Abstract

Little is known about the biological effects of bisphosphonates on primary malignant bone tumors. The purpose of this study was to investigate the antitumor effects of newly developed minodronate (MIN) on a variety of human malignant bone tumors. We examined the effects of MIN and clinically relevant incadronate (INC) on the proliferation, apoptosis, and cell cycle of two osteosarcoma (Saos-2, MG-63), two chondrosarcoma (SW1353, OUMS27), and two Ewing's sarcoma (RD-ES, SK-ES-1) cell lines. Furthermore, we investigated the anti-invasion effects of MIN on sarcoma cells and the effects of MIN on tumor growth in nude mice. MIN inhibited the viability of all six cell lines in a dose-dependent manner with IC_{50} values of 2.7-5.0 μM, which were significantly lower than those of INC. Importantly, both bisphosphonates affected the viability of normal bone marrow stromal cells much less than sarcoma cells. Both bisphosphonates induced cell cycle perturbation in all sarcoma cells tested and apoptosis in Saos-2 and SW1353 cells, although they failed to induce apoptosis in RD-ES and SK-ES-1 cells. MIN significantly suppressed invasion, even at a low concentration of 1 μM ($p < 0.01$). Daily injection of 5 μg of MIN inhibited the growth of SK-ES-1 xenograft sarcoma in nude mice without loss of body weight. These findings suggest that MIN may have a beneficial adjuvant role in the treatment of patients with malignant bone tumors.

Introduction

Osteosarcoma (OS), chondrosarcoma (CS), and Ewing's sarcoma (ES) are among the top three common malignant bone tumors, other than hematological malignancies. Patients with unresectable primary tumors and those with clinically evident metastases still have a poor prognosis even in chemotherapy-responsive OS and ES [1,18]. On the other hand, the primary treatment of CS has been wide surgical resection, whereas chemotherapy has a minimal role in the treatment of CS [17]. More effective chemotherapeutic agents for the treatment of these malignant bone tumors are needed.

Bisphosphonates, stable analogues of pyrophosphonate, are potent inhibitors of osteoclast-mediated bone resorption, and have been widely used as a therapeutic agent for osteoporosis, Paget's disease, and hypercalcemia associated with cancer. Moreover, increasing preclinical evidence that bisphosphonates have direct antitumor effects on a variety of human cancer cells has been reported [14]. Minodronate (MIN), formerly YM529, is a newly developed third-generation nitrogen-containing bisphosphonate. Phase II trials in Japan have shown that MIN was efficacious and safe without serious adverse effects in patients with osteoporosis [13], and Phase III trials are currently underway for patients with osteoporosis in Japan. It has been reported that MIN shows a 100-fold greater potency than pamidronate, and is 10-fold more potent than a clinically relevant third-generation derivative, incadronate (INC). MIN is not only as potent as zolendronate (ZOL) at inhibiting bone resorption in animal studies, but is also as efficacious as ZOL at inhibiting human leukemic cell growth *in vitro* and *in vivo* [20]. Potent antitumor effects of MIN on various hematopoietic tumor cells and cancer cells have been reported [15,24], whereas limited reports have been published about the

antitumor effects of bisphosphonates on malignant bone tumors. The purpose of our study was to investigate the antitumor effects of MIN on common primary malignant bone tumors, including OS, CS, and ES.

Materials and methods

Cell culture and reagents

Saos-2, MG-63, SW1353, RD-ES, and SK-ES-1 cells were purchased from the American Type Culture Collection (Manassas, VA). OUMS27 cells were generously provided by Dr. Ozaki and Dr. Kunisada (Okayama University, Okayama, Japan) [10]. Cells were maintained in the suggested medium with additives in a 5% CO_2 humidified atmosphere at 37°C. Cells were used at passages 20 - 50 for all experiments. Two human bone marrow stromal cells (MSC317 and MSC411) were isolated from bone marrow aspirates of normal human donors (19- and 11-year old male, respectively) during operations for anterior cruciate ligament reconstruction of the knee. Written informed consent was obtained from the patients and their guardians, in accordance with a biology study approved by the Institutional Review Board of Hiroshima University. Primary cultures were established as described previously [9]. All assays were carried out on cell cultures within three passages. MIN (1-hydroxy-2-(imidazo[1,2-a]pyridin-3-yl)ethylidene bisphosphonic acid monohydrate) and INC (cycloheptylamino)methylene bisphosphonic acid) were kindly supplied from Yamanouchi Pharmaceuticals (Ibaragi, Japan).

Cytotoxicity assay

Subconfluent cells harvested by trypsinization were grown in the appropriate medium supplemented with 10% or 15% fetal bovine serum (FBS) in 96–well culture plates (5 X 10^3 cells/well) and allowed to adhere overnight. Cells were treated for 72 h

with 0.1, 1, 10, 100, and 1000 μM bisphosphonates. The viable cell amount was measured by Cell Counting Kit-8 (Dojin, Kumamoto, Japan), according to the instructions. In brief, 10% working solution including 2-(2-methoxy-4-nitrophenyl) -3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt (WST-8) was added into each well and cultures were incubated for 4 h at 37°C. The absorbance was measured at 450 nm (reference wavelength 690 nm) using a microplate reader (MPR-A4i, Tokyo, Japan).

