SPANDIDOS PUBLICATIONS

Zoledronic acid, a third-generation bisphosphonate, inhibits cellular growth and induces apoptosis in oral carcinoma cell lines

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Abstract. Bisphosphonates (BPs) inhibit bone resorption by preventing osteoclast maturation and apoptosis induction. Recently, BPs have also been shown to have antitumor effects against various types of carcinomas *in vitro* and *in vivo*. In this study, we investigated the antitumor effect of zoledronic acid (ZOL), a third generation bisphosphonate, on proliferation, cell cycle and apoptosis of oral cancer cells. Direct antitumor effects of ZOL against four oral carcinoma cell lines (squamous cell carcinoma, HSC3, HSC4, SCCKN; salivary adenocarcinoma, HSY) were measured by WST assay. Apoptosis-related molecules were analyzed by Western blot analysis and cell cycle was analyzed by flow cytometry. ZOL had a dose-dependent antitumor effect in the four oral cancer cell lines. ZOL activated caspase-3, -8 and -9 and induced cellular apoptosis. Western blot analysis showed that ZOL increased cleaved anti-human poly(ADP-ribose) polymerase expression and decreased Bcl-2 and Bid expression. Treatment with ZOL increased the number of cells in apoptosis, sub G1 phase and S phase, and reduced the number of cells in the G0/G1 and G2/M phase in a concentration-dependent manner. ZOL inhibits cell proliferation and induces apoptosis of oral cancer cells *in vitro*. These findings suggest that ZOL might be beneficial in the treatment of oral carcinoma patients.

Introduction

Oral cancer, especially squamous cell carcinoma (OSCC), has a relatively high rate of recurrence and poor prognosis because of nodal and distant metastases (1-4). Although recent advances in radio and/or chemotherapy have improved patients' quality of life, the relative 5-year survival rate has not significantly changed (5). Thus, more effective therapeutic strategies against oral cancers are necessary. A few studies have found that mandibular invasion of oral cancer especially OSCC predicts poor prognosis (6). Rao *et al* reported that the frequency of mandibular invasion of oral carcinomas ranged from 12 to 56% (7). Clinically, bone destruction in SCCs causes many problems regarding the function and prognosis of patients. Bone invasion in OSCCs is a critical determinant of the postoperative functional outcome. So, it is very important to develop new treatments for bone invasion by oral carcinomas.

Bisphosphonates (BPs) are structural analogs of pyrophosphoric acid, a biomedical component. They are potent inhibitors of osteoclastic bone resorption. They have been used for treatment of osteoporosis, Paget's disease, and cancer-induced hypercalcemia (8,9). BPs can be divided into three generation based on differences in the structure of the R_1 side chain. The first generation of BP contains no nitrogen, while the second generation of BP contains nitrogen in the R_1 side chain. The third generation of BP contains nitrogen in circular structure. Zoledronic acid (ZOL) is a nitrogen-containing BP classified as a third-generation of BP that has greater potential for anti-osteoclastic bone resorption than second-generation BPs (10). The nitrogen-containing BPs inhibit protein prenylation in osteoclasts and can induce apoptosis (11).

Recently, BPs have presented *in vitro* and *in vivo* producing direct antitumor activity in a variety of malignant tumors (12-15). However, the effect of BPs on oral cancers is not so manifested. In our study, we demonstrated the antitumor effect of ZOL on the proliferation, cell cycle and apoptosis of oral cancer cell lines.

Materials and methods

Reagents. ZOL was kindly provided as the hydrated disodium salt, molecular weight 401.6, by Novartis Pharma AG (Basel, Switzerland), dissolved in phosphate-buffered saline and stored as a 10 mM stock solution at -20˚C for *in vitro* experiments.

