Antiangiogenic Effects of Bisphosphonates on Laser-Induced Choroidal Neovascularization in Mice

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PURPOSE. To demonstrate that bisphosphonates inhibit laser-induced choroidal neovascularization (CNV) in vivo and down-regulate angiogenic gene expression in retinal pigment epithelial cells in vitro.

METHODS. Male C57BL/6 mice were treated with intraperitoneal injections of alendronate, clodronate, or saline at the onset (day 0) of experiments. CNV was induced by laser photocoagulation the next day, and fluorescein angiography (FA) was performed on experimental days 7 and 14. Histologic and immunohistochemical examinations were performed on day 7. ARPE-19 cells were grown on multi-plate wells coated with type I collagen to induce the gene expression of VEGF and integrins. Alendronate or clodronate was applied for 3 days, and real-time PCR was performed to measure VEGF-A, VEGF-B, and VEGF-C and integrin-αV, integrin-β1, and integrin-β3.

RESULTS. Alendronate and clodronate significantly suppressed the size of laser-induced CNV. Immunoreactivities for VEGF and integrin-αV were remarkably attenuated with alendronate and mildly reduced with clodronate. Alendronate significantly downregulated the gene expression profiles of VEGF and integrins, whereas clodronate had no effect in ARPE-19 cells.

CONCLUSIONS. Although only adverse effects of bisphosphonate have been documented in the ophthalmologic literature, some therapeutic effects of bisphosphonates, including antiangiogenesis, may be expected in ocular diseases. Antiangiogenic mechanisms of bisphosphonates may vary; further investigation is needed. (Invest Ophthalmol Vis Sci. 2007;48:5716–5721) DOI:10.1167/iovs.07-1023

In industrial countries, the number of age-related diseases such as osteoporosis and age-related macular degeneration (AMD) has remarkably increased over the years. It is a matter of urgency to establish preventive strategies for these disorders. Once these diseases have progressed, treatment is difficult, and patients experience serious functional disabilities. Bisphosphonates were developed as powerful inhibitors of osteoclasts and are commonly used to treat and prevent osteoporosis. Many recent reports have suggested multiple pharmacologic effects of bisphosphonates, such as antiangiogenic effects and the induction of apoptosis in tumor cells, which make bisphosphonates widely interesting compounds. In ophthalmology, bisphosphonates are known to cause uveitis or scleritis as adverse effects. However, these effects reversely indicate that bisphosphonates have good ocular tissue permeability. Our purpose in the experiments outlined here was to evaluate the therapeutic effects of bisphosphonates on ocular diseases, especially AMD. There are some reasonable bases for this challenge. First, AMD is an angiogenic disorder accompanied by choroidal neovascularization (CNV), and it causes severe visual loss in the elderly. Second, because many senior patients are affected by both osteoporosis and AMD, the use of bisphosphonates may be beneficial in treating both diseases. Last, current AMD therapies are predominantly based on surgical procedures including laser therapy and intravitreal injections of medicines or antibodies, which require particular equipment and pose a risk for surgical complications. Medications with oral drugs or eye drops more easily treat a broad range of patients.

In this study, we demonstrated the inhibitory effect of bisphosphonates on CNV in vivo and changes in angiogenic gene expression profiles of cultured retinal pigment epithelial cells in vitro.

MATERIALS AND METHODS

Animals

All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male C57BL/6 mice (3–4 weeks old) were purchased from CLEA Japan, Inc. (Tokyo, Japan). For all procedures, animals were anesthetized by intraperitoneal injections of pentobarbital.

Treatment with Bisphosphonates

On experimental day 0, the day before laser administration, alendronate (1 mg/kg; Merck, Tokyo, Japan), clodronate (15 mg/kg; Merck), or saline was injected intraperitoneally. Because bisphosphonates are long-acting drugs in vivo, additional injections of bisphosphonates or saline were performed on day 7 of experiments for analysis on day 14.

Induction of CNV

CNV was generated on experimental day 1 by laser-induced ruptures in Bruch membranes, as previously described. Briefly, three laser photocoagulation (50 μm, 0.05 second, 50 mW) spots were administered in each eye. Air bubbles were observed as a sign of correct rupture in each Bruch membrane.

Fluorescein Angiograms

After pupils were dilated with 0.05% tropicamide/0.25% phenylephrine HCl and subsequent intraperitoneal injections of 0.1 mL of 2.5% fluorescein sodium (Alcon Japan, Tokyo, Japan), fluorescein angiography (FA) was performed with a fundus camera (Canon CF-60UV; Canon Inc., Tokyo, Japan). Digital images were taken at 4 to 6 minutes for comparison. FA was performed on experimental days 7 and 14. For semiquantitative analyses of fluorescein leakage, the largest leakage in each eye was evaluated in a masked fashion by three observers. The area of leakage was outlined and calculated as the number of pixels by...
Histologic Procedures
Mice were humanely killed, and eyes were enucleated and fixed with 4% paraformaldehyde overnight at 4°C. Consecutive cryosections were prepared at 14-μm thickness. Eye sections were thoroughly examined, and the CNV with the greatest thickness was photographed.

