

Role of p53 in Sensing Oxidative DNA Damage in Response to Reactive Oxygen Species-Generating Agents

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ABSTRACT

The tumor suppressor p53 plays an important role in the regulation of cellular response to DNA damage. Recent studies suggest that p53 is able to bind DNA with certain structural alterations in a sequence-independent manner and to interact with several molecules involved in DNA repair. This study was undertaken to test the hypothesis that p53 may participate in sensing oxidative DNA damage, the most frequently occurring spontaneous DNA lesion, and modulate its repair by the base excision repair (BER) machinery. Using synthetic DNA containing 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxoG), we showed that p53 was pulled down together with two BER proteins, human 8-oxoguanine glycosylase (hOGG1) and AP endonuclease (APE). Functional analysis showed that p53 significantly enhanced the sequential activities of hOGG1 and APE in excising the 8-oxoG nucleotide from DNA *in vitro*. The ability of p53 to enhance the removal of oxidized DNA bases was further demonstrated *in vivo* using a pair of p53 isogenic lines. HCT116 p53^{+/+} cells exhibit a more rapid removal of 8-oxoG from DNA than p53^{-/-} cells exposed to the same levels of reactive oxygen species (ROS) stress. Together, these results suggest that p53 participates in sensing oxidative DNA damage and modulates BER function in response to persistent ROS stress.

INTRODUCTION

The tumor suppressor p53 is an important molecule involved in regulating cellular response to DNA damage by genotoxic agents such as UV or ionizing radiation, reactive oxygen species (ROS), and certain anticancer drugs. Depending on the cell type, the nature of the DNA damaging agent, and the extent of DNA damage, p53 induces the cells to undergo arrest to allow repair or alternatively may induce apoptosis. The stabilization and activation of the p53 protein as a transcriptional activator occur through a series of phosphorylation and acetylation events. On activation, p53 transcriptionally activates a number of target genes that are involved in regulating cell cycle checkpoints or in inducing apoptosis (1–3). The p53 protein itself also may be directly involved in recognizing DNA damage. Previous studies have shown that in response to DNA damage, p53 protein physically interacts with certain DNA repair molecules (4–7). Interestingly, p53 also can directly bind single-stranded and double-stranded DNA ends (8, 9), Holliday junctions (10), and extra base bulges (insertion/deletion mismatches; ref. 11). Given the central role of p53 in maintaining genomic integrity, it is not surprising that mutations of this gene are found in >50% of cancers (12) and appear to be associated with a poor clinical response to therapeutic agents (13, 14). Thus, a detailed understanding of the mechanism by which p53 senses DNA damage and its role in signaling DNA repair *versus* apoptosis has important implications in cancer therapeutics.

Cells living in an oxygen-rich atmosphere are constantly exposed to

various endogenous and exogenous ROS. Endogenous ROS are produced by a number of metabolic enzymes and through leakage of electrons from the mitochondrial electron transport chain. Exogenous sources of ROS include UV light, γ -irradiation, and chemicals. Several intracellular defense systems exist that scavenge ROS, protect cells from their harmful effects, and thus maintain the cellular redox homeostasis (15, 16). Agents that disrupt this redox balance may cause accumulation of ROS, leading to cellular damage. A key target of ROS in cells is the DNA. ROS-induced DNA damage occurs in the form of base modifications, apurinic (AP) sites, and DNA strand breaks. A frequent oxidative modification of DNA is the hydroxylation of guanine at C-8, leading to the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxoG; refs. 17, 18). Persistence of ROS-induced DNA damage could result in the generation of deleterious mutations. Therefore, prompt repair of the damaged DNA or, alternatively, the elimination of cells bearing these oxidative lesions is vital to the maintenance of genomic integrity of the organism.

Oxidized bases in DNA such as 8-oxoG are repaired mainly by the base excision repair (BER) pathway, which involves the recognition and removal of the oxidized base by a glycosylase. Human 8-oxoguanine glycosylase (hOGG1) has glycosylase activity that removes the 8-oxoG base, generating an AP site. It also has an AP-lyase activity that cleaves the DNA 3' to the AP site (19). AP endonuclease (APE, or ref-1) recognizes the AP site and cleaves the DNA backbone 5' to the AP site, generating a 3' hydroxyl group and a 5' phosphate group. DNA polymerase β (DNA pol β) inserts the appropriate nucleotides into the nick before the gap is sealed by a DNA ligase (20). Agents such as ionizing radiation, UV, and certain chemicals can cause ROS-induced oxidative DNA damage and trigger apoptosis (21–24). It also has been suggested that p53 is involved in mediating oxidative stress-induced apoptosis in experimental systems (25–28). However, the mechanism by which p53 senses ROS-induced DNA damage and its role in the subsequent cellular responses remain unclear. Recent studies by Offer *et al.* (29, 30) and Zhou *et al.* (31) have suggested that the wild-type p53 protein can markedly stimulate BER of uracil-containing DNA and methylated DNA, and that this is associated with its ability to interact with APE and DNA pol β (31). These studies provide strong evidence for the direct involvement of p53 in BER of damaged DNA.

On the basis of the reported involvement of p53 in cellular responses to oxidative stress, and the requirement of hOGG1 and APE for the removal of 8-oxoG from DNA, we tested the hypothesis that p53 might play a role in sensing ROS-induced DNA damage through interactions with hOGG1 and APE. Our studies showed that p53 was pulled down together with hOGG1 and APE by 8-oxoG-containing DNA, suggesting a physical interaction of p53 with these proteins. p53 also facilitated the excision of 8-oxoG from DNA *in vitro* and in whole cells, indicating a direct role for p53 in sensing oxidative DNA damage and in mediating subsequent cellular responses.