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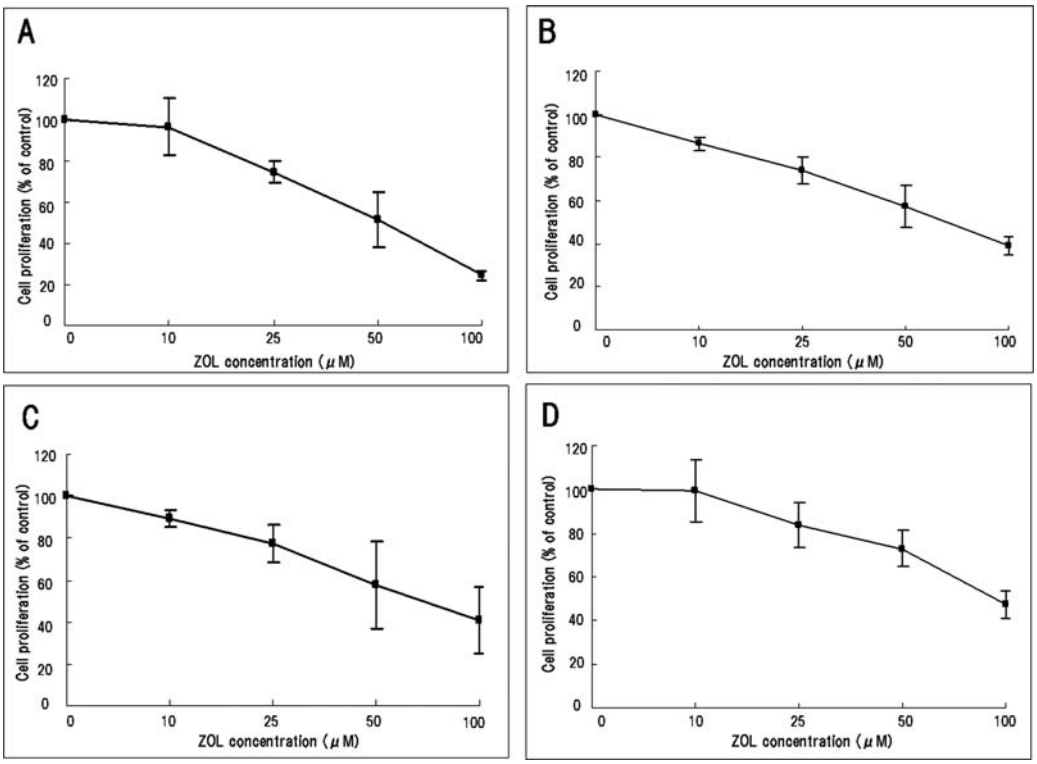

Figure 1. Direct effect of ZOL on four oral carcinoma cell lines HSC-3 (A), HSC-4 (B), SCCKN (C), and HSY (D). Cells were incubated with concentrations of 0 (control), 10, 25, 50, 100 μM of ZOL for 72 h, and cell proliferation was determined by WST-8 assay. Data represent cell numbers during treatment relative to control untreated cells (100%). Values are the means ± SD of three independent experiments.

Cell lines and cell culture. Human OSCC cell lines, HSC3 (poorly differentiated), HSC4 (well differentiated) and SCCKN (moderately differentiated), and the human salivary adenocarcinoma cell line, HSY, 8 were maintained at 37˚C in a fully humidified incubator with 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (JRH, Lenexa, KS, USA), 100 U/ml penicillin, 100 μg/ml streptomycin and 292 μg/ml L-glutamine. HSY cells were kindly provided by Dr Y. Miyamoto (Department of Oral and Maxillofacial Surgery, Tokushima University School of Dentistry).

Determination of cell proliferation in vitro. Tumor cell proliferation was measured using a Cell Counting Kit-8 (CCK-8) according to the manufacturer's instructions. The cells were seeded on 96-well flat-bottomed tissue culture plates at a concentration of $5x10^3$ cells/well with complete culture medium and allowed to adhere to the plate overnight. Then the cells were incubated in the presence of 0 (control), 10, 25, 50 and 100 μM of ZOL for another 72 h. After treatment, 10 μl of WST-8 Solution Reagent was added to each well for 180 min. The samples were directly measured photometrically using a Bio-Rad Model at 450 nm (Bio-Rad Lab, Richmond, CA, USA).

