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Advances in Brief

p53-null Cells Are More Sensitive to Ultraviolet Light Only in the Presence of Caffeine[1]

Jeffrey S. DeFrank, Wei Tang, and Simon N. Powell[2]

Department of Radiation Oncology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114

Abstract

We have shown previously that p53(−/−) fibroblasts show greater sensitization by caffeine to the lethal effects of ionizing radiation compared with p53(+/+) cells. Recently published data have suggested a possible role of p53 in nucleotide excision repair: an association of p53 and xeroderma pigmentosum group B protein and a greater sensitivity to cisplatin of RKO cells transfected with the E6 protein of human papilloma virus (inactivating p53). We show that p53(+/+) and p53(−/−) cells have equal sensitivity to germicidal UV light (as with ionizing radiation). However, the introduction of 2 mM caffeine led to a sensitization enhancement ratio (at 10% survival) of 1.8 in p53(−/−) cells, but only 1.3 in wild-type (p53+/+) cells. Lower doses of caffeine had less effect, and 0.1 mM caffeine resulted in no detectable sensitization of either cell type to UV light in contrast to X-rays. The differential sensitivity of p53(−/−) cells to X-rays and caffeine was thought to be due to override of the G_2-M block to cell cycle progression. In response to UV light, cells accumulate in S phase, and the magnitude of S-phase accumulation was observed to be greater in p53(−/−) cells. Caffeine had little effect on the cell cycle distribution in p53(+/+) cells. However, for p53(−/−) cells, a greater proportion were in S phase after treatment with caffeine, and a complete loss of S-phase delay was observed after UV irradiation. In conclusion, the role of p53 in nucleotide excision repair appears to be of little significance for cell survival. Greater sensitization of p53(−/−) cells to caffeine could be mediated via override of S-phase delay.

Introduction

The tumor suppressor p53 is the protein whose function is most frequently inactivated in malignant cells. There is great interest in the possible role of p53 acting directly in the repair of DNA damage, which may give an opportunity to exploit differences in DNA repair between normal cells and tumor cells. Loss of function of p53 has been shown to prevent apoptosis, and results in relative resistance to cytotoxic agents such as X-rays, etoposide, or bleomycin. This effect is seen frequently in lymphoid lineage cells (1–3), but in many other normal cells and tumor cells p53 has relatively little impact on the magnitude of cell killing in response to DNA damaging agents. Considering the major role played by p53 in the response to DNA damage, it is surprising that it does not contribute significantly to the cytotoxic effect, as was originally expected in the "Guardian of the Genome" model (4).

There have been two reports of primary fibroblasts with and without p53 having equal sensitivity to ionizing radiation (5, 6). There are a number of papers describing tumor cells or transformed cells with inactivated p53 being more resistant to X-rays compared with the same cells containing wild-type p53 (7, 8). Others have reported no difference (9), but there are no reports suggesting that cells with inactivated p53 are more sensitive to X-rays as predicted in the Lane model.

Recently, however, a greater sensitivity to cisplatin and UV light was observed in cells with nonfunctional p53 (10, 11). This raises the the following question: Does p53 play a direct role in the specific DNA repair pathway of NER[3], thought to be the predominant pathway to repair UV light and cisplatin damage? Although UV damage to DNA resulting in pyrimidine dimers appears to be repaired exclusively via NER, certain cisplatin adducts also may be recognized by the mismatch repair pathway (12). However, the loss of function of one component of mismatch repair (hMLH1) resulted in resistance, not sensitivity, to cisplatin (13), suggesting the development of tolerance of the cisplatin adduct. Because p53 has also recently been found to bind to the promoter of the *hMSH2* gene, p53 status may also affect the level of mismatch repair (14). The repair of cisplatin damage may involve more than one repair pathway, and the influence of p53 function could be the opposing effects of impaired NER with sensitization and impaired mismatch repair with resistance. The use of UV damage appears to allow a focus on NER alone.

