Pro-inflammatory and anti-angiogenic effects of bisphosphonates on human cultured retinal pigment epithelial cells

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Abstract

Aim—Bisphosphonates have been shown to induce ocular inflammatory diseases such as uveitis and scleritis, while being protective against angiogenic diseases like neovascular age-related macular degeneration (AMD). Therefore, we studied the effects of bisphosphonates on primary culture of human fetal retinal pigment epithelium (hRPE), a cell type known to secrete both inflammatory and angiogenic factors. Alendronate and etidronate were selected for this experiment as they are members of the two structurally different classes of bisphosphonates.

Methods—Primary cultures of hRPE were serum-starved for 24 h and then treated for 24 h with alendronate (0.0001, 0.1, 100 μ M) or etidronate (0.01, 1 μ M). Cell viability was measured using the MTT assay. Investigation of secreted cytokines induced by bisphosphonates was performed using a human cytokine 29-Plex Panel (Bio-Plex) array and the results were analysed with an analysis of variance (ANOVA).

Results—Etidronate, at the lower concentration, significantly increased the expression of interleukin (IL)-6 ($p=0.03$) and IL-8 ($p=0.04$). At the higher concentration, etidronate significantly decreased the expression of granulocyte macrophage colony-stimulating factor ($p=0.02$) and basic fibroblast growth factor (bFGF) ($p=0.02$). Alendronate, at the highest concentration, significantly increased the expression of IL-8 ($p=0.02$) and decreased the expression of eotaxin ($p=0.02$). Alendronate also significantly decreased the expression of bFGF at all concentrations ($p<0.05$) and demonstrated a trend towards decreasing vascular endothelial growth factor expression at low concentration.

Conclusions—Alendronate and etidronate display dose dependent effects in hRPE cells. Alendronate and etidronate administration resulted in concentration dependent elevations in inflammatory cytokines. Furthermore, alendronate and etidronate administration resulted in reduced expression of a number of angiogenic factors. These findings may explain the increased incidence of ocular inflammation as well as the therapeutic effect on neovascular AMD which have been described with bisphosphonates.

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INTRODUCTION

Bisphosphonates are the most commonly prescribed drugs used to treat osteoporosis. They act to promote osteoclast apoptosis and inhibit osteoclast-mediated bone resorption, thus increasing bone density and decreasing the risk of fractures.¹² Bisphosphonates can be classified as nitrogen-containing bisphosphonates (NBPs) or non-nitrogen-containing bisphosphonates (nNBPs) based on their molecular mechanism of action and structure.³ NBPs induce osteoclast apoptosis by inhibiting the intracellular mevalonate pathway whereas nNBPs act via cytotoxic ATP analogue-type metabolites.³⁴ Bisphosphonates are also used in the treatment of Paget's disease and hypercalcaemia of malignancy, and have recently been shown to exhibit antitumour effects.²⁵ For all the above reasons and the projected increase in the prevalence of osteoporosis in the next decade, the use of bisphosphonates will likely increase in the future.²

Bisphosphonates, particularly NBPs, are known to induce side effects typical of an acute phase reaction, mediated through the activation of $\gamma\delta$ T cells and the release of inflammatory cytokines and mediators.⁶⁷ Although rare, cases of ocular and orbital inflammation following NBPs and nNBPs administration have been reported.¹² Recently, Etminan et al⁴ demonstrated that the use of oral bisphosphonates is associated with an increased risk of scleritis and uveitis. Reports of uveitis recurrence following bisphosphonate rechallenge further support this correlation.⁸ However, the exact mechanism of ocular inflammation following bisphosphonate use remains unknown, though inflammatory mediators are believed to be involved.⁴

While bisphosphonates may help promote ocular inflammation, their effect on angiogenesis appears to be inhibitory. A clinical study has demonstrated a therapeutic effect of bisphosphonates in patients with neovascular age-related macular degeneration (AMD).⁹ In addition, animal models have shown that bisphosphonates can inhibit laser-induced choroidal neovascularisation, and cell culture studies have shown a reduction in vascular endothelial growth factor (VEGF) expression from ARPE-19 cells.¹⁰

