MOLECULAR AND CELLULAR BIOLOGY, Mar. 1994, p. 1815–1823
0270-7306/94/$04.00+0

Vol. 14, No. 3

DNA Strand Breaks: the DNA Template Alterations That Trigger p53-Dependent DNA Damage Response Pathways

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Received 14 September 1993/Returned for modification 5 November 1993/Accepted 24 November 1993

The tumor suppressor protein p53 serves as a critical regulator of a G_1 cell cycle checkpoint and of apoptosis following exposure of cells to DNA-damaging agents. The mechanism by which DNA-damaging agents elevate p53 protein levels to trigger G_1/S arrest or cell death remains to be elucidated. In fact, whether damage to the DNA template itself participates in transducing the signal leading to p53 induction has not yet been demonstrated. We exposed human cell lines containing wild-type p53 alleles to several different DNA-damaging agents and found that agents which rapidly induce DNA strand breaks, such as ionizing radiation, bleomycin, and DNA topoisomerase-targeted drugs, rapidly triggered p53 protein elevations. In addition, we determined that camptothecin-stimulated trapping of topoisomerase I-DNA complexes was not sufficient to elevate p53 protein levels; rather, replication-associated DNA strand breaks were required. Furthermore, treatment of cells with the antimetabolite *N*(phosphonoacetyl)-L-aspartate (PALA) did not cause rapid p53 protein increases but resulted in delayed increases in p53 protein levels temporally correlated with the appearance of DNA strand breaks. Finally, we concluded that DNA strand breaks were sufficient for initiating p53-dependent signal transduction after finding that introduction of nucleases into cells by electroporation stimulated rapid p53 protein elevations. While DNA strand breaks appeared to be capable of triggering p53 induction, DNA lesions other than strand breaks did not. Exposure of normal cells and excision repair-deficient xeroderma pigmentosum cells to low doses of UV light, under conditions in which thymine dimers appear but DNA replication-associated strand breaks were prevented, resulted in p53 induction attributable to DNA strand breaks associated with excision repair. Our data indicate that DNA strand breaks are sufficient and probably necessary for p53 induction in cells with wild-type p53 alleles exposed to DNA-damaging agents.

Living organisms persistently encounter myriad threats to genome integrity. Perhaps to permit repair of DNA template damage before DNA replication and mitotic chromosome segregation, most cells delay transit through the cell cycle in response to DNA damage (36, 39, 49, 59, 62). Cell cycle checkpoints manifest at the transition from G_1 to S and from G_2 to M may facilitate the maintenance of DNA sequence fidelity through repair and prevent the accumulation of heritable DNA template alterations. For example, G_2 checkpoint defects in mutant strains of *Escherichia coli* as well as *Saccharomyces cerevisiae* are accompanied by increased genetic instability (24, 28, 31, 62, 63, 65). Evidence has accumulated to implicate the tumor suppressor protein p53 as a major regulator of the G_1 cell cycle checkpoint in mammalian cells: cells with wild-type p53 genes exhibit transient increases in p53 protein levels accompanying G_1 arrest in response to ionizing radiation, whereas cancer cells harboring mutant p53 genes fail to arrest in G_1 following γ irradiation (34, 38). In addition, while introduction of cDNA encoding wild-type p53 into neoplastic cells lacking p53 genes restores G_1 arrest following DNA damage, overexpression of mutant p53 protein in tumor cells containing wild-type p53 genes abrogates the DNA damage-induced G_1 arrest (38). Furthermore, embryonic fibroblasts derived from mice carrying disrupted p53 genes lack the G_1 cell cycle checkpoint (35).

Recent data have also implicated p53 as a critical determinant of cell fate following certain types of DNA damage (13, 44). For example, while murine thymocytes exposed to ionizing radiation undergo a process resembling programmed cell death (apoptosis), thymocytes carrying disrupted p53 genes appeared incapable of rapidly triggering this cell death pathway (13, 44). Whether DNA damage activates a p53-dependent signal transduction pathway leading to G_1 arrest or to apoptosis could depend on the cell type and physiologic state (55). However, both pathways are likely to share one or more of the steps involved in p53 induction and p53 function following DNA damage.

A critical step in the p53-dependent pathway culminating in cell cycle arrest appears to involve DNA damage-induced increases in nuclear wild-type p53 protein levels by a posttranscriptional mechanism (34, 35, 38). Induced wild-type p53 protein may then function in a cell cycle checkpoint pathway or apoptosis pathway to regulate the expression of genes associated with growth arrest or programmed cell death. For example, *gadd45*, one of several growth arrest and DNA damage-inducible genes thus far identified, is induced in coordination with growth arrest in mammalian cells following serum deprivation or as a consequence of DNA damage (20, 21); *gadd45* induction by ionizing radiation is a p53-dependent process and may be a consequence of direct transcriptional regulation of *gadd45* mRNA synthesis by wild-type p53 protein (35). However, a requirement for *gadd45* in either radiation-induced cell cycle arrest or cell death has not yet been demonstrated.

Several studies have suggested that a variety of DNA-damaging agents act to elevate p53 protein levels and/or p53-specific DNA binding activity in cells with wild-type p53 alleles (23, 34, 47, 58, 67). Unfortunately, the mechanism of p53 induction remains unclear: increases in p53 protein levels caused by DNA-damaging agents could be triggered by a specific DNA lesion (or by several specific DNA lesions), by any general DNA helix distortion, or by some other signal

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transduction process independent of DNA template damage. A requirement for a specific DNA lesion to elevate p53 protein levels might offer a considerable economy to damaged cells by limiting the required repertoire of DNA damage recognition functions necessary for initiation of the p53-dependent cell cycle checkpoint or apoptosis pathway. Furthermore, an understanding of the mechanisms of p53 induction could provide profound new insights into how the p53-dependent G_1 cell cycle checkpoint pathway or p53-dependent apoptosis might be manipulated therapeutically for the purpose of improving human cancer prevention and cancer treatment. To determine whether some specific DNA template lesion(s) might be responsible for elevating wild-type p53 protein levels in cells following exposure to DNA-damaging agents, we assessed p53 protein levels in human cell lines containing wild-type p53 following exposure to a diverse collection of different DNA-damaging agents. We report here that DNA strand breaks may be the critical DNA template insult common to many different DNA-damaging agents capable of triggering p53 induction.

