

Role of Reactive Oxygen Species in Cells Overexpressing Manganese Superoxide Dismutase: Mechanism for Induction of Radioresistance

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Abstract

Manganese superoxide dismutase (MnSOD) catalyzes the dismutation of superoxide anions (O_2^-) into hydrogen peroxide (H_2O_2). We altered the intracellular status of reactive oxygen species by introducing human MnSOD cDNA into the human ovarian cancer cell line SK-OV-3. The overexpression of MnSOD inhibited cell growth and induced a concomitant increase in the level of H_2O_2 in SK-OV-3 cells. The cells overexpressing MnSOD were more resistant to irradiation than parental cells. MnSOD overexpression shortened the G_2 -M duration in irradiated cells. Either inhibition of p38 mitogen-activated protein kinase (p38MAPK) or scavenging free radicals blocked the induction of radioresistance by MnSOD and also abolished the shortening of the G_2 -M duration with concomitant inhibition of p38MAPK phosphorylation. Irradiation increased the generation of H_2O_2 even more in these transfectants. These results suggest that the accumulated H_2O_2 potentiated the activation of p38MAPK after irradiation in cells overexpressing MnSOD, which led to the protection of cells from irradiation-mediated cell death through the G_2 -M checkpoint. SK-OV-3 cells had no constitutive expression of p53, and the overexpression of MnSOD and/or irradiation did not induce p53 or p21^{WAF1}, which causes cell cycle arrest. Thus, our results suggest that MnSOD alters the cell cycle progression of irradiated cells independently of p53 and p21^{WAF1}.

Introduction

Irradiation induces cells to synthesize or activate proteins including the immediate early genes, *c-fos*, and *egr-1*, and NF- κ B and cytokines such as tumor necrosis factor (TNF) and granulocyte macrophage colony-stimulating factor (GM-CSF) (1–3). The cellular responses to ionizing radiation also include activation of cell cycle checkpoints that delay the progression of cell growth (4). Irradiation activates checkpoints at the

transition from the G1 to S phase, in the S phase, and at the transition from the G2 phase to mitosis (G_2 -M) (4). On the other hand, irradiation also induces cell death or apoptosis. However, the mechanisms by which irradiation either activates the checkpoints of surviving cells or induces apoptosis are not clear, although the generation of reactive oxygen species (ROS) seems to be a key factor. Recent studies have shown that irradiation induces the activation of signaling pathways including that of the mitogen-activated protein kinase (MAPK) family (5). Irradiation activates one of the MAPK family members known as a stress-activated protein kinase or c-Jun NH₂-terminal kinase [stress-activated protein kinase (SAPK)/JNK] as well as p38MAPK (6). p38MAPK, a prototypic MAPK member (also known as p38 α), was found to be tyrosine phosphorylated in macrophages on treatment with lipopolysaccharide (7). This protein was also identified as a specific target of a series of anti-inflammatory compounds (8). The p38MAPK pathway is thought to be a primary signaling pathway for cell death that is activated by stressful events such as irradiation (5). The cell cycle is regulated by cyclin-dependent kinases specific for each phase, where they become activated (9). Studies have shown that MAPK is also involved in the cell cycle and is regulated by the phosphorylation of many signaling molecules (5). At G_2 -M transition, one of the regulatory mechanisms is phosphorylation of the retinoblastoma protein (Rb), which regulates the activation of E2F, and another one is the activation of MAPK pathways (10). The p38MAPK pathway is known to be activated at the G_2 -M phase of the cell cycle by DNA damage (5). However, the precise mechanisms that underlie the potentiation of p38MAPK activation in the G_2 -M phase by radiation are not clear.

ROS such as superoxide anions (O_2^-), hydroxyl radicals (OH^\cdot), and hydrogen peroxide (H_2O_2) are physiologically generated as a consequence of aerobic respiration and substrate oxidation in aerobic organisms; the generation is increased in response to external stimuli including irradiation. However, inadequate accumulation of ROS results in oxidative stress that leads to damage to biological macromolecules; the products of lipid peroxidation can cause DNA damage (11). Oxidative stress is often initiated by the overproduction of O_2^- , which is converted to potent oxidants such as OH^\cdot , while it is also dismutated to relatively less reactive H_2O_2 . The formation of O_2^- is also enhanced on pathological activation of enzymatic

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systems such as xanthine and xanthine oxidase. Against the inadequate accumulation of ROS, there are several nonenzymatic and enzymatic antioxidant systems; intracellular homeostasis of ROS is kept by a balance between the generation of ROS and the intracellular activity of the antioxidant system. Superoxide dismutase (SOD, EC 1.15.1.1) catalyzes the dismutation of O_2^- into O_2 and H_2O_2 , and the peroxide can be destroyed by catalase (CAT, EC 1.11.1.6) or glutathione peroxidase (GSH-Px, EC 1.11.1.9) (12, 13). On the other hand, recent studies have shown that ROS are important signaling molecules; ROS modulate Ca^{2+} signaling and protein phosphorylation events, and function as regulators for various biological processes, inducing gene expression, cell growth, differentiation, and apoptosis (12).

The respiratory chain in mitochondria is a major source of oxygen radicals, and manganese superoxide dismutase (MnSOD) is located at the mitochondria and exists as a homotetramer (12). In mammalian cells, there are three types of SOD: cytosolic copper-zinc SOD (CuZnSOD), mitochondrial MnSOD, and extracellular SOD (ECSOD). Previous studies have shown that the lack of MnSOD gene in *Escherichia coli* and yeast leads to hypersensitivity to oxidative stress (14). Homozygous mutant mice lacking MnSOD died within the first 10 days after birth and showed dilated cardiomyopathy, accumulation of lipid in liver and skeletal muscle, and metabolic acidosis (15). Furthermore, mice lacking MnSOD showed degenerative injury of the central nervous system, particularly in the basal ganglia and brain stem associated with damaged mitochondria (16). On the other hand, studies found that transfection of MnSOD cDNA rendered the cells resistant to tumor necrosis factor-, irradiation-, or Adriamycin-mediated cytotoxicity and irradiation-induced neoplastic transformation (17). Moreover, it has been shown that MnSOD-transgenic mice were resistant against oxygen-induced injury and toxicity as compared to the control mice (18). Previously, we showed that irradiation increased the activity of MnSOD in various types of cells (19, 20). Increased levels of MnSOD lead to the accumulation of H_2O_2 if the H_2O_2 cannot be converted, in turn, by increased activity of CAT or GSH-Px. On the other hand, overexpression of MnSOD has been reported to inhibit cell growth with a concomitant accumulation of ROS (21). Further studies found that tumor cells contain elevated levels of H_2O_2 and that the Ras protein induces cellular pathways leading to the production of O_2^- (22, 23). These studies suggest that the intracellular redox status may be one of the important regulators in cell growth, because it is influenced by exposure to external stresses such as oxidative damage. However, the role of ROS in cells is not clear except for their defense against microorganisms in phagocytes, and also the mechanism for rendering the cells overexpressing MnSOD resistant to external stresses remains unresolved.