We hypothesised that bisphosphonates induce ocular inflammation through the release of inflammatory cytokines while being protective against angiogenic diseases by reducing the expression of angiogenic factors. We further hypothesised that the retinal pigment epithelium (RPE) cells may be involved in these processes. Therefore, we studied the effects of alendronate (an NBP) and etidronate (an nNBP) on primary culture of human fetal retinal pigment epithelium (hRPE), a cell type known to secrete inflammatory cytokines and angiogenic factors in the outer retina.^{11–14}

METHODS

Cell culture and materials

Methods for securing human fetal tissue were humane and included proper written informed consent. This study complied with the Declaration of Helsinki and was approved by the Clinical Review Ethics Board of the University of British Columbia. hRPE cells were

isolated from fetal donor eye tissues, as previously described.^{15–17} hRPE cells were cultured and expanded in Dulbecco's modified Eagle's medium (Invitrogen-Gibco, Grand Island, New York, USA) containing 10% fetal bovine serum (Invitrogen-Gibco), 100 µg/mL penicillin (Sigma-Aldrich, St. Louis, Missouri, USA), 100 µg/mL streptomycin (Sigma-Aldrich) and 2 mM l-glutamine (Invitrogen) at 37°C in a humidified atmosphere with 5% CO₂. hRPE cells from the same donor and of passages 5–7 were selectively used in experiments. Alendronate and etidronate used in this experiment were purchased from Sigma Chemicals.

Cell viability determination using an MTT assay

hRPE cells were seeded at a density of 20×10^3 cells per well on a 96-well plate and incubated overnight with medium. Following serum starvation for 24 h, the cells were incubated at 37°C in a humidified atmosphere with 5% CO₂ with or without bisphosphonates (alendronate 0.0001–500 µM; etidronate 0.01–10 000 µM) for 24 h, while using phosphate buffered saline as the vehicle control. Following media aspiration, the cells were subsequently incubated in serum-free medium containing 0.5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for 4 h. The medium was then removed and 200 µL of dimethyl sulfoxide (DMSO) was added to each well. The absorbance at 540 nm was determined using a plate reader and normalised to those of control treatments. Each experiment was conducted in at least four replicates and results were analysed using two-tailed t tests. p Values less than 0.05 were considered significant.

Cytokine analysis

hRPE cells were plated at a density of 0.3×10^6 cells per well in 6-well plates for both control and experimental groups. Following serum starvation when the cells were at near confluence for 24 h, the cells were further incubated with or without bisphosphonates (alendronate 0.0001, 0.1, 100 µM; etidronate 0.01, 1 µM) for 24 h. The stimulation experiments were performed in triplicates. The supernatant was then pooled, isolated, centrifuged (2000 rpm×5 min) and stored for further experiments.

Concentrations of interleukin (IL)-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, IL-18, eotaxin, basic fibroblast growth factor (bFGF), granulocyte colony-stimulating factor, granulocyte macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFNγ), IFN-inducible protein 10, monocyte chemoattractant protein-1, macrophage inflammatory protein-1α (MIP-1α), MIP-1β, regulated on activation normal T cell expressed and secreted (RANTES), platelet-derived growth factor, VEGF, stromal cell-derived factor-1α (SDF-1α) and tumour necrosis factor-α were determined using a commercially available multiplex bead-based sandwich immunoassay kit (Human 27-Plex with an additional 2-Plex kit containing IL-18 and SDF-1α antibodies, Bio-Rad Laboratories). Assays were performed according to manufacturer's instructions. Assays were performed in duplicates according to manufacturer's protocols. Data were analysed using one-way analysis of variance (ANOVA) followed by Tukey multiple comparison tests (Graphpad Software). All data are represented as mean±SD and adjusted p values less than 0.05 were considered significant.

RESULTS

hRPE cell viability following bisphosphonate administration

Alendronate treatment did not significantly affect hRPE cell viability when compared with the control treatment (figure 1). Similarly, etidronate treatment did not significantly affect hRPE cell viability, except at the highest concentration (10 000 μM) where it significantly decreased cell viability by 32% ($p=0.0001$; figure 1). Both agents increased cell viability at lower concentrations, but the results were not statistically significant.

