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Deregulated Manganese Superoxide Dismutase Expression and Resistance to Oxidative Injury in p53-deficient Cells[1]

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ABSTRACT

Loss of function of the tumor suppressor protein p53 represents a very frequent event in human carcinogenesis, but the molecular mechanisms linking impaired p53 activity to increased cell malignancy are still incompletely understood. p53 is normally involved in both cell cycle control and the induction of cell death and is involved in the latter mainly through the transcriptional regulation of pro- and antiapoptotic proteins. Reactive oxygen species are known to be powerful inducers of p53 activity; moreover, they play a role in the execution of p53-dependent apoptosis. Here we show that transformed mouse fibroblasts lacking p53 are significantly more resistant than wild-type (wt) controls to the cytotoxic effect of a number of pro-oxidant treatments. Interestingly, these cells also exhibit deregulated expression of the antioxidant enzyme manganese superoxide dismutase (MnSOD), a protein known to protect cancer cells from the oxidative injury inflicted by antitumoral cytokines and anticancer drugs. MnSOD activity was also increased in liver tissue from p53-deficient mice in comparison with wt tissue. Transient transfection of wt *p53* in HeLa cells led to a significant reduction in steady-state MnSOD mRNA levels and enzymatic activity, confirming that the expression of this antioxidant enzyme is negatively regulated by p53. Forced expression of MnSOD rendered HeLa cells resistant to p53-dependent cytotoxic treatments and, in cotransfection experiments, counteracted the growth-inhibitory effect of p53. Taken together, these data identify MnSOD as a potential target for tumor suppressor protein p53 and underscore the relevance of MnSOD modulation in the context of normal p53 functions because it is consistent with many reports of abnormally increased MnSOD expression in human cancers.

INTRODUCTION

Oxygen radicals are among the most recognized and widely investigated mediators of cell damage (1). Chemical species such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^•$), and nitric oxide (NO) derived from incomplete reduction of molecular oxygen are highly reactive toward key constituents of living cells, including DNA, proteins, and lipids. Genotoxicity, protein denaturation, compromised enzymatic activities, and lipid peroxidation all represent consequences of cell exposure to excess amounts of ROS,[3] a condition usually indicated as oxidative stress. Both exogenous oxygen radicals and oxygen species endogenously derived as a byproduct of mitochondrial respiration, drug metabolism, or any other intracellular redox reaction can exert deleterious effects on cell function and viability, depending on cellular antioxidant defenses and capability to repair oxidative damage (2). Accordingly, a role for ROS has been postulated in the genesis of a large number of human and experimental pathological processes, including chronic inflammation, neurodegeneration, tissue aging, and cancer (3, 4).

Massive cell oxidation leads easily to cell death through a necrotic process in which a main role is played by bioenergetic failure, ATP depletion, and loss of cell membrane integrity. However, milder oxidative insults can also induce cell death, which is mediated mainly by apoptosis. This observation, together with many findings on the increase of intracellular ROS during apoptosis and on the protective effects of antioxidant species in most cellular models of programmed cell death, recently led to the hypothesis that ROS may play a central role in determining the fate of cells committed to die (5, 6). This view has been further strengthened by the discovery of the role of mitochondria and mitochondrial factors [such as cytochrome *c* (7) and apoptosis-inducing factor (8)] in the triggering of the apoptotic process. As an early event after cell exposure to apoptogenic stimuli (including oxidants), cytochrome *c* is released by the mitochondria to the cell cytosol, where it contributes to the activation of the caspase cascade. A direct consequence of cytochrome *c* translocation is the interruption of the respiratory chain, leading to excess production of superoxide anion [O_2^- (9)], which in turn might contribute to mitochondrial damage and accelerate the release of proapoptotic substances. ROS are therefore produced by mitochondria in the early phases of the apoptotic process and are not necessary for apoptosis triggering but likely play a central role in amplifying the catastrophic cascade of events ultimately leading to cell collapse. The general cytoprotective effects of antioxidant species in most models of apoptosis are in agreement with this view.

MnSOD, a M_r 85,000 tetrameric enzyme located in the mitochondrial matrix, is the principal scavenger for superoxide in mitochondria (10). Consistent with the idea of a role for mitochondrial O_2^- in amplifying and accelerating cell response to apoptotic stimuli, this enzyme has been shown to be induced by and protect from a number of cytotoxic and proapoptotic agents, including inflammatory cytokines tumor necrosis factor (11) and interleukin 1, UV and ionizing radiation, anticancer drugs (12), and pesticides (Paraquat; Ref. 13). We have recently shown that MnSOD overexpression in HeLa cells also confers resistance to cell death induced by serum deprivation (14). This evidence, together with recent reports of reduced survival and mitochondrial dysfunction in mice genetically deprived of MnSOD (15), supports the notion that SOD2 acts mainly as a survival protein that is required to maintain mitochondrial integrity in cells exposed to adverse conditions. Consistent with this interpretation, we and others have described an increased expression of this enzyme in certain classes of tumors, such as brain tumors (16), thyroid tumors (17), and colon neoplasm (18) in comparison with corresponding normal tissues.