MATERIALS AND METHODS

Cell lines and cell culture. ML-1 myeloid leukemia cells were propagated in suspension in RPMI 1640 (Gibco BRL) supplemented with 10% fetal calf serum. LNCaP prostatic adenocarcinoma cells (29) (generously provided by William B. Isaacs, Johns Hopkins University School of Medicine, Baltimore, Md.) were also cultivated in RPMI 1640 with 10% fetal calf serum. Both of these human cancer cell lines contain wild-type p53 alleles (32, 34). Normal human lymphoblastoid cell line GM 2184 and xeroderma pigmentosum (XP) lymphoblastoid cell line GM 2250 were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (Camden, N.J.). Suspension cultures of lymphoblastoid cells were maintained in RPMI 1640 with 10% fetal calf serum.

Exposure to DNA-damaging agents. Hydroxyurea was generously provided by John T. Isaacs (Johns Hopkins University), teniposide (VM26) was supplied by Leroy F. Liu (Robert Wood Johnson School of Medicine, New Brunswick, N.J.), *N*(phosphonoacetyl)-L-aspartate (PALA) was provided by Thea Tlsty (University of North Carolina, Chapel Hill), and 4-hydroperoxycyclophosphamide was supplied by O. Michael Colvin (Johns Hopkins University). Cytosine arabinoside was obtained from Upjohn, bleomycin was acquired from Mead Johnson, etoposide (VP-16) was obtained from Bristol Laboratories, and methotrexate, methyl methanesulfonate, camptothecin, doxorubicin, daunorubicin, recombinant human tumor necrosis factor, mitomycin, vinblastine, dimethyl sulfoxide, caffeine, 2-aminopurine, aphidicolin, actinomycin D, and ethidium bromide were all obtained from the Sigma Chemical Co. Cultured human cells were exposed to these different agents in complete growth medium as described in the figure legends. Cells were exposed to ionizing radiation by treatment in a ^{137}Cs γ irradiator at a dose rate of approximately 1 Gy/min. For treatments with UV light, cells washed with phosphate-buffered saline were exposed to a germicidal light source to the desired UV dose (with the flux at 254 nm measured in each experiment with a UV meter [model J-225; UV Products, Inc., San Gabriel, Calif.).

Electroporation of nucleases into ML-1 cells. Nucleases were introduced into ML-1 cells by electroporation (12, 48, 64). Briefly, ML-1 cells suspended in growth medium were collected by centrifugation at 10,000 $\times$ *g*, washed in electroporation buffer (272 mM sucrose and 1 mM $MgCl_2$ in 7 mM potassium phosphate, pH 7.4), and then resuspended in electroporation buffer containing nucleases or appropriate nuclease storage buffers. Four-millimeter-diameter cuvettes containing 10^6 cells in 0.8 ml were treated in a Bio-Rad electroporator set to deliver 300 V at 500 μF. Following electroporation, the cells were incubated at 37°C for 30 min in electroporation buffer and then at 37°C in growth medium before being assessed for wild-type p53 protein levels via immunoblot analysis.

Immunoblot analysis for p53 protein levels. Attached cells were collected for immunoblot analysis following treatment with DNA-damaging agents by scraping monolayer cultures with a rubber policeman. Dislodged monolayer cells in Hanks' balanced salt solution or cells from suspension cultures were then collected by centrifugation at 14,000 $\times$ *g* for 10 min at room temperature. Cell pellets were lysed in sample buffer (2% sodium dodecyl sulfate, 10% glycerol, and 10 mM dithiothreitol in 62 mM Tris-HCl, pH 6.8) by heating to 95°C for at least 10 min. DNA content of cell lysates was estimated by a diphenylamine assay (9). Equivalent cell extracts were electrophoresed on 10% polyacrylamide gels, transferred to nitrocellulose filters (Hybond-ECL; Amersham) by using a semidry electroblotter (Millipore), and then assessed for p53 protein levels by immunoblot analysis using a mixture of anti-p53 protein antibodies Ab-1 and Ab-2 (Oncogene Sciences) in a manner previously described (35, 37). If necessary, immunoblots were reprocessed for expression of DNA topoisomerase I by using human scleroderma antiserum (54) (supplied by William C. Earnshaw, Johns Hopkins University) or for expression of glutathione *S*-transferase π by using specific rabbit antiserum (Oncor).

RESULTS

A variety of agents elevate wild-type p53 levels in human cells. To ascertain what types of DNA damage trigger p53 protein induction in cells containing wild-type p53, we studied the effects of several different agents on wild-type p53 protein expression in the human prostatic adenocarcinoma cell line LNCaP and the human myeloid leukemia cell line ML-1. In previous studies, wild-type p53 elevations were detectable in less than 1 h after exposure of ML-1 cells to γ irradiation (34). In this study, we detected rapid significant elevations in p53 levels within 2 to 3 h after treatment with bleomycin (Fig. 1B, lane 2), actinomycin D (Fig. 1A, lane 6), etoposide (Fig. 1B, lane 5), teniposide (Fig. 1A, lane 8), doxorubicin (Fig. 1A, lane 9), and camptothecin (Fig. 1A, lane 7, and Fig. 1B, lane 8). No significant p53 induction was discernible in these cells at these early time points after treatment with hydroxyurea (Fig. 1A, lane 3), recombinant human tumor necrosis factor (Fig. 1A, lane 5), mitomycin (Fig. 1A, lane 4), vinblastine (Fig. 1A, lane 2), ethidium bromide (Fig. 1A, lane 10), methyl methanesulfonate (Fig. 1B, lane 6), daunorubicin (Fig. 1B, lane 7), or 4-hydroperoxycyclophosphamide (Fig. 1B, lane 3). These studies suggested that agents capable of directly causing DNA strand scission (such as bleomycin or γ irradiation) were more effective at rapidly elevating wild-type p53 levels than agents that create DNA strand cross-links (mitomycin and 4-hydroperoxycyclophosphamide), induce DNA base modifications (methyl methanesulfonate), intercalate into the DNA helix (ethidium bromide), or otherwise interfere with the cell cycle or cellular metabolism (hydroxyurea, vinblastine, and tumor necrosis factor). In addition, topoisomerase-targeted drugs (actinomycin D, doxorubicin, teniposide, and camptothecin) appeared to act rapidly to elevate p53 levels at the doses studied. Of interest, the topoisomerase-targeted drug daunorubicin (Fig. 1B, lane 7), a compound related to doxorubicin

