Advanced Glycation Endproducts and Alendronate Differentially Inhibit early and Late Osteoclastogenesis In vitro

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Abstract

Advanced Glycation Endproducts (AGEs) are greatly elevated in bone extracellular matrix of patients with Diabetes mellitus, and this has been associated with the increased incidence of fractures observed in these patients. AGEs affect the homeostasis of bone cells such as osteoblasts and osteoclasts. Bisphosphonates are first-line anti-osteoporotic drugs that principally exert their effects by inhibiting osteoclastic activity. However, the effect of bisphosphonate treatment on bone quality in patients with Diabetes is uncertain. In the present work we have evaluated the action of AGEs (50-200 μg/ml), with or without Alendronate (10⁻⁶-10⁻⁴M), on osteoclastogenesis induced by co-cultures of Raw 264.7 macrophages and UMR106 osteoblasts. We determined the effects of different culture conditions on osteoclastic recruitment, tartrate-resistant acid phosphatase (TRAP) activity and expression of RAGE (receptor for AGEs); and on the osteoblastic expression of RANK ligand (RANKL). AGEs and Alendronate inhibited the recruitment and TRAP activity of osteoclasts, with an additive effect of both agents at high concentrations of Alendronate. While AGEs prevented the early and late stages of osteoclastogenesis, Alendronate (alone or in co-incubation with AGEs) only inhibited its later stages. In addition, both AGEs and Alendronate increased the osteoclastic expression of RAGE and decreased the osteoblastic expression of RANKL, correlating closely with their inhibition of osteoclastogenesis. If these in vitro results can be extrapolated to a clinical setting, they may be indicating a potentiation of the anti-resorptive effects of Alendronate in the context of bone extracellular matrix with excess accumulation of AGEs, as might occur in a patient with Diabetes mellitus.

Keywords: Advanced glycation endproducts; Osteoclasts; Alendronate; RAGE; RANKL

Introduction

Advanced Glycation Endproducts (AGES) are post-translational, non-enzymatic and irreversible modifications of proteins that can alter their bioactivity. AGEs have been strongly implicated in the pathogenesis of the more prevalent chronic complications of Diabetes mellitus such as atherosclerosis, nephropathy, retinopathy and neuropathy [1]. More recently, their role in diabetic osteopathy has begun to be analyzed. Levels of AGEs are greatly elevated in the bone extracellular matrix of patients with Diabetes mellitus, and this accumulation of AGEs has been associated with the increased fracture rates observed in patients with both types of Diabetes [2,3]. In addition, AGEs have been found to accumulate with physiological ageing in various tissues including bone, and so they have also been implicated in the development of osteoporosis in elderly non-diabetic individuals [4,5].

AGEs can exert deleterious effects on bone-derived cells: they decrease the osteogenic potential of bone marrow mesenchymal cells [6] and inhibit the bone-forming capacity of osteoblasts [7]. Two apparently contradictory studies also indicate that AGEs can directly regulate osteoclastic activity. One study shows that short-term exposure to AGEs can activate osteoclast-mediated bone resorption [8], whereas the other study shows the opposite effect albeit after a longer exposure to extracellular AGEs [9].

Many cellular effects of AGEs occur due to the interaction of these moieties with specific receptors such as RAGE. This receptor is expressed in osteoblasts [10] and osteoclasts [9], and its occupation by AGEs has been postulated to reduce bone remodelling and collagen turnover, thus facilitating the accumulation of more AGEs in bone extracellular matrix [9] that could contribute to the decrease in bone quality associated with ageing and Diabetes mellitus.

Osteoclasts arise from hematopoietic stem cells that, in the presence of the receptor activator of nuclear factor κB (RANK) ligand (RANKL) and macrophage-colony stimulating factor, undergo differentiation and fusion resulting in large multinucleated cells characterized by expression of a series of osteoclast markers, such as tartrate-resistant acid phosphatase (TRAP) [11]. This enzyme, in the context of bone, is a specific and sensitive osteoclastic marker permitting accurate assessment of the abundance of this cell type. RANKL is expressed on the surface of osteoblast-lineage cells, and functions by interacting with its receptor RANK on osteoclast precursors [12].

