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Manganese Superoxide Dismutase Negatively Regulates the Induction of Apoptosis by 5-Fluorouracil, Peplomycin and γ-Rays in Squamous Cell Carcinoma Cells

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We investigated the relationship between manganese superoxide dismutase (Mn-SOD) activity and apoptosis induced by anticancer drugs and radiation. Although the activity of copper, zinc-SOD did not differ greatly among 9 squamous cell carcinoma (SCC) cell lines (OSC-1 to OSC-9), the Mn-SOD activity did differ among the cell lines. The Mn-SOD activity was increased by treatments with 5-fluorouracil (5-FU), peplomycin and ^{137}Cs, reaching plateau levels at 12 h after treatment and then decreasing gradually. When OSC-1 and OSC-3, and OSC-2 and OSC-4 were examined as representative cell lines with low and high Mn-SOD activity, respectively, the decrease was more prominent in OSC-1 and OSC-3 than in OSC-2 and OSC-4. The intracellular levels of superoxide and hydrogen peroxide (H_2O_2) were increased after treatment with the anticancer agents, and the increases were larger in OSC-1 and OSC-3 than in OSC-2 and OSC-4. The decrease of mitochondrial membrane potential ($\Delta\psi_m$) by the anticancer agents was marked in OSC-1 and OSC-3. Correspondingly, the release of cytochrome *c*, the activation of caspase-3 and the cleavage of poly(ADP-ribose)polymerase were stronger in OSC-3 than in OSC-4. In addition, apoptosis induced by the anticancer agents was prominent in OSC-3, exhibiting a close relationship with the $\Delta\psi_m$ and the H_2O_2 level. These results indicate that Mn-SOD in SCC cells modulates apoptosis induction and the inactivation of Mn-SOD might be a promising strategy for SCC treatment.

Key words: Mn-SOD — Apoptosis — Reactive oxygen — Cytochrome *c* — Mitochondrial membrane potential

The induction of tumor cell differentiation and apoptosis are considered to be potentially useful therapeutic strategies for both nonepithelial and epithelial carcinomas.[1–5] The mechanisms of differentiation and apoptosis induced by γ-rays and various anticancer drugs have been examined, and the roles of multiple proteins involved in differentiation and apoptosis have been clarified.[6–10] However, many details remain to be elucidated, and satisfactory induction of apoptosis or differentiation has not been achieved in tumors other than leukemias.[11]

There are many pathways in the induction of apoptosis. The signal arising from the death domains of Fas (CD95/APO-1) is one of the potent apoptosis-inducing pathways.[12, 13] In this Fas-associated pathway, the signal is relayed to the Fas-associated death domain (FADD) and flows downstream, activating interleukin-1 converting enzyme (ICE) family proteases and caspase-activated DNase (CAD).[12–18] The final step of the pathway is DNA fragmentation, which results from the inactivation of the DNAase inhibitor, poly(ADP-ribose)polymerase (PARP) and protein kinase Cδ (PKCδ) by the cleaved product of caspase-3 (apopain).[19–21] In other pathways, cytochrome *c* released from the mitochondria is usually involved under the regulation of Bcl-2 family members.[22–25] The release of cytochrome *c* is also induced by stimuli from the FADD.[26]

The signal from FADD thus appears to play a key role in the induction of apoptosis. The involvement of the CD95 system and caspase-3 in chemotherapy-induced apoptosis of Ewing's sarcoma, colon carcinoma and small cell lung carcinoma cells was reported.[27–29] However, their involvement was not observed in the chemoradiotherapy-induced apoptosis of breast carcinoma or renal cell carcinoma cells.[27] These findings suggest that multiple pathways are involved in the induction of apoptosis of solid tumor cells. Therefore, it may be necessary to identify the main apoptosis-inducing signal in each carcinoma treated with a given anticancer agent.

A reduction of the mitochondrial membrane potential ($\Delta\psi_m$) is required for cytochrome *c* release.[23] A reduction of the $\Delta\psi_m$ is induced by signals from FADD as well as by reactive oxygen intermediates (ROI) which possess

Abbreviations used: Mn-SOD, manganese superoxide dismutase; SCC, squamous cell carcinoma; 5-FU, 5-fluorouracil; PLM, peplomycin; PARP, poly(ADP-ribose)polymerase; FADD, Fas-associated death domain; ICE, interleukin-1 converting enzyme; CAD, caspase-activated DNase; PKCδ, protein kinase Cδ; ROI, reactive oxygen intermediates; DEDTC, diethyl dithiocarbamate; ATZ, aminotriazole; BSO, buthionine sulfoximine; Rh123, rhodamine 123; HE, hydroethidine; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; PI, propidium iodide; MFI, mean fluorescence intensity; Apaf, apoptotic protease-activating factor.

protein phosphorylation-inducing activity and activate transcription factors such as NF-κB and AP-1.[26, 30–32] Therefore, the role of ROI appears to be important for the induction of apoptosis.[33, 34]

Many anticancer drugs, γ-rays and ultraviolet rays induce the generation of ROI in tumor cells.[35–39] When the level of generated ROI exceeds the ROI-scavenging activity of the tumor cells, the cells are impaired to some degree, depending on the amount of the excessive ROI. Superoxide dismutase (SOD), catalase and glutathione peroxidase are important scavengers of ROI. Among these enzymes, manganese-SOD (Mn-SOD), which is located in the mitochondrial matrices, is induced by cytotoxic and cell-impairing stimuli, and protects the cells from generated superoxide ($O_2^{\cdot-}$).[39–42] It is thus reasonable to speculate that tumor cells which have a high level of Mn-SOD can better protect themselves against lethal cytotoxic stimuli as compared to those with low Mn-SOD activity. However, some investigators have reported that tumor cells with high Mn-SOD activity were sensitive to anticancer drugs and γ-rays.[43, 44] In addition, it was recently reported that an antioxidant, *N*-acetylcysteine, induces apoptosis via an increase of p53 protein.[45] To resolve this confusion and to provide a better basis for apoptosis-inducing therapy in solid tumors, we investigated intracellular Cu,Zn-SOD and Mn-SOD activities in several squamous cell carcinoma (SCC) cell lines, and examined the relationship between Mn-SOD activity and apoptosis. The results obtained should be useful in the design of treatment strategies for SCC.

