Oxidants such as H$_2$O$_2$ play a role in the toxicity of certain DNA-damaging agents, a process that often involves the tumor suppressor p53. H$_2$O$_2$ is rapidly degraded by catalase, which protects cells against oxidative injury. To study the effect of catalase on apoptosis induced by DNA-damaging agents, HepG2 cells were infected with adenovirus containing the cDNA of catalase (Ad-Cat). Forty-eight hours after infection, catalase protein and activity was increased 7-10-fold compared with control cells infected with Ad-LacZ. After treatment with Vp16 or mitomycin C, control cells underwent apoptosis in a p53-dependent manner; however, overexpression of catalase inhibited this apoptosis. Basal levels as well as Vp16- or mitomycin C-stimulated levels of p53 and p21 protein were decreased in the catalase-overexpressing cells as compared with control cells. However, p53 mRNA levels were not decreased by catalase. There was no difference in p53 protein synthesis between catalase-overexpressing cells and control cells. However, pulse-chase experiments indicated that p53 protein degradation was enhanced in the catalase-overexpressing cells. Proteasome inhibitors but not calpeptin prevented the catalase-mediated decrease of p53 content. Whereas Vp16 increased, catalase overexpression decreased the phosphorylation of p53. The protein phosphatase inhibitor okadaic acid did not prevent the catalase-mediated down-regulation of p53 or phosphorylated p53. These results demonstrate that catalase protects HepG2 cells from apoptosis induced by DNA-damaging agents in association with decreasing p53 phosphorylation; the latter may lead to an acceleration in the degradation of p53 protein by the proteasome complex. This suggests that the level of catalase may play a critical role in cell-induced resistance to the effects of anti-cancer drugs which up-regulate p53.

Many chemotherapeutic drugs, including Vp16 and mitomycin C, induce cells to undergo apoptosis through damage of nuclear DNA. The activation of wild type p53, a major tumor suppressor protein, which is implicated in cell cycle control, DNA repair, and apoptosis (1–3), has been reported to be involved in the process of apoptosis induced by DNA-damaging agents (3, 4). The increase in p53 levels after DNA damage results in large part from stabilization of the p53 protein against degradation, and accumulation of p53 occurs because of this increased protein stability in the presence of ongoing translation (4). Acute overexpression of wild type p53 mediated by adenovirus facilitates the ability of DNA-damaging drugs to induce death of cancer and normal cells (5). As a transcription factor, p53 protein enhances the transcription rate of several genes including p21$^{WAF1}$, GADD45, and 14-3-3$\zeta$, which are genes involved in cell cycle arrest (6–8), and Bax, CD95, Noxa, PUMA, and p53AIP1, which are related to p53-dependent apoptosis (9–13).

Studies in a variety of cell types have suggested that ROS formation may be directly related to the ability of DNA-damaging agents to induce apoptosis (14). Other studies indicate that ROS are downstream mediators of p53-dependent apoptosis (15, 16). p53 acts to regulate the intracellular redox state and induces apoptosis by a pathway that is dependent on ROS production (17). p53 results in apoptosis through a multi-step process, including the transcriptional induction of redox-related genes, the generation of ROS, and the oxidative degradation of mitochondrial components, leading to apoptosis (15). There is also evidence that ROS could regulate apoptosis as a signal transmitter or mediator (16, 18, 19). However, other studies indicate that apoptosis can be induced in the absence of any detectable oxidative stress when cells are treated with Vp16 and cisplatin (20), and oxidative stress can inhibit apoptosis in human lymphoma cells (21).

Catalase, an anti-oxidative enzyme, is able to degrade hydrogen peroxide (H$_2$O$_2$), which is an important ROS related to p53 activation. Catalase has been shown to protect a variety of cells against oxidative stress-induced toxicity (22). Our laboratory has utilized adenoviral catalase expression systems to study the ability of catalase to prevent CYP2E1-dependent, and ROS-dependent toxicity to HepG2 cells (23, 24). In the current study, catalase adenovirus was used to infect HepG2 cells to study the effects of catalase on the regulation of apoptosis and the modulation of p53 expression induced by DNA-damaging agents.

