p53-Induced Up-Regulation of MnSOD and GPx but not Catalase Increases Oxidative Stress and Apoptosis

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ABSTRACT

p53-mediated apoptosis may involve the induction of redox-controlling genes, resulting in the production of reactive oxygen species. Microarray expression analysis of doxorubicin exposed, related human lymphoblasts, p53 wild-type (WT) Tk6, and p53 mutant WTK1 identified the p53-dependent up-regulation of manganese superoxide dismutase (MnSOD) and glutathione peroxidase 1 (GPx). Consensus p53 binding sequences were identified in human MnSOD and GPx promoter regions. A 3-fold increase in the MnSOD promoter activity was observed after the induction of p53 in Li-Fraumeni syndrome (LFS) fibroblast, TR9-7, expressing p53 under the control of a tetracycline-regulated promoter. An increased protein expression of endogenous MnSOD and GPx also positively correlated with the level of p53 induction in TR9-7 cells. However, catalase (CAT) protein expression remained unaltered after p53 induction. We also examined the expression of MnSOD, GPx, and CAT in a panel of normal or LFS fibroblasts, containing either WT or mutant p53. We found increased MnSOD enzymatic activity, MnSOD mRNA expression, and MnSOD and GPx protein in LFS fibroblasts carrying a WT p53 allele when compared with homozygous mutant p53 isogenic cells. The CAT protein level was unchanged in these cells. We observed both the release of cytochrome C and Ca2+ from the mitochondria into the cytoplasm and an increased frequency of apoptotic cells after p53 induction in the TR9-7 cells that coincided with an increased expression of MnSOD and GPx, and the level of reactive oxygen species. The increase in apoptosis was reduced by the antioxidant N-acetylcysteine. These results identify a novel mechanism of p53-dependent apoptosis in which p53-mediated up-regulation of MnSOD and GPx, but not CAT, produces an imbalance in antioxidant enzymes and oxidative stress.

INTRODUCTION

An increase in the level of reactive oxygen species (ROS), termed as oxidative stress, is controlled by multiple, interacting, low and high molecular weight components. Among them, superoxide dismutase (SOD), glutathione peroxidase 1 (GPx), and catalase (CAT) play a central role (reviewed in Refs. 1, 2). The overall effect of the antioxidant system depends on the intracellular balance between these antioxidant enzymes rather than a single component (3). In the antioxidant enzyme system, SOD catalyzes the dismutation of superoxide radicals to H2O2, which is further metabolized to H2O and O2 by CAT and GPx (4). An imbalance in the coordinated expression/activity of antioxidant enzymes can lead to the generation of oxidative stress (3, 5). An overexpression of Cu-Zn SOD in mouse epithelial cells resulted in the sensitization to oxidative chromosomal aberrations and DNA strand breaks; however, a simultaneous increase in CAT or GPx corrected this hypersensitivity (5).

There are two forms of intracellular SOD in mammalian cells: (a) Cu,Zn-SOD, localized in the cytoplasm and nucleus; and (b) MnSOD, which is found in the mitochondrial matrix. MnSOD (SOD2) is localized to chromosome 6q25, a region that is frequently deleted in certain types of human cancer (6, 7). A decreased level of MnSOD has been reported in tumor cells (8). Overexpression of MnSOD in murine and human cell lines leads to a decrease in the colony-forming ability and a reduction in the tumor formation in athymic nude mice (9–12). Furthermore, both human and mouse cell lines with MnSOD overexpression have reduced growth rate, plating efficiency, and viability compared with the control cells (11, 13). In addition to a slower growth rate, MnSOD-overexpressing cells show increased sensitivity to oxygen radical treatment, and this sensitivity is abrogated by pyruvate, a H2O2 scavenger (14).

GPx is a selenoprotein and occurs in five different isoforms. GPx1 is the major isoform that metabolizes H2O2 to O2 and H2O. GPx overexpression has been found to block MnSOD-induced tumor growth inhibition (15). CAT is a tetramer enzyme with a ferrroporphyrin group in each subunit. Although GPx is found mainly in the cytosol and mitochondrial matrix, CAT is largely present in peroxisomes. The hypersensitivity of Cu-Zn SOD-expressing mouse epithelial cells to H2O2-induced DNA damage is corrected by CAT expression in the double transfectant (5). A decrease in the CAT activity has been found in a variety of animal tumors (16). Fibroblasts from cancer-prone Xerodermia-pigmentosum cases possess a 5-fold lower CAT activity and produces five times more H2O2 upon UV irradiation exposure when compared with cells from noncancer-prone trichothiodystrophy (17). Although both GPx and CAT decompose H2O2, their contributions vary depending on the amount and the site of H2O2 production (18, 19).