MATERIALS AND METHODS

Cell Lines and Chemicals. HCT116 colon carcinoma cells with wild-type p53 and their isogenic derivatives that lack p53 (HCT116 p53^{-/-}) were provided by Dr. Bert Vogelstein (Johns Hopkins University School of Medi-

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cine, Baltimore, MD). The cells were maintained in McCoy's 5A medium containing 10% fetal bovine serum. 2-Methoxyestradiol (2-ME) and hydrogen peroxide (H_2O_2) were purchased from Sigma-Aldrich (St. Louis, MO). A 10-mmol/L stock solution of 2-ME was prepared by dissolving the compound in DMSO. Hydroethidine was obtained from Molecular Probes (Eugene, OR). The 8-oxoG assay kit was purchased from Kamiya Biochemical Company (Seattle, WA). Oligonucleotide containing 8-oxoG and its complementary oligomer were purchased from Trevigen (Gaithersburg, MD). The biotinylated oligomer and other synthetic oligomers indicated in the studies were purchased from Genosys (Woodlands, TX). Antibodies for immunoblot analysis were purchased from the following sources: anti-p53 from Oncogene Research Products (San Diego, CA), anti-APE from Trevigen, anti-hOGG1 from α Diagnostics (San Antonio, TX), and anti-actin from Sigma-Aldrich. Streptavidin-coated magnetic particles and magnetic particle separator were obtained from Roche Diagnostics (Basel, Switzerland). [γ - ^{32}P]ATP was purchased from ICN Radiochemicals (Irvine, CA), and T4 polynucleotide kinase was from USB (Cleveland, OH). Recombinant human p53 protein was obtained from BD Biosciences (San Diego, CA). Purified APE and hOGG1 were purchased from Trevigen.

Measurement of Intracellular Superoxide. The level of intracellular superoxide (O_2^-) was measured using the fluorescent dye hydroethidine (HEt) as described previously (32). Briefly, HCT116 p53^{+/+} and HCT116 p53^{-/-} cells were treated with 2-ME to induce ROS accumulation and then incubated with HEt at a concentration of 100 ng/mL for 1 hour. The cells were harvested for analysis by flow cytometry. The level of superoxide in the cells was expressed as percentage of the control (untreated) samples.

Analysis of 8-oxoG Residues in DNA. To determine the levels of 8-oxoG residues in DNA, HCT116 cells were treated with 3 μ mol/L 2-ME for 3, 6, 9, and 12 hours or alternatively with H_2O_2 at concentrations of 100 μ mol/L and 500 μ mol/L for 1 hour. The levels of 8-oxoG residues in cellular DNA before and following treatment with 2-ME and H_2O_2 were determined using the 8-oxoG assay kit. This assay is based on the principle that FITC-labeled avidin binds with high specificity to 8-oxoguanine in DNA (33). The assay has been successfully used to detect and measure 8-oxoG residues in DNA in several studies (34, 35). Cells were harvested, washed with PBS, and fixed with 2% paraformaldehyde on ice for 15 minutes. The samples then were washed with PBS and fixed in 70% ethanol at 4°C overnight. On the following day, the cells were washed once with PBS and with the wash solution provided by the manufacturer and then incubated with blocking solution, incubated with FITC-labeled probe for 8-oxoG, and analyzed by flow cytometry according to the manufacturer's protocol. The intensity of the FITC fluorescent signal is proportional to the level of 8-oxoG residues in DNA. The levels of 8-oxoG residues in the H_2O_2 - or 2-ME-treated samples were expressed as percentage relative to the untreated control.

Assay of Removal of 8-oxoG Residues from DNA in Whole Cells. A pulse-chase experiment was used to assay the removal of 8-oxoG residues from DNA in whole cells. The p53-isogenic pair of HCT116 cells was treated with 3 μ mol/L 2-ME for 6 hours or 500 μ mol/L H_2O_2 for 1 hour. The cells were rinsed with fresh medium (without H_2O_2 or 2-ME) and then incubated in fresh medium. The cells were harvested before or at various times after incubation in fresh medium. The relative amount of 8-oxoG in cellular DNA then was measured as described previously. The decrease of 8-oxoG signal as a function of the chase time reflects 8-oxoG removal from DNA in whole cells.

Assay of Proteins that Bind to 8-oxoG DNA. HCT116 p53^{+/+} and HCT116 p53^{-/-} cells incubated with or without 2-ME were washed once with ice-cold PBS and lysed in three volumes of buffer containing 10 mmol/L Tris-HCl (pH 7.5), 0.5 mmol/L EDTA, 0.5% NP40, 200 mmol/L NaCl, 1 mmol/L DTT, 5% glycerol, and 2 mmol/L $MgCl_2$. The cells then were ruptured with 20 strokes of homogenization and centrifuged at 8000 \times g for 15 minutes. The supernatants then were used in a pull-down assay. A synthetic 8-oxoG-containing oligonucleotide annealed to its complementary oligomer with biotin at its 3' end was used to pull down the binding proteins. The oligonucleotides contain the following sequence: 5'-GAACTAGTGOATCCCCGGGCTGTC-3' and 3'-Bt-GAATCTTGATCACCTAGGGGGCCCGACG-5', where O represents 8-oxoG and Bt indicates biotin. The 8-oxoG oligonucleotide (2 pmol/ μ L) and its biotinylated template (1 pmol/ μ L) were mixed and heated in an annealing buffer [10 mmol/L Tris-HCl (pH 7.5), 5 mmol/L magnesium chloride, and 30 mmol/L sodium chloride] to 85°C and allowed to gradually cool to room temperature. Streptavidin-coated magnetic particles (1 mg) were

prewashed with a wash buffer [20 mmol/L Tris-HCl (pH 7.5), 0.1 mmol/L DTT, 2 mmol/L magnesium chloride, 100 mmol/L sodium chloride, 1 \times protease inhibitor mixture, and 100 μ g/mL BSA], mixed with the 8-oxoG-containing DNA hybrid, and kept at 24°C for 20 minutes. The beads with the bound DNA then were washed once with annealing buffer and resuspended in annealing buffer, mixed with the protein extracts from the HCT116 p53^{+/+} and HCT116 p53^{-/-} cells in a binding buffer [20 mmol/L Tris-HCl (pH 7.5), 0.1 mmol/L DTT, 2 mmol/L magnesium chloride, 300 mmol/L sodium chloride, and 1 \times protease inhibitors] in the presence of 100 pmol/ μ L competing DNA with identical nucleotide sequence but without 8-oxoG at position 10. The mixture then was incubated on ice for 60 minutes. The magnetic beads with DNA and bound proteins then were pulled down using a magnetic separator, washed three times with wash buffer, and resuspended in lysis buffer (50 mmol/L Tris-HCl, 10% glycerol, 2% SDS, 0.025% bromphenol blue, and 2.5% β -mercaptoethanol), followed by heating at 95°C for 5 minutes. The samples then were loaded on a 10% denaturing polyacrylamide gel. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane and blotted for p53, APE, and hOGG1.