ROS have recently been indicated as downstream mediators of the tumor suppressor protein p53. This nuclear phosphoprotein, whose function is very often lost in human cancers (19), normally acts as a transcription factor, promoting the expression of a number of genes by binding to a specific consensus DNA sequence (20, 21) and repressing the expression of some others, especially those harboring TATA (22)

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[3] The abbreviations used are: ROS, reactive oxygen species; MnSOD, manganese superoxide dismutase; SOD2, superoxide dismutase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CMV, cytomegalovirus; GFP, green fluorescent protein; CuZnSOD, copper/zinc-dependent, cytosolic superoxide dismutase; wt, wild-type; RT-PCR, reverse transcription-PCR; Ab, antibody; DCF-DA, dichlorodihydrofluorescein diacetate.

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or CAAT (23) boxes or SP-1 (24) binding sequences in their promoter regions, mainly through protein-protein interactions with the corresponding DNA binding factors.

A number of stimuli can trigger p53 activation. DNA damage activates p53 by phosphorylation and increased resistance to degradation (25, 26). Furthermore, a number of "nongenotoxic" stresses, such as heat shock and hypoxia (27, 28), can up-regulate p53 function; hypoxia induces *p53* stabilization by the hypoxia-induced factor α (29). The main physiological outcomes of p53 activation, cell growth arrest and apoptosis, justify its tumor suppression activity and reflect the effect of p53 on the transcriptional activation/repression of a multiplicity of target genes.

At present, whereas many p53 target genes (such as *p21/Waf1*, *GADD45*, *cyclin G*, and *MDM2*) involved in the inhibitory effects of the tumor suppressor protein on cell cycle progression have been identified, the mechanisms mediating p53-induced apoptosis are still incompletely understood. Recently, convincing evidence has shown that p53 activation is accompanied by a net increase in intracellular ROS concentration and that the removal of oxygen radicals by antioxidant drugs impedes apoptosis induced by p53 (30, 31). These data suggest that oxygen radicals may play an important role in the apoptotic process triggered by p53, a role that is also supported by the observation that many of the proteins transcriptionally regulated by p53 are somehow involved in redox metabolism (30). To date, however, very little is known about the molecular mechanisms linking p53 activation to cell damage by oxygen radicals and, ultimately, to programmed cell death.

Here we show that in p53-deficient cells, resistance to oxidative stress correlates with increased expression of the mitochondrial scavenger MnSOD and that this enzyme is negatively regulated at a transcriptional level by p53. Because MnSOD is also able to prevent the induction of apoptosis by p53, these data outline a novel mechanism whereby down-regulation of the survival protein MnSOD links p53 function to oxidative mitochondrial damage and cell death.

MATERIALS AND METHODS

Chemicals and Abs. MTT, Adriamycin, and Paraquat-dichloride were obtained from Sigma Aldrich. G418 and Trizol were purchased from Life Technologies, Inc., and dichlorodihydrofluorescein diacetate was obtained from Molecular Probes (Eugene, OR). Anti-MnSOD and Anti-CuZnSOD Abs were purchased from Calbiochem. Anti-p53 monoclonal Abs pAb240 and DO-1 were obtained from Santa Cruz Biotechnology.

Cell Lines, Plasmids, and Animals. Cervical adenocarcinoma HeLa cells (purchased from the American Type Culture Collection) and p53+/+ and p53−/− E1A/Ras-transformed fibroblasts (a generous gift of Dr. Scott Lowe, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) were maintained in RPMI 1640 and DMEM, respectively, supplemented with 10% FCS (Eurobio, Les Ulis Cedex B, France), antibiotics, and L-glutamine (Life Technologies, Inc.) at 37°C in 5% CO_2.

Human MnSOD cDNA (excised by *Eco*RI) and human p53 (excised by *Bam*HI) were obtained from the American Type Culture Collection and cloned into PcDNA3/zeo (zeocine resistance) and PcDNA3/neo (neomycin resistance; Invitrogen), respectively. Mutant p53 (C53–175) in pCMV-neo and the synthetic p53-responsive promoter PG13 were generous gifts of Dr. B. Vogelstein (Howard Hughes Medical Institute, Baltimore, MD). The CMV-GFP and promoterless-GFP plasmids were from Clontech.

p53+/+ and p53−/− C57Bl6-J mice (Jackson Laboratories) used as a source of liver tissue were maintained in the local animal facility.

Cell Transfection. The p53/Neo construct (0.2 μg) or the corresponding empty vector (0.2 μg) was transfected into 50–60 $\times$ 10^4 HeLa cells in a 24-well plate using Effectene (Qiagen, Valencia, CA) according to the manufacturer's recommendations. After 48 h, cells were harvested, and total RNA was extracted with Trizol (Life Technologies, Inc.). Average transfection efficiency was around 40%, as assessed by cytofluorometric analysis of cells transfected with an equivalent amount of a CMV-GFP construct (Clontech).

Stable cotransfection of HeLa cells with p53 (0.2 μg) and MnSOD (0.8 μg) was performed in duplicate in a 24-well plate using the MnSOD/zeo and p53/neo constructs. Empty vectors were used as appropriate controls. Each well received a total amount of 1 μg of DNA.

