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Effect of Extracellular Cations on the Chemotherapeutic Efficacy of Anticancer Drugs

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Cancer development and the efficiency of chemotherapy relies on the patients calcium-related pathological status such as hyper- or hypocalcemia. In the present study, we investigated the effect of extracellular cations such as calcium and magnesium on the therapeutic efficacy of antitumor drugs. The analytic parameters used were cellular drug uptake/excretion and the chemosensitivity of the human breast cancer cell lines, MCF7 and MCF7/ADR. Both calcium and magnesium ions decreased the membrane permeability of cancer cells, which was determined by cell size analysis. These divalent ions also lowered the drug uptake and the cytoplasmic levels of rhodamine 123 and adriamycin, suggesting that they might interfere with the diffusion of these drugs by modifying the physical properties of the cytoplasmic membrane. The acute cytotoxicity of adriamycin after a short period of incubation correlated with changes in its cytoplasmic level. Our results indicate that these extracellular cations might play an important role in the therapeutic activities of anticancer drugs in cancer patients. These results also provide insight a new aspect of chemotherapy, because they suggest that the therapeutic doses of anti-cancer drugs should be modified in cancer-bearing patients presenting with abnormal blood calcium levels.

Key words: Calcium ion, Magnesium ion, Hypercalcemia, Hypocalcemia, MCF7, MCF7/ADR, Cytotoxicity, Chemosensitivity.

INTRODUCTION

The precise fluidity of cell membranes is biologically important. The phase transition from a fluid state to a rigid crystalline state is very important in terms of the transport of drugs and nutrients, which passively diffuse through the plasma membrane. Composition, temperature, and the presence of extracellular ions can change the fluidity of a membrane. This is especially true of ions such as calcium and magnesium, which are able to reduce membrane fluidity, thereby inhibiting membrane transport (Negishi *et al.*, 1983; Beck, 1984; Dordal *et al.*, 1995). Decreased cytosolic calcium augments melphalan uptake (Miller *et al.*, 1992). Crespo *et al.* reported that elevated intracellular calcium decreased red cell filterability and fluidity (Crespo *et al.*, 1988). Changes in extracellular calcium and magnesium were found to affect membrane transport by altering membrane fluidity in primary cultures of proximal rabbit renal cells (Sakhrani *et al.*, 1985).

Chemotherapeutic agents such as hydroxyurea and doxorubicin are transported by passive diffusion. Diffusion across the cytoplasmic membrane is affected by several factors, which include the physicochemical properties of the drugs or the physical status of the membrane (Dordal *et al.*, 1995; Tagger *et al.*, 1987; Speelmans *et al.*, 1995). Based on this background, it was suggested that calcium and magnesium ions might modulate the uptake of anticancer drugs, and induce changes in their therapeutic efficacy.

In the present study, we investigated the effects of calcium and magnesium ions on membrane fluidity and drug uptake and retention. The cytotoxicity of adriamycin was examined in the presence or absence of calcium and magnesium ions. The effects of these ions on chemotherapy are discussed in relation to clinical symptoms including hyper- and hypocalcemia.

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MATERIALS AND METHODS

Materials

All chemicals such as rhodamine 123 (R-123) and adriamycin (ADR) were purchased from Sigma (St. Louis, MO, USA). RPMI 1640 medium and Hanks Balanced Salt Solution (HBSS) were purchased from GIBCO BRL (Grand Island, NY, USA). Two kinds of HBSS were used, HBSS (cat No. 14180), which consisted of KCl, KH_2PO_4, $NaCl_2$, $Na_2HPO_47H_2O$, and D-Glucose, and HBSS (cat No. 14060), which consisted of $CaCl_2$, $MgCl_26H_2O$ and $MgCl_27H_2O$ in addition to the above HBSS. Human cancer cells (MCF7 and MCF7/ADR) were cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS, R10) at 37°C in a humidified atmosphere of 5% CO_2. To produce consistent MDR-1 gene expression, the MCF7/ADR cells were maintained in the presence of adriamycin (ADR, 0.1 mg/ml).

Analysis of cell volume

To assess membrane fluidity, the change of cell size was analyzed using the Cell Counter (Model TTC, Scharfe System, Germany). Cells were suspended in HBSS with calcium and magnesium ions (ion-containing HBSS) or without them (ion-free HBSS). The two types of HBSS were serially diluted in designated concentrations to reduce the osmotic pressure (Endl *et al.*, 1996). 1 × represented normal osmotic pressure and 0.7 × represented hypo-osmotic pressure at 70% of the normal osmotic pressure.

