Alendronate Inhibits Intraperitoneal Dissemination in \textit{In vivo} Ovarian Cancer Model

Kae Hashimoto, \textsuperscript{1} Ken-ichirou Morishige, \textsuperscript{1,2} Kenjiro Sawada, \textsuperscript{1} Masahiro Tahara, \textsuperscript{1} Rikako Kawagishi, \textsuperscript{1} Yoshihide Ikebuchi, \textsuperscript{1} Masahiro Sakata, \textsuperscript{1} Keiichi Tasaka, \textsuperscript{1} and Yuji Murata \textsuperscript{1}

\textsuperscript{1}Department of Obstetrics and Gynecology, Osaka University Graduate School of Medicine and \textsuperscript{2}Department of Gynecology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan

\section*{Abstract}
Ovarian cancer is characterized by diffuse peritoneal carcinomatosis and often by large volumes of ascites. We previously reported that alendronate, a nitrogen-containing bisphosphonate, inhibited ovarian cancer cell migration by attenuating the activation of Rho through inhibiting the mevalonate pathway. However, questions remain about the ability of alendronate to inhibit the invasiveness of cancer cells to the adherent tissues and the growth of disseminated ovarian cancer \textit{in vivo}. We established an \textit{in vivo} ovarian cancer model with i.p. carcinomatosis in athymic immunodeficient mice. In the prevention model, in which alendronate administration started from the day after tumor inoculation, alendronate prevented the stromal invasion, reduced the tumor burden, and inhibited ascites accumulation. Histologic observation revealed that alendronate treatment decreased the stromal invasion of the i.p. tumor while inhibiting the metalloproteinase-2 activity in ascites. This antitumor effect might result from the inhibition of cancer cell migration and proteolytic activity. In the treatment model, in which alendronate was given from 10 days after tumor inoculation when macroscopic tumors are already implanted in the peritoneum, the antitumor effect was weaker but still significant. Furthermore, alendronate administration decreased the serum CA-125 levels of mice bearing disseminated ovarian cancer compared with those of nontreated mice. The potent effects of alendronate in reducing stromal invasion, tumor burden, and ascites suggest that it will be of value in regimens for treatment of women with ovarian cancer. (Cancer Res 2005; 65(2): 540-5)

\section*{Introduction}
Most patients with ovarian cancer will eventually die from their disease, at least in part because 75\% to 80\% of all patients present with late-stage disease, for which current treatment is inadequate. Late-stage ovarian cancer is characterized by widespread peritoneal dissemination and ascites (1). The taxane paclitaxel has shown significant effects against ovarian cancer and somewhat improved response rates compared with some other therapeutic agents (2). Despite these advances, the mortality due to ovarian cancer has not been dramatically improved (2, 3). Therefore, new paradigms and therapeutic approaches need to be investigated to improve the treatment of this neoplasm.

One new therapeutic strategy is to clarify the mechanism of metastasis of cancer cells and to identify agents that prevent cancer cells from invading or migrating into the peritoneum. Among many growth-promoting factors known to be present in ovarian cancer ascites, lysosphosphatic acid (LPA) is found there at significant levels and may play an important role in the development or progression of ovarian cancer (4, 5). Actually, LPA has been reported to induce many cellular effects, including mitogenesis, the secretion of proteolytic enzymes, and migration activity (6–8).

Cell migration is regulated by a combination of different processes: the contraction of actomyosin, the formation of stress fibers, and the turnover of focal adhesions (9). Contraction of the actomyosin system is important for cell migration, and LPA induces myosin light-chain phosphorylation through the activation of small GTP-binding protein (G protein) Rho, leading to the stimulation of cell contractility and motility (10). Another fundamental component affecting cell motility is focal adhesion: cell-extracellular matrix adhesions can alter the cell’s capacity to attach and migrate through surrounding tissues (9). Rho is a key mediator of the assembly of structures involved in focal adhesion. Changes in the expression and activities of the components of focal adhesions may make an important contribution to cancer invasion (11). We previously reported that LPA stimulates cell motility through stress fiber formation and focal adhesion assembly under the control of Rho activation in ovarian cancer (7). Interfering with the LPA signal transduction pathway by modulating the Rho activity is thus an attractive strategy for improving the outcome of this devastating disease.