In vitro Base Excision Assay. The effect of p53 on the activities of APE and hOGG1 in removing 8-oxoG was tested using a [^{32}P]-labeled 8-oxoG-containing oligonucleotide as a substrate for *in vitro* excision assay. The oligonucleotide hybrid contained the following sequence: 5'- ^{32}P GAAC-TAGTGOATCCCCGGGCTGTC-3' and 3'-CTTGATCACCTAGGGGGCCGACG-5', where O represents 8-oxoG. The 8-oxoG oligomer (20 pmol) was labeled with [^{32}P] at the 5' end using T4 polynucleotide kinase and annealed to its complementary oligomer (100 pmol). The *in vitro* base excision reaction mixtures (final volume, 15 μ L) contained buffer 1 [10 mmol/L HEPES-KOH (pH 7.4), 100 mmol/L potassium chloride, and 10 mmol/L EDTA], buffer 2 [10 mmol/L HEPES-KOH (pH 6.4), 100 mmol/L potassium chloride, and 10 mmol/L magnesium chloride], or buffer 3 [10 mmol/L HEPES-KOH (pH 6.8) and 15 mmol/L magnesium chloride] and an appropriate amount of ^{32}P -labeled DNA, hOGG1, APE, and p53 as indicated in each figure legend. The samples were incubated at 37°C for 60 minutes, and by adding one volume of loading buffer (95% formamide, 20 mmol/L EDTA, and 0.05% bromphenol blue) and heating to 90°C for 5 minutes, the reactions were stopped. The samples then were loaded onto a 12% denaturing polyacrylamide gel containing 8.3 mol/L urea. Following electrophoresis, the DNA bands were visualized by exposure to an X-ray film. The intensity associated with each band was quantified using a densitometer.

Immunoblot Analysis of p53 and Proteins. HCT116 p53^{+/+} cells were treated with 3 μ mol/L 2-ME or 500 to 1,000 μ mol/L H_2O_2 for various times, harvested, and washed with ice-cold PBS. The cells then were mixed with lysis buffer [1% Triton X-100, 300 mmol/L sodium chloride, 0.5% DOC, 25 mmol/L HEPES (pH 7.5), 20 mmol/L glycerol phosphate, 0.1% SDS, 1 mmol/L orthovanadate, 0.5 mmol/L DTT, 1.5 mmol/L magnesium chloride, 0.2 mmol/L EDTA, and 1 \times protease inhibitors], vortexed, and incubated on ice for 20 minutes. The lysates were centrifuged at 14,000 rpm at 4°C for 20 minutes. The supernatant was recovered and mixed with an equal volume of loading buffer (50 mmol/L Tris-HCl, 10% glycerol, 2% SDS, 0.025% bromphenol blue, and 2.5% β -mercaptoethanol) and heated to 95°C for 5 minutes. The samples then were loaded onto a 10% denaturing polyacrylamide gel. The proteins were separated by electrophoresis and transferred onto a nitrocellulose membrane. The membrane was blotted with antibodies specific to the proteins of interest.

Immunofluorescent Staining and Microscopic Analysis. HCT116 p53^{+/+} cells were grown on glass coverslips in 100-mm culture dishes. After appropriate drug treatment as indicated, the medium was aspirated, and the cells on glass slips were rinsed briefly with PBS and immediately fixed with a 1:1 mixture of methanol and acetone for 15 minutes. Following fixation, the cells were permeabilized with a mixture of 1% Triton X-100 and 0.5% NP40 in PBS for 30 minutes and blocked with 1% BSA in PBS for 30 minutes. The samples then were incubated with the primary antibody (anti-p53, 1:50 dilution in PBS) for 1 hour at room temperature, washed with PBS twice, and incubated with the secondary antibody (antimouse IgG-rhodamine conjugate, 1:40 dilution in PBS) for 1 hour in the dark. After washing with PBS twice, the coverslips were mounted on microscopic glass slides using Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and visualized and photographed using a Nikon Eclipse TE300 fluorescent microscope (Tokyo, Japan).

RESULTS

Accumulation of p53 Protein and Its Nuclear Localization in Response to ROS Exposure. Because the p53 protein plays a central role in cellular responses to various types of DNA damage and has been shown to physically associate with certain types of DNA lesions, we hypothesized that p53 may be involved in sensing ROS-mediated DNA damage and signaling the downstream cellular responses. To test this hypothesis, we used two agents, H_2O_2 and 2-ME, to cause ROS stress in the cells. H_2O_2 is known to diffuse into cells and in the presence of Fe^{2+} to generate OH^- radicals that damage DNA (15). 2-ME is able to cause O_2^- accumulation in cancer cells through inhibition of the antioxidant enzyme superoxide dismutase (32, 36, 37). We first assayed for changes in p53 protein levels and its localization following treatment with H_2O_2 and 2-ME. HCT116 cells with wild-type p53 were treated with 500 $\mu\text{mol/L}$ H_2O_2 for 1 hour or 3 $\mu\text{mol/L}$ 2-ME for 10 hours, immunostained for p53, and observed under a fluorescence microscope. As seen in Fig. 1A, the control cells expressed a low background level of p53, which was seen mainly in the cytoplasm. Following treatment with H_2O_2 (Fig. 1B) or 2-ME (Fig. 1C), there was a significant increase of p53 protein in the cells, indicated by increased intensity of the fluorescent signal. The p53 protein appears to localize in the nuclei. For reasons that are still unclear, the p53 protein consistently exhibited an uneven or clustered distribution when cells were exposed to H_2O_2 .