The HeLa subclone A12, which overexpresses human MnSOD under the control of the CMV promoter, has been described previously (14).

p53 Reporter Assay. To assess the transcriptional activity of p53 in HeLa cells, a reporter plasmid was created by cloning the synthetic p53-dependent promoter PG13 in the *Hin*dIII site of a promoterless GFP vector (pEGFP-1; Clontech). The reporter construct was cotransfected with wt p53, mutant p53, or empty pCDNA3 in HeLa as described above. After 24 h, cell fluorescence was assessed by flow cytometry. A CMV promoter-driven GFP vector was used as a positive control.

mRNA Evaluation. Levels of MnSOD and actin mRNAs were evaluated by RT-PCR using a kit from Promega according to the manufacturer's recommendations. Primer sequences were as follows: (*a*) MnSOD forward primer sequence, 5′-GGTAGCACCAGCACTAGCAG-3′; (*b*) MnSOD reverse primer sequence, 5′-CTGCAGTACTCTATACCACTACA-3′; (*c*) β-actin forward primer sequence, 5′-TGAGGCTCTTTTCCAGCCTT-3′; and (*d*) β-actin reverse primer sequence, 5′-CTAGAAGCACTTGCGGTGCA-3′.

Protein Expression. MnSOD activity was evaluated by an "in gel" SOD assay on 50 or 100 μg of total protein lysate as described by Beauchamp and Fridovich (32). Immunoreactive MnSOD, CuZnSOD, and p53 were assessed by standard Western blotting analysis of mitochondrial (MnSOD) or total (CuZn, p53) protein lysates, using the appropriate Abs (see above) followed by enhanced chemiluminescence detection. Mitochondria were obtained as described previously (33).

Analysis of ROS Generation. Intracellular ROS concentration under different growth conditions was assessed by flow cytometry on cells loaded with the oxygen radical-sensitive probe dichlorodihydrofluorescein diacetate (Molecular Probes). The dye was added to cell cultures 30 min before analysis, at a concentration of 10 μg/ml. Cells were then trypsinized, and green fluorescence (FL-1 channel, 520 band-pass filter) was analyzed immediately with a Coulter Epics flow cytometer equipped with a 480 nM emission argon laser. Averages $\pm$ SD of mean cell fluorescence in duplicate experiments were calculated.

MTT Reduction Test for Cell Toxicity. To assess cell survival after different cytotoxic treatments, cells were seeded at 4 $\times$ 10^4 cells/well (E1A-Ras fibroblasts) or 2 $\times$ 10^4 cells/well (HeLa cells) in 96-well plates in the presence of the indicated amounts of FCS, Adriamycin, and Paraquat. Forty-eight h later, surviving cells were stained with MTT as described by Mossman (34).

Selection of G418-resistant Cells. Effects of transfected MnSOD and p53 on HeLa cell growth potential were evaluated by counting neomycin-resistant cells after transfection of the selection marker alone or with *p53*, *MnSOD*, or both genes. After 2 weeks of selection in medium containing 800 μg/ml G418, live cells were trypsinized and counted.

RESULTS

Oncogene-transformed mouse fibroblasts easily undergo programmed cell death in response to a multiplicity of cytotoxic stimuli. Convincing evidence exists that this process is dependent, at last to some extent, on the function of the tumor suppressor protein p53 (35–37). Consistent with these reports, the absence of p53 significantly improved the survival of E1A-Ras-transformed fibroblasts in response to serum deprivation, oxygen radicals producing the pesticide Paraquat, and the alkylating agent Adriamycin (Fig. 1), as assessed by the MTT reduction test on cells derived from either wt p53 or p53-null mice and exposed to scalar strengths of the above-mentioned treatments. However, protection was not complete, and the effect of p53 was less evident at the highest levels of cell insult.

Fibroblast exposure to serum-free medium and, albeit to a lesser extent, treatment with Adriamycin or Paraquat were accompanied by a significant increase in the intracellular concentration of ROS, as revealed by cytofluorometric analysis of cells loaded with the ROS-sensitive fluorescent probe DCF-DA (Fig. 2).

No significant differences in intracellular ROS content were ob-

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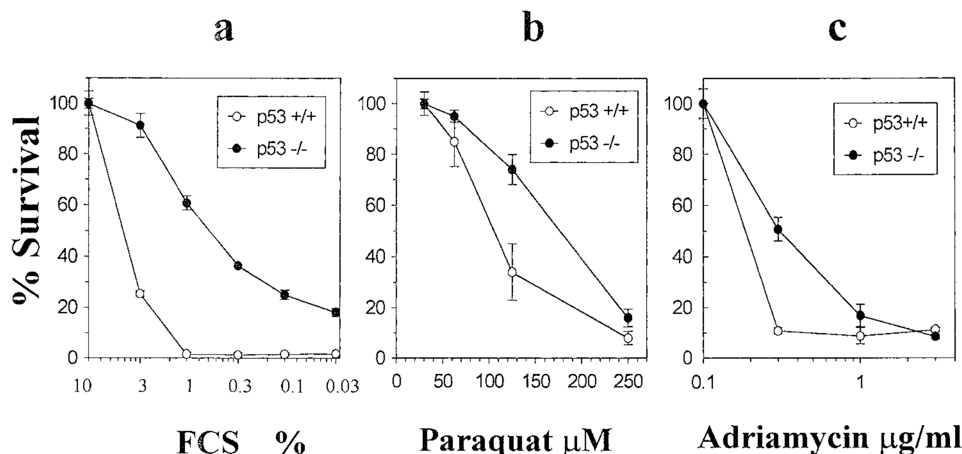

