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Beneficial effects of curcumin on antitumor activity and adverse reactions of doxorubicin

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ABSTRACT

Many medicines used in cancer chemotherapy decrease the quality of life (QOL). It is believed that an increase in food intake during cancer chemotherapy may produce an improvement in QOL. Curcumin is widely used as a coloring and flavoring agent in food. The effects of curcumin in relation to the chemotherapeutic drug doxorubicin (DOX) were examined.

While DOX alone did not decrease tumor weight, the combination of DOX and curcumin significantly reduced tumor weight to 56.5% ($p < 0.05$) of that of the control group. The combined curcumin enhanced apoptosis by DOX and decreased cell viability. The curcumin–DOX combination also suppressed activation of caspase-3, -8, and -9 compared to DOX alone. It is presumed that combining curcumin increased DOX-induced antitumor activity by suppressing the main caspase pathway and activating the main caspase independent pathway. The combination of curcumin and DOX suppressed the reduction of glutathione peroxidase activity and increased lipid peroxide levels in the heart. Therefore, it is expected that curcumin may reduce the adverse reactions associated with DOX. Our results suggest that curcumin can be used as a modulator to enhance the therapeutic index of cancer patients and improve their QOL.

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1. Introduction

Chemotherapy using antitumor drugs plays an important role in clinical cancer treatment. However, these remedies are accompanied by several problems including severe adverse reactions and expression of drug-resistant cells which may necessitate the discontinuation of chemotherapy and contribute to a reduction in the therapeutic index. Biochemical modulation has been postulated as a method to improve the therapeutic index of clinical therapy. The process of biochemical modulation involves alteration of the pharmacological activities of antitumor drugs by combining them with other compounds (modulators) which increase antitumor activity and/or decrease adverse reactions (Konishi and Dezuki, 1992; Sadzuka et al., 2002a).

Patients concerned with adequate nutritional support take supplements or herbs with meals both during and after chemotherapy and irradiation treatments (Auerbach, 2006). In the past few years, complementary medicines have gained significant ground in oncology. Adequate nutritional support is expected to arrest the growth of disease and relieve symptoms without the fear of direct harmful effects and negative interactions with chemotherapy (Weiger et al., 2002).

Doxorubicin (DOX), an anthracycline derivative, is used for the treatment of a variety of malignancies including solid tumors and leukemia due to its broad spectrum antitumor activity. However, the adverse reaction of DOX-induced cardiac toxicity is a dose-limiting factor. It has been reported that theanine, a specific amino acid in green tea, enhanced the antitumor activity of DOX *in vivo* with increasing DOX concentrations in tumors (Sadzuka et al., 1996, 1998; Sugiyama and Sadzuka, 1998, 1999). This action was found to be related to the inhibitory activity of tumor cells *in vitro* toward DOX efflux (Sadzuka et al., 1996, 1998; Sugiyama and Sadzuka, 1998, 1999). In addition, theanine promoted efflux of DOX from normal cells, and suppressed DOX-induced increase in lipid peroxide (LPO) in the hearts of mice (Sugiyama and Sadzuka, 2004). Therefore, theanine reduced DOX-induced adverse reaction in normal tissues. Furthermore, it was reported that taurine, a major amino acid in fish, and cucurbitacin, the bitter tasting component of squash fruit, also enhanced the antitumor activity of DOX (Sadzuka

Abbreviations: Ac-DEVD-MCA, acetyl-Asp-Glu-Val-Asp-MCA; Ac-IETD-MCA, acetyl-Ile-Glu-Thr-Asp-MCA; Ac-LEHD-MCA, acetyl-Leu-Glu-His-Asp-MCA; AIF, apoptosis-inducing factor; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DOX, doxorubicin; EDTA-2Na, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; FDA, fluorescein diacetate; GSHpx, glutathione peroxidase; LPO, lipid peroxide; PI, propidium iodide; QOL, quality of life; TBA, 2-thiobarbituric acid.

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et al., 2009, 2010). Food element modulators can provide a safe, easy to take approach to improve the therapeutic index of cancer chemotherapy without reducing the patients' quality of life (QOL).

Curcumin is widely used as a food coloring and flavoring agent and has wide range of pharmacological activities which include anti-inflammatory, antioxidative, and anti-angiogenic properties. Curcumin effects on cellular enzymes such as cyclooxygenase and glutathione S-transferase (GST) include a potent antioxidant capacity at neutral and acidic pH that inhibits several cell signaling pathways (Sharma et al., 2005). Recent studies have reported that curcumin demonstrated antitumor activity by inhibiting constitutive NF-κB activation and inducing apoptosis in tumor cells (Khar et al., 1999; Anto et al., 2000; Pillai et al., 2004). It is hypothesized that antioxidative components may reduce the antitumor activity of drugs by influencing their antitumor mechanisms. However, intake levels of antioxidative components in various tumor cells vary (Hospers et al., 1999; Connors, 1966); therefore, the effects of curcumin as a modulator should be confirmed to ensure that it does not reduce the antitumor activity of drugs and that other contradictory interactions are weak or non-existent.

In this study, we examined the effects of curcumin with regard to antitumor activity, adverse reactions, and membrane transfer of DOX. We successfully clarified the utility of curcumin as a modulator based on cytotoxicity results and induction of apoptosis.