Bisphosphonates (BP) are first-line anti-osteoporotic drugs that mainly exert their effects by inhibiting osteoclastic recruitment and activity, and secondarily by increasing osteoblast bone-forming activity. Part of these actions may take place by reduction of RANKL expression by osteoblasts and/or bone marrow stromal cells, which in turn decreases osteoclastogenesis and bone resorption [13].

Although the use of BP such as Alendronate has been recommended by some authors for treatment of diabetic osteopathy, this could potentially pose a problem since Diabetes mellitus is...
generally associated with low bone turnover [14]. Thus in this context, BP treatment could further reduce bone remodelling, increase AGEs accumulation and decrease bone quality. In an attempt to address this issue, we previously evaluated the combined in vitro actions of Alendronate and AGEs on bone-forming cells, and demonstrated that Alendronate is able to prevent the anti-osteogenic and pro-apoptotic effects of AGEs on osteoblasts [15]. Additionally, the results of two recent clinical studies support the use of this BP in diabetic patients: Keegan et al. reported that a 3-year treatment with Alendronate was associated with increased BMD in older women with type 2 Diabetes mellitus [16], whereas Ikeda and Iwata showed that treatment with this BP for 5 years inhibited the decrease in radial BMD induced by Diabetes [17]. However, there is still a concern regarding the effects of BP treatment on bone quality in diabetic patients, and this can only be addressed by evaluating fracture incidence in controlled studies.

Although the mechanisms of action of BP on osteoclasts are known with precision [18], their effect on this cell type in the presence of excess AGEs is not. The main hypothesis of our present study was that the inhibitory effect of Alendronate on osteoclastogenesis could be potentiated in the presence of AGEs. In order to test this hypothesis, we investigated the combined effect of AGEs and Alendronate on the recruitment and differentiation of osteoclastic cells obtained by coculture, and on the expression and sub-cellular distribution of RANKL and RAGE.

Materials and Methods

Materials

Alendronate [1-hydroxy-3-aminobutylidene-1,1-bisphosphonic acid] was provided by Elea Laboratories (Argentina); Dulbecco’s modified Eagle’s medium (DMEM), trypsin-EDTA and foetal bovine serum (FBS) were obtained from Gibco (Invitrogen, Buenos Aires, Argentina). Tissue culture disposable material was from Nunc (Tecnolab, Buenos Aires, Argentina). D-glycolaldehyde, D-glycoaldehyde, Triton X-100 were obtained from Sigma-Aldrich (Buenos Aires, Argentina). 4% paraformaldehyde and 412 mM acetic acid, 0.209% Triton X-100, 412 mM NaCl, 4.12 mM EDTA, 10.6 mM ascorbic acid, 10.1 mM 4-nitrophenylphosphate, 41.6 mM Na2tartrate at pH: 5.5) at 37 °C for 1 hour, after which the reaction was stopped with 100 µl of 300 mM NaOH and the product measured at 405 nm [9]. Another aliquot of each extract was used for protein determination by Bradford’s technique [22]. Results are expressed as TRAP specific activity (nmol p-NP/ mg protein x min).

Preparation of advanced glycation endproducts

Advanced glycation endproducts-modified (AGEs) bovine serum albumin (BSA) was prepared by incubation of 10 mg/ml BSA with 33 mM D-glycolaldehyde in 150 mM phosphate-buffered saline pH 7.4 at 37°C for 3 days under sterile conditions [19]. D-glycolaldehyde was used as the glycatin sugar instead of glucose to speed up non-enzymatic glycosylation. Control BSA was incubated in the same conditions without sugar. The unincorporated sugar was removed by centrifugation/filtration with Centricon filter cartridges. The formation of AGEs was assessed with a LUMEX Fluorat®-02-Panorama spectrofluorometer (St. Petersburg, Russia), by their characteristic fluorescence-emission maximum at 420 nm upon excitation at 340 nm. The estimated level of AGEs obtained in this in vitro incubation was 18.5 % relative fluorescence intensity/mg protein, in contrast to 3.2 % for control BSA.