ROS has been implicated in the induction of apoptosis (18, 20, 21). Mitochondria is the major source of superoxide anion (O2·−) production in cells and ~1−2% of oxygen reduced by mitochondria are converted to O2·− (22). The O2·− is converted to H2O2 by MnSOD, which is present in the mitochondria. Although O2·− is primarily confined to the mitochondria, H2O2 is capable of passing through the mitochondrial membrane into the cytoplasm and the extracellular microenvironment (23). Excess of H2O2 that is not reduced by GPxs inside the mitochondria can cross the mitochondrial membrane into the cytosol and is reduced by CAT and GPx to H2O and O2. Another free radical, nitric oxide (NO·) can enhance the generation of O2·− and subsequent generation of H2O2 by inhibiting the cytochrome oxidase and thereby modulating ROS-mediated apoptosis (21). Increased oxidative stress causes the release of cytochrome C from mitochondria into the cytosol. Cytosolic cytochrome c binds to Apaf1 and activate caspase 9 in the apoptosis in response to diverse inducers of cell death (24–26). Cytochrome c is released from the intermembrane space of the mitochondria through openings in the outer membrane, which formed as a consequence of mitochondrial permeability transition, leading to the loss of the mitochondrial membrane potential (reviewed in Refs. 27, 28).
p53-induced apoptosis can be mediated by ROS. One mechanism is p53 regulation of the expression of genes that are related to cellular redox status and involved in apoptosis (Ref. 20; reviewed in Ref. 29). In the present study, we investigated the hypothesis that an oxidative stress induced from an imbalance between H₂O₂-producing MnSOD and the peroxide-removing enzymes, GPx and CAT, plays a role in the p53-mediated apoptosis.

MATERIALS AND METHODS

Cell Lines and Cell Culture. The human lymphoblast cell line, TK6 with wild-type (WT) p53, and the highly related pair, WTK1 with mutated (non-functional) p53 (30), were grown in RPMI medium, supplemented with 15% fetal bovine serum. The cell lines MDAH 041, MDAH 087, and MDAH 172 were derived from fibroblasts of patients with Li-Fraumeni syndrome (LFS; Ref. 31). These cell lines were kindly provided by Michael Tainsky (Case Western Reserve University, Cleveland, OH). The cells were grown in an atmosphere of 5% CO₂ in DMEM, supplemented with 10% fetal bovine serum. LFS fibroblast, (TR9-7), engineered to produce p53 only in the absence of tetracycline (32), was obtained from George Stark (Cleveland Clinic Foundation, OH). The cells were cultured in DMEM plus 10% fetal bovine serum and maintained in the presence of 600 μg/ml G418, 50 μg/ml hygromycin, and 3 μg/ml tetracycline. Under these conditions, p53 expression is undetectable.

Construction of pGL3-MnSOD and Reporter Gene Assay. The MnSOD promoter region was amplified by PCR using DNA, isolated from normal human fibroblasts, as a template. Using the long PCR kit (Perkin-Elmer, Branchburg, NJ), the 2.3-kb MnSOD promoter region (~2215 to +42) was amplified and cloned into the HindIII site of the basic luciferase reporter plasmid pGL3 (Promega, Madison, WI). The nucleic acid sequence of the cloned MnSOD promoter region was confirmed by using “Long distance sequencing” method as described by Hagiwara et al. (33). For transient transfection, TR9-7 cells were grown for 4 days with and without tetracycline. Cells were washed once in serum-free medium (Opti-MEM; Life Technologies, Inc., Rockville, MD) and cotransfected for 2 h in 3 ml of Opti-MEM containing 2 μg of pGL3-MnSOD plasmid, containing the MnSOD promoter linked to the luciferase reporter gene. 2 μg of β-galactosidase-expression plasmid pCMV-β-gal as transfection control, and 40 μl of Lipofectamine (Life Technologies, Inc.). After transfection, the medium was replaced with full growth medium with and without tetracycline. After 48 h of incubation, cells were lysed, and cellular extracts were prepared for luciferase and β-galactosidase assay. Promoter activity is expressed as luciferase activity in relative light units/mU β-galactosidase. Each experiment was repeated three or more times.