Caspase-3, -8, and -9 activation assay. A CPP32/Caspase-3, -8 and -9 Colorimetric Protease Assay Kit (MBL Co., Ltd.) was used to investigate the activation of caspase-3, -8 and -9 expression in ZOL-treated HSC-3 cells. The assay was performed according to the manufacturer's instructions after 72 h of incubation with 0 (control), 10, 25, 50 or 100 μM of ZOL. Caspase-3, -8 and -9 activation led to cleavage of the provided colorimetric substrates, which could be measured photometrically at 405 nm (Bio-Rad Lab).

Western blot analysis. Subconfluent HSC-3 cells were cultured in 60-mm dishes with 0 (control), 10, 25, 50 or 100 μM of ZOL for 72 h. They were harvested and lysates extracted and equal amounts of protein (10 μg) were separated by SDS-PAGE and transferred to PVDF membranes. Rabbit anti-human poly(ADP-ribose) polymerase (PARP) polyclonal antibody (1:500 dilution; Cell Signaling Technology, Danvers, MA), goat anti-Bcl-2 polyclonal antibody (N-19; 1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human Bid polyclonal antibody (1:500 dilution; R&D Systems, Inc., Minneapolis, MN), and ß-actin monoclonal antibody (1:500 dilution; AC15, Sigma, St. Louis, MO, USA) were used as the primary antibodies. Specific bands were detected with an enhanced chemiluminescence system (ECL Detection System; Amersham, UK).

Hoechst 33258 staining. To detect apoptotic cells, both adherent and floating cells were collected, which were fixed with 100 μl of Clarke's fixative (ethanol:acetic acid = 3:1) for 5 min at 4˚C, allowed to dry in air onto glass slides and then stained with 1 mg/ml Hoechst 33258 for 10 min in the dark at room temperature. After staining, the cells were washed three times with distilled water for 5 min each and counted.

Flow cytometric analysis. For cell cycle analysis, subconfluent HSC3 cells were cultured in 60-mm dishes with 0 (control), 25 and 100 μM of ZOL for 24, 48 and 72 h. The

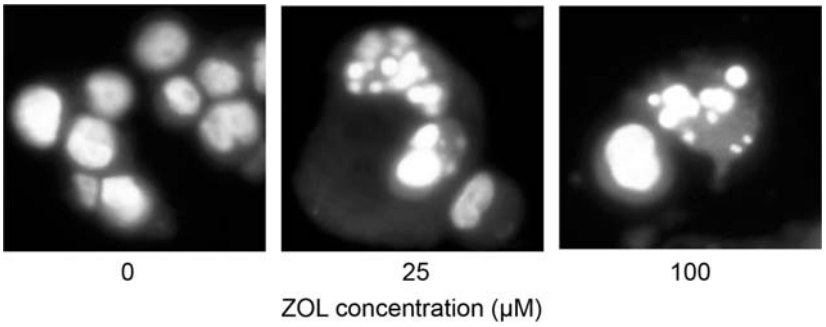

Figure 2. Fluorescent microscopic analysis shows apoptotic morphology of HSC-3 cells treated with a variety of concentrations of ZOL and stained with Hoechst 33258.