MATERIALS AND METHODS

Cells, chemicals, and reagents All nine SCC cell lines used in the present study (OSC-1 to OSC-9) were established from lymph node metastasis of oral SCCs. These carcinomas are transplantable to *nu*/*nu* mice. Except for OSC-4, the cell lines possess a mutated *p53* gene in one or 2 exons. The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 2 m*M* L-glutamine and antibiotics. 5-Fluorouracil (5-FU, Kyowa Hakko Co., Tokyo), peplomycin (PLM, a bleomycin derivative, Nihon Kayaku Co., Tokyo), diethyldithiocarbamate (DEDTC, a Mn-SOD inhibitor, Sigma Chemical, St. Louis, MO), aminotriazole (ATZ, a catalase inhibitor, Sigma), and buthionine sulfoximine (BSO, a cysteine transglutaminase inhibitor, Nacalai Tesque, Kyoto) were purchased. The antibodies used were anti-mouse cytochrome *c* monoclonal antibody (mAb), anti-rabbit PARP polyclonal Ab, anti-goat caspase-3 mAb and anti-mouse Mn-SOD mAb, purchased from PharMingen (San Diego, CA), Boehringer Mannheim (Indianapolis, IN), Transduction Laboratory (Lexington, KY), and Chemicon (Tenecula, CA), respectively. The secondary Ab conjugated to horseradish peroxidase (HRP) was purchased from Bio-Rad (Hercules, CA). Enhanced chemiluminescence (ECL) reagents were bought from Amersham (Amersham, UK). Rhodamine 123 (Rh123), hydroethidine (HE), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), and propidium iodide (PI) were purchased from Molecular Probes (Eugene, OR) and Sigma.

SOD activity SOD activity was measured by Õyanagi's method.[46] The sample (0.1 ml) was mixed with reagent A (0.2 ml: pH 7.0, 0.2 m*M* hydroxylamine plus 0.2 m*M* hypoxanthine, 1.77 m*M* hydroxylamine *O*-sulfonic acid) and 0.1 ml of water. The reaction was started by adding reagent B (0.2 ml: 1.25 U/ml xanthine oxidase and 10^{-4} *M* EDTA-2Na). The mixture was incubated for 30 min at 37°C, and then reagent C (2.0 ml: 30 μg/ml sulfanilic acid, 0.5 μg/ml *N*-1-naphthylethylenediamine and 16.7% acetic acid) was added. The final mixture was allowed to stand for 20 min at room temperature, and the optical absorption was then measured at 550 nm.

Flow cytometry The $\Delta\psi_m$, intracellular $O_2^{\cdot-}$, intracellular H_2O_2 and apoptotic cell death were assessed by measurements of fluorescence intensity with a FACscan (Becton-Dickinson, Mountain View, CA) after staining with Rh123, HE, DCFH-DA and PI, respectively. The harvested cells were stained with Rh123 (10 μ*M*), HE (2 μ*M*), DCFH-DA (5 μ*M*) or PI (10 μ*M*) for 10 to 15 min before analysis.

Subcellular fractionations and western blotting The preparation of mitochondria and cytosol fractions was conducted according to the methods of Tang *et al*.[47] Briefly, cells (3×10^6) at the end of the treatment were harvested and washed with ice-cold PBS. Cells were suspended in 500 μl of buffer A (20 m*M* HEPES-KOH, pH 7.5, 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 1 m*M* sodium EDTA, 1 m*M* sodium EGTA, 1 m*M* dithiothreitol) containing 250 m*M* sucrose and a mixture of protease inhibitors (1 m*M* PMSF, 1% aprotinin, 1 m*M* leupeptin, 1 μg/ml pepstatin A and 1 μg/ml chymostatin). The cells were homogenized in the same buffer solution, with a glass Pyrex homogenizer using a type B pestle (40 strokes). Unbroken cells, large plasma membrane fragments and nuclei were removed by centrifuging the homogenates at 1,000*g* at 4°C for 10 min. The resulting supernatant was subjected to centrifugation at 10,000*g* at 4°C for 20 min. The pellet fraction (mitochondrial fraction) was washed with buffer A containing sucrose and then solubilized in 50 μl of TNC buffer (10 m*M* Tris-acetate, pH 8.0, 0.5% NP-40, 5 m*M* $CaCl_2$). The supernatant was recentrifuged at 100,000*g* for 1 h at 4°C to precipitate the cytosol fraction. The protein concentration was determined by the Lowry method,[48] then 37 and 25 μg aliquots of each fraction were subjected to 15% SDS-PAGE. Western blotting was performed as described elsewhere.

PARP cleavage assays Equal numbers (3×10^6) of cells

treated with 5-FU, PLM or γ-radiation were each lysed in 2× sample buffer (125 m*M* Tris HCl, pH 6.8, 10% β-mercaptoethanol, 4% SDS, 20% glycerol, and 0.001% bromophenol blue). The lysates were boiled for 10 min, then equal amounts of proteins were fractionated on a 7.5% polyacrylamide gel and electroblotted onto nitrocellulose membranes. Immunoblotting was performed using a rabbit polyclonal anti-PARP antibody and goat anti-rabbit IgG conjugated with HRP. Protein bands (the 116 kDa parental PARP band and the 85 kDa cleavage product) were revealed with ECL.