The increase in p53 protein levels in response to 2-ME and H_2O_2 treatment was further confirmed by immunoblot analysis. As shown in Fig. 1D, there was a significant increase in p53 protein levels after HCT116 cells were incubated with H_2O_2 . Similarly, a time-dependent increase of p53 protein also was seen when the HCT116 cells were incubated with 2-ME (Fig. 1E). A similar increase in p53 protein levels and its nuclear localization also was observed in the myeloid leukemia ML-1 cells (wild-type p53) after treatment with 2-ME (data not shown). These results suggest the possible involvement of p53 in cellular responses to ROS-inducing agents.

p53^{+/+} Cells Accumulate Lower Levels of Oxidative DNA Damage Compared with Their Isogenic p53^{-/-} Cells. We next examined whether the p53 response seen after treatment of HCT116

cells with H_2O_2 and 2-ME was associated with oxidative DNA damage and evaluated the possible role of p53 in affecting the removal of such DNA damage in intact cells. HCT116 p53^{+/+} cells were compared with their isogenic derivatives (HCT116 p53^{-/-} cells). The extent of oxidative DNA damage was assessed by measuring the levels of 8-oxoG residues in DNA, a commonly used marker of oxidative DNA damage (17, 18), using an assay kit as described in Materials and Methods. Treatment of cells with 100 and 500 $\mu\text{mol/L}$ of hydrogen peroxide (H_2O_2) resulted in an increase in 8-oxoG in DNA, as indicated by the increase in fluorescence of the 8-oxoG signal. However, there was a significant difference in the levels of 8-oxoG in the HCT116 p53^{+/+} and HCT116 p53^{-/-} cells. p53^{-/-} cells consistently exhibited higher 8-oxoG content than the p53^{+/+} cells (Fig. 2A; $P < 0.01$). Similar results were obtained following incubation of the cells with 2-ME, which caused a statistically significant increase in 8-oxoG in DNA in both cell lines (Fig. 2C). As in the case of the H_2O_2 -treated cells, 2-ME caused consistently higher levels of 8-oxoG in the p53^{-/-} cells than in the p53^{+/+} cells at all of the time points tested (Fig. 2B; $P < 0.01$). Interestingly, quantitation of the cellular superoxide contents showed that 2-ME induced similar increase of ROS in both cell lines at all of the time points tested (Fig. 2D), indicating that 2-ME-induced ROS accumulation is independent of p53 status. Therefore, the lower levels of 8-oxoG in p53^{+/+} cells than in p53^{-/-} cells exposed to the same levels of ROS stress suggest the possibility that the p53 protein may facilitate the removal of 8-oxoG from DNA.

Pulse-chase experiments then were performed to test whether p53 enhances the removal of 8-oxoG residues from DNA. Following induction of 8-oxoG accumulation in HCT116 p53^{+/+} and p53^{-/-} cells by a short-term incubation with H_2O_2 or 2-ME, the ROS-generating agents were washed out, and the cells were incubated in fresh medium (without H_2O_2 or 2-ME) for various times. The amount of 8-oxoG remaining at each time point was quantified to evaluate the rate of 8-oxoG elimination. As shown in Fig. 3, there was a more rapid removal of 8-oxoG from DNA in p53^{+/+} cells compared with p53^{-/-} cells. The rapid elimination of 8-oxoG from p53^{+/+} cells was consistently observed when the cells were treated with 2-ME (Fig. 3A and

Fig. 1. Accumulation of p53 protein in response to ROS stress in HCT116 cells. HCT116 cells (wild-type p53) were left untreated as control (A), treated with 500 $\mu\text{mol/L}$ H_2O_2 for 1 hour (B), or incubated with 3 $\mu\text{mol/L}$ 2-ME for 10 hours (C). The cells were fixed, permeabilized, and incubated with a mouse anti-human p53 antibody, followed by rhodamine-labeled anti-mouse IgG. Cells were visualized using a Nikon immunofluorescent microscope at 40 \times magnification. Bright field (top) and fluorescent images (bottom) were captured for the same fields. D, HCT116 cells were incubated with H_2O_2 , and total p53 protein was assayed by immunoblot analysis. Actin was used as a loading control. Lane 1, control; Lane 2, 500 $\mu\text{mol/L}$ H_2O_2 for 2 hours; Lane 3, 500 $\mu\text{mol/L}$ H_2O_2 for 5 hours; Lane 4, 1000 $\mu\text{mol/L}$ H_2O_2 for 2 hours. E, HCT116 cells were incubated with 3 $\mu\text{mol/L}$ 2-ME for various times, and the total p53 protein was assayed by immunoblot analysis. Lane 1, untreated control; Lanes 2–6, cells treated with 2-ME for 3, 6, 10, 14, and 24 hours, respectively.