Cellular cultures

Co-culture of raw 264.7 and UMR106 cells: As a model system of osteoclastogenesis, we used a co-culture of the Raw 264.7 a murine monocyte/macrophage cell line with UMR106 rat osteosarcoma cells [20]. For different experiments, Raw 264.7 and UMR106 were co-cultured on 6 or 24-well plates in DMEM supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% FBS, with or without different doses of AGEs (50, 100, 200 µg/ml) and/or Alendronate (10⁻¹M, 10⁻²M, 10⁻³M, 10⁻⁴M). The culture medium was replaced every 48 h. Co-cultures were grown for 8 days.

Osteoblast cultures: The UMR106 rat osteosarcoma-derived cell line has been shown to conserve certain characteristics of differentiated osteoblastic phenotype [21]. In order to assess RANKL expression in UMR106 osteoblasts, cells were seeded on 6-well plates and incubated in DMEM with different doses of BSA or AGEs, with or without Alendronate, for the periods of time indicated in the legends of figures. At the end of the culture period, the monolayer was evaluated by indirect immunofluorescence to determine RANKL expression.

Quantitation of the development of multinucleated giant cells

Co-cultures grown in 24-well plates and submitted to different experimental conditions were washed with PBS and solubilized in 0.1% Triton X-100. An aliquot of the total cell extract was used to assess TRAP activity. For this, each aliquot was incubated in the reaction buffer (412 mM acetic acid, 0.209% Triton X-100, 412 mM NaCl, 4.12 mM EDTA, 10.6 mM ascorbic acid, 10.1 mM 4-nitrophenylphosphate, 41.6 mM Na2tartrate at pH: 5.5) at 37°C for 1 hour, after which the reaction was stopped with 100 µl of 300 mM NaOH and the product measured at 405 nm [9]. Another aliquot of each extract was used for protein determination by Bradford’s technique [22]. Results are expressed as TRAP specific activity (nmol p-NP/ mg protein x min).

Expression of RAGE and RANKL by immunofluorescence analysis

Cells grown on coverslips in 6-well plates were washed in cold PBS, fixed with 4% paraformaldehyde in PBS (10 minutes at room temperature), and permeabilized with methanol for 4 minutes at –20°C [23]. Nonspecific binding sites were blocked with 1% BSA in PBS for 2 hours. Cells (co-cultures or osteoblasts) were then incubated with anti-RAGE or anti-RANKL antibody (1:300 in blocking buffer) overnight at 4°C. After washing, cells were exposed to a FITC-conjugated secondary antibody (1:200) for 1 hour at room temperature. Coverslips were mounted in PBS-glycerol and observed with a fluorescence confocal Leica TSC SP5 AOBs microscope for visualisation of sub-cellular distribution for each specific protein. Images were analysed using Image J program.
Statistical analysis

The results are expressed as the mean ± S.E.M. and were obtained from three separate experiments performed in triplicate. Differences between groups were assessed by One-way ANOVA with Tukey post-hoc test. For non-normally distributed data, non-parametrical Kruskal Wallis with Dunn post-hoc test was performed using GraphPad in Stat version 3.00 (Graph Pad Software, San Diego, California, USA). A p-value < 0.05 was considered statistically significant.

Results

Effect of AGEs and alendronate on osteoclast recruitment and differentiation

To evaluate the possible action of AGEs and Alendronate on osteoclastogenesis, we used a co-culture model with Raw 264.7 macrophages and UMR106 osteoblastic cells, evaluating TRAP activity after 8 days. As can be seen in Figure 1, this co-culture induced the formation of giant multinucleated TRAP (+) osteoclasts. When TRAP enzymatic activity was quantified in co-culture lysates as described in Materials and methods, this marker of osteoclast differentiation was decreased after exposure to AGEs in a dose-dependent manner (10-30% reduction versus control BSA) (Figure 2A). We also investigated the effect of different doses of Alendronate (included in the culture media together with either BSA or AGEs) on osteoclastic differentiation. For these experiments we chose a dose of 100 µg / ml of AGEs, which on its own reduces TRAP activity by 13% (Figure 2A). As can be seen in Figure 2B, low doses of Alendronate (10^{-5}M-10^{-4}M) co-incubated with BSA induced a significant decrease in TRAP activity (about 10% reduction versus BSA alone). However when these low doses of Alendronate were co-incubated with AGEs, TRAP activity showed no difference versus AGEs alone. High levels of Alendronate (10^{-3}M-10^{-2}M) also reduced TRAP activity, although in this case both when co-incubated with BSA (25% decrease versus BSA alone) or with AGEs (17% decrease versus AGEs alone).