Western blotting for Mn-SOD and caspase-3 After cell treatment with each agent, proteins were extracted as described above, and the Mn-SOD and caspase-3 levels were assessed by western blotting, essentially as was done in the above PARP cleavage assay.

Northern blot analysis Total RNA was isolated by the method of Chirgwin *et al.*[49] RNA was estimated spectrophotometrically at 260 nm. Twenty micrograms of total

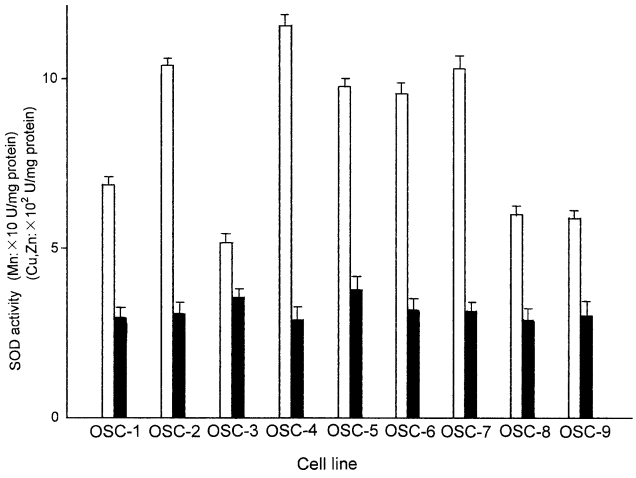


Fig. 1. Steady-state SOD activity in SCC cells. The Mn-SOD (□) and Cu,Zn-SOD (■) activities of nine SCC cell lines were measured as described in "Materials and Methods." All data are means of quadruplicate counts.

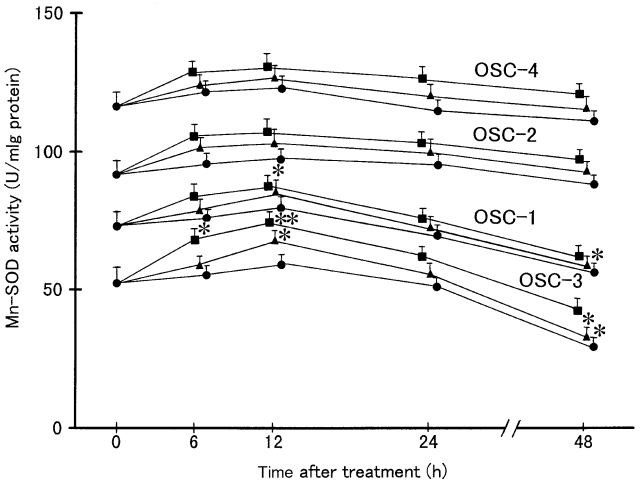


Fig. 2. Mn-SOD activity after treatment with anticancer agents. OSC-1, OSC-2, OSC-3 and OSC-4 cells were treated with 100 μg/ml 5-FU (●) or 10 μg/ml PLM (▲) for the indicated times or irradiated with 20 Gy of ^{137}Cs (■). After 6, 12, 24, and 48 h, the cells were harvested and the Mn-SOD activity was measured. All data are the mean of four experiments. * P<0.05, ** P<0.01 (vs control, by t test).

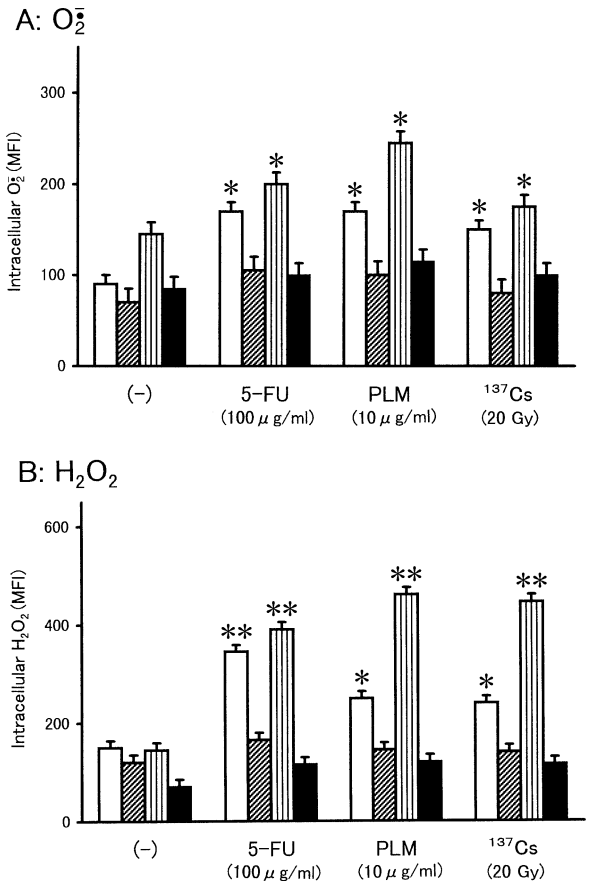


Fig. 3. ROI in SCC cells before and after treatment with anticancer agents. OSC-1 (□), OSC-2 (▨), OSC-3 (▥) and OSC-4 (■) cells were treated with 100 μg/ml 5-FU or 10 μg/ml PLM or were irradiated with 20 Gy of ^{137}Cs γ radiation. After 24 h, the cells were harvested and stained with HE (A) or DCFH-DA (B) for 15 min and then subjected to flow cytometry. Intracellular O_2^- and H_2O_2 levels are expressed as the mean fluorescence intensity of three experiments. * P<0.05, ** P<0.01 (vs time 0, by t test).

