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Comparative effects of five bisphosphonates on apoptosis of macrophage cells *in vitro*

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ARTICLE INFO

Article history:
Received 27 July 2006
Accepted 21 September 2006

Keywords:
Bisphosphonates
Apoptosis
Necrosis
Macrophage
Flow cytometry

ABSTRACT

Bisphosphonates (BPs) inhibits bone resorption by reducing osteoclastic activity; they induce osteoclast apoptosis. Pathophysiology of prostheses loosening is complex and implies an inflammatory reaction secondary to the phagocytosis of wear debris by macrophages with a secondary increased bone resorption by osteoclasts. BPs inhibit proliferation and cause cell death in macrophages by induction of apoptosis. We have used mouse macrophage-like J774.1 cells to evaluate the effects of five BPs.

J774A.1 cells were cultured in a standard culture medium for 2-days. BPs (alendronate, pamidronate, etidronate, risedronate, zoledronic acid) were added in the medium at concentration of 10^{-6} to 10^{-4} M during 3 days. Cells were studied by fluorescence microscopy after staining with the fluorescent dye Hoescht H33342 and the percentage of apoptotic cells was determined on 300 nuclei. Cells were analyzed by flow cytofluorometry after staining with annexin V-FITC (for counting apoptotic cells) and propidium iodide (for necrotic/late-apoptotic cells) on 2000 cells.

Etidronate did not cause significant apoptosis or necrosis, at any concentration. Alendronate and pamidronate caused apoptosis and death only at very high concentration [10^{-4} M]. On the contrary, apoptotic and necrotic cells were evidenced with risedronate or zoledronic acid at lower concentrations. These effects were dose-dependant and occurred when concentration reached [10^{-5} M]. The number of apoptotic cells was higher with zoledronic acid and then with risedronate. Cytofluorometry appeared superior to cytologic analysis in the investigation of macrophage apoptosis, since necrotic cells loose contact with the glass slides and are not identifiable in cytological counts. Some amino-BPs appear to induce apoptosis in macrophages.

1. Introduction

Migration of polyethylene wear debris is recognized to induce periprosthetic bone loss after total hip arthroplasty. Particles are generated in the joint by daily joint motion that provoke erosion of the polymer and they can migrate far from the area where they are generated [1]. Particles are then phagocytosed by macrophages that release large amount of inflammatory cytokines such as TNFα, IL-6 or IL-1 [2]. These cytokines induce a local fibrotic process associated with osteoclastogenesis

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doi:10.1016/j.bcp.2006.09.031

leading to periprosthetic bone loss [3]. Inflammatory cytokines have also been found to activate osteoclast differentiation by the NFκB pathway *via* the RANKL–RANK factors in periprosthetic tissues [4,5]. It appears that macrophages play a key role in periprosthetic bone loss, leading progressively to aseptic loosening [6]. Bisphosphonates (BPs) are a group of pharmacological compounds that have powerful anti-osteoclast activity. They are now widely used in the treatment of various bone diseases where an increased osteoclastic activity is encountered (e.g., Paget bone disease, osteoporosis, fibrous dysplasia, myeloma and bone metastases) [7]. BPs formula is based on a P–C–P backbone, the geminal carbon having the potency to bind two radicals: R1 and R2. When R1 is a hydroxyl group, the binding of the compound to the hydroxyapatite of bone is increased. The R2 radical supports the antiosteoclastic effects. The first BP was etidronate, in which R2 was a methyl group ($-CH_3$). Increasing the length of R2 was found to considerably enhance the antiosteoclastic properties; the introduction of an amino group and a cyclic ring further increased the antiosteoclastic properties [8]. Several generations of BPs are now on the market with different antiosteoclastic activities.