Fig. 1. Resistance of p53-deficient fibroblasts to the cytotoxic effects of pro-oxidant stimuli. p53+/+ (clone C8, ○) and p53−/− (clone A9, ●) E1A/Ras-transformed fibroblasts were seeded at 40,000 cells/well in a 96-well plate in the presence of decreasing amounts of FCS (*a*) or increasing concentrations of either Paraquat (*b*) or Adriamycin (*c*). Forty-eight h later, cytotoxicity was evaluated by MTT reduction test as described by Mossman (34). Values are the mean ± SD of triplicate cultures. Each *panel* is representative of at least two independent experiments.

served between wt and p53−/− cells. It should be noted that the production of oxygen radicals was observed 16 h after the beginning of treatment, *i.e.*, before morphological signs of cell death could be appreciated.

Because oxygen radicals are almost universal mediators of cell damage (1), and evidence exists that p53 is involved in the apoptotic response to oxidative stress (37), these findings are consistent with a causal role for ROS in the cytotoxic effects observed on oncogene-transformed fibroblasts, and by extension, with the idea that the relative resistance in p53-deficient cells may depend, at least in part, on increased cell resistance to oxidative stress.

We have reported recently that overexpression of the mitochondrial superoxide scavenger MnSOD increases the resistance of HeLa cells to apoptosis on serum withdrawal. This observation contributed to the idea that MnSOD may act as a survival protein in maintaining cellular and mitochondrial integrity under adverse growth conditions, a role already recognized for other mitochondrial proteins such as bcl-2 and bcl-X_L (38). MnSOD has also been reported to protect mouse fibroblasts from Paraquat cytotoxicity (13) and cardiomyocites from the adverse effect of Adriamycin (39), probably by removing harmful

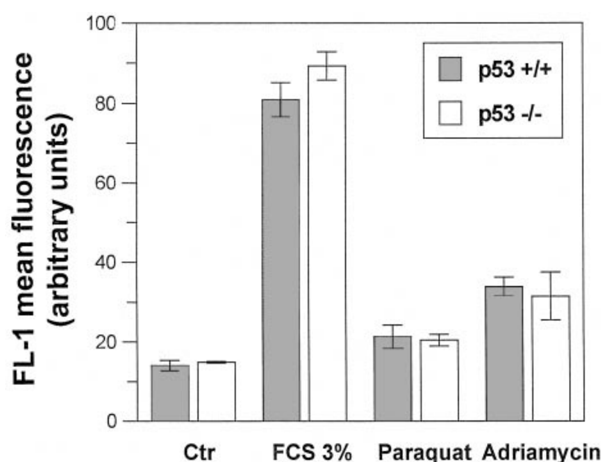

Fig. 2. Increased intracellular concentration of ROS by treatment with serum starvation, Paraquat, and Adriamycin. DCF-DA-loaded p53+/+ fibroblasts were tested for oxygen radical production as described in "Materials and Methods." Cells (p53+/+, ▨; p53−/−, □) were exposed to the indicated stimuli (3% FCS, 0.3 μg/ml Adriamycin, and 125 μM Paraquat) for 16 h, followed by 30-min loading with 5 μg/ml DCF-DA, trypsinization, and cytofluorometric analysis. Values are the mean ± error spreading of duplicate samples. Increases in intracellular ROS after different treatments were all significant (at least $P < 0.5$, Student's *t* test), whereas differences between p53+/+ and p53−/− cells where not significant.

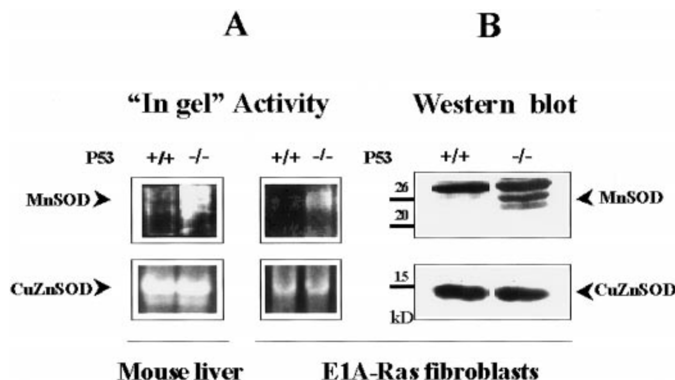

Fig. 3. Deregulated expression of MnSOD in p53−/− cells. *A*, MnSOD activity in E1A/Ras fibroblasts and mouse liver protein extracts was assessed by an in gel SOD assay, as described by Beauchamp and Fridovich (32), in the presence of 1 mM $MnCl_2$. The *broad white band* corresponding to MnSOD is indicated by the *arrow*. *B*, anti-MnSOD immunoblot of protein lysates from p53+/+ and p53−/− fibroblasts. Mitochondria were purified as described in Ref. 33. Mitochondrial proteins (100 μg) were loaded in each lane. MnSOD appears as a doublet band immediately below the M_r 26,000 marker and is indicated by the *arrow*. The *top band*, which is present in both p53+/+ and p53−/− samples, is nonspecifically stained by rabbit IgG. *Bottom panel*, anti-CuZnSOD immunoblot analysis of total protein lysates (100 μg protein/lane).

oxygen radicals generated by the intracellular metabolism of these drugs.