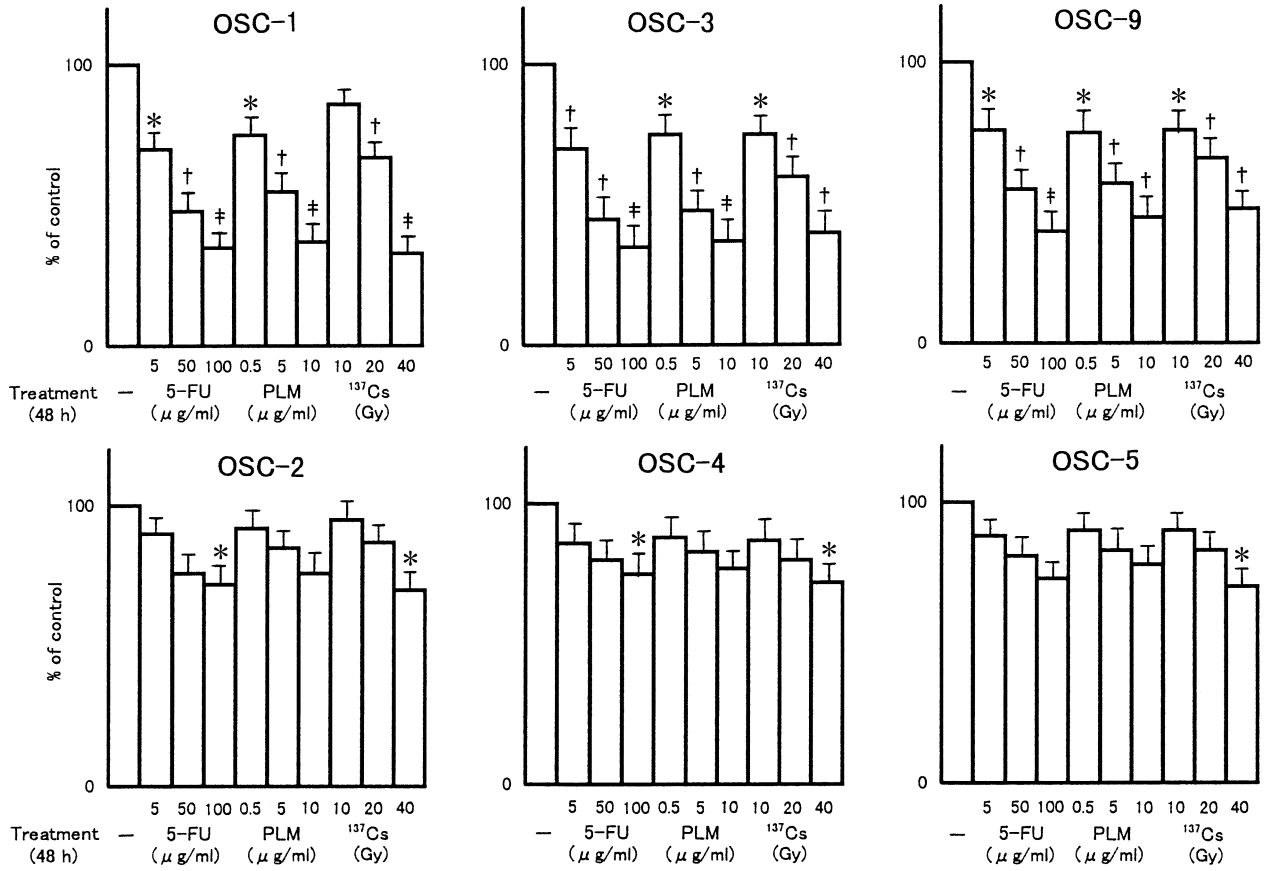


Fig. 4. Influence of 5-FU, PLM and ^{137}Cs on tumor cell viability. OSC-1, -2, -3, -4, -5 and -9 cells were pretreated with 5 to 100 μg/ml of 5-FU, 0.5 to 10 μg/ml of PLM, or 10 to 40 Gy of ^{137}Cs γ radiation. After 48-h cultivation, the cells were harvested and the cell viability was examined in terms of trypan-blue exclusion. Each column with bar is the mean±1 SD of quadruplicate determinations. * P<0.05, † P<0.01, ‡ P<0.001 (vs time 0, by t test).

RNA was loaded onto 1% agarose-formaldehyde gel for electrophoresis and bands were transferred to a nylon membrane (Nytran-plus; Schleicher & Schuell, Keene, NH). The membrane was baked at 80°C for 2 h, prehybridized at 42°C in prehybridization solution (50% formamide, 5× saline-sodium phosphate-EDTA (150 m*M* NaCl, 10 m*M* NaH_2PO_4, H_2O, and 1 m*M* EDTA), 5× Denhardt's solution, 0.1% SDS, and 100 μg/ml sonicated salmon sperm DNA), and hybridized for 72 h at 42°C. The probes were ^{32}P-labeled using the random priming method. The filters were washed at room temperature twice for 15 min with 2× SSC (150 m*M* NaCl and 15 m*M* sodium citrate), 0.1% SDS, and 0.1% NaPPi, and twice for 20 min at 65°C with 0.1× SSC, 0.1% SDS, and 0.1% NaPPi. The blots were then exposed to X-ray film at −70°C for an appropriate time. The probes used were a human Mn-SOD cDNA probe and a chicken β-actin cDNA probe (OnCor, Inc., Gaithersburg, MD).

