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Polyoxyethylene-modified Superoxide Dismutase Reduces Side Effects of Adriamycin and Mitomycin C

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Polyoxyethylene-modified superoxide dismutase (SOD-POE) is a newly developed long-acting superoxide dismutase. Adriamycin (ADR) and mitomycin C (MMC) generate superoxide, which contributes to their cytocidal effects or side effects. We examined whether SOD-POE could prevent the side effects induced by superoxide generated by antitumor agents, and the following results were obtained. SOD-POE did not influence the antitumor effects of ADR and MMC either *in vitro* or *in vivo*, but prevented the toxic death of BALB/c, *nu/nu* male mice caused by overdoses of ADR or MMC. As for its effective sites, SOD-POE prevented a decrease in the specific activity of rotenone-sensitive NADH-ubiquinone oxido-reductase (complex I) in heart muscle mitochondrial respiratory chain function in BALB/c male mice administered 10 mg/kg ADR, and prevented damage to the sarcoplasmic reticulum and mitochondria of mouse heart muscle by ADR as observed by electron microscopy. Furthermore, SOD-POE prevented bone marrow suppression induced by MMC in Donryu rats. The above results suggest that combination chemotherapy with SOD-POE would make it possible to increase the maximum permissible doses of antitumor agents, improving the efficacy of these agents.

Key words: Superoxide — Superoxide dismutase — Antitumor agent — Mitochondrial respiratory chain function — Electron microscope

The formation of oxygen radicals in the human body causes peroxidative damage to biomembranes and destroys cells, and free radicals are believed to be involved in aging, inflammation, cataract formation, autoimmune diseases, ischemic diseases, carcinogenesis and many other diseases.[1)] The many antioxidants in the body may be classified into 2 groups, enzymes and non-enzymes. Antioxidant enzymes include superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione-*S*-transferase and peroxidase, while antioxidant non-enzymes include vitamin C, vitamin E, glutathione, metallothionein, uric acid, ceruloplasmin, transferrin, albumin and many other substances. The anticancer agents adriamycin (ADR), mitomycin C (MMC), bleomycin and VP-16 generate superoxide and it plays an important role in their cytocidal effects and also their side effects.[2–5)]

SOD was discovered by McCord and Fridovich in 1969,[6)] and forms a part of the defense mechanisms of living organisms against oxygen toxicity. There are four types of SOD; Fe-, Mn-, Cu,Zn- and EC-SOD. Cu,Zn-SOD was first discovered in calf red blood cells, and its molecular weight is 31,200. Mn-SOD was found in *E. coli*. Its molecular weight is 40,000 and 80,000.[7)] Fe-SOD was also found in a bacillus,[8)] and EC-SOD (extracellular) was found in extracellular fluid.[9)]

SOD-POE is a newly developed recombinant human Cu,Zn-SOD with polyoxyethylene (POE), and it has a longer residence time in blood than conventional SOD. Since we hypothesized that the mechanisms of the antitumor effects and the side effects of superoxide-generating anticancer agents might be distinct, in this work we examined whether SOD-POE could selectively decrease the side effects of several such agents without reducing the effectiveness of their antitumor action.

MATERIALS AND METHODS

SOD and SOD-POE Free SOD and SOD-POE were supplied by Ajinomoto Chemical Co., Inc., Tokyo. Three g (0.094 m*M*) of human recombinant Cu,Zn-SOD (4170 units/mg) was dissolved in 300 ml of K_2PO_4 (0.1 *M*, pH 7.2), then 3.56 g of α-carboxymethyl, ω-carboxy-methoxy-polyoxyethylene (1.19 m*M*, Japan Oil Chemical Co., Ltd. Tokyo) was added. The mixture was stirred and left for 1 h, then 0.34 g of glycine (4.5 m*M*) was added to stop the reaction. This solution was passed through an ultrafiltration membrane, YM 30 (cut-off 30,000 daltons, American Corp., Danvers, CO) and 200 ml of SOD-POE solution was obtained. The SOD concentration in this solution was 10.2 mg/ml and its activity was 96.4% (4,020 units/mg). The average number of polyoxyethylene molecules bound to one SOD molecule

