Incadronate Induces Cell Detachment and Apoptosis in Prostatic PC-3 Cells

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Abstract. *Background: Bisphosphonates are widely used for the treatment and prevention of osteoporosis and are also effective in the treatment of bone metastasis of prostate cancer. Several mechanisms underlying the antitumor effect of bisphosphonates have been proposed, including direct effects on tumor cells, such as induction of apoptosis and inhibition of invasion. Materials and Methods: The detached and adherent cells after incadronate treatment were collected separately and stained with trypan blue solution. Results: It was found that incadronate induced cell detachment with dephosphorylation of focal adhesion kinase (FAK). The induction of cell detachment by incadronate was prevented by coincubation with geranylgeraniol. The activation of caspase-3 was observed in incadronate-treated floating cells, but not in the adherent cells. A caspase inhibitor did not inhibit cell detachment by incadronate but it markedly prevented cell death. Conclusion: These results suggest that incadronate induces cell detachment, followed by caspase-dependent apoptosis.*

Bisphosphonates are analogues of pyrophosphate and act as potent inhibitors of bone resorption (1, 2). They are widely used for the treatment of bone diseases, such as Paget's disease, osteoporosis, and hypercalcemia with malignant tumors (2). Moreover, there are now many experimental and clinical studies showing that bisphosphonates are also useful for the treatment of cancer patients with bone metastases. Experimental studies have indicated that bisphosphonates inhibit cell growth, cell invasion, and cell adhesion to non-mineralized bone matrices, and induce apoptosis in various cultured cell lines (3-7). Clinical studies have shown that treatment with bisphosphonate decreased skeletal complications and improved the quality of life in patients with bone metastatic cancer (8-12). Adjuvant treatment of breast cancer patients with bisphosphonate in combination with hormonal therapy or chemotherapy has reduced the incidence of bone metastasis (11, 12).

In patients with prostate cancer, bone metastases often occur in the advanced stages of the disease, therefore, a beneficial effect of bisphosphonates is expected in patients with this cancer. In fact, the result of a clinical study has shown that significant pain reduction was observed in prostate cancer patients with bone metastases after receiving bisphosphonates (13). Moreover, bisphosphonate treatment has been reported to decrease serum prostate-specific antigen levels in a case of hormone-refractory prostate cancer (14).

The antitumor effects of bisphosphonates are mainly explained by the inhibition of enzymes of the mevalonate pathway (15-17). Furthermore, decreased expression of a variety of genes involved in tumor cell function has been reported (18-20). We reported that incadronate reduced caveolin-1 expression in PC-3 cells (21). Since caveolin-1 is the principal component of caveolae and interacts with numerous signaling proteins involved in integrin signaling (22), the reduction of caveolin-1 expression by incadronate in PC-3 cells would lead to a disruption of focal adhesion and then cell death mediated by cell detachment. In this study, we examined the effect of incadronate on cell death of prostatic PC-3 cells.

Materials and Methods

Materials. Incadronate was kindly supplied by Astellas Pharma Inc. (Tokyo, Japan). Farnesol and geranylgeraniol were from Sigma-Aldrich (St. Louis, MO, USA) and MP Biomedicals (Aurora, OH, USA), respectively. Acetyl-Asp-Glu-Val-Asp α-(4-methyl-coumaryl-7-amide) (Ac-DEVD-MCA) and benzyloxycarbonyl-Asp-[(2,6-dichlorobenzoyl)oxy]methane (Z-Asp-CH_2-DCB) were purchased from the Peptide Institute (Osaka, Japan). Mouse anti-FAK monoclonal antibody and mouse anti-phosphotyrosine antibody (4G10) were purchased from Transduction Laboratories (Lexington, KY, USA) and Upstate Biotechnology (Charlottesville, VA, USA), respectively. Rabbit anti-caveolin-1 polyclonal antibody was from Sigma (Saint Louis, MO, USA). Protein A/G PLUS-Agarose was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals were of analytical grade.