For immunohistochemistry, sections were blocked with 5% normal goat serum in 0.1 M PBS, then incubated in 200× rabbit antihuman vascular endothelium growth factor (VEGF) antisera or 200× mouse antihuman integrin αV antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) with normal goat serum overnight at 4°C. For secondary antibodies, incubations with either 200× goat anti-rabbit IgG/Cy3 or anti-mouse IgG/FITC were applied for 1 hour at room temperature. Bright-field images and immunoreactivities were observed with a fluorescence microscope (Olympus Provis AX80; Olympus Optical, Tokyo, Japan).

Cell Cultures
The routine maintenance of a human retinal pigment epithelium (RPE) cell line ARPE-19 was described previously. Cultures from passages 18 to 22 were used in experiments.

Type I collagen induces RPE angiogenic gene expression; hence, 35-mm culture dishes coated with type 1 collagen (BioCoat Collagen Matrix; BD Biosciences, Bedford, MA) were provided. Thin-layer basement membrane matrix (BioCoat Matrigel Matrix; BD Biosciences) was used as a control. RPE cells were seeded at 600,000 cells/cm² in each 35-mm dish with Dulbecco modified Eagle medium/nutrient mixture F12 with 15 mM HEPES buffer (DMEM/F12; Gibco, Grand Island, NY) plus 10% fetal bovine serum (FBS; UBI Upstate, Lake Placid, NY), 0.548% additional sodium bicarbonate, and 2 mM L-glutamine solution, (Gibco) and were kept at 37°C in 10% CO₂ for 3 days. Serum was withdrawn and kept incubated for 48 hours to quiescent cells. Cells were further incubated with or without bisphosphonate for 24 hours and then subjected to the following experiments.

Drug Toxicity
Cells were washed twice in Hanks balanced salt solution (HBSS) and were harvested with 0.5% trypsin/EDTA solutions. Cell viability was estimated by the exclusion of 0.4% trypan blue solution. The number of living cells was determined using a hemocytometer. Cells were washed twice in HBSS and fixed by 10% formalin for 1 hour. Then they were washed twice in HBSS and stained with Hoechst dye for 1 hour. Pyknosis cells were counted, and the apoptosis rate of cells was evaluated. To ensure reproducibility, experiments were repeated three times.

RNA Extraction
Total RNA was extracted from cells (RNeasy Mini-Kit; Qiagen, Valencia, CA) according to the manufacturer’s recommendations. Total RNA was eluted from the column in 50 μL RNase-free water. The purity and concentration of RNA was determined by measuring absorbance at 260 nm and 280 nm.

Real-Time Quantitative PCR
Total RNA was reverse transcribed into cDNA in a total volume of 100 μL using random hexamers as primers (Senscript RT Kit; Qiagen) according to the manufacturer’s instructions.

Real-time PCR was performed in 96-well plates (ABI Prism 7700 Sequence Detection System; Applied Biosystems, Foster City, CA) using dilutions of first-strand cDNA with a final concentration of assay (1× Assays-On-Demand; Applied Biosystems) and master mix (1× TaqMan Universal PCR Master Mix; Applied Biosystems) according to the manufacturer’s instructions. Final reaction volumes were 25 μL. Each sample was analyzed in triplicate. Thermal cycler conditions were 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Sequence detection software (Applied Biosystems) was used to extract PCR data, which were then exported to software (Excel; Microsoft, Redmond, WA) for further analyses. The amount of targeted gene expression was normalized to an endogenous reference and relative to a calibrator. The endogenous reference used in the experiments reported herein was cyclophilin A. Basement membrane matrix (Matrigel; BD Biosciences) cDNA was used as a calibrator in all the experiments. The target threshold cycle (Ct) and endogenous control Ct were calculated for each sample. The Ct of the endogenous control was then subtracted from the Ct of the target gene. This value was defined as ΔCt. The ΔCt of each sample was then subtracted from the ΔCt calculated for the calibrator, and this value was defined as ΔΔCt. The amount of target gene expression normalized to the endogenous control and relative to the calibrator was calculated using the formula ΔΔCt.

Statistical Analysis
Unpaired Student’s t-tests (two-tailed) were used for all statistical analyses in the present study. P < 0.05 was considered statistically significant.

RESULTS
Fluorescein Leakage in Angiographies
Laser spots in control mice showed remarkable leakage in dye, indicating the existence of CNV on experimental day 7 that naturally decreased on day 14. Mice treated with clodronate or alendronate showed weaker visual leakages on experimental day 7 and day 14 (Fig. 1). Semiquantitative analyses revealed a significantly smaller leakage area in mice treated with alendronate or clodronate than in control mice (Fig. 2).