There has been some *in vivo* and human trials of BPs to limit bone resorption due to polyethylene particles. The primary goal of these studies was to limit periprosthetic bone resorption by inducing osteoclast apoptosis. However, BPs have also been found to be active on other cell types: they present a proapoptotic activity on malignant plasma cells in myeloma [9], in breast adenocarcinomas and antiangiogenic properties have been reported for some aminobisphosphonates. It thus appears important to analyze the effect of these pharmacological compounds on macrophages (since their effects on osteoclasts have been well characterized by others) [10].

The aim of the present *in vitro* study was to use different types of commercially available BPs, in culture with a well defined macrophage cell line, to quantify their respective apoptotic power. These mechanisms were evaluated separately by cytological and cytometric analyses.

2. Materials and methods

2.1. Cell culture

The murine macrophage-like cells J774 A.1 was obtained from the European collection of animal cell cultures (Salisbury, UK). Cells were cultured in Dulbecco's modified Eagle's medium (Eurobio, France) containing 10% heat-inactived fetal calf serum (Seromed-Strasbourg-France), 10 U/ml penicillin, 100 μg/ml streptomycin and 1 mM L-glutamine, in a 5% CO_2 atmosphere.

2.2. Reagents

Stock solutions (10^{-2} M) were prepared in PBS, pH 7.4 and filter-sterilized using a 0.2 μm filter and stored at −20 °C until use.

Etidronate (ETI), alendronate (ALN) and risedronate (RIS) were provided by Procter and Gamble Pharmaceuticals (Cincinnati, OH, USA). Zoledronic acid (ZOL) and pamidronate (APD) were provided by Novartis Pharma AG (Basel, Switzerland). All bisphosphonates were added in the culture medium at concentration of 10^{-6}, 5×10^{-6}, 10^{-5}, 5×10^{-5} and 10^{-4} M.

2.3. Fluorescence microscopy

Cells were seeded onto 24-wells plates (Costar, Cambridge, MA) on glass slides at a density of 2×10^4 cells/well and let overnight to adhere. The medium was replaced with a freshly prepared one, containing one of the BPs (ETI, ALN, APD, RIS, ZOL) or an equivalent volume of PBS. Cultures were maintained during 3 days. Cells were analyzed for apoptosis, following visualization of nuclear morphology with the fluorescent DNA-binding dye Hoescht H33342. The monolayers were fixed in a mixture composed of ethanol 95°/acid acetic (3:1, v/v), rinsed with PBS and then incubated with 2 μg/ml Hoescht H33342 for 10 min. After rinsing in PBS, the slides were mounted in a high viscosity aqueous medium (Apathy's sirup) and observed using a fluorescent microscope. An average of 300 nuclei was analyzed in each case on random fields. All experiments were done in triplicate.

2.4. Flow cytofluorometry

Cells were seeded onto 6-wells plates (Costar, Cambridge, MA) at a density of 5×10^4 adherents cells/well and let overnight to adhere. The medium was replaced with the same medium containing one of the BPs (ETI, ALN, APD, RIS, ZOL) or an equivalent volume of PBS and treated in triplicate for up to 3 days. Adherent and non-adherent cells were harvested by scrapping and pooled for quantitation of apoptosis or death according to the method of Vermes et al. [11]. The cells were washed twice with cold PBS and then resuspended in a buffer. For each concentration of BPs, the cells were dispatched onto 96-well plates and processed with one of the following treatment: no staining, staining with annexin V-FITC (BD Biosciences Pharmingen™, San Jose, CA), staining with propidium iodide (Sigma P4170), staining with annexin V-FITC and propidium iodide. Stainings were done by incubation for 15 min at room temperature (20–25 °C) in the dark. Analysis by flow cytometry was done within 1 h after staining. The respective proportions of apoptotic cells and necrotic/late-apoptotic cells were measured on 2000 cells with a FACScan cytofluorometer (Beckton Dickinson, Mountainview, CA, USA). Annexin V-FITC was used as a marker for the identification of apoptotic cells; annexin V-FITC plus propidium iodide labeled the necrotic/late-apoptotic cells.