Measurement of apoptosis by flow cytometry

Seventy-two hours after incubation with 50 μM bisphosphonates in a serum-containing medium, apoptotic and dead cells were determined using the Annexin V-FITC Apoptosis Detection kit (Oncogene Research Products, San Diego, CA), as described previously [16]. Stained cells were analyzed by flow cytometry using the FACSCalibur (Becton Dickinson, Bedford, MA).

Cell cycle analysis

Cells serum-starved for 36 h were treated with 50 μM bisphosphonates in a serum-containing medium for 36 h. Adherent cells were harvested by trypsinization, washed with cold PBS, then fixed with ice-cold 70% ethanol at –20°C overnight. Cells were washed with PBS, treated with RNAse A (200 μg/ml), and stained with propidium iodide (50 μg/ml) at room temperature for 30 min in the dark. Cell cycle distribution was determined by the FACSCalibur flow cytometer (Becton Dickinson). DNA histogram was analyzed using ModFit LT (Verity Software House Inc., Topsham, ME).

Matrigel invasion assay

Invasion assays were performed using Bio-Coat invasion chambers (BD Biosciences,

Bedford, MA), which consist of a 24-well companion plate with cell culture inserts containing 8 μm pore size filters coated with the basement membrane Matrigel. Cells were treated with 1 and 10 μM MIN in a serum-containing medium for 72 hours. Cells were then trypsinized, washed twice with a serum-free medium, and seeded in the upper chamber in a serum-free medium (5×10^4/500 μl/chamber). Ten % FBS-containing medium was added to the bottom chamber as a chemoattractant to induce cell invasion through a porous membrane. Incubation was carried out for 72 h at 37°C in a humidified 5% CO_2 incubator. Cells that had invaded through a porous Matrigel-coated membrane were fixed with methanol and stained with hematoxylin. The number of invading cells was determined by counting five high-powered fields (x200 magnification).

In vivo effects of minodronate

Xenografts of human SK-ES-1 cells were initiated by subcutaneous injections of 1×10^7 cells into the right flanks of 6 athymic nude mice at the age of 6 weeks (CLEA, Tokyo, Japan). The mice received daily intraperitoneal injections of 5 μg of MIN or physiological saline solution. The smallest and largest diameters of tumors, and the body weights were measured once a week. Tumor volumes were calculated using the following formula: volume (mm^3) = (smallest diameter)^2 X (largest diameter)/2. All animal experiments were conducted according to the guidelines of the Institutional Animal Care and Use Committee and the protocol was approved by the Ethics Committee for Experimental Animals of Hiroshima University.

Statistical analysis

Statistical significance was determined by Student's *t*-test, using SPSS (Chicago, IL). $p < 0.05$ was considered significant.

Results

Antiproliferative effects of MIN and INC on malignant bone tumor cells

Following treatment with MIN or INC for 72 h, cell viability of two human OS cell lines (Saos-2 and MG-63), two human CS cell lines (SW1353 and OUMS27), two human ES cell lines (RD-ES and SK-ES-1), and two human bone marrow stromal cells as normal controls (MSC317 and MSC411) was assessed by the WST-8 method. Both bisphosphonates inhibited the viability of all eight cell lines in a dose-dependent manner (Fig. 1*A-D*). In the six sarcoma cell lines, IC_{50} values of MIN were 2.7 – 5.0 μM, which were significantly lower than those of INC (18.5 –50.0 μM) ($p < 0.01$, Student's *t*-test). Bone marrow stromal cells were remarkably refractory to both bisphosphonates (IC_{50} of MIN, 84 and 92 μM; IC_{50} of INC, 290 and 300 μM). There were no significant differences of IC_{50} values of MIN among the different types of sarcoma cells.

MIN and INC induce cell cycle inhibition with or without apoptosis in sarcoma cells

Cell cycle analyses were performed on adherent cells alone after short treatment periods (36 h) to observe early events on DNA histogram of viable cells. Cell populations were synchronized in G1 by serum starvation to allow a more distinct representation of their progression through the individual cell cycle phases. MIN or INC treatment resulted in S phase arrest and apoptosis in Saos-2 cells and SW1353 cells (Fig. 2 and 3). MIN induced larger amounts of viable apoptotic cells than INC in both cell lines. In contrast, the treatment of RD-ES and SK-ES-1 cells with MIN or INC for 72 h caused dead cells

without the induction of apoptosis (Fig. 2). MIN and INC increased in the population of RD-ES and SK-ES-1 cells at G_0/G_1 peak and S phase, respectively (Fig. 3).

MIN suppresses invasive capacity of malignant bone tumor cells

We observed a significant decrease in the invading capacity of all 6 tumor cell lines exposed to 1 or 10 μM MIN, shown in Fig. 4. Ten μM MIN treatments almost fully blocked invasion through Matrigel of all cell lines (less than 5% of the controls). Among the cell lines, there were no significant differences in the relative number of invading cells after incubation with an identical concentration of MIN.

