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Resistance of Mitochondrial DNA-depleted Cells against Cell Death

ROLE OF MITOCHONDRIAL SUPEROXIDE DISMUTASE*

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We have shown that mitochondrial DNA-depleted (ρ^0) SK-Hep1 hepatoma cells are resistant to apoptosis, contrary to previous papers reporting normal apoptotic susceptibility of ρ^0 cells. We studied the changes of gene expression in SK-Hep1 ρ^0 cells. DNA chip analysis showed that MnSOD expression was profoundly increased in ρ^0 cells. O_2^- contents increased during ρ^0 cell derivation but became normalized after establishment of ρ^0 phenotypes, suggesting that MnSOD induction is an adaptive process to increased O_2^-. ρ^0 cells were resistant to menadione, paraquat, or doxorubicin, and O_2^- contents after treatment with them were lower in ρ^0 cells compared with parental cells because of MnSOD overexpression. Expression levels and activity of glutathione peroxidases were also increased in ρ^0 cells, rendering them resistant to exogenous H_2O_2. ρ^0 cells were resistant to p53, and intracellular ROS contents after p53 expression were lower compared with parental cells. Other types of ρ^0 cells also showed increased MnSOD expression and resistance against ROS. Heme oxygenase-1 expression was increased in ρ^0 cells, and a heme oxygenase-1 inhibitor decreased the induction of MnSOD in ρ^0 cells and their resistance against ROS donors. These results indicate that ρ^0 cells are resistant to cell death contrary to previous reports and suggest that an adaptive increase in the expression of antioxidant enzymes renders cancer cells or aged cells with frequent mitochondrial DNA mutations to resist against oxidative stress, host anti-cancer surveillance, or chemotherapeutic agents, conferring survival advantage on them.

Since the first report of mitochondrial DNA (mtDNA)[1]-depleted mammalian cells, a number of mtDNA-depleted cells (ρ^0 cells) have been produced by long-term treatment of cells with ethidium bromide (EtBr) or other reagents that inhibit replication of mitochondrial genes (1). Such cells and their cybrids provided valuable cell models for studying important functions of mitochondria such as oxidative phosphorylation, ATP production, electron transport, and reactive oxygen species (ROS) generation. In addition to such well known functions, recent investigations revealed that mitochondria are the master players in both apoptosis and necrosis, two classical modes of cell death (2, 3).

Because of the essential role of mitochondria in cell death, ρ^0 cells could be employed as important tools for the study of cell death. However, most ρ^0 cells have been reported to undergo apoptosis as efficiently as their parental cells (4–6). In contrast, we have reported that mtDNA-depleted hepatoma cells (SK-Hep1 ρ^0 cells) are resistant to TRAIL-induced apoptosis, which is consistent with the importance of mitochondria in cell death (7). However, it is not clearly elucidated how those cells with mitochondrial dysfunction resist cell death. It is even not well understood how such cells with defective oxidative phosphorylation survive.

We studied the changes of the gene expression profile in SK-Hep1 ρ^0 cells that allow them to survive in the absence of oxidative phosphorylation and to resist against apoptotic stimuli. We observed that manganese superoxide dismutase (MnSOD) and other antioxidant enzymes such as glutathione peroxidases (GPx) are up-regulated in those cells leading to an efficient disposal of increased oxidative stress and increased resistance against ROS or p53, which might be related to the resistance of cancer cells (8, 9) or aged cells (10, 11) with mtDNA mutations against apoptosis and their avoidance of the host surveillance. Our results indicate that ρ^0 cells are resistant to various forms of cell death contrary to previous reports and that intact mitochondrial function is crucial for the execution of cell death.

EXPERIMENTAL PROCEDURES

MtDNA-depleted ρ^0 Cells—SK-Hep1 ρ^0 cells were derived from SK-Hep1 hepatoma cells by culturing in the presence of 100 ng/ml EtBr for more than 20 generations (7, 12). MIN6N8 ρ^0 cells were developed by treating MIN6N8 murine insulinoma cells (13) with EtBr for more than 20 generations. PCR analysis using primers specific for human or murine mtDNA, measurement of cytochrome *c* oxidase activity and ATP contents all indicated that SK-Hep1 ρ^0 cells (12) and MIN6N8 ρ^0 cells are authentic ρ^0 cells. MDA-MB-435 ρ^0 cells of a breast cancer cell origin were derived by essentially the same

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[1] The abbreviations used are: mtDNA, mitochondrial DNA; ROS, reactive oxygen species; MnSOD, manganese superoxide dismutase; GPx, glutathione peroxidases; Cu,Zn-SOD, copper,zinc superoxide dismutase; cGPx, classical GPx; PHGPx, phospholipid hydroperoxide GPx; HO-1, heme oxygenase-1; ZnPP, Zn(II) protoporphyrin IX; O_2^-, superoxide anion; RT, reverse transcriptase.

method (14). All ρ^0 cells were maintained in the presence of 50 μg/ml uridine.

RT-PCR—Total RNA was extracted from parental cells and SK-Hep1 ρ^0 cells by using TRIzol reagent (Invitrogen). Total RNA was digested by RNase-free DNase I treatment for 20 min, extracted using phenol-chloroform, and then precipitated with 2.5 volumes of 100% ethanol. Reverse transcription was carried out using Superscript II (Invitrogen) and oligo(dT)$_{12–18}$ primer. PCR amplification using primer sets specific for each target gene was carried out at 59 °C annealing temperature for 25 cycles. Nucleotide sequences of the primers were based on published cDNA sequences (MnSOD forward, GGTAGCACCAGCACTAGCAG; MnSOD reverse, GTGCAGTACTCTATACCACTACA; copper,zinc superoxide dismutase (Cu,Zn-SOD) forward, CAGTGCAGGTCCTCACTTTA; Cu,Zn-SOD reverse, CCTGTCTTTGTACTTTCTTC; classical GPx (cGPx) forward, AAGGTACTACTTATCGAGAATGTG; cGPx reverse, GTCAGGCTCGATGTCAATGGTCTG; phospholipid hydroperoxide GPx (PHGPx) forward, TGTGCGCGCTCCATGCACGAGT; PHGPx reverse, AAATAGTGGGGCAGGTCCTTCTCT; catalase forward, TTAATCCATTCGATCTCACC; catalase reverse, GGCGGTGAGTGTCAGGATAG; glyceraldehyde-3-phosphate dehydrogenase forward, ACCCAGAAGACTGTGGATGG; glyceraldehyde-3-phosphate dehydrogenase reverse, TTCTAGACGGCAGGTCAGGT); heme oxygenase-1 (HO-1) forward, CAGGCAGAGAATGCTGAGTTC; HO-1 reverse, GATCTTGAGCAGGAACGCAGT.

