p53R2-dependent Pathway for DNA Synthesis in a p53-regulated Cell Cycle Checkpoint

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ABSTRACT

A recently identified ribonucleotide reductase (RR), p53R2, is directly regulated by p53 for supplying nucleotides to repair damaged DNA. We examined the role of this p53R2-dependent pathway for DNA synthesis in a p53-regulated cell cycle checkpoint, comparing it to R2-dependent DNA synthesis. The elevation of DNA synthesis activity through RR in response to γ-irradiation was closely correlated with the level of expression of p53R2 but not of R2. The p53R2 product accumulated in nuclei, whereas R2 levels in cytoplasm decreased. We found a point mutation of p53R2 in cancer cell line HCT116, which resulted in loss of RR activity. In those cells, DNA damage-inducible apoptotic cell death was enhanced through transcriptional activation of p53AIP1. The results suggest that p53R2-dependent DNA synthesis plays a pivotal role in cell survival by repairing damaged DNA in the nucleus and that dysfunction of this pathway might result in activation of p53-dependent apoptosis to eliminate dangerous cells.

INTRODUCTION

RRs catalyze conversion of rNDPs to the corresponding deoxyribonucleotides and provide a balanced supply of precursors for DNA synthesis (1). An RR molecule consists of two dissimilar dimeric protein components, i.e., a large regulatory subunit (R1) and a small catalytic subunit (R2; Ref. 1). The enzyme is activated in S phase, where it contributes to DNA synthesis and replication. Neither the mRNA nor protein levels of R1 change throughout the cell cycle, whereas levels of both R2 mRNA and R2 protein increase during S phase and decrease outside of S phase (2). These facts suggest that the expression of R2 might be regulated in a cell cycle-dependent manner and that R2 might be a rate-limiting factor for supplying dNTPs for DNA synthesis in S phase.

On the other hand, the role of R2 in DNA repair seems to differ among species. In yeast, expression of RNR2, the homologue of human R2, is induced in response to DNA damage and plays a significant role in DNA repair (3–5). Moreover, yeast chromosomes encode two kinds of large subunit; RNR2, the homologue of human R1, and RNR3 is inducible by DNA damage (6). The homologue of RNR3 has never been reported in mouse or human. This implies that the mechanism of dNTP supply in the mammalian DNA damage-signaling pathway might be quite different from that in yeast. Several studies have indicated a possibility that murine or human R2 might be related to DNA repair (2, 7, 8), but those data seem much less convincing than data regarding the process in yeast. The mechanism of the DNA damage-signaling pathway in humans remains largely unknown.

We identified recently a novel RR gene, p53R2, which encodes a product showing a high degree of identity to R2 except in the NH2-terminal portion. The NH2-terminal difference could imply different roles for p53R2 and R2 (9). We found that the expression of p53R2 was induced by wt p53 in response to various genotoxic stresses, including γ-irradiation, UV-irradiation, or exposure to adriamycin, whereas expression of R2 occurred in a cell cycle-dependent manner and was in fact suppressed in response to DNA damage. We demonstrated that DNA synthesis in cells arrested in G1 or G2 after DNA damage was mediated through the RR activity of p53R2 and concluded that p53R2 might be directly involved in processes designed to repair the damage (9). Furthermore, p53R2 was located in nuclei of the damaged cells, in striking contrast to previous reports that R2 is present only in cytoplasm (10, 11). Those results prompted us to infer that two independent pathways for supply of dNTPs might exist, one involved in normal maintenance of dNTPs for DNA replication during S phase and the other supplying dNTPs for urgent repair of DNA damage.