FIGURE 1. Fluorescein angiograph images on experimental day 7 (A, C, E) and day 14 (B, D, F). The control mouse (A, B) shows a larger leakage area than the mouse treated with alendronate (C, D) or clodronate (E, F).
Histologic Findings

A thick, wide CNV was found at the sub-RPE and the subretinal space with ruptured Bruch membranes on day 7 in control mice. CNVs found in alendronate- or clodronate-treated mice were obviously smaller than those in control animals (Fig. 3). Immunoreactivities of VEGF and integrin αV were found at the innermost layer of CNV components in control mice (Fig. 4). Distributions of VEGF and integrin αV immunoreactivities were similar, whereas retinal ganglion cells were only immunopositive for VEGF (Fig. 4, arrowheads) that excluded possible cross-reactions. Immunoreactivities of VEGF and integrin αV were remarkably attenuated in mice treated with alendronate. Mice treated with clodronate showed weak VEGF and integrin αV immunoreactivities.

Cell Viabilities

There was no difference in cell viability among control, alendronate, and clodronate groups under our experimental conditions. Few pyknosis cells were found under all studied conditions; this did not differ among experimental groups (data not shown).

In Vitro RPE Gene expression

VEGF-A, -B, and -C gene expression was upregulated by type I collagen. Alendronate suppressed the gene expression of all VEGF subtypes dose dependently except for VEGF-B, which showed a moderate rebound with 10 μM alendronate (Fig. 5). The whole integrin gene family was upregulated by type I collagen and inhibited by alendronate in a dose-dependent manner (Fig. 6). In contrast, clodronate did not show any effect on selected RPE gene expression patterns grown on type I collagen (data not shown).

**Figure 2.** Semiquantitative analyses revealed a significant reduction of leakage by clodronate and alendronate on experimental day 7 (A) and day 14 (B). Values are presented as mean ± SD of 8 subjects. *P < 0.05; **P < 0.01.

**Figure 3.** The histopathology of a control mouse (A), a mouse treated with clodronate (B), or a mouse treated with alendronate (C) on experimental day 7. Treatment with each bisphosphonate remarkably reduced the size of the CNV lesion.
DISCUSSION

In the present study, the suppression of laser-induced CNV by the administration of bisphosphonates was demonstrated in vivo. The inhibitory effect of alendronate on a number of angiogenic gene expression profiles was also revealed in cultured RPE.

Bisphosphonates accumulate predominantly in bone tissue and regulate the function of osteoclasts, including the induction of apoptosis. Recent studies have demonstrated that antitumor and antiangiogenic effects are associated with bisphosphonates, which propose novel possibilities for this drug class. In some cases, bisphosphonates also induce uveitis or scleritis, which leads to the discontinuation of bisphosphonate drug use in some patients. Bisphosphonates are also associated as drugs that cause concern, especially in the eyes. However, our clinical data showed a significantly lower incidence of AMD in patients with osteoporosis treated with bisphosphonates than those who were not (Honda S, unpublished data, 2007). Thus, we are trying to uncover the beneficial effects of bisphosphonates, if any, for the eyes.
Our study revealed almost equivalent inhibitory effects of clodronate and alendronate on CNV formation in vivo. However, in vitro studies showed distinct results for each drug. The mechanism in which bisphosphonates exhibit their pharmacologic effects depends on the molecular structure of each compound and largely depends on the existence or absence of nitrogen residues. Clodronate is a non-nitrogen-containing bisphosphonate (NN-BP) metabolized to a non-hydrolyzable cytotoxic ATP analogue, adenosine 5′-(β,γ-dichloromethylene) triphosphate (AppCC12p), and induces apoptosis. In contrast, alendronate is a nitrogen-containing bisphosphonate (N-BP) which inhibits famesyl diphosphonate synthetase in the biosynthetic mevalonate pathway. Similarly, statins are thought to inhibit CNV through the inhibition of mevalonate synthesis. Recently, many differences were disclosed in terms of suppress CNV through the inhibition of mevalonate synthetase. Similarly, statins are thought to inhibit CNV through the inhibition of mevalonate synthesis. Finally, the expression of matrix metalloproteinase (MMP) and integrins to inhibit angiogenesis in vitro and in vivo. The involvement of MMP, integrins, and VEGF in CNV formation through direct inhibition of the proliferation of vascular endothelial cells and by possible effects that regulate the function of mononuclear cells. Overall mechanisms of inflammation induced by macrophages. Such anti-inflammatory effects are also expected with alendronate by inhibiting the function of mononuclear cells. Overall mechanisms of bisphosphonates to inhibit angiogenesis, however, have not been fully elucidated and must be investigated further.

This is the first report to introduce bisphosphonates as possible drugs for the prevention and treatment of AMD in addition to osteoporosis. A number of bisphosphonates have been identified by the National Registry of Drug-Induced Ocular Side Effects. The authors thank Noriko Ishibashi and Kyoko Iseki (Riken, Kobe, Japan) for technical assistance.

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