2. Materials and methods

2.1. Chemicals

DOX injection was purchased in 10 mg/vial (Adriacin) from Kyowa Fermentation Inc. (Tokyo, Japan). Curcumin, 2-thiobarbituric acid (TBA), and fluorescein diacetate (FDA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). RPMI 1640 medium was obtained from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from JRH Biosciences (Kansas, USA). Penicillin–streptomycin, propidium iodide (PI), and bisbenzimide (Hoechst 33342) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Caspase-3, -8, and -9 substrates; acetyl-Asp-Glu-Val-Asp-MCA (Ac-DEVD-MCA); acetyl-Ile-Glu-Thr-Asp-MCA (Ac-IETD-MCA); and acetyl-Leu-Glu-His-Asp-MCA (Ac-LEHD-MCA) were obtained from Peptide Institute Inc. (Osaka, Japan). Thiazole blue (MTT, lyophilized) and ethylenediaminetetraacetic acid (EDTA, 2Na) were purchased from Dojindo Laboratories (Kumamoto, Japan). Trypsin was obtained from Invitrogen (California, USA). All other chemicals used in this study were of the highest purity available.

2.2. Animals

Male CDF_1, BDF_1, and C57BL/6 mice (4–5 weeks old, weighing 2025 g) were obtained from Japan SLC, Inc. (Hamamatsu, Japan). The animals were housed in a room maintained at $25 \pm 1\,^{\circ}C$ and $55 \pm 5\%$ relative humidity, with free access to regular chow pellets and water. Animal experiments were approved by the institutional animal care and use committee at Iwate Medical University.

2.3. Effect of curcumin on DOX-induced antitumor activity in vivo

M5076 ovarian sarcoma cells (5.0×10^5 cells/animal) were transplanted onto the backs of BDF_1 mice. DOX (2.0 mg/kg/day for 4 days) was intraperitoneally (i.p.) injected at 14, 16, 18, and 20 days after tumor inoculation. Curcumin (10 mg/kg/day or 100 mg/kg/day for 4 days) was administered i.p. to tumor-bearing mice at 15, 17, 19, and 21 days. The mice were sacrificed by cervical dislocation on the 22nd day after inoculation at which time the solid tumors, heart, lungs, liver, and kidneys were immediately removed and weighed. Tissue samples were homogenized in 10 volumes (w/v) of 10 mM phosphate-buffered saline (PBS, pH 7.8). Each suspension (1.0 ml) was mixed for 60 s with 5.0 ml of chloroform–methanol (4:1, v/v) and then centrifuged ($1200 \times g$, 15 min). The concentration of DOX in the organic phase was determined with a fluorescence spectrophotometer (excitation wavelength: 470 nm, emission wavelength: 585 nm).

2.4. Effect of curcumin on DOX-induced adverse reactions in vivo

Curcumin (10 or 100 mg/kg/day, i.p.) was administered to CDF_1 mice and DOX (15 mg/kg, i.p.) was injected on the 2nd day 3 h after curcumin treatment. Blood samples were collected from the hearts of anesthetized mice on the 6th day, and liver, heart, and kidneys were immediately removed and weighed. The tissue samples were homogenized in buffer and LPO levels and glutathione peroxidase (GSHpx) activity in each sample were estimated using TBA fluorophotometric method (Sadzuka et al., 1981) and the method of Hafeman et al. (1974), respectively. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured using a commercial kit (Transaminase C II-Test, Wako Diagnostics). Protein levels in the tissue samples were estimated according to the method of Lowry et al. (1951).

2.5. Effect of curcumin on DOX concentration in M5076 ovarian sarcoma cells in vitro

M5076 ovarian sarcoma cells (1.0×10^6 cells/animal) were transplanted i.p. into C57BL/6 mice. Ascites fluid was collected on the 14th day after transplantation. The M5076 ovarian sarcoma cell suspensions (1.0×10^7 cells/ml medium) were incubated with 5.0 μg/ml of DOX at 37 °C for 60 min in the presence or absence of curcumin (1.0 or 5.0 μM).

To examine the effect of curcumin on DOX efflux from M5076 ovarian sarcoma cells, the cell suspensions were preincubated with 10 μg/ml DOX at 37 °C for 30 min. After incubation, the medium was cooled on ice and then centrifuged at $150 \times g$ for 3 min. The cells were then washed and resuspended in fresh medium. This cell suspension (1.0×10^7 cells/ml) was incubated at 37 °C for 120 min in the presence or absence of curcumin (1.0 or 5.0 μM). After incubation, the medium was cooled on ice and then centrifuged at $150 \times g$ for 3 min. The cells were washed and resuspended in ice-cold PBS (10 mM, pH7.8), and DOX concentration was determined with a fluorescence spectrophotometer (excitation wavelength: 470 nm, emission wavelength: 585 nm).

2.6. Effect of curcumin on DOX-induced cytotoxicity in M5076 ovarian sarcoma cells

M5076 ovarian sarcoma cells were seeded in 96-well plates (1.0×10^5 cells/well). After 24 h of incubation, the cells were treated with different concentrations of curcumin (0–20 μM) and/or DOX (0–50 μM). After 48 h incubation at 37 °C in a humidified atmosphere containing 5% CO_2 in air, they were treated with MTT and incubated for 4 h. The amount of formazan produced was determined by absorbance at 570 nm. Cytotoxicity was expressed as a percentage of the absorbance measured in control cells.

To detect morphological changes associated with cell death, M5076 ovarian sarcoma cells were seeded in 24-well plates (1.0×10^6 cells/well) and treated with different concentrations of curcumin (0–20 μM) for 24 h at 37 °C in humidified atmosphere containing 5% CO_2 in air. The cells were treated with different concentrations of DOX (0–5.4 μM). At pre-determined times, cells were resuspended in PBS containing FDA (0.1 μg/ml) and PI (0.5 mg/ml) and incubated for 10 min at 37 °C. Viability and morphological

changes associated with cell death were determined using a flow cytometer (FCM) (Epics XL; Coulter, Hialeah, FL, USA).