The above-mentioned considerations, together with the finding of increased resistance of p53-defective cells to typical superoxide-producing cytotoxic treatments, prompted us to investigate SOD2 expression in wt and p53-deficient cells.

An in gel SOD assay as described by Beauchamp and Fridovich (32) was used to assess SOD2 activity in protein extracts from wt and p53−/− ras-transformed mouse fibroblasts. As shown in Fig. 3*A*, MnSOD activity, seen as an unstained area in the context of a dark nondenaturing gel, was significantly higher in p53-deficient cells than in wt controls. This difference was mirrored by the content of immunoreactive protein, which was evaluated by Western blot analysis of protein lysates obtained from purified mitochondria (Fig. 3*B*). No significant differences were found in the expression of CuZnSOD (Fig. 3*B*) or of the antiapoptotic protein bcl-2 between p53+/+ and p53−/− cells (data not shown). These data suggest that MnSOD enzyme is selectively up-regulated in p53-deficient cells, consistent with the finding of increased resistance of these cells to the cytotoxic effects of ROS-producing stimuli.

MnSOD expression is strongly affected by cell transformation (40, 41), and evidence also exists that its cellular content may vary according to the phase of the cell cycle (42). MnSOD up-regulation in

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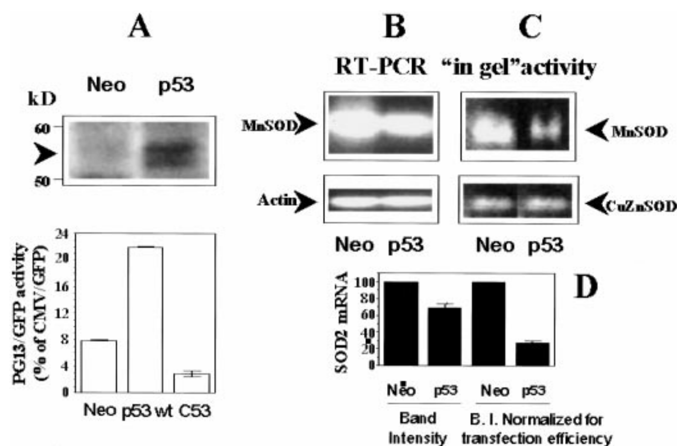

Fig. 4. MnSOD down-regulation by p53. HeLa cells (50–60 × 10^4 cells/well in a 24-well plate) were transiently transfected with either wt p53 (200 ng) or empty vector (pCDNA3, 200 ng), and MnSOD expression was evaluated 48 h later. *A*, expression of transfected p53 in HeLa cells. *Top*, p53 immunodetection in total protein lysates from neo- and p53/neo-transfected cells. *Bottom*, PG13-GFP reporter construct *trans*-activation by endogenous and transfected (C53, transcriptionally inactive dominant negative) p53. *B*, RT-PCR of MnSOD transcript from mock- and p53-transfected HeLa cells. The MnSOD band is 40% reduced in the p53-transfected sample. The actin band is indicated as a RNA loading control. *C*, MnSOD activity in mock- and p53-transfected HeLa cells. SOD enzymogram was performed as described in the Fig. 3 legend, except that $MnCl_2$ was omitted. The MnSOD and CuZnSOD activity bands are indicated. Transfection efficiency was about 40%, as assessed by GFP expression. *D*, densitometric analysis of MnSOD mRNA bands in neo- and p53-transfected HeLa cells. Values are the mean ± SD of two independent experiments. Normalization for the efficiency of transfection was calculated as follows: (percentage decrement of band intensity/percentage of transfected cells) × 100.

p53-deficient cells was also observed in p53−/− normal liver in comparison with wt tissue (Fig. 3*A*), suggesting that this difference is not limited to transformed cells and is not likely to reflect different cell distribution through the cell cycle but is directly linked to the functional status of p53.