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was 4.3. SOD activity was measured by the method of McCord *et al.*[6] and the number of POE molecules bound to SOD was calculated from the C/N ratio obtained from atomic analysis. SOD activity in blood was measured after intravenous administration of SOD and SOD-POE in rats. SOD activity in blood disappeared quickly, whereas that of SOD-POE remained for 96 h after intravenous administration.[10]

Cell line and culture conditions WS-SC is a human esophageal squamous cell carcinoma cell line established by Professor H. Watanabe (National Cancer Center, Tokyo) and was provided by him. This cell line had been maintained as xenografts in BALB/c, *nu/nu* mice (Shizudokyo, Shizuoka) and was converted to *in vitro* culture by Akiyama. Cells were cultured in RPMI-1640 (Nissui Pharmaceutical Co., Ltd., Tokyo) supplemented with 10% fetal calf serum (Irvine Scientific, Santa Ana, CA) and incubated at 37°C in an atmosphere of 5% CO_2 and 95% air.

***In vitro* SDI assay with MTT** The sensitivity of WS-SC to antitumor agents with or without SOD-POE *in vitro* was tested by SDI assay with MTT.[11, 12] WS-SC was cultured in 96-well tissue culture plates (Falcon, Becton Dickinson and Co., NJ) with antitumor agents. The concentrations of the anticancer agents ADR and MMC were 1.0 and 0.1 μg/ml, respectively, and that of SOD-POE was 10 or 1 μg/ml. The cells (3×10^5 cells/ml) were cultured with these agents for 72 h. The medium of each well was aspirated and the cells were washed with PBS. Ten μl of MTT (0.4%) and the same volume of sodium succinate (0.1 *M*) were poured into each well, then the plates were allowed to stand for 1 h at room temperature and 200 μl of isopropanol was added to each well. Optical density at 540 nm was measured with an EASY READER (SLT-LABINSTRUMENTS GmbH, Australia) after formazan had dissolved. Anticellular activity was expressed as IC_{50} values.

***In vivo* nude mice assay** BALB/c, *nu/nu* male were inoculated with 2 mm^3 of WS-SC, and ADR or MMC was administered intraperitoneally (ADR, 10 mg/kg, MMC, 10 mg/kg). SOD-POE was given at 100 U/body intraperitoneally twice a day for 5 days. Body weight and tumor size were measured, and tumor weight was approximately calculated by using the formula, $1/2LW^2$ (L: length, W: width). The survival rate was determined in each group of 10 mice. Statistical significance was assayed using the chi-square test or Fisher's exact probability test.

Influence of SOD-POE on heart muscle mitochondrial respiratory chain function in mice given ADR Experiments were carried out on BALB/c male mice. Ten mg/kg of ADR was administered to mice intraperitoneally. SOD-POE was given at 100 U/body intraperitoneally twice a day for 5 days after administration of ADR. Mice were fasted for 24 h before the experiments and allowed free access only to water.

Cytochrome *c* (Type III), EDTA (ethylenediaminetetraacetic acid) and soybean phospholipid mixture (containing 22% L-α-phosphatidylcholine) were obtained from Sigma, St. Louis, MO. NADH (nicotinamide adenine dinucleotide, reduced form) was purchased from Oriental Yeast Co., Tokyo, and DCIP (2,6-dichloroindophenol) was from Wako Chemical Co., Osaka. Coenzyme Q_1 and coenzyme Q_2 were supplied by Eisai Co., Tokyo. Other chemicals used were of reagent grade. Absorbance measurements were performed using a Shimadzu UV-visible recording spectrophotometer, model UV-250 (Shimadzu, Kyoto).

Preparation of mouse heart muscle mitochondria Mice were killed by cervical dislocation and the heart was removed. The tissue was put into ice-cold 50 m*M* Tris-HCl buffer, pH 7.4 containing 0.25 *M* sucrose, minced with a pair of scissors and then gently homogenized with a Physcotron Handy Micro Homogenizer (Niti-on, Tokyo) for 30 s. The homogenates were centrifuged at 3,000 rpm for 20 min and the supernatants obtained were centrifuged at 10,000 rpm for 15 min. Then the pellets were washed with 10 m*M* Tris-HCl buffer, pH 7.8 containing 0.25 *M* sucrose and finally resuspended in the same buffer. Protein in the mitochondrial preparations was measured by the bicinchoninic acid method according to Smith *et al.*[13]