Nitrogen-containing bisphosphonates (N-BPs) are potent inhibitors of bone resorption used for the treatment and prevention of osteoporosis. N-BPs have been shown to inhibit the cholesterol biosynthesis pathway as well as isoprenylation (farnesylation and geranylgeranylation) by inhibiting either isopentenyl diphosphate synthase or a downstream enzyme, farnesyl diphosphate synthase, or both (12). Protein targets of isoprenylation include small G proteins such as Rho, Ras, Rac, and Rab, which require the posttranslational modification to undergo a series of changes that lead to their attachment to the plasma membrane and their full activation. The activation of small G proteins is essential for cancer cell growth and invasion (13, 14). Accordingly, N-BPs have the potential to inactivate small G proteins that regulate cancer cell growth, motility, and invasion (15, 16).

We previously studied the effect of alendronate, a N-BP, on the \textit{in vitro} migration of human ovarian cancer cells (17). Treatment of ovarian cancer cells with alendronate resulted in the inactivation of Rho, changes of cell morphology, loss of stress fiber formation and focal adhesion assembly, and
suppression of phosphorylation or tyrosine phosphorylation of focal adhesion proteins, which are essential processes for cell migration. These results suggest that alendronate inhibits Rho activation by preventing geranylgeranylation, resulting in inhibition of the cell motility and invasiveness of human ovarian cancer cells.

We have developed a model of i.p. ovarian carcinomatosis in athymic immunodeficient mice. This model closely resembles stage III ovarian cancer, with both extensive dissemination of ovarian carcinoma cells to peritoneal surfaces and the development of massive ascites. In this study, we analyzed the in vivo effects of alendronate in the prevention protocol (treatment starting from the day after tumor inoculation) and in the treatment protocol (treatment starting 10 days after tumor inoculation when visible tumors are already spread in the peritoneum similar to stage III ovarian cancer) and showed that alendronate markedly inhibited the invasiveness of human ovarian cancer cells, which resulted in the suppression of ascites formation and decrease of the tumor burden.

Materials and Methods

Materials. Mice received injections of alendronate sodium hydrate (monosodium-4-amino-1-hydroxybutylidene-1, 1-diphosphonate trihydrate, Teijin Limited, Chiyoda-ku, Tokyo, Japan). Alendronate was solubilized in citrate solution at a concentration of 10 mM/mL and adjusted to neutral pH. DMEM was purchased from Life Technologies, Inc. (Gaithersburg, MD), cultured in DMEM supplemented with 10% (volume for volume) fetal bovine serum, penicillin (10 units/mL)-streptomycin (10 mg/mL) in 95% air and 5% CO2 at 37°C, and used within 15 passages after initiation of the culture.

Experimental Animals. Six- to 8-week-old female nude mice (BALB-c nu/nu, Japan SLIC, Inc., Hamamatsu, Japan) were housed in filtered-air laminar-flow cabinets and were manipulated using aseptic procedures. Procedures involving animals and their care were conducted in conformity with the guidelines of our university.