Human bone marrow stromal cells are not included in the invasion assay, since no bone marrow stromal cells, even untreated, could invade through a Matrigel-coated membrane in this experimental condition.

MIN inhibited sarcoma growth in nude mice

Instead of orthotopical implantation of SK-ES-1 cells into the tibia of nude mice, we used subcutaneous implantation of SK-ES-1 cells in nude mice, because there was no evidence of intraosseous tumor formation at 6 weeks after intratibial implantation.

All mice survived without significant differences of body weights between the MIN-treated group and the control group (data not shown). SK-ES-1 xenograft sarcoma cells in the control group grew rapidly in nude mice. In contrast, the daily intraperitoneal injections of 5 μg of MIN inhibited sarcoma growth (Fig. 5*A* and *B*). Four weeks after tumor inoculation, the mean tumor volume was 114 mm^3 in the MIN-treated group, which was significantly smaller than that of the control group (1261 mm^3) ($p < 0.01$, Student's *t*-test).

Discussion

To date, little is known about the antitumor effects of bisphosphonates on malignant bone tumors cells. In most of the previous studies, first-generation or second-generation bisphosphonates were used for the treatment of OS cells, with relatively high concentrations of bisphosphonates required for the inhibition of cell proliferation [12,21]. More recently, Evdokiou et al. [5] published one study using a third-generation bisphosphonate, ZOL. ZOL induced a dose- and time-dependent decrease in cell proliferation in a panel of human OS cell lines, although it was variably effective in reducing cell number in the different cell lines. The concentrations at which ZOL exerted half-maximal effects on cell proliferation at 72 h were 2-3 μM in sensitive OS cell lines, and 10-50 μM in less sensitive OS cell lines. Our findings demonstrated that MIN and INC dose-dependently inhibited the proliferation of two OS cell lines. MIN was a superior inhibitor with an IC_{50} after 72-h of treatment of 3.4 μM and 4.3 μM in Saos-2 and MG-63, respectively. Both Saos-2 and MG-63 belong to less sensitive OS cell lines in Evdokiou's experiments. There are no English language articles investigating the direct antitumor effects of bisphosphonates on CS and ES cells, except for a very recent report by Sonnemann et al. [22]. They noted that after a 72-h incubation with 50 μM pamidronate, a second-generation bisphosphonate, cell numbers were reduced by up to 80%. We demonstrated that third-generation MIN more efficiently decreased cell growth in CS and ES cell lines in a dose-dependent manner, and affected the viability of normal bone marrow stromal cells much less than sarcoma cells. Furthermore, daily injections of MIN inhibited Ewing's sarcoma growth in nude mice without loss of body weight.

Nitrogen-containing bisphosphonates, including MIN and INC, are generally

considered to inhibit the mevalonate pathway, ultimately leading to apoptosis in tumor cells as well as in osteoclasts [7]. However, several studies have provided evidence that bisphosphonates fail to induce apoptosis in some prostate cancer and breast cancer cell lines [3,11]. We have demonstrated that MIN or INC treatment results in S phase arrest and apoptosis in Saos-2 (OS) cells and SW1353 cells (CS), but causes dead cells without the induction of apoptosis in both ES cells. The reasons for different responses of tumor cells to bisphosphonate treatment are unclear, but may reflect differences in the status of p53. The S phase arrest and apoptosis induction indicate that bisphosphonates might be causing DNA damage. DNA damage has been shown to activate p53, regulating the process of apoptosis. Several studies have shown that stable transfection of p53 mutants into p53-null cells results in a protective effect against DNA damage-induced apoptosis [2,23]. In this study, MIN or INC induced apoptosis in p53-null Saos-2 cells, but not in RD-ES and SK-ES-1 cells having a mutated p53 gene [8,23]. Further studies are underway to clarify the underlying molecular mechanisms of antiproliferative action of bisphosphonates in malignant bone tumors.

Many studies have shown that bisphosphonates inhibit the invasion of prostate and breast cancer cells through extracellular matrices. Interestingly, some of these activities were observed at very low bisphosphonate concentrations in the range of 10^{-12} to 10^{-6} M, suggesting that inhibition of adhesion and invasion are among the most potent biological effects of bisphosphonates [6]. Consistent with previous studies, this study has shown that MIN can significantly suppress the invasion of malignant bone tumor cells, even at a

low concentration of 1 μM. Although this reduced invasion may in part reflect the effects of MIN on the apoptotic or non-apoptotic death of tumor cells, cell death alone does not account for the loss of invasive potential. For example, when Saos-2 cells were treated with MIN at 1 μM for 72h, 79.2% cells lost their invasive ability, whereas cell viability decreased by only 0.6 %. It is, therefore, highly likely that the inhibitory effects of MIN on cell invasion may be due to the inhibition of the invading process itself and not so much from the cytotoxic effects of MIN on tumor cells.