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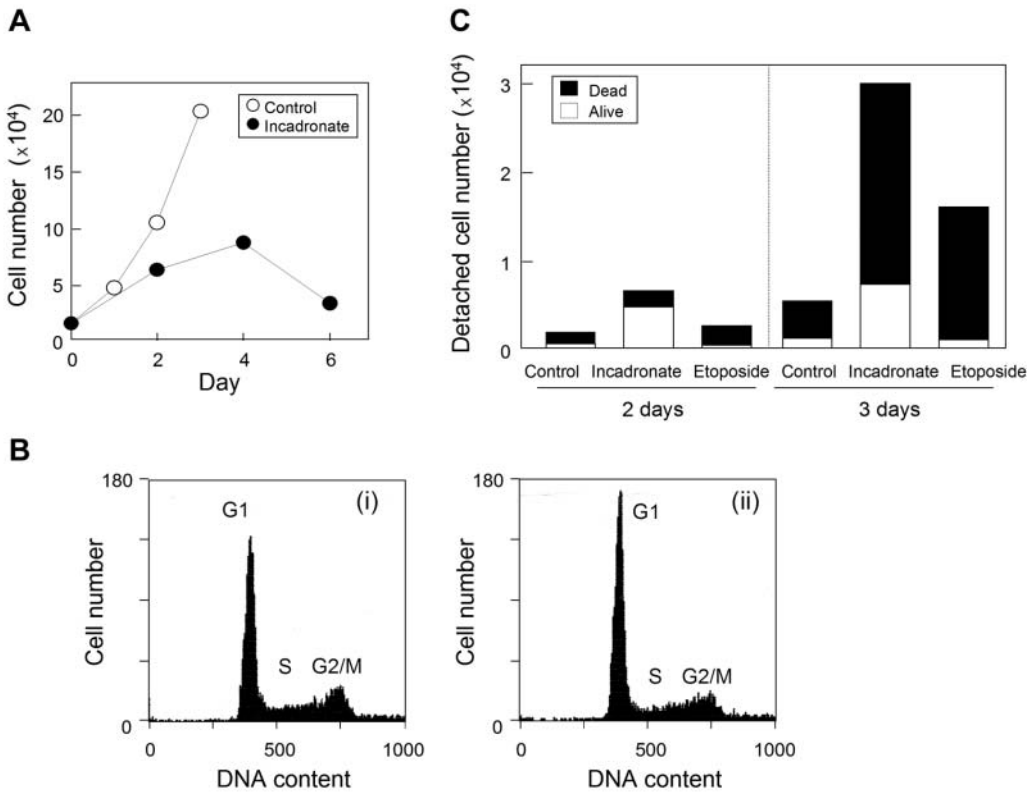

Figure 1. *Effect of incadronate on the growth of human prostate cancer PC-3 cells. A) Cell proliferation. PC-3 cells were seeded at a density of 1x10⁴ cells, and treated with or without 200 μM incadronate for the period indicated. After incubation, the viable cell number was determined by trypan blue exclusion. B) Flow cytometric analysis of the DNA content of incadronate-treated PC-3 cells. The cells were treated without (i) or with (ii) 200 μM incadronate for 3 days. C) Cell viability in incadronate or etoposide-treated floating PC-3 cells. The cells were plated at a density of 6x10⁴ cells in 35-mm dishes and treated with 200 μM incadronate or 10 μM etoposide for the period indicated. The detached cells were collected, stained with trypan blue and counted under a microscope using a hemocytometer.*

Cell culture. Human prostatic carcinoma PC-3 cells were cultured in RPMI-1640 medium containing 10% fetal calf serum under a humidified atmosphere with 5% CO_2 at 37˚C.

Trypan blue exclusion assay. Cells were seeded at a density of $1x10^4$ cells/well of 24-well plates or $6x10^4$ cells per 35-mm dishes. The cells were treated with 200 μM incadronate or 10 μM etoposide (a known apoptosis inducer) for the indicated period of days. In some cases, incadronate-treated cells were coincubated with the intermediates of the mevalonate pathway (farnesol and geranylgeraniol) or a caspase inhibitor (Z-Asp-CH_2-DCB). The detached cells were collected from the medium and adherent cells were trypsinized and collected. The detached and adherent cells were stained with a 0.1% trypan blue solution and counted under a microscope using a hemocytometer.

Cell cycle analysis. Cells were treated with 200 μM incadronate for 3 days. After the incubation, the cells were harvested and suspended in phosphate-buffered saline (PBS) and fixed in 70% ethanol for 1 h at –20˚C. The fixed cells were then treated with 200 μg/mL of propidium iodide and 10 μg/mL of RNase A for 1 h at 37˚C, and analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA).