2.7. Determination of apoptotic cells

Apoptotic cells were determined on the basis of characteristic changes in nuclear morphology after staining with the DNA-binding fluorochrome Hoechst 33342. To detect chromatin-condensed cells, different concentrations of curcumin and/or DOX were added in 2.0% paraformaldehyde for 5 min and then fixed in cold methanol at −20 °C for 1 h and suspended in PBS containing 0.5 mM Hoechst 33342. After incubation for 30 min at 37 °C, the cells were microscopically observed.

2.8. Determination of caspase activity in M5076 ovarian sarcoma cells

Caspase activities were measured using synthetic fluorogenic substrates (Ac-DEVD-MCA, Ac-IETD-MCA, and Ac-LEHD-MCA; substrates for caspase-3, -8, and -9). The cells were lysed in lysis buffer (10 mM Tris–HCl, pH 7.5, 130 mM NaCl, 1.0% Triton X-100, 10 mM $Na_4P_2O_7$, and 10 mM Na_2HPO_4). Aliquots of cell lysate were incubated in reaction buffer (20 mM HEPES, 10% glycerol, and 2.0 mM dithiothreitol [DTT]) in the presence of each fluorogenic substrate (200 μM) for 1 h at 37 °C. The amount of fluorogenic MCA moiety released was measured using a spectrofluorometer (excitation wavelength, 380 nm; emission wavelength, 460 nm). The fluorescence intensity was converted to micromoles of MCA released using the standard curve of 7-amino-4-methylcoumarin.

2.9. Statistical analysis

Results were expressed as a mean ± S.D. Statistical analysis was performed using a Student's *t*-test (between two groups) or one way analysis of variance (ANOVA) (among three or more groups).

3. Results

3.1. Effects of curcumin on DOX-induced antitumor activity in M5076 ovarian sarcoma bearing mice

The effects of curcumin on DOX-induced changes in tumor weight and DOX concentrations in the tumors are shown in Fig. 1. In M5076 bearing mice, DOX did not decrease tumor weight, compared to the control group (0.65 ± 0.74 g). On the other hand, the combination of DOX and curcumin significantly reduced tumor weight to 56.5% ($p < 0.05$) of the control level. The DOX concentration in the tumors was 0.136 ± 0.032 μg/g protein in the DOX alone group. There was no change in the DOX concentration (0.140 ± 0.026 μg/g protein) when combined with curcumin. In contrast, the DOX concentration in the heart in the curcumin–DOX group was reduced to 74.2% compared to that in the DOX alone group (10.14 ± 3.81 μg/g protein).

3.2. Effects of curcumin on DOX-induced adverse reactions

The effects of curcumin on serum AST and ALT levels induced by DOX, as makers of cardiac damage are shown in Fig. 2. DOX increased AST level by 2.9 times of the normal level, while curcumin–DOX combination had no effect. On the other hand, DOX alone increased ALT level by 1.6 times of normal level, and the addition of curcumin significantly suppressed this level to 87.1% ($p < 0.05$) of that in the DOX alone group.

The effects of curcumin on DOX-induced increase in LPO are shown in Fig. 3. LPO levels in the mice hearts increased 1.2 times to that in normal level after DOX administration, while curcumin inhibited the DOX-induced increase in LPO levels. Cardiac GSHpx activity in mice in the DOX alone group was reduced to 46.4% of the normal level (0.100 ± 0.014 unit/g protein), while the curcumin–DOX combination recovered GSHpx activity to 0.101 ± 0.004 unit/g protein (data not shown). The treatment with curcumin normalized DOX-induced increase of LPO levels in the liver and kidneys, as well as AST and ALT activity as makers of cardiac damage and LPO levels in the heart (data not shown).

3.3. Effects of curcumin on DOX concentration in M5076 ovarian sarcoma cells in vitro

The effects of curcumin on DOX influx and efflux in M5076 ovarian sarcoma cells are shown in Fig. 4. Intracellular uptake of DOX increased in a time-dependent manner. The curcumin–DOX combination had no effect on DOX intracellular uptake. Similarly, curcumin did not change DOX efflux from M5076 ovarian sarcoma cells.

3.4. Effects of curcumin on DOX induced cytotoxicity

As shown in Fig. 5, DOX decreased cell viability in a dose dependent manner in the presence and absence of curcumin. Curcumin (5.0 μM and 10 μM) had no effect on DOX cytotoxicity whereas 20 μM of curcumin enhanced DOX-induced cytotoxicity. The IC_{50} of DOX decreased from 3.83 μM to 0.78 μM by combining it with 20 μM curcumin. At concentrations of 0.1–5.0 μM DOX, cell viability significantly decreased when combined with curcumin ($p < 0.01$ and $p < 0.001$). Curcumin alone caused cytotoxicity in a dose-dependent manner and the IC_{50} of curcumin was 56.3 μM in this study. The combination of curcumin (0–20 μM) with DOX (0–1.0 μM) at a non-cytotoxic concentration significantly reduced cell viability compared to DOX alone.

Morphological changes associated with DOX-induced cell death are shown in Fig. 6. Histograms depict the characterization of cells and cellular constituents of M5076 ovarian sarcoma cells treated with curcumin and/or DOX for 12 h. In the control and curcumin-treated groups, cell size was larger than in other groups and complexity was lower. In addition, fluorescence intensity of FDA was high while PI was low. These results suggest that cell viability in the curcumin alone group was retained higher than in the DOX alone and combined curcumin–DOX groups. On the other hand, it was observed that some cells in the DOX-treated (5.4 μM) group were smaller, with a higher complexity and lower FDA fluorescence intensity than control and curcumin alone groups. The occurrence of apoptosis was particularly shown in the DOX-treated group. Furthermore, the frequency of apoptosis was increased by treatment with both DOX and curcumin. Low dose (1.8 μM) DOX treatment did not influence cell viability, but in the group of cells treated with a combination of curcumin and DOX, the frequency of apoptosis increased in a dose-dependent manner associated with curcumin (data not shown).