RESULTS

SOD activities and ROI levels in OSC cell lines The activities of Cu,Zn-SOD in the nine cell lines were almost the same, at about 3×10^2 U/mg protein (Fig. 1). The Mn-SOD activity differed according to the cell line. In OSC-1, -3, -8 and -9, the Mn-SOD activity was about 5×10^1 U/mg protein. In OSC-2, -4, -5, -6 and -7 lines, it was about 1×10^2 U/mg protein. OSC-3 and OSC-4 were chosen as representative cell lines with low and high Mn-SOD activity, respectively, and used in the present examinations. When tumor cells were treated with 100 μg/ml 5-FU or 10 μg/ml PLM for the indicated times or irradiated with ^{137}Cs (20 Gy), the Mn-SOD activity in OSC-4 increased from 116 U/mg protein at time 0 to 124, 127, and 131 U/mg protein at 6 h, respectively (Fig. 2). Similarly, the Mn-SOD activity in OSC-3 was increased from the initial level of 55 U/mg protein to 61, 64 and 67 U/mg

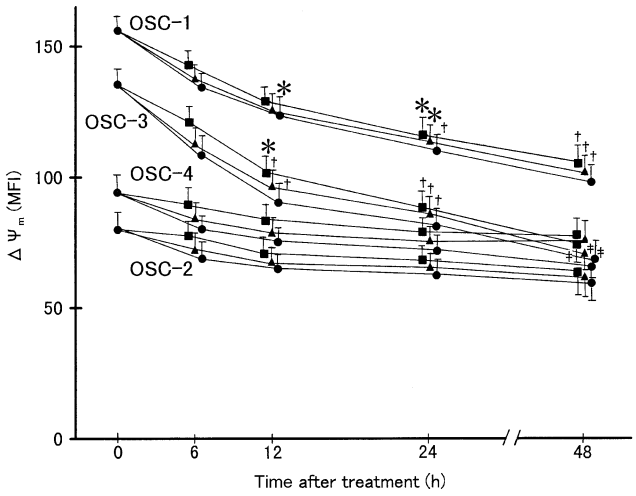
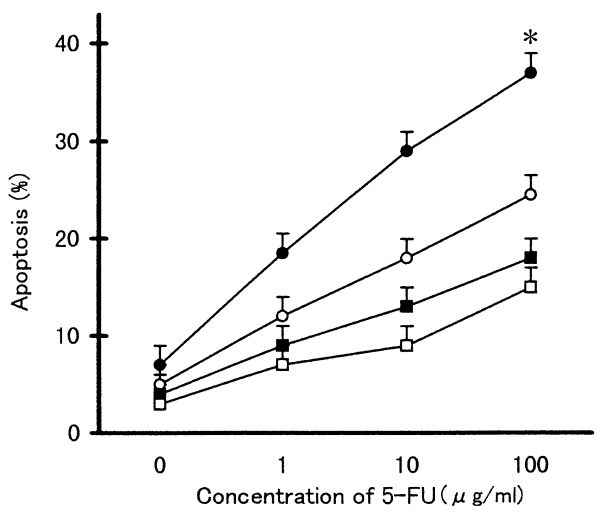
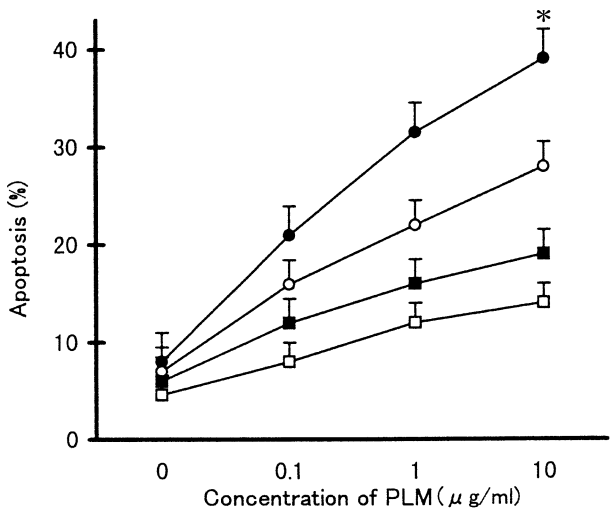


Fig. 5. Kinetics of $\Delta\psi_m$ after treatment with anticancer agents. OSC-1, OSC-2, OSC-3 and OSC-4 cells were treated with 100 μg/ml 5-FU (●) or 10 μg/ml PLM (▲) or irradiated with 20 Gy of ^{137}Cs γ radiation (■). After cultivation for the indicated times, the cells were harvested and stained with Rh123 for 15 min. Each $\Delta\psi_m$ value is the mean fluorescence intensity of four experiments. * P<0.05, † P<0.01, ‡ P<0.001 (vs control, by t test).





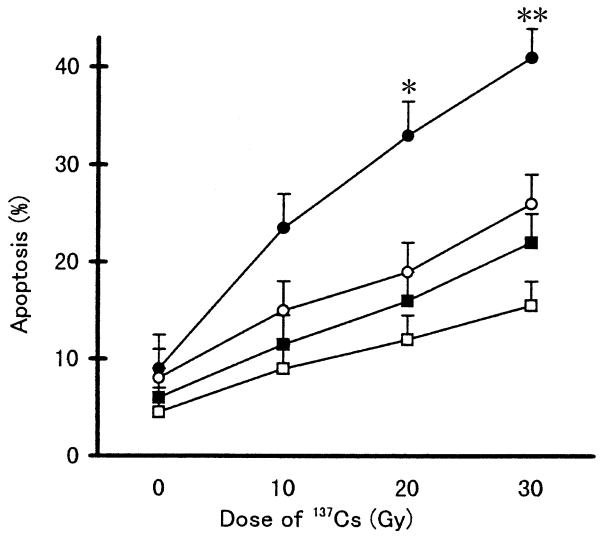


Fig. 6. Influence of inhibitors of ROI scavengers on apoptosis. OSC-3 and OSC-4 cells were treated with the indicated doses of inhibitors of ROI scavengers. After 48 h, the cells were harvested and stained with PI for 10 min. Apoptotic cells were then observed using flow cytometry, and the mean percentages of apoptotic cells were calculated from the results of four experiments. ○ OSC-3 without inhibitors of ROI scavengers, ● OSC-3 with inhibitors of ROI scavengers, □ OSC-4 without inhibitors of ROI scavengers, ■ OSC-4 with inhibitors of ROI scavengers. ROI scavengers: ATZ, 100 μM; BSO, 250 μM; DEDTC, 10 μM. * P<0.05, ** P<0.01 (vs without inhibitors, by t test).