Experimental Design. The in vitro ovarian cancer models using Caov-3 cells were prepared as previously described (18). Caov-3 cells were injected i.p. as a cell suspension into 10 nude mice (10 × 106 cells per animal) for each experimental group. The treatment regimens started the day after tumor inoculation and continued for 7 weeks in the prevention protocol. This model corresponds to remission cases after the primary treatment of patients with stage III ovarian cancer, who respond well to taxane-containing chemotherapy after the debulking surgery (response rate, 60-80%; refs. 19, 20). Because most remission cases relapse (3), preventive consolidation therapy to avoid recurrence is necessary. On the other hand, in the treatment protocol, administration of alendronate started 10 days after tumor inoculation and continued for 5 weeks. On the 10th day after inoculation, visible tumors are already disseminated i.p. (data not shown). Alendronate was delivered using a saline vehicle and given i.p. every day. The daily doses of alendronate used were as follows: 0, 0.1, and 1.0 mg/kg/d. The dosage levels were determined on the basis of the s.c. dose at which the pharmacologic effect appears in ovariecotomized rats with osteopenia (21). At the end of the treatment period, the mice were sacrificed. The volume of ascites was measured, and tumor tissue was excised, weighed, fixed in 4% paraformaldehyde, and embedded in paraffin. Paraffin sections (5 μm) were used for histologic analysis. Sections were stained with H&E and examined. Ascites samples were collected and centrifuged at 3,000 × g for 10 minutes and then stored at −20°C. Matrix metalloproteinase (MMP)-2 concentration was determined by using MMP-2 activity assay system (Amersham, Piscataway, NJ) according to the manufacturer’s instructions. This assay system can detect both endogenous levels of free active MMP-2 and total levels of free MMP-2. Blood samples were collected from the left heart ventricle and assayed for serum CA-125 and plasma calcium.

Results

Effects of Alendronate in Caov-3 Ovarian Cancer Model. We previously showed that alendronate inhibited LPA-induced ovarian cancer cell migration in vitro (17). To assess the effect of alendronate on tumor invasion in vivo, we used a model of i.p. ovarian carcinoma in athymic immunodeficient mice. To assess the anti-invasive/antimigratory effect of alendronate, we started the administration of the drug from the day after cancer cell inoculation in the prevention protocol. Preliminary experiments revealed that tumor-bearing mice began to exhibit abdominal swelling with ascites around 4 weeks after the cancer cell inoculation and died with cachexia after the 8th week without any treatment. Therefore, we sacrificed and examined the mice 7 weeks after cancer cell inoculation. At postmortem examination, tumors were found on the surface of the peritoneum, diaphragm, intestines, omentum, and uterus, with massive ascites in the control group. Furthermore, we also assessed the antitumor effect of alendronate in the treatment protocol, in which tumors had already spread to the peritoneum 10 days after inoculation, similar to previous studies that examined the inhibitory effect on tumor growth in vivo models (22, 23). This model corresponds to stage III ovarian cancer, which is the stage at which the majority of patients with ovarian cancer are first treated. The extent of tumor burden varied among the different treatment groups (Fig. 1A). The control mice of the prevention protocol were given injections of saline for 10 more days than the control animals of the treatment protocol. As a result, the tumor burden and ascites of the control animals were not different between the prevention protocol and the treatment protocol. Therefore, the data of both control groups were combined. i.p. tumor burden and ascites were both quantified. Mean tumor burden in the control group (2.18 ± 0.60 g) was significantly reduced (by ~37%) in the group given 0.1 mg/kg/d (1.38 ± 0.20 g; P < 0.001) in the prevention protocol. Strikingly, alendronate treatment at 1 mg/kg/d reduced the tumor burden by ~88% (0.48 ± 0.42 g; P < 0.001; Fig. 1B). Fig. 1C shows the effects of the alendronate treatment on ascites formation. The mean volume of ascites in the control group was 7.20 ± 3.34 mL. In contrast, very little ascites developed in alendronate-treated (1.0 mg/kg/d) mice. The inhibitory effect of alendronate on ascites formation was also dose dependent. In the treatment model, the inhibitory effects on tumor burden and ascites accumulation were less marked than those in the prevention models but were significant and dose dependent (Fig. 1). The difference of the inhibitory effects between these two models might suggest that the effect of alendronate in this experiment acts mainly through an anti-invasion/antimigration mechanism rather than through an antiproliferative mechanism. Alendronate in this dose range did not impair the viability of mice and did not change the actual body weight because the difference in body weight between the control group and treated group corresponded to the tumor burden and ascites (Fig. 1D).