**MATERIALS AND METHODS**

**Reagents**—Propidium Iodide was purchased from Molecular Probes (Eugene, OR). Polyclonal antibody raised in rabbit against human catalase was obtained from Calbiochem, and antibodies raised in rabbit against p53, phosphorylated p53 on serine 20, p21, Bcl-2, and Bcl-xL were obtained from Santa Cruz (Santa Cruz, CA). Horseradish peroxidase conjugated to goat anti-rabbit IgG, MEM, and fetal bovine serum were purchased from Sigma.

**Cell Culture and Recombinant Adenovirus Infection**—HepG2 cells...
were cultured in MEM containing 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine in a humidified atmosphere in 5% CO₂ at 37 °C. Ad-LacZ, a β-galactosidase-expressing replication-deficient adenovirus, and Ad-Cat, a human catalase-expressing replication-deficient adenovirus, were constructed as described previously (23). Before infection, cells were seeded onto dishes or plates, grown to 60% confluence, and infected with Ad-LacZ or Ad-Cat with 100 m.o.i. Forty-eight hours after infection, cells were collected and assayed for catalase expression or treated with DNA-damaging agents.

Cell Transfection—HepG2 cells were seeded onto 10-cm culture dishes and grown to 80% confluence. The expression plasmid vector pCMV-p53mt135 (Clontech), containing mutant p53 or empty vector pCMV, was transfected into HepG2 cells using Electroporation reagent (Qiagen) according to the instructions provided by the manufacturer. This mutant p53 expression vector encodes a mutant p53 protein that combines with wild type p53 protein in a dominant-negative manner and blocks the DNA binding domain on wild type p53, therefore inhibiting the activity of wild type p53. Cells were selected by MEM containing 1 mg/ml G418. Two weeks after transfection, the surviving clones were isolated and grown to large scale. Stable cell lines with overexpression of mutant p53 (HepG2-mtp53) as well as cells transfected with empty vector (HepG2-neo) were selected and maintained in MEM containing 1 mg/ml G418.

Western Blotting—Cell lysates were prepared by sonicating cells followed by centrifugation. The protein concentration of the supernatant was measured (DC protein assay reagent, Bio-Rad), and 10 μg of denatured protein was resolved on 10% SDS-PAGE and electroblotted onto nitrocellulose membranes (Bio-Rad). The membranes were incubated with rabbit anti-human catalase polyclonal antibody (1:1000) followed by incubation with horseradish peroxidase conjugated to goat anti-rabbit IgG (Sigma) (1:5000). Detection by the chemiluminescence reaction was carried out for 1 min using the ECL kit (Amersham Biosciences) followed by exposure to Kodak X-Omat x-ray film (Eastman Kodak Co.). Similar Western blots were carried out to detect P53, phosphorylated p53 (pS53), P21, Bcl-2, and Bcl-xL.

Catalase Activity Assay—To validate expression of functional catalase in HepG2 cells, HepG2 cells were inoculated with various reagents were scraped and centrifuged at 1200 × g, the cell pellet was resuspended in 80% ethanol and stored at 4 °C for 24 h. Cells were washed twice with PBS. The pellet was resuspended in PBS containing 100 μg/ml RNase A, incubated at 37 °C for 30 min, stained with propidium iodide (50 μg/ml), and analyzed by flow cytometry DNA analysis as described previously (24).

RESULTS

Overexpression of Catalase in HepG2 Cells—To examine the capacity of Ad-Cat to enhance the expression and activity of catalase in cells, HepG2 cells were infected with 100 m.o.i. of Ad-Cat or Ad-LacZ. By 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) assay it was determined that more than 98% of the HepG2 cells were infected with the adenovirus under these reaction conditions. 48 h later, cell lysates were prepared and subjected to Western blot analysis. Fig. 1A shows the expression of catalase in the total cell extract from these cells. Results from densitometric analyses of the intensity of the various bands indicated that the expression of catalase in total cell extracts of cells infected with Ad-Cat was 7–10-fold higher than that in uninfected HepG2 cells or HepG2 cells infected with Ad-LacZ. Similar results were obtained by measuring catalase activity (Fig. 1B). In total cellular extracts of HepG2 cells infected with Ad-Cat, catalase activity was 7–10-fold higher than in uninfected HepG2 cells. This result indicates that overexpression of catalase in HepG2 cells enhances the catalase activity.
higher than that in uninfected HepG2 cells or HepG2 cells infected with Ad-LacZ.