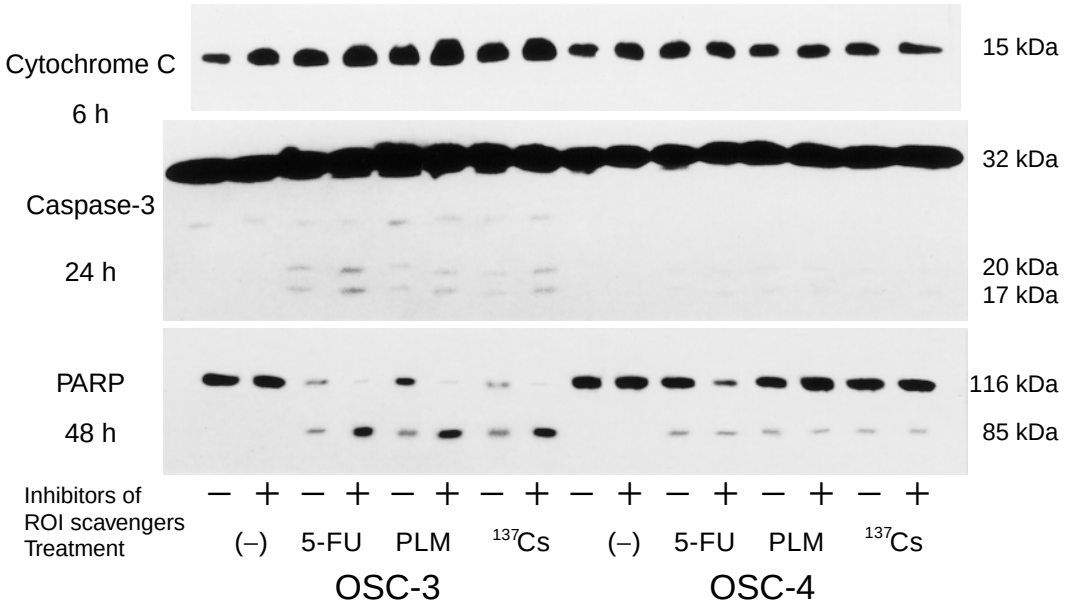


Fig. 7. Influence of 5-FU, PLM and ^{137}Cs on cytochrome *c*, caspase-3 and PARP. OSC-3 and OSC-4 cells were treated with the indicated agents for the indicated periods. Cytochrome *c* release into the cytosol and caspase-3, and PARP cleavage were determined by western blotting.

protein by 6 h treatment with 5-FU, PLM or irradiation with ^{137}Cs, respectively. The Mn-SOD activity continued to increase for 12 h and reached a plateau, then decreased gradually. Compared to OSC-2 and OSC-4, OSC-1 and OSC-3 showed a stronger decline.

Intracellular $O_2^{\bullet-}$ and H_2O_2 were increased by the anticancer agents in all examined cell lines (Fig. 3). Treatment with 100 μg/ml 5-FU caused the intracellular $O_2^{\bullet-}$ and H_2O_2 levels in OSC-3 to increase markedly from the control mean fluorescence intensities (MFI) of 137±18 and 127±17 to 227±16 and 403±14, respectively. Similarly, OSC-1 showed high $O_2^{\bullet-}$ and H_2O_2 levels after 5-FU treatment. OSC-2 and OSC-4 revealed slight increases of the MFI. The MFI of $O_2^{\bullet-}$ and H_2O_2 in OSC-4 were increased from 93±13 and 64±8 to 117±18 and 120±14, respectively, by 5-FU treatment. The increase of the MFI in OSC-2 was almost the same as that in OSC-4. After treatments with PLM and ^{137}Cs, greater increases of $O_2^{\bullet-}$ and H_2O_2 were observed, with more prominent increases in OSC-1 and OSC-3 than in OSC-2 and OSC-4.

Cell viability after treatment with anticancer agents Compared to OSC-2, -4 and -5 cell lines, OSC-1, -3 and -5 exhibited marked reductions in viability after treatment with 5-FU, PLM and ^{137}Cs, with an inverse relationship between viability and Mn-SOD activity (Fig. 4). In the cell lines with low Mn-SOD activity (OSC-1, -3 and -5), the viability was decreased to 40–50% of the control by 100 μg/ml 5-FU, 10 μg/ml PLM and 40 Gy of ^{137}Cs γ radiation. In the cell lines with high Mn-SOD activity, the decrease in viability was only 20–30%.

$\Delta\psi_m$, release of cytochrome *c* and apoptosis in OSC lines with low and high Mn-SOD activity A reduction of $\Delta\psi_m$ was observed after treatment with the anticancer agents in cell lines with low (OSC-1 and OSC-3) and high (OSC-2 and OSC-4) Mn-SOD activity (Fig. 5). However, the $\Delta\psi_m$ in OSC-1 and OSC-3 was reduced more than that in OSC-2 and OSC-4. The MFI in OSC-4 decreased slightly from 90 to 60 after treatment with 100 μg/ml 5-FU, while the MFI in OSC-3 decreased from 130 to 54.