Determination of R- 123 uptake and Release

For R-123 uptake experiments, cancer cells (3×10^5 cells/well of 6 well plate) were stabilized by incubation in R10 for 4 hr and then washed three times with pre-warmed ion-free HBSS or ion-containing HBSS. After washing, cells were suspended in each HBSS and R-123 (1 μg/ml) was added. After incubation, cells were washed three times and intracellular R-123 was extracted twice with 0.2 ml n-butanol. Fluorescence intensity was measured with a Luminescence Spectrometer (Model LS50B, Perkin Elmer, UK) at an excitation wavelength of 518 nm and an emission wavelength of 532 nm. The amount of R-123 was calculated using the R-123 standard curve (Chieli *et al.*, 1993). For R-123 release experiments, cells were incubated for 15 min in each HBSS containing R-123 (1 μg/ml). The medium was then changed to either HBSS without R-123. R-123 was extracted every 5 min and quantified using a Luminescence Spectrometer.

Determination of adriamycin uptake and release

For ADR uptake experiments, cancer cells (3×10^5 cells/well of 6 well plate) were stabilized by incubation in R10 for 4 hr and washing three times with pre-warmed ion-free HBSS or ion-containing HBSS. After washing, cells were suspended in each HBSS and ADR (10 μg/ml) was added. Intracellular ADR was extracted with 1 ml of 0.3 N HCl-50% ethanol (Kato *et al.*, 1992) and the amount of ADR was determined by high-performance liquid chromatography (HPLC). For ADR release experiments, cells were incubated for 15 min in each HBSS containing ADR (10 μg/ml). The medium was then changed to each HBSS without ADR. ADR was extracted every 5 min and quantified by HPLC. Chromatographic separations were accomplished on a Waters Millipore model 510 liquid chromatography (Millipore, Mariborough, MA) equipped with Lichrosorb RP-18 column of 10 μm particle size (Waters assoc. Waltham, MA). An isocratic solvent system (mobile phase) which consisted of 0.05 M NaH_2PO_4 (70%) and methanol (30%) was used. The flow rate was 1.6 ml/min. Fluorescence was measured with a Waters 470 liquid chromatography fluorometer (Millipore). The excitation wavelength was 470 nm and the emission wavelength 585 nm.

Determination of chemosensitivity

Cells (8×10^3 cells/well of 96 well plated) were stabilized by incubation in RPMI 1640 containing 5% FCS medium (R5) for 24 h. The medium was then changed to ion-free HBSS or ion-containing HBSS and ADR (10 μg/ml) was added for 5 min. A sulfor-hodamin B (SRB) assay was performed immediately to determine the acute toxicity of ADR. In other experiments, the medium was changed to R5 without ADR, and the cells were further incubated up to 72 h. SRB assay was performed every day to determine the growth inhibitory activity of ADR. SRB assay was performed by the previously described methods (Wu *et al.*, 1992; Skehan *et al.*, 1990; Monks *et al.*, 1991). After incubation, cells were fixed with 50% (w/v) trichloroacetic acid (TCA) solution for 1 h at 4 °C. They were then washed with water and the TCA-fixed cells stained with 0.4% SRB solution dissolved in 1% acetic acid for 30 min at room temperature. After staining, the unbound dye was washed out with 1% acetic acid and Tris base (0.1 ml, 10 mM, pH 10.5) added. The optical density (OD) was then measured with a microtiter plate reader (Molecular Devices, Model Emax, CA) at 540 nm. Results are presented as per-centages relative to the control.

Measurement of intracellular calcium ions

Intracellular calcium concentration was measured by the fluorescence method using Fura-2/AM. MCF7 and MCF7/ADR cells (2×10^6 cells/ml) were labeled with 5 μM Fura-2/AM for 30 min at 37°C and washed

with phosphate buffered saline (PBS) twice. $CaCl_2$ (10 mM) was loaded to change extracellular calcium level. Calcium-bound Fura-2 and calcium-unbound free Fura-2 were excited at 340 nm and 380 nm and the emission was measured at 510 nm with a Luminescence Spectrometer (Model LS50B, Perkin Elmer, UK). Intracellular calcium levels were calculated as the ratio of bound/unbound Fura-2 fluorescence (Todd and Mikkelsen, 1994).