Intracellular ROS—We evaluated ROS generation by measuring dichlorofluorescin fluorescence after loading the cells with 5 μM DCF-DA. It is recognized that dichlorofluorescin may be oxidized by several oxidants including H₂O₂, superoxide radical, lipid hydroperoxides, lipid peroxyl and alkoxyl radicals, and by cellular peroxidases (25, 26) and is not specific for e.g. H₂O₂ but serves as a general index for ROS production. In the absence of VP16, control fluorescence was 150 arbitrary units (Fig. 2). Infection with Ad-LacZ had no effect on DCF production, but infection with Ad-Cat decreased fluorescence by about 45%. The addition of 50 μM VP16 increased DCF production by about 60%. Ad-LacZ again had no effect, whereas Ad-Cat decreased fluorescence by about 40%. Thus, VP16 increased production of ROS by HepG2 cells, and catalase decreased the basal as well as the VP16-stimulated ROS generation.

Overexpression of Catalase Protects HepG2 Cells from Apoptosis Induced by DNA-damaging Reagents—HepG2 cells were plated separately in 10% fetal bovine serum, MEM; after overnight incubation, cells were infected with Ad-Cat and Ad-LacZ adenovirus with 100 m.o.i. Forty-eight hours later, 500 μM Vp16 or 10 μg/ml mitomycin C were added, and the cells were incubated for a further 24 h. DNA fragmentation and DNA analysis by flow cytometry as well as cellular morphology changes were carried out as described under "Material and Methods." Fig. 3A shows the results of DNA fragmentation. No DNA ladder was seen in either Ad-LacZ (lane 2) or Ad-Cat (lane 3)-infected HepG2 cells without any treatment. When treated with Vp16 (lanes 4 and 5) or mitomycin C (lanes 6 and 7), HepG2 cells infected with Ad-LacZ showed a clear DNA ladder (lanes 4 and 6), whereas only a very weak DNA ladder was seen in HepG2 cells infected with Ad-Cat (lanes 5 and 7). DNA analysis by flow cytometry after staining with propidium iodide was used to measure the percentage of apoptotic cells (Fig. 3B). After 48 h infection with either Ad-LacZ or Ad-Cat, the percentage of apoptotic cells was less than 3% in the absence of any treatment with DNA-damaging agents (Fig. 3B, panels a and b). The percentage of apoptotic cells increased to 25 and 21% after 24 h of treatment with 500 μM Vp16 or 10 μg/ml mitomycin C, respectively (Fig. 3B, panels c and e). The HepG2 cells infected with Ad-Cat were resistant to 500 μM Vp16 or to 10 μg/ml mitomycin C-induced apoptosis, displaying only 5 and 7% apoptotic cells, respectively (Fig. 3B, panels d and f). Similar results were observed when studying morphologic changes (Fig. 3C). Cells infected with Ad-LacZ lost normal morphology when treated with 500 μM Vp16 or 10 μg/ml mitomycin C for 24 h, becoming round in shape and shrinking in size (panels c and e), whereas most of the cells infected with Ad-Cat retained their shape and structure (panels d and f).