3.5. Effects of DOX and curcumin on chromatin condensation

Fig. 7 shows chromatin condensation 12 h and 24 h after incubation of M5076 ovarian sarcoma cells in the presence or absence of DOX and curcumin. In the control and curcumin alone groups, chromatin condensation did not occur but was observed in the DOX-treated group. The combination of curcumin with DOX increased the frequency of chromatin condensation. In particular, combining curcumin with DOX enhanced apoptosis.

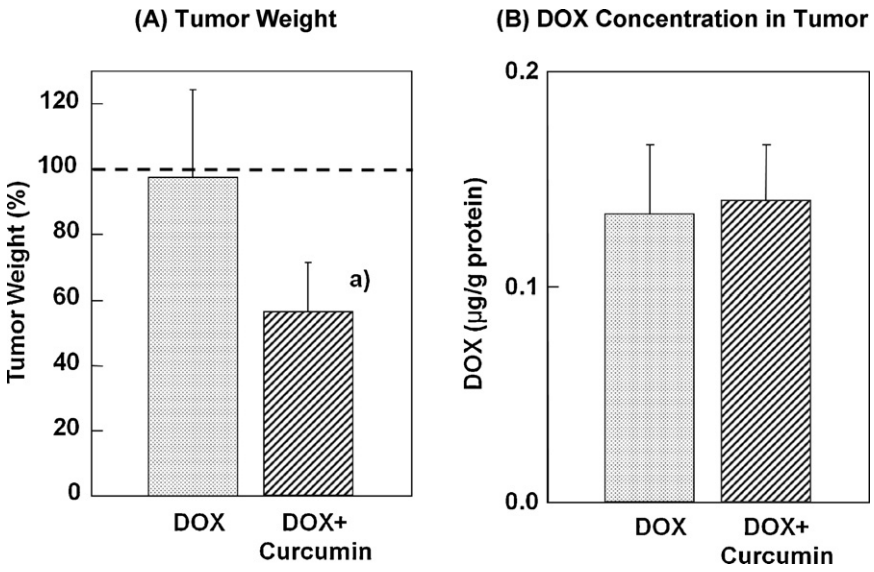

Fig. 1. Effect of curcumin on DOX induced antitumor activity in M5076 ovarian sarcoma tumor-bearing mice. DOX (2.0 mg/kg/day) and curcumin (100 mg/kg/day) were intraperitoneally injected for 4 days. Each column is the mean ± S.D. (n = 4–6). A significant difference from the DOX alone group is indicated by 'a)' $p < 0.05$.

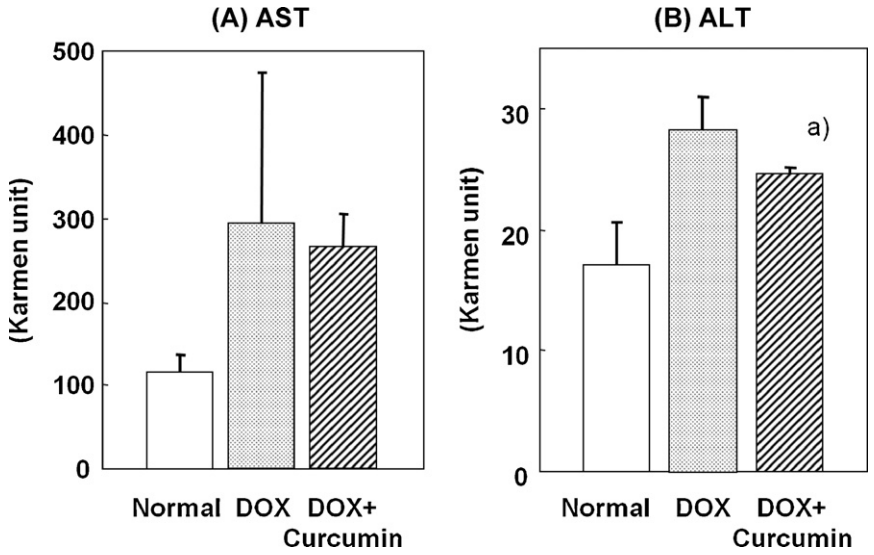

Fig. 2. Changes in AST and ALT levels induced by the administration of DOX and curcumin. Curcumin (100 mg/kg, i.p.) was administered for 5 days, and DOX (15 mg/kg, i.p.) was injected into the mice on the second day. Serum AST and ALT levels were measured as described in Section 2, and are expressed as Karmen units. Each column is the mean ± S.D. (n = 4–6). A significant difference from the level of the DOX alone group is indicated by 'a)' $p < 0.05$.

3.6. Effects of DOX and curcumin on caspase activity in M5076 ovarian sarcoma cells

Activity of caspase-3, -8, and -9 in M5076 ovarian sarcoma cells is shown in Fig. 8. The cells were incubated for 8 h with DOX and/or curcumin. In the control and curcumin alone groups, activity of caspase-3 did not change, but it increased in a dose-dependent manner with DOX. On the other hand, curcumin–DOX combination decreased caspase-3 activity significantly ($p < 0.001$). Furthermore, DOX-induced increase in activity of caspase-8 and -9 were suppressed by the combination with curcumin.

