A p53-independent Pathway for Induction of p21waf1/cip1 and Concomitant G1 Arrest in UV-irradiated Human Skin Fibroblasts

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Abstract

Largely on the basis of studies using the potent clastogen ionizing radiation, it has been widely assumed that up-regulation of the cyclin-dependent kinase inhibitor p21waf1/cip1 in cultured cells exposed to DNA-damaging agents is contingent upon the presence of functional p53 tumor suppressor protein. Nevertheless, we demonstrate here that the model mutagen 254-nm UV light induces p21waf1/cip1 protein and concomitant G1 arrest in normal human skin fibroblasts, as well as in p53-deficient fibroblasts derived from cancer-prone Li-Fraumeni syndrome patients. However, as expected, following exposure to ionizing radiation, elevated p21waf1/cip1 protein levels and G1 arrest were observed only in normal fibroblasts. These data provide a prominent and clinically relevant example in which p21waf1/cip1-mediated growth arrest occurs independently of p53 in human cells treated with a model DNA-damaging agent.

Introduction

For cells undergoing genotoxic stress, actively regulated periods of growth arrest that maintain genomic stability and protect against neoplastic transformation are induced throughout the cell cycle. It is now firmly established that the Cdk" inhibitor p21waf1/cip1, a primary target for transcriptional activation by the p53 tumor suppressor protein, plays an essential role in the initiation of G1 arrest in cultured cells following exposure to DNA damaging agents. In the case of human diploid fibroblasts, as well as tumor cell lines, treated with the potent clastogen IR, induction of p21waf1/cip1 appears to be absolutely dependent on the presence of functional p53 protein (1—6) and engages G1 arrest either indirectly by preventing phosphorylation of Rb protein (2, 6, 7) or directly by binding the DNA polymerase processivity factor proliferating cell nuclear antigen (8). Notwithstanding the importance of p53-dependent, p21waf1/cip1-mediated pathways in growth arrest, it should be emphasized that in many instances the presence of wild-type p53 is not sufficient for the initiation of G1 delay in mutagenized cells. For example, many IR-exposed, p53-proficient human tumor cell lines exhibit impaired G1 arrest (6), perhaps indicating a role for p53-independent pathways in the generation of hypophosphorylated Rb [e.g., involving Rb phosphatases (9) or Cdk inhibitors other than p21waf1/cip1 (10, 11)]. In the case of HSFs, however, it appears that p53 may serve a more essential function, because all p53-proficient, IR-exposed HSF lines examined to date exhibit a prolonged G1 arrest (2, 6), which can be abolished by transfection of oncogenes encoding proteins that bind and inactivate wild-type p53 (6).

The clear demonstration of p53 dependence for the induction of p21waf1/cip1 in IR-treated cells, coupled with the perception of IR exposure as a consummate paradigm for the cellular response to DNA damage, has led to the widespread notion that p21waf1/cip1-mediated growth arrest following exposure to genotoxic agents is generally p53 dependent (12). Nonetheless, we demonstrate here that the model mutagen 254-nm UV light induces p21waf1/cip1 protein and concomitant G1 arrest not only in normal HSFs homozygous for wild-type p53 but also in skin fibroblasts derived from cancer-prone LFS patients that are either heterozygous or hemizygous for mutant p53. On the other hand, as expected, following exposure to IR, induction of p21waf1/cip1 and G1 arrest were observed only in normal fibroblasts. These data support previous investigations implicating a critical role for p21waf1/cip1 in growth control for UV-exposed, as well as IR-exposed, cultured cells. Most notably, however, our results clearly indicate the existence of distinct p53-dependent and p53-independent pathways for p21waf1/cip1-mediated growth arrest in cells exposed to DNA-damaging agents, as exemplified in IR-exposed and 254-nm UV-exposed HSFs, respectively.

Materials and Methods

Cell Strains. The normal primary (p53 +/+ ) HSF strain CRL1474 was obtained from the American Type Culture Collection. Low passage number LFS primary skin fibroblast strains MDAHO41 and MDAHO87 (<30 population doublings; designated 04lpd<30 and 087pd<30) are p53 heterozygotes (+/-), manifesting a frameshift mutation at codon 184 and a single base substitution at codon 248, respectively (13). After 30—35 population doublings, the LFS strains enter a period of crisis analogous to cellular senescence in normal fibroblasts. However, unlike normal fibroblasts, which lose their capacity to proliferate, LFS fibroblasts eventually grow out. The resulting post-crisis high passage number derivatives (>200 population doublings; designated 041pd>200 and 087pd>200) have lost their respective wild-type p53 alleles and are considered spontaneously immortalized. Pre- and post-crisis LFS strains were kindly provided by Dr. Michael Tainsky (M. D. Anderson Cancer Center, Houston, TX). All skin fibroblasts were maintained in modified Eagle’s medium with Earle’s salts (Life Technologies, Inc.) supplemented with 10% FCS and antibiotics.