To further confirm that the negative control of p53 on MnSOD expression represents a general regulatory mechanism, SOD2 activity was evaluated in a number of human cancer cell lines with different p53 functional status. This analysis revealed that MnSOD content is in an inverse correlation with p53 function, with the enzyme being expressed at considerable levels in cells in which p53 is either mutated (HT29 colon carcinoma) or virtually absent (HeLa cervical carcinoma) but barely detectable in cell lines expressing high amounts of wt p53 such as the breast carcinoma MCF7 cell line (Ref. 27; data not shown). In HeLa cells, in particular, very low p53 expression is due to protein degradation driven by human papillomavirus protein E6 (43). However, these cells retain normal responsiveness to p53-dependent regulatory mechanisms and rapidly undergo apoptosis when p53 is artificially overexpressed by transfection (44, 45). Consistent with a previous report (46), HeLa cells transiently transfected with wt p53 cDNA display p53 protein accumulation and increased activity of a p53-dependent reporter construct, despite accelerated p53 turnover in this cell line (Fig. 4*A*, *top* and *bottom panels*). Concomitantly, SOD2 mRNA was significantly reduced in comparison with mock control (Fig. 4*B*). Densitometry of the RT-PCR SOD2 bands consistently revealed a 30–40% decrease of the steady-state MnSOD messenger level; the percentage of inhibition becomes at least 70% when normalized for the efficiency of cell transfection, which was reproducibly around 40%, according to GFP expression (Fig. 4*D*.) This finding therefore supports the hypothesis that MnSOD expression is negatively regulated at a transcriptional level by tumor suppressor protein p53. Accordingly, MnSOD activity was also significantly decreased in HeLa cells by p53 overexpression as assessed by in gel SOD assay, whereas no obvious effect was observed on the activity of cytosolic CuZnSOD (Fig. 4*C*).

If down-regulation of MnSOD plays a role in the biochemical cascade linking activation of p53 to cell death, forced overexpression of this enzyme is expected to interfere with p53-dependent cytotoxicity and therefore mimic, at least to some extent, the loss of functional p53. Exposure of HeLa cells to serum deprivation, Paraquat, and Adriamycin led to intracellular accumulation of p53, suggesting that p53 is also involved in the physiological response to oxidative injury in these cells (Fig. 5*B*). When challenged with the above-mentioned stimuli, HeLa cells overexpressing MnSOD showed significantly improved survival in comparison with mock-transfected cells (Fig. 5, *A* and *C*). Intriguingly, this difference closely resembles the effect of p53 deletion on the survival of E1A-Ras-transformed fibroblasts (Fig. 1).

To directly assess the consequences of MnSOD overexpression on the growth-inhibitory effects of tumor suppressor protein p53, human wt p53 cDNA was cloned into a vector containing the neomycin resistance selection marker (neo) and transfected into HeLa cells together with an excess of human MnSOD cDNA or the corresponding empty vector; after 2 weeks of selection in neomycin-containing medium, cell survival was evaluated by simple counting of live cells. In agreement with previous reports (45), p53 overexpression resulted in a significant decrease in HeLa cell survival in comparison with the empty vector control. Cotransfection of MnSOD cDNA significantly improved cell resistance to p53 transfection (Fig. 6), suggesting that this mitochondrial superoxide scavenger can protect cells from p53-induced growth suppression/apoptosis, albeit with some variability among different experiments. MnSOD cDNA alone did not affect cell selection in G418-containing medium (Fig. 6), or significantly influence the efficiency of p53 transfection, as assessed by Western blot analysis 48 h after transfection (data not shown). However, it should be noted that the protective effect of MnSOD was not complete (Fig. 6) and was not sufficient to allow the generation of stable clones overexpressing p53. Despite these limitations, the beneficial effect of MnSOD on survival of p53-transfected cells confirms that ROS are not only potent inducers but are also downstream effectors of p53

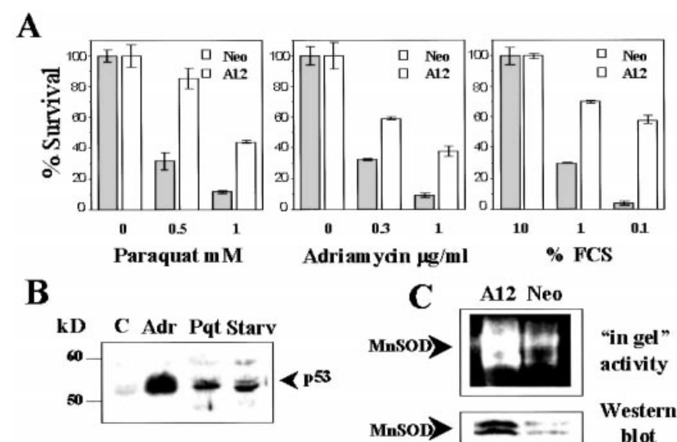

Fig. 5. Increased resistance to Paraquat, Adriamycin, and serum deprivation of HeLa cells overexpressing MnSOD. *A*, cytotoxicity of Paraquat, Adriamycin, and serum deprivation on HeLa cells stably transfected with MnSOD (clone A12) or PcDNA3 empty vector (neo) was evaluated by MTT reduction assay, as described in "Materials and Methods." The MnSOD-positive clone A12 has been characterized previously (14). *B*, p53 induction by pro-oxidant stimuli in HeLa cells. Cells (5 × 10^5) were treated with 1 μg/ml Adriamycin (24 h), 500 μM Paraquat (48 h), or low serum (48 h). Total lysates (80 μg) were immunoblotted with a mixture of anti-p53 Abs [DO-1 (1:2000) + pAb240 (1:2000)], and immunocomplexes were revealed by enhanced chemiluminescence. The p53 doublet band is indicated by *arrow*. *C*, increased levels of MnSOD activity (*top*) and immunoreactive protein (*bottom*) in A12 cells *versus* neo control cells.