2.5. Statistical analysis

The statistical analysis was performed using the Systat® statistical software, release 11. (Systat, Inc.). Results were expressed in mean ± the standard error of mean (S.E.M.). A variance analysis (ANOVA) followed by a Bonferroni's post hoc test was used to test the existence of significant differences between treatments. A difference was regarded as statistically significant for a value $p < 0.05$.

3. Results

3.1. Microscopic analysis

J774A.1 are highly adherent cells that appeared firmly attached onto the glass slides. Cells that are undergoing apoptosis demonstrated nuclear condensation (a mechanism related to the DNA fragmentation at the molecular level) that can be easily identified by the Hoechst 33342 staining under fluorescence microscopy (Fig. 1). Mitotic figures were also easily identified but were not taken into account during measurements. An insignificant increase in apoptotic cell count was found for the highest concentrations of ETI (10^{-4} and 5×10^{-5} M). With amino-BPs, apoptosis was evidenced at much lower concentrations (Fig. 2). RIS was found to be the most pro-apototic compound since a significant increase was found as from 5×10^{-6} M. ZOL and APD were found to present a noticeable apoptotic potency but when high drug concentrations were used, no cell could be found at the surface of glass slides, indicating a complete loss of adherence and detachment.

3.2. Flow cytofluorometry

The annexin-V binding to phosphatidyl serine translocated to the outer side of the plasma membrane characterizes an early event in the apoptosis pathway [11]. The annexin-V-FTIC labeled cells, which were also propidium iodide negative, were considered as early apoptotic cells; they were separated on the flow cytometric dot plots. Similarly, the double positivity for propidium iodide and annexin-V-FTIC characterized necrotic or late-apoptotic cells that are evidenced in another gate on the scattergrams (Fig. 3) [12]. The five BPs exhibited very different activities on the J774A.1 cells (Fig. 4). ETI presented a small but significant increase in the number of necrotic/late-apoptotic cells for the highest concentrations. ALE significantly increased early apoptosis at the highest concentration and necrotic/late-apoptotic cells became significantly more numerous than in controls as from 5×10^{-6} M. The effects were more pronounced with RIS which induced early apoptosis as from 5×10^{-5} M concentration and necrosis/late-apoptosis was significantly higher at 5×10^{-6} M. APD induced necrosis as from 5×10^{-6} M but the increase remained modest even at the highest 10^{-4} M concentration. ZOL was found to induced early and late-apoptosis at 10^{-5} M and the highest values for early apoptosis and necrosis were obtained at 10^{-4} M.

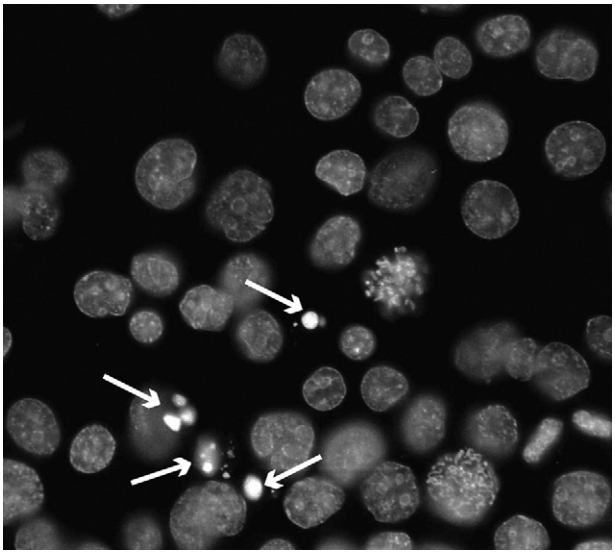

Fig. 1 – J774 cells stained with Hoechst 33342 and examined under fluorescence microscopy. The apoptotic bodies are easily identified (→). Original magnification 1000×.