MIN inhibited cell growth in malignant bone tumor cell lines with an IC_{50} of 2.7-5.0 μM. These IC_{50} values are higher than the potentially achievable serum concentration of 1 - 2 μM, based on the pharmacokinetics of clinical trials with ZOL for cancer patients with bone metastases [4]. In rat experiments, Sato *et al.* [19] suggested that the effective local concentrations of bisphosphonates at sites of active bone resorption could reach up to 1 mM, due to their great avidity for bone. Although there is no convincing evidence that MIN can have a direct effect on tumor cells themselves within the bone microenvironment, the invasive capacity of malignant bone tumors may be a more favorable target of MIN. MIN significantly suppressed their invasion, even at a low concentration of 1 μM.

In conclusion, the present study suggests that MIN may have the most powerful antitumor effects on a variety of malignant bone tumor cells among currently available bisphosphonates. These findings indicate that MIN may have a beneficial adjuvant role in the treatment of malignant bone tumors.

Acknowledgements

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Figure legends

Fig. 1. Minodronate (MIN) and incadronate (INC) more efficiently inhibited the viability of all malignant bone tumor cell lines than that of normal bone marrow stromal cells. Two human osteosarcoma cell lines, Saos-2 and MG-63 (*A*); two human chondrosarcoma cell lines, SW1353 and OUMS27 (*B*); two human Ewing's sarcoma cell lines, RD-ES and SK-ES-1 (*C*); two human bone marrow stromal cells (MSC317 and MSC411) were treated for 72 h with the indicated concentrations of MIN or INC. The viable cell amount was assessed by the WST-8 method. Cell viability was expressed as a percentage of the absorbance of bisphosphonate-treated cells vs. untreated control cells. Data shown are mean ± SD (n = 4 wells).

Fig. 2. Induction of apoptosis and necrosis in malignant bone tumor cells treated with minodronate (MIN) or incadronate (INC). Cells were incubated with 50 μM bisphosphonates for 72 h. Apoptotic and dead cells were analyzed by flow cytometry using dual staining with propidium iodide (PI) and Annexin V-FITC. MIN and INC induced viable apoptotic cells stained with Annexin V alone (lower right quadrant) in Saos-2 and SW1353, but only dead cells stained with PI (both upper left and right quadrants) were induced in RD-ES and SK-ES-1. Results shown are representative of three independent experiments and values (%) indicate mean ± SD. **, $p < 0.01$ vs. control group (untreated cells) as determined by Student's *t*-test.

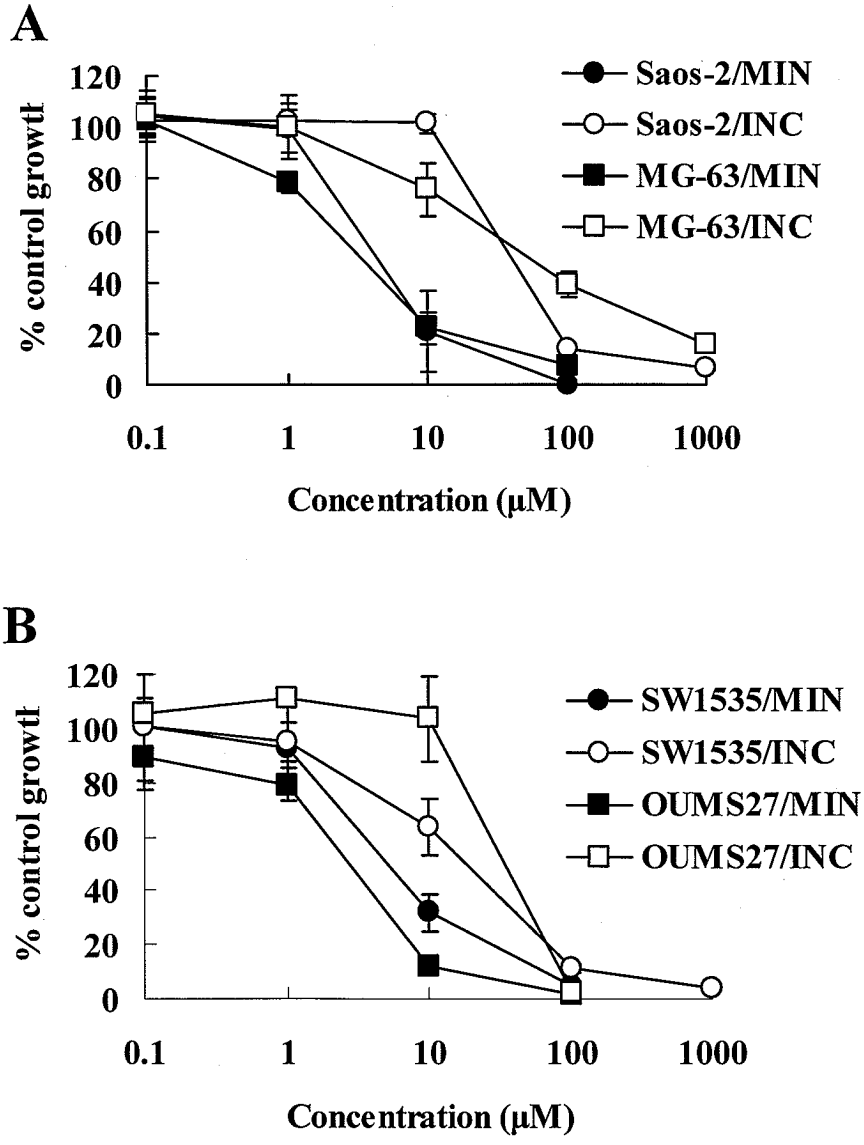
Fig. 3. Minodronate (MIN) and incadronate (INC) caused S phase arrest in Saos-2, SW1353, and SK-ES-1, but induced G_0/G_1 phase arrest in RD-ES. Serum-starved cells

