Inhibitory Effects of Bisphosphonates on the Proliferation of Human Ovarian Cancer Cell Lines and the Mechanism

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Abstract: Bisphosphonates are now well established as successful agents for the prevention and treatment of postmenopausal osteoporosis and corticosteroid-induced bone loss. Bisphosphonates have also recently become important in the management of cancer-induced bone disease, and they now have a widely recognized role for patients with multiple myeloma and bone metastases secondary to breast cancer and prostate cancer. Recent studies suggest that, besides the strong antiosteoclastic activity, the efficacy of such compounds in the oncological setting could also be due to direct anti-tumor effect. However, the effect of bisphosphonates to ovarian and endometrial cancers has not been elucidated. Thus, we examined the direct effect of bisphosphonates on the various ovarian cancer cell lines. Except for etidronate, all of bisphosphonates examined had the direct inhibitory effects on proliferation of all ovarian cancer cell lines used. Especially, pamidronate had the most marked inhibitory effect and inhibited dose-dependently the proliferation of ovarian cancer cell lines. KFr 13 cells among ovarian cancer cell lines used was the most sensitive to pamidronate and the caspase 3 activity was markedly stimulated by treatment with pamidronate, suggesting induction of apoptosis.

Key Word: Ovarian cancer cells, bisphosphonates, cell proliferation, apoptosis.

INTRODUCTION

Bisphosphonates are potent inhibitors of bone resorption; therefore, they are used widely and successfully for treating or preventing malignant hypercalcemia, tumoral osteolysis, Paget's disease, and osteoporosis [1, 2]. Bisphosphonates are now well established as successful agents for the prevention and treatment of postmenopausal osteoporosis, since they have been shown to increase bone mass and diminish by half, fracture rates at the spine, hip and other sites in postmenopausal osteoporosis [3, 4]. Despite this wide use, the mechanism of their actions is not yet fully understood. Recent studies have suggested that, besides the strong antiosteoclastic activity, the efficacy of such compounds in the oncological setting could be also due to a direct anti-tumor effect [5]. In fact, the apoptotic and antiproliferative effects of bisphosphonates on osteoclasts may be exerted also on macrophages and tumor cells [6-8]. This antiproliferative mechanism of bisphosphonates has been attributed to a cytotoxic/cytostatic or proapoptotic effect [7, 8].

Thus, we attempted to elucidate direct antiproliferative effects of bisphosphonate using various ovarian cancer cell lines and the mechanism.

MATERIALS AND METHODS

Agents

Etidronate disodium, pamidronate disodium, alendronate sodium hydrate and incadronate disodium were supplied from Sumitomo Pharmaceutical Company Japan, Novartis Pharma Co. Ltd, Japan, Banyu Pharmaceutical Company Japan and Astellas Pharmaceutical Company Japan, respectively.

Cell Lines

Cell lines used in the present study are described in Table **1**. KF28 cells are a single-cell clone of the human ovarian carcinoma cell line KF [9]. KFr13 is a cisplatin-resistant subline derived from KF28 cells [10]. KF28TX and KFr13TX are paclitaxel-resistant sublines derived from KF28 and KFr13 cells, respectively. MH and HT cell lines were derived from patients who did not respond to primary cisplatin-based chemotherapy [12]. TYK cell line was established from undifferentiated carcinoma of the ovary and TYK/R is a cisplatin-resistant cell line established by exposure of TYK to escalating doses of cisplatin [13]. RTSG was derived from poorly differentiated adenocarcinoma of the ovary. KK and RMG-II were established from clear cell carcinoma of the ovary [12]. RMUG-S and RMUG-L were derived from mucinous cystadenocarcinoma of the ovary [14]. These cell lines were grown as monolayer cultures in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units penicillin/ml, and 100 μg streptomycin/ml (Grand Island Biological Co., Island, N.Y.) in a humidified atmosphere of 5% CO_2 at 37ºC, and were usually subcultured once a week with 0.25% trypsin.