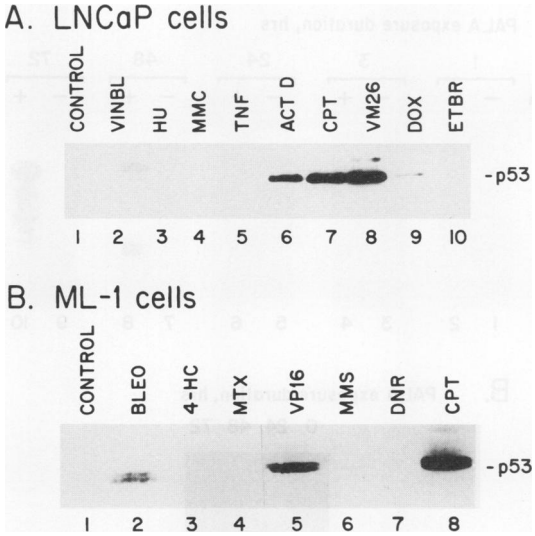

FIG. 1. Several different agents trigger p53 elevations in human cancer cells with wild-type p53 alleles. Protein-containing extracts were prepared from LNCaP prostatic adenocarcinoma cells (A) and ML-1 myeloid leukemia cells (B) exposed to various agents in complete growth medium. Extracts containing 10 μg of cellular DNA (A) or prepared from 2×10^6 cells (B) were electrophoresed on polyacrylamide gels, transferred to nitrocellulose filters, and then evaluated for p53 protein expression by immunoblot analysis with anti-p53 antibodies as described in Materials and Methods. (A) Immunoreactive p53 levels in LNCaP extracts following treatment for 2 h with 0.1% dimethyl sulfoxide (CONTROL, lane 1), 1 μM vinblastine (VINBL, lane 2), 2 mM hydroxyurea (HU, lane 3), 1 μg of mitomycin per ml (MMC, lane 4), 100 Units of recombinant human tumor necrosis factor per ml (TNF, lane 5), 1 μM actinomycin D (ACT D, lane 6), 1 μM camptothecin (CPT, lane 7), 1 μM teniposide (VM26, lane 8), 1 μM doxorubicin (DOX, lane 8), and 1 μM ethidium bromide (ETBR, lane 10). (B) p53 levels detected in ML-1 cells after 3 h of treatment with growth medium alone (CONTROL, lane 1), 0.06 U of bleomycin per ml (BLEO, lane 2), 0.5 μg of 4-hydroperoxycyclophosphamide per ml (4-HC, lane 3), 10 nM methotrexate (MTX, lane 4), 10 ng of etoposide per ml (VP16, lane 5), 10 μg of methyl methanesulfonate (MMS, lane 6), 10 nM daunorubicin (DNR, lane 7), and 10 μM camptothecin (CPT, lane 8).

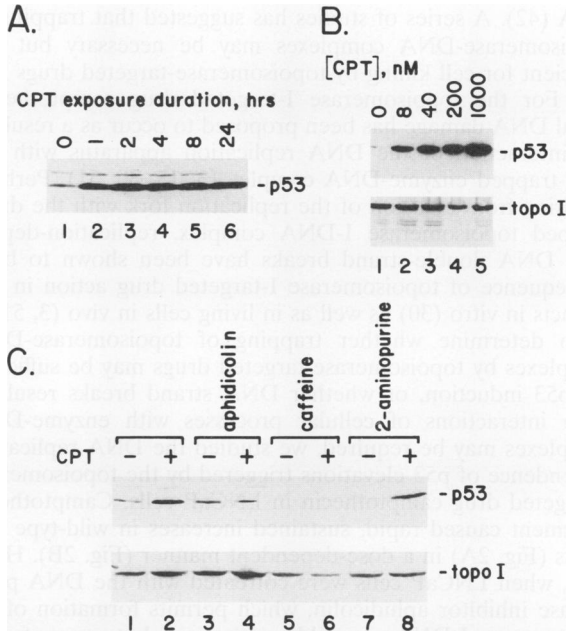

FIG. 2. Replication dependence of camptothecin-induced p53 elevations. Extracts prepared from LNCaP prostatic adenocarcinoma cells treated with camptothecin (CPT) and other agents were prepared and analyzed for p53 protein expression by immunoblot analysis as described for Fig. 1 and in Materials and Methods. (A) p53 levels for LNCaP cells treated with 20 nM camptothecin for 0 (lane 1), 1 (lane 2), 2 (lane 3), 4 (lane 4), 8 (lane 5), and 24 (lane 6) h. (B) p53 levels for LNCaP cells treated for 2 h with no added camptothecin (lane 1) and for 2 h with 8 nM (lane 2), 40 nM (lane 3), 200 nM (lane 4), and 1,000 nM (lane 5) camptothecin. (C) Effects of aphidicolin, caffeine, and 2-aminopurine on camptothecin-induced p53 elevations. LNCaP cells were treated for 2 h with (lanes 2, 4, 6, and 8) and without (lanes 1, 3, 5, and 7) 10 nM camptothecin. For lanes 3 and 4, 10 μM aphidicolin was present 15 min before camptothecin addition and throughout the 2-h incubation. Similarly, for lanes 5 and 6, 4 mM caffeine was included, and for lanes 7 and 8, 10 mM 2-aminopurine was present. As controls in panels B and C, immunoblots were reprobed with antibodies specific for topoisomerase (topo) I.

(Fig. 1A, lane 9), failed to trigger significant p53 protein increases in these cells at the time and low (10 nM) dose studied. Furthermore, actinomycin D (Fig. 1A, lane 6) treatment of LNCaP cells (with 1 μM actinomycin D for 2 h) appeared to result in a more rapid induction of wild-type p53 levels than had been observed for ML-1 and RKO cells previously (treated with less than 1 nM actinomycin D for up to 24 h [34]). Taken together, these data suggest that topoisomerase-targeted drugs may have different p53 induction activities in different cells, at different doses, and after different exposure durations.