MnSOD and GPx mRNA Expression. For the analysis of MnSOD and GPx mRNA expression, 2 x 10⁵ TR9-7 cells were plated in a 10-cm dish in growth medium containing 3 μg/ml tetracycline. Forty-eight h after plating, the medium containing tetracycline was replaced by growth medium containing no tetracycline. In the control plates, the medium was changed with complete growth medium containing tetracycline. One, 2, 4, and 5 days after the withdrawal of tetracycline from the growth medium, the cells were lysed, and total RNA was prepared according to the procedure of Chomczynski et al. (34). Total RNA (10 μg) was used for Northern blot analysis using MnSOD, GPx, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) riboprobes (3, 35, 36).

MnSOD, GPx, CAT, and p53 Protein Expression. TR9-7 cells were cultured in the absence of tetracycline for 2, 4, and 5 days. In a control dish, cells were cultured in the presence of 3 μg/ml tetracycline. Other LFS fibroblasts were grown to 75–80% confluency. Protein lysate was prepared in radiomunoprecipitation assay buffer and subjected to Western blot analysis, as described earlier (37), using one of the following: anti-MnSOD monoclonal antibody (Chemi-Con International, Temecula, CA), anti-GPx monoclonal antibody (MBL Corporation, Boston, MA), anti-CAT (gift from Alain Baret (Nante, France) and Cortex Biochem (San Leandro, CA)), or anti-p53 polyclonal antibody CM-1 (Signet Pathology System, Dedham, MA).

MnSOD Enzymatic Activity Measurement. MnSOD activity was determined by activity gel assay (38). Cells were collected in cold PBS and sedimented at 1600 x g for 5 min. The cell pellet was then resuspended in 200 μl of 50 mM potassium phosphate buffer with a pH of 7.8 and followed by sonication for four 15-s bursts on a Branson sonicator B15 (position 2, continuous setting). Aliquots were removed from each sample to determine the protein concentration with the BCA reagent (Pierce, CA). Extracts were then centrifuged at 8000 x g for 5 min, and the supernatant was used. An aliquot of the cleared extract corresponding to 50 μg of protein was applied to a nondenaturing 10% polyacrylamide gel to localize MnSOD activity previously described (38) with the exception that no N,N,N',N'-tetramethylethylenediamine was used for staining.

RESULTS

Induction of MnSOD and GPx after Doxorubicin Treatment in TK6 Cells with WT p53. We examined the induction of antioxidant enzymes MnSOD, GPx, and CAT in TK6 and WTK1 cells, after

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treatment with doxorubicin, using microarray hybridization technique. We have earlier reported that treatment of TK6 cells (functional p53) with 0.5 μg/ml doxorubicin induces apoptosis in 39 ± 2% (mean ± SD) cells by 24 h; however, WTK1 cells (mutant p53) were completely resistant to the same treatment (40). Both MnSOD and GPx were found among the gene cluster showing increased induction in TK6 cells compared with WTK1 cells after 24 h exposure to doxorubicin (Supplemental Table 1). CAT mRNA expression was not found to be altered in this assay.

MnSOD, GPx, and CAT Expression in Isogenic Pairs of LFS Fibroblasts. To determine the effect of p53 on the up-regulation of MnSOD, we measured MnSOD expression and activity in LFS cell lines, carrying different p53 genotypes, as well as TR9-7 cells in which p53 expression is regulated by tetracycline. MnSOD activity, mRNA and protein expression, and GPx and CAT protein expression were compared in three pairs of LFS cell lines, expressing either WT p53, mutated p53, or were null for p53 (Fig. 1, A and B). The 041+/− cell line expresses only WT p53 because there is a frameshift mutation of one p53 allele at codon 184, whereas in 041−/− cells, the normal p53 allele is lost. The 041+/− cells showed more MnSOD mRNA and enzymatic activity, and MnSOD and GPx protein expression when compared with the p53-null 041−/− cells. Similar results were obtained with 087 cells. The 087+/− cells contain a codon 248Tp heterozygous mutation, whereas 087−/− cells lack the remaining WT allele. In 172+/− cells that contain a codon 175His heterozygous mutation, MnSOD mRNA expression is increased when compared with 172−/− cells that lack the remaining WT allele; however, no change in MnSOD enzymatic activity nor MnSOD and GPx protein expression was observed. These results indicate that MnSOD and GPx expression in LFS cells correlates with the p53 genotype. However, no change in CAT expression was observed in these isogenic LFS cells.