The involvement of p53 in NER is suggested by direct and indirect observations. Direct evidence is the observation that MCF-7 cells are more resistant to cisplatin compared with MCF-7 cells transfected with the human papilloma virus E6 protein (10). Similar findings have been reported with MCF-7 transfected with a plasmid containing a dominant-negative mutation of p53 (alanine-143), and RKO cells transfected with E6 are more sensitive to UV light and cisplatin (11). Furthermore, using a host-cell reactivation assay and a chloramphenicol acetyl transferase gene in a reporter plasmid, the MCF-7 cells with wild-type p53 repaired the UV light-treated plasmid 2-fold more efficiently than the MCF-7-E6 cells. Thus, differences in expression of the E6 protein, which results in differences in the levels of p53 protein and loss of p53 function, leads to greater sensitivity to UV light and less efficient repair of UV light-damaged plasmid. The conclusion was that p53 was contributing to NER. Another possible explanation is that E6 protein is changing other factors involved in the response to DNA damage in addition to inactivating p53.

Indirect evidence for the involvement of p53 in NER is the association of p53 and the excision repair cross-complementing protein ERCC3 (15) seen by immunoprecipitation. The hepatitis B virus protein X was shown to inhibit p53 function and, furthermore, inhibited the association of p53 with ERCC3 (which is xeroderma pigmentosum group B). The functional consequences of the association of p53 and ERCC3 are unknown. It was suggested that it may be regulating the helicase activity of ERCC3. However, in Li-Fraumeni fibroblasts containing no functional alleles of p53, transcription-coupled repair was found to be normal, whereas the ability to remove UV damage (cyclobutane dimers) from the overall genome was decreased (16). The influence of p53 has not been tested directly in an *in vitro*

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[2] To whom requests for reprints should be addressed, at Department of Radiation Oncology, Cox 302, 100 Blossom Street, Massachusetts General Hospital, Boston, MA 02114. Phone: (617) 726-8669; Fax: (617) 726-3603; E-mail: powell.simon@mgh.harvard.edu.

[3] The abbreviation used is: NER, nucleotide excision repair.

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excision repair assay. There may be limited opportunity for changes in helicase activity to modify the *in vitro* assay, as the plasmid substrate contains largely unwound DNA. A downstream effector of p53, GADD45, has also been shown to interact with proliferating cell nuclear antigen (17). The addition of recombinant GADD45 enhanced the excision repair assay, suggesting that p53 may influence NER indirectly by a transactivation-mediated effect. However, these latter observations have not been reproduced by others (18, 19).

Caffeine has long been known to impair the cell cycle arrest in response to DNA damaging agents (20–22). X-irradiation tends to cause an arrest of the cell cycle at both the G_1-S and the G_2-M boundaries. UV irradiation causes a predominant S-phase delay, rather than a G_2-M delay (23). The sensitization of X-ray-induced cell killing by caffeine has been shown to be greater in cells lacking the function of p53 (9, 24), and the proposed explanation of the caffeine effect is override of G2 delay. The quality of the G2 block in p53(−/−) cells was thought to be more susceptible to the effects of caffeine, but there was no proof that this was the mechanism of differential sensitivity to cell killing. Other reports have confirmed the abnormal G2 block in cells lacking the function of p53 (25).

The current question addressed in this report is: Does p53 influence cell survival following UV radiation? Furthermore, are p53(−/−) cells more sensitized by caffeine after UV light, as found for X-rays? Because UV light does not induce a G2 delay, these experiments seek to test the mechanism of caffeine sensitization. To eliminate the possible confounding effects of working with cells transfected with the HPV-16-E6 protein, we measured UV sensitivity with and without caffeine in murine embryonic fibroblasts, in which the endogenous *p53* gene had been inactivated by gene knockout in embryonic stem cells.