DNA fragmentation assay. PC-3 cells were seeded at a density of $4x10^5$ cells per 100-mm dish and incubated with or without 200 μM incadronate for the indicated period of days. The detached and adherent cells were collected separately and washed with ice-cold phosphate-buffered saline (PBS). Aliquots of $1x10^4$ cells were lysed in a buffer (10 mM Tris-HCl, 10 mM EDTA, and 1% Triton X-100) and treated with 100 μg/mL of Proteinase K for 12 h at 37˚C and then with 50 μg/mL of RNase A for 6 h at 37˚C. The sample was electrophoresed and visualized by staining with ethidium bromide.

Caspase-3-like protease activity. PC-3 cells were seeded on 60-mm dishes and incubated with 200 μM incadronate or 10 μM VP-16. After incubation, the detached and adherent cells were collected separately and resuspended in a buffer (25 mM HEPES, 2 mM $MgCl_2$, 5 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), and 10 μg/ml pepstatin) and then sonicated. The lysates were centrifuged at 160,000 xg for 20 min. Aliquots (6 μg) of the extracted proteins were incubated at 37˚C for 20 min in a buffer consisting of 25 mM HEPES, 2 mM $MgCl_2$, 5 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM DTT and 10 μg/ml pepstatin with 50 μM Ac-DEVD-MCA. The release of amino-4-methylcoumarin was monitored with a

spectrofluorometer at an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

Immunoprecipitation analysis. Immunoprecipitation was performed as previously described (23). PC-3 cells were seeded on 60-mm dishes and incubated with or without 200 μM incadronate. The cells were pelleted and resuspended in buffer A (20 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 1 mM $NaVO_4$, 5 mM NaF, 1 mM PMSF, 1 μg/mL leupeptin and 1 μg/mL pepstatin) and then homogenized by passage through a 26-gauge needle 10 times. The cell lysates were centrifuged at 800 xg for 5 min and the supernatants were incubated with a 20-μL aliquot of protein A/G agarose at 4˚C overnight and then centrifuged. The protein A/G-treated cell lysates were used for immunoprecipitation.

A 20 μL aliquot of protein A/G-agarose was incubated with 5 μL of anti-FAK mouse monoclonal antibody and mouse IgG at 4˚C for 2 h, respectively. The antibody-protein A/G agarose beads were pelleted by centrifugation. After four washes with buffer A containing 1 μg/mL of bovine serum albumin (BSA), the antibody-protein A/G agarose-treated beads were incubated with the protein A/G-agarose-treated cell lysates at 4˚C for 4 h and then washed three times with buffer A and ice-cold PBS, respectively. The precipitated beads were dissolved in SDS sample buffer and subjected to SDS-PAGE with 7.5% polyacrylamide gels. The proteins were transferred to a PVDF membrane (Millipore, Billerica, MA, USA) and the membrane was incubated with anti-phosphotyrosine mouse monoclonal antibody 4G10. Detection was accomplished with a horseradish peroxidase-conjugated secondary antibody and an ECL detection system (Amersham Biosciences, Piscataway, NJ, USA).

Protein assay. The protein assay was carried out using the Bradford method with BSA as a standard (24).

Results

Incadronate induced apoptosis after cell detachment. We first examined the effect of incadronate on cell proliferation in prostatic PC-3 cells. PC-3 cells were treated with 200 μM incadronate for a specified period and counted. The viable attached cell number in incadronate-treated cells increased up to the 4th day of treatment, but the maximum cell number was still much lower than that of the untreated control (Figure 1A). In order to examine the effect of incadronate on the cell cycle of PC-3 cells, a flow cytometric analysis of propidium iodide-stained cells was performed. Figure 1B (ii) shows that the cells treated with incadronate were arrested in the G1 phase. The percentage of cells in the G1 phase increased from 55% to 64% with 200 μM incadronate treatment in PC-3 cells.

PC-3 cells were treated with incadronate for 2 or 3 days and then floating cells were collected and the numbers of live and dead cells were counted. As shown in Figure 1C, the treatment with incadronate increased the number of floating cells in a time-dependent manner and a significant number of live floating cells were observed. The percentage of live cells was approximately 75% for 2-day incubation and 25% for 3-day. In the case of etoposide (a known apoptosis inducer) treatment, the percentage of live cells was less than 10% among the floating cells.