4. Discussion

In our previous studies on biochemical modulation, caffeine, a xanthine derivative, and theanine, a glutamate derivative, were shown to be useful modulators which increased DOX-induced antitumor activity (Sugiyama et al., 2001; Sadzuka et al., 2000, 2002b).

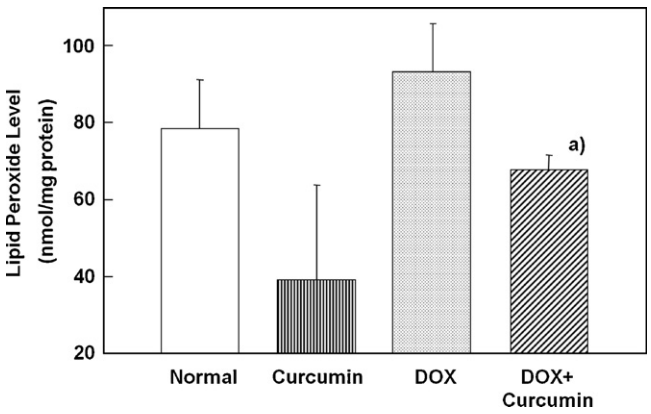

Fig. 3. Effect of curcumin on DOX-induced changes in LPO levels in the heart. Curcumin (100 mg/kg, i.p.) was administered for 5 days, and DOX (15 mg/kg, i.p.) was injected to mice on the second day. Each column is the mean ± S.D. (n = 4–6).

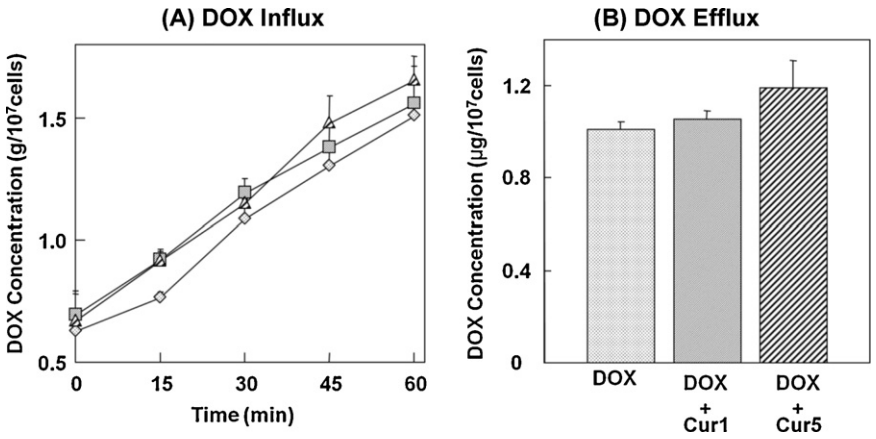

Fig. 4. Effects of curcumin on DOX influx and efflux in M5076 ovarian sarcoma cells. [A] M5076 ovarian sarcoma cells (5.0×10^6 cells/ml) were incubated with DOX (5 μg/ml) in the presence or absence of curcumin (1.0 (Cur1) or 5.0 (Cur5) μM) at 37 °C for 60 min. Each point is the mean ± S.D. ($n = 4$). —◇—: DOX; —△—: DOX + Cur5; —▨—: DOX + Cur1. [B] M5076 ovarian sarcoma cells (5.0×10^6 cells/ml) were preincubated with DOX (10 μg/ml) at 37 °C for 30 min. After the incubation, the cells were washed and resuspended in fresh medium. This medium was incubated in the presence or absence of curcumin (1.0 (Cur1) or 5.0 (Cur5) μM) at 37 °C for 30 min. Each column represents the efflux level of DOX and is the mean ± S.D ($n = 4$).

It appears that these effects were mediated by increased DOX concentration in the tumors through suppression of DOX efflux from the tumor cells. Curcumin (diferuloylmethane) is a phenolic compound from the plant *Curcuma longa* (*Linn*) widely used as a coloring and flavoring materials in food. Its anti-inflammatory activity and antioxidative effects are known and utilized (Radha et al., 2006). Recent studies have reported antitumor activity of curcumin and its mechanism, one of which is the ability to induce apoptosis in cancer cells (Moragoda et al., 2001; Shishir et al., 2005). Therefore, it is expected that curcumin can improve cancer prevention efforts and chemotherapy results. In this study, we anticipated positive effects of curcumin when combined with DOX such as enhancing antitumor activity, and reducing adverse reactions attributed to DOX.

After an injection of curcumin or DOX to M5076-bearing mice, tumor weight was not reduced in the DOX alone group as M5076 ovarian sarcoma has a low sensitivity to DOX treatment. In contrast, the combination of DOX with curcumin significantly reduced the tumor weight to 56.5% ($p < 0.05$) of that of the control group, thereby significantly enhancing the antitumor activity of DOX. However, the addition of curcumin did not increase DOX concentration in the tumors as compared to the DOX alone group; thus, it is presumed that curcumin enhanced the antitumor activity of DOX by another mechanism.