In the present study, we modulated the cellular redox status by the introduction of human MnSOD cDNA into human ovarian cancer cells, SK-OV-3, and investigated the mechanisms of the growth of suppression and the altered sensitivity to irradiation in these cells. We found that the accumulated H_2O_2 potentiated the activation of p38MAPK after irradiation in cells overexpressing MnSOD, which led to the protection of cells from irradiation-mediated cell death through the G2 checkpoint.

Results

Levels and Activities of MnSOD in SK-OV-3 Cells Overexpressing MnSOD

To investigate the role of ROS in cell growth and the cell cycle, SK-OV-3 ovarian cancer cells were transfected with a pCR3.1/Neo construct containing a full-length human MnSOD cDNA. As control, cells were transfected with a pCR3.1/Neo vector. We obtained several clones with different levels and activities of MnSOD. Fig. 1 shows two of these clones. Western blot analysis showed that the clones s10 and s20 had almost 4- and 5-fold higher levels of the MnSOD protein, respectively, as compared to that of the control (Fig. 1A). We also studied the MnSOD activity on these cells by nondenatured polyacrylamide gel fractionation of the lysate followed by nitro blue tetrazolium (NBT) staining (Fig. 1B). As reported previously, eukaryotes have two types of SOD in cells; the bands of high molecular mass are MnSOD and the very faint bands at low molecular mass correspond to CuZnSOD (12). The results obtained were almost identical to those described above. There were no differences in the levels of CuZnSOD in the MnSOD-transfected cells and in the control transfected cells (Fig. 1B). The levels of CAT and GSH-Px were not altered in these cells (data not shown), an observation consistent with previous studies (21). We used the s20 clone of MnSOD transfectants from this experiment in the present study.

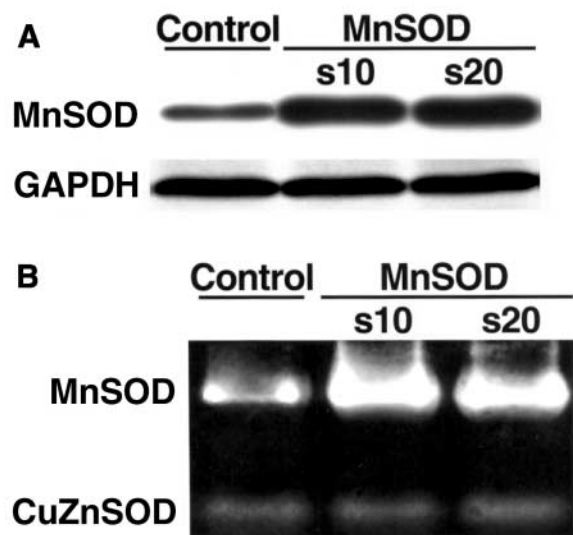


FIGURE 1. Levels and activities of MnSOD in the SK-OV-3 cells overexpressing MnSOD. SK-OV-3 cells were transfected with 2 μ g of either control vector pCR3.1 or an expression vector of MnSOD. Stable transfectants were obtained by G418 selection (0.7 mg/ml). s10 and s20 cells were stable transfectants of MnSOD. **A.** Western blot analysis of SK-OV-3 cells transfected with expression vector of MnSOD. The cells were harvested and 50 μ g samples of whole cell protein were subjected to SDS-PAGE (12%) and transferred to polyvinylidene difluoride (PVDF) membranes. Levels of MnSOD protein were determined using an anti-human MnSOD antibody. Glyceraldehyde-3-phosphate dehydrogenase was used for loading control. **B.** Activities of SOD in cells overexpressing MnSOD. Cells were sonicated in ice-cold potassium phosphate buffer and 50 μ g samples of protein were electrophoresed in nondenaturing 12% polyacrylamide gel, and then stained with NBT.

Effect of MnSOD Overexpression on Growth of SK-OV-3 Cells

To study the effect of MnSOD overexpression on cell growth, cells were synchronized for 48 h in medium without FCS, and 1×10^5 cells of each cell line were seeded in 60-mm tissue culture plates and cultured in the presence of FCS for 9 days. Cells were trypsinized at each point. The numbers of cells were counted and the viability was determined by trypan blue exclusion method. The control cells grew in a time-dependent manner and the cell number reached a plateau by day 7; the cell number increased to $4.7 \pm 0.1 \times 10^6$ cells/plate (Fig. 2; also see Table 1). As for the MnSOD transfectants, they grew in a similar pattern until day 3, after which their growth rate became significantly reduced; their number was 65% of the control cells on day 5 and leveled off after day 6. Moreover, the maximum number of MnSOD transfectants was less than 40% of that of the parental SK-OV-3 cells.

Effect of Irradiation on Growth of Cells Overexpressing MnSOD

We determined whether MnSOD overexpression affected cell growth in irradiated SK-OV-3 cells. To study their growth after irradiation, 1×10^5 cells of each cell line were seeded in 60-mm tissue culture plates. Cells were synchronized for 48 h in medium without FCS. After incubating in FCS-containing medium for 2 h, cells were irradiated with 5 Gy of X-ray and cultured in the presence of FCS for 7 days. Cells were harvested at various points; the cell numbers were counted and the cell viability was determined by trypan blue exclusion method (Table 1). Irradiation markedly inhibited the growth rate of both cell lines. In the control cells, irradiation reduced the cell numbers by almost 75% and 85% on days 3 and 5, respectively. In MnSOD transfectants, on the other hand, irradiation reduced the cell numbers by 55% and 57%. The growth of both cell lines followed a similar pattern after irradiation, with the cell number reaching a plateau on day 6. However, there were significant numerical differences between the two cell lines because at each point, the number of stable transfectants with MnSOD was significantly greater than that of the control cells.

To examine the sensitivity of the MnSOD transfectants to radiation, we also determined the effect of irradiation on clonal cell growth. Irradiation at 2 and 5 Gy inhibited colony formation by 37% and 86%, respectively, in parental control cells (Fig. 3). In contrast, almost 89% and 42% of the cells overexpressing MnSOD survived 2 and 5 Gy of irradiation.