AGEs and Alendronate additionally affected the recruitment (i.e., number) of multinucleated cells. As can be seen in Figure 3B, the average number of osteoclasts per field was significantly inhibited by the presence of AGEs in the culture media (45% reduction versus BSA alone). Figure 3A shows representative fields of TRAP-stained macrophage/osteoblast co-cultures, incubated either with BSA (left) or with AGEs (right), in which inhibition of the number of multinucleated cells by AGEs can be observed. Alendronate, when co-incubated with BSA, also induced a significant and dose-dependent decrease in the number of osteoclasts. However when Alendronate was co-incubated with AGEs, only high doses (10^{-3}M) of this BP induced an additional effect on the inhibition of osteoclastic recruitment (16% decrease versus AGEs alone), whereas low doses did not.

Figure 1: Environmental-Scanning Electron Microscopy (E-SEM) images of co-cultures of Raw 264.7 macrophages and UMR106 osteoblastic cells. Left: Image showing a dark giant cell (osteoclast) and smaller high-contrast Raw 264.7 cells (1400x). Middle: Magnification of the same image showing the osteoclast (3000x). Right: Osteoclast stained by TRAP cytochemistry (Obj. 40x).

Figure 2: Effect of AGEs and/or alendronate on osteoclastogenesis. (A) UMR-106 osteoblastic cells and Raw 264.7 macrophages were co-cultured for 4 days until formation of multinucleated cells. They were then incubated with different doses (50 µg/ml, 100 µg/ml or 200 µg/ml) of BSA or AGEs for an additional 4 days. TRAP activity was determined in cell lysates by a colorimetric method. Results are expressed as % of basal (BSA alone), and are shown as the mean ± S.E.M. (* p<0.05, ** p<0.01). (B) UMR-106 osteoblastic cells and Raw 264.7 macrophages were co-cultured for 4 days until formation of multinucleated cells. They were then incubated for an additional 4 days with 100 µg/ml of BSA or AGEs, and/or different doses of alendronate (10^{-3}M - 10^{-2}M). TRAP activity was determined in cell lysates by a colorimetric method. Results are expressed as % of basal (BSA alone), and are shown as the mean ± S.E.M. (# p<0.001 versus BSA alone) ($\# p<0.001$ versus AGEs alone).

AGEs and alendronate inhibit different stages of osteoclastic induction

To evaluate whether AGEs and/or high doses (10^{-3}M) of Alendronate preferentially affect early or late osteoclastogenesis, these agents were added to the co-culture at different time points. As can be seen in Figure 4, AGEs significantly inhibited osteoclastic TRAP activity at all time points. On the other hand, high-dose Alendronate only appears to decrease osteoclastogenesis in its later stages since its inclusion in the culture media during the first four days of co-culture did not induce an inhibition of TRAP activity, both when co-incubated with BSA or with AGEs. However, the addition of Alendronate to the culture media during the last four days of co-culture significantly inhibited osteoclastic TRAP activity (25% decrease versus BSA alone, and 30% reduction versus AGEs alone).

AGEs and alendronate modulate RAGE expression in osteoclastic cells

The effect of AGEs and/or Alendronate on RAGE expression was evaluated by indirect immunofluorescence. The inclusion of AGEs in the co-culture media (Figure 5Ad) induced a significant increase in osteoclastic RAGE-associated fluorescence (152% versus BSA alone).
(Figure 5B). Similarly, inclusion of low doses (10^-5M) of Alendronate plus BSA (Figure 5Ab) induced a significant increase in RAGE expression (130% versus BSA alone). However, high doses (10^-3M) of Alendronate plus BSA did not alter the osteoclastic expression of RAGE (Figure 5Ac and B).