Overexpression of Catalase Down-regulates p53 and p21 Protein but Not Bcl-2 and Bcl-xL in HepG2 Cells—We investigated the expression of selected proteins related to apoptosis in cells infected with Ad-LacZ and Ad-Cat for 48 h with or without further treatment with 50 μM Vp16 or 5 μg/ml mitomycin C for 16 h. The basal level of p53 was low in HepG2 cells, most likely due to the short half-life of wild type p53. This basal level of p53 was even lower in cells infected with Ad-Cat than in cells infected with Ad-LacZ (Fig. 4, panel a). After treatment with Vp16 or mitomycin C, the p53 protein level was dramatically increased in cells infected with Ad-LacZ but only slightly increased in the cells infected with Ad-Cat. Because p21 is one of the downstream genes of p53, which is induced by activation of p53, p21 protein level was also detected by Western blot. Coincident with p53, the p21 protein level was dramatically increased in cells infected with Ad-LacZ after treatment with Vp16 or mitomycin C (Fig. 4, panel b). Infection with catalase
FIG. 3. Expression of catalase protects HepG2 cells from apoptosis induced by Vp-16 or mitomycin C. HepG2 cells were infected with Ad-Cat and Ad-LacZ adenovirus at 100 m.o.i. Forty-eight hours later, 500 μg Vp16 or 10 μg/ml mitomycin C were added and incubated for a further 24 h. DNA fragmentation and DNA analysis by flow cytometry as well as morphology changes were carried out as described under “Material and Methods.” A, DNA fragmentation. Lane 1, 100-bp DNA marker; lane 2 (Ad-LacZ) and lane 3 (Ad-Cat) were HepG2 cells infected with adenovirus without any treatment of Vp-16 or mitomycin C; lane 4 (Ad-LacZ) and lane 5 (Ad-Cat) were HepG2 cells treated with Vp16 after infection; lane 6 (Ad-LacZ) and lane 7 (Ad-Cat) were HepG2 cells treated with mitomycin C after infection. B, DNA analysis by flow cytometry. Panels a (AD-LacZ) and b (AD-Cat) were HepG2 cells infected with adenovirus without any treatment of Vp-16 or mitomycin C; panels c (AD-LacZ) and d (AD-Cat) were HepG2 cells treated with Vp16 after infection; panels e (AD-LacZ) and f (AD-Cat) were HepG2 cells treated with mitomycin C after infection. One of the three independent experiments is shown. C, morphologic change. Panels a (AD-LacZ) and b (AD-Cat) were HepG2 cells infected with adenovirus without any treatment of Vp-16 or mitomycin C; panels c (AD-LacZ) and d (AD-Cat) were HepG2 cells treated with Vp16 after infection; panels e (AD-LacZ) and f (AD-Cat) were HepG2 cells treated with mitomycin C after infection (100×).
a decrease in normal p53 function confers resistance to Vp16-induced apoptosis. HepG2-neo and HepG2-mtp53 cell lines were produced as described under "Materials and Methods." Cells were treated with 0, 50, 250, or 500 μM Vp16 for 24 h. DNA fragmentation was carried out as described in Fig. 3A. One of two independent experiments is shown.

Fig. 6. Expression of catalase and/or treatment with Vp-16 does not change the p53 mRNA levels. Wild type HepG2 cells (W) and HepG2 cells infected with Ad-Cat (C) or Ad-LacZ (Z) for 48 h were incubated in the absence or presence of 50 μM Vp16 for 16 h. RNA was isolated, and Northern blot analysis was performed using [32P]dCTP-labeled p53 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes. Two independent experiments were carried out.

Fig. 4. Expression of catalase modulates p53 and p21 but not Bcl-2 and Bcl-xL protein level. HepG2 cells were infected with Ad-Cat (C) or Ad-LacZ (Z) at a m.o.i of 100 for 48 h with or without further treatment by adding 50 μM Vp16 or 5 μg/ml mitomycin C (Mito) for 16 h. 25 μg of protein were loaded onto each lane for 10% SDS-polyacrylamide gel electrophoresis followed by Western blot analysis with polyclonal rabbit anti-human p53 (a), p21 (b), Bcl-2 (c), and Bcl-xL (d) antibody as described under "Materials and Methods." Three independent experiments were carried out.

Adenovirus lowered the basal as well as the stimulated levels of p21, analogous to results with p53 levels. Bcl-2 and Bcl-xL are two anti-apoptotic proteins. The expression of these proteins was not different between Ad-LacZ and Ad-Cat infection with or without treatment by Vp16 or mitomycin C (Fig. 4, panels c and d).

Apoptosis Induced by Vp16 Is p53-dependent—To evaluate whether catalase protects HepG2 cells from apoptosis induced by DNA-damaging agents through down-regulation of p53 protein, it was important to first demonstrate that p53 was required for the developing apoptosis. HepG2-mtp53 and HepG2-neo cell lines were set up by stable transfection of HepG2 cells with the expression plasmid vector containing mutant p53 or empty vector. This mutant p53 expression vector encodes a mutant p53 protein, which combines with wild type p53 protein in a dominant-negative manner and blocks the DNA binding domain on wild type p53, therefore inhibiting the activity of wild type p53. After treatment with different concentrations (50, 250, and 500 μM) of Vp16 for 24 h, HepG2 cells transfected with the empty vector (HepG2-neo) underwent DNA fragmentation (Fig. 5). However, no or very weak DNA fragmentation was observed in HepG2 cells transfected with the mutant p53 expression vector (HepG2-mtp53), even at the maximum concentration of Vp16 (500 μM) studied (Fig. 5). These results indicate that p53 is necessary for the Vp16-induced apoptosis in the HepG2 cells and suggest that one mechanism by which catalase protects against this apoptosis is to lower the intracellular levels of p53.