were treated with 50 μM bisphosphonates for 36 h. Adherent cells alone were harvested, fixed, and stained with propidium iodide. Cell cycle distribution was determined by a flow cytometer, and analyzed using ModFit LT. Results shown are representative of three independent experiments and values (%) indicate mean ± SD. *, $p < 0.05$; **, $p < 0.01$ vs. control group (untreated cells) as determined by Student's *t*-test.

Fig. 4. Minodronate (MIN) suppresses the invasive capacity of all malignant bone tumor cell lines. Cells were treated with the indicated concentration of bisphosphonates for 72 h. Cell invasive capacity was assessed using Bio-Coat invasion chamber systems, as described in "Materials and methods". The number of invading cells was calculated as a percentage of untreated Saos-2 cells. Data are mean ± SD obtained from three separate experiments. **, $p < 0.01$ vs. control group (untreated cells) as determined by Student's *t*-test.

Fig. 5. Effects of Minodronate (MIN) on sarcoma growth in nude mice. Nude mice bearing xenografts of human SK-ES-1 cells received daily intraperitoneal injections of 5 μg of MIN or physiological saline solution. The daily injections of MIN inhibited sarcoma growth (*A*). Data are mean ± SD obtained from 6 athymic mice. **, $p < 0.01$ vs. control group as determined by Student's *t*-test. Representative photographs were taken after 4 weeks of tumor inoculation. Arrows indicate tumors (*B*).

Fig. 1.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% control growth
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Concentration (μM)
Saos-2/MIN
Saos-2/INC
MG-63/MIN
MG-63/INC
B
120
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SW1535/MIN
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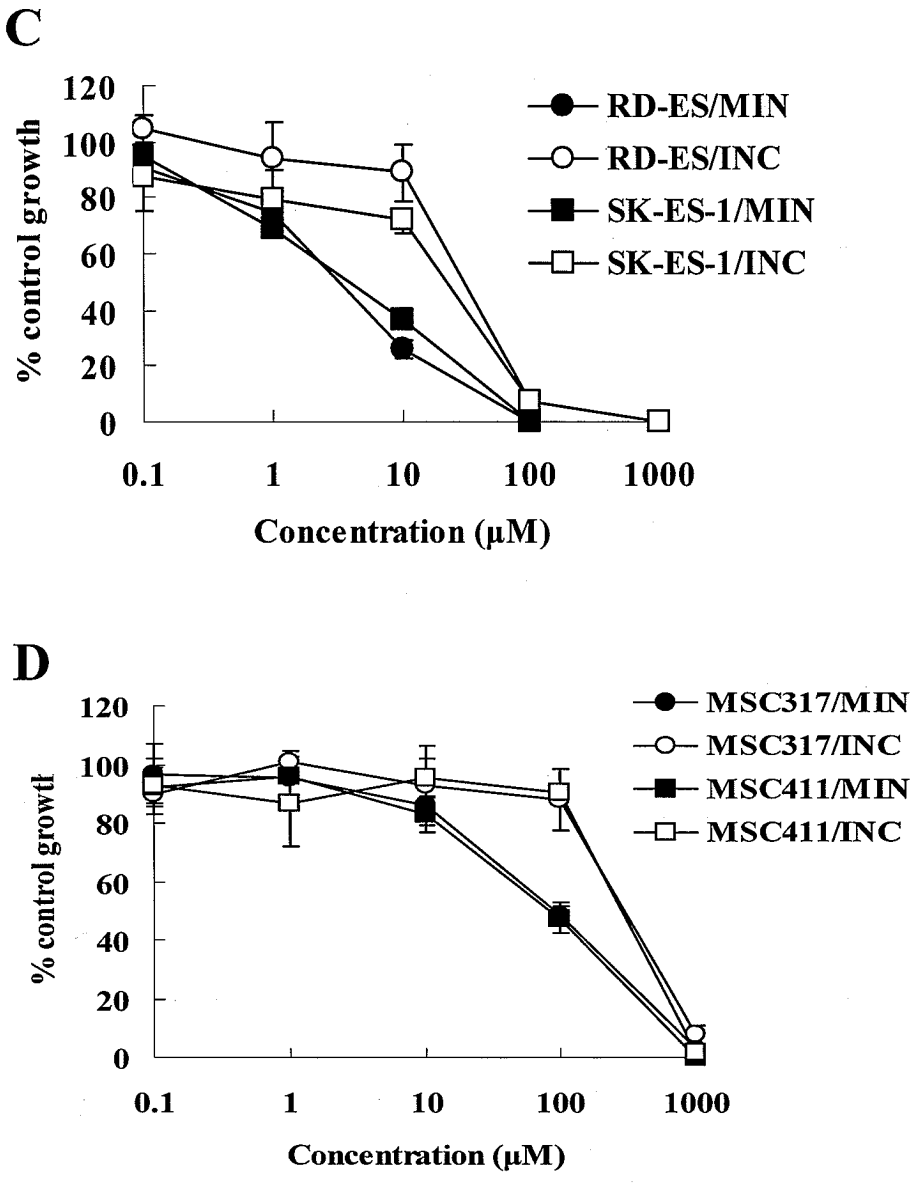
OUMS27/INC