Irradiation Conditions. Confluent cell monolayers growing in 100-mm dishes were washed with PBS, covered with 5 ml of PBS, and exposed to 254-nm UV light using a Philips G25T8 germicidal lamp at a fluence of 0.2 Jm-2s-1. In the case of IR, cell monolayers in 60-mm dishes were washed with PBS, covered with 2 ml of PBS, and irradiated using a cesium-137 source (Gamma Cell; Atomic Energy Canada) at a dose rate of 6.3 rad/s.

Cell Cycle Analysis. Normal and LFS fibroblasts were maintained at confluence in 60-mm culture dishes for approximately 1 week with medium changes every 2 days. Under these conditions, at least 83% of the cells had accumulated in G1-G0. Following irradiation with either 10 Jm-2 of 254-nm UV or 5 Gy of IR, cellular proliferation was stimulated by dilution and replating of cells in fresh complete medium (i.e., release from confluence). At various time points postirradiation, the cells were trypsinized, stained with modified Krishan buffer (0.05 mg/ml PI, 0.1% sodium citrate, 0.2 mg/ml RNase, and 0.3% NP40), and immediately analyzed using a FACSscan flow cytometer.
cytometer (Becton Dickinson). The percentage of cells in each phase of the cell cycle was determined as a function of DNA content using ModFit software (Becton Dickinson).

The proportion of S-phase cells in normal and LFS fibroblast strains was also assessed following incorporation of BrdUrd. Confluent cell monolayers were irradiated as described above, diluted, and replated on glass slides. Thirty min prior to harvest at the various time points, slides were placed in fresh complete medium containing 10 μg/ml BrdUrd, followed by washing with PBS and fixation in ice-cold methanol for 30 min. The slides were immersed in 2 × HCl for 15 min at room temperature, washed with PBS containing 0.5% HSA, and incubated in PBS-HSA containing monoclonal anti-BrdUrd antibody for 45 min at room temperature. Samples were then reacted with fluorescein-conjugated antimouse antibody for 45 min and washed with PBS-HSA, and the nuclei were stained with 10 μg/ml PI. The proportion of S-phase nuclei among at least 300 total nuclei was counted for each determination.

**Western Analysis.** In parallel with analysis of cell cycle progression, replicate cultures were processed for determination of p53 and p21waflc1p protein expression by Western blotting. At the appropriate time points postirradiation, cells were extracted in radioimmunoprecipitation assay buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP40, and 1% sodium deoxycholate) containing 1 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfanyl fluoride, and 1 μM Na2VO4, followed by sonication and clarification by centrifugation. Aliquots containing equal amounts of total protein were then resolved by SDS-PAGE and electroblotted onto nitrocellulose membranes. Membranes were incubated with primary antibody for 1–2 h at room temperature or overnight at 4°C, followed by enhanced chemiluminescence using a secondary antibody coupled to horseradish peroxidase. The primary monoclonal antibodies used were anti-p53 (DO-1; Santa Cruz Biotecology) and anti-p21waflc1p (Ab-1; Calbiochem).