It is feared that an increase in DOX concentration in normal tissues could enhance the adverse effects attributed to DOX, especially the cardiotoxicity associated with increasing DOX concentrations in the heart which is a dose-limiting factor (Rlum and Carter, 1974; Doroshow, 1991). The DOX concentration in the heart in the curcumin–DOX group was reduced to 74.2% compared with that in the DOX alone group. This decrease in the DOX concentration in the heart may suppress the adverse reactions associated with DOX. Furthermore, the antioxidative effect of curcumin was expected to reduce the adverse reactions of DOX. We measured LPO levels and GSHpx activity in the heart as indicators of DOX-induced cardiotoxicity and AST and ALT levels as indicators of tissue damage. The AST and ALT levels in the serum were increased by DOX which indicates DOX-induced cardiotoxicity. On the other hand, the addition of curcumin had a tendency to suppress the DOX-induced increase in AST and ALT activity. In the heart, the reduction in GSHpx activity following administration of DOX caused an increase in LPO levels. In contrast, the addition of curcumin suppressed these DOX-induced changes. The cardiotoxicity is caused by active oxygen species that are formed during DOX metabolism process (Oliveira et al., 2004). The DOX induced active oxygen species oxidize lipids in the myocardial membrane (Sazuka et al., 1987). It has been reported that the reduction of GSHpx activity is particularly connected with increasing LPO levels (Sazuka et al., 1989). Curcumin with

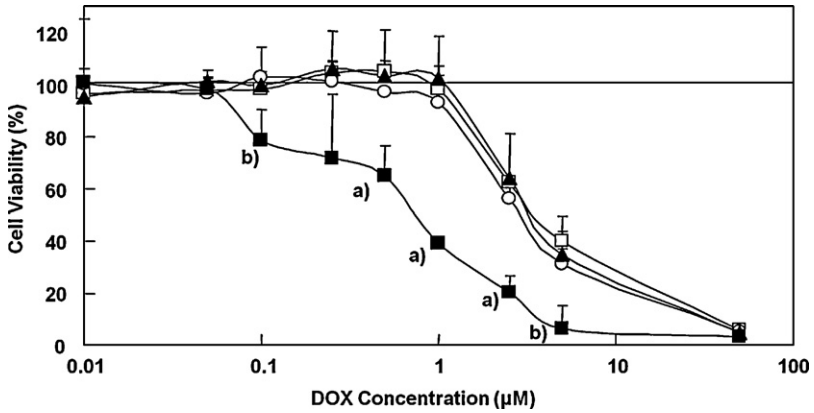

Fig. 5. Effect of curcumin on DOX-induced cytotoxicity in M5076 ovarian sarcoma cells. M5076 ovarian sarcoma cells were plated on 96-well plates and on the second day, cells were treated with DOX and/or different concentrations of curcumin for two days. Experiments were done in quadruplicate. Cell viability was determined by MTT assay. Each point represents the mean ± S.D. ($n = 2$–4). Cell viability is a percentage of control sample values. Significant differences from the DOX alone group are indicated by 'a)' $p < 0.001$ and 'b)' $p < 0.01$. —□—: DOX; —○—: DOX + Cur5; —▲—: DOX + Cur10; —■—: DOX + Cur20.

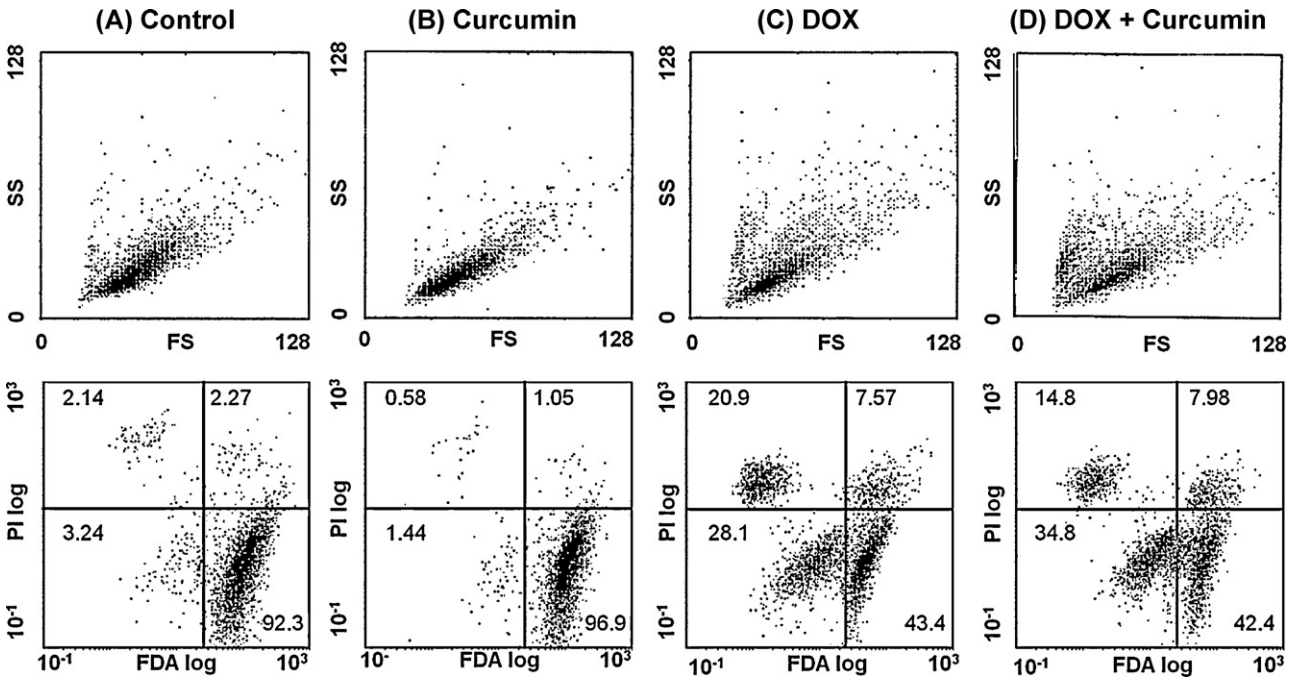

Fig. 6. Flow cytometric histograms of M5076 ovarian sarcoma cells treated with different concentrations of curcumin and/or DOX. M5076 ovarian sarcoma cells were plated on 24-well plates in the presence or absence of curcumin (10 μM). On the second day, cells were treated with DOX (5.4 μM). Following incubation for 12 h, cells were stained and analyzed using a flow cytometer.

antioxidative property was able to interfere with the adverse effects of DOX attributed to the accumulation of LPO in the heart.