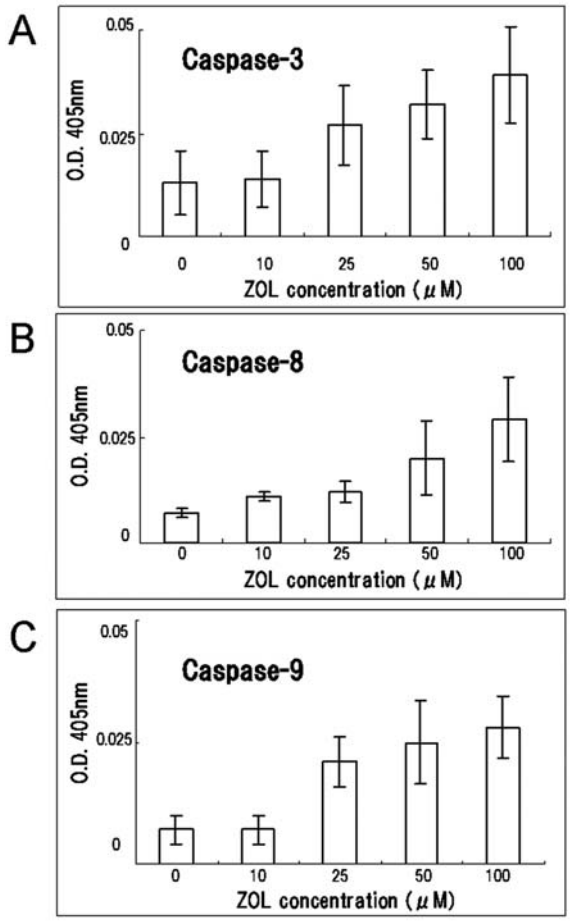

Figure 3. Colorimetric assay of caspase-3, -8, -9 activation. The activity of caspase-3, -8, -9 was increased in HSC-3 cells treated with ZOL for 72 h. Values are the means ± SD of three independent experiments.

cells were trypsinized, fixed in 70% ice-cold ethanol, and stored at 4˚C for 72 h. After fixation, cells were suspended in 100 μl phosphate-citrate buffer (0.19 M Na_2HPO_4, 4 mM citric acid) and incubated for 30 min at room temperature, and then resuspended in 1 ml of PBS containing 10 μg/ml of propidium iodide (PI) and 10 μg/ml of RNase. The PI stained cell samples were analyzed using FACSCalibur (Beckman-Coulter Epics Altra) and examined to determine the proportion of the population of cells in sub-G1, G0/G1, S and G2/M phases of the cell cycle.

Results

Zoledronic acid inhibits growth of oral cancer cells. We used the WST-8 assay to investigate the growth inhibitory effect of ZOL on oral cancer cells. ZOL (10-100 μM) inhibited cell growth of all four oral cancer cell lines in a concentration-dependent manner. HSC-3, which is a poorly differentiated human OSCC cell line with a potential for high lymph node metastasis, and ZOL inhibited most effectively the proliferation of HSC-3 of the four oral cancer cell lines (Fig. 1). HSC-3 cells were studied in the following experiments.

Induction of apoptosis. We performed DNA staining of ZOL-treated HSC-3 cells using Hoechst 33258 staining. Fluorescent microscopic analysis demonstrated apoptotic cells by the presence of condensation and segmentation of the nuclei in HSC-3 cells treated with a variety of concentrations of ZOL (Fig. 2).

Caspase-3, -8 and -9 activation assay. The activity of caspase-3, -8 and -9 in HSC-3 cells treated with ZOL at a variety of concentrations increased in dose-dependent manner after 72 h (Fig. 3). Therefore, the activation of caspase-3, -8 and -9 correlated with the induction of apoptosis.

Western blot analysis. Additionally, we performed Western blot analysis to demonstrate PARP activity with inducted apoptosis by ZOL. We screened whole protein extracts from HSC-3 treated ZOL. PARP is a representative substrate for the effector caspase, and its cleavage is a marker of caspase-dependent apoptosis (16). PARP cleavage could be detected in proteins extracted from HSC-3 cells treated with ZOL for 72 h, indicating that the decrease in viable cells after ZOL treatment was accompanied due to an increase in tumor cell apoptosis (Fig. 4). Caspase-8 is known to activate the proapoptotic Bcl-2 family member Bid by cleavage, so we also analyzed the amount of full length Bid protein in whole cell extracts. While 25 μM of ZOL reduced the amount of Bid protein, 100 μM of ZOL reduced the amount of full length Bid to almost undetectable levels. In addition, we analyzed the same whole cell extracts for expression of antiapoptotic Bcl-2 family members. Bcl-2 levels decreased substantially in the ZOL treated groups. These findings suggest that ZOL induced apoptosis of HSC-3 through the caspase-3, -8 and -9 pathway.

