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High doses of bisphosphonates reduce osteoblast-like cell proliferation by arresting the cell cycle and inducing apoptosis

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ABSTRACT

Objectives: The study objective was to evaluate the effect on osteoblast growth of high concentrations of three nitrogen-containing bisphosphonates (pamidronate, alendronate, and ibandronate) and one non-nitrogen-containing bisphosphonate (clodronate), using the MG-63 cell line as an osteoblast model, in order to determine the role of osteoblasts in bisphosphonate-related osteonecrosis of the jaw (BRONJ).
Materials and methods: Osteoblasts were incubated in culture medium with different doses of pamidronate, alendronate, ibandronate or clodronate. The proliferative capacity of the osteoblasts was determined by spectrophotometry (MTT-based) at 24 h of culture. Flow cytometry was used to determine the percentage of cells in each cell cycle phase (G0/G1, G2/M, and S) and to discriminate apoptotic cell death from necrotic cell death in the cell cycle at 24 h of treatment.
Results: All the bisphosphonates assayed produced a significant and dose-dependent reduction in MG-63 proliferation at the high doses assayed (10^{-4} and 5×10^{-5} M) in comparison with controls ($p < 0.001$). Cell cycle study revealed that all assayed bisphosphonates significantly arrested the cell cycle in phase G0/G1 at doses of 10^{-4} and 5×10^{-5} M, increasing the percentage of cells in this phase ($p < 0.05$). Apoptosis/necrosis studies showed significant changes compared with control cells, with an increased percentage of cells in apoptosis after treatment with 10^{-4} or 5×10^{-5} M of pamidronate, alendronate, ibandronate, or clodronate ($p < 0.05$).
Conclusions: High doses of nitrogen-containing or non-nitrogen-containing bisphosphonates can reduce the proliferation of MG-63 osteoblast-like cells by arresting the cell cycle and inducing apoptosis/necrosis.

1. Introduction

Bisphosphonates (BPs) are a family of pyrophosphate synthetic analogues in which the oxygen linking the phosphates has been replaced by carbon. They are widely used to treat bone disorders, including osteoporosis, Paget's disease, hypercalcemia of malignancy, fibrous dysplasia, and the bone metastases of breast and prostate cancer (Ralston et al., 1989; Eggelmeijer et al., 1994; Lala et al., 2000; Rodan and Martin, 2000; Lane et al., 2001). BPs can be divided between nitrogen-containing and non-nitrogen-containing forms. Nitrogen-containing BPs, such as pamidronate, alendronate, or ibandronate, interrupt the mevalonate pathway of cholesterol synthesis, inhibiting the enzyme farnesyl diphosphate synthase and blocking prenylation of small GTPases, leading to the impairment of osteoclast function (Lane et al., 2001; Reszka and Rodan, 2004). For their part, non-nitrogen-containing BPs such as clodronate suppress bone resorption by being metabolized into non-hydrolysable ATP analogues that have no releasable energy content, producing osteoclast death (Frith et al., 1997, 2001).

BPs bind to hydroxyapatite crystal but vary in the strength of their binding, which may play an important role in the duration of their action. Differences in the severity of adverse effects on bone

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tissue in long-term BP treatment have been attributed to the lesser or greater binding of the different BPs to hydroxyapatite (Nancollas et al., 2006; Idris et al., 2008). According to some authors, although therapeutic doses are not very high (10^{-5} to 10^{-9} M) (Chen et al., 2002), very high concentrations may reach the bone after prolonged treatment because of the strong binding of these drugs to hydroxyapatite (Marx, 2014).

BPs are widely prescribed, and their ability to inhibit osteoclast formation and activity in vitro and in vivo is well documented (Russell, 2007; Silverman and Maricic, 2007). However, the mechanisms underlying their action on bone and their effects on osteoblasts are not fully understood, although various proposals have been made, including: a decrease in bone turnover and subsequent accumulation of microfractures; a toxic effect on osteoblasts; a decrease in collagen production of osteoblasts (Açil et al., 2012); an anti-angiogenic effect producing avascular necrosis; and a reduction in the viability of fibroblasts and oral keratinocytes (Santini et al., 2002; Mashiba et al., 2005; Landesberg et al., 2008). Although useful in the treatment of some bone diseases, BPs have also been associated with the development of BP-related osteonecrosis of the jaw (BRONJ) (Marx, 2003; Ruggiero et al., 2004). Probably, the infectious conditions that often precede the onset of BRONJ support recent pathogenesis theories stating that local inflammation and associated pH-changes may trigger the release and activation of nitrogen-containing bisphosphonates ultimately resulting in necrosis (Otto et al., 2012).