The DNA damage checkpoint plays a critical role in preventing genomic instability by regulating the cell cycle and DNA repair (12). Inactivation of the checkpoint may impair the DNA repair mechanism and increase susceptibility of cells to genotoxic agents (13). p53, one of the critical checkpoint genes, is frequently mutated in cancers of various types. Genomic instability is often observed in cancers carrying p53 mutations, but its mechanism is not fully understood; however, the discovery of p53R2 provided an important clue for clarifying it. That is, p53 appears to participate directly in DNA repair by inducing p53R2 in response to DNA damage. Thus, inactivation of p53 could directly interfere with damage-induced transcription of p53R2, enhance mis-incorporation of dNTPs, and increase the frequency of mutations, resulting in genomic instability in cancers where p53 has undergone mutation.

Here we report evidence to support our hypothesis that two independent pathways exist in human cells to supply dNTPs for DNA synthesis, one involving R2 and the other, p53R2. Our results indicate that inactivation of either p53 or its transcriptional target, p53R2, should interfere with regulation of the p53-dependent DNA repair pathway and increase the sensitivity of cells to anticancer drugs.

MATERIALS AND METHODS

Cell Culture. Normal human fibroblast cell line NHDF4042, derived from neonatal skin, was purchased from Clonetics, Inc., Lovo, HCT116 (colorectal adenocarcinomas), and MCF7 (human mammary carcinoma) cells were purchased from American Type Culture Collection. All cells were cultured under conditions recommended by their respective depositories.

Cell Cycle Analysis. γ-irradiated cells were trypsinized, washed in PBS, and fixed with 70% ethanol at the indicated times. Fixed samples were centrifuged, treated with RNase (1 mg/ml), and resuspended in propidium iodide (50 μg/ml). The stained cells were analyzed on a Becton Dickinson FACScan flow cytometer.

Antibodies. Polyclonal antibodies against p53R2 and R2 proteins were generated in rabbits, using recombinant p53R2 and R2 proteins synthesized in...
bacteria. The antibodies were affinity purified using the same recombinant proteins. Anti-p21 Waf1 antibody was purchased from Transduction Laboratories (Lexington, KY). Mouse monoclonal anti-ß-actin antibody was purchased from Sigma Chemical Co.

Immunocytochemistry. Cells were plated on poly-D-lysine-coated multi-well chamber slides (Becton Dickinson), and 24 h later, the cells were damaged by 14 Gy of y-irradiation. The cells were fixed 72 h later with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS for 3 min at 4°C. The cells were covered with blocking solution (3% BSA and 2.5% goat serum in PBS) for 1 h at room temperature and incubated for 1 h at room temperature with a rabbit anti-p53R2 or anti-R2 antibody diluted 1:1000 in blocking solution. This antibody was stained with a goat antirabbit secondary antibody conjugated to FITC and viewed with an ECLIPSE E800 microscope (Nikon).

Immunoblotting. For preparation of whole-cell lysates, adherent and detached cells were collected and resuspended in chilled lysis buffer containing 150 mM NaCl, 50 mM Tris (pH 8.0), 1% NP-40, 0.5% deoxycholate, 0.1% SDS, and 0.5 mM phenylmethanesulfonyl fluoride hydrochloride. Collected cells were allowed to lyse for 30 min on ice. The lysates were spun for 5 min in a microcentrifuge at 4°C, and the supernatants were measured for protein concentration by detergent-compatible protein assay (Bio-Rad), then boiled in SDS sample buffer. A 20-µg aliquot of each sample was loaded onto a 10% SDS-PAGE gel and blotted onto a nitrocellulose membrane (Amersham Pharmacia Biotech). Protein bands on Western blots were visualized by enhanced chemiluminescence detection (Amersham Pharmacia Biotech).

Preparation of Subcellular Fractions. Cells were suspended in 10 volumes of a hypotonic buffer [0.01 M NaCl, 1.5 mM MgCl2, and 0.01 M Tris-HCl (pH 7.4)], incubated for 10 min at 4°C, and homogenized by 10 strokes in a Dounce homogenizer. The homogenates were centrifuged at 1,000 g for 3 min at 4°C to collect nuclei; then the supernatants were centrifuged at 14,000 g for 30 min at 4°C to collect the heavy membrane pellet. The final supernatants were considered cytosolic fractions. The nuclei and heavy membrane preparations were washed with a hypotonic buffer and suspended in lysis buffer.