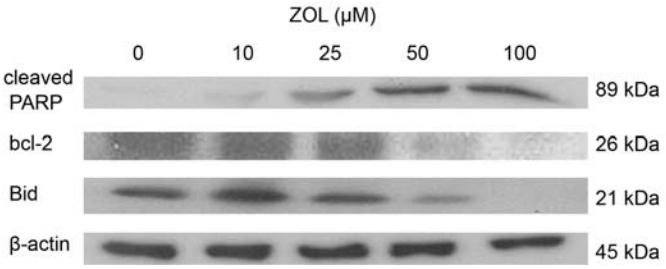

Figure 4. Protein expression in HSC-3 cells treated with ZOL. HSC-3 cells were incubated with ZOL for 72 h, and protein expression was analyzed by Western blotting.

Cell cycle distribution. We performed flow cytometry analysis to investigate the cell cycle and apoptosis of HSC-3 cells after ZOL treatment. HSC-3 cells were treated with 25 and 100 μM of ZOL for 24, 48 and 72 h. ZOL treatment increased the number of cells in sub G1 and S phase, and decreased the number of cells in G0/G1 and G2/M phase in a time- and concentration-dependent manner (Fig. 5, Table I).

Table I. The population of cell cycle in HSC-3 (%).

	Control	ZOL (25 μM)	hZOL (100 μM)
subG1			
24 h	2.2	14.2	10.1
48 h	5.3	35.5	29.7
72 h	3.5	35.8	42.8
G0/G1			
24 h	58.0	43.8	46.6
48 h	71.5	26.8	33.3
72 h	68.0	26.6	22.0
S			
24 h	23.4	24.6	36.4
48 h	11.8	26.0	31.5
72 h	17.1	30.8	30.3
G2/M			
24 h	16.4	17.4	6.9
48 h	11.4	11.7	5.5
72 h	11.4	8.7	4.9

Discussion

This study suggests that ZOL inhibited cell proliferation and induced apoptosis in oral cancer cell lines. Our study demonstrated that ZOL had a direct antitumor effect, in addition to the inhibition of osteoclast-mediated bone invasion in oral cancer. This is the first study to show that ZOL has antitumor effects on cultured oral cancer cell lines.

The mechanism of action of nitrogen-containing BPs is thought to be via inhibition of the activity of farnesyl diphosphate (FPP) synthase and geranylgeranyl diphosphate (GGPP) synthase, which are part of the mevalonate pathway, resulting in the inhibition of prenylation of small GTP proteins such as Ras, Rap1A, and Rho, (17,18) which then act as antitumor effectors. Recently, BPs have been shown to have direct antitumor effects in multiple types of cancer cells *in vitro* and i*n vivo* (12-15). It has been shown that admini-