RESULTS

Effect of calcium and magnesium ions on cell volume

In order to show the effect of ions on membrane permeability, we changed the osmotic pressure of the medium. MCF7 and MCF7/ADR cells were placed in a hypo-osmotic condition and the change in cell size was monitored. Fig. 1 shows that calcium and magnesium ions prevent changes of cell volume by decreasing the membrane permeability. In the absence of extracellular cations, the size of the MCF7 cells gradually increased when the osmotic pressure of the medium was lowered. However, in the presence of cations, the size was only slightly changed (Fig. 1A). The MCF7/ADR cell also responded to hypo-osmotic conditions in a manner similar (Fig. 1B). These results demonstrated that the cytoplasmic membrane permeability was dependent upon the presence of divalent ions in the extracellular environment. These ions possibly make the cytoplasmic membrane rigid and prevent the hypo-osmotic swelling.

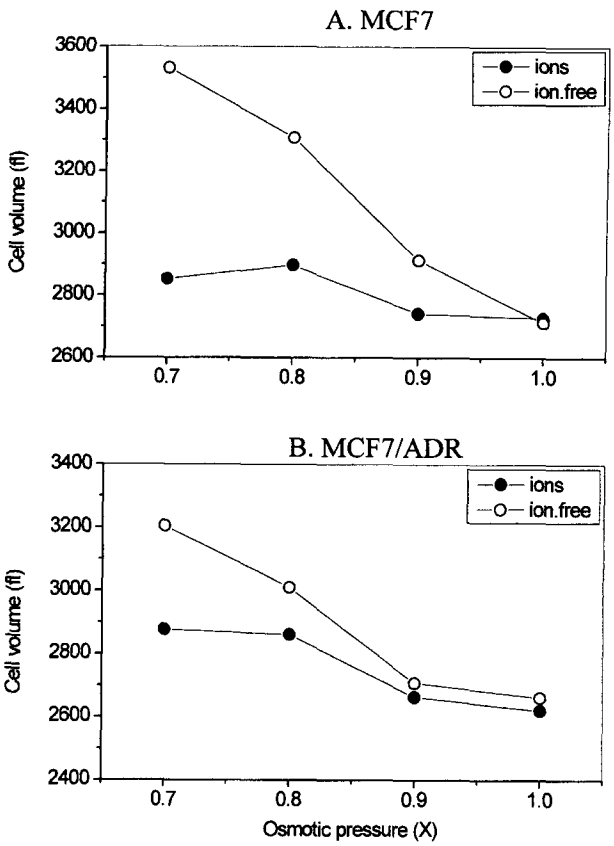

Fig. 1. Changes of cell volume under hypoosmotic conditions. MCF7 (A) and MCF7/ADR cells (B) were suspended in an ion-containing HBSS (closed circles, ions) or ion-free HBSS (open circles, ion.free) with different osmotic pressures. 1 × represents normal osmotic pressure and 0.7 × represents a hypoosmotic condition 70% of normal. The change in cell volume was determined using a CASY-1 cell counter. The data represents one of three independent experiments performed during the analysis of 10,000 cells.

Effect of calcium and magnesium ions on uptake and retention of R-123 and adriamycin

The decreased membrane permeability caused by ions also affected the uptake of drugs. Calcium and magnesium ions impeded the cytoplasmic uptake of R-123 in MCF7 cells (Fig. 2A). MCF7/ADR cells responded in a similar way as their MCF parents-(Fig. 2C). The release rate of R-123 was also high in the absence of calcium and magnesium ions in MCF7 (Fig. 2B) and MCF7/ADR (Fig. 2D). The uptake and release of ADR in MCF7 and MCF7/ADR showed similar patterns to those of R-123 (Fig. 3). These results suggested that the ions decreased membrane permeability and prevented the diffusion of R-123 and ADR through the membrane.