Antisense Oligonucleotides. To inhibit the expression of endogenous p53R2 and R2, we prepared high-performance liquid chromatography-purified antisense oligonucleotides (p53R2AS, ACATTTACCTCATCCT; R2AS, GGCCCTACCGTGTTCT) and as controls, sense oligonucleotides (p53R2S, AGGATGAGGTAAATGT; R2S, AGAACACGGTGAGCCC), according to the sequences of the p53R2 and R2 genes. The oligonucleotides (1 µM each) were transfected with Lipofectin reagent (Life Technologies, Inc.) for 4 h, after which the transfected cells were damaged by y-irradiation (14 Gy).
**Assay of DNA Synthesis.** We evaluated RR activity by means of a DNA synthesis assay coupled with measurement of reduction of rNDP, with some modifications. For the DNA damage experiments, $2 \times 10^6$ cells were plated on 10-cm dishes, and after 24 h, the cells were damaged by 14 Gy of $\gamma$-irradiation. RR activity was analyzed at the indicated times after DNA damage. To standardize the activity, we counted the number of live cells by trypan blue staining immediately after harvesting and used $1 \times 10^6$ cells in each experiment to quantify DNA repair activity. For permeabilization, cells with or without DNA damage were washed twice in solution “A” [150 mM sucrose, 80 mM KCl, 35 mM HEPES (pH 7.4), 5 mM potassium phosphate (pH 7.4), 5 mM MgCl2, and 0.5 mM CaCl2], suspended in cold solution “A” containing 0.25 $\mu$g/ml lysolecithin (Sigma Chemical Co.), and incubated for 1 min at 4°C.

![Fig. 3. Immunostaining of p53R2 (top panel) and R2 (bottom panel) proteins in wt p53 cell lines with or without DNA damage from 14 Gy of $\gamma$-irradiation. Cells were stained with rabbit polyclonal anti-p53R2 or anti-R2 antibodies 72 h after irradiation.](image)
To measure the degree of rNDP reduction and DNA synthesis, 1 × 10⁶ permeabilized cells were incubated at 37°C for 10 min in 300 µl of 50 mM HEPES (pH 7.4) containing 10 mM MgCl₂, 8 mM DTT, 0.06 mM FeCl₃, 7.5 mM potassium phosphate (pH 7.4), 0.75 mM CaCl₂, 10 mM phosphoenolpyruvate, 2 mM ATP, 0.2 mM [³H]rCDP, 0.2 mM rGDP, 0.2 mM rADP, and 0.2 mM dTDP. Then a 300-µl aliquot of each incubation mixture was added to 60 µl of 60% percholic acid/0.1% sodium PP i and kept on ice for 15 min. This mixture was diluted by adding 1 ml of distilled H₂O and centrifuged to precipitate acid-insoluble material. The pellet was extracted with 0.1 ml of 0.2 N NaOH and incubated at 37°C for 30 min. The entire process of percholic acid precipitation and NaOH extraction was repeated, and 75-µl aliquots of the NaOH suspensions in 5 ml of AQUASOL-2 (Packard) were counted for radioactivity with an Aloka LSC-5100 liquid scintillation counter.

Mutant (V115L) Construct of a p53R2 Expression Plasmid. The entire coding sequence of p53R2 cDNA was amplified by PCR using 1 µl/plaque-forming unit DNA polymerase (Stratagene) and inserted between the EcoRI and XhoI sites of the pcDNA 3.1 mammalian expression vector, which contains a cytomegalovirus promoter. The QuikChange Site-directed Mutagenesis Kit (Stratagene) was used according to the manufacturer’s instructions, with oligonucleotides 5'-CGCTTTAGTCAGGAGCTGCAGGTTCCAGAGG-3' and 5'-CCTCTGGAACCTGCAGCTCCTGACTAAAGCG-3' to introduce a single-nucleotide change in codon 115 of human p53R2 from Valine to Leucine. The presence of the changes was confirmed by DNA sequencing.