Materials and Methods

Cell Culture. Murine embryonic fibroblasts derived from mice in which there were two normal alleles of *p53(+/+)* and mice in which both *p53* alleles were disrupted (−/− or null) were obtained from T. Jacks (Massachusetts Institute of Technology, Cambridge, MA). Cells were grown in DMEM with 15% calf serum supplemented with penicillin (100 units/liter), streptomycin (1 mg/ml), HEPES buffer (20 mM), and glucose (5 g/liter), and maintained at 37°C with 5% CO_2. Cells were used at low passage (5–20) from the initial derivation to avoid senescence.

The cells were UV irradiated in a laminar air flow tissue culture hood equipped with a UV light source. This source was calibrated for irradiation at 254 nm, with a dose rate to give 10 Jm^{-2} in 22 s. The cells were washed in PBS before UV irradiation to eliminate absorption from the phenol red indicator in DMEM. Caffeine was dissolved in PBS to 1 M stock solution and then filter-sterilized using a 0.22-μm filter. Caffeine was added to final concentrations of 0.1, 0.5, 1.0, 2.0, and 5.0 mM.

Cell Survival. Cell survival was measured using a standard clonogenic assay. A single-cell suspension was obtained, counted by hemocytometer, serially diluted, and then seeded according to the expected surviving fraction. Heavily X-irradiated feeder cells consisting of p53(±) cells exposed to 50 Gy were used to enhance the plating efficiency of the fibroblasts from 5 to 15%. Colonies, defined as >50 cells, developed after 16–20 days. The surviving fraction was measured as the number of surviving viable colonies divided by the number of cells seeded and corrected by the observed plating efficiency for the cell. Triplicate plates were seeded for each dose point in each experiment, and survival was measured in four independent experiments.

Cell Cycle Analysis. To ensure exponential growth at the time of analysis, 5×10^{-5} cells were seeded 24–48 h before UV irradiation with 20 Jm^{-2}. Cells were harvested for flow cytometric analysis after 0, 3, 6, 9, 12, and 24 h. For each time point, single-cell suspensions were collected into a pellet by centrifugation at 1500 rpm for 5 min, washed twice in PBS, and then fixed in ice-cold 70% ethanol. The cells were washed twice more in PBS and then resuspended in propidium iodide (20 μg/ml) and RNase (500 μg/ml). The samples were stored at 4°C and protected from light exposure.

Flow Cytometry. DNA analysis was performed using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA) emitting a 488-nm beam. The FACScan was calibrated for linearity using chicken erythrocyte nuclei. Aggregated nuclei and clumps were omitted from analysis with pulse area *versus* pulse gating. Data from 20,000 nuclei were saved in a list mode and displayed as a frequency distribution histogram of propidium iodide fluorescence at 575 nm. Analysis of histograms was performed using a mathematical curve-fitting program model that calculated the percentage of cells in each phase of the cell cycle and coefficient of variation around G_0/G_1 mean peak (RFIT, Becton Dickinson).

Results

UV Light Survival. Four replicate experiments were carried out for the two cell types: p53(+/+) and p53(−/−). The logarithm of the surviving fraction plotted against UV light dose is shown in Fig. 1, and reveals no significant difference between the cells. It was noted that curves were concave upward (less relative kill as dose increases) in contrast to X-ray survival.

The effect of caffeine on the UV light survival of p53(+/+) cells is shown in Fig. 2*A*. Only three doses of caffeine are shown (0, 0.5, and 2 mM), which demonstrated a small degree of sensitization at 2 mM caffeine of borderline significance. In contrast, when the p53(−/−) cells were treated under identical conditions, the effect of caffeine was clearly different from no caffeine treatment (see Fig. 2*B*). At each of the three dose points, a statistically significant difference was obtained comparing 2 mM caffeine with no caffeine ($P = <0.001$). The sensitization enhancement ratio (ratio of UV light doses without and with caffeine to produce 10% survival) of 2 mM caffeine in p53(−/−) cells was 1.8. The ratio for p53(+/+) cells was 1.3, which would result in a therapeutic gain factor (ratio of enhancement in the "tumor" cell to enhancement in the normal cell) of 1.4.