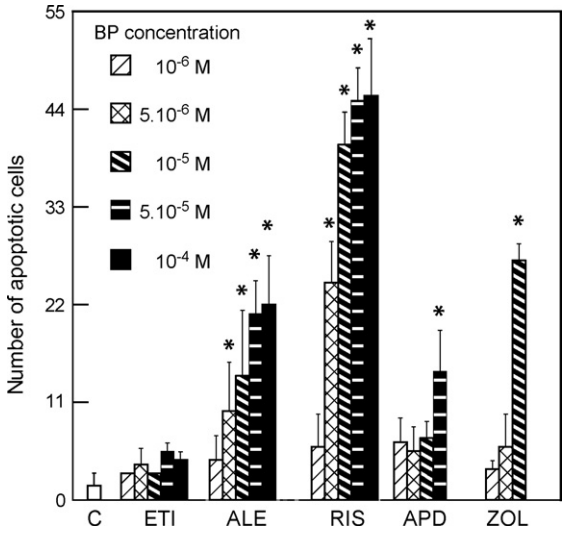

Fig. 2 – Cytologic effect of the five bisphosphonates after Hoechst 33342 staining in presence of various BPs concentration (* = amount of apoptotic cells significant from control cultures without BP (C) $p > 0.05$).

4. Discussion

In the present study, differences were noted between cytology and cytofluorometry in the potency for BPs to induce apoptosis in macrophages. Cytological analysis was based on the identification of condensed apoptotic nuclei in these adherent cells. This phenomenon is considered as an early cytologic event in the apoptotic cascade before contact loosening [13,14]. However, changes in the plasma membrane are one of the earliest identifiable feature at the molecular level. In apoptotic cells, the membrane phospholipid phosphatidylserine is tranlocated from the inner to the outer leaflet of the plasma membrane, thus exposing phosphatidylserine to the external cellular environment [11,15]. When J774 apoptotic cells were counted with Hoechst H33342, the apoptotic cells that had detached from the glass slides could not be taken into account. RIS appeared to be the most potent BPs since almost all cells seemed to have been removed from the glass slides with ZOL and APD at high concentrations. In the cytofluorometric assay, early apopotic cells were positive for annexin-V and negative for propidium iodide; necrotic/late-apoptotic cells were double positive. In this condition, this analysis appeared superior in the study of macrophage cells since both types of populations could be identified at the same time (together with living cells).