For transfection, 1 × 10⁶ cells were seeded into 10-cm dishes and transfected with 4 µg of plasmid mixture preincubated for 15 min with 25 µl of FuGENE6 Transfection Reagent (Roche). After 12 h of incubation at 37°C, transfected cells were damaged by γ-irradiation (14 Gy) and analyzed for RR activity 48 h later.

RESULTS

Indispensability of p53R2 for DNA Synthesis after DNA Damage. Using four cell lines containing wt p53, we examined DNA synthesis activity through RR in response to 14 Gy of γ-irradiation. All four cell lines revealed time-dependent induction of RR, although the levels of enzyme activity varied significantly from one line to another (Fig. 1). To determine whether this RR activity reflected p53R2 or R2, we examined the levels of both proteins by Western blot analysis. As Fig. 1 shows, p53R2 was induced by DNA damage in all four cell lines in a time-dependent manner, whereas the amounts of R2 proteins decreased. The RR activity in each line except for HCT116 was proportional to the amount of p53R2 protein but not to the amount of R2. These results supported our hypothesis that there are two independent pathways for supply of deoxyribonucleotides, one through activity of R2 at S phase and the other through p53R2 for DNA repair in cells arrested at G₁ or G₂.

To confirm that the increased RR activity in wt p53 cell lines after DNA damage had originated in enhanced expression of p53R2, we prepared antisense oligonucleotides p53R2-AS and R2-AS, corresponding respectively to the sequences of the p53R2 and R2 genes, and then examined their effects on the RR activity in MCF7 cells with or without DNA damage. Treatment with p53R2-AS inhibited damage-induced RR activity in these cells (Fig. 2A); as expected, although R2-AS inhibited RR activity in undamaged MCF7 cells, it did not inhibit total RR activity in damaged MCF7 at all. The expressions of p53R2 and R2 proteins were confirmed to be inhibited by antisense oligonucleotides, p53R2-AS and R2-AS, respectively (Fig. 2B). These results, taken together, suggest that the increased RR activity after DNA damage totally depends on p53R2.

Subcellular Localization of p53R2 and R2. In a previous report, we demonstrated nuclear accumulation of p53R2 in response to DNA damage and suggested that R2 may produce deoxynucleotides in the cytoplasm, whereas p53R2 may synthesize dNTPs from rNDPs in nuclei and supply them for repairing the genome (9). To evaluate this hypothesis additionally, we examined the subcellular locations of R2...
and p53R2 in the four cell lines with wt p53, with or without DNA damage, using anti-R2-specific or anti-p53R2-specific antibodies. p53R2 was slightly detectable in perinuclear and other cytoplasmic regions of undamaged cells, but in addition, we clearly observed its induction and accumulation in nuclei in response to DNA damage (Fig. 3, top panel). In contrast, R2 staining was clear in the cytoplasm of cells without DNA damage but very weak after exposure to /H9253-irradiation (Fig. 3, bottom panel). Moreover, to confirm the damage-inducible nuclear localization of p53R2, cell lysates were fractionated by differential centrifugation to separate the nuclear and cytoplasmic components. Then, those components were subjected to Western blot analysis with anti-p53R2 or anti-R2-specific antibody. In the /H9253-irradiated (14 Gy) MCF7 cells used in this experiment, nuclear localization of p53R2 was confirmed by immunostaining and immunoblotting with anti-p53R2-specific antibody (Fig. 4). These experiments clearly indicated that p53R2, but not R2, increased in nuclei in response to DNA damage in a time-dependent manner.