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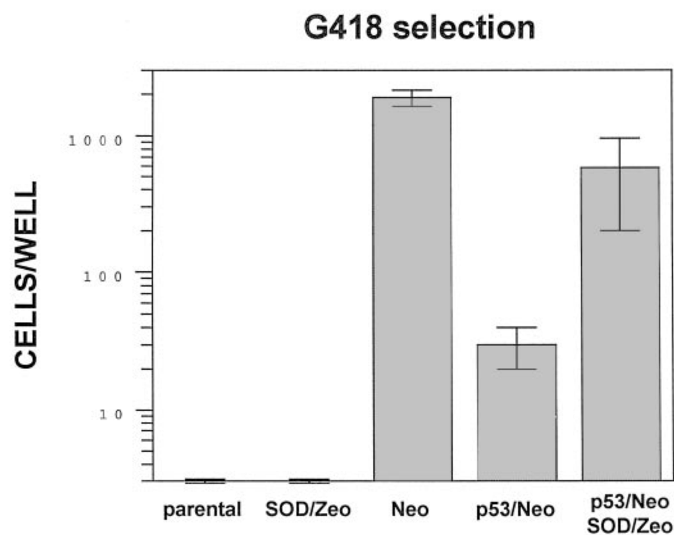

Fig. 6. MnSOD antagonizes the growth-inhibitory effect of p53. Effects of p53 and MnSOD on HeLa cell growth in G418-containing selective medium. 48 h after transfection with the indicated constructs, cells were plated in selective medium (G418, 800 μg/ml). Fourteen days later, surviving cells were counted, trypsinized, and counted. *parental*, no vector. *SOD/Zeo*, MnSOD/zeo (zeocine resistance gene). *Neo*, pCDNA3/neo (neomycin resistance gene). *p53/Neo*, wt p53/neo + pCDNA3/zeo (1:4). *p53/Neo/SOD/Zeo*, p53/neo + MnSOD/zeo (1:4). Note the increase in survival of p53/neo + MnSOD/zeo-transfected cells *versus* the p53/neo + pCDNA3/zeo transfectants. Bars are the means ± SD of two independent transfections. *Neo, p53/Neo,* and *p53/Neo/SOD/Zeo* are significantly different by at least $P < 0.5$ (Student's *t* test).

activity (30, 31) and identify the superoxide anion as an important radical species involved in p53 cytotoxicity.

DISCUSSION

Despite the enormous amount of information available on the genetics, structure, and function of the tumor suppressor protein p53, the cascade of molecular events linking p53 activation to cell death is still incompletely understood. The identification of biochemical and functional alterations specifically associated with loss of function of p53 therefore represents a front line in the current understanding of tumor biology.

The major findings of the present work are the identification of the mitochondrial superoxide scavenger MnSOD as an enzyme whose cellular content is negatively regulated by p53 and the ability of this enzyme to counteract the growth-inhibitory effects of p53 on cervical carcinoma HeLa cells.

Many studies suggest that MnSOD (or SOD2) could play a general role as a cell survival protein; in fact, in cell culture models, SOD2 overexpression has proven to be protective against the cytotoxicity (either by apoptosis or necrosis) of a number of agents including lipopolysaccharide and tumor necrosis factor α, ionizing and UV radiations, anticancer drugs, pro-oxidant chemicals, and serum deprivation (12, 14). Interestingly, many of these treatments are well-known inducers of p53. As for *in vivo* studies, mice genetically deprived of this enzyme exhibit severe cardiomyopathy and neurodegenerative lesions, likely related to impaired mitochondrial function by unscavenged oxygen radicals. Recent discoveries on the central role of mitochondria in the initiation of apoptosis and on the generation of ROS in mitochondria after cytochrome *c* release have added new support to the notion that MnSOD can play an important role in the maintenance of cell viability under stressful conditions and, through the surveillance on mitochondrial integrity, can directly influence cell fate toward different possible outcomes represented by recovery from stress, apoptosis, or necrosis (47). In view of these properties of SOD2, the finding that this enzyme is negatively controlled by p53 could significantly contribute to the understanding of how p53 activation is coupled to the triggering of the apoptotic process and, eventually, to cell death.

Important evidence exists that modification of the cellular redox balance represents a crucial event downstream of p53 activation. p53 retroviral transfer to cultured cells is accompanied by a significant production of oxygen radicals (30, 31); furthermore, the expression of several redox active proteins is transcriptionally regulated by p53 (31). Among these, the mitochondrial scavenger glutathione peroxidase is transcriptionally induced by the tumor suppressor protein (48). To date, however, inhibition of antioxidant defenses by p53 activation has not been reported, despite the fact that this is a very common mechanism of oxidative stress in mammalian cells.

HIV protein Tat, for example, determines an oxidative stress in infected cells by down-regulating the expression of MnSOD (49). We suggest that, in a similar fashion, MnSOD down-regulation contributes, at least in part, to the increase of oxygen radicals reported in p53-transduced cells as necessary for p53-mediated apoptosis. Whereas previous studies have not identified the source and nature of these radical species, the present data showing decreased MnSOD expression in p53-transfected cells together with a protective effect of SOD2 toward p53-mediated apoptosis indicate mitochondrial superoxide (O_2^-) as a potentially critical effector in this process.