Furthermore, plasma calcium was not altered by the continuous administration of alendronate [control group, 9.35 ± 0.90 mg/dL; alendronate (1.0 mg/kg/d) group,
9.23 ± 0.23 mg/dL in the prevention model, 9.20 ± 0.74 mg/dL in the treatment model]. These data suggest that these doses of alendronate did not induce pathologic features such as hypocalcemia and are appropriate for in vivo studies.

Stromal Invasion Is Inhibited by Alendronate. It has been speculated that the suppression of tumor growth and ascites formation by alendronate might result, at least in part, from the inhibition of cancer cell migration by alendronate, whose effects on cell migration were previously shown in vitro by Sawada et al. (17). To test this possibility, we examined resected tumor sections under a microscope. In control animals of the prevention and treatment model, stromal invasion to surrounding tissues was detected at several sites by microscopic observation (Fig. 2A). In contrast, in alendronate-treated animals of the prevention model, we could detect only a few sites where the tumors invaded the host tissue, even with intensive and careful observation (Fig. 2B and C), although the anti-invasive effect of alendronate was weaker but still significant in the treatment model (Fig. 2C). The anti-invasive activity is known to be exerted mainly through the inhibition of migratory activity and the suppression of the expression of proteolytic enzymes, including plasminogen activators and MMPs that degrade cell-extracellular matrix proteins. Especially, MMP-2 is one of the potent MMPs in ovarian cancer progression (6, 24, 25). We examined MMP-2 concentrations in the ascites of Caov-3-inoculated animals. The active MMP-2 concentration was 8.58 ± 7.91 ng/mL (n = 15) in the control (Caov-3–inoculated group) and was reduced to 3.72 ± 3.60 ng/mL (n = 10) in the alendronate-treated group (1.0 mg/kg/d) of the prevention protocol and 3.89 ± 1.70 ng/mL (n = 10) in the alendronate-treated (1.0 mg/kg/d) group of the treatment model (Fig. 3). The total MMP-2 concentrations were not significantly different between the groups (96.46 ± 43.06, 76.12 ± 22.51, and 74.56 ± 14.28 ng/mL, respectively). These results suggest that the anti-invasive mechanism of alendronate might mainly involve its antimigratory activity to ovarian cancer cells and inhibition of MMP-2 activation.

Serum CA-125 Level Is Reduced by Alendronate. Caov-3 cells express the CA-125 antigen, a clinically sensitive marker of ovarian cancer, and the change of tumor mass in patients with ovarian cancer.
The serum CA-125 level of normal 7-week-old female nude mice was 1.57 ± 0.15 units/mL ($n = 3$). By comparison, the CA-125 level was extremely high in the nontreated group (897 ± 923 units/mL, $n = 10$). Alendronate treatment (1 mg/kg/d) significantly reduced the serum CA-125 level (115 ± 79.1 units/mL in the prevention protocol, 220 ± 225 units/mL in the treatment protocol, $n = 8$; $P < 0.05$) in tumor-bearing mice (Fig. 4). These data are in accord with the reduction of tumor burden and ascites by alendronate treatment.

**Discussion**

During the progression of ovarian carcinomas, cancer cells released from the surface of the tumor can adhere to and invade tissues and organs in the peritoneal cavity. Consequently, the poor outcome is, at least in part, due to peritoneal dissemination caused by the aggressive migration activity of ovarian cancer cells (1). To undergo peritoneal dissemination, ovarian cancer cells need to detach from the primary tumor, attach to the cell-extracellular matrix of other tissues, and migrate into stroma. Once cancer cells migrate into stroma, angiogenesis occurs from preexisting capillaries or venulae. Currently available anticancer drugs, even if they are very effective at killing cancer cells, can be used only at limited concentrations because of their toxicity to normal cells. Accordingly, it is worthwhile looking for drugs that inhibit the progression of ovarian cancer without affecting cell viability.