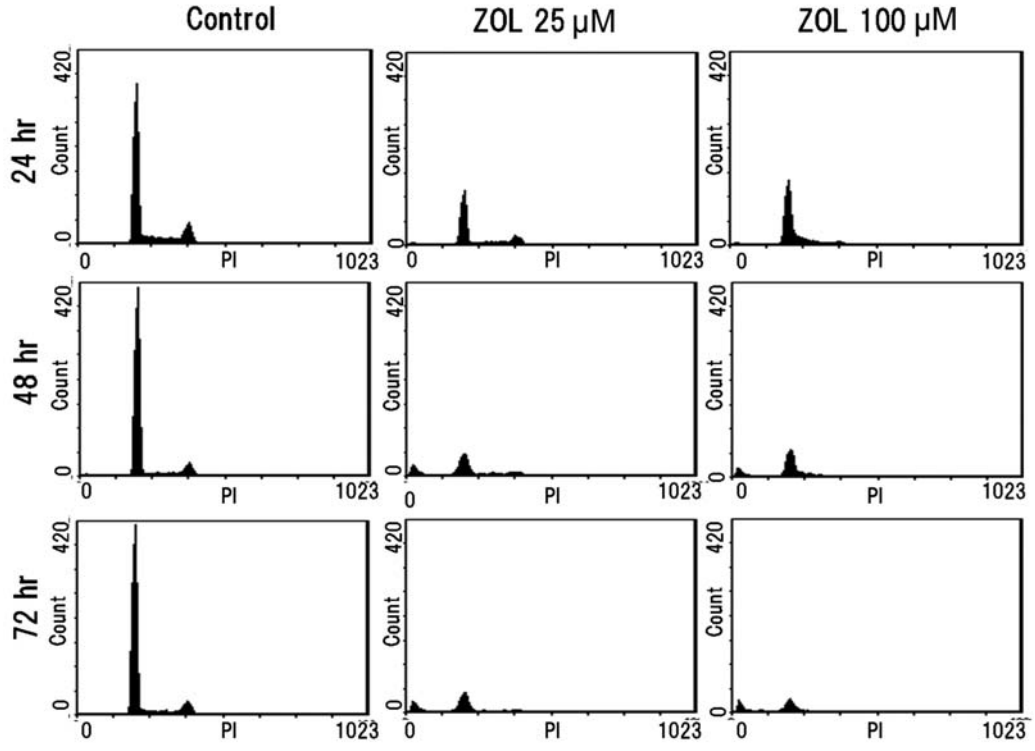

Figure 5. Flow cytometric analysis of DNA content in HSC-3 cells treated with of ZOL for 24, 48, or 72 h.

of BPs to patients with prostate cancer with bone s improves pain and tumor markers clinically (19).

Apoptosis requires the activation of caspases, which can be divided into initiator caspases and effector caspases. It is well known that there are many signal transduction pathways in apoptosis (20,21). Activation of caspase-3, a component of the downstream of apoptosis pathway, is essential for the induction of apoptosis (20). Cleavage of PARP, an endogenous substrate of caspase-3, provides definite evidence of apoptosis (21). In the present study, caspase-3 was activated and cleavage of PARP was identified in the HSC-3 cells treated with ZOL by the caspase protease assay and Western blot analysis, confirming activation of the apoptosis signal pathway at the molecular level. Caspase-9 is the most critical upstream molecule of the apoptotic protease cascade. Bcl-2 is mainly located on the outer membrane of mitochondria and prevents the release of cytochrome c, thereby preventing caspase activation (22,23). Caspase-8 activates downstream caspases by direct or indirect cleavage of Bid and induces cytochrome c release from mitochondria (24). The present study revealed the cleavage of these molecules in the HSC-3 cells treated with ZOL, suggesting that they participate in apoptosis induced by ZOL.

Several studies have indicated the benefits of the combined use of BPs with other cytotoxic agents (15,26-28). Romani *et al* indicated that ZOL induces S-phase arrest in cholangiocarcinoma cells. Thus, ZOL could represent a reliable adjuvant chemotherapeutic agent which is able to sensitize cells to cytotoxic agents and/or radiation (29). Our flow cytometric analysis also showed that ZOL induced S-phase arrest in HSC-3 cells. This might suggest the synergistic effect of ZOL with other cytotoxic agents in oral carcinomas.

In conclusion, we confirmed that ZOL inhibits the proliferation of oral carcinoma cells *in vitro* by inducing apoptosis and/or cell cycle arrest. Therefore, it is suggested that ZOL treatment may be beneficial for patients with oral cancer. Further study should be conducted to clarify the mechanism of the antitumor effects of ZOL.

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