Western Blot Analysis—Cells were lysed in a buffer containing 100 mM NaCl, 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40. Protein concentration in cell lysates was determined using a commercial protein assay kit (Bio-Rad). After adding a loading buffer containing 50 mM Tris-HCl, pH 6.8, 2% SDS, 8% glycerol, and 0.4% β-mercaptoethanol, an equal amount of protein for each sample was separated by 12% SDS-PAGE and transferred to Hybond enhanced chemiluminescence (ECL) membranes (Amersham Biosciences). After incubation in 1:1,000 dilution of primary antibodies to MnSOD (Calbiochem), Cu,Zn-SOD(Upstate USA Inc., Charlottesville, VA) or cGPx (Labfrontier Co., Seoul, Korea), membranes were probed with appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences). Bound antibody was visualized using an ECL reagent (Amersham Biosciences).

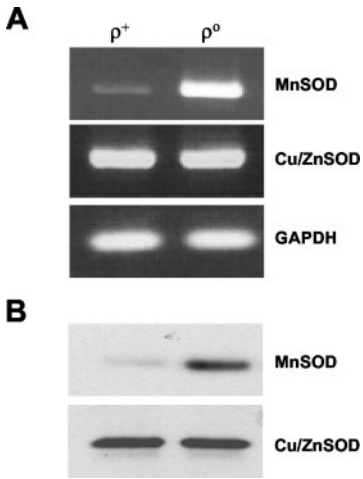

FIG. 1. **Markedly increased expression of MnSOD in SK-Hep1 ρ^0 cells.** *A*, RT-PCR analysis showed profoundly increased expression of MnSOD compared with parental cells at the RNA level, whereas the expression of Cu,Zn-SOD and a control gene (glyceraldehyde-3-phosphate dehydrogenase, *GAPDH*) was not different between the two cells. *B*, Western blot analysis confirmed the increased MnSOD in ρ^0 cells at the protein level. The expression of Cu,Zn-SOD was not different between the two cells.

Measurement of Cell Death—After treatment of cells with 25 to 75 mM menadione (Sigma), 1 to 20 mM paraquat (Sigma), 50 to 100 mM H_2O_2 (Sigma), 100 to 200 nM doxorubicin (Donga Pharmaceuticals, Seoul, Korea), or infection with Avpp53 (15), the trypan blue exclusion test was performed by incubating cells in 0.02% trypan blue solution (Invitrogen) for 5 min. Avpp53 and Ad5CMVSOD2 (Gene Transfer Vector Core, University of Iowa, Iowa City, IA) were propagated in HEK 293 cells and concentrated by the standard CsCl ultracentrifugation method. Cells seeded on 96-well plates were infected with Avpp53 at a multiplicity of infection of 50 in a serum-free medium for 1 h. For multiple adenoviral transduction, Ad5CMVSOD2 infection at a multiplicity of infection of 50–100 was performed 24 h before Avpp53 infection. For some experiments, preincubation with 1 mM *N*-acetylcysteine (Sigma) or 1 mM reduced GSH (Sigma) for 30 min or with Zn(II) protoporphyrin IX (ZnPP, Frontier Scientific Inc., Logan, UT) for 24 h was done before treatment with test reagents.

ROS Measurement—For intracellular superoxide anion (O_2^-) measurement, cells were incubated with 1 μg/ml fresh dihydroethidium (Molecular Probes, Eugene, OR) in Hanks' balanced salt solution at 37 °C for 15 min. Flow cytometry was conducted after washing in Hanks' balanced salt solution (excitation, 518 nm; emission, 605 nm). In the case of intracellular H_2O_2, cells were incubated with 10 μg/ml fresh 2′,7′-dichlorofluorescein diacetate (Molecular Probes) in Hanks' balanced salt solution at 37 °C for 30 min for flow cytometry (excitation, 485 nm; emission, 530 nm).

GPx Activity—cGPx activity was measured using a published protocol with modifications (16). In short, postnuclear fractions were prepared by douncing cells in an isotonic buffer (10 mM HEPES, pH 8.0, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, and 100 μg/ml leupeptin) and spinning down at 800 × *g*. Supernatant comprising both mitochondrial and cytoplasmic fraction was sonicated in a Branson sonifier 450 (Danbury, CT) and saved for measurement of enzymatic activity. Appropriately diluted protein samples were added to 700 μl of the reaction mixture containing 50 mM Tris-HCl, pH 7.6, 5 mM EDTA, 1 mM GSH, 0.2 mM NADPH, and 0.5 unit of glutathione reductase (Calbiochem, La Jolla, CA). The reaction was started by adding 350 μl of 0.22 mM *t*-butyl hydroperoxide as a substrate at 25 °C. The decrease in absorbance at 340 nm was monitored by a UV spectrophotometer for conversion to the enzyme activity. The rate of the decrease in A_{340} was converted to the activity using the following formula: 1 milliunit/ml = 1 nmol of NADPH/min/ml = ΔA_{340}/min/0.00622.

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared as previously described (17). Synthetic double-stranded oligonucleotides of the consensus NF-κB binding sequence, AGTTGAGGGGACTTTCCCAGGC (Promega), were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Nuclear extract was incubated with the labeled probe in the presence of poly(dI-dC) in a binding buffer containing 20 mM HEPES at room temperature for 30 min. DNA-protein complexes were resolved by electrophoresis in a 5% non-denaturing polyacrylamide gel and visualized by autoradiography.

RESULTS

Increased MnSOD Expression in SK-Hep1 ρ^0 Cells—First, we studied the changes of gene expression in SK-Hep1 ρ^0 cells

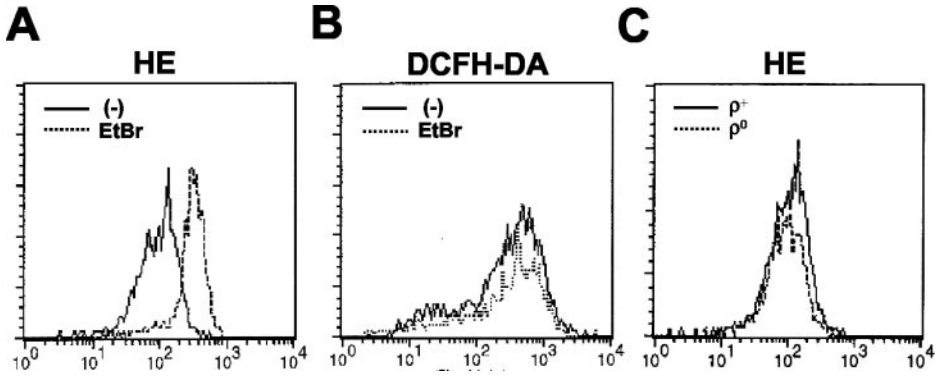

FIG. 2. **Increased ROS production by EtBr treatment of parental cells.** *A*, flow cytometric analysis after dihydroethidium (*HE*) loading showed that EtBr treatment for 10 days resulted in a marked increase in O_2^- contents compared with untreated parental cells. *B*, contents of H_2O_2 stained by 2′,7′-dichlorofluorescein diacetate (*DCFH-DA*) were not changed by the same treatment. *C*, O_2^- contents in fully established SK-Hep1 ρ^0 cells cultured with EtBr for more than 2 years were not different from that in parental cells.