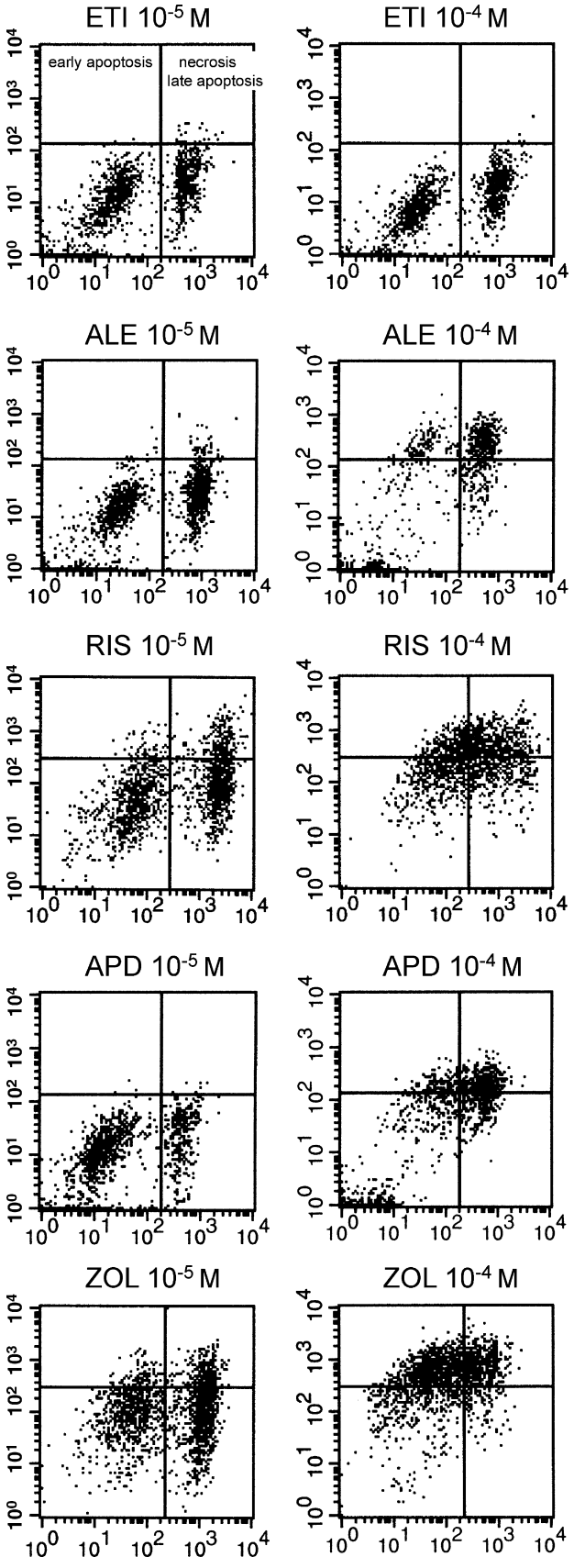

Fig. 3 – Cytofluorometric assay with the annexin V-prodidium iodide. The annexin-V positive cells are illustrated in the upper left dial on the scatterplot and correspond to early apoptotic cells. Necrotic/late-apoptotic cells are on the upper right dial. The scattergrams are prodidec for etidronate (ETI), risedronate (RIS) and zoledronic acic (ZOL) at 10^{-5} and 10^{-4} M.

Two classes of BPs exist and are based on differences in the structure of the R2 chain on the geminal carbon. Amino-BPs (ALN, RIS, APD, ZOL) have a basic nitrogen-containing moiety in the R2 side chain. Non-amino-BPs (such as ETI) do not contain an amino group in the side chain. Although both groups of BPs are effective in the bone-resorbing activity, it has been shown that there are different mechanisms of action at the molecular level [8,16–18]. Because the monocyte/macrophage is a precursor cell for osteoclast when activated by M-CSF (macrophage colony stimulating factor) and RANKL [19], it was important in this study to compare the effect of BPs on macrophages *in vitro*. Amino-BPs prevent the post-translational prenylation of small GTPases by a potent inhibition of the farnesyl pyrophosphatase (FPP) [20–22]. This enzyme plays a key role in the cholesterol synthesis (mevalonate pathway) and is essential for the synthesis of small GTPase signalling proteins, that are vital for function and survival [23]. The amino-BPs inhibit FPP in the following order of potency: ZOL > RIS > ibandronate > ALE; clodronate and ETI (non-amino BPs) do not have any significant inhibitory effect [24]. On the other hand, non-amino BPs can be metabolically incorporated into a nonhydrolyzable analog of ATP that accumulates in the cytoplasm of osteoclasts [25] and induces osteoclast apoptosis [26] by inhibiting the mitochondrial adenine nucleotide translocase (ANT) [27]. Recently, amino-BPs were also found to increase the synthesis of an ATP analog (Appp1). Appp1 is converted into AMP and IPP (isopentenyl diphosphate); IPP is also a strong inhibitor of ANT. It appears that the most recent amino-BPs can induce apoptosis by both inhibition of FPP and excess production of IPP [28]. The efficiency of Appp1 production with different BPs was found identical to their order of potency inhibition of FPP with ZOL > RIS > ALE > clodronate. In the present study, the effect of the various BPs was found similar this classification, when considering the cytofluorometric assay where the efficiency (combining early and late-apoptosis) was ZOL > RIS > ALE > APD > ETI. ETI has been found to have no effect on FPP [23]. We are not aware of comparisons between APD and ZOL in their respective efficiency on FPP; both BPs are used by infusion for the treatment of malignant hypercalcemia and tumor-induced osteolysis.