**Results**

*p53 Expression in Normal and LFS Fibroblasts following Irradiation with 254-nm UV or IR.* Previous investigations of diverse cell types have demonstrated intracellular accumulation of p53 protein through posttranscriptional stabilization following exposure to DNA damaging agents (14). Normal skin fibroblasts manifested increased p53 protein following irradiation with either 5 Gy IR or 10 Jm−2 254-nm UV, with levels peaking at approximately 6 h in each case (Fig. 1B). [In the case of normal HSFs, it should be emphasized that 5 Gy IR and 10 Jm−2 254-nm UV represent equitoxic treatments, each yielding a relative survival of approximately 10–15% (6, 37).] For LFS strains 087pd<30 and 087pd>200, high basal levels of constitutive p53 protein expression were documented (Fig. 1A), which was expected in view of the increased stability exhibited by most mutant forms of p53. Unexpectedly, however, no full-length protein could be detected in 041pd<30, which had previously been characterized as p53+/-; rather, these cells expressed comparatively low levels of a putative truncated p53 protein (i.e., with a relative molecular weight of approximately 44,000). Curiously, this is not consistent with the predicted weight (roughly 25,000) of the mutant polypeptide that would be expected based on the location of the first stop codon activated by the frameshift mutation. Nonetheless, the data do suggest that the wild-type p53 allele in our sample of strain 041pd<30 had already been lost by passage 30, heralding the onset of crisis. Finally, as previously shown (13), no basal p53 protein expression could be detected in 041pd>200 cells, which are known to have lost wild-type p53 while still retaining the frameshifted mutant allele. Irradiation with either 5 Gy IR or 10 Jm−2 254-nm UV also failed to yield any detectable p53 protein in this strain (data not shown).

**Cell Cycle Analysis in Normal and LFS Fibroblasts following Irradiation with 254-nm UV or IR.** The entry of cells into S phase as a function of time postirradiation was monitored in UV- and IR-treated fibroblasts by flow cytometry analysis of PI-stained cells and by immunohistochemical staining following incorporation of BrdUrd. Only S-phase cells were measured because the high passage number LFS strains exhibited significant tetraploid/aneupeloid populations as indicated by flow cytometry (Fig. 2), rendering impossible the precise quantitation of G1 cells by flow cytometry. Tetraploidy/ aneupeoidy represents a particular manifestation of genetic instability that is characteristic of transformed LFS fibroblasts, as well as other p53-deficient tumor cell lines (15, 16). Moreover, our sample of 041pd<30, which had been presumed to be heterozygous for wild-type p53 (i.e., precrisis stage), had in fact apparently already entered the crisis period. Indeed, this strain also exhibited a substantial 8N population at the time of analysis and, as mentioned earlier, did not express any detectable full-length p53 protein. Normal HSFs, however, as well as 087pd<30, did not manifest any noticeable degree of tetraploidy/aneuploidy as indicated by flow cytometry analysis.

The cell cycle profiles of PI-stained cells obtained by flow cytometry (raw data) and corresponding graphical representations depicting the entry of cells into S phase as determined either by flow cytometry or BrdUrd immunohistochemistry are shown in Figs. 2 and 3, respectively. In all cases there was excellent agreement between the quantitation of S-phase cells using either technique. In accord with previous investigations (17, 18), normal p53+/- fibroblasts irradiated with 5 Gy IR followed by release from confluence remained arrested in G1 for as long as 5 days (data not shown for times >32 h), whereas all the LFS fibroblast strains entered S phase at a rate similar to the nonirradiated controls (Fig. 3). In contrast, exposure to 10 Jm−2 of 254-nm UV induced a transient G1 arrest lasting approximately 8 h in all LFS fibroblast strains and somewhat longer (at least 12 h) in normal fibroblasts. Furthermore, the UV-induced G1 arrest response exhibited dose dependence in the case of at least one LFS fibroblast strain (i.e., 041pd>200), increasing in duration by approximately 8 h for each 5 Jm−2 increment in dose until at least 20 Jm−2 (data not shown).

**Expression of p21waflc1p Protein in Normal and LFS Fibroblast Lines following Irradiation with 254-nm UV or IR.** As expected (18), following exposure to 5 Gy IR, appreciable induction of p21waflc1p protein was documented only in normal p53+/- fibroblasts, although very low levels of p21waflc1p expression were also observed in sham-irradiated and IR-irradiated LFS fibroblasts (Fig. 4). This latter effect may be attributable to some minor up-regulation of p21waflc1p through growth or serum stimulation (i.e., in our case, due to release from confluence), as previously documented in tumor cell lines (19). Following treatment with 10 Jm−2 of 254-nm UVC, in

![Image](https://cancerres.aacrjournals.org)
CONTROL IR 254nm UV

wt MDAHO87 MDAHO41 wt MDAHO87 MDAHO41 wt MDAHO87 MDAHO41

0hr

24hr

28hr

32hr

UV-INDUCED G, ARREST IN HSFs

Fig. 2. Cell cycle progression in normal and LFS human fibroblasts. Synchronized fibroblasts were irradiated with IR (5 Gy) or 254-nm UV (10 J/m²), or were sham irradiated and then immediately released from confluence. At the indicated times postirradiation, cell cycle progression was analyzed by flow cytometry following staining with propidium iodide. Cell number (Y axis) is plotted versus DNA content. As described in the text, strains O4lpd<30, 087pd>200, and O4lpd>200 exhibited a considerable degree of aneuploidy/tetraploidy; therefore, the first peak corresponds to diploid G0-G1 cells and the second peak to the diploid G2-m plus tetraploid/aneuploid G0-G1, wt, wild type.