Effect of bisphosphonates on cytokine production

Etidronate, at the lower concentration (0.01 μM), significantly increased the expression of IL-6 ($p=0.03$) and IL-8 ($p=0.04$) (figure 2). At the higher concentration (1 μM), etidronate significantly decreased the expression of GM-CSF ($p=0.02$) and bFGF ($p=0.02$) (figure 2). Alendronate, at the highest concentration, significantly increased the expression of IL-8 ($p=0.02$) and decreased the expression of eotaxin ($p=0.02$) (figure 3). Alendronate also significantly decreased the expression of bFGF at all concentrations in a concentration dependent fashion ($p<0.05$) (figure 3). Alendronate and etidronate did not significantly alter the expression of the other cytokines measured. Of note, alendronate at lower concentrations (0.0001 and 0.1 μM) decreased VEGF expression, though the changes did not reach statistical significance (data not shown).

DISCUSSION

Ocular inflammatory diseases can be vision threatening and include syndromes such as scleritis and uveitis.¹⁸ Scleritis and uveitis are inflammatory ocular diseases that can lead to severe vision impairment and can be associated with various systemic diseases.¹⁹ Similar to other inflammatory diseases such as rheumatoid arthritis and systemic lupus erythematosus,²⁰ IL-6, an inflammatory cytokine produced by T cells and other activated inflammatory cells, has been proposed to contribute to the development of experimental autoimmune uveoretinitis, a model of uveitis,^{21,22} through its effects on Th17 cell differentiation and proliferation.^{23,24} Th17, a subset of IL-17-producing CD4 T cells,²⁵ plays a central role in autoimmune diseases, as IL-17 mediates chronic inflammation by stimulating the production of inflammatory cytokines such as IL-6 and IL-8 from epithelial and endothelial cells and promoting neutrophil and macrophage recruitment into the retina.^{26–28} IL-6 likely plays a role in the early phase of Th17 cell differentiation and the suppression of autoimmune disease development following blockade of IL-6 activity further supports its role in the pathogenesis of auto-immune diseases such as experimental autoimmune uveoretinitis.²⁸ Proposed to be major inflammatory cytokines in uveitis, elevated levels of IL-6 and IL-8 have been found in intraocular fluids of patients with active or chronic uveitis of different aetiologies, such as ocular toxoplasmosis, viral uveitis, Fuchs hetero-chromic uveitis syndrome, intermediate uveitis and Behcet's disease.^{29–32} IL-8, which acts to promote neutrophil and T cell recruitment to the eye, has also been proposed to contribute to the development of uveitis.²⁸ Therefore, elevations in IL-6 and IL-8 expression in RPE cells, observed in this study following administration of etidronate at a lower concentration and alendronate at a higher concentration, may help explain the increased incidence in ocular

inflammation reported following bisphosphonate use. Further studies are necessary to confirm this result. In addition, the exact mechanism by which both bisphosphonates induce the production and release of pro-inflammatory cytokines in RPE cells remains to be elucidated, though it may be the result of their respective intracellular actions.

AMD is a condition that affects the central retina and choroid, leading to central vision loss. It is the most common cause of vision impairment in the elderly and its prevalence is on the rise.³³ AMD can be classified as being non-neovascular or neovascular.³⁴ The non-neovascular form is characterised by drusen deposits, along with RPE cell atrophy, geographic atrophy and gradual visual loss.³⁴ Angiogenesis, defined as the growth of new vessels from existing blood vessels, is an important process in neovascular AMD. Migration of endothelial cells into the sub-RPE space or subretinal space results in the formation of immature and leaky vessels, leading to bleeding, fluid exudation below the retina and macular oedema.³⁴ As wet AMD progresses, proliferation of fibrotic tissue and progressive neural tissue loss can occur, which could lead to severe vision loss.³⁵ Several angiogenic factors have been implicated in the pathogenesis of AMD, including VEGF and bFGF.³⁶