Overexpression of Catalase Down-regulates p53 Protein by Potentiating p53 Protein Degradation but Not by Decreasing p53 Protein Synthesis or mRNA Levels—The catalase down-regulation of the expression of p53 protein under basal conditions or after treatment with DNA-damaging agents could be due to an effect on p53 transcription or translation or protein degradation. HepG2 cells were infected with Ad-LacZ and Ad-Cat for 48 h with or without further treatment by 50 μM Vp16 for 16 h. RNA was isolated, and a Northern blot was performed using [32P]dCTP-labeled p53 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes. There was no significant difference in p53 mRNA levels between cells infected with Ad-LacZ and Ad-Cat either under basal conditions or after treatment with Vp16 (Fig. 6). Vp16 treatment, which dramatically elevated p53 protein levels (Fig. 4), did not have any effect on p53 mRNA levels (Fig. 6). This result indicates that overexpression of catalase does not decrease transcription of the p53 gene into mRNA; therefore, changes in mRNA are not responsible for the inhibitory effect of catalase on the level of p53 protein.

Two other possibilities that might relate to the inhibitory effect of catalase on the level of p53 protein were considered, which are that catalase inhibits p53 protein synthesis or catalase increases the degradation of p53 protein. Pulse-labeling and pulse-chase assays were performed to investigate these possibilities. HepG2 cells were infected with Ad-LacZ and Ad-Cat for 48 h and then treated with 50 μM Vp16 for 16 h followed by pulsing with [35S]methionine for 0.5, 2, and 4 h. p53 protein was immunoprecipitated, and the incorporated label was quantified by SDS-PAGE and recorded on x-ray film. The intensity of the p53 signal increased after 2 h of pulsing as compared with 0.5 h (Fig. 7A). To validate the assay, the effect of cycloheximide was determined; p53 synthesis was inhibited by adding 40 μM cycloheximide. There was no decrease in the p53 signal intensity in cells infected with Ad-Cat compared with cells infected with Ad-LacZ at any time point (Fig. 7A). This indicates that overexpression of catalase does not change the rate of p53 protein synthesis.

To determine the rate of p53 protein degradation, pulse-chase was performed by adding cold methionine for 0, 2, and 4 h after a 4-h pulse with [35S]methionine. Compared with cells infected with Ad-LacZ, the p53 signal in cells infected with Ad-Cat was considerably lower after the 2- and 4-h chase (Fig. 7B; quantification in Fig. 7C). This indicates that the rate of p53 degradation was potentiated by overexpression of cata-
The control or calpeptin-treated cells but only 20% of Vp16-treated cells, catalase lowered p53 levels by 50% compared to the Ad-LacZ-infected cells (Fig. 8). In the presence of lactacystin or MG115, which may reflect that both treatments result in decreasing p53 turnover (further described below, Fig. 9). The administration of lactacystin or MG115 increased the p53 protein level 3- to 4-fold in Ad-LacZ-infected cells not treated with Vp16. This likely reflects the prevention of p53 degradation. The elevated level of p53 in the Vp16-treated cells was not further increased by lactacystin or MG115, which may reflect that both treatments result in decreasing p53 turnover (further described below, Fig. 9). Calpeptin did not affect the basal level of p53 or the Vp16-stimulated levels in the Ad-LacZ-infected cells (Fig. 8). In the Vp16-treated cells, catalase lowered p53 levels by 50–60% in the control or calpeptin-treated cells but only 20–25% in the lactacystin- or MG115-treated cells. Thus, the lowering of p53 levels by catalase was partially prevented or diminished by administration of proteasome inhibitors but not by calpeptin. Because of toxicity, higher concentrations of lactacystin or MG115 could not be evaluated; however, these results are consistent with role for the proteasome in p53 turnover and in the catalase enhancement of this turnover.