Measurement of electron transport activity The specific activity of rotenone-sensitive NADH-ubiquinone oxido-reductase (complex I) was determined by a modification of the method of Hatefi and Rieske.[14] The reaction mixture consisted of 20 μl of potassium phosphate buffer (1.0 *M*, pH 8.0), 20 μl of NaN_3 (0.1 *M*), 50 μl of coenzyme Q_1 (1.0 m*M*), 10 μl of asolectin suspension (15 mg/ml in 1 m*M* EDTA) and 880 μl of distilled water. The reaction mixture was incubated at 38°C before use. The reaction was initiated by adding 12 μl of NADH (10 m*M*) and 40 μl of mitochondrial suspension, successively. After 15 s, 10 μl of rotenone was added. The reaction rate was followed for 1 min by recording the decrease in absorbance at 340 nm. The specific activity of NADH-ubiquinone oxido-reductase was deduced from the rate of decrease in absorbance.

The specific activity of succinate-ubiquinone oxido-reductase (complex II) was determined by a modification of the method of Hatefi and Stiggall.[15] The reaction mixture consisted of 20 μl of sodium succinate (1.0 *M*, pH 7.4), 0.5 μl of EDTA (0.2 *M*, pH 7.0), 20 μl of NaN_3 (0.1 *M*) and 920 μl of potassium phosphate buffer (50 m*M*, pH 7.4). The reaction mixture was incubated at 38°C before use. The reaction was initiated by adding 16 μl of DCIP (4.65 m*M*), 20 μl of coenzyme Q_1 (2.5 m*M*) and mitochondrial suspension. The reaction rate was

followed for 3 min by recording the decrease in absorbance at 600 nm. The specific activity of succinate-ubiquinone oxido-reductase was deduced from the rate of decrease in absorbance.

The specific activity of ubiquinol-cytochrome *c* oxido-reductase (complex III) was determined by the method of Shimomura *et al.*[16] Reduced coenzyme Q_2 was prepared by the method of Rieske.[17] The reaction mixture consisted of 200 μl of cytochrome *c* (30 μM), 100 μl of soybean phospholipid mixture, 700 μl of reaction buffer containing 25 mM potassium phosphate, pH 7.5, 25 μM EDTA and 0.02% Tween 20. Before the start of the enzymic reaction, reduced coenzyme Q_2 was added as a concentrated ethanolic solution to give a concentration of 63 μM, and nonenzymic reduction of cytochrome *c* was measured at 550 nm for 30 s. Then the reaction was started by adding the enzyme and the reaction rate was followed by recording the increase in absorbance at 550 nm. The specific activity of ubiquinol-cytochrome *c* oxido-reductase was estimated from the rate of increase in absorbance.

The specific activity of cytochrome *c* oxidase (complex IV) was determined by a modification of the method of Wharton and Tzagoloff.[18] To prepare ferrocytochrome *c*, 1% ferricytochrome *c* was reduced completely by dithionite, and excess dithionite was removed by passing the solution through a column of Sephadex G-25 (fine). Potassium phosphate buffer (2.67 ml; 50 mM, pH 7.0) and 30 μl of 10% Triton X-100 were added to 100 μl of ferrocytochrome *c* solution. Immediately after the addition of 0.1 ml of mitochondrial suspension, the reaction was followed for 15 s by recording the decrease in absorbance at 550 nm. The specific activity of cytochrome *c* oxidase was estimated from the rate of decrease in absorbance.

All analyses were performed in duplicate, and mean values were recorded. Since each group (ADR group, ADR+SOD-POE group and control group) consisted of 6 mice, 12 measurements were made for each complex.

Effect of SOD-POE on ADR damage to mouse heart muscle observed by electron microscopy ADR was administered to BALB/c male mice intraperitoneally at 2 mg/kg 4 times every other day. SOD-POE was given subcutaneously at 100 U once a day for 7 days after the first injection of ADR. Three weeks after the last injection of ADR, each mouse was anesthetized with ethanol, the chest was opened, and the heart was immediately extirpated entirely and fixed with phosphate buffer solution for 5 min. Then, several small tissue blocks of the left ventricular free wall were excised. These specimens were fixed in ice-cold 3% glutaraldehyde in phosphate buffer, then postfixed with 2% osmium tetroxide in phosphate buffer and embedded in Epon. Sections (1 μm thick) were cut from each Epon-embedded block. Sections of each group were stained with uranyl acetate and lead, and examined in an Hitachi 11B or an Hitachi 12 electron microscope.