Regulation of MnSOD, GPx, and CAT Expression by p53. The promoters of human MnSOD and GPx, but not CAT, contain sequences that correspond to the consensus p53 binding element, as described earlier (Ref. 41; Fig. 1C). These p53 consensus binding sites are present in the absence of tetracycline in the presence of tetracycline, TR9-7 cells were cotransfected with pGL3-MnSOD-Luc and pCMV-βgal, as described in “Materials and Methods.” Cells were incubated in the presence or absence of tetracycline and used for the measurement of luciferase activity. Removal of tetracycline from the growth medium (induction of p53) increased the MnSOD promoter activity by 3-fold when compared with the controls in the presence of tetracycline in the growth medium (repression of p53; P = 0.002, Fig. 1D). A p21 reporter construct was used as a positive control, which resulted in a 5-fold increase in the promoter activity in the presence of p53 (P = 0.0001, Fig. 1E). These results indicate that p53 transactivates the MnSOD promoter. Similar results for GPx have been reported previously (42).

To further investigate the association between WT p53 and H2O2-generating MnSOD, we used TR9-7 cells, which express p53 in the absence of tetracycline in the culture medium. Fig. 2A compares MnSOD mRNA expression of both 1- and 4-Kb mRNA species, generated by alternate splicing and polyadenylation in TR9-7 cells grown either in the presence or absence of tetracycline. The induction of p53 increased MnSOD mRNA expression in a time-dependent manner, reaching a maximum after 5 days. As shown in Fig. 2C, MnSOD protein expression was positively correlated with the change in MnSOD mRNA expression. Furthermore, increased MnSOD expression was consistent with the increase in p53 induction. We also performed immunofluorescent staining for MnSOD in TR9-7 cells. Cells that were induced to express p53 by the removal of tetracycline for 4 days showed an intense staining pattern for MnSOD when compared with cells without p53 expression (Supplemental Fig. 1).

We next determined if p53 expression affects the expression of hydrogen peroxide-removing enzymes, GPx and CAT, which act downstream to MnSOD activity. The balance between hydrogen peroxide-producing MnSOD and peroxide-removing GPx and CAT can influence the availability of ROS in the cellular environment. We determined GPx mRNA and protein expression in TR9-7 cells in the presence (p53 suppressed) or absence of tetracycline (p53 induced; Fig. 2, B and C). We observed an increase in GPx mRNA and protein expression after the induction of p53 in a time-dependent manner. However, we did not find any change in CAT expression up to 5 days after p53 induction.

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Increased p53 Expression Induces ROS Production and Apoptosis. We examined the effect of p53 expression on apoptosis in TR9-7 cells. Cells grown in either the presence or absence of tetracycline were immunostained for p53 with polyclonal anti-p53 antibody CM-1 and followed by 4′,6-diamidino-2-phenylindole staining to score for apoptotic nuclei (Fig. 3A). Cells with condensed chromatin and fragmented nuclei were considered apoptotic. At least 100 cells, treated with or without tetracycline for 4 days, were examined for apoptosis. Cells that were grown in the absence of tetracycline showed positive p53 immunostaining and apoptotic nuclei when compared with the control cells grown in the presence of tetracycline. The addition of 5 mM N-acetyl cystein (NAC), an antioxidant, in the culture medium of cells expressing p53, decreased the number of p53-positive apoptotic cells. Fig. 3, B and C, compares the frequency of cells expressing p53 with the frequency of cells undergoing apoptosis. Sixty-eight percent of cells grown in the absence of tetracycline expressed p53 (Fig. 3B), and only 33% of all of the cells were apoptotic (P = 0.001, Student t test; Fig. 3C). Presence of NAC lead to a significant decrease in apoptosis (P = 0.002). Cells grown in the presence of tetracycline showed only a small level of background p53 staining (3%) as well as apoptosis (2%). These results indicate that the expression of p53 correlates with apoptosis and that ~45% of the p53-expressing cells undergo apoptosis.