Caffeine has been found both to abrogate the G_1 (34) and G_2 (39, 61) cell cycle checkpoints activated by DNA-damaging agents and to increase the cytotoxicity of many DNA-damaging agents (10, 39, 55). We have previously shown that caffeine blocks p53 protein increases in ML-1 cells induced by ionizing radiation (34). Similarly, in studies of LNCaP cells treated with camptothecin and caffeine, we found that caffeine appeared both to lower basal p53 levels in the LNCaP cells and to abrogate camptothecin-induced p53 increases (Fig. 2C, lanes 1, 2, 5, and 6). Both the mechanism by which caffeine affects the signal transduction pathway culminating in p53 protein elevations and the mechanism of caffeine-mediated enhancement of cytotoxicity remain to be elucidated. Interestingly, 2-aminopurine, another agent reported to inhibit cell cycle arrest in G_1 and G_2 (2), did not appear to affect camptothecin-triggered p53 protein induction (Fig. 2C, lanes 7 and 8). Undoubtedly, 2-aminopurine must affect G_1 cell cycle arrest by a mechanism distinct from that used by caffeine. It may be that the agent undermines the p53-dependent G_1 cell cycle checkpoint by affecting the checkpoint pathway at a step distal to p53 protein induction (35).

Wild-type p53 induction by the topoisomerase I-targeted drug camptothecin requires replicative DNA synthesis and DNA strand breaks. A striking finding of our limited screen of p53 induction by potential DNA-damaging agents was the reproducible elevation of p53 protein levels triggered by topoisomerase-targeted antineoplastic drugs (Fig. 1). These drugs interact with DNA topoisomerases in such a way as to trap the enzymes in complexes with cellular DNA that resemble intermediates in enzyme catalysis (11, 41, 42, 57). Treatment of the trapped enzyme-DNA complexes (often referred to as cleavable complexes) with protein denaturants yields protein (enzyme)-associated DNA strand breaks from which the enzymes can be recovered in covalent linkage with broken

DNA (42). A series of studies has suggested that trapping of topoisomerase-DNA complexes may be necessary but not sufficient for cell killing by topoisomerase-targeted drugs (15, 41). For the topoisomerase I-targeted drug camptothecin, lethal DNA damage has been proposed to occur as a result of the interaction of the DNA replication apparatus with the drug-trapped enzyme-DNA complex (3, 15, 30, 51). Perhaps resulting from collision of the replication fork with the drug-trapped topoisomerase I-DNA complex, replication-dependent DNA double-strand breaks have been shown to be a consequence of topoisomerase I-targeted drug action in cell extracts in vitro (30) as well as in living cells in vivo (3, 51).

To determine whether trapping of topoisomerase-DNA complexes by topoisomerase-targeted drugs may be sufficient for p53 induction, or whether DNA strand breaks resulting from interactions of cellular processes with enzyme-DNA complexes may be required, we studied the DNA replication dependence of p53 elevations triggered by the topoisomerase I-targeted drug camptothecin in LNCaP cells. Camptothecin treatment caused rapid, sustained increases in wild-type p53 levels (Fig. 2A) in a dose-dependent manner (Fig. 2B). However, when LNCaP cells were cotreated with the DNA polymerase inhibitor aphidicolin, which permits formation of topoisomerase I-DNA cleavable complexes but prevents the generation of replication-dependent DNA strand breaks (3, 15, 30, 51), camptothecin was unable to elevate p53 protein levels (Fig. 2C, lanes 3 and 4). This observation suggests that DNA strand breaks are critical for p53 protein induction by the topoisomerase-targeted drug camptothecin. Whether this mechanism is generalizable to all topoisomerase-targeted drugs remains to be determined.

Increased wild-type p53 levels accompany DNA strand breaks associated with cell death induced by antimetabolites. Although antimetabolites such as methotrexate have not been thought to modify DNA directly, a body of evidence has accumulated to suggest that such agents may cause cell death by fostering an accumulation of DNA strand breaks in treated cells through a mechanism which remains unclear (19, 40). DNA strand breaks occurring as a result of methotrexate treatment generally appear slowly and accompany loss of cell viability (40). In studies of p53 induction in ML-1 cells by methotrexate, we were unable to detect p53 elevations until after continuous exposure to drug for 48 h, at which time only weak induction was detectable (not shown). This time course of p53 induction was reminiscent of the time course of DNA fragmentation reported as a consequence of methotrexate treatment (40). Amplification of the *CAD* gene in response to treatment with the inhibitor PALA has been shown to occur in cells with mutant or disrupted p53 alleles but not in cells expressing wild-type p53 (43, 66). To determine whether PALA might induce p53 in coordination with causing DNA strand breaks, we evaluated p53 levels and genomic DNA integrity in ML-1 cells treated for various periods of time with PALA (Fig. 3). We detected increases in p53 levels in ML-1 cells accompanying DNA strand breaks only after 24 to 48 h of continuous exposure to PALA (Fig. 3A), a time frame consistent with the appearance of numerous DNA strand breaks (Fig. 3B). These studies suggest that antimetabolites such as methotrexate and PALA lead to elevated p53 levels as DNA strand breaks accumulate within cells.

DNA strand breaks are sufficient for wild-type p53 induction. After finding a close correlation between induced DNA strand breaks and increases in wild-type p53 protein levels following treatment with a variety of DNA-damaging agents, we sought to determine whether DNA strand breaks alone were sufficient to trigger p53 elevations. Nucleases have been found to cleave genomic DNA when introduced into mammalian cells by electroporation (12, 48, 64). When DNase I (Fig. 4B) or the restriction endonuclease *Alu*I (Fig. 4A) was introduced into ML-1 cells by electroporation, increases in p53 protein levels were detected by immunoblot analysis. Such increases in wild-type p53 levels were not observed when albumin (Fig. 4A, lane 2), heat-inactivated *Alu*I (Fig. 4A, lane 5), or RNase A (Fig. 4B, lane 2) was introduced. Of interest, depending on conditions used, electroporation itself frequently reduced p53 levels in ML-1 cells (Fig. 4A, lanes 1 and 2, and data not shown). DNase I-induced increases in p53 protein levels became evident with the introduction of increasing amounts of nuclease (Fig. 4B), suggesting that some threshold level of DNA strand breaks might be required for eliciting rapid p53 induction. Interestingly, high levels of DNase I electroporated into ML-1 cells caused less dramatic increases in p53 protein (Fig. 4B, lane 7). Whether this phenomenon resulted from accelerated cell destruction by nuclease activity,