Point Mutation of p53R2 in the HCT116 Cell Line. The amount of p53R2 protein detected by Western analysis in HCT116 cells did not reflect the degree of RR activity, which was lower than in the other wt p53 cell lines, although HCT116 actually contained a larger amount of the protein than the others (see Fig. 1). However, we had detected two forms of p53R2 protein in HCT116, each showing a different mobility on Western blots. Hence, we considered that enzymatic activity of p53R2 might have been lost because of a genetic alteration in HCT116 cells. By sequencing the entire p53R2 cDNA prepared from mRNA, we found a point mutation at codon 115 that caused an amino acid substitution from valine to leucine (Fig. 5A).

This mutation was not present in genomic DNAs isolated from 100 control individuals. Moreover, as this valine residue is conserved in the RR small subunit from yeast to human, this nucleotide change would be likely to affect RR activity.

As was evident in Fig. 1, the faster mobility form of p53R2 protein in HCT116 cells was more abundant than the slower form, implying greater stability of the smaller molecule. To determine which form corresponded to the mutant p53R2 protein, we performed Western blot analysis of MCF7 cells transfected with either wt or mutant p53R2 expression vector, using anti-p53R2 polyclonal antibody. As shown in Fig. 5B, the faster form was observed only in lysates of COS7 and MCF7 cells transfected with the mutant vector. Although the expression levels of wt and mutant p53R2 mRNAs in HCT116 were similar according to reverse transcription-PCR experiments...
In Relation to Activation of p53-dependent Apoptosis.

We had demonstrated previously that inhibition of p53R2 expression enhanced the sensitivity of cells to diverse agents of DNA damage (9). Hence, we reasoned that HCT116 cells containing a point mutation of p53R2 might reveal increased sensitivity to DNA damage. Thus, we examined the cell cycle profiles of the four wt p53 cell lines after DNA damage induced by 14 Gy γ-irradiation. As shown in Fig. 6A, the cell cycles of the three cell lines without mutation of p53R2 were arrested at G1 and G2 until 72 h, whereas remarkable apoptosis occurred at 12 h in HCT116. Moreover, expression of p53AIP1, a pivotal mediator for p53-dependent apoptosis in these cell lines, was strikingly induced in HCT116 cells (Fig. 6B, bottom panel) but not at all in MCF7 (Fig. 6B, top panel), LoVo, or NHDF cells (data not shown). These results suggest that inactivation of p53R2 might cause activation of a p53-dependent apoptotic pathway to eliminate the dangerous cells with unrepaired DNA (Fig. 7).

DISCUSSION

RNR2 in yeast and R2 in mouse, as well as R2 in human cells, have each been induced after DNA damage, suggesting that R2 might have a role in DNA repair (2–5, 7). The RR activity of Caski cells (a cervical cancer cell line) was shown to increase 4-fold after γ-irradiation (2). R2 protein is stabilized by post-translational, but not transcriptional, regulation after UV irradiation (8). We were able to observe induction of R2 protein until 12 or 24 h after γ-irradiation, especially in cells bearing mutant p53 genes (data not shown). The induction of R2 protein by DNA damage appeared to be correlated with dysregulation of the cell cycle in our system, because mutant p53 cell lines had revealed progression of the cell cycle in the face of DNA damage signaling. In the wt p53 cell lines where the cell cycle was regulated normally and p53R2 was induced by γ-irradiation, we were unable to detect induction of R2 protein (Fig. 3). Although the involvement of R2 in DNA repair remains to be clarified, our results have indicated that p53R2, a transcriptional target of p53, is the major provider of nucleotides for the DNA repair process. Indeed, recent evidence that mammalian p53R2 forms an active RR in vitro with the R1 protein strongly supports this notion (14).