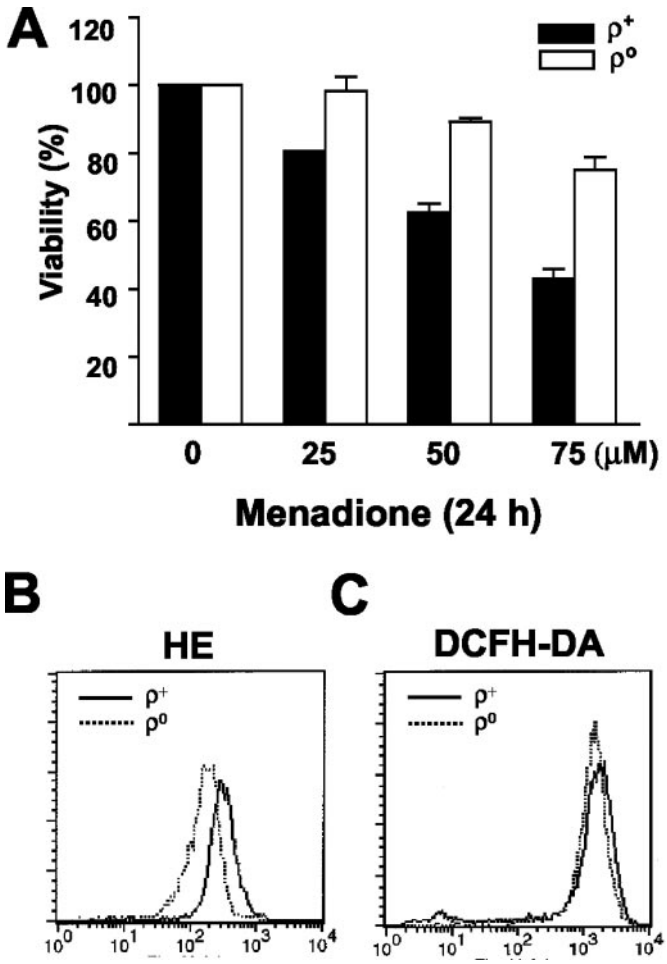

FIG. 3. **Resistance of SK-Hep1 ρ^0 cells against menadione.** *A*, Trypan blue staining showed that ρ^0 cells were resistant to menadione, an ROS stressor producing O_2^- (mean ± S.D.). *B*, intracellular O_2^- contents after menadione treatment were lower in ρ^0 cells compared with parental cells. *C*, intracellular H_2O_2 contents after menadione treatment were not different between the two cells. *HE*, dihydroethidium; *DCFH-DA*, 2′,7′-dichlorofluorescein diacetate.

using DNA chip analysis (Genomic Tree, Taejon, Korea). Among the genes that were overexpressed in SK-Hep1 ρ^0 cells, *MnSOD* was one of the genes that showed the most striking increase in the expression levels compared with parental cells (-fold increase of 20.9). The increased expression of MnSOD in SK-Hep1 ρ^0 cells was confirmed by RT-PCR and Western blot analysis (Fig. 1, *A* and *B*). In contrast to MnSOD that is expressed in mitochondria and metabolizes O_2^- produced during mitochondrial electron transfer, the expression of Cu,Zn-SOD, another major superoxide dismutase expressed in cytoplasm was not changed as demonstrated by RT-PCR and Western blot analysis (Fig. 1, *A* and *B*).

Increased ROS Production during ρ^0 Cell Derivation but Not in Established ρ^0 Cells—The increased expression of MnSOD could be an adaptive response to the failure of mitochondrial electron transport and an increase in the leakage of O_2^- from mitochondria of SK-Hep1 ρ^0 cells (18). As SK-Hep1 ρ^0 cells might be in a new equilibrium between increased O_2^- production and increased MnSOD expression, they might not be good models to study the changes in ROS contents by the interruption of mitochondrial electron transfer (see Fig. 2*C*). Thus, we reiterated the derivation of mtDNA-depleted SK-Hep1 ρ^0 cells by culturing parental cells with 100 ng/ml EtBr and studied if the blockade of the mitochondrial electron transfer by the inhibition of mtDNA replication with EtBr leads to an increased intracellular ROS. As hypothesized, intracellular O_2^- contents measured by dihydroethidium were increased after incubation of parental cells with EtBr for 10 days compared with untreated cells (Fig. 2*A*), suggesting that the inhibition of mitochondrial respiratory complexes blocks mitochondrial electron transport between mitochondrial respiratory complexes and enhances the production of O_2^- (18). Intracellular H_2O_2 measured by 2′,7′-dichlorofluorescein diacetate was not increased by SK-Hep1 parental cell treatment with EtBr probably because the compensatory increase in MnSOD and the adaptive increase in the dismutation of O_2^- to H_2O_2 had not yet occurred after 10 days of treatment (Fig. 2*B*). O_2^- contents in fully established SK-Hep1 ρ^0 cells that have been maintained for more than 2 years was not different from that in parental cells probably because of the equilibrium between the increased O_2^- production and increased O_2^- dismutation (Fig. 2*C*).

Resistance of SK-Hep1 ρ^0 Cells against ROS—Next, we studied if SK-Hep1 ρ^0 cells were resistant to ROS by metabolizing ROS efficiently because of the increased expression of MnSOD. We first treated SK-Hep1 ρ^0 cells and parental cells with menadione, a prototypic oxygen stressor of the quinonoid type producing O_2^- (19, 20). We employed a trypan blue exclusion assay as a measure of cell death because ROS induces cell death of mixed patterns showing both necrosis and apoptosis depending on the experimental condition and differentiation between necrosis, and apoptosis on the morphological ground does not have much significance when the death effector is already known as ROS (21, 22). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was not employed because such assay testing of mitochondrial succinate dehydrogenase activity was not considered appropriate when studying cells with mtDNA depletion. Treatment with 25–75 μM menadione for 24 h induced death of SK-Hep1 parental cells in a dose-dependent manner. In contrast, SK-Hep1 ρ^0 cells were resistant to treatment with menadione (Fig. 3*A*). Because these results suggested the possibility that SK-Hep1 ρ^0 cells acquired resistance to menadione by metabolizing O_2^- more rapidly because of the increased MnSOD, we next measured the intracellular O_2^- contents after treatment with menadione. As hypothesized, intracellular O_2^- contents after treatment of SK-Hep1 ρ^0 cells with 1 mM menadione for 20 min were significantly lower than that after the same treatment of parental cells (Fig. 3*B*). O_2^- contents before menadione treatment was not different between the two cells probably because of the equilibrium between the increases in O_2^- production and that in disposal (Fig. 2*C*). Contents of H_2O_2 after menadione treatment that is produced from O_2^- by SOD were not significantly different between SK-Hep1 ρ^0 cells and parental cells (Fig. 3*C*), suggesting a new equilibrium between the increased dismutation of O_2^- and an increased processing of H_2O_2 in SK-Hep1 ρ^0 cells (see Figs. 5 and 6).