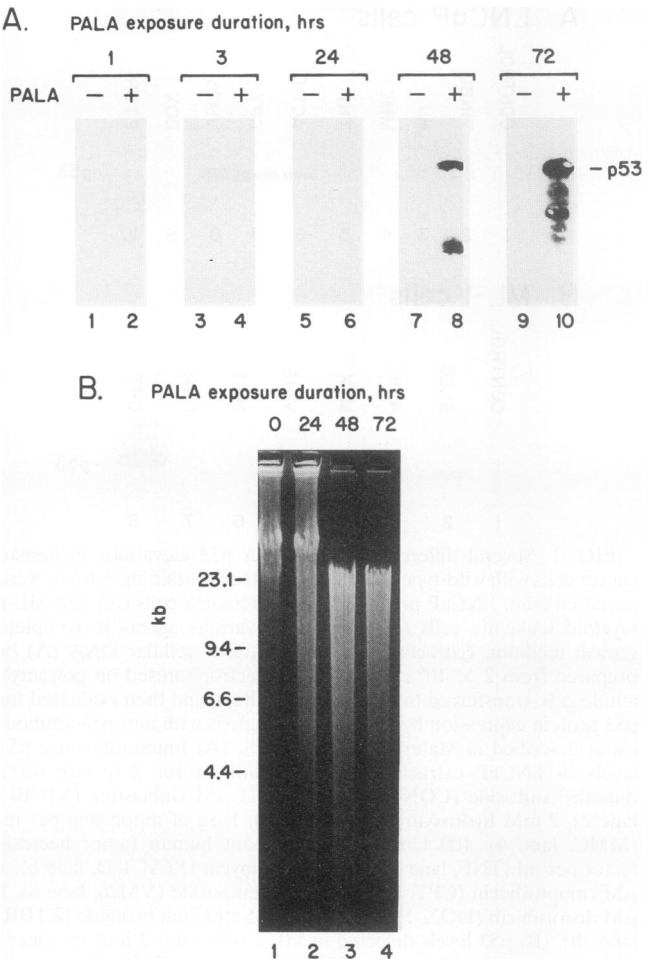

FIG. 3. Increases in p53 protein levels accompany DNA strand breaks following prolonged exposure of ML-1 cells to PALA. (A) ML-1 cells treated by constant exposure to 100 μM PALA for 1 (lane 2), 3 (lane 4), 24 (lane 6), 48 (lane 8), and 72 (lane 10) h were evaluated for p53 expression by immunoblot analysis. (B) DNA was extracted from ML-1 cells similarly exposed to 100 μM PALA for 0 (lane 1), 24 (lane 2), 48 (lane 3), and 72 (lane 4) h and analyzed by agarose gel electrophoresis followed by ethidium bromide staining. The migration positions of *Hin*dIII-digested lambda DNA molecular size markers are indicated.

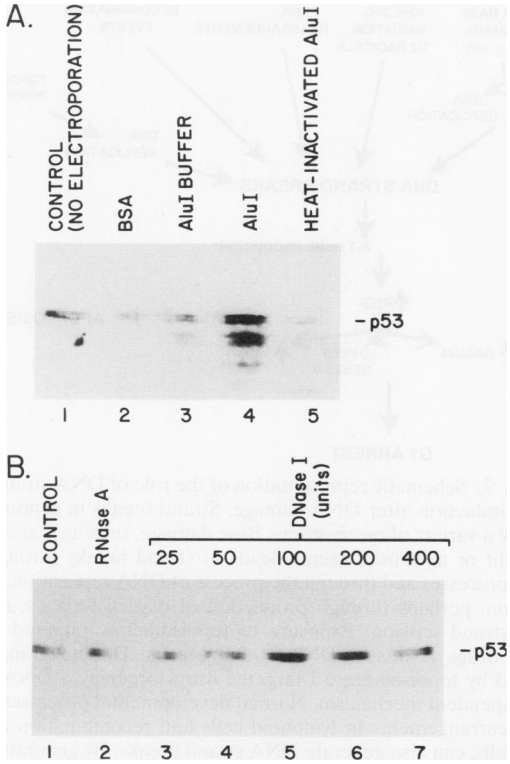

FIG. 4. Electroporation of nucleases into ML-1 cells causes p53 induction. Nucleases *Alu*I and DNase I were introduced into ML-1 cells by electroporation as described in Materials and Methods, and p53 protein levels were assessed 1 h later by immunoblot analysis using anti-p53 antibodies. (A) p53 expression was evaluated following electroporation of ML-1 cells in the presence of bovine serum albumin (BSA; 12.5 μl in 0.8 ml of electroporation buffer; lane 2), *Alu*I storage buffer (12.5 μl of 10 mM Tris-HCl–100 mM NaCl–0.1 mM EDTA–1 mM dithiothreitol–200 μg of bovine serum albumin per ml–50% [vol/vol] glycerol at pH 7.4; lane 3), *Alu*I (12.5 μl containing 100 U of nuclease activity in *Alu*I storage buffer; lane 4), and heat-inactivated *Alu*I (12.5 μl containing 100 U of enzyme activity heated to 95°C for 30 min; lane 5). As a control, p53 levels in protein extracts from ML-1 cells not treated by electroporation are shown in lane 1. (B) p53 levels were assessed following electroporation of ML-1 cells with no added nucleases (lane 1), with RNase A (6.25 μg; lane 2), and with DNase I (25, 50, 100, 200, and 400 U of nuclease activity; lanes 3 to 7). Each of the electroporation experiments in panel (B) was performed in the presence of DNase I storage buffer (40 μl of 20 mM Tris-HCl–50 mM NaCl–1 mM dithioerythritol–0.1 mg of bovine serum albumin per ml–50% [vol/vol] glycerol, pH 7.6, in 0.8 ml of electroporation buffer).