Fig. 1.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RD-ES/MIN
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MSC317/MIN
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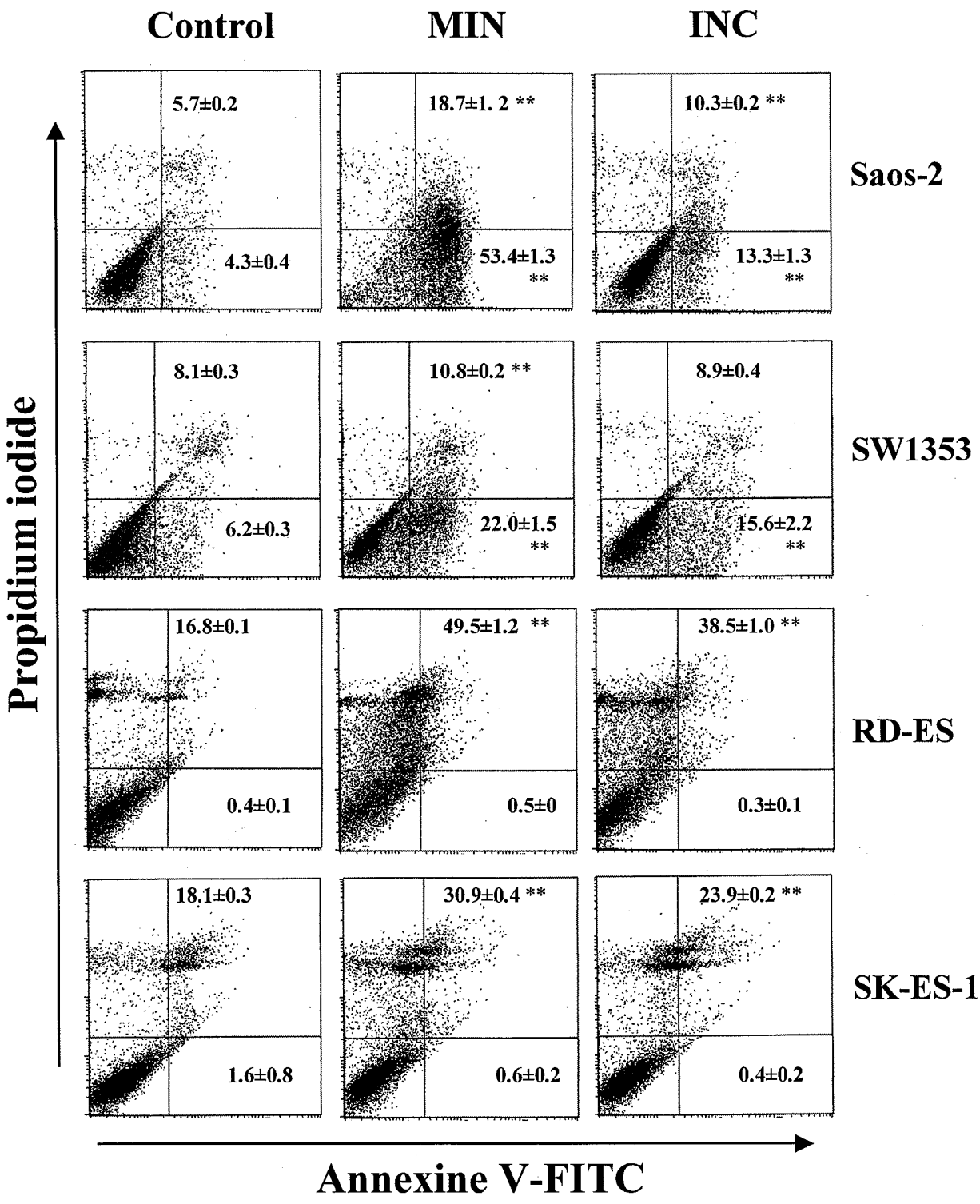
MSC411/MIN
MSC411/INC