B contrast to the situation for 5 Gy IR, p21waflclPl protein was rapidly induced in all fibroblast strains regardless of p53 status (Fig. 4). In the UV-irradiated wild-type strain, protein induction was observed at 3 h and remained elevated until at least 24 h postirradiation, whereas in the LFS strains, p21waflclPl was also induced by 3 h, but at lower levels compared with p53-proficient cells. Furthermore, in the LFS strains, p21waflclPl protein levels had abated completely by 12—24 h. In any case, it is particularly significant that induction of p21waflclPl protein correlated with the passage of cells from G1 to S for normal, as well as LFS, fibroblasts. Indeed, the wild-type p53 cells remained in G1 for at least 32 h post-UV irradiation, whereas the LFS strains began to enter S phase somewhat earlier (i.e., by 24 h post-UV irradiation; Fig. 3).

Discussion

Two distinct modes of p21waflclPl-mediated growth arrest have thus far been characterized in cultured cells: a p53-dependent mode for cells that have sustained DNA damage (12) and a p53-independent mode for cells responding to various stimuli in the apparent absence of genotoxic stress [e.g., following the application of growth factors (20) or hormones (21) or serum stimulation (19)] and for terminally differentiated cells (12). Although a limited number of investigations have reported up-regulation of p21waflclPl in p53-deficient cells following exposure to certain DNA damaging agents (22—24), a plethora of studies have demonstrated the virtual universality of p53 dependence for p21waflclPl induction in IR-exposed human and murine lines. This has resulted in the emergence of a widely held notion that p21waflclPl-mediated growth arrest is generally p53-dependent in cells exposed to genotoxic agents. In disagreement with this prevailing view, our data strongly indicate that induction of p21waflclPl and concomitant G1 arrest can occur regardless of p53 status in HSFs irradiated with the model mutagen 254-nm UV light. This reveals a novel mode of DNA damage-inducible, p21waflclPl-mediated growth arrest.

Fig. 3. Graphical representation of cell cycle progression in normal versus LFS fibroblasts. Cells were analyzed via flow cytometry alone (A), BrdUrd immunohistochemistry alone (B), or BrdUrd immunohistochemistry in addition to flow cytometry (C–F). Each time point represents an average of at least three independent experiments. A and B, normal fibroblasts; C, 087pd<30 LFS fibroblasts; D, 041pd<30 LFS fibroblasts; E, 041pd>200 LFS fibroblasts; F, 087pd>200 LFS fibroblasts. □, IR-irradiated; ◇, sham-irradiated; ●, 254-nm UV-irradiated.
arrest, as exemplified in UV-irradiated human cells, which is p53-independent and therefore distinct from the IR-induced pathway. The observed UV-induced growth delay is likely ancillary to the p53-dependent pathway because significantly greater levels and duration of p21expression were evident in normal versus LFS skin fibroblasts. It is also important to emphasize that p21 induction was documented in UV-irradiated 041pd>200 LFS fibroblasts, which are null for p53 expression following irradiation with either UV or IR as determined by Western blotting. This suggests that the observed up-regulation of p21 is not attributable to any residual transactivation potential of, or gain-of-function for, mutant p53 proteins in the LFS fibroblasts.

Although the data base on DNA damage-inducible growth arrest is considerably smaller for cells treated with UV versus IR, there are indications that the initiation of G1 arrest in 254-nm UV-exposed cultured cells is p53-dependent. For example, p53-proficient normal human oral keratinocytes manifested G1 arrest following UV irradiation, whereas an oral cancer cell line expressing mutant p53 did not (25). Nevertheless, in accord with the results presented here, there is also evidence that G1 arrest can occur independently of p53 in certain UV-irradiated cell lines. Indeed, transient UV-induced G1 delay in some human and murine cell lines was shown to correlate with hypophosphorylated Rb, whereas p53 did not begin to accumulate until the onset of S phase (26). In addition, Orren et al. (27) demonstrated transient G1 arrest after treatment with 254-nm UV, but not with IR, in p53-deficient Chinese hamster ovary cells. However, in contrast with the results presented in our study, Liu et al. showed that UVB treatment of normal mouse keratinocytes results in rapid intracellular accumulation of p53 protein and concomitant induction of p21, an effect that was abolished in the case of p53-deficient keratinocytes (28, 29). In any case, the overall importance of p21 in UV-induced growth arrest has been demonstrated in both human and murine cells. Indeed, transient G1 delay in UVB-irradiated rat keratinocytes coincided with induction of p21, which was accompanied by inhibition of cyclin-dependent kinase activity (30). In addition, UVB treatment inhibited human melanocyte proliferation by causing arrest in G1, and this correlated with expression of p21 and concomitant Rb hypophosphorylation (31).

