* to increasing concentrations of zoledronate (25, 50, 100 µM) for 15 min after which chemotaxis was assessed using a Zigmond chamber assay. A dose-dependent decrease in *in vitro* neutrophil chemotaxis speed was observed with increasing concentrations of zoledronate (Figure 1A). There was no zoledronate-induced cell death as assessed by Trypan blue uptake in cells during chemotaxis. Since the respiratory burst of neutrophils is an essential mechanism to eliminate pathogens, we quantified fMLP-induced NADPH oxidase activity in neutrophils treated with zoledronate (Figure 1B). NADPH oxidase activity was significantly reduced from baseline with increasing concentrations of zoledronate ($P < 0.05$). To determine if zoledronate treatment had an effect on *in vivo* neutrophil recruitment, mice were treated with a single dose of either 2 or 20 µg of zoledronate 4 weeks before PMN isolation for *in vitro* chemotaxis experiments or induction of sodium periodate peritonitis. Intriguingly, *in vitro* migration velocity of isolated PMN was still decreased 4 weeks after systemic zoledronate treatment. Sodium periodate-induced *in vivo* neutrophil recruitment was also diminished in these mice (Figure 1D). This suggests that zoledronate has a residual effect on neutrophil effector functions. To test if the effect of zoledronate on PMN function was drug-specific, we tested another bisphosphonate, pamidronate. Quantification of *in vitro* chemotaxis of PMN pretreated with various concentrations of pamidronate showed a dose-dependent effect on migration speed (Figure 2A). Also, fMLP-induced respiratory burst in isolated PMN decreased upon pamidronate treatment (Figure 2B), and pamidronate inhibited sodium periodate-induced *in vivo* PMN recruitment 1 month after pamidronate treatment (Figure 2C). These results strongly suggest that inhibition of PMN effector functions in these experiments is a general effect of (nitrogen) bisphosphonates.

It has been reported that bisphosphonates can cause neutropenia as a rare side effect in some patients (Gutteridge *et al.*, 1996; Coukell and Markham, 1998; Russell *et al.*, 2010). To test whether zoledronate caused neutropenia in our mouse model, mice were treated with a single dose of zoledronate (2 µg) 2 weeks before counting neutrophil numbers in the blood. While control mice showed normal neutrophil counts similar to those found in a previous study (Nemzek *et al.*, 2001), zoledronate pretreated mice had significantly lower neutrophils counts (Table 1). This finding led us to consider the possibility that zoledronate may decrease neutrophil survival and/or neutrophil proliferation/differentiation in the bone marrow.

To assess the effect of zoledronate on neutrophil differentiation, we used an *in vitro* neutrophil differentiation assay.