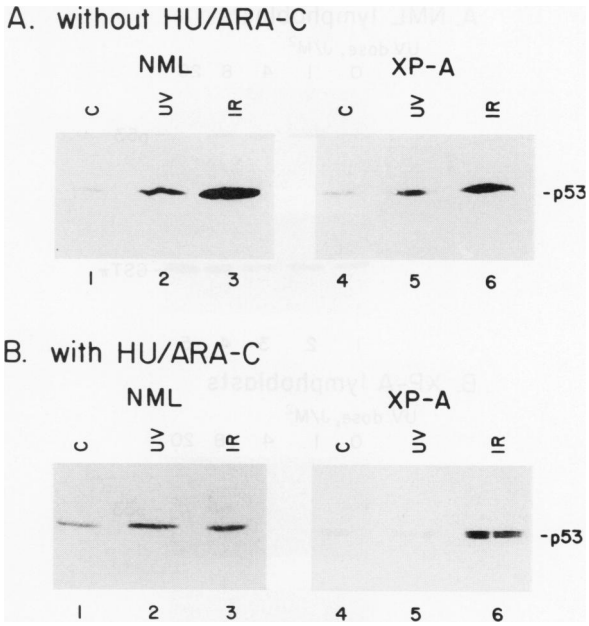

FIG. 5. Preferential induction of p53 by UV light in normal lymphoblastoid cells versus XP group A lymphoblasts under conditions in which excision repair-associated DNA strand breaks occur. Immunoblot analysis for p53 protein expression was performed on protein-containing extracts prepared from normal (NML) (GM 2184; lanes 1 to 3) and XP complementation group A (XP-A) (GM 2250; lanes 4 to 6) lymphoblastoid cells 3 h after treatment with UV radiation (20 J/m^2 for panel A, lanes 2 and 5, and 2 J/m^2 for panel B, lanes 2 and 5) or ionizing radiation (IR) (2 Gy; lanes 3 and 6) under normal growth conditions (A) or in the presence of 2 mM hydroxyurea (HU) and 10 μM cytosine arabinoside (ARA-C) (B).

from disruption of major physiologic cell processes and signal transduction pathways, or by some other mechanism is not clear. Rapid elevation of wild-type p53 levels triggered by nucleases demonstrates that DNA strand breaks are sufficient for p53 induction.

DNA base damage inflicted by UV light may not be the trigger for p53 induction. Maltzman and Czyzyk first identified increases in p53 protein levels in nontransformed rodent cells in response to treatment with UV light (47). Subsequently, several studies have reported elevations in p53 levels following UV irradiation (23, 27, 67). Treatment of living cells with UV light results in a variety of DNA lesions, including modified DNA bases and DNA strand breaks (14, 22, 60). To determine whether creation of DNA base modifications, such as thymine dimers, by UV irradiation was sufficient to raise p53 levels, or whether DNA strand breaks were required, we studied UV light effects on p53 levels in normal human lymphoblastoid cells and in lymphoblastoid cells derived from a patient afflicted with XP. XP describes a family of inherited syndromes resulting from defects in the excision repair of UV photoproducts such as thymine dimers (50). We found that when normal or XP (group A exhibiting less than 3% of normal excision repair activity [50]) lymphoblasts were treated with UV irradiation or γ irradiation under normal growth conditions, increases in p53 protein levels were detected (Fig. 5A). These increases in wild-type p53 levels detected after UV exposure might have occurred as a result of DNA photoproducts, replication- or repair-induced DNA strand breaks, or some DNA template-independent mechanism. However, when both types of cells were treated with UV light under conditions in which UV-induced replication dependent strand breaks were inhibited (18, 22), UV-stimulated p53 induction was detected in normal but not XP lymphoblasts (Fig. 5B, lanes 2 and 5). As a control, ionizing radiation, which directly induces DNA strand breaks, elevated p53 levels in both types of lymphoblastoid cells (Fig. 5B, lanes 3 and 6) under the same growth-inhibitory conditions. These results suggest that DNA strand breaks occurring as part of the excision repair process can trigger p53 protein induction.

Of interest, while group A XP cells are quantitatively defective in the repair of UV photoproducts, the cells are capable of a low level of excision repair of UV photoproducts (18, 22, 50). Following exposure to UV irradiation, Fornace and Seres (22) detected repair-induced strand breaks in group A XP cells at a level of 0.1 to 0.2 single strand break per 10^9 Da of DNA for each joule of incident UV light per square meter.

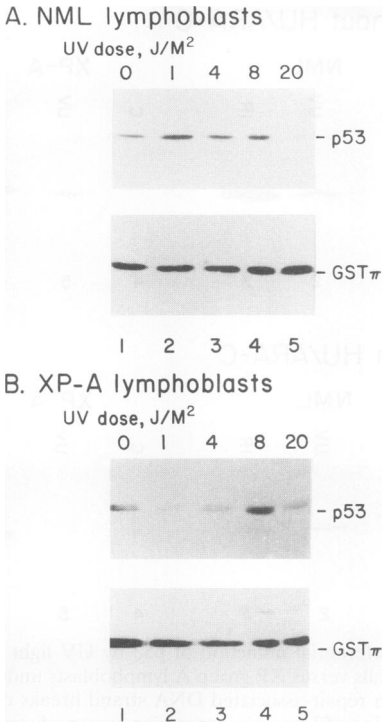

FIG. 6. UV light dose response for p53 induction in normal and XP lymphoblasts. Immunoblot analyses for p53 protein expression were performed on protein-containing extracts prepared from normal (NML; A) and XP group A (XP-A; B) lymphoblasts 3 h after treatment with UV light at doses of 0, 1, 4, 8, and 20 J/m^2 in the presence of 2 mM hydroxyurea and 10 μM cytosine arabinoside. As a control, immunoblots were reprobed with antibodies specific for glutathione *S*-transferase π (GSTπ).