Cell Cycle Analysis and p53/p21^{WAF1} Expression After Irradiation in MnSOD Transfectants

We next determined whether the overexpression of MnSOD altered the cell cycle distribution in irradiated SK-OV-3 cells. Cells were synchronized by serum depletion for 48 h as described above, and the medium was then replaced by FCS-containing medium. After being fixed in 70% ethanol at various time points after irradiation, the cells were stained with propidium iodide and cell cycle progression was determined by flow cytometry. When the control transfectants were released from the block because of serum deprivation, the cell cycle

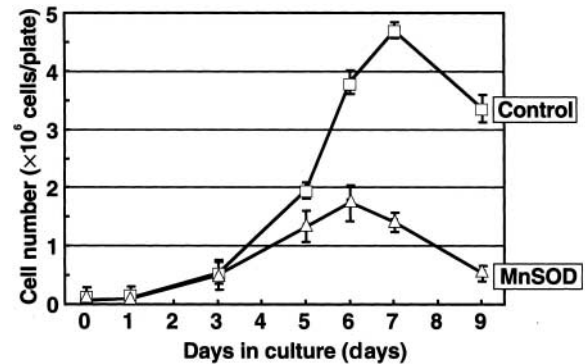


FIGURE 2. Effect of MnSOD overexpression on growth of SK-OV-3 cells. Cells (1×10^5) were seeded in 60-mm tissue culture plates and cultured for 48 h without FCS. Then, cells were cultured in medium containing 7% FCS for indicated durations. Cells were harvested and counted by trypan blue exclusion method. The graph shows mean values in triplicates. Day 5, $P < 0.005$; Day 6, $P < 0.001$; Day 7, $P < 0.001$.

progressed together with an increase in the number of cells in the S phase by 18 h (Table 2). Thereafter, the cells gradually re-entered the G₁ phase. Cell cycle progression of the MnSOD transfectants was similar to that of the control cells. After irradiation with 5 Gy, 74% of the control cells entered the G₂-M phase by 18 h as expected, and 62% of the cells still remained at the G₂-M phase 24 h after irradiation. The cell cycle profiles of MnSOD-overexpressing cells postirradiation were similar to those of the control cells until 6 h (Table 2). However, MnSOD-overexpressing cells cycled more rapidly after irradiation and left the G₂-M phase earlier than the control cells; 65% of cells had progressed into the G₁ phase by 24 h, whereas 62% of the control cells remained at the G₂-M phase.

SK-OV-3 cells lack the p53 protein (24). We confirmed that these cells did not have the constitutive expression of p53 and that irradiation and/or overexpression of MnSOD did not induce the p53 expression (Fig. 4). Furthermore, irradiation failed to induce the p21^{WAF1} expression in both cell lines.

Inhibition of p38MAPK Increased Radiation Sensitivity in MnSOD Transfectants After Irradiation

Irradiation activates the p38MAPK-signaling pathway to a level similar to that observed by physiological stimulation (5). To determine whether the p38MAPK signaling pathway is involved in cell growth and survival of the SK-OV-3 cells overexpressing MnSOD after irradiation, we used a specific inhibitor of p38MAPK, SB203580. This compound has high specificity for most p38MAPK isozymes (25). Cells were seeded in 60-mm tissue culture plates and cultured without FCS for 48 h. They were then cultured with the FCS-containing medium for 1 h, and incubated with the inhibitor for 1 h followed by 5 Gy of irradiation. The numbers of colonies were counted 2 weeks later. Colony-forming assays showed that treatment with 10 μ M of SB203580 increased the radiation sensitivity of MnSOD transfectants, reducing the colony-forming capacity by 50%, whereas incubation with the compound did not affect the colony-forming capacity in the irradiated control cells (Fig. 5). Furthermore, pretreatment with a ROS scavenger, NAC, also significantly reduced the ability of

Table 1. Cell Growth of SK-OV-3 Cells Overexpressing MnSOD

	Untreated		Irradiated	
	Control	MnSOD	Control	MnSOD
Day 0	1 × 10 ⁵ cells/plate			
Day 1	1.0 ± 0.5	1.0 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
Day 3	5.5 ± 0.5 ^a	5.1 ± 0.3 ^a	1.4 ± 0.2 ^b	2.3 ± 0.1 ^b
Day 5	19.5 ± 0.4 ^c	13.3 ± 0.9 ^c	3.5 ± 0.4 ^d	5.7 ± 0.6 ^d
Day 6	38.2 ± 1.3 ^e	17.5 ± 2.0 ^e	5.6 ± 0.4 ^f	10.1 ± 0.9 ^f
Day 7	47.0 ± 0.9 ^g	14.1 ± 1.1 ^g	5.3 ± 0.7 ^h	9.5 ± 1.2 ^h

Note: Cells (1 × 10⁵) were plated and cultured for 48 h without FCS. Then, cells were cultured in medium with FCS for 2 h and irradiated with 5 Gy of X-ray. Cells were collected on indicated days by trypsinization and counted. Results represent the mean and SE of triplicate cultures. ^a*P* > 0.1; ^b*P* < 0.01; ^c*P* < 0.005; ^d*P* < 0.01; ^e*P* < 0.001; ^f*P* < 0.005; ^g*P* < 0.001; ^h*P* < 0.05.

colony formation in cells overexpressing MnSOD (*P* < 0.01). In contrast, pretreatment with 10 μM of GF109203X, a selective inhibitor of PKC, had no significant effect on the number of colonies in either of these cell lines. No effect of any compound on the ability of colony formation was observed in untreated cells (data not shown).

Effects of p38MAPK Inhibition on Cell Cycle Progression in Irradiated Cells Overexpressing MnSOD

To examine whether the activation of p38MAPK also affects the cell cycle progression of MnSOD-overexpressing cells in irradiation, we treated the cells with either SB203580 or NAC. Cells were synchronized in the G1 phase by serum depletion for 48 h and then cultured in medium with FCS for 1 h. Cells were preincubated with either of these compounds for 1 h and then harvested 24 h postirradiation (Table 3). Treatment with the compound alone did not affect cell cycle progression in either cell line. Treatment with SB203580 had almost no effect on cell cycle progression in the irradiated control cells. However, treatment with 10 μM of SB203580 delayed cell cycle progression of SK-OV-3 cells overexpressing MnSOD after irradiation; the earlier exit from the G2 phase was blocked by pretreatment with the p38MAPK inhibitor, and these cells had almost the same cell cycle profile as the parental control cells. Furthermore, pretreatment with NAC effectively inhibited this earlier exit from the G2 phase in MnSOD transfectants, whereas the treatment had no influence on cell cycle progression in the control cells.