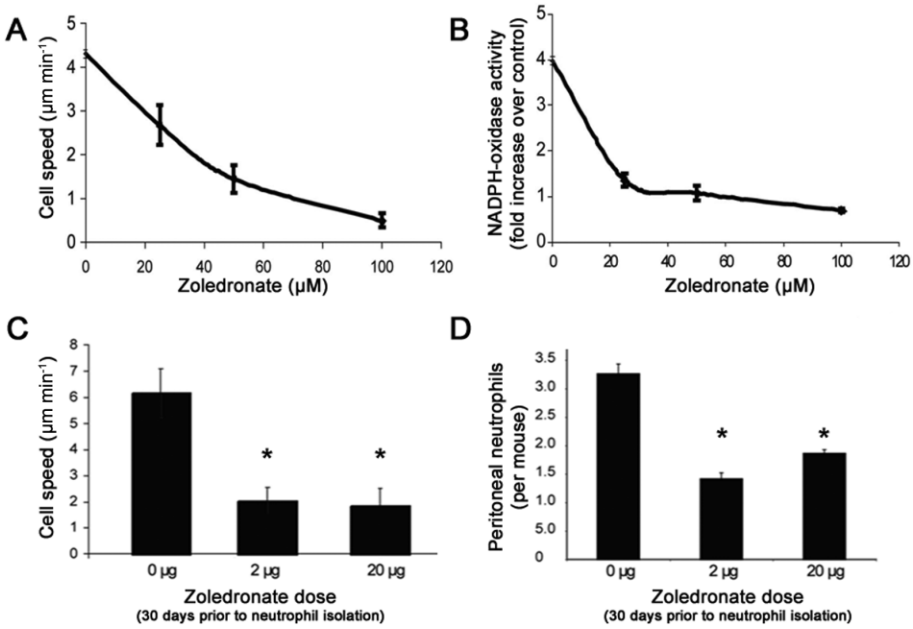

Figure 1

Effect of zoledronate exposure on neutrophil function. Neutrophils were treated for 15 min with the indicated concentration of zoledronate followed by the assessment of either (A) fMLP-mediated chemotaxis in a Zigmond chamber or (B) fMLP-mediated NADPH oxidase activity. Zoledronate inhibited both fMLP mediated chemotaxis and NADPH oxidase activity in a dose-dependent manner. (C) In order to determine if *in vivo* zoledronate treatment had a residual effect on *in vitro* neutrophil functions, mature neutrophils were isolated from bone marrow of 4 month old mice treated 30 days earlier with 0, 2 or 20 µg of zoledronate. fMLP-mediated neutrophil chemotaxis was assessed in a Zigmond chamber. Neutrophils from zoledronate -treated mice demonstrated a residual inhibitory effect on neutrophil chemotaxis. (D) *In vivo* neutrophil recruitment to sites of inflammation was assessed in a sodium periodate-induced peritonitis model. Three hours after sodium periodate injection, the cellular infiltrate was collected and quantified. In this model, more than 95% of cells collected were neutrophils. Mice previously treated with zoledronate display a clear reduction in neutrophil recruitment to the inflamed peritoneum. Data shown are means ± SD; $*P < 0.01$, significantly different from control (0 µg); $n = 3$ with three mice per experiment.

Control stem cells were differentiated into neutrophils for 12 days in the presence of G-CSF. During this differentiation period, cells were treated with varying concentrations of zoledronate (0, 1, 10, 50, 200 µM). After 12 days of differentiation, cells were analysed by Diff-Quick staining. Figure 3A illustrates a dose-dependent decrease in viable mature neutrophils (dark blue nucleus) after 12 days with increasing zoledronate concentration, suggesting that zoledronate exposure leads to increased cell death during neutrophil differentiation *in vitro* ($P < 0.05$). Although zoledronate depressed survival of mature neutrophils, it does not appear to have any effect on neutrophil progenitor proliferation and no significant differences were observed when quantifying the numbers of metamyelocytes, or banded and segmented neutrophils between control and zoledronate differentiated cells, and zoledronate did not affect proliferation of haematopoietic progenitors *in vitro* (data not shown).

To determine if the effect of zoledronate on neutrophil survival during differentiation was irreversible, stem cells were isolated from mice 4 weeks after a single dose of zoledronate and differentiated *in vitro*. Figure 3C illustrates that stem cells from zoledronate-pretreated mice were able to differentiate normally *in vitro*, with similar survival rates to control cells . However, addition of zoledronate to control stem cells during *in vitro* differentiation increased the number of apoptotic events (Figure 3C; $P < 0.05$). These results suggest that zoledronate must be present during neutrophil differentiation to affect neutrophil survival. Since mature neutrophils derived from zoledronate-treated mice would be exposed to zoledronate in the bone marrow space, we expected them to have lower survival rates. To test this, mature neutrophils derived from zoledronate-treated mice were cultured for 24 h, which induces spontaneous apoptosis in neutrophils. In contrast to our data with the *in vitro* differentiation assay, we did observe reduced survival of neutrophils from zoledronate-treated mice (Figure 3D). As *in vitro* differentiated neutrophils are cultured in the presence of G-CSF, we repeated the mature neutrophil survival assay in the presence of G-CSF. Intriguingly, under these conditions, zoledronate decreased the life span of mature neutrophils (Figure 3E). Since G-CSF is known to prolong neutrophil survival (Colotta *et al.*, 1992), we conclude that zoledronate does not affect neutrophil survival *per se* but decreases the positive effect of G-CSF on neutrophil life span.