Effect of calcium and magnesium ions on acute cytotoxicity of adriamycin

The increase of cellular drug uptake in ion-free HBSS resulted in a strong growth inhibition of MCF7 and MCF7/ADR. These cells were treated with ADR (10 mg/ml) for 5 min in ion-free or ion-containing medium. The cytotoxicity of ADR was determined immediately 24, 48, and 72 hr after drug washout. The acute cytotoxicity of ADR to MCF7 cells was more potent in the absence of ions than in their presence, whereas the cytotoxicity was not affected by ions after 24 hr (Fig. 4A). The cytotoxicity of adriamycin to MCF7/ADR cells was also stronger in ion-free HBSS up to 24 hr (Fig. 4B). This observation indicated that ions influenced the acute cytotoxicity of drugs during the first 24 hr. The drug resistant properties of MCF7/ADR cells were observed from 48 hr, but not less than 24 hr, compared with their parent MCF7 cells. These results suggest that extracellular ions can modulate drug uptake, and that this could alter the chemosensitivity of cancer cells to drugs. It would also seem to be the case that multidrug resistance cells have a higher recovery rate from acute

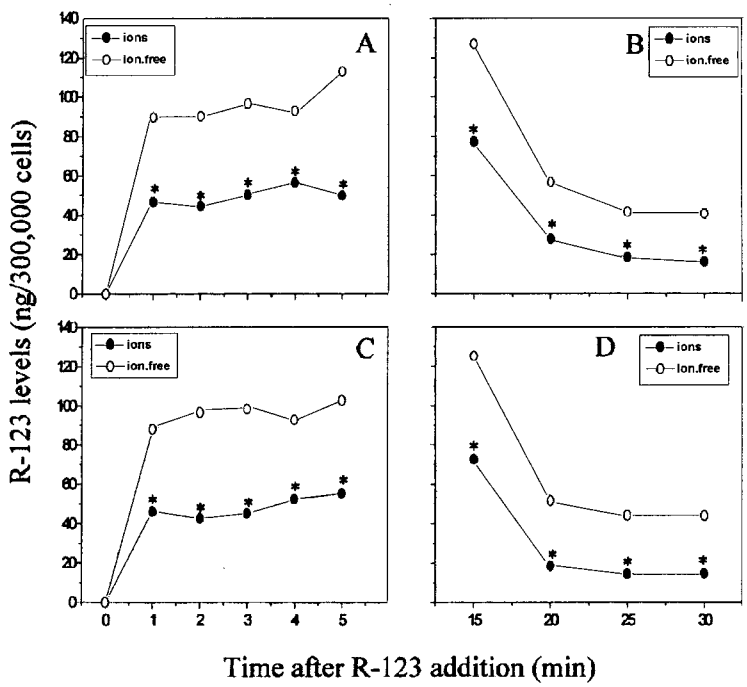

Fig. 2. Uptake and release of R-123. The rates of uptake (A and C) and release (B and D) of R-123 in MCF7 and MCF7/ADR were determined respectively. Cells were incubated with R-123 (1 μg/ml) in ion-containing HBSS (ions, closed circles) or ion-free HBSS (ion.free, open circles). To determine the uptake level, intracellular concentrations were determined every min for 5 min. Similarly, to determine the release levels, cells were incubated with R-123 for 15 min, and then thoroughly washed. Intracellular concentrations were determined every 5 min for 15 min. The data represents the mean +/- standard deviation of three independent experiments that were obtained from four separate analyses. Students *t* test was used to determine the significance of differences between closed and the corresponding open circles ($*p<0.01$).