Fig. 2.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INC
Saos-2
SW1353
RD-ES
SK-ES-1
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18.7±1. 2 **
53.4±1.3 **
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Propidium iodide
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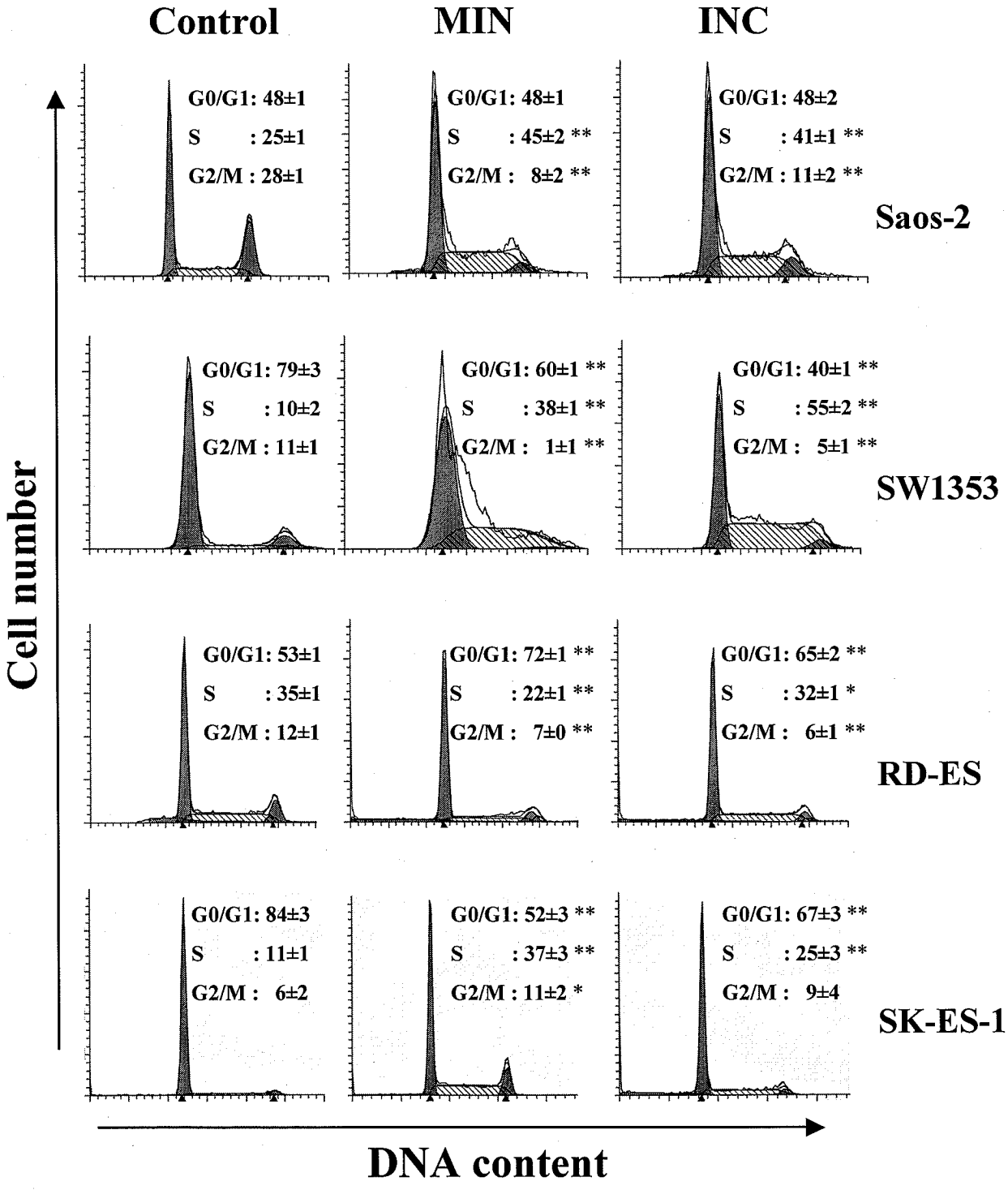
Fig. 3.