To determine whether p53-induced apoptosis is associated with an increased production of ROS, we measured intracellular dichlorofluorescin (DCF)-fluorescence in TR9-7 cells, grown either in the presence (p53 repressed) or absence of tetracycline (p53 expressed). DCFH-DA diffuses into cells where it is deacetylated to DCFH, which fluoresces upon reaction with ROS. TR9-7 cells grown in the absence of tetracycline (p53 expressed) for 4 days showed strong DCF fluorescence (P = 0.0003, Fig. 4, A and B) when compared with cells in the presence of tetracycline, where only background fluorescence is detected. Using the NIH Image analysis software (39), relative DCF fluorescence was compared in ~60 cells, cultured in the absence or presence of tetracycline. The results in Fig. 4B show quantitatively increased DCF fluorescence in cells expressing p53 when compared with cells without p53 expression. To confirm that the increase in DCF fluorescence in p53-expressing cells was due to the increased production of ROS, we measured DCF fluorescence in the presence of an antioxidant, NAC. NAC treatment reduced the p53-induced increase in DCF fluorescence (P = 0.004). These data are consistent with the hypothesis that increased ROS production, after p53 expression in TR9-7 cells, can mediate p53-induced apoptosis.

CAT Overexpression Inhibited p53-Mediated Apoptosis in TR9-7 Cells. Infection with adeno-CAT increased the expression of CAT in TR9-7 cells at different time points (Fig. 5A). Nuclei stained with 4′,6-diamidino-2-phenylindole were analyzed for apoptosis at 24, 48, 96, and 120 h after infection. CAT overexpression significantly inhibited apoptosis at 96 and 120 h (P < 0.01; Fig. 5, B and C).

Cytochrome c Is Released from Mitochondria into the Cytosol after Induction of p53. We measured cytochrome c expression both in mitochondria and cytosol, after p53 induction in TR9-7 cells (Supplemental Fig. 2). The complete release of cytochrome c from the mitochondria 3 days after removal of tetracycline corresponded with an increase of cytochrome c in the cytosol.

Ca2+ Mobilization Occurs Late in p53-Induced Apoptosis. To examine the question whether Ca2+ release from the mitochondria was the cause or the consequence of p53-induced oxidative stress and apoptosis, we measured intracellular calcium concentration Ca2+ as a function of time after p53 induction. As shown in Supplemental Fig. 3, Ca2+ began to increase by day 3 after the removal of tetracycline and induction of p53 and continued to rise past the last measurement at day 4. This result, combined with our observation that ROS production is high 2 days after p53 induction, indicates that changes in mitochondrial calcium homeostasis occur as a consequence of ROS generation at a later stage in apoptosis rather than participating in its initiation.

DISCUSSION

Several agents that induce p53-dependent apoptosis also generate oxygen radical species, which is consistent with the hypothesis that ROS might play a critical role in p53-mediated apoptosis (20, 43). A model for p53-dependent apoptosis was proposed (20), consisting of (a) the transcriptional induction of redox-related genes, p53-induced genes; (b) the formation of ROS; and (c) oxidative degradation of mitochondrial components. The electron transport chain in the mitochondria is the major source of intracellular ROS production, which makes it likely that alterations in mitochondrial function are responsible for the increase in ROS production during p53-induced apoptosis. In support of a role of mitochondrial ROS generation in p53-induced apoptosis, it was shown that bongkrekic acid, an inhibitor of mitochondrial permeability transition, abolished p53-induced apoptosis and that cells undergoing p53-mediated apoptosis showed increased mitochondrial lipid peroxidation (20). Furthermore, p53-dependent apoptosis has been shown to be associated with the loss of mitochondrial membrane potential and increased ROS production (43).