However, curcumin has been reported to have very poor bioavailability due to its rapid metabolism in the liver and intestines (Shoba et al., 1998). Therefore, repeated treatment with curcumin is required to induce significant effects. At present, the recommended human dose of curcumin is 500 mg/day p.o. (Hendler, 2001). The antitumor activity of curcumin was reported with 20 μM of curcumin *in vitro* (Notarbartolo et al., 2005). However, the concentration of curcumin in the plasma of patients after oral administration of 3.6 g/day was 11.1 ± 0.6 nmol/L (Sharma et al., 2004). It is difficult to obtain 10–20 μM of curcumin in plasma or tissues *in vivo* after oral administration. We selected the concentration of curcumin for this study based upon the appropriate intake for humans and the difference in metabolism between humans and mice. Therefore, we expect the effect of curcumin reported in this paper to be clinically relevant.

We examined the effects of curcumin on DOX influx and efflux *in vitro* to clarify the mechanism responsible for the enhancement of DOX-induced antitumor activity. In both the DOX alone group and the curcumin–DOX group, the uptake of DOX in tumor cells was increased in a time-dependent manner, but there was no significant difference between these groups. Furthermore, curcumin did not suppress DOX efflux from tumor cells. It was shown that curcumin did not have any effect on DOX transport across the tumor cell membrane. These results supported the fact that the DOX concentration in the tumor did not change *in vivo*. Since curcumin enhanced the effect of DOX antitumor activity, it was assumed that it performed these actions *via* a mechanism other than membrane transport of DOX.

It has been reported that curcumin caused the apoptosis of tumor cells through various pathways (Pillai et al., 2004; Mishra et al., 2005). Therefore, we examined the effect of curcumin on DOX cytotoxicity in tumor cells. Cell viability was decreased with DOX in a dose-dependent manner. In addition, curcumin–DOX significantly decreased cell viability by DOX. Histograms of FCM indicated that the curcumin–DOX combination induced more apoptosis than DOX alone. The decrease in cell viability and the enhancement of apoptosis were shown by fluorescence intensity after treatment with FDA and PI. The observation of chromatin condensation with Hoechst 33342 stain shows that treatment with curcumin and DOX induced chromatin condensation in a time dependent manner suggesting that cells were undergoing apoptosis.

There are two main pathways by which apoptosis can occur. One is the pathway which activates caspase-3 through activation of caspase-8 by a signal from the death receptor. Another is the pathway which activates caspase-3 through activation of caspase-9 with cytochrome c released from the mitochondria. In our study, DOX treatment of M5076 ovarian sarcoma cells enhanced the activation of caspase-3 in a time- and condensation dependent manner (data not shown) and the combination of curcumin with DOX suppressed activation of caspase-3 compared with DOX alone. This effect was speculated to involve the anti-oxidative effects of curcumin.

The antitumor activity of DOX is shown to be due to topoisomerase II inhibition and DNA damage through radical reactions (Chabner et al., 1996; Gewirtz et al., 1999; Jung and Reszka, 2001; Hurley, 2002) and apoptosis. The mechanism of apoptosis involving DOX has been determined to be due to oxidative DNA damage by DOX-induced hydrogen peroxide (H_2O_2) generation, although DOX-induced apoptosis may involve topoisomerase II inhibition (Mizutani et al., 2005). H_2O_2 generated through both pathways leads to an increase in mitochondrial membrane potential and subsequent caspase-3 activation through caspase-9 activation in DOX-induced apoptosis. Thus, we postulate that antioxidative properties of curcumin inhibit H_2O_2 release from DOX leading to suppressed activation of caspase-9 and -3. Curcumin suppressed the activation of caspase-9 to 91.6% ($p < 0.001$) and caspase-8 to 64.3% ($p < 0.001$) compared to DOX alone which suggests that more detailed studies are necessary to clarify the mechanism by which curcumin suppresses the DOX-induced activation of caspase-3.

Viability of cells treated with curcumin and DOX was significantly lower than with DOX alone and the occurrence of apoptosis