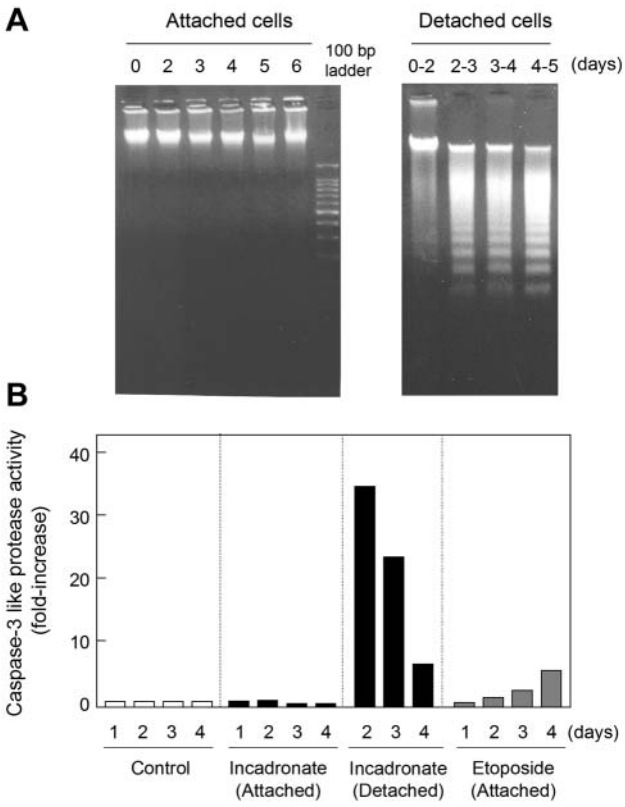

Figure 2. *Induction of apoptosis in incadronate-treated floating PC-3 cells. A) DNA fragmentation analysis of attached and detached PC-3 cells after incadronate treatment. PC-3 cells were treated with 200 μM incadronate for the period indicated. After incubation, attached and detached cells were collected separately and cellular DNA was electrophoresed. B) Caspase-3-like protease activity in incadronate or etoposide-treated PC-3 cells. The cells were treated with 200 μM incadronate or 10 μM etoposide for the period indicated. The attached and detached cells after treatment were collected separately and cell lysates were prepared. The caspase-3-like protease activity was determined by measuring the cleavage of the fluorogenic substrate Ac-DEVD-MCA.*

Nitrogen-containing bisphosphonates have been shown to induce apoptosis in a variety of tumor cells (5, 7, 16). In order to determine whether incadronate induces apoptotic cell death in PC-3 cells, an oligonucleosomal DNA fragmentation assay and caspase-3-like protease assay were performed. As shown in Figure 2A, the oligonucleosomal DNA fragmentation was observed only in detached cells treated with incadronate, not in attached cells. Furthermore, caspase-3-like protease activity also increased in the detached cells after incadronate treatment, but not in the attached cells (Figure 2B). In the case of etoposide treatment, caspase-3-like protease activity increased in the attached cells (Figure 2B).

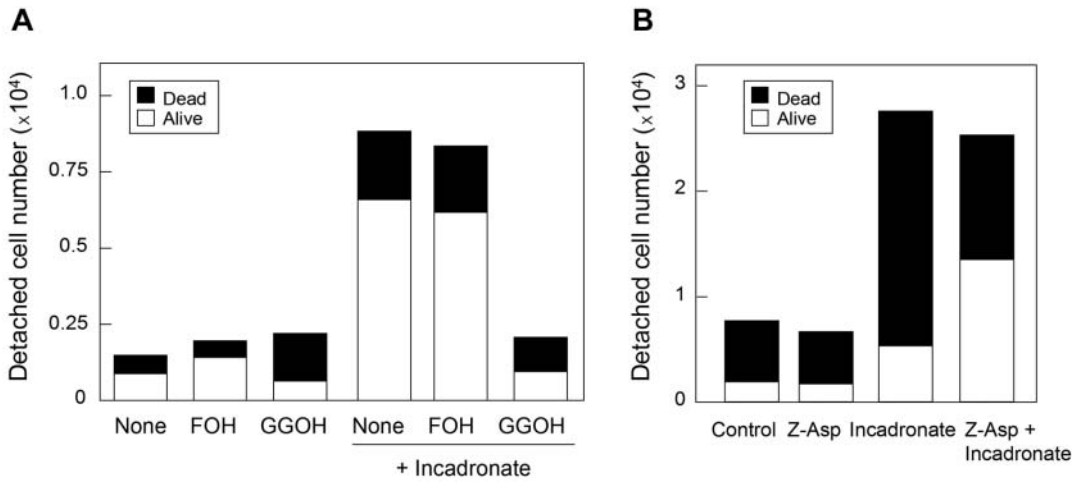

Figure 3. *Effect of isoprenoids and a caspase inhibitor in incadronate-treated PC-3 cells. A) PC-3 cells were treated with 20 μM farnesol or geranylgeraniol with or without 200 μM incadronate for 2 days. B) PC-3 cells were treated with 50 μM Z-Asp-CH2-DCB with or without 200 μM incadronate for 3 days. The detached cells were collected, stained with trypan blue and counted under a microscope using a hemocytometer.*