Bisphosphonates are known to inhibit a crucial step in the prenylation of Rho GTPases (Amin *et al.*, 1992), and these proteins are important for neutrophil chemotaxis, respiratory burst and survival (Dinauer, 2003; Glogauer *et al.*, 2003; Gu *et al.*, 2003; Cancelas *et al.*, 2006). To test if zoledronate treatment affects the activity of these key signalling proteins in neutrophils, primary cells were pretreated with zoledronate (20 µM) or vehicle before stimulation with fMLP. Activity of

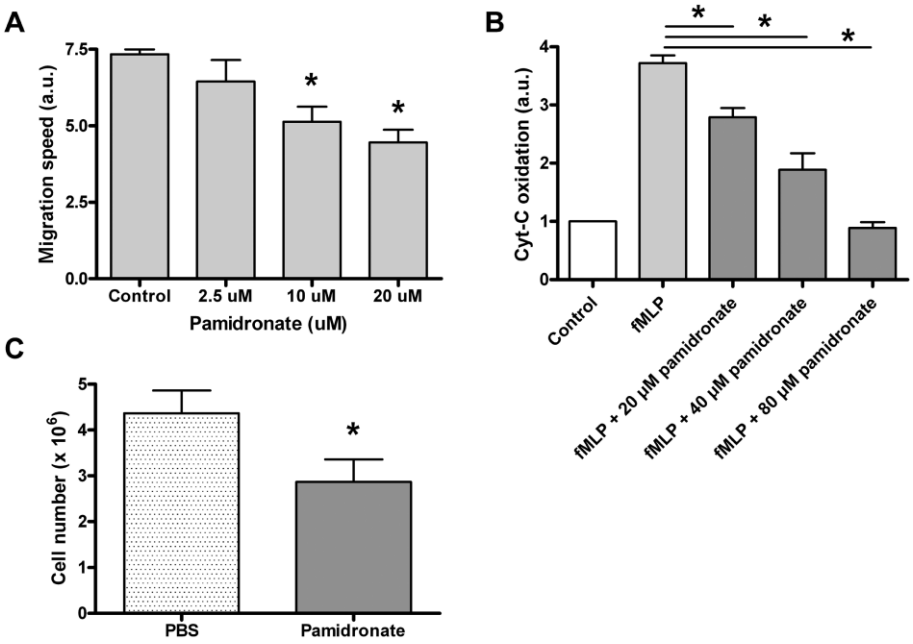

Figure 2

Effect of pamidronate exposure on neutrophil function. Experiments were carried out as presented in Figure 1, but with the change that pamidronate was used as the bisphosphonate. (A) *In vitro* chemotaxis experiments (Zigmond chamber) were performed with PMN pretreated with pamidronate (2.5, 10 or 20 µM). *$P < 0.05$, significantly different from control. (B) Respiratory burst in pamidronate -treated PMN was quantified using a cytochrome C oxidation assay. *$P < 0.05$, significantly different from fMLP alone. (C) *In vivo* recruitment of PMN was quantified in pamidronate-treated mice or carrier (PBS)-treated mice. *$P < 0.05$, significantly different from PBS. Data shown are means ± SD; $n = 3$ with three mice per experiment.

Table 1

Effect of zoledronate exposure on the number of circulating blood cells

	Control ($\times 10^3$ cells·µL^{-1})	Zoledronate ($\times 10^3$ cells·µL^{-1})
Lymphocytes	1.65 ± 0.67	1.56 ± 0.49
Monocytes	0.17 ± 0.05	0.38 ± 0.51
Eosinophils	0.07 ± 0.03	0.07 ± 0.02
Basophils	0.02 ± 0.01	0.02 ± 0.01
Neutrophils	0.72 ± 0.22	0.31 ± 0.12*
Erythrocytes	6320 ± 2420	4360 ± 670

Data shown are means ± SD; *$P < 0.05$, significantly different from control; $n = 6$.

the small Rho GTPases Rac1, RhoA and Cdc42 was analysed by a GTP-dependent pulldown assay. While fMLP activation of Rac1, and Cdc42 was normal in cells pretreated with zoledronate (20 µM) for 15 min i*n vitro*, RhoA activity downstream of fMLP was depressed by almost 50% (Figure 4).