The objective of this study was to evaluate the effect on osteoblast growth of high concentrations of three nitrogen-containing BPs (pamidronate, alendronate, and ibandronate) and one non-nitrogen-containing BP (clodronate), using the MG-63 cell line, in order to determine the role of osteoblasts in BRONJ. The MG-63 cell line is commonly used as an osteoblast model because it shares the same characteristics.

2. Materials and methods

We studied three nitrogen-containing BPs, pamidronate (Sigma–Aldrich, St. Louis, MO, USA), alendronate (Sigma), and ibandronate (Sigma), and one non-nitrogen-containing BP, clodronate (Sigma).

2.1. Cell culture

Human MG-63 osteosarcoma cell line was purchased from American Type Cultures Collection (ATCC, Manassas, VA, USA) and maintained as described by De Luna-Bertos et al., 2013, in Dulbecco's modified Eagle medium (DMEM; Invitrogen Gibco Cell Culture Products, Carlsbad, CA, USA) with 100 IU/mL penicillin (Lab Roger SA, Barcelona, Spain), 50 μg/mL gentamicin (Braum Medical SA, Jaen, Spain), 2.5 μg/mL amphotericin B (Sigma), 1% glutamine (Sigma), and 2% HEPES (Sigma), supplemented with 10% fetal bovine serum (Gibco, Paisley, UK). Cultures were kept at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cells were detached from the culture flask with a solution of 0.05% trypsin (Sigma) and 0.02% ethylenediaminetetraacetic acid (EDTA) (Sigma) and were then washed and suspended in complete culture medium with 10% fetal bovine serum.

2.2. Cell proliferation assay

Osteoblasts were seeded at 1×10^4 cells/mL per well into a 24-well plate (Falcon, Becton Dickinson Labware, NJ, USA) and cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 for 24 h. Next, the medium was replaced with DMEM containing pamidronate, alendronate, ibandronate, or clodronate at a dose of 10^{-4}, 5×10^{-5}, or 10^{-5} M. After 24 h of culture, the cell proliferation was measured by MTT assay, as described by Manzano-Moreno et al. (2013), replacing media with phenol red-free DMEM containing 0.5 mg/mL MTT (Sigma) and incubating for 4 h. Cellular reduction of the MTT tetrazolium ring resulted in the formation of a dark-purple water-insoluble deposit of formazan crystals. After incubation, the medium was aspirated, and dimethyl sulfoxide (DMSO, Merck Biosciences, Darmstadt, Germany) was added to dissolve the formazan crystals. Absorbance was measured at 570 nm with a spectrophotometer (Sunrise, Tecan, Männedorf, Switzerland.) The results were reported as mean absorbance (570 nm) ± SD. At least three separate experiments were conducted for each treatment, using the mean value in the analysis.

2.3. Cell cycle

Cultured human MG-63 cells treated for 24 h with 10^{-4}, 5×10^{-5}, or 10^{-5} M of pamidronate, alendronate, ibandronate, or clodronate, or without BP (control), were detached from the culture flask by treatment with a solution of 0.05% trypsin (Sigma) and 0.02% EDTA (Sigma) and were then washed and suspended in PBS and prepared for study of the cell cycle as reported by García-Martínez et al. (2011). The suspension obtained was placed in 200 μL PBS with 2 mL ice-cold 70% ethanol and 30% distilled H_2O and vigorously mixed. Cells were left for at least 30 min in the cold and then harvested by centrifugation and resuspended in 800 μL PBS. Cells were microscopically examined and, if clumped, passed through a 25-gauge syringe needle. Cells were then incubated at 37 °C for 30 min with 100 μL ribonuclease (RNase) (1 mg/mL) and 100 μL propidium iodine (PI). Finally, samples were analyzed by using an argon-ion laser tuned to 488 nm (Facs Vantage, Becton Dickinson, Palo Alto, CA, USA), measuring forward and orthogonal light scatter and red fluorescence, determining both the area and peak of the fluorescent signal.