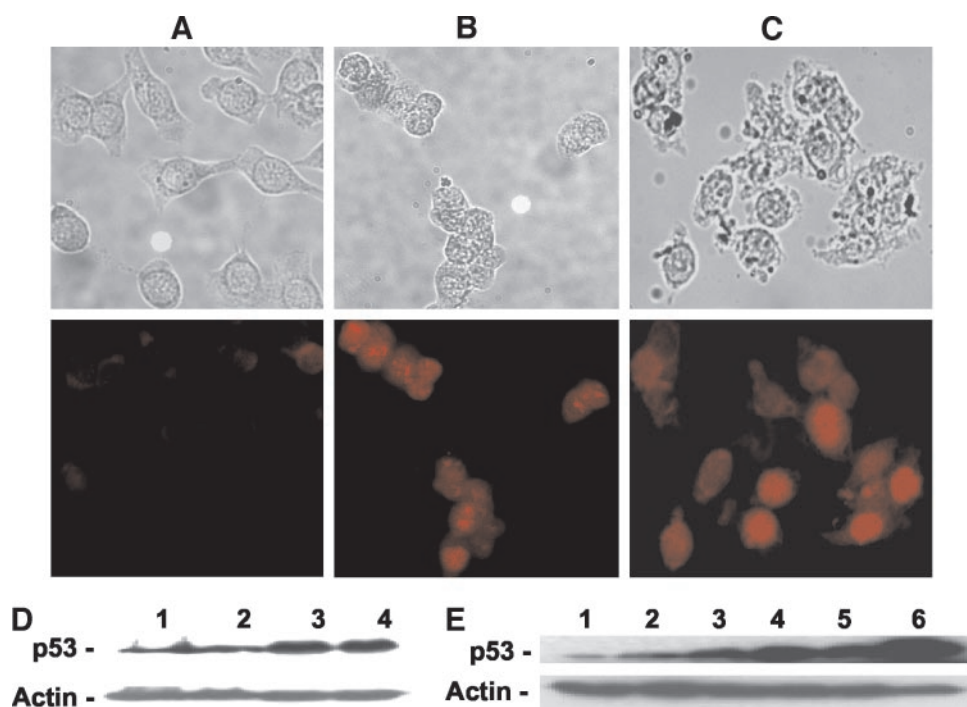
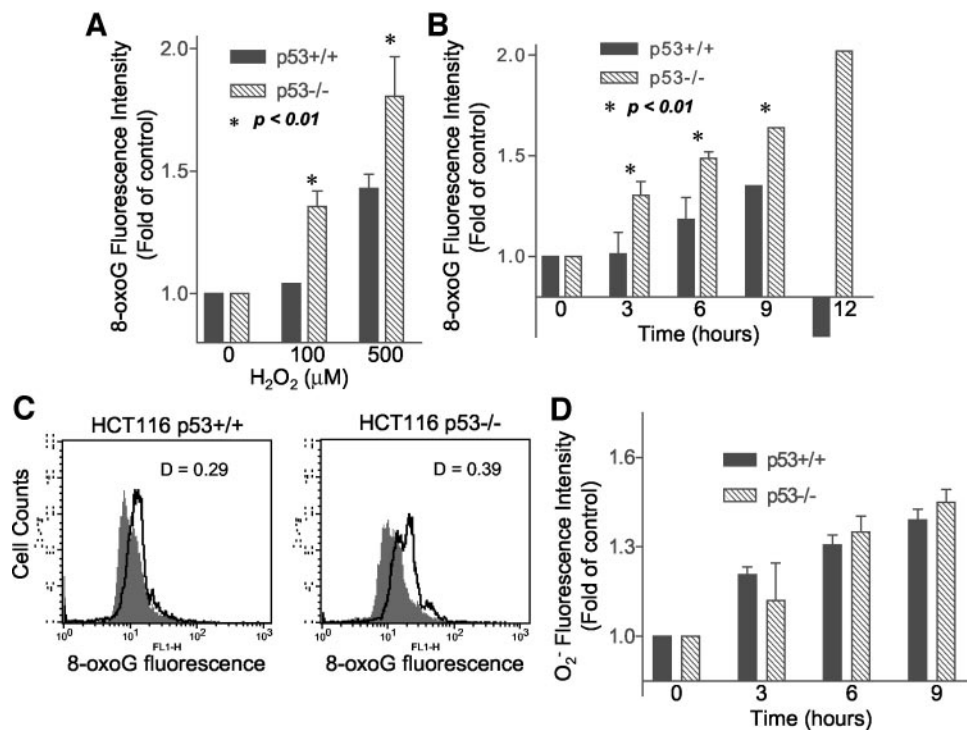


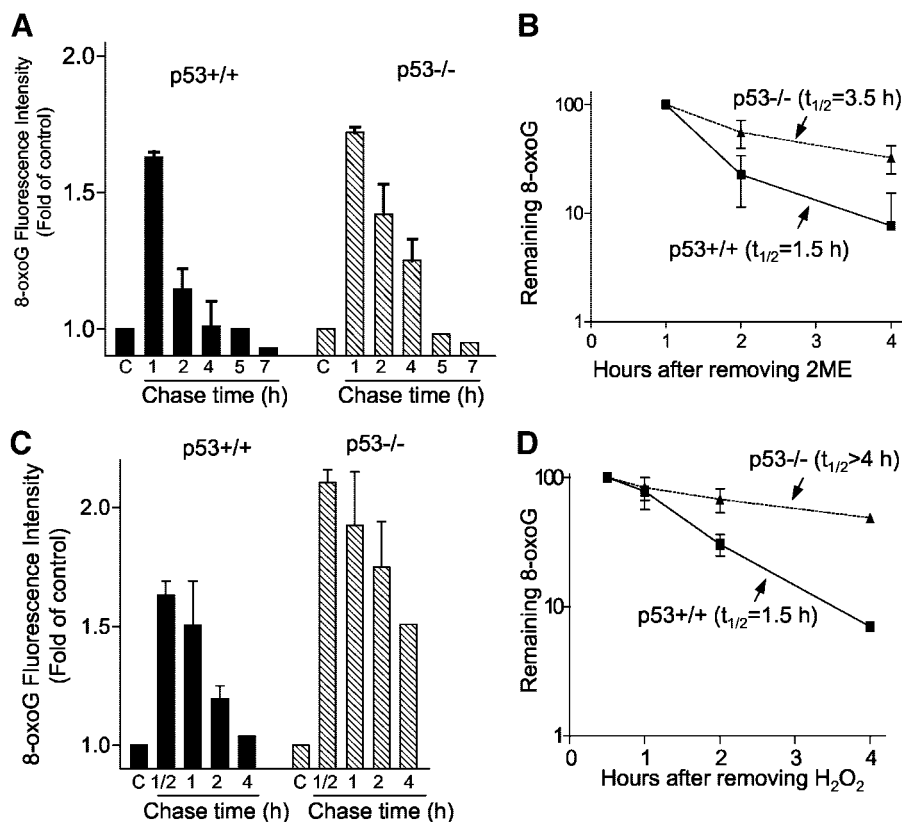
Fig. 2. Induction of oxidative DNA damage by H_2O_2 and 2-ME in HCT116 cells. HCT116 p53^{+/+} and p53^{-/-} cells were treated with the indicated concentrations of H_2O_2 for 1 hour (A) or with 3 $\mu\text{mol/L}$ 2-ME for various times (B). The cells were fixed, and 8-oxoG residues in DNA were quantified by flow cytometry analysis as described in Materials and Methods. The relative amount of 8-oxoG is expressed as fold of the control samples. Representative flow cytometric histograms are shown in C. Solid histogram, control; open histogram, cells treated with 3 $\mu\text{mol/L}$ 2-ME for 6 hours. The *D* value is calculated using Kolmogorov-Smirnov statistical method. A *D* value >0.15 indicates statistically significant difference between the control and treated samples. *D*, HCT116 p53^{+/+} and p53^{-/-} cells were incubated with 3 $\mu\text{mol/L}$ 2-ME for the indicated time periods, and the accumulation of superoxide ($O_2^{\cdot-}$) was determined as described in Materials and Methods and expressed as fold of untreated control samples.