We then studied the effect of another oxidative stressor producing superoxide, paraquat (23, 24). Treatment with 1–20 mM paraquat induced death of SK-Hep1 parental cells in a dose-dependent manner; however, SK-Hep1 ρ^0 cell death after paraquat treatment was markedly less compared with parental cells (Fig. 4*A*). Similar to the menadione treatment, intracellular O_2^- contents after treatment of SK-Hep1 ρ^0 cells with 10 mM paraquat were lower than that after the same treatment of parental cells, which is consistent with our hypothesis that SK-Hep1 ρ^0 cells are able to metabolize O_2^- more rapidly by increased MnSOD and acquired resistance to O_2^- (Fig. 4*B*). H_2O_2 contents after paraquat treatment were not significantly different between SK-Hep1 ρ^0 cells and parental cells, similar to menadione treatment (Fig. 4*C*).

Next we studied the expression of H_2O_2 metabolizing en-

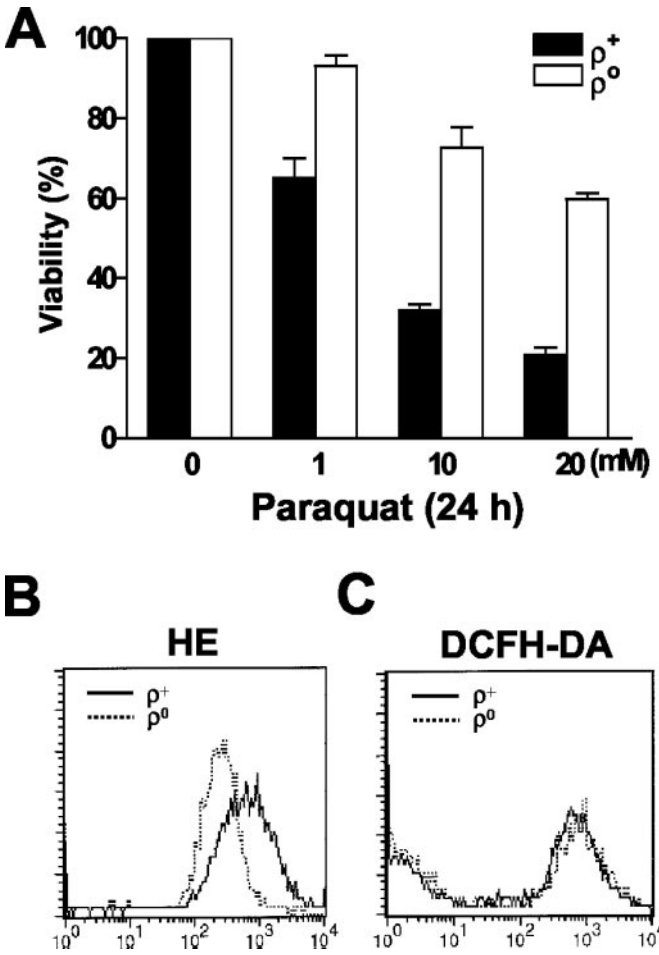

FIG. 4. **Resistance of SK-Hep1 ρ^0 cells against paraquat.** *A*, ρ^0 cells were resistant to paraquat (mean ± S.D.). *B*, O_2^- contents after paraquat treatment were lower in ρ^0 cells compared with parental cells. *C*, H_2O_2 contents after paraquat treatment were not different between the two cells. *HE*, dihydroethidium; *DCFH-DA*, 2′,7′-dichlorofluorescein diacetate.

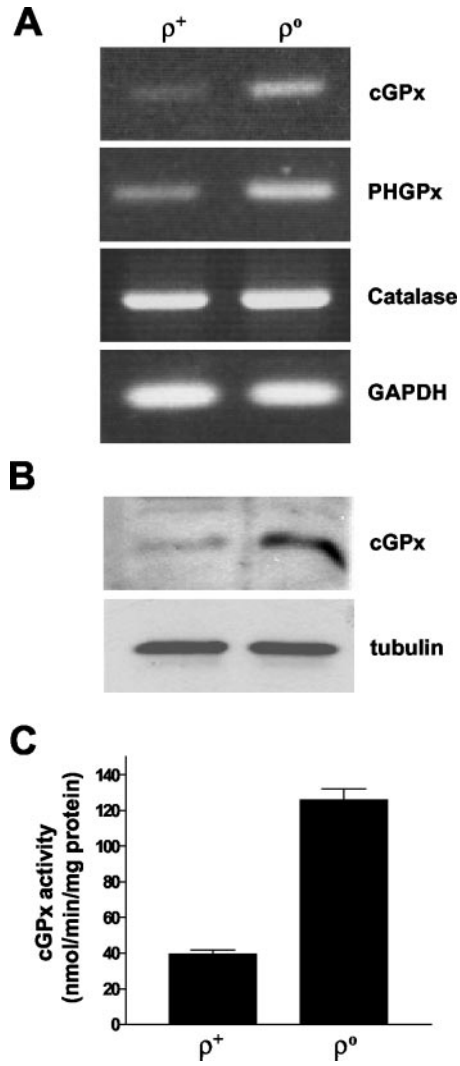

FIG. 5. **Increased GPx expression in SK-Hep1 ρ^0 cells.** *A*, RT-PCR analysis showed that the expression of cGPx and PHGPx was increased in ρ^0 cells compared with parental cells at the RNA level, whereas the expression of catalase was not different between the two cells. *B*, immunoblot analysis showed that cGPx protein expression was increased in ρ^0 cells. *C*, cGPx activity was also increased in ρ^0 cells compared with parental cells (mean ± S.D.). *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

zymes and the susceptibility of SK-Hep1 ρ^0 cells to H_2O_2 because SK-Hep1 ρ^0 cells may need to process increased intracellular H_2O_2 by MnSOD overexpression. We studied the expression of GPx that catalyzes the conversion of H_2O_2 to H_2O and O_2 in various compartments of the cells including mitochondria (25). RT-PCR analysis showed that the expression of 2 types of GPx (cGPx and PHGPx) that are expressed in mitochondria (25) was increased in SK-Hep1 ρ^0 cells compared with parental cells (Fig. 5*A*). In contrast, the expression of catalase that metabolizes H_2O_2 in peroxisome was not increased (Fig. 5*A*). Western blot analysis also demonstrated that the expression of the cGPx protein was increased in SK-Hep1 ρ^0 cells (Fig. 5*B*). The increased cGPx expression was functional because cGPx activity in the postnuclear fraction of SK-Hep1 ρ^0 cells comprising both cytoplasmic and mitochondrial fractions was significantly increased compared with parental cells (Fig. 5*C*).