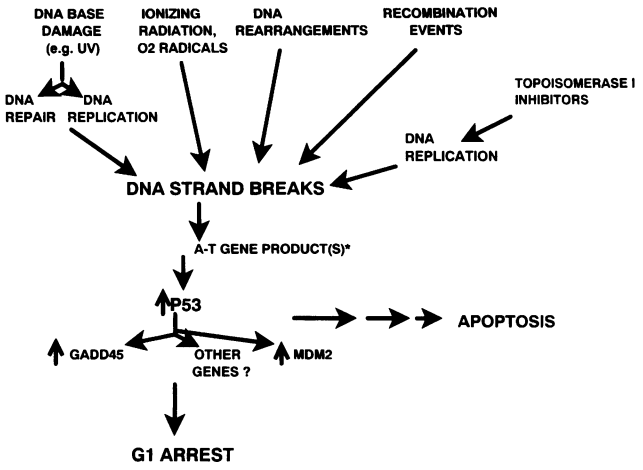

FIG. 7. Schematic representation of the role of DNA strand breaks in p53 induction after DNA damage. Strand breaks in genomic DNA arise by a variety of mechanisms. Base damage, such as that caused by UV light or alkylating agents, leads to strand breaks through DNA repair processes and through the process of DNA replication. Ionizing radiation, perhaps through production of oxygen radicals, results in DNA strand scission. Exposure to topoisomerase-targeted antineoplastic drugs results in DNA strand breaks. Double-strand breaks induced by topoisomerase I-targeted drugs occur by a DNA replication-dependent mechanism. Normal developmental processes, such as gene rearrangements in lymphoid cells and recombination events in germ cells, can also generate DNA strand breaks. By generating DNA strand breaks, all of these threats to genome integrity lead to p53 induction and initiation of p53-dependent response pathways. The asterisk adjacent to "A-T gene products" indicates that these functions may be involved in responses to only certain types of DNA strand breaks, such as those initiated by ionizing radiation. The cellular response to p53 induction may be G_1 arrest or apoptotic cell death, depending on cell type and other undefined parameters of the physiologic state of the cell. Whether *gadd45* plays a critical role in G_1 arrest, apoptosis, or both functions has not yet been demonstrated.

We reasoned that if strand breaks inflicted on UV-damaged DNA by excision repair processes were capable of triggering p53 protein elevations, treatment of XP cells with high levels of UV light under growth-inhibitory conditions might result in p53 protein increases as greater amounts of repair-induced strand breaks appear. In an effort to test this prediction, normal and XP lymphoblasts were treated with increasing doses of UV irradiation under conditions in which UV-induced replication-dependent DNA strand breaks were inhibited (Fig. 6) (18, 22). Increases in p53 protein levels were readily detected in normal lymphoblasts even after exposure to low to modest UV doses (Fig. 6A, lanes 1 to 4). In contrast, XP lymphoblasts exposed to different amounts of UV light did not display detectable p53 protein increases until after treatment with UV to a dose of 8 J/m^2. For both types of cells, exposure to a UV light at a dose of 20 J/m^2 resulted in little if any increase in p53 protein levels under these conditions (Fig. 6A and B, lanes 5). This abrogation of p53 induction at high levels of exposure to UV radiation may result from UV light-mediated inhibition of protein synthesis (up to 80% inhibition of [^{35}S]methionine incorporation at these doses; data not shown); previous studies have revealed that the protein synthesis inhibitor cycloheximide prevents p53 elevations triggered by ionizing radiation (34). All of our results on p53 induction by UV light suggest that the presence of UV photoproducts in the DNA is not sufficient to elevate p53 levels. Rather, p53 induction may occur as a result of DNA strand breaks arising both from replication of a damaged DNA template and from excision repair of damaged nucleotides. These findings also suggest that the cellular response to damaged DNA may be a critical determinant leading to p53 induction. Thus, the different physiologic states of different cells may affect the timing or magnitude of p53 induction following various types of DNA template damage.

DISCUSSION

Major functions of wild-type p53 protein appear to include the regulation of a G_1/S cell cycle checkpoint activated by DNA damage and the mediation of apoptosis in certain situations in response to DNA damage (13, 34, 35, 38, 44). DNA-damaging agents activating the p53-dependent cell cycle checkpoint pathway typically cause wild-type p53 protein to accumulate to high levels within damaged cells. In our study of p53 induction by various DNA-damaging agents, we have found that DNA strand breaks appear to be the critical DNA lesions responsible for triggering elevations in p53 protein levels (Fig. 7). Of the agents that we studied, rapid significant p53 increases were observed following treatment with ionizing radiation, with the DNA strand-breaking drug bleomycin, and with DNA topoisomerase-targeted drugs. Of interest, the topoisomerase I-targeted drug camptothecin triggered p53 induction only under conditions permitting the occurrence of replication-dependent DNA strand breaks. Agents such as ionizing radiation, which cause DNA strand breaks in nonreplicating cells, generated p53 increases in a DNA replication-

independent manner. Antimetabolite exposure resulted in gradual delayed increases in p53 levels accompanying DNA fragmentation. Introduction of nucleases into cells with wild-type p53 alleles resulted in rapid and specific p53 protein elevations, implicating DNA strand breaks as sufficient to trigger p53 induction. Finally, studies of the effects of UV exposure on normal and XP lymphoblasts suggested that the presence of DNA photoproducts is not adequate to increase p53 levels; rather, DNA strand breaks resulting from replicating UV-damaged DNA or DNA strand breaks resulting from excision repair are required for p53 induction.

DNA strand breaks, especially DNA double-strand breaks, have been found to occur in mammalian cells following exposure to a variety of agents (19). Such lesions present cells with several types of problems. Breaks in the DNA helix would relax any torsional superhelical strain in the DNA template that might be required for transcriptional regulation, resulting in alterations in gene expression. Diminished transcriptional activity in specific genes has been found after treatment of cells with ionizing radiation at a dose consistent with generating one strand break within the chromosomal domain containing the transcriptional unit (45). In addition to effects on gene expression, strand breaks may predispose to genomic alterations associated with neoplastic transformation and progression. Broken DNA ends may be potentially recombinogenic, resulting in chromosome segment translocations, deletions, or amplifications in cells that traverse the cell cycle. When genomic DNA strand breaks have been generated by the introduction of restriction endonucleases into cells, a strikingly increased frequency of chromosomal aberrations has been observed in cells reaching mitosis (6, 7). In addition, restriction enzyme-induced strand breaks have been found to increase neoplastic transformation (5, 8, 68). Finally, unrepaired or irreparable DNA strand breaks may trigger cell death. A variety of mammalian strand break repair-deficient mutant cell lines display increased sensitivity to the lethal effects of clastogenic insults (25, 33). Clearly, DNA strand breaks constitute a considerable threat to cell viability and cell genome integrity. The need for mammalian cells to deal with such a threat may be why strand breaks appear to serve as the genomic DNA lesion responsible for initiating the p53-dependent DNA damage response pathway(s).