B) or H_2O_2 (Fig. 3C and D). For instance, the time for the level of 8-oxoG to decrease by 50% ($t_{1/2}$) was ~ 1.5 hours in p53^{+/+} cells compared with the $t_{1/2}$ value of 3 or 4 hours in p53^{-/-} cells (Fig. 3A). These results suggest that p53 may facilitate the removal of 8-oxoG lesions from cellular DNA.

Binding of p53, hOGG1, and APE to 8-oxoG-Containing DNA *In vitro*. The accelerated removal of 8-oxoG lesions from DNA in p53^{+/+} cells suggests the possibility that p53 may interact with BER machinery, which includes hOGG1 and APE that are involved in the removal 8-oxoG from DNA. To test this possibility, a synthetic

Fig. 3. p53 facilitates the removal of 8-oxoG residues from DNA in whole cells. A, HCT116 p53^{+/+} and p53^{-/-} cells were treated with 3 $\mu\text{mol/L}$ 2-ME for 6 hours. The cells were washed by brief rinsing with fresh medium and then incubated in drug-free medium for the indicated times. The levels of 8-oxoG residues remaining in cellular DNA were measured at each chase time point as indicated and expressed as fold of the control sample. B, The percentage of the remaining 8-oxoG residues in cellular DNA was plotted as a function of chase time. The $t_{1/2}$ values indicate the estimated time for the level of 8-oxoG residues to decrease by 50%. C, HCT116 p53^{+/+} and p53^{-/-} cells were treated with 500 $\mu\text{mol/L}$ H_2O_2 for 1 hour. The cells were washed and chased, and 8-oxoG residues remaining in cellular DNA were measured as described previously. The percentage of the remaining 8-oxoG residues in cellular DNA was plotted as a function of chase time (D).



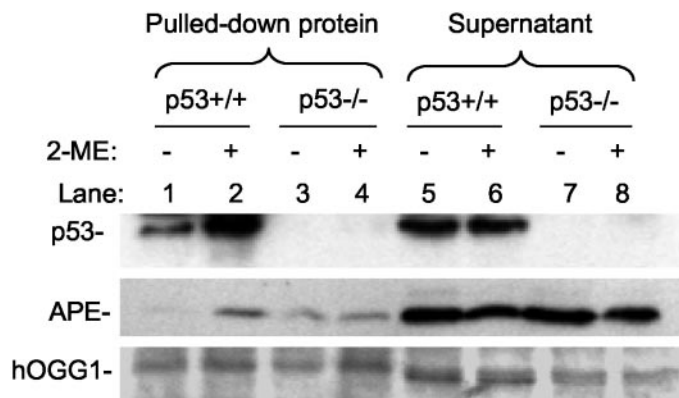


Fig. 4. Physical interaction of 8-oxoG-containing DNA with p53, APE, and hOGG1 *in vitro*. Streptavidin-coated magnetic beads carrying biotinylated oligomers containing 8-oxoG were used to pull down binding proteins from cell extracts in the presence of 100-fold competing DNA with identical sequence but without 8-oxoG. The pulled-down proteins were washed, eluted from the beads, and subjected to Western blot analysis for hOGG1, APE, and p53 as described in Materials and Methods. Lanes 1 and 2, pulled-down products from protein extracts of HCT116 p53^{+/+} cells treated without and with 2-ME (3 μ mol/L for 9 hours), respectively; Lanes 3 and 4, pulled-down products from protein extracts of HCT116 p53^{-/-} cells treated without and with 2-ME (3 μ mol/L for 9 hours), respectively; Lanes 5–8, supernatants of the pull-down samples corresponding to Lanes 1–4, respectively.

oligonucleotide containing 8-oxoG was used to pull down the proteins that preferentially bind 8-oxoG-containing DNA in the presence of 100 folds competing DNA as described in Materials and Methods. The pulled-down protein components subsequently were eluted and analyzed by immunoblot analysis. As shown in Fig. 4, p53 protein along with APE and hOGG1 were pulled down by the 8-oxoG-containing oligonucleotide. In the protein extracts from cells pretreated with 2-ME, there was a significant increase of p53 bound to 8-oxoG-containing DNA, accompanied by a greater binding of APE (Lane 2). The binding of hOGG1 to 8-oxoG appeared not to be significantly affected by the p53 status (Lanes 1 through 4) but was increased by pretreatment of the cells with 2-ME (Lanes 2 and 4).