Cytotoxicity Assay

Cytotoxicity assays were performed in 96-well microtiter plates by seeding 2000 cells/well for KFr13, KFr13TX, KK and MH, 2500 cells/well for TYK/R, HT and RMUG-S,

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Table 1. Cell Lines Used in the Present Study

Cell lines	Histologic types	References
KF28	Serous cystadenocarcinoma	[9]
KFr13	Serous cystadenocarcinoma	[10]
KF28TX	Serous cystadenocarcinoma	[11]
KFr13TX	Serou cystadenocarcinoma	[11]
MH	Serous cystadenocarcinoma	[12]
HT	Serous cystadenocarcinoma	
TYK	Undifferentiated carcinoma	[13]
TYK/R	Undifferentiated carcinoma	[13]
RTSG	Poorly differentiated adenocarcinoma	
KK	Clear cell adenocarcinoma	[12]
RMG-II	Clear cell adenocarcinoma	
RMUG-S	Mucinous cystadenocarcinoma	[14]
RMUG-L	Mucinous cystadenocarcinoma	[14]

3000cells/well for KF28, KF28TX, TYK and RTSG, 4000 cells/well for RMUG-L and 5000 cells/well for RMUG-II, respectively and they were incubated in the absence or presence of drugs in a humidified atmosphere of 5% CO_2 at 37ºC. After incubation for 5 days, cytotoxicity was evaluated by crystal violet staining according to a previous report (15). Briefly, an equal volume of 10% formalin in phosphate-buffered saline containing 0.2% crystal violet was added to each well and, after incubation at room temperature, the absorbance at 570 nm of stained cells in each well was measured with an automatic microtest-plate reader (Multi-scan MCC/340, Titertek, Flow Laboratories Inc., Va.). The average absorbance of the control wells in the absence of drugs was regarded as 100%, and the percentage cell growth in the each well was calculated. The concentrations of drugs that inhibited the growth of cells to the level of 50% of the control growth (IC_{50}) were obtained from graphical plots. The viability was assessed by trypan blue dye exclusion.

Caspase-3 Activation Assay

Cell death caspase-3 activity in drug-treated cell lysates was determined using a colorimetric assay kit (R&D Systems, Minneapolis, MN). Briefly, KFr cells were treated with IC_{50} doses of cisplatin, paclitaxel, pamidronate and etidronate alone for 4 hr. The amount of chromophore p-NA released from DEVD-pNA substrate peptide was read in a plate reader as per the supplier's instructions.

Statistical Analysis

Quadruplicate samples were assayed in these *in vitro* experiments.

Statistical differences were determined by Chi-square test and P value less than 0.05 was considered statistically significant.

RESULTS

As shown in Table **2**, we examined inhibitory effects of bisphosphonates on proliferations of various ovarian cancer cell lines described in Table **1**. Etidronate among bisphosphonates used in the present study failed to obtain the IC_{50} dose in the all cell lines used. Pamidronate had the most marked inhibitory effect on proliferations of all cell lines, showing significantly smaller IC_{50} dose (40.5 ± 13.3 μM) than incadronate and alendronate. In addition, we examined the inhibitory effect according to histological types (Table **3**). Although pamidronate showed the marked inhibitory effect on the proliferation in any histological type, it is noteworthy that the inhibitory effect on proliferations of clear cell carcinoma, which has been reported to be chemoresistant [16], was prominent not only in pamidronate but also in incadronate and alendronate (Table **3**). It was also demonstrated that the strong inhibition of cell proliferation

Table 2. Inhibitory Effects of Bisphosphonates on Proliferations of Ovarian Cancer Cell Lines

Agents	IC_{50} μM (Mean ± SD)
Pamidronate	40.5 ± 13.3[a] (13)[b]
Incadronate	128.5 ± 105.8 (12) (IC_{50} was not obtained in 1 cell line)
Alendronate	119.6 ± 109.2 (8) (IC_{50} was not obtained in 5 cell lines)
Etdronate	IC_{50} was not obtained in all 13 cell lines examined)

[a]P<0.01 (Chi-square test), compared to incadronate and alendronate.

[b]Number of cell lines examined.

Table 3. Inhibitory Effects of Bisphosphonates on Proliferations According to Histologic Types of Ovarian Cancer Cell Lines

Histology	IC_{50} μM (Mean ± SD)		
	Pamidronate	Incadronate	Alendronate
Serous	32.9 ± 14.7 (6)[a]	128.1 ± 131.0 (6)	61.6 ± 29.6 (4)
Clear	39.8 ± 5.7 (2)	57.9 ± 16.4 (2)	72.9 ± 43.9 (2)
Mucinous	61.9 ± 8.3 (2)	210.7 ± 71.6 (2)	Not obtained
Undifferentiated	38.5 ± 4.5 (3) (not obtained in 1 cell line)	118.3 ± 91.2 (2) (not obtained in 1 cell line)	259.7 ± 146.6 (2)

[a]Number of cell lines examined.

by pamidronate resulted in induction of caspase-3 activity, suggesting induction of apoptosis (Table **4**).