To directly prove that SK-Hep1 ρ^0 cells have an increased ability to process H_2O_2, we studied susceptibility of SK-Hep1 ρ^0 cells to exogenous H_2O_2 that readily permeates across biological membranes (26). Similar to other oxidative stressors, 50–100 μM H_2O_2 induced SK-Hep1 parental cell death in a dose-dependent manner. However, SK-Hep1 ρ^0 cells were markedly resistant to H_2O_2, suggesting a more rapid disposal of exogenous H_2O_2 by the increased GPx expression (Fig. 6*A*). Intracellular H_2O_2 contents after H_2O_2 treatment were also lower in SK-Hep1 ρ^0 cells compared with parental cells (Fig. 6*B*), which is consistent with our hypothesis that SK-Hep1 ρ^0 cells acquired resistance to H_2O_2 by the increased expression of cGPx or PHGPx. H_2O_2 contents before H_2O_2 treatment were not different between the two cells (Fig. 6*C*).

Resistance of SK-Hep1 ρ^0 Cells to p53—Next we studied the possible resistance of SK-Hep1 ρ^0 cells against p53 because several previous reports indicated the role of ROS in p53-induced apoptosis (27–29). SK-Hep1 cell death after infection with adenovirus expressing p53 (Avpp53) was dependent upon ROS because antioxidants such as *N*-acetylcysteine or GSH inhibited p53-induced SK-Hep1 parental cell death (Fig. 7*A*). Trypan blue exclusion assay showed that SK-Hep1 ρ^0 cells were markedly resistant to adenoviral transduction of p53, consistent with our hypothesis (Fig. 7*B*). Both intracellular O_2^- and H_2O_2 contents in SK-Hep1 parental cells were increased after adenoviral expression of p53, consistent with the inhibition of p53-induced SK-Hep1 cell death by antioxidants (Fig. 7*C*). Intracellular O_2^- and H_2O_2 contents after adenoviral expression of p53 in SK-Hep1 ρ^0 cells were not apparently different from that before infection, suggesting that the increased expression of MnSOD

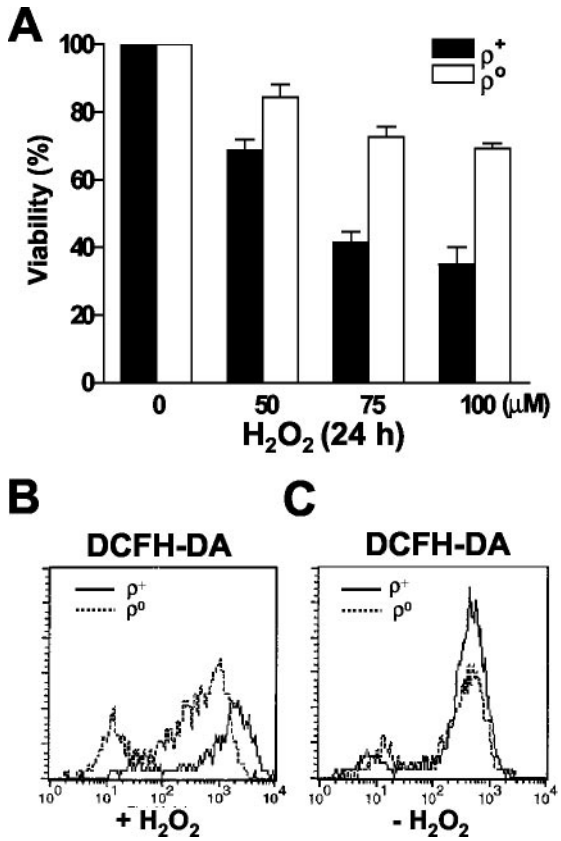

FIG. 6. **Resistance of SK-Hep1 ρ^0 cells against H_2O_2.** *A*, SK-Hep1 ρ^0 cells were resistant to exogenous H_2O_2 (mean ± S.D.). *B*, H_2O_2 contents after H_2O_2 treatment were lower in SK-Hep1 ρ^0 cells compared with parental cells. *C*, H_2O_2 contents before H_2O_2 treatment were not different between the two cells. *HE*, dihydroethidium; *DCFH-DA*, 2′,7′-dichlorofluorescein diacetate.

and GPx protected SK-Hep1 ρ^0 cells from p53-induced death by attenuating ROS production (Fig. 7*C*). In addition to p53, SK-Hep1 ρ^0 cells were resistant to doxorubicin that induces target cell death by multiple mechanisms including DNA breakage, p53 induction (30, 31), and ROS production (32, 33) (Fig. 7*D*).

Protection of Parental Cells by MnSOD Overexpression—To directly prove the role of MnSOD in the resistance against ROS or p53, we infected parental cells with adenovirus expressing MnSOD (Ad5CMVSOD2). MnSOD expression was strongly induced by infection with Ad5CMVSOD2 (Fig. 8*A*). Adenoviral overexpression of MnSOD partly but significantly reduced target cell death by menadione, paraquat, adenoviral p53 expression, and doxorubicin, suggesting that the increased dismutation of O_2^- by adenoviral MnSOD expression decreased cell death by ROS donors or p53. However, SK-Hep1 cell death by H_2O_2 that occurs downstream of MnSOD was not decreased by adenoviral expression of MnSOD (Fig. 8*B*).

Resistance of Other Types of ρ^0 Cells against ROS—To investigate if the increased expression of MnSOD and the resistance against ROS are restricted to a specific type of ρ^0 cells (SK-Hep1 ρ^0 cells) or relevant to other types of ρ^0 cells, we examined the expression of MnSOD in MIN6N8 ρ^0 cells and MDA-MB-435 ρ^0 cells. Both cells had increased expression of MnSOD compared with their respective parental cells and showed resistance against menadione (Fig. 9, *A* and *B*), paraquat, or doxorubicin (data not shown), suggesting that the induction of MnSOD and the resistance against ROS are general phenomena common to a wide variety of ρ^0 cells.

Role of HO-1 in MnSOD Induction—To address the mechanism of antioxidant enzyme induction in ρ^0 cells, we studied the possible activation of NF-κB that is reportedly activated by ROS and induces MnSOD expression. However, our electrophoretic mobility shift assay analysis clearly showed that NF-κB was not activated in untreated SK-Hep1 ρ^0 cells, whereas treatment with 10 ng/ml tumor necrosis factor α for 15 min induced strong NF-κB activation in SK-Hep1 ρ^0 cells as well as parental cells (Fig. 10*A*). We next studied the role of HO-1 whose expression was increased more than 4-fold in our DNA chip analysis and which has been reported to induce MnSOD in response to oxidative stress (34, 35). A strong induction of HO-1 in SK-Hep1 ρ^0 cells was confirmed by RT-PCR analysis (Fig. 10*B*). Treatment with the HO-1 inhibitor ZnPP for 24 h decreased the expression of MnSOD and cGPx at RNA and protein levels in a dose-dependent manner (Fig. 10, *C* and *D*). Pretreatment of SK-Hep1 ρ^0 cells with ZnPP for 24 h also markedly attenuated the resistance of SK-Hep1 ρ^0 cells against menadione, doxorubicin, paraquat, adenoviral p53 infection, and H_2O_2, strongly indicating the role of HO-1 in the induction of antioxidant enzymes in SK-Hep1 ρ^0 cells and their resistance against ROS (Fig. 10*E*).