One mechanism for the increased production of ROS is the imbalance in the antioxidant enzyme system. Both MnSOD and GPx showed a 2-fold higher induction in the TK6 (functional p53) cell line after 24 h of doxorubicin treatment when compared with WTK1 (mutant p53). However, CAT induction was not observed in the expression analyses. The p53-dependent increase in GPx expression in our study is consistent with the earlier finding (42). Earlier studies have shown that genes transactivated by p53 contain the consensus p53 binding element (41). We identified potential p53 consensus binding sites in human MnSOD and GPx promoters. When we transfected the MnSOD-luciferase promoter construct into TR9-7 cells, we
found a 3-fold increase in the MnSOD promoter activity after p53 expression, in the absence of tetracycline, when compared with the control cells that do not express detectable p53. p53 expression and the activation of a downstream target was also confirmed by transfecting a p21/waf1-reporter construct, containing consensus p53 binding element. p53-induced up-regulation of luciferase activity, under the control of the GPx promoter, has been reported previously (42). Our result, showing the up-regulation of human MnSOD promoter activity by p53 in human lymphoblastoid cells and fibroblasts, contrasts a recent study that describes down-regulation of rat MnSOD promoter activity by p53 in human MCF-7 cancer cells (44). Although, the rat MnSOD promoter shows 36% similarity with the human MnSOD promoter, it contains only one-half of the consensus p53 binding sequence. Furthermore, overexpression of p53 in rat smooth muscle cells did not generate ROS, whereas human smooth muscle cells showed strong DCF fluorescence generated from the oxidative stress (45). Also, transient transfection of HeLa cells with WT p53 showed a reduction in the MnSOD level (46). These results indicate that p53-mediated production of ROS may be cell type and species dependent.

We found that TR9-7 cells, when grown in the absence of tetracycline (p53 expressed), showed strong DCF fluorescence, a marker of increased oxidative stress when compared with cells in the presence of tetracycline (p53 suppressed). These data support the earlier findings that an imbalance in the levels of antioxidant enzymes can lead to the production of oxidative stress (3, 5) and are consistent with our hypothesis that p53-induced up-regulation of MnSOD and GPx, without any alteration in CAT, can produce oxidative stress in the cells. When we induced p53 in TR9-7 cells, ~33% of the cells expressing p53 underwent apoptosis. However, the number of apoptotic cells were reduced substantially in the presence of an antioxidant, NAC, which supports the functional role of ROS in p53-mediated apoptosis in the present study. NAC has been found to react with H₂O₂ but not O₂⁻ (47). CAT treatment, which degrades H₂O₂ to water and molecular oxygen, inhibits p53-mediated apoptosis in human smooth muscle cells (45). It can be argued that CAT plays a significant role in reducing p53-mediated oxidative stress, thus inhibiting apoptosis. In most cells, mitochondria lack CAT, and the H₂O₂ that is generated inside mitochondria can be degraded by GPx. However, the activity of GPx depends on the level of glutathione and NADPH and may not be sufficient for complete removal of H₂O₂ under oxidative stress. Excess H₂O₂ can cross the mitochondrial membrane and can be degraded by CAT. Engineered HepG2 cells overexpressing CAT in the mitochondria are protected from oxidative injury by H₂O₂ (48). CAT-overexpressing thymocytes also are resistance to dexamethasone-induced apoptosis (49). Furthermore, ceramide-induced inhibition of CAT and increased oxidative stress causes apoptosis in myeloid cells treated with vesnarinone, an inotropic agent used for treating heart failure (50). CAT overexpression also inhibited Vp16 or mitomycin C-induced apoptosis in HepG2 cells by decreasing phosphorylation and accelerating the degradation of p53 (51). In the present study, we tested the protective role of CAT in oxidant-induced apoptosis by its overexpression in TR9-7 cells. Consistent with the hypothesis, the overexpression of CAT significantly

Fig. 3. p53 and 4′,6-diamidino-2-phenylindole (DAPI) staining of TR9-7 cells (A). Cells that were grown in the absence of tetracycline showed positive p53 immunostaining and apoptotic nuclei when compared with the control cells grown in the presence of tetracycline. The addition of N-acetyl cysteine (NAC), an antioxidant, in the culture medium of cells expressing p53, decreased the number of p53-positive apoptotic cells. Quantitation is presented as the percentage of p53-expressing cells (B) and the percentage of apoptotic cells (C) for 600 cells counted.
reduced p53-mediated apoptosis. These and other observations propose a protective role of CAT in oxidant-mediated apoptosis. Based on our results, showing an increase in MnSOD and GPx levels, but not in the CAT level, after p53 expression leading to the production of ROS and apoptosis, it can be hypothesized that an increase in GPx alone (without CAT) does not suffice for the degradation of H$_2$O$_2$ produced by MnSOD. Furthermore, apoptosis induced by H$_2$O$_2$ and oxidized low-density lipoprotein in macrophages is associated with increased expression of p53 and MnSOD (52).