In three cell lines with wt p53 that were tolerant to γ-irradiation in the experiments reported here (MCF7, NHDF, and LoVo), p53R2 protein was induced in a time-dependent manner and accumulated in nuclei. In these three lines, R2 was strongly expressed in cytoplasm in (data not shown), the amount of mutant p53R2 was much greater than that of wt p53R2. This result indicated that the mutant (V115L) p53R2 protein might be more stable than the wt.

Although the other allele contained wt p53R2, we speculated that the mutant allele might influence the wt in a dominant-negative manner because the RR small subunit is known to form homo-dimers.

To confirm the physiological significance of this mutation, we prepared expression vectors for wt p53R2 and the mutant allele (p53R2-V115L) in pcDNA3.1 and examined the RR activity of MCF7 cells transfected with either vector. Transfection of wt p53R2, but not the mutant p53R2, clearly revealed enhancement of damage-induced RR activity (Fig. 5C). The RR activity of MCF7 cells transfected with the mutant (V115L) p53R2 expression vector was nearly equivalent to that of mock vector, suggesting that the mutation of valine to leucine at codon 115 was likely to result in loss of the RR activity of p53R2 without causing a dominant-negative effect on endogenous p53R2 protein. Although the mechanism behind this phenomenon is unclear, this result may explain why the RR activity of HCT116 was not proportional to the amount of p53R2 protein present.

Inactivation of the p53R2-dependent DNA Synthesis Pathway in Relation to Activation of p53-dependent Apoptosis. We had demonstrated previously that inhibition of p53R2 expression enhanced the sensitivity of cells to diverse agents of DNA damage (9).
the absence of DNA damage, but its expression was significantly repressed, and the protein disappeared from cytoplasm after γ-irradiation. These results supported our hypothesis that there are two independent pathways for nucleotide supply, R2 and p53R2: R2 is involved in DNA replication at S phase, and p53R2 is involved in DNA repair at arrested G1 and G2 phases (9).

We found a point mutation of the p53R2 gene at codon 115 in the HCT116 cancer cell line, which caused an amino acid substitution from valine to leucine; this mutant had no RR activity. To our knowledge, this is the first evidence that p53R2 may be inactivated in human cancers. The sensitivity of HCT116 cancer cells to γ-irradiation appeared to increase much more than that of the three cell lines without mutation of p53R2. Furthermore, in HCT116 cells, the expression of p53AIP1 in response to DNA damage with γ-irradiation was strikingly enhanced, although it was not induced in all three cell lines whose cell cycles were arrested at G1 and G2. These facts are consistent with the notion that p53 is a cell cycle checkpoint that determines cell survival or cell death through transcriptional regulation of either p53R2 or p53AIP1, as illustrated in Fig. 7 (15). We speculate that inactivation of the p53R2-dependent pathway of DNA synthesis would cause failure of the normal repair system; the p53-dependent apoptotic pathway might be activated instead, resulting in apoptosis to eliminate dangerously damaged cells. As we reported earlier, phosphorylation of p53, especially at Ser46, would drive that “decision” by p53 (15, 16).

With regard to cancer therapy, R2 has been considered an important target for development of anticancer agents. However, although R2 is essential for supplying nucleotides for DNA synthesis under normal cellular conditions, it does not appear to contribute much to DNA repair. Cancer cells are often burdened by mechanisms other than inactivation of p53 that affect the checkpoint machinery. We would expect, e.g., that inactivation of p53R2 would enhance genotoxic insults in cancer cells. Especially in cells that retain wt p53, we would expect inactivation of the p53R2-dependent DNA synthesis pathway to activate p53-dependent apoptosis. In fact, inhibition of p53R2 expression by antisense oligo increased the sensitivity of MCF7 cells (containing the wt p53 gene) to diverse genotoxic stresses, such as γ-irradiation, UV irradiation, and exposure to adriamycin treatment (9). If one could develop a highly specific inhibitor for p53R2 and deliver it directly to cancer cells along with anticancer drugs or agents, the combination might serve as an effective system for killing cancer cells selectively.

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