The apoptotic cell death after treatment with 5-FU or PLM or γ-irradiation was more prominent in OSC-3 than in OSC-4 (Fig. 6). When inhibitors of ROI scavengers, DEDTC, ATZ and BSO, were individually added to the culture medium, apoptotic cell death was enhanced in both OSC-3 and OSC-4. The addition of these inhibitors strongly induced apoptosis in OSC-3. The western blotting revealed a strong release of cytochrome *c* into the cytosol and activation of caspase-3 by the anticancer agents in OSC-3 (Fig. 7). Following treatment with the anticancer agents, a decrease of the 32-kDa band and the appearance of cleaved product bands at 20 and 17 kDa were observed in both cell lines, especially in OSC-3. The cleavage of caspase-3 was enhanced by ROI scavenger inhibitors. In addition, the cleavage of PARP by the anticancer agents was more prominent in OSC-3 than in

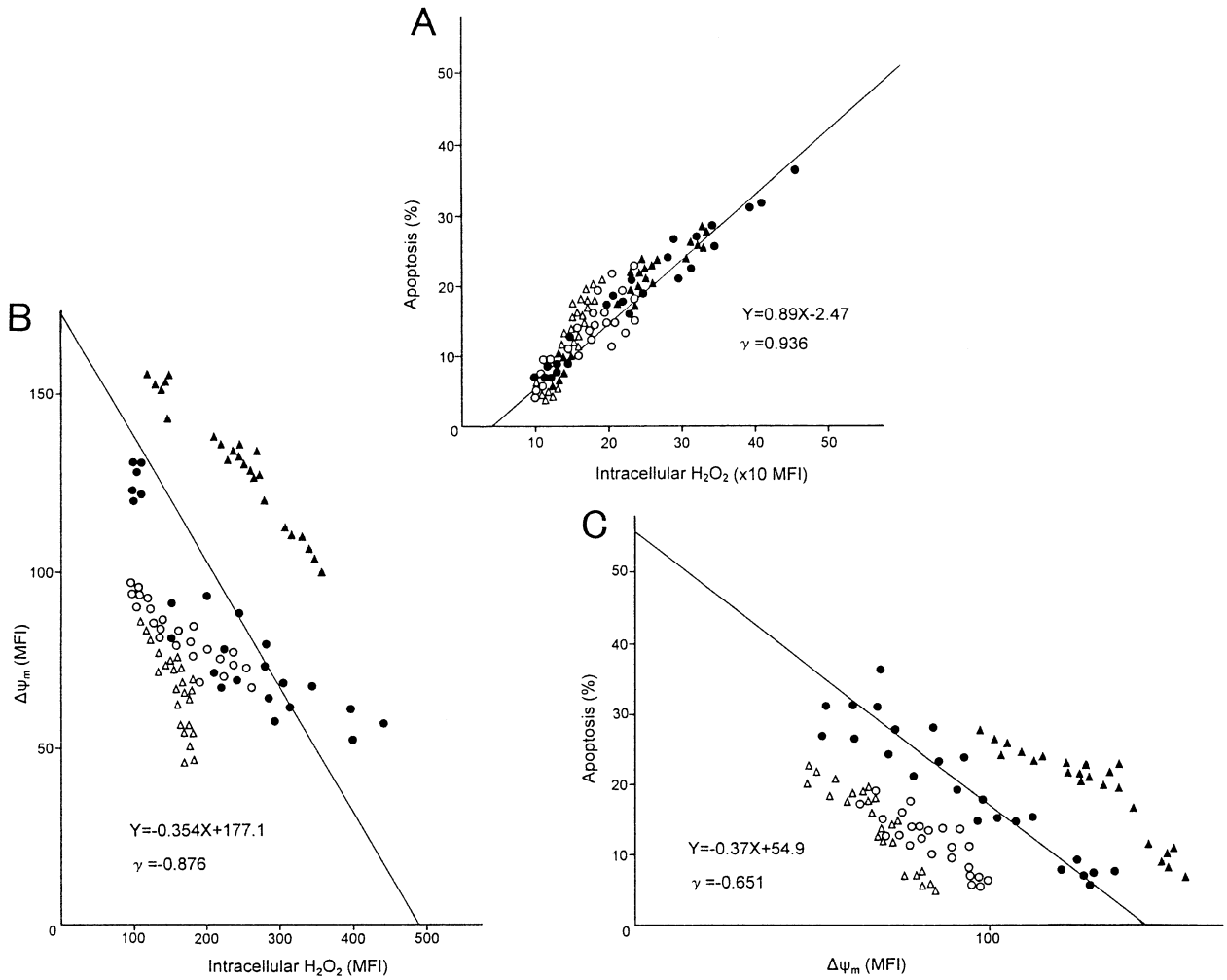


Fig. 8. Correlations among H_2O_2, $\Delta\psi_m$ and apoptosis. All plots were obtained from experiments using OSC-1 (closed triangles), OSC-2 (open triangles), OSC-3 (closed circles) and OSC-4 (open circles). A. Correlation between intracellular H_2O_2 level and apoptosis. B. Correlation between intracellular H_2O_2 level and $\Delta\psi_m$. C. Correlation between $\Delta\psi_m$ and apoptosis.

OSC-4. The ROI scavenger inhibitors upregulated the cleavage of PARP. These changes appeared to be coordinated with the levels of apoptosis in both cell lines.