Cell Cycle Analysis. One reason to use UV exposure to cells was to test the hypothesis that caffeine sensitization was mediated via G_2-M override, as UV damage is reported to cause S-phase delay (26) rather than G2 block. Fig. 3 shows the effect of UV exposure on the cell cycle distribution of p53(+/+) cells and p53(−/−) cells. The only detectable change following UV irradiation was an accumulation of cells in S phase. Fig. 3*A* shows that before irradiation, the S-phase fraction was 28% in p53(+/+) cells, and 9 h after UV light it increased to 34%. The G_2 population remained relatively constant over the 24 h after irradiation at 8–12%. The proportion of p53(−/−) cells in S phase before irradiation was 30%, and 9 h after irradiation it was 47%. Again, the G_2 population remained constant, but at a higher level of 14–18%. The proportion of cells in S phase before irradiation was not significantly different between the cell types.

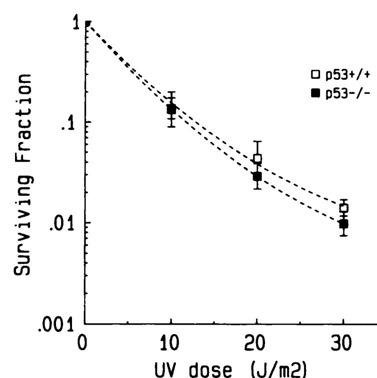

Fig. 1. The surviving fraction of cells in response to UV irradiation using a clonogenic assay. Surviving fraction is shown on a logarithmic axis against UV light dose (in Jm^{-2}) plotted linearly. □, p53(+/+) cells; ■, p53(−/−) cells. Data points are the mean of four replicate values and 95% confidence intervals. *Bars*, 2 × SE.

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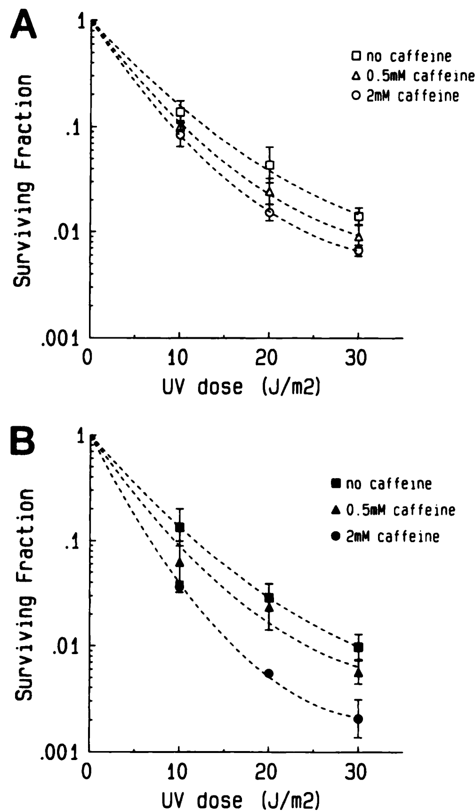

Fig. 2. *A*, surviving fraction of p53(+/+) cells to UV light without caffeine (□); with 0.5 mM caffeine (△); and with 2 mM caffeine (○). *B*, surviving fraction of p53(−/−) cells to UV light shown similarly with *solid symbols*. *Bars*, 2 × SE.

However, the cell cycle distribution in response UV irradiation showed a higher proportion of p53(−/−) cells accumulating in S phase 9 h after UV irradiation.

The effect of caffeine on the cell cycle distribution after UV irradiation is shown in Fig. 3, *C* and *D*. In p53(+/+) cells, the starting S-phase fraction was 25% (not significantly different from 28% in the previous experiment), which remained constant for the 3- and 6-h time points. At 9 h, S phase increased to 34% briefly, and by 12 h it was back to 26%. Thus, the S-phase delay was not significantly altered by 2 mM caffeine. In p53(−/−) cells, the starting S-phase fraction was elevated at 44%, reflecting exposure to 2 mM caffeine for 24 h. After UV irradiation, there was no further increase in S-phase cells, and by 9 h the S-phase fraction had decreased to 28% with G_1 cells accumulating to 52%, from 36% initially.