BPs inhibit the adhesion of breast cancer and prostate cancer cells to bone matrix (27). In a mouse model of breast cancer, BPs inhibited the progression of the cancer and the development of bone metastasis (28). In prostate cancer cells, BPs have shown a remarkable inhibitory effect on cell proliferation via the induction of cell death and cytoplasia in bone (29). Thus, BPs are known to inhibit bone metastasis without affecting visceral metastasis. In contrast, another study showed an inhibitory effect of the BP minodronate on the growth of soft-tissue metastases when this compound was used in a preventive manner (30). More impressively, alendronate treatment of rats bearing the syngenic 13762 mammary tumor in bone substantially reduces the formation of spontaneous pulmonary metastasis in the animals (31). Furthermore, a clinical study showed that the BP clodronate suppresses distant metastases to both bone and visceral organs in patients with breast cancer (32). Thus, BPs may exert direct antitumor effects outside the bone.

The direct effects of N-BPs on cancer cell growth and invasion have been examined in *in vitro* experiments. Previous reports about the effects of N-BPs on cancer cells mainly focused on cell viability, and the effective concentrations of N-BPs were relatively high (27, 33, 34). According to those reports, the concentrations of pamidronate, zoledronate, or ibandronate that induced apoptosis of breast cancer and prostate cancer cells were around 100 μmol/L. Considering that the concentration of alendronate that significantly inhibits bone resorption is 0.1 μmol/L, which is a clinically used concentration of alendronate (35), effects of alendronate other than the induction of apoptosis of cells seem likely to be clinically important. The anti-invasive effect of alendronate might result from the inhibition of migratory activity and proteolytic activity. A recent report showed that alendronate inhibits the *in vitro* invasion of prostate cancer cells at low concentrations (15). Furthermore, we
have reported that the inhibitory effect of alendronate on migration occurs without enhancing the apoptosis of cells in vitro (17). These results suggest that alendronate might be effective in vivo at moderate concentrations.

Actually, it is difficult to obtain rather high concentrations of BPs in nonskeletal organs (36). Recent studies using 14C-labeled BPs proved that BPs were accumulated in the aortas of even healthy rabbits and in human arteries at moderate concentrations (37). In addition, chronic i.v. N-BP therapy was reported to increase high-density lipoprotein cholesterol and decrease low-density lipoprotein cholesterol in postmenopausal women (38). These facts suggest that BPs, especially N-BPs, may have the potential to be present at moderate concentrations in peritoneal tumors, especially with daily i.p. administration. Further experiments using 14C-labeled alendronate will be necessary for examining the concentrations of alendronate in peritoneal tumors in nude mice. Moderate concentrations of N-BPs might inhibit the migratory activity of ovarian cancer cells without inducing apoptosis in normal cells. Therefore, we conducted this study applying alendronate to control the peritoneal dissemination of ovarian cancer, using the i.p. administration method.

We also examined the alendronate effect against the proteolytic activity of cancer cells. In this study, the active MMP-2 concentrations in ascites were reduced by alendronate treatment without modulating the total MMP-2 concentrations. Although the mechanism of inhibiting MMP-2 activity has not been elucidated, Boissier et al. (39) suggested that N-BPs might suppress the MMP-2 activity through zinc chelation without attenuating the total MMP-2.

Strikingly, alendronate inhibited not only the invasiveness of ovarian tumors but also ascites formation and tumor growth. This antitumor effect may also derive from other mechanisms than the suppression of cancer cell migration and proteolytic activity: BPs might exert an antiangiogenic effect through lowering of the vascular endothelial growth factor expression (40) and might cause direct inhibition of endothelial cell migration, proliferation, sprouting, and tubulogenesis (36, 41). The fact that alendronate accumulates in vessels (37) strongly suggests that alendronate has the potential ability to prevent angiogenesis during tumor growth. Furthermore, N-BPs might exert the antiproliferative effects on cancer cells through inhibiting other members of the Ras superfamily than Rho (16, 42). Actually, alendronate also inhibited the proliferation of Caov-3 cells through the suppression of Ras in vitro (data not shown).

This is the first report showing that N-BPs inhibit cancer cell invasion to visceral organs in vivo and may provide the basis for a new therapy to control the dissemination of ovarian cancer.

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References


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