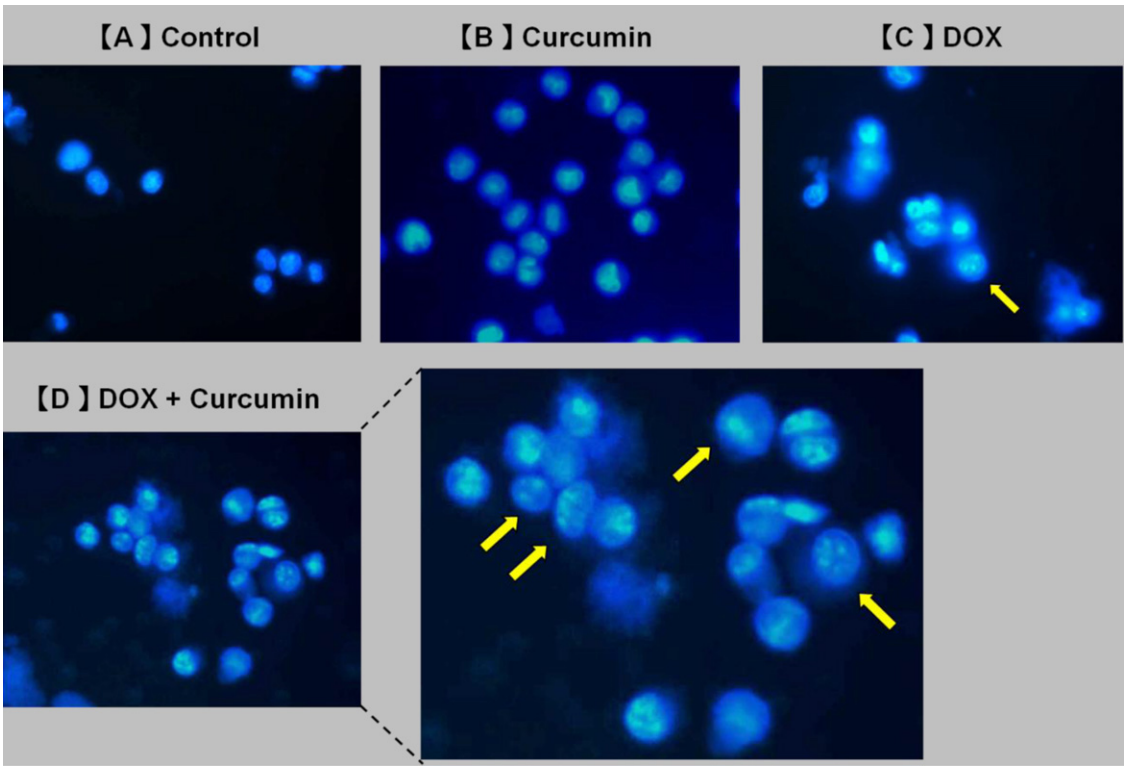

Fig. 7. Effects of DOX and curcumin on chromatin condensation in M5076 ovarian sarcoma cells. M5076 cells were seeded on 24-well plates in the presence or absence of curcumin (10 μM). On the second day, cells were treated with DOX (5.4 μM). Following incubation for 12 h, chromatins were stained with Hoechst 33342. Arrow indicates apoptotic cells.

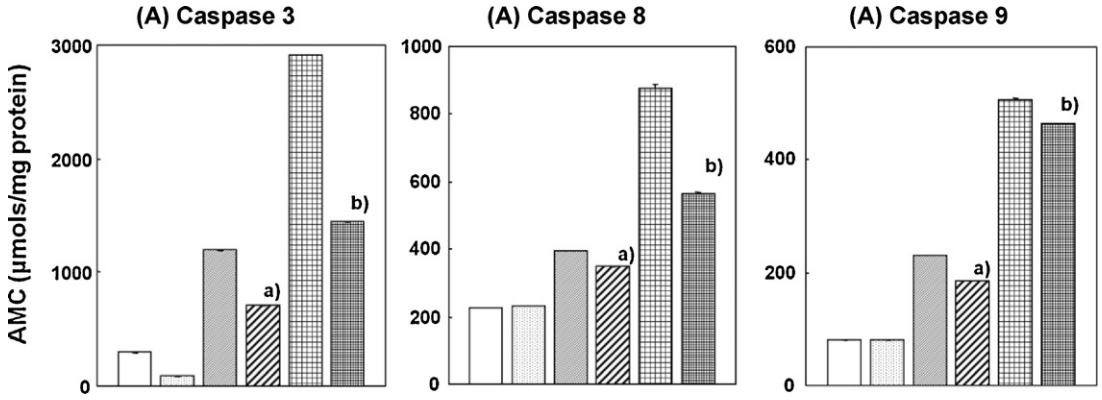

Fig. 8. Effects of DOX and curcumin on caspase activity in M5076 ovarian sarcoma cells. M5076 cells were plated on 24-well plates in the presence or absence of curcumin. On the second day, cells were treated with DOX (1.8 or 5.4 μM). Following incubation for 8 h, cells were lysed. Aliquots of cell lysate were added to the reaction buffer containing fluorogenic substrate (Ac-DEVD-MCA [A], Ac-IETD-MCA [B], Ac-LEHD-MCA [C]) and the mixtures were incubated for 1 h. The amounts of fluorogenic MCA moiety released were measured by spectrofluorometry. The fluorescence intensity was converted to micromoles of MCA released using the standard curve of 7-amino-4-methylcoumarin. Each column represents the mean ± S.D. ($n = 3$). Significant difference from the 1.8 μM DOX group is indicated by 'a)' $p < 0.001$. Significant difference from the 5.4 μM DOX group is indicated by 'b)' $p < 0.001$. □: Control; ▨: curcumin 10 μM; ▨: DOX 1.8 μM; ⊞: DOX 5.4 μM; ▨: DOX 1.8 μM + curcumin 10 μM; ▦: DOX 5.4 μM + curcumin 10 μM.

was enhanced. We hypothesized that curcumin combined with DOX suppresses the main caspase pathway and activates the main caspase independent pathway involving apoptosis-inducing factor (AIF) (Candé et al., 2002; Arimura et al., 2003) and caspase-12 through endoplasmic reticulum stress (Li et al., 2006).

In conclusion, curcumin is a useful modulator to enhance DOX-induced antitumor activity while reducing the adverse effects by suppressing lipid peroxidation in normal tissue. The mechanism by which curcumin enhances DOX antitumor activity is considered to involve main caspase-independent cell death. We expect that the addition of curcumin may enable a reduction in the effective dose of DOX for treatment. It is our hope that continued basic study of useful food elements such as curcumin will bring about an improvement in clinical cancer therapy and QOL of patients who take advantage of the benefits of these substances.

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