We also studied the phosphorylation of p38MAPK followed by irradiation. Western blot analysis using an antibody that recognizes the phosphorylated form of p38MAPK showed that activation of p38MAPK was evident from 24 h after exposure to radiation in MnSOD transfectants, and the level of the phosphorylated p38MAPK was slightly higher in irradiated control cells than in untreated cells (Fig. 6). These results were in agreement with previous findings by other investigators (5, 26). Treatment with either SB203580 or NAC inhibited the phosphorylation of p38MAPK after irradiation in both cell lines. However, the levels of p38MAPK were not altered by SB203580 or NAC.

Enhanced Production of ROS in Stable Transfectants With MnSOD

MnSOD scavenges O₂⁻ to H₂O₂ (5). We compared the rate of H₂O₂ production in control cells and cells overexpressing MnSOD. The production of ROS was assessed using the fluorescent probe specific for H₂O₂, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), at a concentration of 5 μM and the fluorescent intensity was evaluated by flow-cytometric analysis (27). Treatment of cells with 0.02 mM of H₂O₂ caused a marked increase in 2',7'-dichlorofluorescein (DCF) fluorescence after 15 min (positive control of the assay, Fig. 7A). To compare the formation of intracellular ROS transfectants at a steady state in control cells and MnSOD transfectants, the DCF-fluorescent intensities of both cell lines were measured (Table 4). We repeated these experiments three times; each experiment showed that the generation of ROS at a steady state was increased in MnSOD transfectants compared to that in control cells. Furthermore, treatment of control cells with nonspecific radical scavenger NAC reduced the mean fluorescent intensity by 7% (Fig. 7B). In contrast, NAC attenuated the intensity by 13% in MnSOD transfectants. Moreover, treatment with the radical scavenger specific for H₂O₂, CAT, reduced ROS production in the control cells and the cells overexpressing MnSOD by 3% and 31%, respectively. Thus, the reducing rates of the mean fluorescent intensities by radical scavengers were larger in MnSOD transfectants. These results suggest that the generation of ROS at a steady state is relatively increased in MnSOD transfectants than in control cells and that the ROS responsible for MnSOD overexpression is likely H₂O₂.

To further study the effect of ionizing irradiation on the generation of H₂O₂ in MnSOD-overexpressing cells, cells were irradiated with 5 Gy of X-ray. After 15 min, cells were stained with DCFH-DA. Irradiation increased the H₂O₂ production in both cell lines; exposure of cells to irradiation increased the fluorescent intensities of the control and MnSOD-transfected

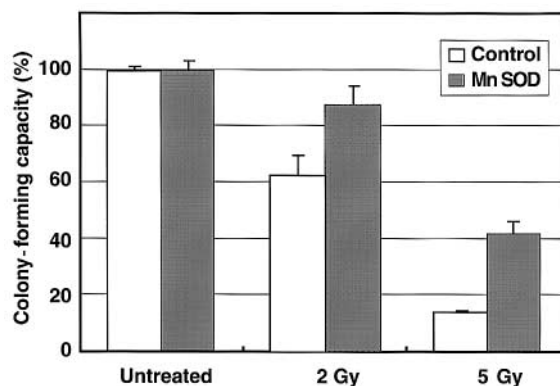


FIGURE 3. Effect of irradiation on colony-forming capacity of MnSOD transfectants. One hundred cells each of control and 2 Gy-irradiated growth, and 200 cells of 5 Gy-irradiated growth, were plated and cultured for 48 h without FCS. Then, cells were cultured in medium with FCS for 2 h and irradiated. The numbers of colonies were counted 2 weeks after irradiation. Experiments were triplicated and results were expressed as percentage colony-forming capacity of untreated cells. Plating efficiencies of untreated control cells and MnSOD transfectants were 82% and 76%, respectively. 2Gy, *P* < 0.05; 5 Gy, *P* < 0.01.

Table 2. Cell Cycle Analysis in MnSOD Stable Transfectants After Irradiation

		Distribution of Cells in Each Stage (%)						
		Stage	0 h	6 h	12 h	18 h	24 h	30 h
Control	G1		66.1	62.6	58.1	52.7	63.0	60.8
	S		16.9	17.8	18.6	21.8	16.7	18.3
	(G ₁ -S ratio)		3.9	3.5	3.0	2.4	3.8	3.3
	G ₂ -M		17.0	19.6	23.3	25.5	20.3	20.9
MnSOD	G1		73.0	72.0	66.6	53.0	58.9	64.6
	S		12.7	12.7	18.0	24.1	14.5	15.1
	(G ₁ -S ratio)		5.7	5.7	3.7	2.2	4.1	4.3
	G ₂ -M		14.3	15.3	15.4	22.9	26.6	20.3
Control + IR	G1		67.1	50.2	20.9	20.8	32.7	67.3
	S		8.2	23.3	35.6	5.4	5.2	5.6
	(G ₁ -S ratio)		8.2	2.2	0.6	3.9	6.3	12.0
	G ₂ -M		24.7	26.5	43.5	73.8	62.1	27.1
MnSOD + IR	G1		70.2	59.6	25.1	30.3	64.8	49.9
	S		7.1	19.6	28.2	5.9	5.5	18.2
	(G ₁ -S ratio)		9.9	3.0	0.9	5.1	11.8	2.7
	G ₂ -M		22.7	20.8	46.7	63.8	29.7	31.9

Note: Cells were plated and cultured for 48 h without FCS. After culturing in FCS-containing medium for 2 h, cells were irradiated with 5 Gy of X-ray (IR) and harvested at indicated points. Cells were collected and fixed with 70% ethanol. Then, cells were treated with RNase A, and their DNA content was examined by propidium iodide staining. The cells were analyzed by FACScaliber using Cell Quest and Modifit programs.

cells by 13% and 22%, respectively (Fig. 7C). The specificity of ROS was also determined by irradiation in the presence of CAT in cells overexpressing MnSOD (Fig. 7C). The treatment with CAT abolished the irradiation-induced increase in the fluorescence.

Effect of H₂O₂ on Activation of p38MAPK in SK-OV-3 Cells

Our findings in the present study strongly suggest that the potentiation of p38MAPK activation in MnSOD transfectants is dependent on ROS production. To determine if H₂O₂ affects the phosphorylation of p38MAPK in SK-OV-3 cells, cells were cultured for 10 min with different concentrations of H₂O₂. The levels of phosphorylated p38MAPK were determined by Western blot analysis using a phosphorylated p38MAPK specific antibody (Fig. 8). An increased level of phosphorylated p38MAPK was observed at a concentration of 0.02 mM of H₂O₂ in control cells, and the phosphorylation of p38MAPK occurred almost in a dose-dependent manner up to 0.1 mM.