2.4. Apoptosis and necrosis analysis

The study of apoptosis and necrosis was performed as described by De Luna-Bertos et al. (2014). Osteoblasts were previously treated with 10^{-4}, 5×10^{-5}, or 10^{-5} M of pamidronate, alendronate, ibandronate, or clodronate for 24 h at 37 °C. Next, cells were detached from the culture flask, washed, suspended in 300 μL PBS, and then labeled with annexin V and PI (Immunostep S.L., Salamanca, Spain), incubating 100 μL aliquots of the cell suspension with 5 μL annexin V and 5 μL PI for 30 min at 4 °C in the dark. Cells were then washed, suspended in 1 mL PBS, and immediately analyzed in a flow cytometer with argon laser (Facs Vantage, Becton Dickinson) at a wavelength of 488 nm to determine the percentage of fluorescent cells. We calculated the percentage of annexin-positive (apoptotic) cells and PI-positive (necrotic) cells from counts of 2000–3000 cells.

2.5. Statistical analysis

SPSS 22.0 (IBM, Chicago, IL) was used for all data analyses. Mean values (±SD) were calculated for each variable. A two-way repeated-measures analysis of variance (ANOVA) was performed to examine the effects on proliferation, apoptosis/necrosis induction, and cell cycle as a function of the BP type (pamidronate, alendronate, ibandronate, or clodronate), treatment duration, and concentration. When a significant interaction was identified, the Bonferroni correction was applied for planned pair-wise comparisons. $P < 0.05$ was considered significant. At least three separate experiments were performed for each assay.

3. Results

3.1. Cell proliferation assay

Compared with untreated cells (controls), all the BPs studied reduced osteoblast proliferation capacity at doses of 10^{-4} and 5×10^{-5} M in a dose-dependent fashion at 24 h of treatment (p <0.001) (Fig. 1). At a dose of 10^{-5} M, no significant difference was found between treated and untreated cells for any of these BPs.

3.2. Cell cycle

The percentage of cells in each cell cycle phase (G0/G1, G2/M and S) was determined by flow cytometry. Results are depicted in Table 1. No significant effect on the MG-63 cell cycle ($p > 0.05$) was observed after treatment for 24 h with 10^{-5} M of pamidronate, alendronate, ibandronate, or clodronate. The percentage of cells in G0/G1 phase was significantly increased by doses of 10^{-4} and 5×10^{-5} M of each BP tested (p values of 0.049 to <0.001). In parallel, the percentage of cells in G2/M phase was significantly reduced by doses of 10^{-4} M of pamidronate and doses of 10^{-4} and 5×10^{-5} M of ibandronate and clodronate.

3.3. Apoptosis and necrosis analysis

Flow cytometry with annexin V and PI labeling were used to discriminate apoptotic cell death from necrotic cell death in the cell cycle. Fig. 2 shows the percentage of viable cells, necrotic cells, and cells in early and late apoptosis after culture for 24 h in the presence of different doses of pamidronate alendronate, ibandronate, or clodronate.

In comparison with controls, pamidronate treatment significantly increased the percentage of apoptotic cells at doses of 10^{-4} and 5×10^{-5} M ($p = 0.011$ and $p = 0.013$ respectively). Alendronate treatment significantly increased the percentage of apoptotic, late apoptotic, and necrotic cells at a dose of 10^{-4} M (p <0.001, $p = 0.001$ and $p = 0.02$ respectively), whereas treatment with 5×10^{-5} M only increased the percentage of apoptotic cells ($p = 0.007$) compared with controls. Treatment with 10^{-4} and 5×10^{-5} M of ibandronate increased the percentage of apoptotic cells ($p = 0.038$, $p = 0.021$, respectively) compared with controls. Finally, treatment with doses of 10^{-4} and 5×10^{-5} M of clodronate significantly increased the percentage of apoptotic and necrotic cells compared with controls (p <0.05). At a dose of 10^{-5} M, no significant differences were found between untreated cells and those treated with any of the BPs.