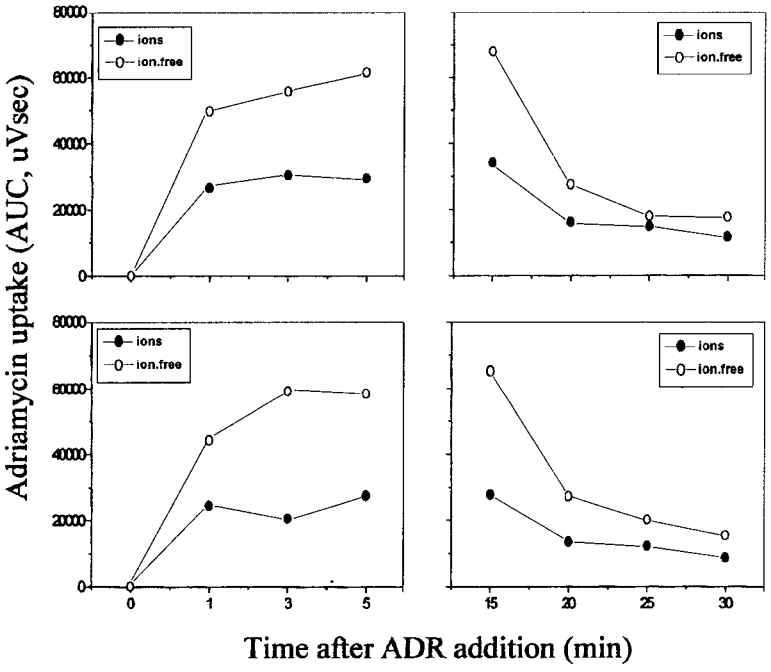

Fig. 3. Uptake and release of ADR. The rates of uptake (A and C) and release (B and D) of ADR in MCF7 and MCF7/ADR were determined respectively. Cells were incubated with ADR (10 μg/ml) in ion-containing HBSS (ions, closed circles) or ion-free HBSS (ion.free, open circles). To determine the uptake levels, intracellular concentrations were determined for 5 min. Similarly, to determine the release levels, cells were incubated with ADR for 15 min, and washed thoroughly. Intracellular concentrations were determined every 5 min for 15 min. The data represents one of three independent experiments.

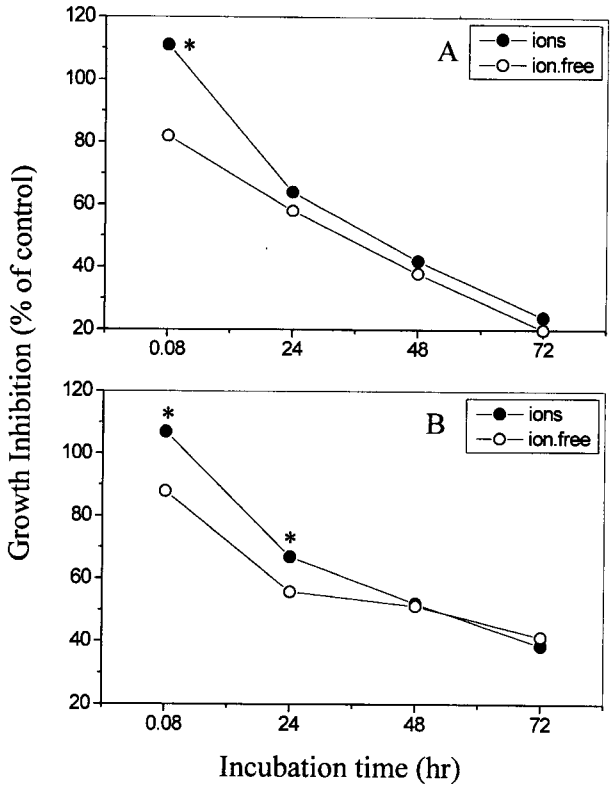

Fig. 4. Chemosensitivity of MCF7 (A) and MCF7/ADR (B) cells. Cells were incubated for 0.08 hr (5 min) with 10 μg/ml ADR in ion-containing HBSS (ions, closed circles) or ion-free HBSS (ion.free, open circles). The cytotoxicity of adriamycin was determined by SRB assay. The data presented represents the mean +/- standard deviation of three independent experiments that were performed in four separate analyses. Significant differences between closed and open circles were determined using the Student *t* test (*$p<0.01$).

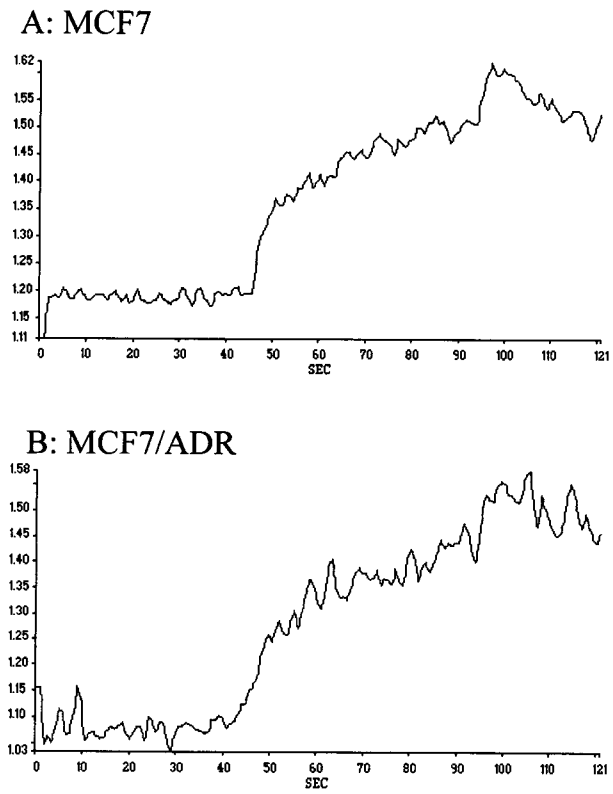

Fig. 5. Effect of extracellular calcium ions on intracellular ion concentration. MCF7 (A) and MCF7/ADR cells (B) were labeled with 5 μM Fura-2/AM. After a fluorimeter was located and ion concentration determined, 10 mM of $CaCl_2$ was added. Results are presented as the ratio of the fluorescence of calcium bound-Fura-2/free Fura-2. The data represents one of three independent experiments.

cytotoxic damage than their parent cells.