Increased expression of the mitochondrial scavenger MnSOD in p53-deficient fibroblasts could appear somehow inconsistent with the fact that we did not detect significant differences in the intracellular content of oxygen radicals between p53−/− and wt cells. This apparent contradiction can be easily reconciled when one considers that in presence of stimuli such as serum deprivation or redox cycling drugs, massive amounts of ROS are generated in the cells, but only one part of those is likely to involve mitochondria and can be targeted by SOD2. Nevertheless, as shown in Fig. 5, improved mitochondrial antioxidant defense translates into a dramatic increase in cell resistance to the above-mentioned treatments in HeLa cells. In view of this evidence, we conclude that overall cellular capability to generate ROS is unaffected by loss of p53, whereas cellular resistance to oxidative stress is markedly increased in p53-deficient cells, likely due to up-regulation of MnSOD.

The molecular mechanism through which p53 represses MnSOD gene expression has not been addressed in the present study. p53 has been reported to negatively regulate a number of promoters, especially those harboring either TATA (22) or CAAT (23) boxes; moreover, p53 interaction with SP-1 results in reduced transcriptional activity by this factor, thereby inhibiting the expression of many SP-1 target genes (24). Human MnSOD promoter does not contain TATA or

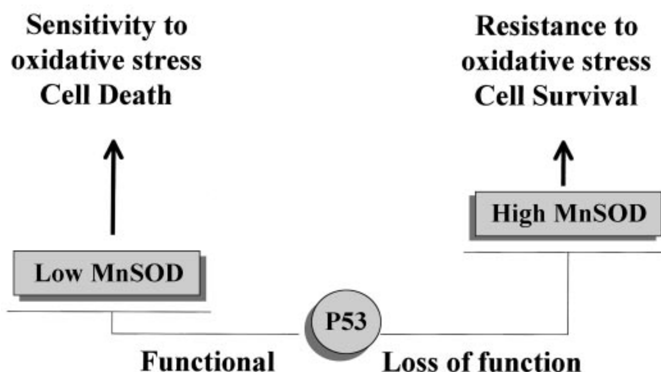

Fig. 7. In this model, deregulated MnSOD expression links loss of p53 function with increased resistance to oxidative stress and possibly with impaired apoptosis.

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CAAT regions but harbors seven potential SP-1 binding sites (50) that are probably involved in the basal level expression of the enzyme. Although not proven in this study, it is therefore conceivable that the inhibitory effects of p53 on SOD2 expression are mediated by transcriptional complex SP-1, as shown, for instance, for insulin receptor expression in mouse liver (51).

MnSOD is a highly inducible gene, and its expression is strongly up-regulated by oxidative stress (52). Whereas increased content of MnSOD in p53-deficient tissues could reflect an increase in cell exposure to oxygen radicals, this mechanism appears to be unlikely because intracellular ROS are not significantly different in wt and p53−/− cells (see above) and in view of recent findings suggesting the pro-oxidant effects of p53 (30).

However, whereas we favor the hypothesis of SP-1 involvement in MnSOD down-regulation by p53, this regulatory circuit certainly deserves more detailed investigation.

The finding of deregulated MnSOD expression in p53-deficient cells might have important ramifications for the understanding of some aspects of tumor biology. Loss of function of p53 in tumors is associated with increased resistance to adverse growth conditions, as those created by exposure to anticancer drugs, radiotherapy, and nutrient restrictions. Many of these conditions are accompanied by increased production of oxygen radicals. We suggest that this resistance could be due, at least in part, to increased expression of the mitochondrial scavenger SOD2 (Fig. 7). In keeping with this model, we and others have described exaggerated expression of MnSOD in some classes of tumors, including brain tumors, cervical carcinoma, and colon and thyroid carcinoma. Moreover, high expression of MnSOD has been associated with an accumulation of mutated p53 in cervical carcinoma samples (53), and a similar association has also been observed in brain tumors.[4] More intriguingly, because SOD2 can at least partially counteract the growth-suppressive effect of p53 (Fig. 6), abnormal expression of MnSOD could confer a "p53 deficient-like" phenotype on tumor cells, even in the presence of a normal tumor suppressor protein. Data reported in Fig. 5 showing increased resistance to Paraquat, Adriamycin, and serum deprivation in HeLa cells overexpressing MnSOD, together with previously reported observations (11, 12, 14), clearly support this hypothesis.

Finally, MnSOD down-regulation by p53 could have important implications for the understanding of the process of cellular aging. Aging in fibroblasts is accompanied by an increased production of oxygen radicals in mitochondria and by oxidative damage to mitochondrial DNA (54). Moreover, ras mutations accelerate the aging of normal fibroblasts (55), likely via a ROS-dependent mechanism (56).

In view of the presented data, delayed aging and increased susceptibility to Ras transformation of p53−/− fibroblasts could be a consequence, at least in part, of MnSOD up-regulation and increased resistance of mitochondria to oxidative stress (Fig. 7).

Whereas many of these considerations are still speculative, the finding of SOD2 modulation by p53 strongly supports the role of this enzyme as a survival protein involved in cell resistance to stress and, as such, as a potential tumor-promoting factor involved in the mechanism linking loss of function of p53 to altered cell growth regulation, tumorigenesis, and resistance to chemotherapy.

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