4. Discussion

The results of this in vitro study demonstrate that high doses of pamidronate, alendronate, ibandronate, or clodronate have a negative effect on osteoblasts, reducing their proliferative capacity by arresting the cell cycle in the G0/G1 phase and inducing apoptosis. This implies major changes in the complex physiology of the osteoblast, which is regulated by multiple local and systemic factors that may regulate the activity of a specific transcription factor (Pérez et al., 2006; Ruiz et al., 2007; Krischak et al., 2007).

The concentrations of BPs used in this study were considerably higher than the therapeutic dose range of 10^{-5} to 10^{-9} M (Chen et al., 2002) but are comparable with the doses that reach the bone in long-term treatment due to the strong binding of these drugs to hydroxyapatite crystals (Marx, 2014). Nevertheless, various in vitro studies have shown that when nitrogen-containing BPs such as zoledronate bind with calcium phosphate, they lose their adverse effect on different cell populations, including osteoblasts (Schindeler and Little, 2005), dental pulp-derived cells (Cvikl et al., 2011), and human fibroblasts from gingiva and periodontal ligament cells (Agis et al., 2010). However, prolonged or localized acidification may favor the release and activation of the drug

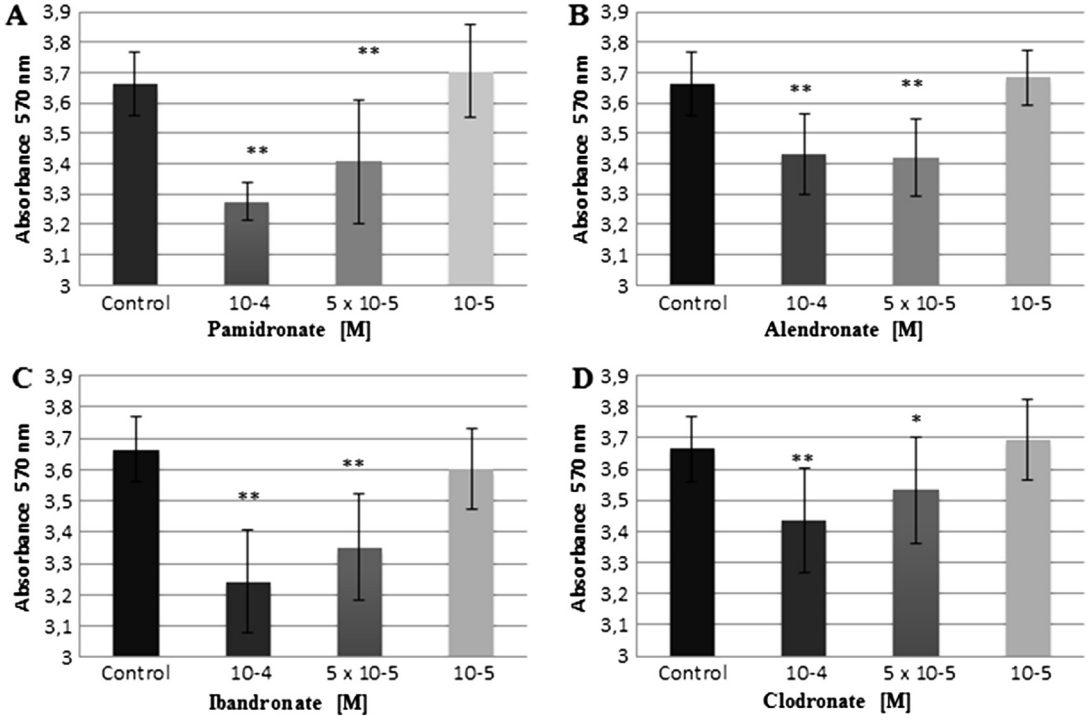

Fig. 1. Effect of four bisphosphonates at different doses (10^{-4}, 5×10^{-5}, 10^{-5} M) on osteoblast proliferation in MG-63 cell line after 24 h of incubation: A) pamidronate; B) alendronate; C) ibandronate; D) clodronate. Data are expressed as means ± SD. *p <0.05; **p <0.001.

