Functional Analysis of the p53 Pathway in Response to Ionizing Radiation in Uveal Melanoma

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PURPOSE. Uveal melanomas are notoriously radioresistant and thus necessitate treatment with extremely high radiation doses that often cause ocular complications. The p53 tumor suppressor pathway is a major mediator of the cellular response to radiation-induced DNA damage, suggesting that this pathway may be defective in uveal melanoma. The current study was conducted to analyze the functional integrity of the p53 pathway in primary uveal melanoma cells.

METHODS. The p53 gene was sequenced in three primary uveal melanoma cell lines. Cultured primary uveal melanoma cells (MM28, MM50, Mel202, Mel270, and Mel290), MCF7 breast carcinoma cells, normal uveal melanocytes (UM47), and normal human diploid fibroblasts (NHDFs) were irradiated at 250 kVp and 12 mA at a dose rate of 1.08 Gy/min for a total dose of up to 20 Gy. Cell viability was analyzed with trypan blue exclusion. Western blot analysis was used to analyze the expression of p53, p53-phospho-Ser15, p21, Bax, PUMA, and Bcl-xL.

RESULTS. No p53 gene mutations were found in MM28, MM50, or Mel270 cells. Upstream signaling to p53 was intact, with normal induction of p53 and phosphorylation of p53-Ser15, in all five cell lines. Radiation-induced downstream activation of p21 was defective in MM28 and MM50 cells, and activation of Bax was defective in MM50 and Mel290 cells. MM28, MM50, and Mel202 cells failed to deamidate Bcl-xL in response to radiation-induced DNA damage. Overall, four of the five uveal melanoma cell lines exhibited at least one downstream defect in the p53 pathway.

CONCLUSIONS. Expression of p53 and upstream signaling to p53 in response to radiation-induced DNA damage appear to be intact in most uveal melanomas. In contrast, functional defects in the p53 pathway downstream of p53 activation appear to be common. Further elucidation of p53 pathway abnormalities in uveal melanoma may allow therapeutic interventions to increase the radiosensitivity of the tumors. (Invest Ophthalmol Vis Sci. 2005;46:1561–1564) DOI:10.1167/iovs.04-1362

Primary uveal melanomas are highly radioresistant.1 Nevertheless, these cancers are most commonly treated with episcleral plaque brachytherapy or charged-particle radiotherapy at very high doses (85–100 Gy).2 This high-dose radiotherapy usually results in good local control but frequently leads to vision-threatening radiation complications, such as cataract, retinopathy, papillopathy, and neovascular glaucoma.3,4 A better understanding of the molecular basis for radioresistance in uveal melanoma could lead to novel therapeutic interventions to render tumors more radiosensitive.

Radiation therapy generates DNA damage, which leads to cell cycle arrest and/or apoptosis, depending on the cellular context.5 The p53 tumor suppressor plays an important role in the cellular response to radiation-induced DNA damage.1,6 Cellular p53 levels are very low in normal cells, due to an inhibitory interaction with HDM2 that targets p53 for degradation.6 After radiation-induced DNA damage, p53 undergoes posttranslational modifications, such as phosphorylation at Ser15, that stabilize the p53 protein by inhibiting its binding to HDM2.6 Consequently, p53 accumulates rapidly and activates downstream target genes encoding p21 (a cell cycle inhibitor), Bax (a proapoptotic protein), and other proteins.8

Over half of human cancers contain p53 mutations, and most others contain genetic lesions in the p53 pathway that render p53 ineffective as a tumor suppressor.10 Untreated uveal melanomas rarely contain p53 mutations,11–13 which suggests that they may contain functional defects that interfere with the p53 pathway. Such defects could contribute to the radioresistant phenotype of uveal melanoma. The Bcl2 family of apoptotic arbitrators is a critical downstream target of p53 signaling.14 Activated p53 signals directly to Bcl2 family proteins by activating Bax and other proapoptotic family members. Disruption of this signaling from p53 to the Bcl2 family proteins can block the proapoptotic p53 stimulus and is encountered commonly in cancer. Deamidation of Bcl-xL at asparagines 52 and 66 in the unstructured loop of the protein has recently been shown to be a hallmark of an intact p53-Bcl2 response to DNA damage.15 This deamidation confers susceptibility to DNA damage-induced apoptosis by abrogating the ability of Bcl-xL to block proapoptotic BH3 domain-only proteins. There is growing evidence that disruption of this deamidation may be a common mechanism by which tumor cells acquire resistance to DNA damage-induced apoptosis.16

In this study, we evaluated the functional status of the p53 pathway in response to ionizing radiation in primary uveal melanoma cell lines by analyzing key molecular events upstream and downstream of p53.