p53 Enhances the Activities of hOGG1 and APE in Removal of 8-oxoG from DNA. To test the ability of p53 to functionally affect the removal of 8-oxoG from DNA by hOGG1 and APE, we carried out an *in vitro* base excision assay. A synthetic 8-oxoG-containing oligonucleotide was labeled with [³²P] at its 5' end and annealed to a complementary oligomer to generate a hybrid as the substrate for *in vitro* excision by hOGG1 and APE in the presence and absence of p53. As illustrated in the top panel of Fig. 5A, the biochemical activity of these proteins alone or in combination was measured by their ability to remove the modified base and cleave the DNA backbone to generate cleaved products that can be revealed on a denaturing polyacrylamide gel. Because hOGG1 is known to remove 8-oxo-guanine to generate an AP site and can then cleave the DNA backbone 3' to the AP base, the action of hOGG1 is expected to produce a [³²P]-labeled 10-nucleotide product. In contrast, APE cleaves the DNA backbone 5' to the AP site to generate a 9-nucleotide band. The combined activities of hOGG1 and APE on the 8-oxoG-containing oligonucleotide construct would result in a major 9-nucleotide band caused by the sequential activity of both enzymes. As shown in Fig. 5A, the 8-oxoG-containing oligonucleotide hybrid alone or its incubation with reaction buffer exhibited a single band at the 24-nucleotide position (Lanes 1 and 2). Addition of hOGG1 to the reaction generated a predominant 10-nucleotide band (Lane 3). The presence of p53 did not increase the intensity of the 10-nucleotide band, indicating that p53 did not affect hOGG1 activity (Lane 5). p53 or APE alone did not cause any DNA cleavage (Lanes 4 and 6). Combination of hOGG1 and APE resulted in a major cleaved band at the

9-nucleotide position (Lane 7). p53 showed minimum effect in enhancing the activity of APE when 8-oxoG oligonucleotide was used as the substrate (Lane 8). However, when the 8-oxoG-containing oligomer was incubated with a mixture of hOGG1, APE, and p53, a

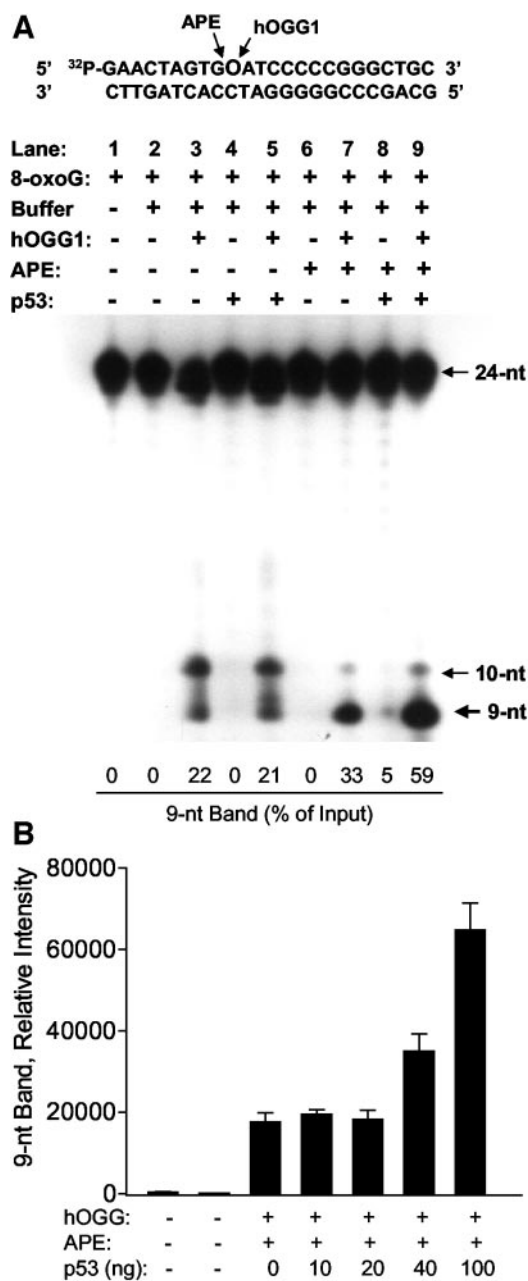


Fig. 5. Removal of 8-oxoG-nucleotide by hOGG1 and APE is enhanced by p53 *in vitro*. **A**, 8-oxoG removal/incision was carried out in 15- μ L reactions containing the [³²P]-labeled 8-oxoG-containing oligodeoxyribonucleotide (incision sites indicated) and various combinations of the protein components as indicated (hOGG1, 66 ng; APE, 74 ng; p53, 40 ng). The samples were incubated at 37°C in appropriate buffer for 60 minutes and terminated by adding an equal volume of loading buffer containing 20 mmol/L EDTA and 95% formamide. The samples were run on a 12% denaturing polyacrylamide gel as described in Materials and Methods. The number below each lane represents the relative radioactivity of the 9-nucleotide band (cleaved product) expressed as percentage of the total input. **B**, concentration-dependent effect of p53 on 8-oxoG removal by hOGG1 and APE. The reaction mixtures (15 μ L) containing [³²P]-labeled 8-oxoG-DNA, hOGG1 (66 ng), APE (74 ng), and various amounts of p53 (0 to 100 ng) were incubated at 37°C for 60 minutes. The reaction products were analyzed by gel electrophoresis as described previously. The band intensity of the 9-nucleotide product was measured by densitometry analysis, and the background density of the same band area in the negative control lane was subtracted from each lane. The error bars represent the mean and SE of three separate gels.

striking increase of 9-nucleotide cleaved band was produced (*Lane 9*). Comparison of the 9-nucleotide band intensity in *Lane 4* (p53 alone), *Lane 7* (hOGG1 and APE), and *Lane 9* (hOGG1, APE, and p53) suggests that p53 is able to significantly enhance the combined activity of hOGG1 and APE in the removal of the nucleotide containing 8-oxoG.