Correlations among the intracellular H_2O_2 level, $\Delta\psi_m$, cytochrome *c* release and apoptosis An inverse correlation was observed between the H_2O_2 level and $\Delta\psi_m$ and between $\Delta\psi_m$ and apoptosis, with correlation coefficients of −0.876 and −0.651, respectively (Fig. 8). The level of H_2O_2 was strongly correlated with apoptosis, with a correlation coefficient of 0.936.

DISCUSSION

Many researchers have investigated the mechanisms of apoptosis induced by γ-rays, ultraviolet rays and anticancer drugs.[6–10, 37, 38] The caspase cascade and the complex signal transduction cascade have been well resolved.[50] Apoptosis-inducing signals are transduced via multiple pathways. The pathways of the signal transduction are broadly divisible into two routes. In the first, the signal is directly transduced from FADD and its adapter proteins to caspase-3.[51] In the second, a series of caspases is activated by cytochrome *c*, which is released from the mitochondria.[24] In the latter pathway, superoxide plays an important role. When certain apoptosis-inducing stimulants are added to the mitochondria, superoxide begins to be generated through the respiratory chain complexes I and III.[25] Superoxide molecules change the electric potential of the mitochondrial inner membrane to release cytochrome *c*.[52] The increased cytochrome *c* molecules then pass through the gates of the outer membrane, which are controlled by Bcl-2 family proteins, Bax and Bcl-2 and

others.[25] The released cytochrome *c* (apoptotic protease-activating factor-2; Apaf-2) cleaves and activates Apaf-1 in the presence of dATP, and the activated Apaf-1 cleaves caspase-9 (Apaf-3). Finally, caspase-3 is activated by the cleaved caspase-9.[52–54]

As noted above, superoxide triggers the cytochrome *c* release, but the exact role of ROI in apoptosis has not yet been identified. However, the involvement of ROI in apoptosis is illustrated by the present finding that antioxidants inhibited the induction of apoptosis. ROI exhibit multiple biological activities; they change the $\Delta\psi_m$ and intracellular calcium ($[Ca^{2+}]_i$) level, induce protein-tyrosine phosphorylation and activate transcription factors by changing the redox status.[30–32] It was reported that in some cell lines, a low level of ROI which does not impair the cells activated NF-κB and AP-1.[55] Therefore, the role of ROI in apoptosis appears to be important.

The activity levels of Cu,Zn-SOD in the 9 cell lines were not very different, whereas the Mn-SOD activities were quite different among the cell lines. In view of the report that leukemic cell lines had greatly different Mn-SOD activities, differences in Mn-SOD activities appears to be ubiquitous.[44] In all cell lines, the Mn-SOD activities were increased by treatment with 5-FU, PLM and radiation during a 12-h period after the treatment. Interestingly, the degree of increase was nearly the same in all cell lines. However, the Mn-SOD activity in each cell line then decreased, and the decrease was more prominent in OSC-1 and OSC-3, which originally possessed lower activity levels than OSC-2 and OSC-4. As expected, the ROI levels after treatment with 5-FU, PLM and radiation were high in OSC-1 and OSC-3. We estimated the glutathione peroxidase activity in each SCC cell line, but no difference in the activity was observed (data not shown). Therefore, the ROI levels appear not to be regulated by glutathione peroxidase, though a role of the enzyme in H_2O_2 generation in tumor necrosis factor-α-treated breast cancer cells was reported.[56]

ROI decrease the $\Delta\psi_m$ and impair the membranes by inhibiting the transfer of electrons among the complexes I, III, IV and flavoprotein, which support the $\Delta\psi_m$.[25] Therefore, we expected that the $\Delta\psi_m$ in OSC-1 and OSC-3 with low Mn-SOD activity would be more decreased by treatment with 5-FU, PLM and radiation than that in OSC-2 and OSC-4 with high Mn-SOD activity, because more ROI would be generated in the former cell line. The results confirmed this idea, revealing high intracellular H_2O_2 and a marked decrease of $\Delta\psi_m$ in OSC-1 and OSC-3. As is well known, decrease of $\Delta\psi_m$ is inhibited by Bcl-2, and Bax enhances the reduction of $\Delta\psi_m$.[25, 57] We therefore speculate that there is an association between ROI and the role of these Bcl-2 family proteins in the cytochrome *c*-releasing mechanism. However, as we reported previously, Bcl-2 was scarcely expressed in these SCC cell lines, and the expression of both proteins was not increased after treatment with 5-FU, PLM or radiation.[58]

The release of cytochrome *c* is not essential for apoptosis, and cytochrome *c*-independent apoptosis has been reported in some cell lines.[47, 59] The dependency on cytochrome *c* seems to differ according to the apoptosis-inducing stimulus. In the cell lines examined here, 5-FU and PLM as well as radiation induced the release of cytochrome *c*, and apoptosis was highly induced by these anticancer agents in OSC-3, associated with a prominent cytochrome *c* release and a strong activation of caspase-3. In addition, a cytochrome *c* release inhibitor, cyclosporin A, nearly completely inhibited the induction of apoptosis, as reported by others.[60] Therefore, the release of cytochrome *c* in SCC cells appears to be an essential event in the signal transduction induced by 5-FU, PLM and radiation.

The intracellular H_2O_2 levels, $\Delta\psi_m$ and apoptosis were correlated with each other. The relationship reveals that an increase in the H_2O_2 level in the mitochondria induces a decrease of $\Delta\psi_m$, and an impairment of the membranes occurs, which is followed by apoptosis via the cleavage of caspase-3 and PARP. However, the pathway of the apoptosis-inducing signals appears to be complex. In fact, there is a report that caspase-8 stimulates cytochrome *c* release.[61] More detailed examinations, including a study of the influence of ROI on CAD and inhibitors of CAD, are required to elucidate the role of ROI in apoptosis.

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