Discussion

ROS are ubiquitously generated mainly by oxidative phosphorylation at mitochondria and released during ATP synthesis (28). On the other hand, exposure to oxidative stress such as ionizing radiation increases the production of ROS, leading to cell damage (29). Recent studies have shown that low levels of ROS can modify cell-signaling proteins important for cell growth and survival (30). Identifying the target proteins for redox modification is key to understanding how signals are transduced in cells. In the present study, we found that alteration of the intracellular redox status by MnSOD overex-

pression affected cell growth and induced the resistance to irradiation through regulation of checkpoints of the cell cycle. We also showed that the resistance to irradiation in cells overexpressing MnSOD is produced by the potentiation of the p38MAPK pathway by ROS generation.

Most of the cellular O₂⁻ is constitutively generated from the mitochondrial electron transport chain and is detoxified by SODs, which convert O₂⁻ to H₂O₂ (31). H₂O₂ is also produced by a variety of intracellular reactions and is normally present in cells at a concentration of as low as approximately 1.0 × 10⁻⁸ M (32). H₂O₂ plays an important role in the normal metabolism such as thyroid hormone biosynthesis and the microbiocidal activity of macrophages (33, 34). However, little is known regarding the other functions of ROS in nonphagocytes. In the present study, the overexpression of MnSOD inhibited the cell growth of SK-OV-3 cells. These results were consistent with previous reports concerning other types of cells (21). Further study showed that H₂O₂ production at a steady state was larger in the MnSOD-overexpressing SK-OV-3 cells than in parental SK-OV-3 cells, and exogenously added H₂O₂ caused growth retardation of these cells (data not shown), indicating that the production of H₂O₂ can modify the regulation of cell growth. On the other hand, overexpression of MnSOD reduced the sensitivity to irradiation in these cells. However, treatment of cells with the radical scavenger NAC abolished the radioresistance induced by MnSOD. Irradiation enhances the generation of O₂⁻ and induces the expression of MnSOD in various types of cells (19, 20, 35), and MnSOD converts O₂⁻ to H₂O₂ (12). In our study, H₂O₂ production was enhanced more in the MnSOD-overexpressing cells than in the parental SK-OV-3 cells following irradiation. ROS have been shown to be involved in diverse aspects of stress response, including the induction of cell death (36), and H₂O₂ by itself is relatively nonreactive toward DNA. Our results suggest that the overexpression of MnSOD affects cell growth and changes the cellular sensitivity to irradiation probably through production of H₂O₂.

External stress such as irradiation induces a complex response manifested by perturbation of the cell cycle. ROS, including H₂O₂, have been demonstrated to induce G1 and G2 checkpoint responses in eukaryotic cells (37). To study the mechanism for the radioresistance in cells overexpressing

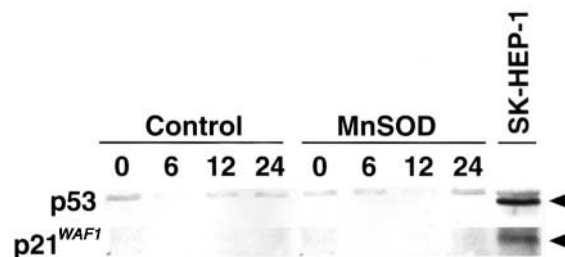


FIGURE 4. Effect of irradiation on p53 and p21^{WAF1} expression in SK-OV-3 cells overexpressed with MnSOD. Cells were cultured for 48 h without FCS, and then FCS was added. After 2 h, cells were irradiated with 5 Gy. Whole cell lysates (50 μg/lane) were prepared at each time, electrophoresed on 12% SDS-polyacrylamide gel and transferred to PVDF membrane. Western blot analysis was performed using anti-p53 and anti-p21^{WAF1} antibodies. As positive control, the hepatoma cell line SK-HEP-1 was used (last lane).

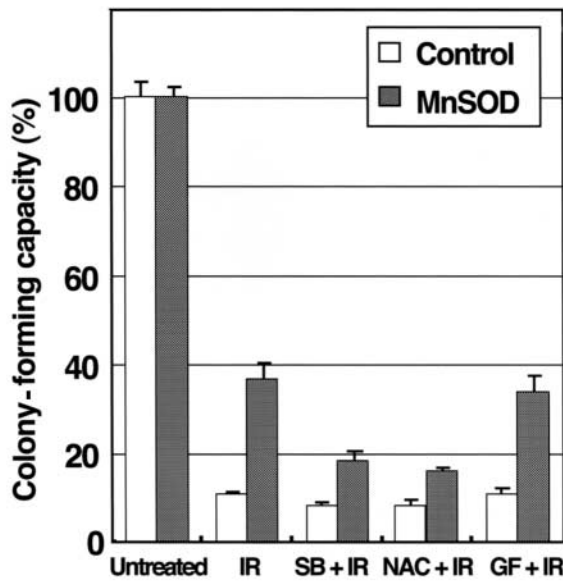


FIGURE 5. Inhibition of p38MAPK or scavenging ROS abolishes induction of radiosensitivity by MnSOD. Two hundred cells were cultured for 48 h without FCS. After culturing in FCS-containing medium for 1 h, cells were incubated with either 10 μM of SB203580 (SB, a p38MAPK inhibitor), 25 mM of *N*-acetyl-L-cysteine (NAC, a ROS scavenger), or 100 μM of GF109203X (GF, an inhibitor of PKC) for 1 h, and then irradiated with 5 Gy of X-ray. The numbers of colonies were counted after culturing for 2 weeks. The results were the mean of triplicated experiments and expressed as percentage colony-forming capacity of untreated cells.

MnSOD, we studied the cell cycle progression after irradiation. In response to DNA damage including irradiation, the p53 tumor suppressor, which is a universal sensor of genotoxic stress, is phosphorylated and stabilized, leading to regulation of the gene expressions required for cell cycle arrest and apoptosis (38). On exposure to irradiation, cells enter into the G1 phase; G1 arrest is induced and repair of DNA is performed in association with p53 (39). In contrast, cells without functional p53 tumor suppressor are arrested in the G₂-M phase by exposure to DNA-damaging reagents (40). However, studies using the human papilloma virus E6 protein (HPV-E6) and SV40 large T antigen have found that G2 arrest occurs through p53-dependent and also -independent mechanisms; this means that the effect of p53 on the G₂-M transition in response to DNA damage depends on the cell type (41). Further studies have shown that mammalian cells can be arrested in the G1 or G2 phase of the cell cycle, depending on the type of damage and the cell cycle stage in which the damage is sensed (42, 43). Thus, cells do not enter DNA synthesis or a mitotic phase initially after exposure to irradiation if the checkpoints are intact.