Table 1
Percentage of cells in phase G0/G1, G2/M and S of cell cycle MG-63 cells treated for 24 h with 10^{-4}, 5×10^{-5}, or 10^{-5} M of pamidronate, alendronate, ibandronate, or clodronate, or without BP (control).

Treatment	% of cells in phase G0/G1		% of cells in phase G2/M		% of cells in phase S	
	Mean ± SD	*p*-value ≤	Mean ± SD	*p*-value ≤	Mean ± SD	*p*-value ≤
Control	33.07 (0.21)	–	12.46 (0.13)	–	54.45 (0.32)	–
Pamidronate						
10^{-4} M	43.23 (2.53)	**0.001**[a]	6.36 (2.8)	**0.02**[a]	50.41 (15.14)	0.68
5×10^{-5} M	36.78 (0.72)	**0.001**[a]	8.48 (2.68)	0.12	58.73 (2.86)	0.06
10^{-5} M	37.53 (2.48)	0.08	9.83 (2.14)	0.16	52.62 (0.68)	0.089
Alendronate						
10^{-4} M	37.36 (1.27)	**0.03**[a]	10.11 (1.93)	0.16	52.52 (2.13)	0.19
5×10^{-5}M	37.68 (1.74)	**0.04**[a]	12.01 (1.12)	0.56	50.3 (1.2)	**0.005**[a]
10^{-5}M	35.2 (1.25)	0.63	11.68 (0.66)	0.11	53.11 (1.81)	0.32
Ibandronate						
10^{-4} M	38.18 (2.13)	**0.025**[a]	11.09 (0.75)	**0.036**[a]	50.72 (4.96)	0.32
5×10^{-5}M	39.84 (4.24)	**0.049**[a]	9.51 (1.47)	**0.026**[a]	50.64 (5.42)	0.34
10^{-5}M	35.9 (3.7)	0.06	8.46 (3.09)	0.089	48.63 (3.54)	0.1
Clodronate						
10^{-4}M	42.77 (0.9)	<**0.001**[a]	5.54 (1.79)	**0.003**[a]	51.68 (0.89)	**0.007**[a]
5×10^{-5}M	50.91 (2.19)	**0.005**[a]	1.41 (1.67)	<**0.001**[a]	47.67 (2.86)	**0.001**[a]
10^{-5}M	36.28 (0.85)	0.064	8.99 (3.52)	0.053	47.72 (2.91)	**0.023**[a]

[a] Significant differences compared with control.

sequestered in the hydroxyapatite crystals of the bone, thereby activating the cascade of events that contribute to the development of BRONJ. This acidification can be produced by infections, hypoxia, co-medication, or microtraumas, among other causes (Otto et al., 2010a, 2010b).

Our results are in agreement with previous in vitro findings that BPs exert a pro-osteoblastogenic effect at lower concentrations (10^{-5} M to 10^{-9} M) and an inhibitory effect at higher concentrations (Im et al., 2004; Idris et al., 2008; Xiong et al., 2009; Kim et al., 2009) but the mechanisms underlying these effects are poorly understood. We also found that high doses of the studied BPs exerted their inhibitory effect on growth by arresting the cell cycle, inducing apoptosis in the cells, similar to the mechanism reported by Tenta et al. (2006) to underlie the effect on the MG-63 cell line of zoledronate, another nitrogen-containing BP. Furthermore, we observed that both nitrogen-containing and non-nitrogen-containing BPs had a toxic effect on the osteoblast through growth inhibition, although the effect of the non-nitrogen-containing BP was less potent. However, in a previous study by our group, low BP doses produced an increase in osteoblast proliferation in parallel with a reduction in their mineralization capacity (Manzano-Moreno et al., 2014). These findings were