On the other hand, although the AGEs-induced up-regulation of RAGE was unmodified by low doses of Alendronate (Figure 5Ac and B), it was completely blocked in the presence of high doses of Alendronate (Figure 5Af and B).

**AGEs and alendronate modulate RANKL expression in osteoblastic cells**

We next investigated the modulation of RANKL expression (by immunofluorescence) in osteoblasts cultured with AGEs and/or Alendronate, as a possible mechanism by which these agents might be regulating osteoclastic recruitment and differentiation. As can be seen in Figure 6, 100 µg / ml AGEs significantly inhibited osteoblastic RANKL expression (50% versus BSA alone). Similarly, Alendronate (10^-3M-10^-2M) dose-dependently inhibited osteoblastic RANKL-associated fluorescence (65-47% versus BSA alone). However, when osteoblasts were co-incubated with AGEs and Alendronate (10^-3M-10^-1M) no further inhibition of RANKL expression was observed (59-45% versus BSA alone).

**Discussion**

N-containing bisphosphonates (BP) are one of the most widely used classes of anti-osteoporotic drugs. Although their primary action is to inhibit osteoclastic functionality and survival, they have also been found to promote osteoblastic development and osteogenic potential. At present the prescription of BP in patients with diabetic osteopathy has not been firmly recommended, since results of clinical studies tend to be contradictory [24,25,14]. These contradictions may be due to different causes, and since AGEs are greatly increased in the extracellular matrix of bone tissue in patients with poorly compensated Diabetes mellitus [2], one of these causes could be possible interactions between the effects of BP and AGEs on bone cells.

Although the mechanisms of action of BP on osteoclasts have been firmly established, the effect of AGEs on this cell type has been poorly defined and published reports are controversial [8,9]. Using unfractinated mouse bone marrow cultures that included osteoclasts among other cell types, Miyata and co-workers found an increase in resorption pits (but not in osteoclast number) when the cultures were incubated for 4 days on AGEs-modified dentin slices. In the other published report on this issue, Valcourt et al. found that both human and rabbit osteoclasts, when cultured on ivory slices modified with pentosidine (an established AGEs structure), transiently increased their resorptive action after 3 days but were greatly inhibited if cultures were extended to 8 days [9].

In our present study with macrophage/osteoblast co-cultures, we
have found that soluble extracellular AGEs dose-dependently inhibit osteoclast number and TRAP activity, in both the early (4 days) and later (8 days) stages of osteoclastogenesis. Our results for 8-day cultures agree with those of Valcourt et al. the differences observed in the early stages of osteoclast induction could be due to the fact that we used soluble AGEs in the culture media instead of growing cells on an AGEs-modified mineralized substratum [9].

In further experiments we set out to evaluate the effect on osteoclastic induction and TRAP activity, of different doses of the N-containing BP Alendronate alone or in combination with soluble AGEs. On its own, Alendronate inhibited both parameters of osteoclastogenesis. This was observed both at low doses of the BP (10⁻⁸M-10⁻⁶M, such as might be expected in the extracellular fluid of a patient treated with Alendronate) and at high doses of the BP (10⁻⁵M-10⁻⁴M, similar to the concentration that can be attained in resorption pits). We also found that Alendronate inhibited osteoclastogenesis in its later stages, but not in its early stages. When we co-incubated Alendronate with AGEs we found that this BP was able to potentiate the inhibition of osteoclast induction by AGEs, although only at high doses of Alendronate and in the later stages of osteoclastogenesis. However, these are exactly the conditions that would be expected in the resorption pits of a patient with Diabetes who is treated with Alendronate, thus theoretically inducing an even greater inhibition of their associated osteoclasts.