Human ovarian carcinoma SK-OV-3 cells lack p53, and Western blot analysis showed that irradiation did not induce the p53 expression (18, 19, 24, 26). We analyzed the cell cycle profile of synchronized cells by FACS. In response to irradiation, parental SK-OV-3 cells did not become arrested in the G1 phase, but they entered into the G2 phase. On the other hand, the overexpression of MnSOD shortened the G2 duration in these cells following irradiation; cells overexpressing MnSOD exited faster from the G2 phase as compared to the

control cells, and then progressed into the G1 phase within 24 h. Prior studies showed that reconstitution of p53-null cells with functional p53 shortened G2 arrest on irradiation (44) and also that bone marrow cells enriched in normal myeloblasts entered mitosis more frequently than p53-null cells after exposure to irradiation (45, 46). In the pathway where p53 is inactivated, such as ataxia telangiectasia (AT), the DNA damage-dependent G2 arrest is longer than in normal cells (47). Therefore, the prolonged G2 phase following DNA damage suggests that checkpoints are not intact in the SK-OV-3 cells and also reflects a lower repair efficiency. Pharmacological agents such as caffeine that force cells to pass the G2 block after DNA damage or induce premature exit from G2 arrest by irradiation increase cell death (48). In the present study, however, the overexpression of MnSOD induced resistance to irradiation in these cells. Taken together, our results indicate that the shortened duration of the G2 phase observed in MnSOD transfectants was not a premature exit. Moreover, the length of G2 arrest has been reported to correlate with the radioresistance of the cell (49). Therefore, our results suggest that MnSOD effectively protects cells from DNA damage induced by irradiation through initiating a certain signal transduction pathway that minimizes the damage to irradiation and leads to shortening of the G2 duration, because MnSOD is unlikely to function as a DNA repair protein. p21^{WAF1} also participates in the G2 checkpoint; p21^{WAF1} has been shown to associate with the cyclinB/cdc2 complex, and p21^{WAF1} delays cyclinB/cdc2 activation (50). It has been also reported that cells lacking p21^{WAF1} did not become arrested in the G2 phase after exposure to irradiation and that failure to arrest was associated with cdc2 kinase activity higher than that observed in cells with p21^{WAF1} (51). Thus, p21^{WAF1} contributes to the G2 checkpoint response. On the other hand, the expression of p21^{WAF1} is induced independent of p53 by 12-*O*-tetradecanoyl phorbol-13-acetate in SK-OV-3 cells as well as other cells (19, 52). In the present study, however, the overexpression of MnSOD and/or irradiation

Table 3. Cell Cycle Analysis of Cells Overexpressing MnSOD Treated With a p38MAPK Inhibitor

Stage	Distribution of Cells in Each Stage (%)							
	Untreated	IR	SB	SB + IR	NAC	NAC + IR		
	0 h	24 h						
Control	G1	67.6	64.7	33.8	67.0	37.7	68.4	35.9
	S	16.2	12.4	4.1	12.7	4.9	11.5	5.2
	(G ₁ -S ratio)	4.2	5.2	8.2	5.3	7.7	5.9	6.9
MnSOD	G ₂ -M	16.2	22.9	62.1	20.3	57.4	20.1	58.9
	G1	70.3	69.9	61.6	71.5	38.9	69.5	39.3
	S	14.9	8.3	4.9	6.8	4.0	7.1	4.9
(G ₁ -S ratio)		4.7	8.4	12.6	10.5	9.7	9.8	8.0
	G ₂ -M	14.8	21.8	33.5	21.7	57.1	23.4	55.8

Note: Cells were cultured for 48 h without FCS and incubated in FCS-containing medium for 1 h with either 10 μM of a p38MAPK inhibitor (SB, SB203580) or 25 mM of a ROS scavenger (NAC). Then, cells were irradiated with 5 Gy of X-ray (IR). Twenty-four hours later, cells were collected and fixed in 70% ethanol and treated with RNase A, and then stained with propidium iodide. DNA contents of the cells were analyzed by FACScaliber using Cell Quest and Modifit programs.

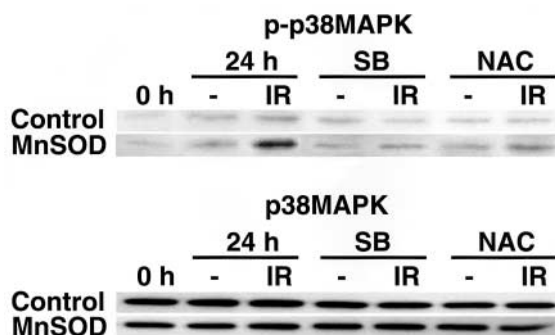


FIGURE 6. Effects of p38MAPK inhibitor and ROS scavenger on phosphorylation of p38MAPK by irradiation in cells overexpressing MnSOD. Cells were cultured for 48 h without FCS. After incubation with SB203580 (10 μ M) or NAC (25 mM) for 1 h in FCS-containing medium, cells were irradiated with 5 Gy of X-ray and then harvested 24 h after irradiation. Whole cell lysates (50 μ g/lane) were prepared, and electrophoresed on 12% SDS-polyacrylamide gel and transferred to PVDF membrane. Western blot analysis was performed using an antibody specific for phosphorylated p38MAPK or p38MAPK antibody.

ation did not induce p21^{WAF1} expression in these cells. Our results also indicate that the induction of radioresistance by MnSOD with concomitant shortening of the G₂ duration is an event independent of p53 or p21^{WAF1}.

Progression of the cell cycle is dependent on the presence of growth factors and internal or external stress stimuli; deregulation in the integration of these signals into the cell cycle leads to neoplastic transformation. The MAPK pathway is one of the key mechanisms by which signals are transduced through the cell (5). Signal transduction through MAPK consists of two highly homologous yet distinct cascades (5). One is the MAPK/ERK pathway strongly activated by mitogens and growth factors and results in activation by MAPK kinase1/2 (MEK1/2). Furthermore, MEK2 activity was recently reported to be necessary for G₂ arrest in mammalian cells (5), whereas MEK1 activity is required for the G₂-M transition (10). The other cascade is mainly activated by stresses and inflammatory cytokines, leading to activation of SAPK/JNK and p38MAPK. SAPKs have been implicated in several diverse processes such as proliferation, apoptosis, response to stress, and production of cytokines. p38MAPK, a serine/threonine protein kinase, is one of the SAPKs and is phosphorylated by MKKK-6 (53). The involvement of p38MAPK in the establishment of G₁-S cycle arrest has been documented (53, 54). On the other hand, there is accumulating evidence of the p38MAPK pathway being activated in the G₂-M phase by DNA damage (5). To determine whether the activation of p38MAPK is required for the earlier exit from G₂ phase in irradiated MnSOD transfectants, we used the specific inhibitor SB203580. The inhibition of p38MAPK resulted in prolonged G₂ duration of MnSOD transfectants after irradiation. Thus, our results show that the earlier exit from G₂ phase occurred, at least in part, through activation of p38MAPK in these cells. Importantly, scavenging ROS with NAC also inhibited shortening of the G₂ phase by MnSOD in the irradiated SK-OV-3 cells. Moreover, untreated MnSOD transfectants had a higher level of phosphorylated p38MAPK than the control cells (data not shown) and H₂O₂ activated p38MAPK in SK-OV-3 cells. Further study found

that treatment with either SB203580 or NAC abrogated the rescue effect of MnSOD on the colony-forming capacity in irradiated cells. Radiation-induced MAPK signaling has been shown to be important for cell growth or apoptosis (5), and increased ROS production leads to the potentiation of p38MAPK activation (22). Thus, our study showed that