Catalase Overexpression Decreases the Phosphorylation of p53—p53 is activated through phosphorylation. Once phosphorylated, p53 is imported into the nucleus and can now activate downstream genes such as p21 and Bax, acting as a transcription factor. In addition, phosphorylated p53 has a longer half-life than unphosphorylated p53, therefore leading to p53 accumulation. This phosphorylation can occur on several serine residues including serine 9, 15, 20, and 392 of the p53 protein. One possible mechanism by which catalase increases turnover of p53 could be by decreasing formation of the more stable phosphorylated p53. Using anti-p53 antibody and a specific antibody for recognition of phosphorylation of p53 on serine 20, the pp53 level was compared with the total p53 level in cells treated with Vp16 or lactacystin. Both Vp16 and lactacystin increased the pp53 level; however, only Vp16 increased the pp53 level (Fig. 9A). This suggests that although lactacystin and Vp16 each increase the pp53 level by decreasing the degradation of p53, they do so by different mechanisms. Lactacystin inhibits the degradation of p53, whereas Vp16 results in formation of the more stable pp53. To determine the effect of catalase on p53 phosphorylation, HepG2 cells infected with Ad-LacZ or Ad-Cat were treated with Vp16 for different times (0, 8, 16, and 24 h), and immunoblots to detect p53 and pp53 were carried out. Catalase overexpression decreased the phosphorylated form of p53 in association with the decrease in total p53 levels as compared with Lacz expression (Fig. 9B).

DISCUSSION

Overexpression of Catalase Protects HepG2 Cells from Apoptosis Induced by DNA-damaging Agents—DNA ladder and flow cytometry experiments as well as morphology changes indicated that HepG2 cells infected with Ad-Cat were less sensitive to Vp16 and mitomycin C-induced apoptosis than HepG2 cells infected with Ad-LacZ. This suggests that ROS, especially H2O2, might be involved in the process of apoptosis induced by these DNA-damaging drugs. Studies in a variety of cell types suggest that cancer chemotherapeutic drugs induce tumor cell
apoptosis in part by increasing formation of ROS (14). However, other studies indicate that apoptosis can be induced in the absence of any detectable oxidative stress when tumor cells were treated with VP-16 and cisplatin (20). ROS may not necessarily be the direct factor to cause apoptosis induced by DNA-damaging reagents, but intracellular ROS may modulate apoptotic gene expression, which subsequently regulates apoptosis. Thus, although catalase was protective against VP-16 or mitomycin C-induced apoptosis, it is not clear if \( \text{H}_2\text{O}_2 \) directly responsible for inducing toxicity or \( \text{H}_2\text{O}_2 \) is upstream of p53.

Catalase Protects HepG2 Cells from Apoptosis by Lowering the p53 Protein Level as a Consequence of Increased Degradation—P53 is a critical apoptotic gene that is activated before and during apoptosis. The overexpression of wild type p53 constructs in tumor cells was able to inhibit cell growth or to initiate apoptosis. p53 gene status modulates the chemosensitivity of non-small cell lung cancer cells. DNA damage induced by the DNA-damaging agents VP-16 or mitomycin C activates p53 mainly through protein modification events such as phosphorylation, which results in p53 protein stabilization and accumulation of p53 protein due to the decrease of its degradation (4) (Fig. 9, A and B). HepG2 cells transfected with a mutant p33 expression vector (HepG2-mtp53), which encodes a mutant p53 protein, decreasing wild type p53 function, are more resistant to VP16-induced apoptosis than HepG2 cells transfected with empty vector (HepG2-neo) (Fig. 5). This indicates that apoptosis induced by VP16 in HepG2 cells is p53-dependent. HepG2 cells overexpressing catalase as mediated by adenovirus-mediated gene transfer (Ad-Cat) show a lower level of basal or induced p53 protein than control (Ad-LacZ) cells. Lowering of p53 levels is likely to be the mechanism whereby catalase overexpression protects HepG2 cells from apoptosis induced by the DNA-damaging agents, whereas the latter induces apoptosis by elevating p53 levels. There are no differences in p53 mRNA levels between catalase-overexpressing cells and control cells with or without treatment by DNA-damaging agents, and the p53 mRNA level did not change after treatment with VP16 even though p53 protein levels were strongly increased. Thus, the decrease in p53 protein levels by catalase overexpression or the increase in p53 levels by treatment with VP16 is due to posttranscriptional effects. P21 is a downstream gene target of p53 and is regulated by p53 (27). HepG2 cells overexpressing catalase also display a lower level of p21 protein than control cells either in the absence or presence of treatment with DNA-damaging agents. Lower levels of p21 protein in the presence of catalase may reflect the lower levels of p53 protein in the presence of catalase. The results of synthesis experiments indicate that overexpression of catalase does not change the rate of p53 protein synthesis; however, pulse-chase experiments did reveal that p53 protein degradation was increased in cells infected with Ad-Cat compared with cells infected with Ad-LacZ.