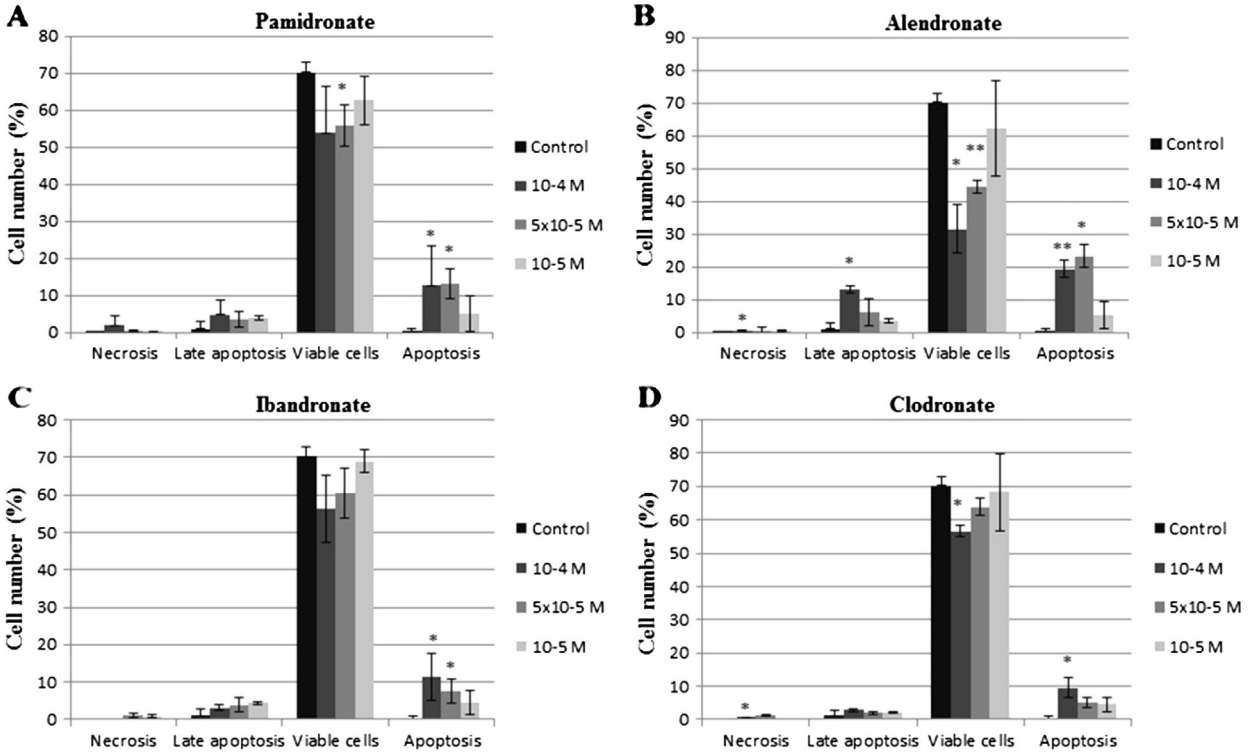

Fig. 2. Percentage of annexin V binding and propidium iodine (PI) uptake of MG-63 cells at 24 h of culture with bisphosphonates at different doses (10^{-4}, 5×10^{-5}, 10^{-5} M): A) pamidronate; B) alendronate; C) ibandronate; D) clodronate. Data are expressed as means ± SD. *p <0.05; **p <0.001.

consistent with the report by Koch et al. (2011) of an increase in osteoblast differentiation after the administration of 10^{-5} M zoledronate or ibandronate, although a reduced expression of differentiation markers was observed at low doses.