Previously, alendronate has been shown to downregulate the gene expression of VEGF in ARPE-19 cells while significantly suppressing the size of laser-induced choroidal neovascularization, suggesting anti-angiogenic properties of bisphosphonates.¹⁰ Our study supports and expands on this finding. Etidronate, at a higher concentration, and alendronate, in a concentration dependent fashion, decreased bFGF expression. bFGF is a potent angiogenic molecule that promotes VEGF secretion by glial cells and stimulates cell proliferation.^{37,38} bFGF is known to exert its angiogenic effect either directly or in a synergistic fashion with VEGF, and has been suggested to play a role in the pathogenesis of AMD.³⁶ Alendronate also decreased VEGF expression at low concentrations, though the difference was not significant. VEGFs, which are the targets for modern therapy of neovascular AMD, are potent and specific regulators of angiogenesis and have been shown to be crucial to the development of CNV and AMD.³⁹⁻⁴¹ A significant reduction in eotaxin expression was also observed following administration of alendronate at a high concentration. Eotaxin, a chemoattractant for eosinophils, has recently been reported to be an early biomarker in AMD.⁴² Secreted by endothelial cells, RPE cells and cells of the neurosensory retina and choroid, eotaxin was proposed to be an angiogenic factor and suggested to be an important contributor to AMD neovascularisation, as blockade of its associated receptor resulted in a reduction in CNV.^{42,43} Etidronate at a high concentration also significantly decreased GM-CSF expression, though its exact role in the pathogenesis of AMD is currently unknown. Though its relevance to AMD remains to be investigated, studies have reported that GM-CSF promotes microglia proliferation and survival,⁴⁴ enhances activity of infiltrating inflammatory cells,⁴⁵ and induces inflammatory cytokine release.⁴⁵ Anti-angiogenic effects of bisphosphonates could therefore be attributed to the reduced expression of multiple angiogenic factors, especially those implicated in the pathogenesis of AMD. Interestingly, these effects may classify bisphosphonates as being protective against AMD. Though the anti-angiogenic effects of bisphosphonates observed in this study may support the notion that bisphosphonates attenuate the development of neovascular AMD, the net effect in conjunction with the observed pro-inflammatory effects

is currently unclear and further studies are warranted to better delineate the clinical relevance of these findings.

A potential limitation of this study is the usage of hRPE cells from a single donor. However, ARPE-19 cells, which are widely accepted and used in research, are also derived from a single donor. Thus, we feel that using cells derived from a single donor still afforded us adequate ability to assess the effect of bisphosphonates on RPE cells.

In conclusion, alendronate and etidronate display dose dependent effects in hRPE cells. Alendronate and etidronate administration result in concentration dependent elevations in IL-6 and IL-8 levels, which could explain the increased incidence of ocular inflammatory events associated with bisphosphonate use. With respect to angiogenesis, the effects of alendronate and etidronate on RPE cells are mainly anti-angiogenic, as indicated by the reduced expression of angiogenic factors such as bFGF and eotaxin. Our results support and provide additional evidence on the previously reported anti-angiogenic effects of bisphosphonates.

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Figure 1.

Effect of alendronate or etidronate on human fetal retinal pigment epithelium (hRPE) cell viability. hRPE cells were seeded at a density of 20×10^3 cells per well on a 96-well plate and incubated overnight. After serum starvation for 24 h, alendronate (0.0001–500 µM) or etidronate (0.01–10 000 µM) were administered for 24 h. Cell viability was measured using the MTT assay, and expressed as percentage of control considering control as 100%.

*Denotes a statistically significant difference ($p < 0.05$).

Figure 2.

Effect of etidronate on IL-6, IL-8, macrophage colony-stimulating factor and basic fibroblast growth factor expression in human fetal retinal pigment epithelium (hRPE) cells. hRPE cells of passage 5–7 cells were plated at 0.3×10^6 cells per well in 6-well plates and incubated overnight. After serum starvation for 24 h, etidronate (0.01 and 1 μM) was administered for 24 h. Concentrations of cytokines were measured in the supernatant using a 29-Plex Bioplex kit (BioRad Laboratories). *Denotes a statistically significant difference ($p < 0.05$). All data are represented as mean $\pm$ SD.

Figure 3.

Effect of alendronate on IL-8, eotaxin and basic fibroblast growth factor expression in human fetal retinal pigment epithelium cells. Cells of passage 5–7 cells were plated at 0.3×10^6 cells per well in 6-well plates and incubated overnight. After serum starvation for 24 h, alendronate (0.0001, 0.1 and 100 μ M) was administered for 24 h. Concentrations of cytokines were measured in the supernatant using a 29-Plex Bioplex kit. *Denotes a statistically significant difference ($p < 0.05$). All data are represented as mean $\pm$ SD.