By adenovirus-mediated gene transfer, Johnson et al. (17) find that cells sensitive to p53-mediated apoptosis produced ROS concomitantly with p53 overexpression, whereas cells resistant to p53 failed to produce ROS. In sensitive cells, both ROS production and apoptosis were inhibited by antioxidant treatment. These results suggest that p53 acts to regulate the intracellular redox state and induces apoptosis by a pathway that is dependent on ROS production (17). In our experiments, removing \( \text{H}_2\text{O}_2 \) by expression of catalase causes the p53 protein level to decrease, and this is associated with resistance to apoptosis induced by VP16 or mitomycin C. This suggests that, at least in HepG2 cells, \( \text{H}_2\text{O}_2 \) regulates apoptosis by modulating p53 levels, and \( \text{H}_2\text{O}_2 \) acts upstream of p53.

As a result of a high turnover rate, the p53 protein has a half-life of ~30–60 min and is maintained at low levels in normal proliferating cells (28). In response to genotoxic stress or oncogenic signaling, p53 levels rapidly increase, mainly through protein stabilization. An important regulator of the p53 level is MDM2, which possesses intrinsic E3 ligase activity and, thus, promotes p53 ubiquitination and subsequent degradation via proteasome-mediated proteolysis (29). Overexpression of catalase did increase p53 protein degradation, and the proteasome inhibitors lactacystin and MG115 but not the calpain inhibitor calpeptin could partially block the p53 protein lowering caused by overexpression of catalase. This is consistent with the overall mechanism that catalase lowers p53 levels by accelerating p53 degradation.

How overexpression of catalase specifically down-regulates p53 protein or enhances p53 turnover is not clear. Because \( \text{H}_2\text{O}_2 \) is a specific substrate of catalase, catalase down-regulation of p53 protein would appear to involve some function or action of \( \text{H}_2\text{O}_2 \). One hypothesis is that \( \text{H}_2\text{O}_2 \) may increase the level of phosphorylated p53 by stimulating certain protein ki-
nases implicated in the phosphorylation of p53 or by inhibiting the activity of protein phosphatases that convert pp53 to the less stable nonphosphorylated state. Once p53 protein is phosphorylated, its half-life is increased, which causes p53 accumulation (30). If ROS indeed modulate the actions of p53 kinases or p53 phosphatases, overexpression of catalase may decrease p53 accumulation by removing ROS such as H$_2$O$_2$. Using a specific antibody for detecting phosphorylated p53 on serine 20, we found that overexpression of catalase did prevent phosphorylation of p53 induced by Vp16, and low levels of pp53 were associated with low levels of total p53. However, the general phosphatase inhibitor, okadaic acid, did not prevent the effect of catalase on down-regulation of p53 or pp53. This suggests that catalase may inhibit activity of p53 kinases, which may be activated by H$_2$O$_2$. Recent studies indicate that ROS such as H$_2$O$_2$-induced phosphorylation of p53 can be mediated in part by polo-like kinase-3 (30). Further studies will be necessary on the effects of ROS and of catalase on the activities of protein kinases implicated in phosphorylation of p53 and on protein phosphatases implicated in the dephosphorylation of pp53. Chromium and vanadate metals that can cause oxidative stress, e.g. thiol oxidation, such that the protein is not an effective substrate for proteolysis. Such putative, stabilizing modifications would be prevented by rapid removal of H$_2$O$_2$ by catalase.

In summary, these results demonstrate that catalase protects HepG2 cells from apoptosis induced by DNA-damaging agents in association with decreasing the level of pp53, which leads to accelerating the degradation of p53 protein. The level of catalase may therefore play an important role in cell-induced resistance to the effects of anti-cancer drugs by modulating the cellular levels of p53 and p53 targets such as p21.

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