Table 4. Induction of Caspase-3 Activity in KFr Cells by Pamidronate

Pmol pNA liberated/hour/μg protein	
Cisplatin	629.3[a]
Paclitaxel	552.2
Pamidronate	398.0
Etidronate	1.1

[a]Average value from four independent experiments.

IC_{50} dose of each treatment drugs was used.

DISCUSSION

Bisphosphonate efficacy for the treatment of bone metastases was initially thought to depend only on the antiosteoclast activity of such compounds. Antiresorptive properties were considered to be sufficient by themselves to explain their ability to reduce skeletal morbidity in patients with lytic bone disease. Bisphosphonates were shown to inhibit establishment and growth of osteoblastic bone metastases from prostate cancer; the efficacy was attributed to the observation that abnormal osteoblastic bone formation within metastases is preceded by osteoclastic activation [17]. However, several lines of evidence suggesting a direct antitumor effect of bisphosphonates have progressively accumulated [18, 19]. On the other hand, such effects of bisphosphonates have not been reported in the gynecological cancers. In the present study, we examined the direct effect of etidronate, incadronate, alendronate and pamidronate (zoledronate has not been approved and obtained in Japan) on proliferation of various human ovarian cancer cell lines. Etidronate, the first bisphosphonate used to treat a human disease, was synthesized exactly 100 years ago [20]. It consists of a simple chemical structure in which the R1 side chain is a methyl group ($-CH_3$) and the R2 side chain is a hydroxyl (-OH). When the length of the R2 side chain was increased from a simple methyl group to longer alkyl chains, significantly more potent compounds were obtained [21]. An up to 1000-fold increase in potency was achieved by the introduction of a primary amino group ($-NH_2$) at the extremity of the R2 alkyl chain, to form the amino-bisphosphonates (e.g. alendronate, pamidronate and neridronate) [22]. Amino-bisphosphonates with a secondary amino group (e.g. incadronate) and a tertiary amino group (e.g. olpadronate) are even more effective, and potency reaches the peak when the tertiary nitrogen is included within a ring structure in the R2 side chain (as in risedronate and zoledronic acid) [23, 24]. As described in Table **2**, pamidronate showed the most potent inhibitory effect among bisphosphonates examined. Interestingly, it was demonstrated that bisphosphonates, except for etidronate, have the potent inhibitory effect in the clear cell carcinoma cell lines, which has been suggested to be chemoresistant (Table **3**) [16]. It is well known that bisphosphonates can be divided in two different classes (amino-bisphosphonates and non-amino-bisphosphonates), not only because of the molecular structures but also the intracellular mechanism of action. Early studies suggested that non-amino-bisphosphonates, such as etidronate and clodronate, can affect a large number of metabolic processes, including glycolysis, fatty acid oxidation and lactate production [25, 26]. Non-amino-bisphosphonates can be metabolized to methylene-containing analogs of ATP, which are extremely resistant to hydrolysis [27, 28]. On the other hand, the more potent amino-bisphosphonates (such as alendronate, risedronate, pamidronate ibandronate and zoledronic acid) are not metabolized to ATP analogs, and show a completely different mechanism of action. Thus, a difference in efficacy was observed between the first generation bisphosphonate etidronate and the amino-bisphosphonates.

Furthermore, we examined the effect of pamidronate on caspase-3 activity by using cisplatin-resistant KFr cell line. Although IC_{50} dose of etidronate showed no effect on the caspase-3 activity, IC_{50} dose of pamidronate as well as IC_{50} doses of cisplatin and paclitaxel stimulated markedly the caspase-3 activity (Table **4**). At present, bisphosphonates are used clinically once a patient has presented with metastatic bone disease. As preclinical evidence has suggested that in addition to their effect on bone resorption, bisphosphonates have anti-tumor effects, the use of bisphosphonates as an adjuvant therapy (in combination with anticancer drugs) is under investigation to target tumor cells systemically. Clinical trials to date assessing potential adjuvant use of bisphosphonates have nevertheless reported conflicting results, with studies monitoring survival and development of bone metastases of patients with primary operable breast

cancers after treatment with clodronate [29-31]. Further investigation of bisphosphonates in the adjuvant setting is still required.

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