Effect of SOD-POE on bone marrow suppression induced by MMC MMC was administered to Donryu rats intraperitoneally at 10 mg/kg, and 5 U/g SOD or SOD-POE was given intraperitoneally twice a day for 5 days. White blood cells (WBC), red blood cells (RBC) and platelets (PLT) in the peripheral blood were counted in each group of 5 rats.

RESULTS

***In vitro* SDI assay with MTT** The differences of IC_{50} among the respective groups of ADR or MMC were not significant. Thus, SOD-POE had no influence on the anticellular activity of ADR or MMC *in vitro* (Table I).

***In vivo* nude mice assay** Tumor weights of the ADR group and the ADR+SOD-POE group were significantly ($P<0.01$) lower than those of the control group on days 11, 13 and 18, but the difference of tumor weights between the ADR group and the ADR+SOD-POE group was not significant. Tumor weights of the MMC group and the MMC+SOD-POE group were significantly ($P<0.01$) lower than those of the control group after day 9, but the difference between the MMC group and the MMC+SOD-POE group was not significant. SOD-POE did not affect the antitumor effect of ADR and MMC (Fig. 1).

Body weight of nude mice was measured. The difference of body weights between the ADR+SOD-POE group and the ADR group was significant at $P<0.05$ on day 13 and at $P<0.01$ on day 17, but the difference of body weights between the MMC group and the MMC+SOD-POE group was not significant. SOD-POE prevented weight loss caused by ADR, but could not prevent weight loss caused by MMC (Fig. 2), probably due to the high dose of MMC employed.

Table I. Effect of SOD-POE on Anticellular Activity of ADR or MMC against WS-SC[a]

Compounds	IC_{50} (μg/ml)[b]
ADR	0.893±0.023
ADR+SOD-POE 1 μU/ml	0.887±0.032
ADR+SOD-POE 10 μU/ml	0.910±0.010
MMC	0.510±0.056
MMC+SOD-POE 1 μU/ml	0.487±0.032
MMC+SOD-POE 10 μU/ml	0.617±0.015

a) The cells (3×10^5/ml) were cultured for 72 h with the test compounds. Cell survival was measured as described in "Materials and Methods."
b) Mean±SD for triplicate cultures.