DISCUSSION

In our DNA chip analysis to elucidate the mechanism of cell survival without mitochondrial oxidative phosphorylation and their resistance against various cell death modes, we observed a striking elevation of MnSOD expression in mtDNA-depleted SK-Hep1 ρ^0 cells but no change in Cu,Zn-SOD expression. This observation is consistent with previous papers showing an MnSOD expression in tissues from patients with various types of mtDNA mutation/deletion (36, 37) and those reporting the ability of MnSOD to suppress several types of cell death (38, 39). Besides SK-Hep1 ρ^0 cells, we also observed the increased expression of MnSOD in other types of ρ^0 cells. The increased expression of MnSOD in various types of ρ^0 cells and in the tissues with mitochondrial dysfunction is most likely an adaptive process to the overproduction of ROS. Increased ROS production in cells or tissues with mitochondrial mutations or dysfunction has been shown (36, 40), which is probably because of the blockade of mitochondrial electron transfer within or between mitochondrial respiratory complexes and subsequent leakage of electrons as O_2^-. We observed no increase of basal ROS levels in SK-Hep1 ρ^0 cells, which might represent a new equilibrium between the overproduction of ROS and the induction of MnSOD. As we could not demonstrate the increased ROS levels in ρ^0 cells, we had to repeat EtBr treatment of parental cells again to prove that the blockade of mitochondrial electron transfer increases ROS production. As a 10-day period of EtBr treatment was long enough to inhibit transcription/replication of mitochondrial DNA but not long enough for the development of cell clones with the adaptive MnSOD overexpression, we were able to demonstrate the increase in O_2^- production before reaching a new equilibrium. Intracellular H_2O_2 contents were not increased after 10 days of EtBr treatment probably because MnSOD overexpression and increased dismutation of O_2^- to H_2O_2 had not yet occurred.

We reasoned that the increased MnSOD expression in SK-Hep1 ρ^0 cells would affect the intracellular metabolism of exogenous ROS and the outcome of the cells after treatment with ROS donors. Consistent with our hypothesis, SK-Hep1 ρ^0 cells showed remarkable resistance to menadione and paraquat, both of which produce superoxide anion through redox cycles (19, 23). Much less intracellular O_2^- levels were also observed after treatment of SK-Hep1 ρ^0 cells with menadione or paraquat, suggesting an increased dismutation of intracellular O_2^-. However, increased dismutation of O_2^- may not necessarily confer a beneficial effect on host cells because of the increased

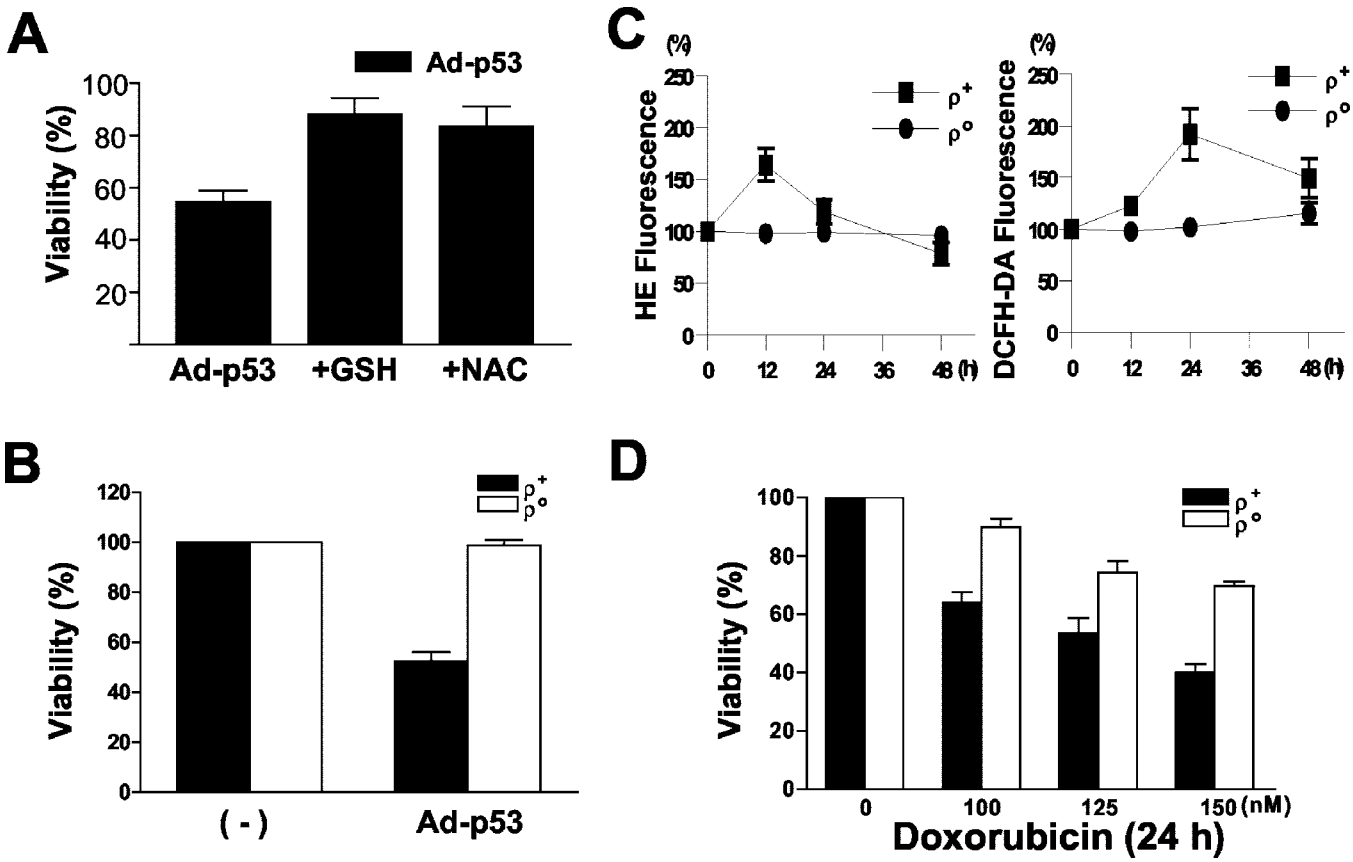

FIG. 7. **Resistance of SK-Hep1 ρ^0 cells against p53 (mean ± S.D.).** *A*, death of SK-Hep1 parental cells by adenoviral p53 expression was inhibited by GSH or *N*-acetylcysteine (*NAC*). *B*, SK-Hep1 ρ^0 cells were resistant to adenoviral expression of p53. *C*, intracellular contents of O_2^- or H_2O_2 after adenoviral p53 expression were lower in SK-Hep1 ρ^0 cells compared with parental cells (% of control fluorescence). *D*, SK-Hep1 ρ^0 cells were resistant to doxorubicin that induces cell death in part through p53 activation. *DCFH-DA*, 2′,7′-dichlorofluorescein diacetate.