![Fig. 4. Induction of p21 protein in normal versus LFS fibroblasts irradiated with 5 Gy IR, 10 Jm-2 254-nm UV, or sham irradiated. Replicate cultures were processed for Western blotting in parallel with determination of cell cycle progression, as described in “Materials and Methods.”](image_url)

Our results with HSFs confirm and extend the above investigations by highlighting, for the first time, the potential importance of p53-independent, p21-mediated growth arrest in human cells subsequent to UV exposure.

Although the biochemical basis for the differential mode of p21 induction in UV- versus IR-irradiated human fibroblasts remains to be elucidated, we speculate that it might somehow be related to some intrinsic properties of the two model mutagens. In fact, the two agents exhibit highly disparate genotoxic and metabolic effects, and it may not be unreasonable to hypothesize that this would result in dissimilar patterns of gene expression. DNA is the primary target for 254-nm UV, which produces almost exclusively replication-blocking, dipyrimidine-type photoproducts (see below), and relatively very few DNA strand breaks. Moreover, 254-nm UV alters the redox state of the cell only very minimally (32). In contrast, IR induces primarily oxidative stress, generating double and single strand DNA breaks and oxidative base adducts and, unlike 254-nm UV, attacks a very broad range of cellular macromolecules in addition to DNA (33). Thus, compared with IR, irradiation with 254-nm UV may represent a more appropriate paradigm for the cellular response to genotoxic agents per se.

What is the potential physiological relevance of the observed p53-independent UV-induced G1 delay in human skin fibroblasts? Cyclobutane pyrimidine dimers and 6-4 pyrimidine pyrimidone photoproducts, which constitute the major premutagenic lesions in cells irradiated with 10 Jm2 of 254-nm UVC in cellular products, which constitute the major premutagenic lesions in cells irradiated with either monochromatic 254-nm UVC or broad-spectrum sunlight (34), are clearly implicated in the etiology of skin cancer (35, 36). We estimate from our cell cycle profiles that the duration of the UV-induced checkpoint in both normal and LFS fibroblasts irradiated with 10 Jm-2 is such that cells remain arrested in G1 for at least 24 h postirradiation. It should be noted that the yield of premutagenic UV photoproducts generated by 10 Jm-2 of 254-nm UVC in cellular DNA is in the range of that which could be induced in human skin during a casual exposure to sunlight (34).] In the case of normal human primary fibroblasts treated with 40 Jm-2 of 254-nm UV, it was shown that during the first 24 h postirradiation, most of the highly premutagenic CPDs (up to 80%) and 6-4PPs (over 90%) are removed via nucleotide excision repair (37). This apparent correlation between virtually complete photoproduct removal and resumption of cell cycle progression strongly suggests that the transient UV-induced G1 delay may have significant impact with respect to the avoidance of skin carcinogenesis in normal, as well as LFS, individuals.
Indeed, p53-deficient LFS patients, although highly susceptible to a wide variety of internal malignancies, are not predisposed to nonmelanoma skin cancer (38). This is rather surprising because mutated p53 alleles have been recovered from up to 50 and 90% of squamous and basal cell carcinomas of the skin, respectively (35, 36). Furthermore, we note the demonstrated outstanding importance of two major p53-regulated processes [i.e., apoptosis (39) and nucleotide excision repair (33)] in the development of skin cancer and the fact that each of these vital processes is known to be at least partially defective in UV-irradiated LFS fibroblasts (40). As such, we speculate that competency in a third major (previously thought to be strictly) p53-dependent process [i.e., DNA damage-inducible p21\textsuperscript{waf1/-mediated} G\textsubscript{1} arrest] actually provides significant protection for LFS patients specifically against sunlight-induced mutation and cancer.

References


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