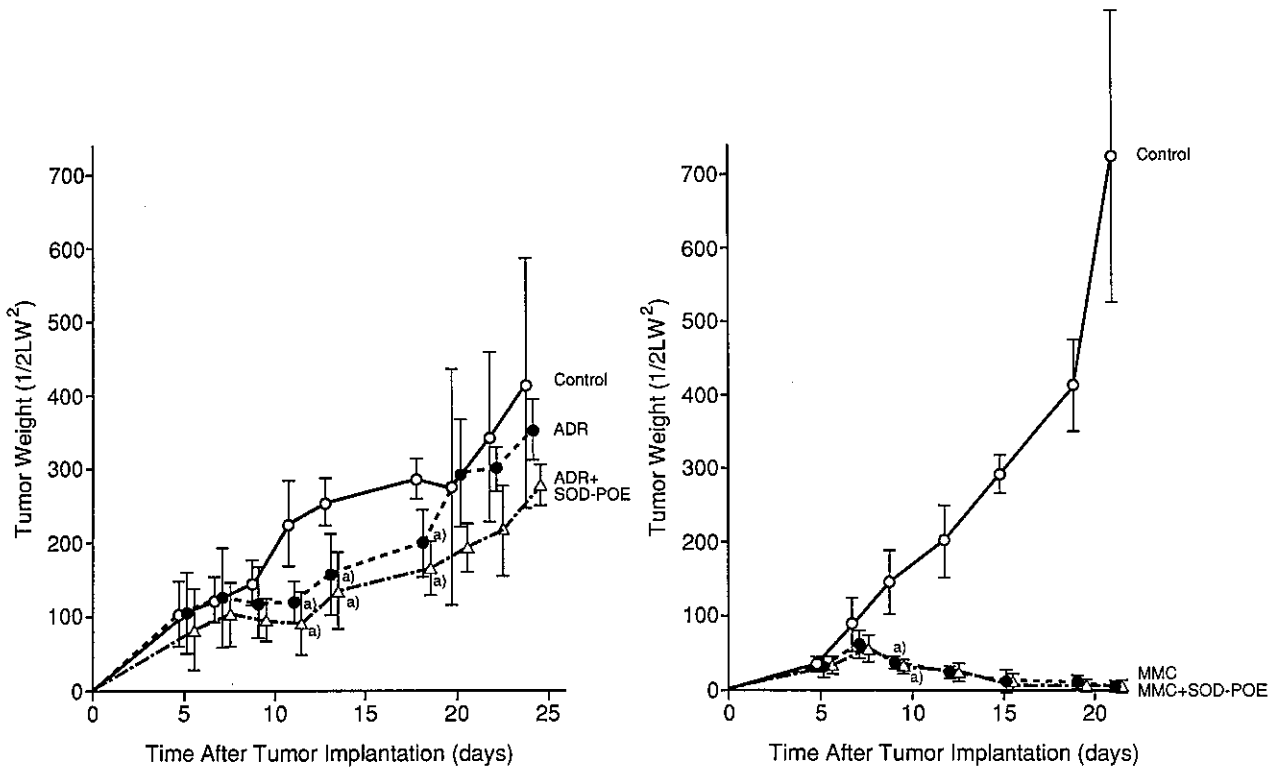

Fig. 1. Effect of SOD-POE on the antitumor activity of ADR or MMC. Bars indicate mean ± SD a) $P<0.01$ compared to the control group.

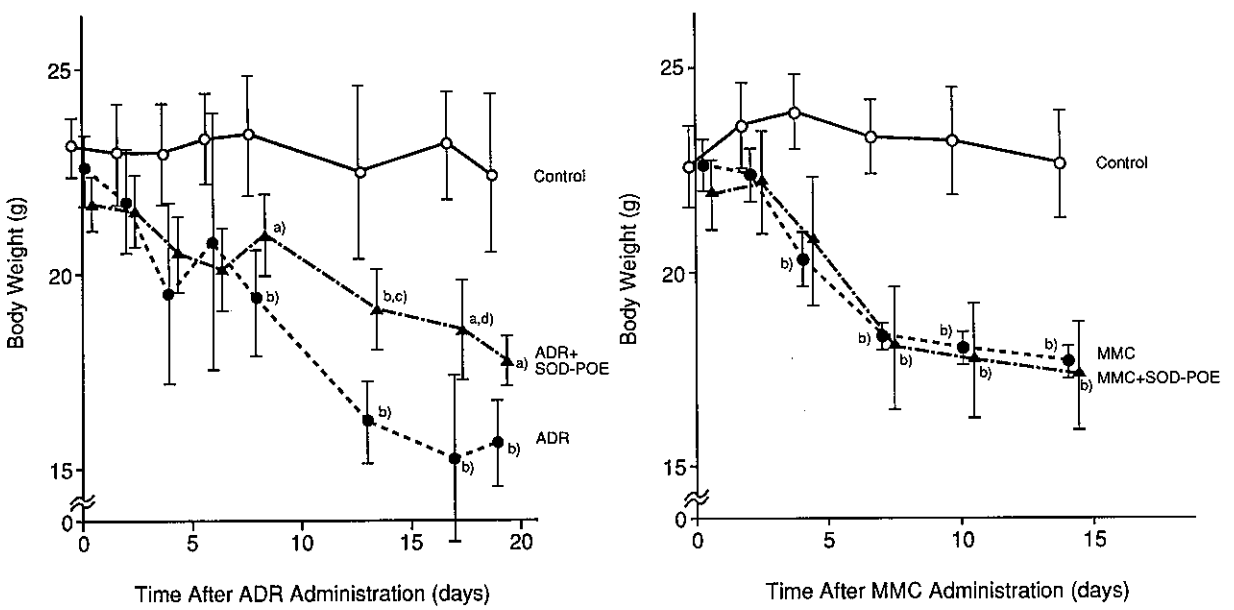

Fig. 2. Effect of SOD-POE on body weight of mice treated with ADR or MMC. Bars indicate mean ± SD. a), $P<0.05$ compared to the control group; b), $P<0.01$ compared to the control group; c), $P<0.05$ compared to the ADR group; d), $P<0.01$ compared to the ADR group.