Discussion and conclusions

A great deal of apprehension exists in the dental community regarding the proper management of patients who have a history of oral or i.v. bisphosphonate exposure because of the emergence of a debilitating condition known as BRONJ (Walter *et al.*, 2008). The observation that a significant number of cases of BRONJ can present with an active infection lead us to suspect that the innate immune system may be perturbed in the small subset of individuals who develop BRONJ. A state of innate immune suppression can result from a decrease in the functional capability of the immune cells, a decrease in the number of immune cells that are present to fight off the infection or a combination of both. We chose to test this hypothesis in mice by first performing functional test on neutrophils following both *in vitro* and *in vivo* exposure to bisphosphonates.

The results of this study show that both *in vitro* and *in vivo* exposure to zoledronate and pamidronate leads to diminished neutrophil chemotaxis *in vitro*, diminished neutrophil recruitment to the peritoneum *in vivo* as well as diminished NAPDH oxidase activity *in vitro*. Notably, the effect on chemotaxis persisted up to 4 weeks after the single zoledronate dose. Since mature neutrophils were isolated from bone marrow, it is likely that that they were exposed to zoledronate that is effectively stored in bone (Green and Rogers, 2002) and released during normal bone remodelling. Because zoledronate inhibits the mevalonate pathway, which is important for prenylation of small Rho GTPases, we also measured its effect on GTPase activity. Of the three tested GTPases, Rac, RhoA and Cdc42, only the activity of RhoA was decreased. RhoA plays an important role during neutrophil migration by regulating contraction and detachment of the