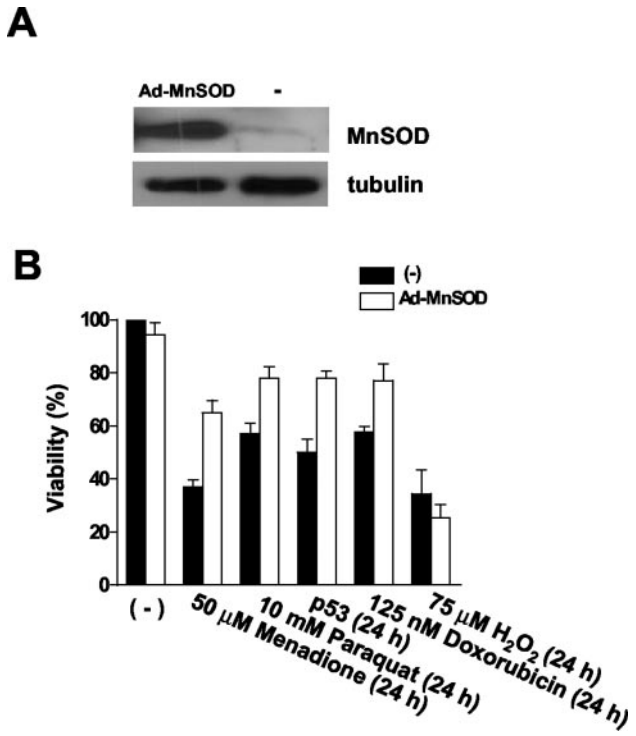

FIG. 8. **Effect of MnSOD overexpression on cell death.** *A*, strong MnSOD expression was observed by Western blot analysis after infection of parental cells with adenovirus expressing MnSOD. *B*, adenoviral MnSOD expression partially inhibited SK-Hep1 cell death by menadione, paraquat, doxorubicin, and p53 adenoviral expression but not by exogenous H_2O_2 (mean ± S.D.).

generation of other ROS such as to H_2O_2 (41). Thus, the resistance of SK-Hep1 ρ^0 cells against menadione or paraquat may be due not only to the overexpression of MnSOD but to the overexpression of GPx. Whereas the -fold increase of GPx expression was less than that of MnSOD, it was functional as shown in our assay of GPx enzyme activity. Furthermore, SK-Hep1 ρ^0 cells were markedly resistant to exogenous H_2O_2, and intracellular H_2O_2 levels after H_2O_2 treatment were less compared with parental cells, consistent with the increased GPx expression. We observed up-regulation of 2 forms of GPx (cGPx and PHGPx) that are expressed in both mitochondria and cytoplasm (25, 42). As H_2O_2 readily moves across intracellular compartments, both cytoplasmic and mitochondrial forms of GPx would participate in the metabolism of H_2O_2.

In addition to the resistance to ROS donors, SK-Hep1 ρ^0 cells showed marked resistance to p53-induced death, which constitutes one of the most important host surveillance against cancers. Consistent with our results, MnSOD overexpression has been reported to inhibit p53-induced death (39). Multiple mechanisms have been proposed for p53-induced apoptosis (43), and ROS has been reported as an important downstream effector of p53 (27–29). In our model also, p53-induced cell death was dependent on ROS as it was inhibited by antioxidants. Because SK-Hep1 cell death by p53 was dependent on ROS, we could test if SK-Hep1 ρ^0 cells were resistant to p53-induced apoptosis and indeed they were consistent with previous reports by others (29). Adenoviral p53 expression in SK-Hep1 ρ^0 cells also did not increase O_2^- or H_2O_2 contents, suggesting that the increased expression of both MnSOD and GPx is involved in the resistance of SK-Hep1 ρ^0 cells against p53. These results suggest an intriguing possibility that various cancers with frequent

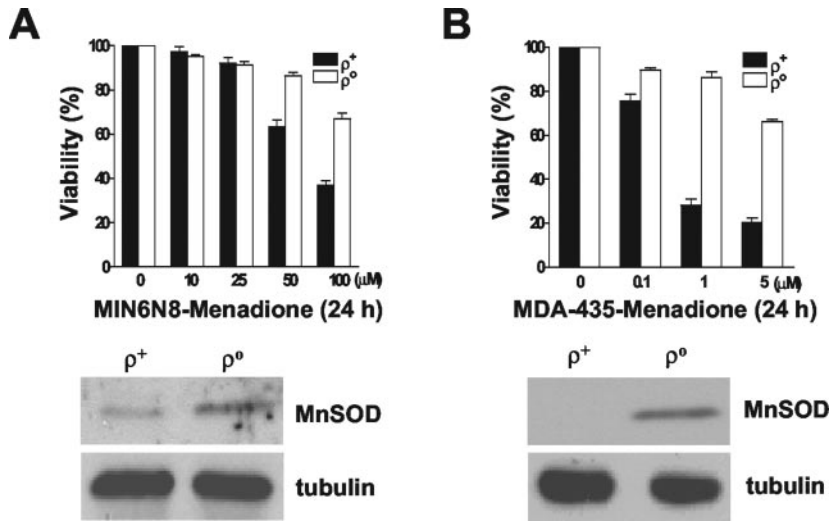

FIG. 9. **Increased expression of MnSOD in other types of ρ^0 cells.** Both MIN6N8 ρ^0 cells of an insulinoma cell origin (*A*) and MDA-MB-435 ρ^0 cells of a breast cancer cell origin (*B*) showed increased MnSOD expression. They were also resistant to menadione, like SK-Hep1 ρ^0 cells.