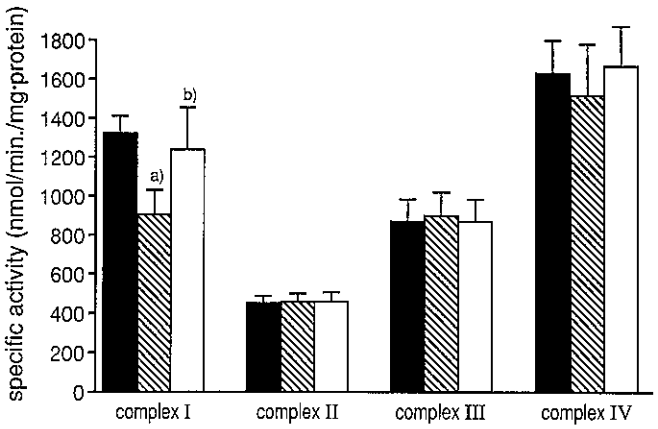

Fig. 3. Influence of SOD-POE on electron transport activity in heart muscle mitochondrial respiratory chain function of mice given ADR. Bars indicate mean±SD. (■), control group; (▨), ADR group; (□), ADR+SOD-POE group; a), $P<0.01$ compared to the control group; b), $P<0.01$ compared to the ADR group.

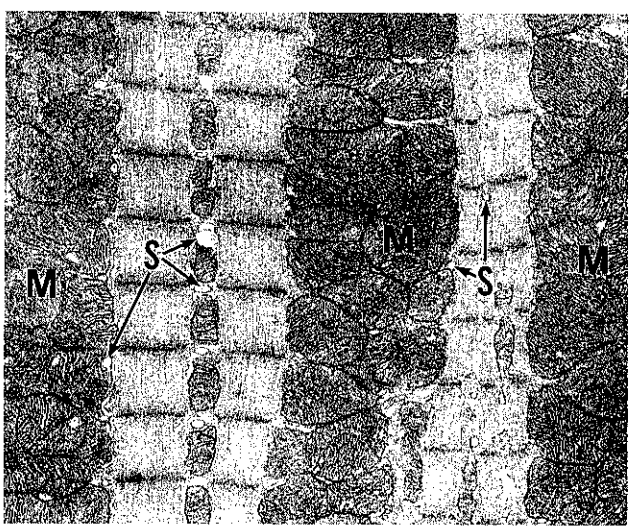

Fig. 4. Thin-section electron microscopy of a normal cardiac muscle cell from a control mouse showed that sarcoplasmic reticulum (S) was a closed intracellular network of tubules and cisternae surrounding myofibrils, mitochondria (M) were regular in shape, and the intercristal spaces were closed. Fixed wth glutaraldehyde-paraformaldehyde mixture and stained with uranyl acetate and lead. ×10,000.

The survival rates of tumor-bearing nude mice at the 14th and 21st days after ADR administration were 80% and 30%, while those in the case of ADR+SOD-POE were 100% and 80%. The difference of the survival rate between the ADR group and the ADR+SOD-POE

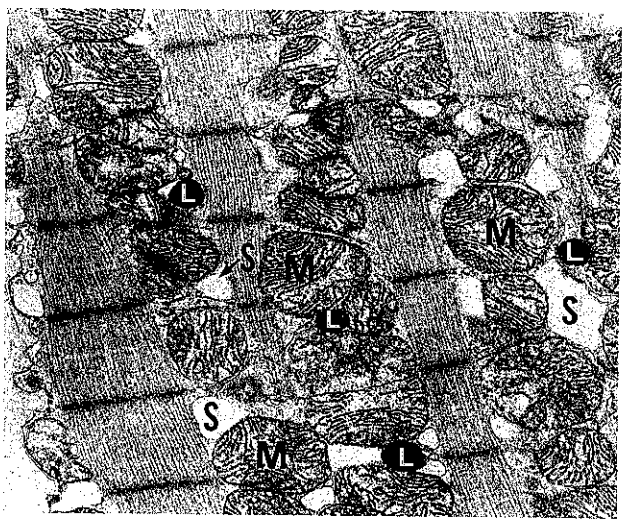

Fig. 5. A cardiac muscle cell from a mouse treated with ADR. Mild dilatation of sarcoplasmic reticulum (S) and an increased number of lipid droplets (L) were observed. Mitochondrial cristae (M) were oriented irregularly and the intercristal spaces were substantially enlarged. Fixed with glutaraldehyde-paraformaldehyde mixture and stained with uranyl acetate and lead. ×10,000.

group at the 21st day was significant at $P<0.05$. The survival rates of nude mice at the 7th and 14th days after MMC administration were 60% and 40%, while those in the case of MMC+SOD-POE were 100% and 100%. The difference of the survival rate between the MMC group and the MMC+SOD-POE group at the 14th day was significant at $P<0.01$.