We next examined whether the effect of p53 on the combined activities of hOGG1 and APE in removal of 8-oxoG is concentration dependent. The labeled 8-oxoG-containing oligonucleotide hybrid was incubated with hOGG1, APE, and varying concentrations of p53 (up to 100 ng/15 μ L reaction) under the conditions described in Materials and Methods. The effect of p53 on the combined activities of hOGG1 and APE was determined as described previously, and the band density of each 9-nucleotide band was quantified. As shown in Fig. 5B, p53 at the concentration <20 ng/reaction (15 μ L) did not significantly affect the activity of hOGG1 and APE in cleaving the 8-oxoG DNA. A high concentration of p53 (40 ng/reaction) caused a substantial increase in the combined enzyme activities, which was further enhanced when a higher concentration of p53 (100 ng/reaction) was added. Thus, p53 is able to increase the activity of hOGG1 and APE in a concentration-dependent manner.

DISCUSSION

The role of p53 in mediating DNA damage response is primarily achieved through the activation of p53 as a transcriptional factor and induction of target genes involved in cell cycle arrest, DNA repair, or apoptosis. Thus, p53 prevents the propagation of deleterious lesions in DNA and is considered as a guardian of the genome. p53 also has been shown to directly recognize and bind DNA lesions and activate cellular responses. However, this latter function of p53 as a potential sensor of DNA damage is less understood. We previously have shown that p53, along with DNA-dependent protein kinase, is involved in sensing DNA strand termination induced by incorporation of anticancer nucleoside analogs in DNA and in triggering apoptosis (38). The study presented here has shown that DNA containing an 8-oxoG residue pulled down p53 protein together with hOGG1 and APE. Importantly, p53 significantly enhanced the combined activity of APE and hOGG1 in excising the 8-oxoG residue. This was consistent with the more rapid removal of these oxidized residues from cellular DNA in whole cells with wild-type p53. Together, these results suggest that p53 plays a role in directly interacting with ROS-damaged DNA and the BER molecules, and in facilitating cellular responses to ROS-induced DNA damage.

Our study suggests a possible physical and functional interaction between p53, hOGG1, and APE. hOGG1 and APE are important components of the BER machinery for the repair of oxidative DNA damage. Recent studies have shown the interaction of p53 with APE *in vivo* using a cell line with inducible p53 (39) and with APE and DNA pol β in the presence of uracil-containing DNA *in vitro* (31). In our study, extracts from p53^{+/+} cells treated with the ROS-generating agent 2-ME showed a significant increase of p53 binding to 8-oxoG-containing DNA (Fig. 4, *Lane 2*). In this pull-down experiment, the amount of p53 protein remaining in the supernatants was similar in the extracts of the untreated and treated cells (*Lanes 5 and 6*). These data suggest that most of the increased p53 protein in the treated cells may be functionally activated and able to bind 8-oxoG-containing DNA, leaving only a smaller portion of p53 protein in the supernatant, which is comparable with the amount of the unbound p53 protein in the untreated cells. Thus, it is likely that the increase of p53 protein in the 2-ME-treated cells reflects the *in vivo* activation of p53 and its accumulation, with a large portion of p53 protein being in an active state.

Earlier studies have shown that p53 enhances BER of DNA containing uracil or methylated bases, and this was shown to occur through the interaction of p53 with APE and pol β (29–31). Consistent with these findings, our study further demonstrated that p53 is able to enhance the combined activities of hOGG1 and APE in excising the 8-oxoG residue and to remove the damaged nucleotide from DNA *in vitro* (Fig. 5). It appears that p53 is unable to enhance the activity of hOGG1 alone. The inability of APE alone to cleave 8-oxoG-containing DNA is consistent with the earlier reports that hOGG1 is required to first remove the 8-oxoG base to generate an AP site for APE action (40, 41). The ability of p53 to significantly enhance the combined activities of hOGG1 and APE is striking. Yet, the detailed molecular interactions among these three molecules require further characterization. The ability of activated p53 to increase binding of APE to 8-oxoG-containing DNA may contribute to this functional enhancement. It is important to note that this enhancement of activity is not caused by the effect of p53 on APE alone because there is a significant difference in the removal of 8-oxoG in the presence and absence of hOGG1 (Fig. 5A). The enhancement of the combined activities of hOGG1 and APE occurs in a concentration-dependent manner. It is unclear at the present time whether there may be a stoichiometric ratio of p53, hOGG1, and APE required for optimal activity. The ability of p53 to enhance the combined activities of hOGG1 and APE in the removal of 8-oxoG from DNA *in vitro* is likely responsible, at least in part, for the more rapid removal of 8-oxoG from cellular DNA in p53^{+/+} cells exposed to H₂O₂ or 2-ME (Fig. 3).

On the basis of our data, we postulate that in response to oxidative DNA damage, p53 localizes to the nucleus and physically interacts with hOGG1, APE, and 8-oxoG-containing DNA. The p53 protein stimulates hOGG1 and APE to rapidly excise the 8-oxoG residues from DNA. In the absence of p53, 8-oxoG residues are excised by the action of hOGG1 and APE but at a lower rate. Thus, p53 plays an important role in sensing ROS-induced 8-oxoG lesions in DNA and in facilitating the removal of these lesions from DNA.

Because p53 seems to play an important role in DNA BER, it is possible that the enhanced removal of the 8-oxoG residues by p53 would minimize gene mutations under oxidative stress. The association of p53 with DNA repair proteins at sites of DNA damage and its activation by DNA repair intermediates also has been demonstrated in other DNA repair systems (38, 42). Because p53 also has been shown to promote apoptosis in response to DNA damage, it also is possible that when cells are exposed to severe ROS stress, the high level of DNA damage may cause persistent activation of p53, leading to induction of apoptosis in the damaged cells. This also may help to eliminate the cells with DNA damage and thus maintain the genetic integrity of the whole cell population.

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