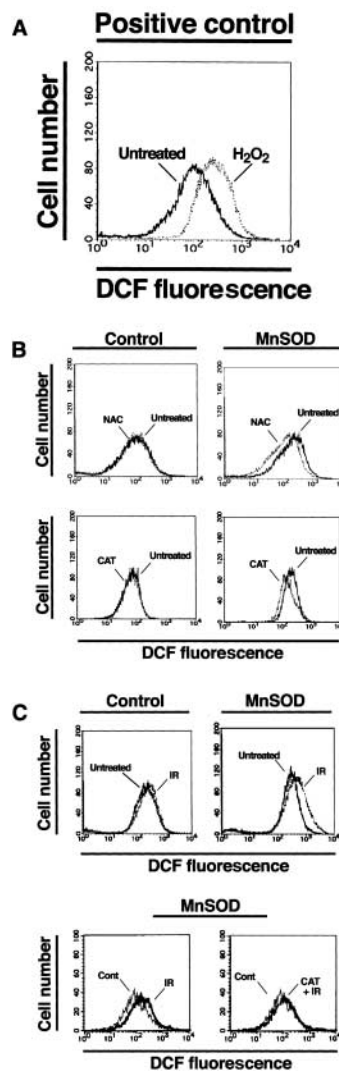


FIGURE 7. Production of ROS in MnSOD-overexpressing cells. Cells were stained with 5 μ M of DCFH-DA, a peroxide-sensitive fluorescent probe DCFH-DA in PBS⁻ for 15 min at 37°C, and the fluorescent intensities were measured using flow cytometry. **A.** Positive control. For positive control of the assay system, cells were treated with 0.02 mM of H₂O₂ for 15 min. **B.** Generation of ROS in control and MnSOD-overexpressing cells at a steady state. Control cells and cells overexpressing MnSOD were treated with either 25 mM of NAC or 1000 units/ml of CAT for 1 h at 37°C, and then stained with 5 μ M DCFH-DA. Resuspended cells were subjected to flow-cytometric analysis. **C.** Effect of irradiation on production of H₂O₂ in MnSOD-overexpressing cells. Cells were plated and cultured without FCS for 48 h and then irradiated with 5 Gy of X-ray after culturing in medium with FCS for 2 h. Fifteen minutes later, cells were stained with DCFH-DA. Resuspended cells were subjected to flow-cytometric analysis. To determine the specificity of ROS, cells were irradiated at 5 Gy in the presence or absence of CAT (1000 units/ml).

Table 4. ROS Production in Stable Transfectants With MnSOD

		DCF Fluorescence	
		Peak Fluorescence	Mean Fluorescence
Experiment 1	Control	109.8 ± 6.9 ^a	105.4 ± 5.9 ^b
	MnSOD	125.5 ± 3.9 ^a	118.2 ± 3.8 ^b
Experiment 2	Control	97.1 ± 9.6 ^c	92.3 ± 8.7 ^d
	MnSOD	161.5 ± 10.4 ^c	159.1 ± 9.9 ^d
Experiment 3	Control	89.1 ± 7.5 ^e	92.3 ± 8.6 ^f
	MnSOD	207.5 ± 15.3 ^e	230.1 ± 14.9 ^f

Note: Cells were stained with 5 μM DCFH-DA in PBS⁻ for 15 min at 37°C. Resuspended cells were subjected to flow-cytometric analysis. Results are means \pm SE of three assays. ^a $P < 0.05$; ^b $P < 0.05$; ^c $P < 0.05$; ^d $P < 0.05$; ^e $P < 0.05$; ^f $P < 0.05$.

MnSOD effectively rescues cells from radiation-mediated cell death through the G2 checkpoint requiring p38MAPK activation and that p38MAPK is activated by increased H₂O₂ generation in these cells.

In the present study, we demonstrated that increased ROS production rendered cells resistant to irradiation through p38MAPK activation. ROS are physiologically produced in cells. However, ROS are reactive and cause DNA damage, and it has been shown that ROS are involved in a variety of pathological processes, including aging and cancer (55). In the present study, however, we showed evidence that ROS activated cell survival signals. The fact that the generation of ROS leads to cell survival signals appears to be a paradox. In response to cytotoxic stresses including irradiation, cells activate or produce proteins that protect themselves from external insults, implying that these stresses may have a self-limiting effect on their toxicity due to activation of the survival pathway. It has also been shown that the overexpression of MnSOD increases cellular resistance to oxidative damage (16, 56). On the other hand, there are certain studies that showed that MnSOD sensitized cells to oxidative stress including radiation (16). The precise mechanisms responsible for the discrepancies among these various studies are not known. Stresses and inflammatory cytokines activate SAPK/JNK and p38MAPK, and there is extensive evidence for the role of the SAPK/JNK and p38MAPK pathway in cell death. In contrast, there are several lines of evidence supporting the cytoprotective effect of the activation of p38MAPK (57). The role of ROS varies in cell types and their concentrations produced (58). Because p38MAPK activation illustrates the complex regulation of diverse biological outcomes such as cell growth and cell death, and irradiation activates multiple signal transduction, the role of p38MAPK activation may be one of the keys for cell survival/death when exposed to external stress (59).

In summary, we showed that overexpression of MnSOD shortened the G2 duration and also induced the radioresistance. However, the earlier exit from the G2 phase in irradiated cells with subsequent increase in cell survival is contrary to prior studies by other investigators of the cell cycle delays and the development of resistance. When exposed to stress, cells do not enter the S or M phase; cell cycle is arrested in the G1 or G2 phase of the cell cycle and become relatively less sensitive to

irradiation. The potential involvement of the pathway activated by MnSOD in induction of radioresistance may be more complex. MnSOD converts O₂⁻ to H₂O₂ and H₂O₂ is less toxic toward DNA than O₂⁻. Therefore, overexpression of MnSOD reduces the magnitude of radiation toxicity, which may also lead to, in part, both the increased survival of cells and the earlier exit from the G2 phase.