Influence of SOD-POE on heart muscle mitochondrial respiratory chain function in mice given ADR The specific activity of complex I in the ADR group was significantly ($P<0.01$) lower than that in the control group or the ADR+SOD-POE group. The specific activities of complexes II, III and IV showed no change among the three groups. SOD-POE nullified the decrease in the specific activity of complex I induced by ADR (Fig. 3).

Effect of SOD-POE on ADR damage to mouse heart muscle observed by electron micrpscopy Thin-section electron microscopy of control myocytes showed that the sarcoplasmic reticulum was a closed intracellular network of tubules and cisternae surrounding myofibrils, the mitochondria were regular in shape and the intercristal spaces were closed (Fig. 4). In the ADR group, mild dilatation of sarcoplasmic reticulum and an increased number of lipid droplets were observed. Mitochondrial cristae were oriented irregularly and the intracristal spaces were substantially enlarged (Fig. 5). In the

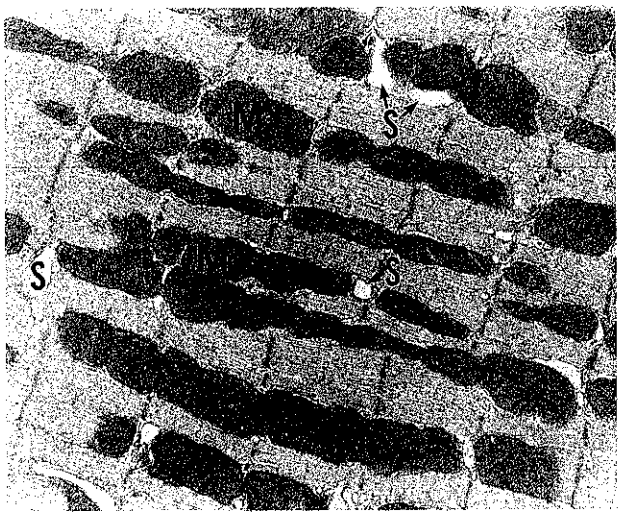

Fig. 6. A cardiac muscle cell from a mouse treated with ADR and SOE-POE. There was no prominent change in sarcoplasmic reticulum (S) or mitochondria (M). ×10,000.

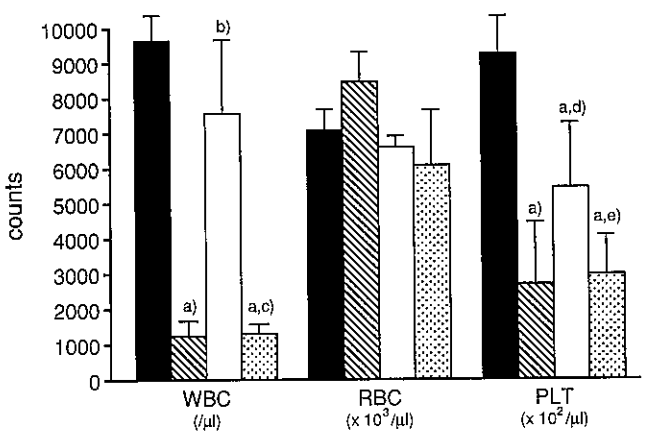

Fig. 7. Effect of SOD-POE on bone marrow suppression induced by MMC. Bars indicate mean±SD. (■), the control group; (▨), the MMC group; (□), the MMC±SOD-POE group; (⊡), the MMC+SOD group; a), $P<0.01$ compared to the control group; b), $P<0.01$ compared to the MMC group; c), $P<0.01$ compared to the MMC+SOD-POE group; d), $P<0.05$ compared to the MMC group; e), $P<0.05$ compared to the MMC+SOD-POE group.

ADR+SOD-POE group, there were no prominent changes in sarcoplasmic reticulum or mitochondria. Lipid droplets were not observed (Fig. 6).