J774A.1 cells have been largely used in the study of apoptosis induced by BPs since this cell line exhibits several characteristics of osteoclasts including the possibility to form polykarions, to express TRAcP (tartrate resistant acid phosphatase) and to resorb particules or biomaterials [29]. This cell line provides similar results than THP-1 human monocytic leukaemia cell lines that are sometimes used in the study of particles [12,30]. In addition, the J774A.1 assay is the reference cell line used for the detailed analysis of bisphosphonate metabolism [31]. These cells also react with wear debris (polyethylene or metal) and have been extensively used to study the effects of particles or metal ion that can induce apoptosis or necrosis in the vicinity of prostheses [32,33]. BPs can limit osteolysis induced by wear debris in animal models

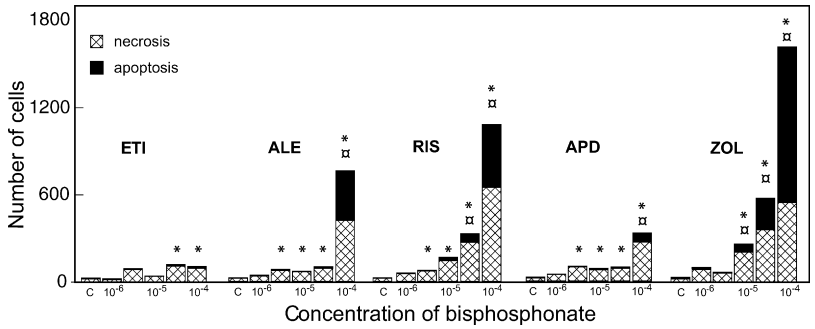

Fig. 4 – Cytofluorometric assay with the annexin V-prodidium iodide. The early apoptotic (■) and necrotic/late-apoptotic cells (⊠) are figured separately for etidronate (ETI), alendronate (ALE), risedronate (RIS), pamidronate (APD) and zoledronic acic (ZOL) at various concentrations ranging for control (C) to 10^{-4} M. * = amount of necrotic/late-apoptotic cells significant from C $p > 0.05$; (¤) = amount of early apoptotic cells significant from C $p > 0.05$.

(after polyethylene particles injection) [34,35] and favourable results have also been presented in men on a limited number of patients [36,37]. However, if BPs are to be proposed for preventing prosthesis loosening, the mode of administration may differ from those proposed in the treatment of metabolic or malignant bone diseases. In these cases, BPs bind to the mineral phase of the bone matrix and they exert their apoptotic effect only when osteoclasts have internalised the compound. The intracellular concentration increases up to 10^{-3} M [31]. Dosages of BPs in the blood seem compatible with the results presented here: a 40 mg dose of pamidronate induces a 2 μg/ml blood level [38]. A 30 mg oral dose of risedronate is associated with a 1 ng/ml blood dosage during at least 8 h [39]. However, comparisons between *in vivo* and *in vitro* dosages are difficult. With the two amino-BPs ZOL and APD, intra-venous infusion is the only way of administration. A single infusion of 4 mg ZOL per year was reported to be effective in the treatment of osteoporosis [40]. Due to the high capacity of ZOL to bind bone hydroxyapatite and its superior apoptotic power on osteoclasts, this regimen could be suitable for osteoporosis; however, it is likely that serum concentration of the BP will not be sufficient to induce macrophage apoptosis over such a long period. With RIS or ALE, various oral regimens are available as daily, weekly and monthly dosing formulations with equal results on fracture prevention [41]. The daily formulation could be interesting to have sufficient and regular serum concentrations, suitable for inducing the apoptosis of activated macrophages around prostheses [39]. *In vivo* experiments in animal models of particles osteolysis are necessary to test this hypothesis. Amino-BPs are pharmacological compounds that appears active on both osteoclasts and macrophages. However, their potency differ and the mode of administration could not be the same in the prevention of prosthesis loosening than in osteoporosis.

Acknowledgments

This work was supported by funds from ''Pays de la Loire''—Axe Biomatériaux and INSERM.

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