Materials and Methods

Cells and Cell Culture

SK-OV-3, a cell line derived from a diffuse human ovarian cancer (American Type Tissue Culture Collection, Rockville, MD), was cultured in α -MEM (Cosmo Bio Co. Ltd., Tokyo, Japan) supplemented with 7% FCS (Intergen, Purchase, NY) in a humidified atmosphere containing 5% CO₂.

Reagents

Anti-human MnSOD polyclonal antibody was purchased from MEDOR (Munich, Germany). Polyclonal anti-p38MAPK and anti-phospho-p38MAPK antibodies were purchased from New England Biolabs (Beverly, MA). NAC and a selective PKC inhibitor, GF109203X (60), were purchased from Sigma (St. Louis, MO). Human CAT from erythrocytes and a selective p38MAPK inhibitor, SB203580, were purchased from Calbiochem (La Jolla, CA).

Construction of Expression Vectors

For MnSOD cDNA, the two 20-mer oligodeoxynucleotides, the forward primer (5'-GGTAGCACCAGCACTAGCAG-3') and the reverse primer (5'-ACTCAGCATAACGATCGTGG-3'), were synthesized according to the nucleotide sequence of human MnSOD cDNA. The 711-bp DNA fragment of human MnSOD was amplified from total RNA of human granulocytes by PCR in a Thermal Cycler MP (Takara, Tokyo, Japan) using EXTaq DNA polymerase (Takara). The resulting PCR product was subcloned into pCR2.1 vector (Invitrogen Inc., Carlsbad, CA). DNA sequence analysis of the cloned DNA confirmed that it contained a complete sequence of human MnSOD cDNA. Furthermore, MnSOD cDNA was cloned in the pCR3.1 eukaryotic expression vector (Invitrogen) containing the cytomegalovirus (CMV) promoter and the neomycin resistance gene (*Neo*) expressed under the SV40 promoter.

DNA Transfection and Selection of Transformed Cells

Two micrograms of plasmid were introduced into 3×10^5

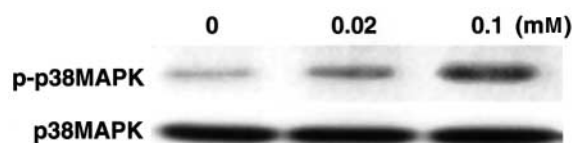


FIGURE 8. Effect of H₂O₂ on activation of p38MAPK. SK-OV-3 cells were cultured for 10 min with H₂O₂ at various concentrations. After harvesting, whole cell lysates (50 $\mu\text{g}/\text{lane}$) were electrophoresed on 12% SDS-polyacrylamide gel and transferred to PVDF membrane. Western blotting was performed using phospho-specific anti-p38MAPK and anti-p38MAPK antibodies.

cells using FuGENE6, lipofection reagent (Boehringer Mannheim Biochemicals, Indianapolis, IN). Forty-eight hours after exposure to DNA, cells were cultured in selection media supplemented with 0.7 mg/ml of G418 for 3 weeks. Single colonies of G418-resistant cells were isolated and then screened for levels of the expression of MnSOD protein by Western blot analysis. These transformants were maintained in complete media containing 0.3 mg/ml of G418.

Western Blot Analysis

Cells were lysed in buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaN₃, 0.1% SDS, 100 mg/ml phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1% NP40, and 0.5% sodium deoxycholate]. After debris had been removed by centrifugation, protein concentrations were measured by the method of Bradford (Bio-Rad, Hercules, CA) (61). Equal amounts of protein were dissolved in SDS-PAGE sample-loading buffer and electrophoresed in a polyacrylamide gel (12%). On transfer to a PVDF membrane (Immobilon, Millipore, Bedford, MA), immunoblotting was performed using rabbit anti-human MnSOD antibody (1:1000). After washing the blots, horseradish peroxidase-conjugated donkey anti-rabbit IgG diluted 1:1000 was added. Immunoreactivity on blots was detected by enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). The relative density of bands was determined by Intelligent Quantifier program (Bio Image, Ann Arbor, MI) and normalized to the loading control.

Native Gel Assay

Cells were collected and suspended in ice-cold potassium phosphate buffer (0.05 M, pH 7.8) with diethylenetriaminepentaacetic acid. After sonication on ice, the supernatants were analyzed for SOD activities. For SOD activity assay, proteins of each sample (50 µg) were loaded and fractionated on a 12% native polyacrylamide gel. The gel was stained by incubation on 2.45 mM NBT for 15 min in the dark followed by 36 mM riboflavin for 15 min in the dark in the potassium phosphate buffer. The gels were exposed to fluorescent light to photogenerate ROS. Chromatic bands corresponding to the SODs appeared against a blue background (reduced products of NBT). Protein levels in the cell lysate were measured by the method of Bradford (61). The relative density of bands of Native gel assay was determined by Intelligent Quantifier program (Bio Image).

Analysis of Sensitivity to Irradiation

One hundred cells each of control and 2 Gy-irradiated growth, as well as 200 cells of 5 Gy-irradiated growth, were seeded in 60-mm-diameter tissue culture plates and cultured without FCS for 48 h. Cells were next cultured with FCS for 2 h, and then irradiated with either 2 or 5 Gy of X-ray. After 2 weeks, cells were washed and plates were stained with Giemsa. The surviving fraction was calculated by counting the numbers of colonies.

Cell Cycle Analysis

For the cytometric analysis of DNA content, cells were collected by trypsinization and fixed with 70% ethanol. Cells

were incubated for 30 min at 37°C with 1 µg/ml RNase A. After washing, cells were resuspended in 500 µl PBS⁻ containing 25 µg propidium iodide and stained for 1 h at room temperature. Cells were filtered through 50–70 µm pore size nylon mesh, and then cell distribution in the cell cycle was analyzed with a FACScaliber flow cytometer (Becton Dickinson, Bedford, MA).

Analysis of ROS Production

Intracellular ROS production was assessed by oxidation of DCFH-DA (Molecular Probes, Eugene, OR) to fluorescent products DCF as measured by flow cytometry (62). Cells were incubated with 5 µM DCFH-DA in PBS⁻ for 15 min at 37°C and harvested. Resuspended cells were subjected to flow-cytometric analysis. A FACScaliber was used for the flow-cytometric analysis. The excitation wavelength was 488 nm, and the observation wavelength was 530 nm for green fluorescence.

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