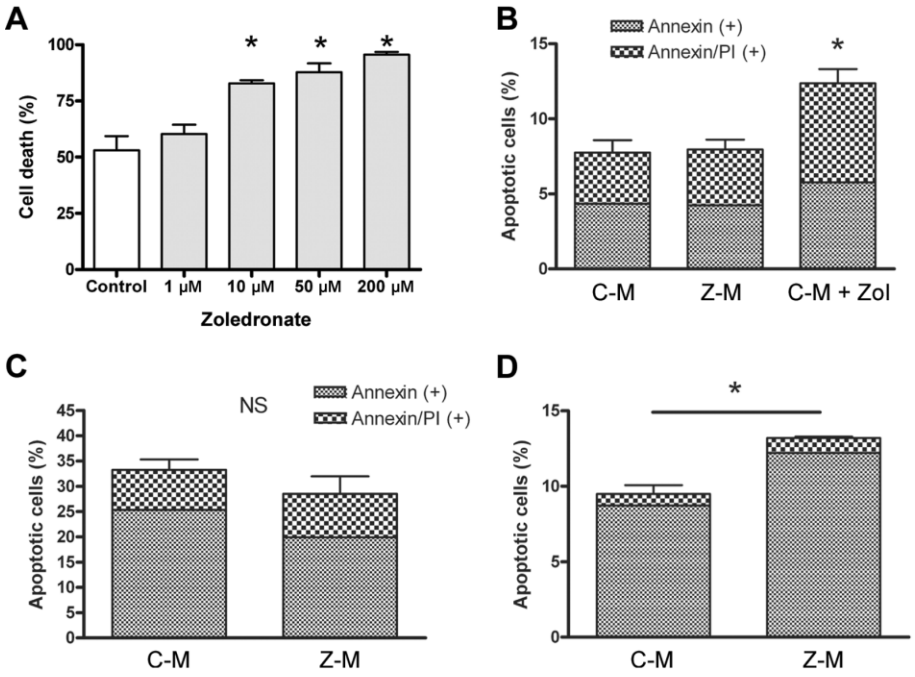

Figure 3

Effect of zoledronate on differentiation and apoptosis of mature neutrophils. (A) In order to determine if zoledronate could affect neutrophil differentiation in the bone marrow, haematopoietic progenitor cells were differentiated into neutrophils, in the presence of zoledronate (0–200 μM) and after 12 days were assessed by Diff-quick staining. Cells were counted in four random fields, and the percentage of dead cells was calculated. As shown in this figure, zoledronate reduced mature neutrophil survival in a dose-dependent manner but did not affect neutrophil differentiation as similar numbers of total cells are present (live and dead), compared with control. (B) Haematopoietic progenitors were isolated from zoledronate treated mice (Z-M) or control mice (C-M) and differentiated *in vitro* into neutrophils without zoledronate. Cells from control mice were also differentiated in the presence of zoledronate (C-M + ZOL). At day 10, the percentage of apoptotic cells was determined by FACS analysis of Annexin–Fitc/PI. (C) Mature neutrophils were extracted from the bone marrow of mice that had been pretreated 2 weeks earlier with a single dose of 2 μg of zoledronate, and cultured for 20 h in RPMI + 10% FCS, whereby cells undergo spontaneous apoptosis. The levels of apoptosis were not significantly different (NS) between mature neutrophils from control (C-M) and zoledronate pre-treated mice (Z-M). (D) Conditions as for C, but G-CSF (30 ng·mL^{-1}) was added to the culture medium. Zoledronate pretreated mice (Z-M) in the presence of G-CSF showed significantly higher levels of apoptosis of mature neutrophils compared with control mice (C-M). These levels of apoptosis are similar those observed during the *in vitro* differentiation of precursors cells in (B). Data shown are means ± SD; *$P < 0.05$, significantly different from control; $n = 3$–4).

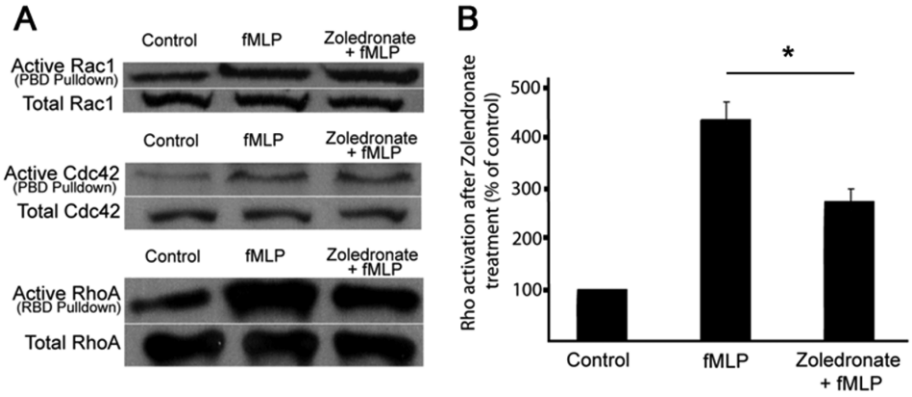

Figure 4

Zoledronate affects Rho small GTPase activity. In order to verify that zoledronate could affect key signalling proteins involved in neutrophil functions, we measured fMLP-mediated Rho small GTPase activity in neutrophils treated with zoledronate *in vitro*. (A) As indicated in this figure, fMLP-induced activation of Rac1 and Cdc42 was identical in control cells and cells pretreated with zoledronate (20 μM) for 15 min. (A,B) RhoA activity downstream of fMLP was depressed by almost 50%. Data shown are means ± SD; *$P < 0.05$, significantly different from control $n = 3$.

uropod (Alblas *et al.*, 2001) and activation of mDia1 (Shi *et al.*, 2009). In contrast to Rac, RhoA is not directly responsible for NADPH-oxidase activation via a direct interaction. However, RhoA can modulate the recruitment of essential cytosolic components of the oxidase, such as p47phox (Kim *et al.*, 2004). Interestingly, it was recently shown that another group of drugs that inhibit the mevalonate biosynthetic pathway, the statins, also inhibit neutrophil function and RhoA activity (Maher *et al.*, 2009).