Discussion

Clonogenic survival to UV light alone showed no difference between p53(+/+) and p53(−/−) cells, implying that p53 is not playing a significant functional role in NER. p53 has been reported to associate with xeroderma pigmentosum group B protein (15), suggesting a possible role in NER. This idea was supported by the observation of a greater sensitivity to both cisplatin and UV light of the RKO cell line transformed by the E6 protein of human papilloma virus type 16 (11), and greater sensitivity of an E6-transformed MCF-7 cell line to cisplatin (10). However, our findings with p53 knockout primary embryonic fibroblasts suggest that p53 alone does not confer detectable UV light sensitivity, and further suggests that E6 transformation may be affecting other proteins that play a role in NER. The association of p53 with xeroderma pigmentosum group B (ERCC3) may be regulating the role of this protein as a helicase. p53 has not been tested in an *in vitro* assay of excision repair, but p53 would only be expected to modify the unwinding (helicase)

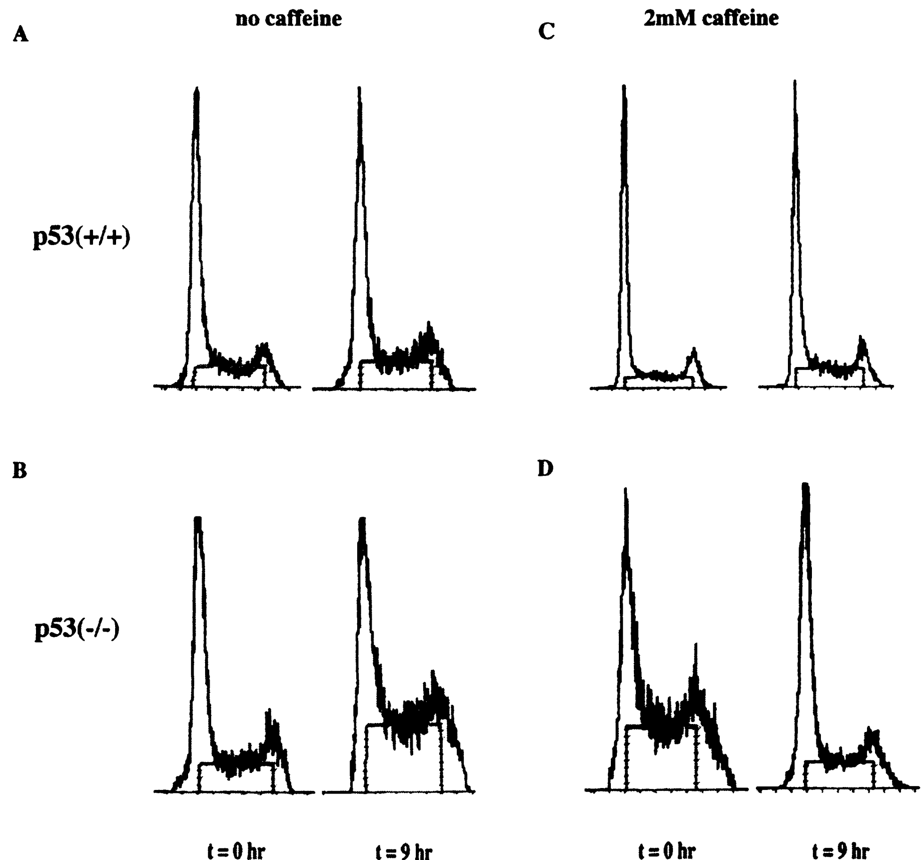

Fig. 3. Flow cytometric profiles of cells before and after UV irradiation. Cell number is plotted vertically, and DNA content is plotted horizontally. The *rectangular lines* denote the DNA content consistent with S phase. The *left* peak reflects the G_1 population, and the *right* peak reflects the G28 population. *A*, p53(+/+) cells immediately before UV irradiation and 9 h after irradiation. *B*, p53(−/−) cells shown similarly. *C*, p53(+/+) cells irradiated after 24 h pretreatment with 2 mM caffeine and 9 h after irradiation. *D*, p53(−/−) cells shown similarly.