Effects of intermediates of the mevalonate pathway and a caspase inhibitor on incadronate-induced cell detachment and cell death. Nitrogen-containing bisphosphonate inhibits the mevalonate pathway by inhibiting farnesyl diphosphate synthase and geranylgeranyl diphosphate synthase (15-17). To examine whether the mevalonate pathway was involved in the cell detachment by incadronate, the effect of the intermediates in the pathway (farnesol and geranylgeraniol: a cell-permeable form of farnesyl diphosphate and geranylgeranyl diphosphate) on the detachment of incadronate-treated PC-3 cells was examined. As shown in Figure 3A, the cell detachment by incadronate was recovered by coincubation with geranylgeraniol, but not farnesol.

We next examined whether the cell death after cell detachment was dependent on caspase activation. Figure 3B shows that the coincubation of PC-3 cells with a caspase inhibitor, Z-Asp-CH_2-DCB, and incadronate increased the percentage of live floating cells from about 20% with incadronate alone to 51%. These results strongly suggest that the cell detachment was associated with the inhibition of the mevalonate pathway and then resulted in caspase-dependent cell death in incadronate-treated PC-3 cells.

Incadronate induced the dephosphorylation of FAK in PC-3 cells. Since incadronate induced cell detachment in PC-3 cells, we examined whether it affected the status of adhesion-associated molecules. We focused on the phosphorylation of focal adhesion kinase (FAK) because FAK acts as a key regulator of the focal adhesion complex by binding to integrin in the membrane (22, 25, 26). As shown in Figure 4, FAK tyrosine-phosphorylation in incadronate-treated PC-3 cells was inhibited by approximately 50% compared with that in the control.

Discussion

In this study, we found that apoptosis was induced in incadronate-treated, detached PC-3 cells. In incadronate-treated cells, a decrease in FAK tyrosine-phosphorylation was observed, suggesting that incadronate induced cell detachment, followed by apoptotic cell death (anoikis-like cell death; see following section).

The attachment of cells to matrix components is important for proliferation, and the loss of cell-to-matrix interaction causes an apoptosis termed "anoikis" (27, 28). From our results, incadronate appeared to lead to anoikis-like cell death in PC-3 cells, as indicated by the following observations. First, we found that incadronate induced the dephosphorylation of FAK. Anoikis is due to the interruption of integrin-mediated signal transduction pathways and FAK is a putative regulator of this signal transduction. FAK activation by phosphorylation leads to signals being transmitted from the focal adhesion sites to the cytoplasm upon cell adhesion, and its dephosphorylation is closely associated with the loss of cell-to-matrix contact (29, 30). Second, incadronate-treated floating cells contained a significant proportion of live cells. Finally, caspase-3 activation was observed only in the incadronate-treated floating cells, not in the adherent cells, whereas etoposide treatment induced caspase-3 activation even in adherent cells. Furthermore, treatment with the caspase inhibitor did not inhibit cell detachment by incadronate but it markedly reduced the cell death, suggesting that the detachment was not caused by induction of cell death but that it did subsequently induce cell death.

Nitrogen-containing bisphosphonates are known to inhibit the key enzyme in the mevalonate pathway (15-17).