Bone is a highly complex tissue in which bone formation and bone remodeling processes go beyond osteoblast proliferation and differentiation and are regulated by a large number of paracrine, autocrine, and endocrine factors, including growth factors, hormones, and cytokines (Eriksen, 2010; Neve et al., 2011). Various authors have reported that BPs can modulate or regulate these factors, thereby affecting the functional capacity of the osteoblast. Thus, Naidu et al. (2008) found that high concentrations of alendronate and zoledronate reduced the viability of osteoblasts but increased TGF-β1 expression. TGF-β1 is known to play an important role in regulating and stimulating the differentiation of osteoprogenitors during fracture repair (Eingartner et al., 1999) and has been described as one of the growth factors responsible for the biostimulatory effect on osteoblast cells of various treatments (Wang et al., 2002; Chen et al., 2004). This growth factor has been found to enhance proliferation and promote osteoblast differentiation in its initial phase but inhibit its differentiation and maturation and suppress matrix mineralization in later phases (Gebken et al., 1999; Balooch et al., 2005). García-Moreno et al. (1998) evaluated the cytotoxic effect of alendronate on primary human osteoblasts and detected no viable cells at 48 h in culture at high concentrations and found a total inhibition of type I collagen synthesis. Pamidronate and zoledronate have also been associated with an acute reaction phase in some patients, who develop fever with an increase in circulating inflammatory cytokines such as interleukin-6 and tumor necrosis factor-α (Kinney et al., 2000; Boskey et al., 2002; Mohammad et al., 2009). It has been established that inflammation can inhibit bone regeneration capacity (Mundy and Bonewald., 1990; Balooch et al., 2005; Alliston, 2006).

The present data and previous findings suggest that high BP concentrations suppress osteoblast growth, inducing apoptosis as a consequence of changes in osteoblast physiology, possibly by preventing their differentiation through inhibition of type I collagen production, a vital component of bone extracellular matrix (Açil et al., 2012). This may result from changes in the expression of one or more of the three main receptors (types I, II, and III) by which TGF-β1 exerts its action on the osteoblast, given that BPs have been reported to increase TGF-β1 synthesis (Naidu et al., 2008; Jia et al., 2013), although the effect of BPs at the level of TGF-β1 receptors is unknown. Moreover, it is possible that the increase in TGF-β1 levels may inhibit osteoblast differentiation and maturation at bone level over the long term.

Numerous studies have associated mandibular osteonecrosis with BP treatment (Marx, 2003; Ruggiero et al., 2004; Boonyapakorn et al., 2008; Walter et al., 2010). The underlying mechanism has not been elucidated, but the lack of vascular supply or bone remodeling and/or regeneration have been implicated as possible causes. Bone tissue loses its capacity to remove bone areas that are unhealthy or have completed their useful life, limiting or reducing its functional adaptation capacity (Yépez-Guillén et al., 2013). In addition, data published by Stockmann et al. (2013) on cancer patients receiving intravenous BPs suggest that MHC class II polymorphisms are genetic risk factors for the development of BRONJ, in line with recent findings on the potential role of inflammation and infection in the pathogenesis of BRONJ.

The BPs most frequently related to BRONJ are those that contain nitrogen (Marx, 2003; Ruggiero et al., 2004; Walter et al., 2007; Marx et al., 2007), although the long-term administration of non-nitrogen-containing BPs such as clodronate can also produce BRONJ and give rise to an elevated accumulation of the drug in bone (Montazeri et al., 2007). The high BP doses reaching the bone in prolonged treatments may have dual adverse effects on the osteoblast: a direct cytotoxic effect, in which the cell dies by apoptosis; and an indirect effect, in which osteoblast differentiation and maturation are inhibited. Thus, the drug accumulated in the bone through its binding to hydroxyapatite can be released and therefore activated, mainly due to local factors such as an infection. High concentrations of the free drug may exert adverse effects on cell populations in the oral cavity, including osteoblasts, hence contributing to the onset of BRONJ (Otto et al., 2010a,b).

One study limitation is that the MG-63 human osteosarcoma cell line was used as the osteoblast model, although this is the most widely used cell line in studies on the effects of drug or other treatments on the osteoblast (Lee et al., 2013; Boanini et al., 2014; Mattioli-Belmonte et al., 2014). It would also be of interest to study the effects of these BPs on primary osteoblast culture lines or other typed osteoblast lines in order to verify the mechanism of action of these drugs on osteoblast growth.

5. Conclusion

In conclusion, the results of this study suggest that high doses of BPs reduce the proliferative capacity of osteoblasts by arresting their cell cycle and inducing apoptosis, explaining the impaired repair capacity of treated cells. Further studies are required to clarify the mechanisms underlying this effect and thereby increase understanding of the development of BRONJ. However, we think that BRONJ is a multifactorial disease in which the type of bisphosphonate and the dose of the drug plays a decisive role.

Conflicts of interest statement

The authors declare that they have no conflict of interest.

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