Our observation that DNA strand breaks appear far more efficient at triggering p53 protein increases than other types of cell and cell genome insults suggests that mammalian cells may exhibit a measure of economy in initiating the p53-dependent damage response pathway. DNA strand breaks accompany the exposure of many different mammalian cells to many different DNA-damaging agents. Undoubtedly, some of the strand breaks occur as a result of excision repair processes. Our studies of UV triggering of p53 protein induction suggest that such attempts at excision repair might be coupled to the p53-dependent DNA damage response pathway via DNA strand breaks, as nonreplicating XP cells incapable of generating strand breaks necessary for DNA photoproduct repair fail to elevate p53 protein levels following UV exposure. We speculate that despite the need for a complex array of DNA damage recognition functions for DNA repair, activation of the p53-dependent cell cycle checkpoint pathway may require a signal transduction system that need only respond to DNA strand breaks.

Different cells and cell types appear to be endowed with different repair capacities for certain DNA lesions, as well as different detoxification capacities for potential DNA-damaging species. Such cells vary in the propensity to generate DNA strand breaks after exposure to cytotoxic or genotoxic agents; this may account for the many of the apparent differences in reports of p53 protein induction by different DNA-damaging agents (23, 58, 67). In addition, as illustrated by the behavior exhibited by XP lymphoblasts in response to UV light, different proliferative states of certain cell types could also influence p53 induction by some DNA-damaging agents which trigger replication-dependent DNA strand breaks (19, 53).

The identification of DNA strand breaks as sentinel lesions in the orchestration of an important cell injury response has profound implications for further studies concerning signal transduction through the p53-dependent G_1 cell cycle checkpoint pathway. In a study of c-*jun* induction by UV radiation, DeVary et al. (16) found that UV light triggered a signal transduction cascade that appeared to originate near the plasma membrane, to involve Src family tyrosine kinases, and to culminate in increased phosphorylation of c-Jun protein, resulting in enhanced transcription factor activity. This damage response pathway has been proposed to act independently of damage to the DNA template itself. In contrast, our data demonstrate that the p53-dependent cell cycle checkpoint pathway appears to be triggered by a specific lesion in the DNA itself, almost certainly a DNA strand break. Thus, the p53-dependent damage response may complement other cell injury response pathways. The identification of DNA strand breaks as critical lesions in transducing the signal leading to p53 induction could facilitate future studies undertaken to elucidate the G_1 cell cycle checkpoint pathway or apoptotic cell death pathway. Enzymes such as poly(ADP-ribose) polymerase and the DNA-dependent protein kinase exhibit greater activity with broken DNAs as cofactors than with covalently closed DNAs as cofactors (4, 26). These enzymes might be candidate participants in the p53-dependent cell cycle checkpoint or cell death pathways.

Finally, DNA strand breaks do not arise solely from pathological insults. Physiologic DNA strand breaks must occur during somatic gene rearrangement processes such as during T- and B-lymphocyte development as well as during germ line gene rearrangement processes such as meiosis in oogenesis and spermatogenesis. Lower eucaryotes such as yeasts contain no p53 genes and exhibit no obvious ionizing radiation-induced G_1 cell cycle checkpoint (62). Perhaps p53-dependent pathways evolved in higher eucaryotes to help regulate physiologic processes associated with DNA strand breaks. For instance, the G_1 checkpoint may prevent attempted DNA replication in lymphoid cells containing inadequately rejoined immunoglobulin or T-cell receptor genes which might result in translocation of segments of these genes to other chromosomal loci and a high risk for neoplastic transformation. Alternatively, inadequately rejoined strand breaks might commit such lymphoid cells to death by a p53-dependent apoptosis pathway. In addition, a G_1 checkpoint might prevent replication of damaged template DNA in germ cells progressing to first meiotic prophase in order to minimize nonhomologous meiotic recombinations and other heritable DNA template alterations. Elevated p53 protein levels have been detected in developing spermatocytes which undergo such meiotic recombinational events (1, 52). In support of these possibilities is the increased incidence of lymphoid and germ cell neoplasms in mice homozygous for disrupted p53 genes (17).

Characterization of the molecular controls of cell cycle checkpoint and apoptosis pathways via elevations of p53 protein levels could prove useful in the formulation of new strategies for cancer prevention and treatment. Identification of the DNA lesion(s) initiating these signal transduction pathways could prove critical in this regard. For example, people afflicted with inherited cancer-prone syndromes, such

as ataxia telangiectasia (35) or Li-Fraumeni syndrome (46, 56), which have potential G_1 cell cycle checkpoint or cell death defects, might be counseled to avoid all environmental agents capable of DNA strand scission or might benefit from treatment with antioxidants or other protective agents to reduce the incidence of DNA strand breaks. Also, many antineoplastic drugs clearly kill replicating cancer cells more effectively than nonreplicating cancer cells. Perhaps the p53-dependent cell cycle checkpoint or apoptosis pathways could be therapeutically activated or inhibited for the purpose of protecting normal cells and selectively killing cancer cells containing mutant p53 alleles. This will not prove trivial, as recent studies have suggested that current treatment strategies using ionizing radiation and topoisomerase-targeted drugs do not directly exploit the p53-dependent cell cycle checkpoint pathway for therapeutic advantage in cancer cell killing in all cell types (55). Undoubtedly, further studies of the p53-dependent cell cycle checkpoint and p53-dependent apoptosis will facilitate future attempts at manipulating these pathways to improve cancer care.

ACKNOWLEDGMENTS

We thank Beverly S. Plunkett and Wen-Hsiang Lee for excellent technical assistance and Albert J. Fornace, Donald S. Coffey, and Kathleen R. Cho for helpful discussions.

This work was supported by National Cancer Institute Prostate Cancer SPORE grant CA58236, grant ES05777 from the National Institutes of Health, and grant 3187 from the Council for Tobacco Research.

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