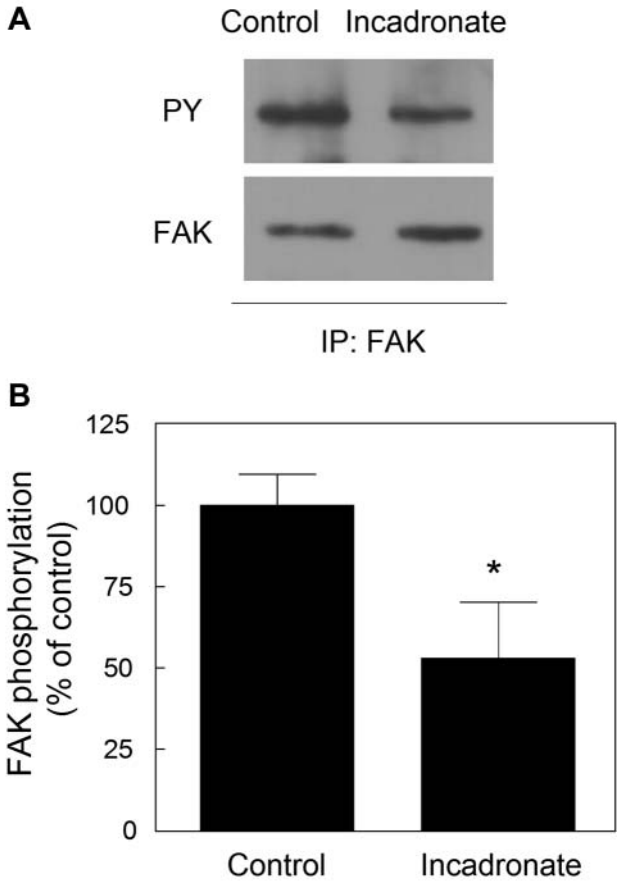

Figure 4. *Effect of incadronate on the phosphorylation of FAK in PC-3 cells. PC-3 cells were treated with 200 μM incadronate for 4 days. A) After incubation, the cells were lysed, immunoprecipitated with anti-FAK antibody, and immunoblotted with anti-phosphotyrosine antibody 4G10 (PY, phosphotyrosine). The blot was then stripped and reprobed with anti-FAK antibody (FAK). B) Band intensity was quantified using the Scion Image program. The intensity of tyrosine phosphorylated FAK was normalized to the intensity of total FAK. * $p<0.05$ versus control.*

We examined whether the induction of cell detachment by incadronate was involved in inhibition of the mevalonate pathway and found that the induction of cell detachment was inhibited by coincubation with the isoprenoid derivative geranylgeraniol but not with farnesol. This result indicates that the cell detachment induced by incadronate in PC-3 cells was involved in the reduction of level of geranylgeranyl diphosphate. There is no clear evidence to explain how a reduced geranylgeranyl diphosphate level induces cell detachment in incadronate-treated PC-3 cells. It should be noted, however, that the down-regulation of caveolin-1 expression by incadronate (21) may be involved in the cell detachment; that is, the downregulation disrupts the association of Src kinases with beta1 integrins, and the phosphorylation of FAK, and results in adhesion to the matrix (26, 31). Zoledronic acid was shown to induce the dephosphorylation of FAK and modulate integrin-mediated adhesion of HUVECs (32). The effects of incadronate on Src kinase and beta integrins are worth examining.

Since incadronate induces apoptotic cell death even in suspension-cultured cells (*e.g.* promyelocytic leukemia HL-60; data not shown), the mechanism of cell death induced by incadronate does not appear to be restricted to detachment. There are at least two distinct mechanisms by which bisphosphonate induces apoptosis: (i) by altering cell cycle progression (6, 16) and (ii) by inducing mitochondrial dysfunction involving changes in the Bcl-2/Bax ratio (16, 18). In fact, G1 cell-cycle arrest by incadronate was observed in PC-3 cells. Moreover, when PC-3 cells were treated with a variety of bisphosphonates, the percentage of live cells among the floating cells after treatment differed markedly depending on the bisphosphonate used (etidronate, pamidronate, alendronate, risedronate, and incadronate); for example, about 50% of incadronate-treated cells were alive, while less than 10% of pamidronate-treated cells were alive (data not shown). This result might suggest that pamidronate causes cell death directly rather than cell detachment, while incadronate induces detachment of PC-3 cells. Taken together, bisphosphonate could induce apoptosis in cancer cells by different mechanisms: cell-cycle arrest (6, 16), mitochondrial damage (16, 18), cell detachment (this study), and the main pathway, probably dependent on cancer cell lines and the bisphosphonate used.

The adhesion of cancer cells to the bone matrix is a key step in bone metastasis. Bisphosphonates have been shown to inhibit the adhesion of cancer cells to the bone matrix (3). Although it is unclear whether the detachment from bone matrix by bisphosphonates occurs in the same way as that from a plastic surface, the results of this study suggest that in already-attached cells, bisphosphonates induce cancer cell detachment and this results in cell death *via* apoptosis. There are numerous reports indicating the effectiveness of bisphosphonate therapy in the management of bone metastases (8-14). Our results could explain one of the mechanisms of inhibition of bone metastasis by treatment with bisphosphonate.

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