Effect of SOD-POE on bone marrow suppression induced by MMC The WBC count in the MMC group was significantly lower ($P<0.01$) than that in the control group, and that in the MMC group was significantly lower ($P<0.01$) than that in the MMC+SOD-POE group. There was no significant differnce in RBC count among the respective groups. The platelet counts in the MMC group and the MMC+SOD-POE group were significantly lower ($P<0.01$) than that in the control group, and that in the MMC group was significantly lower ($P<0.05$) than that in the MMC+SOD-POE group. Thus, SOD-POE prevented the decreases of WBC and platelet count induced by MMC. There was no effect of unmodified SOD on bone marrow suppression induced by MMC (Fig. 7).

DISCUSSION

Free radicals are inevitably formed in aerobic organisms and are not harmful under normal conditions, causing damage only when produced excessively, or when the capacity to deal with them is impaired. The antitumor agents ADR and MMC act by generating superoxide, which damages the DNA of cancer cells. This superoxide may also be resposible for the dose-limiting toxicities of these chemicals.

Our hypothesis that the mechanisms responsible for the antitumor effects and side effects are distinct was supported by the results of this study. SOD-POE suppressed only the side effects of the antitumor agents but not the antitumor effects themselves. Hauser *et al.* reported that free SOD increased the survival rate of tumor-bearing mice treated with tumor necrosis factor (TNF) by decreasing the toxicity of TNF and did not interfere with its antitumor efficacy.[19] However, free SOD is excreted from the kidney and most SOD in blood disappears within a few minutes.

Some attempts have been made to prolong the half life of free SOD. By binding SOD to Ficoll, polyethylene glycol and albumin, McCord and Wong succeeded in prolonging its half life in blood, but failed to obtain tissue permeability.[20] Michelsen encapsulated SOD in liposomes and obtained a long life with good tissue permeability.[21] SOD-POE was also developed for the same purpose. Our experiments showed that effective sites of SOD-POE were heart muscle in mice given ADR or bone marrow in rats given MMC. As regarde side effects, ADR was found to damage the sarcoplasmic reticulum and mitochondria, based on our observation of heart muscle by electron microscopy, and to decrease the specific activity of complex I, which is the most important respiratory enzyme that initiates electron transport in oxidative phosphorylation, based on our measurement of electron transport activity in heart muscle mitochondrial respiratory chain function. However, SOD-POE prevented such damage. A possible mechanism of ADR cardiotoxicity is as follows. One electron reduction of

ADR forms the semiquinone radical which, in the presence of molecular oxygen, leads to the formation of superoxide anion radical, This superoxide is converted to hydrogen peroxide. Superoxide itself or hydroxy radical, which is formed from superoxide anion radical and hydrogen peroxide, can trigger free radical reactions leading to the peroxidation of poly-unsaturated fatty acid components of membrane lipids and inhibition of the respiratory chain, damaging complex I existing at the inner membrane of mitochondria. SOD-POE prevents these free radical reactions by scavenging superoxide, the key mediator of these reactions. Some authors[22-26] have reported ultrastructural alterations of the myocardium induced by ADR: dilation of the sarcoplasmic reticulum, mitochondrial swelling, separating of mitochondrial cristae and/or an increased number of lipid droplets. Myers *et al.*[27] indicated that an important mechanism of ultrastructural degenerative change is a change in cell membrane permeability owing to lipid peroxidation mediated by free radicals.

We think the changes in the dilatation of sarcoplasmic reticulum, irregularly oriented mitochondrial cristae and/or enlarged intercristal spaces that we observed in heart muscle by electron microscopy are a consequence of a change in cell membrane permeability induced by promotion of lipid peroxidation mediated by free radicals. Among the hypotheses proposed to explain the cardiotoxicity are: 1) inhibition of DNA, RNA and protein synthesis,[28-30] 2) peroxidation of membrane lipids,[27] and 3) inhibition of enzyme systems in the mitochondrial respiratory chain.[31, 32] Our results are consistent with hypotheses 2 and 3.

Means to ameliorate the side effects of antitumor agents are an important aspect of chemotherapy. Our results suggest that it will be possible to raise the maximum permissible doses of antitumor agents by using combined chemotherapy with SOD-POE, making these agents even more effective.

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