Apoptosis, induced by a variety of stimuli, coincides with the release of cytochrome c from mitochondria into the cytoplasm where it activates a cascade of proteolytic enzymes crucial for the execution of apoptosis (20, 45). Our results are consistent with the previous studies showing that p53 induction leads to the release of cytochrome c from mitochondria into the cytosol (53). Cytochrome c release is a necessary intermediate in the p53 activation of downstream apoptotic targets as shown in a recent study, demonstrating that caspase-9 and its cofactor, Apaf-1, are essential downstream components of p53 in myc-induced apoptosis (54). Cytochrome c has been shown to be essential for the Apaf-1 binding to procaspase-9 promoting its dimerization and activity by autocatalysis (55, 56). Furthermore, p53 transcriptionally transactivates Apaf-1 (40, 57), which is a major downstream component in the p53-mediated apoptotic pathway (58). Although, p53-mediated apoptosis involves the generation of ROS, the role of MnSOD may appear to be contradictory in the literature. One scenario is that the decrease in the MnSOD level leads to oxidative stress from the accumulation of superoxide anion (O$_2^-$), which can mediate the p53-dependent apoptotic pathway (44). Other studies have suggested that a decrease or inhibition of MnSOD expression leads to the reduction in apoptosis (52), and its induction is associated with apoptosis (59). All-trans-retinoic acid induces MnSOD expression and apoptosis in acute myeloblastic leukemia cells (60). Overexpression of c-myc or E2F-induced ROS accumulation and enhanced serum-deprived apoptosis in NIH 3T3 and Saos2 cells (61). In the same study, E2F1 was found to suppress ROS-induced MnSOD expression by inhibiting nuclear factor-$\kappa$B activity (61). However, it is not clear from this study how an increase in MnSOD expression relieves the cells from total ROS accumulation without any alteration in the peroxide removing enzymes. Furthermore, overexpression of c-myc, followed by the generation of ROS, DNA damage, and p53 accumulation in normal human fibroblast did not affect apoptosis. None of the studies, thus far, have addressed the imbalance of antioxidant enzymes that determines the generation of oxidative stress associated with p53-mediated apoptosis. As discussed earlier, the study of a single component of the antioxidant system does not yield a clear understanding of the overall effect. Although many antioxidant components are involved in the regulation of cellular redox status, in the present study, we have addressed this issue by investigating the regulation of MnSOD, GPx, and CAT expression by p53 and showed that their imbalance leads to oxidative stress and apoptosis. However, the degree of contribution and interdependence of several different redox-related genes in p53-induced apoptosis has yet to be determined. The imbalance in the antioxidant cascade described here, the previous studies demonstrating p53 regulation of genes encoding proteins that cause mitochondrial depolarization, and the release of cytochrome c or an essential component of the apoptosome, i.e., Apaf1, are all consistent with oxidative stress as the major mechanism of p53-dependent apoptosis.

**Fig. 4.** The production of ROS was determined by dichlorofluorescin (DCF) fluorescence. DCF fluorescence was measured with a confocal microscope equipped with a laser at an excitation wavelength of 488 nm and emission wavelength of 525 nm (A). DCF fluorescence was quantified with the “NIH Image Analysis” software. B shows the mean of fluorescence of 50–100 cells.

**Fig. 5.** Expression of CAT at different time points in TR9-7 cells following infection with Adeno-CAT expression vector or Adeno-GAL control in the absence of tetracycline (A). Immunocytochemical staining of TR9-7 cells infected with either Adeno-CAT or Adeno-GAL control (B). CAT overexpressing cells show significant inhibition of apoptosis after 96 and 120 h.
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