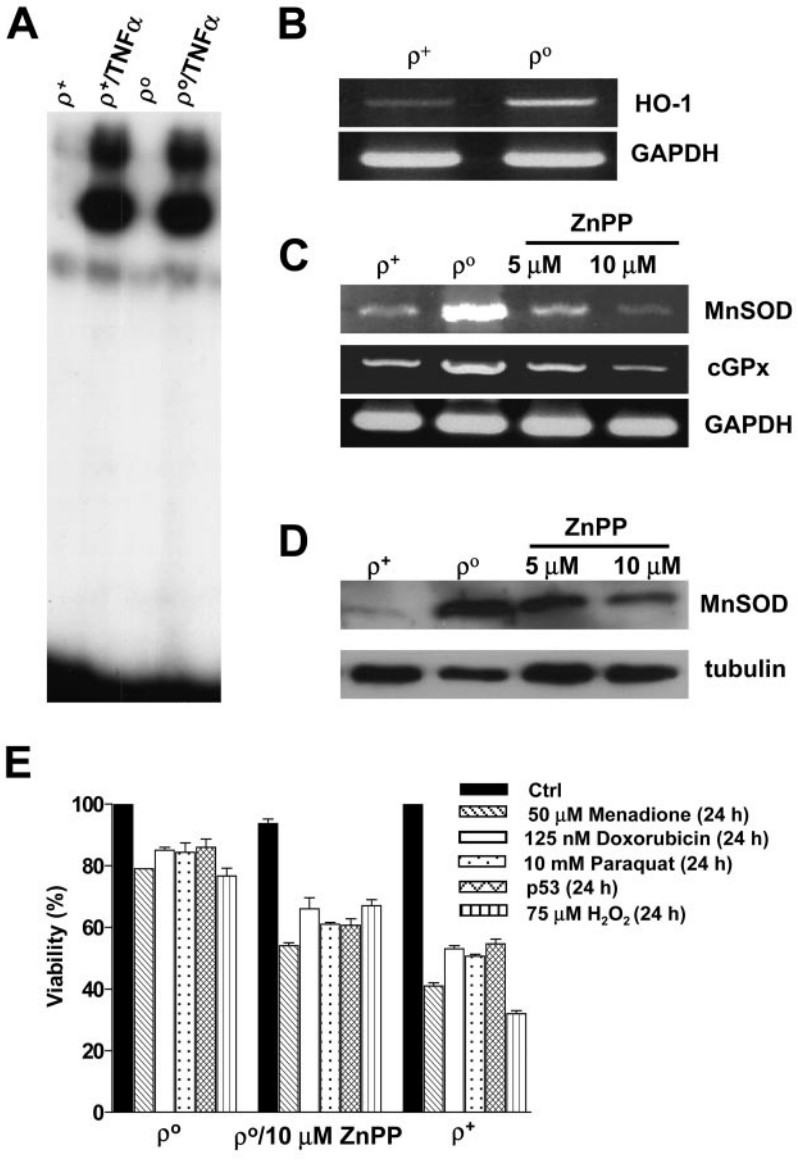

FIG. 10. **Role of HO-1 in MnSOD induction.** *A*, NF-κB was not activated in untreated SK-Hep1 ρ^0 cells, whereas tumor necrosis factor α treatment for 15 min induced strong NF-κB activation in both parental cells and ρ^0 cells. *B*, RT-PCR analysis showed that HO-1 was upregulated in SK-Hep1 ρ^0 cells. Treatment of SK-Hep1 ρ^0 cells with ZnPP, an HO-1 inhibitor, markedly decreased the expression of MnSOD and cGPx at RNA (*C*) and protein levels (*D*). *E*, ZnPP also attenuated the resistance of SK-Hep1 ρ^0 cells against menadione, paraquat, doxorubicin, p53, and H_2O_2.

mtDNA mutations (8, 9) avoid host anti-cancer surveillance imposed by p53, whereas the complete depletion of mtDNA in ρ^0 cells will not be comparable with the effect of multiple deletions in mtDNA of cancer cells.

The resistance of SK-Hep1 ρ^0 cells against doxorubicin is consistent with a previous report using HeLa ρ^0 cells (44) and might be explained by the combined effect of the resistance against p53 and that against ROS, the two main but not mu-

tually exclusive mechanisms of doxorubicin effect (30–32). This result suggests the possibility that the mtDNA mutation observed in cancers is one of the important causes of the resistance against chemotherapeutics frequently observed in advanced cancers.

Aged cells or cancer cells have increased frequency of mtDNA mutation/deletion. The percentage of individual mutation/deletion may be low in aged cells but the total frequency of various kinds of mutations/deletions including known and unknown ones may be high (10, 11). Several specific mutations have been reported to occur up to 50% in aged fibroblasts (11). Mitochondrial abnormalities were also found in almost 80% of some types of cancers (8, 9). A significant proportion of those aged cells or cancer cells with mitochondrial abnormalities would have increased intracellular ROS contents. Most of such cells will be dead because of the increased ROS; however, some of them will adapt to the increased ROS production by inducing antioxidant enzymes. Once survived, such cells would have a survival advantage over normal cells with intact mitochondrial function because they may be able to endure endogenous or exogenous oxidative stress and avoid cancer surveillance by p53 or TRAIL (7). This suggestion is consistent with previous papers showing increased MnSOD and GPx expression in aged or senescent tissue (45–47) and also a recent paper showing the decreased apoptosis of aged cells to genotoxic stress (48). In cancer cells, most previous reports (49, 50) showed low MnSOD expression. However, several recent papers (51–53) reported an increased expression of MnSOD in advanced cancer tissue, particularly in relation to poor prognosis or metastasis. Most cancer tissues might not have an increased expression of antioxidant enzymes; however, some selected cancer cells might have an increased expression of antioxidant enzymes leading to resistance against exogenous stress and poor prognosis (54).

Regarding the molecular mechanism of MnSOD induction in ρ^0 cells, NF-κB does not appear to play a role. Whereas NF-κB is one of the most extensively studied among the known inducers of MnSOD and has been reported to be activated by ROS (55, 56), we could clearly demonstrate that NF-κB was not activated in untreated SK-Hep1 ρ^0 cells. A recent paper demonstrated that ROS itself does not mediate NF-κB activation (57), contrary to previous reports. Our observation that HO-1 was markedly overexpressed in SK-Hep1 ρ^0 cells and that ZnPP as an HO-1 inhibitor decreased the expression of MnSOD/cGPx and also the resistance of SK-Hep1 ρ^0 cells against various ROS donors strongly suggests that HO-1 is an important mediator of the induction of MnSOD and cGPx in SK-Hep1 ρ^0 cells. These results are consistent with previous reports that HO-1 is rapidly induced by various pro-oxidants and serves to normalize the redox microenvironment by catabolizing pro-oxidant metalloporphyrins to bile pigment and also by inducing antioxidant enzymes such as MnSOD (35, 58). Whereas our results suggest a mechanism of the induction of antioxidant enzymes in response to ROS, further studies will be necessary to understand the biological significance and detailed biochemical mechanism of these observations and to extrapolate these findings from artificial ρ^0 cells to the resistance of natural senescent cells or cancer cells against various types of cell death.

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Mechanisms of Signal Transduction:

Resistance of Mitochondrial DNA-depleted Cells against Cell Death: ROLE OF MITOCHONDRIAL SUPEROXIDE DISMUTASE

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