Some patients treated with bisphosphonates exhibit neutropenia as a side effect (Gutteridge *et al.*, 1996; Coukell and Markham, 1998; Russell *et al.*, 2010). Interestingly, we observed a significant reduction in circulating neutrophils levels (mild form of neutropenia) in zoledronate-treated mice. The number of circulating neutrophils is determined by the balance between generation and release of new cells from the bone marrow and spontaneous apoptosis of mature neutrophils (Christopher and Link, 2007). Our results indicate that zoledronate influences neutrophil life span in a G-CSF-dependent manner. Activation of the G-CSF receptor by G-CSF triggers an intracellular cascade of signalling events that ultimately activate proliferation and differentiation of neutrophil progenitors and enhanced survival of mature neutrophils. Indeed, other groups have shown that G-CSF signals via the G-CSF receptor through various GTPases, including Ras and Rap1 (de Koning *et al.*, 1998), which require proper prenylation for their functioning. Our results suggest that zoledronate may alter G-CSF receptor signalling, impeding the antiapoptotic effect of G-CSF on neutrophils, which results in a shorter life span. Whether lower circulating neutrophil numbers after zoledronate treatment are caused by a decreased life span and/or other mechanisms, such as neutrophil release from the bone marrow, remains to be resolved. Whether and how a decreased level of circulating PMNs contributes to BRONJ is unclear. However, it interesting to note that the presence of *Actinomyces*, a pathogen commonly found in BRONJ lesions (Hansen *et al.*, 2006), seems to increase upon decreasing numbers of blood PMN in patients receiving chemotherapy (Majda-Stanislawska *et al.*, 1998). It is relevant that, during the time course of our experiments, we did not observe obvious signs of spontaneous infection in zoledronate-treated mice. This study was carried out using mice that facilitates study of bisphosphonate effects on the innate immune system. It must, however, be noted that there are limitations in this model when making comparisons with the human innate immune system. While human blood is neutrophil-rich (50–70% neutrophils), mouse blood has a strong preponderance of lymphocytes (75–90% lymphocytes, 10–25% neutrophils) (Doeing *et al.*, 2003). It is not clear what, if any, functional consequence this shift towards neutrophil-rich blood in humans has, although it does mean that while the vasculature serves as a storage site for neutrophils in humans, the bone marrow is the primary neutrophil storage site in mice. This may mean that murine neutrophils have a higher direct 'lifetime' bisphosphonate exposure than human neutrophils, due to their storage in the bone marrow compartment. This is particularly relevant as earlier studies (Russell, 2007) suggested that bone acts as a reservoir for bisphosphonate and neutrophils differentiate within the bone marrow. Moreover, it is tempting to speculate that the effect of bisphosphonate is exacerbated when neutrophils are recruited to infections near bone tissue, such as in BRONJ. We believe that the neutrophil defect observed in bisphosphonate users is quite mild and under normal circumstances does not predispose the patient to increased risk of systemic infections. However, in those cases where the jaw of a bisphosphonate-treated patient is subjected to trauma, immune cells that are normally recruited as part of the healing process, may exhibit decreased life span and reduced functionality. This could predispose the patient to increased risk of local impaired bone healing, particularly in light of the oral bacterial biofilm and the reduced tissue vascularity that is also a secondary effect of bisphosphonate therapy. This reduced innate immune functionality could contribute to the 'triple hit' or 'perfect storm' situation required for BRONJ where oral trauma coupled with reduced vascularity, slow soft tissue healing and reduced innate cellular immune functions increase the risk of delayed healing and subsequent bone necrosis. Further studies will be required to determine the specific contribution of bisphosphonate-induced repression of PMN function in the pathophysiology of BRONJ. We and others note that there is certainly bacteria present in the BRONJ sites. Whether it is a true infection or only colonization with a reduced inflammatory response, due to the suppressed neutrophil response will need to be addressed in clinical studies we are undertaking.

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Conflicts of interest

None.

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