Diverse actions of AGEs can be mediated by specific cell-surface receptors such as RAGE. This protein is expressed in many cell types, and bone cells such as osteoblasts [10] and osteoclasts [9] are no exception. Additionally, AGEs-RAGE interaction has been found to directly modulate osteoclastic recruitment and function [26]. Based on these observations, in the present study we evaluated by immunofluorescence whether exposure to AGEs and/or Alendronate could induce changes in osteoclastic RAGE expression. As previously described by other authors, incubation with AGEs alone induced an up-regulation of RAGE in this cell type. Low-dose Alendronate on its own also increased RAGE expression, but when co-incubated with AGEs it did not induce further changes in RAGE-associated fluorescence. High-dose Alendronate alone did not modify the expression of RAGE in osteoclasts, although when this dose of BP was co-incubated with AGEs it completely prevented the up-regulation of RAGE induced by AGEs.

Altogether, our present results suggest that the inhibitory effects of low-dose Alendronate on osteoclastogenesis might be partially mediated by changes in the expression of RAGE. On the other hand and in the presence of AGEs, high-dose Alendronate shows an inhibitory effect that does not depend on the modulation of RAGE expression.

An indirect mechanism by which AGEs and/or Alendronate could
inhibit osteoclast recruitment and differentiation is by down-regulation of RANKL in osteoblasts/stromal cells. In experiments with UMR106 osteoblastic cells, we found that the expression of RANKL was greatly reduced by AGEs alone or by Alendronate alone (in this case dose-dependently), although co-incubation with both agents did not further inhibit RANKL. This decrease in osteoblastic RANKL expression closely correlated with the inhibition of osteoclastogenesis induced by AGEs and/or Alendronate. Other authors have found opposite effects of AGEs on the expression of RANKL, however for their study they used primary bone explant cultures that include different cell types [27]. On the other hand, our results for RANKL inhibition by Alendronate are in close agreement with other studies that report similar in vitro results for osteoblasts of human and rat origin [28-30]. Additionally, a recent clinical study has demonstrated that Alendronate treatment of women with post-menopausal osteoporosis reduces the expression of RANKL by osteoblasts and/or bone marrow stromal cells, which in turn can decrease osteoclastogenesis and bone resorption [13].

Diabetic osteopathy has been proposed to be an adynamic disease, caused by low bone turnover and/or formation. In a young growing subject with type 1 Diabetes, net low bone formation causes a decrease in bone mineral density (BMD) and a propensity to fractures. In patients with type 2 Diabetes, BMD tends to be normal or increased and this could be due to a decrease in bone remodeling frequency in an age group where there is usually a negative balance for individual remodeling cycles. Thus, BMD measurements in these patients can be misleading since decreased remodeling reduces bone quality at a micro-structural level, and this alone can increase fracture incidence.

We have previously found that the accumulation of AGEs on a collagenous matrix decreases the integrin-mediated proliferation and bone-forming capacity of osteoblasts [7,31]. In addition, we have demonstrated that Alendronate can prevent these AGEs-induced alterations in osteoblastic function [15]. If our past and present in vitro results could be extrapolated to an in vivo setting, they could be indicating that Alendronate on one hand opposes the deleterious actions of AGEs on osteoblasts, but on the other hand potentiates the effects of AGEs on osteoclasts. This duality of action of Alendronate on the consequences of AGEs accumulation in bone (one of the potential pathogenic mechanisms of diabetic osteopathy) is probably the cause of contradictory results that have been found in patients with Diabetes mellitus treated with this BP.

In conclusion, we have found that both AGEs and Alendronate can inhibit the recruitment and differentiation (TRAP activity) of osteoclasts in culture, with an additive effect of both agents at high concentrations of Alendronate. While AGEs prevent the early and late stages of osteoclastogenesis, Alendronate can only inhibit the latter. These results could be indicating a potentiation of the anti-resorptive effects of Alendronate when bone extracellular matrix accumulates excess AGEs, as occurs in a patient with poorly compensated Diabetes mellitus. In addition, AGEs as well as low doses of Alendronate can increase the expression of RAGE in osteoclasts, an effect that correlates closely with their inhibition of osteoclastogenesis. In cultured osteoblasts, AGEs and Alendronate reduce RANKL expression, which could also partly explain the inhibitory effects of these agents on the recruitment and differentiation of osteoclasts.

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