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function, which is not tested by these assays. Li-Fraumeni fibroblasts with no functional *p53* allele were found to be no more sensitive to UV light than a heterozygote fibroblast (16). A deficiency in the removal of UV damage was found in the p53-null cell, but it was suggested that increased cell killing was not seen because of an inhibition of apoptosis. This suggests that a repair difference does exist between p53+ and p53− cells, but the consequences are differences in genetic instability, not in cell survival.

Our previous data with X-rays (9) showed greater sensitization by caffeine for p53(−/−) compared with p53(+/+) fibroblasts. The suggested explanation of this effect of caffeine was override of G_2 delay. Overall, the greater effect of caffeine on p53(−/−) cells was thought to be due to both a quantitative reason (more cells at the G_2-M checkpoint because of lack of the G_1-S checkpoint) and a qualitative reason (caffeine more readily overrides G_2-M in p53(−/−) cells). Coincident with this report, two other groups reported an equivalent finding: greater sensitization by methyl-xanthines in cells with disrupted function of p53 (24, 27). Further data, using a temperature-sensitive mutant of p53, also confirmed the abnormal quality of the G_2-M checkpoint when p53 function is abrogated (25).

However, when cells were exposed to UV irradiation to avoid causing a G_2-M delay, the p53(−/−) cells remained more sensitive to caffeine compared with p53(+/+) cells. It is noted that the dose threshold of caffeine for UV light sensitization is higher than for X-rays; 1 mM caffeine was required to show detectable and significant sensitization for UV light compared with 0.1 mM for X-rays. Caffeine is likely to have a diverse spectrum of action and affect multiple biochemical pathways. The higher dose threshold for UV light sensitization may limit the ultimate therapeutic application of methyl-xanthines to achieve therapeutic gain, but the observation of greater sensitization in cells lacking the function of p53 is potentially exploitable.

The mechanism of greater sensitization by caffeine after exposure to UV light in p53(−/−) cells has not been determined with confidence. The suggestion is that, as for X-ray sensitization, there is a lower threshold to override cell cycle arrest. In p53(−/−) cells, although X-rays resulted in a G_2 block that was decreased by caffeine, UV light resulted in a predominant S-phase delay that was decreased by caffeine. The effect of caffeine without UV exposure was to increase the number of cells in S phase in p53(−/−) cells, but the same dose of caffeine in p53(+/+) cells caused no significant change. When UV irradiation was given in the presence of caffeine, the p53(−/−) cells no longer retained the capacity to delay in S phase, and 9 h after irradiation the proportion of cells in S phase had decreased. The data does not distinguish whether the effect of caffeine results in a shorter time in G_1 or a longer time in S phase. However, the combined UV light and caffeine data suggest a shortening of G1 and an override of S-phase delay. The differential response to caffeine of p53(−/−) compared with p53(+/+) cells was clear.

It is suggested that a p53-dependent response to UV light results in slowing of the transit of cells from G_1 and into the S phase. UV light is known to induce p53 (28) and, relative to X-rays, causes an S-phase delay. S-phase delay was initially thought to be due to persistent DNA damage causing a block to replication, although, in addition, it may be mediated by a checkpoint mechanism. The replication machinery can bypass DNA damage, but the presence of DNA damage can trigger signals to arrest cell cycle progression. Why UV light leads to a different pattern of cell cycle arrest compared with X-rays is not clear. It suggests that DNA damage recognition and the effector pathways are different with the two modes of irradiation.

In summary, we have provided data that show that p53-null cells are more susceptible to the sensitizing effects of the methyl-xanthine caffeine in combination with both UV light and, previously, with ionizing radiation. The loss of checkpoint control in p53-null cells appears to make these cells more vulnerable to override of remaining the non-p53-dependent checkpoints.

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