Control
MIN
INC
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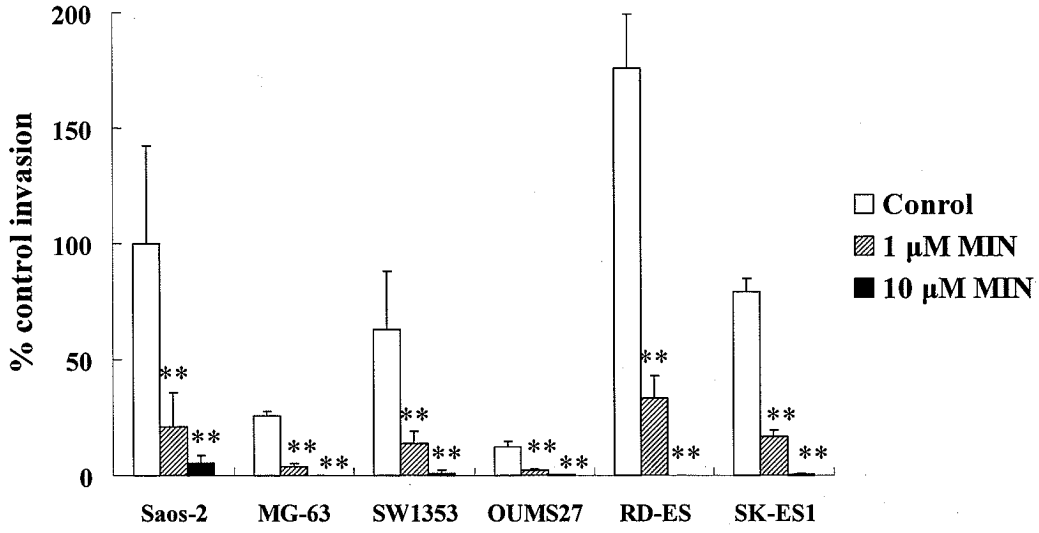
S : 25±1
G2/M : 28±1
G0/G1: 48±1
S : 45±2 **
G2/M : 8±2 **
G0/G1: 48±2
S : 41±1 **
G2/M : 11±2 **
Saos-2
G0/G1: 79±3
S : 10±2
G2/M : 11±1
G0/G1: 60±1 **
S : 38±1 **
G2/M : 1±1 **
G0/G1: 40±1 **
S : 55±2 **
G2/M : 5±1 **
SW1353
G0/G1: 53±1
S : 35±1
G2/M : 12±1
G0/G1: 72±1 **
S : 22±1 **
G2/M : 7±0 **
G0/G1: 65±2 **
S : 32±1 *
G2/M : 6±1 **
RD-ES
G0/G1: 84±3
S : 11±1
G2/M : 6±2
G0/G1: 52±3 **
S : 37±3 **
G2/M : 11±2 *
G0/G1: 67±3 **
S : 25±3 **
G2/M : 9±4
SK-ES-1
Cell number
DNA content

Fig. 4.


200
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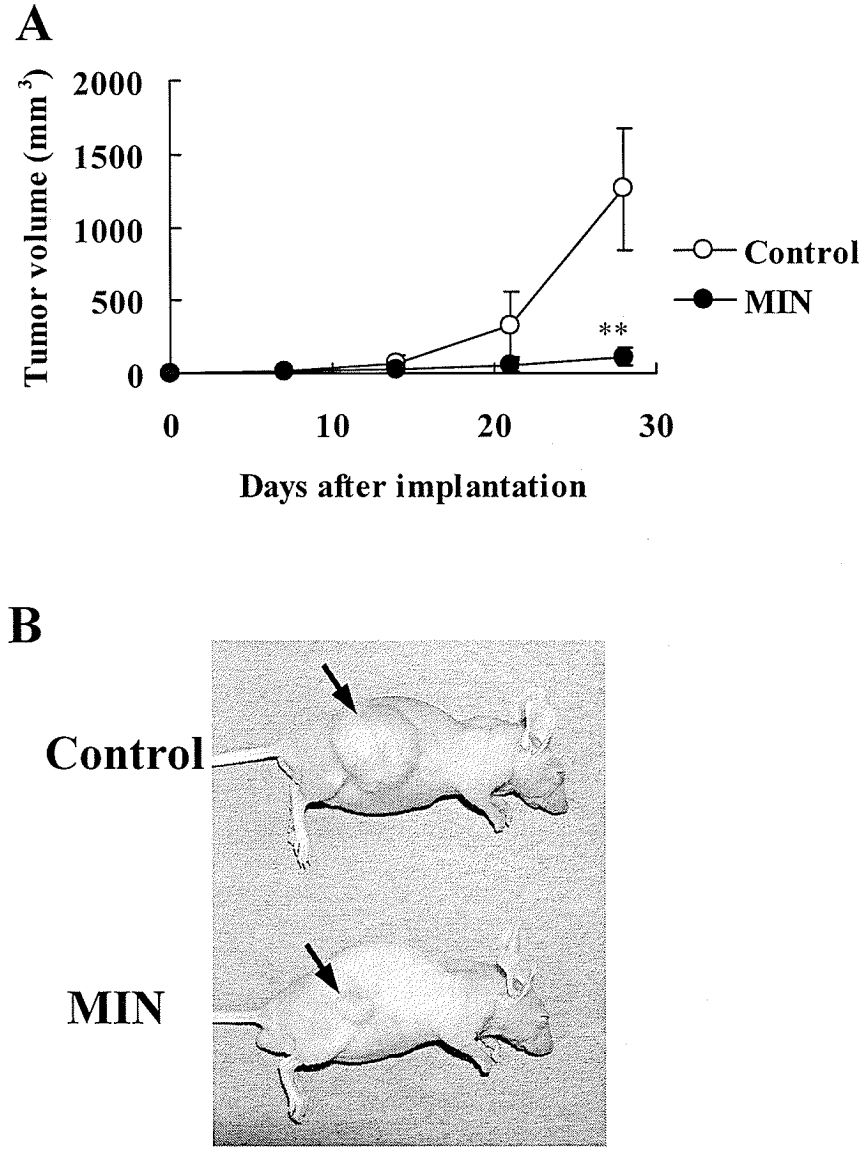
10 μM MIN

Fig. 5.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Tumor volume (mm3)
2000
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Days after implantation
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MIN