Effect of extracellular divalent ions on intracellular ion concentrations

Fig. 5 shows that the acute exposure of MCF7 and MCF7/ADR cells to calcium ions caused a rapid increase in the intracellular concentration of calcium ions. By loading 10 mM calcium chloride, the intracellular calcium concentration was rapidly increased. Accordingly, the effect of calcium ions on drug uptake and chemosensitivity might be anticipated from the interaction between extra- and/or intracellular calcium ions and the cytoplasmic membrane.

DISCUSSION

The present results demonstrate that extracellular calcium and magnesium ions can influence cellular drug uptake and chemosensitivity of cancer cells. Our observation might provide a new perspective into the chemotherapeutic efficacy of drugs. The blood calcium level is changed during the development and remedy of neoplastic diseases. In chemotherapy with anticancer drugs, the acute tumor lysis syndrome has been commonly observed in diseases with large tumor burdens and high proliferative fractions that are exquisitely sensitive to cytotoxic treatment (Gomez and Han, 1987; Boccia *et al.*, 1985; Vogelzang *et al.*, 1983). The syndrome occurs as a result of the rapid release of intracellular components into the blood stream, which may increase to life-threatening concentrations (Boles *et al.*, 1984; Wollner *et al.*, 1986). The syndrome is characterized by hyperuricemia, hyperkalemia, hyperphosphatemia, and hypocalcemia, and may be induced by high-grade lymphomas and is increasingly being observed in solid tumors such as metastatic breast carcinoma and medulloblastoma (Stark *et al.*, 1987; Tomlinson and Solberg, 1984). If one considers the present results which show that the removal of calcium ions increases the cytotoxicity of anticancer drugs, it becomes clear that the antitumor action of chemotherapeutic agents can be increased.

In this context, it has previously been reported that tumors will disappear effectively in acute tumor lysis syndrome (Keating *et al.*, 1977). On the other hand, other life-threatening adverse effects of acute tumor lysis syndrome might be aggravated. To prevent these side effects, the doses of anticancer agents should be reduced although the therapeutic efficacy will weaken. Accordingly, before chemotherapy, the delicately determined dosage of anticancer agents must be pre-determined as a suboptimal therapeutic dose, so as to avoid severe side effects.

Hypercalcemia is the most common life-threatening metabolic disorder associated with cancer. The incidence of hypercalcemia varies with the underlying cancer diagnosis; it is highest in myeloma and breast cancer, intermediate in non-small cell lung cancer, and rare in small cell carcinoma of the lung and colon cancer (Brada *et al.*, 1990; Heath *et al.*, 1980; Axelrod *et al.*, 1987). Hypercalcemia may influence the drug uptake and cytotoxicity of anticancer drugs. In the hypercalcemic condition, cancer cells show drug-resistance to anticancer agents by lowering drug uptake and so reduce therapeutic efficacy. For this reason, cancer patients with hypercalcemia should be administered high doses of anticancer drugs to achieve the expected therapeutic outcome.

We previously reported that the drug resistance mechanism of MCF7/ADR cells was not related with P-gp regulated drug release (Kim *et al.*, 1997). On the basis of the present results, which indicate that MCF7 and MCF7/ADR cells showed similar drug uptakes and retentions in the presence or absence of calcium and magnesium ions, cations concentrations are not related with the drug resistant phenotype. Although the drug resistance mechanism of MCF7/ADR was unclear, it might be related to the repair activity of cells. Acute cytotoxicity to adriamycin was similar in both cell types. However, though the toxicity was reduced or even eliminated in resistant cells, it was maintained and resulted in higher levels of cytotoxicity in sensitive cells.

From our observations, it is clear that extracellular ions such as calcium and magnesium affect the therapeutic efficacy of anticancer drugs, indicating that drug dosage should be carefully predetermined in accor-dance with the blood calcium level of cancer patients.

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