MATERIALS AND METHODS

Cell Cultures

These studies were approved by the Washington University Institutional Review Board and conformed to the tenets of the Declaration of Helsinki. The cell lines used included normal human diploid fibroblasts (NHDFs; from ATCC, Manassas, VA), MCF7 breast carcinoma cells (ATCC), and Mel202, Mel270, and Mel290 uveal melanoma cells (gift of Bruce Ksander, Harvard Medical School, Boston, MA). Cell lines developed in our laboratory included UM47 normal uveal melanocytes and MM28 and MM50 uveal melanoma cells. MM28 and MM50 cells were maintained in Ham’s F-12 medium (BioWhittaker, Walkersville, MD), supplemented with 2 mM t-glutamine, 10% fetal bovine serum, 50 µg/mL gentamicin, 10 ng/mL cholela toxin, 20 ng/mL human growth factor, and 0.1 mM isobutylmethylxanthine. Mel202, Mel270, and
Mel290 cells were maintained in complete RPMI 1640 supplemented with 10% fetal bovine serum. Other cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen). The cells were plated at 10⁵ cells/100-mm tissue culture dish, grown to plateau phase, and irradiated at 250 kVp and 12 mA at a dose rate of 1.08 Gy/min for a total radiation dose as indicated (see Fig. 2). They were then placed back in the incubator, and lysates were collected at the indicated time points (see Fig. 3).

**Western Blot Analysis**

Western blot analysis was performed, as previously described,¹⁷ using antibodies that recognize p53 (1:1000; DO-1 monoclonal; Santa Cruz Biotechnology, Santa Cruz, CA), p53-phospho-Ser15 (1:1000; Cell Signaling Technology, Beverly, MA), p21 (1:1000; F-5; Santa Cruz Biotechnology), HDM2 (1:1000; N-20; Santa Cruz Biotechnology), Bax (1:1000; Cell Signaling), PUMA (1:500; C-100; Oncogene, Manhasset, NY), Bcl-x, (1:1000; Cell Signaling Technology) and α-tubulin (1:1000; Sigma-Aldrich, St. Louis, MO).

**Cell Viability Assays**

Trypan blue exclusion assays were used to measure cell viability after ionizing radiation. For these assays, MCF7 or Mel202 cells (1 × 10⁴) were plated in triplicate in 24-well plates. At various time points after radiation treatment with 0, 9, or 20 Gy, cells were trypsinized and counted with a hemocytometer.

**p53 Gene Sequencing**

DNA was obtained from Mel270, MM28, and MM50 cells using the DNeasy tissue kit (Qiagen, Valencia, CA) and submitted to the Washington University/Siteman Cancer Center Molecular Core Laboratory for sequencing. Exons 2 to 11 and introns 2 to 11 were sequenced with PCR primer sets, as previously described.¹⁸

**RESULTS**

In most studies a very low frequency of p53 gene mutations has been found in primary, untreated uveal melanomas.¹¹,¹³ Consistent with these previous reports, we sequenced the p53 coding sequence in MM28, MM50, and Mel270 cells and found no DNA mutations (data not shown). To evaluate the functional status of p53, we first examined its cellular protein levels after irradiation. In normal cells, ionizing radiation causes the p53 protein to undergo various posttranslational modifications that lead to protein stabilization and accumulation. At 8 hours after irradiation (9 Gy), Western blot analysis showed that p53 accumulated in MM28, MM50, Mel202, Mel270, and Mel290 uveal melanoma cells at levels similar to its accumulation in NHDFs and UM47 normal uveal melanocytes (Fig. 1). Similar results were seen when protein was analyzed at 1 hour after irradiation and when a dose of 20 Gy was used (data not shown).

Phosphorylation of Ser15 is one of the key posttranslational modifications to p53 that occur in response to radiation-induced DNA damage.¹⁹ We evaluated the phosphorylation status of Ser15 at 8 hours after irradiation, using a phosphospecific antibody that recognizes p53 that has been phosphorylated at Ser15. All five uveal melanoma cell lines exhibited robust phosphorylation of Ser15 in response to 9 Gy of irradiation at levels comparable to those in NHDF and UM47 cells (Fig. 1). These results indicate that upstream signaling to p53 in response to radiation-induced DNA damage is intact in these uveal melanoma cells.

To evaluate downstream signaling in the p53 pathway, we examined protein levels of p21 and Bax, which are direct transcriptional targets of activated p53.²⁰,²¹ By Western blot analysis, p21 was induced 8 hours after 9 Gy of irradiation in NHDF, UM47, Mel202, Mel270, and Mel290 cells, but not in MM28 or MM50 cells (Fig. 1). Under the same conditions, Bax was induced in Mel202 and Mel270 cells, but not in Mel290 or MM50 cells (Fig. 1).

Mel202 cells were one of the two uveal melanoma cell lines that demonstrated normal upstream and downstream signaling in response to radiation treatment. Therefore, we examined this cell line in more detail for other possible defects in the p53 pathway. For these studies, we compared Mel202 cells to MCF7 breast carcinoma cells, which are relatively radiosensitive and often used for studying cellular response to ionizing radiation.²² Mel202 cells were significantly more resistant to ionizing radiation than MCF7 cells, as measured by cell viability (Fig. 2). To look for defects in the kinetics of p53 activation in Mel202 cells, we examined them over a time course after irradiation with 20 Gy. The same results were noted between the two cell lines. Similarly, p21 levels increased by 8 hours after irradiation in both cell lines with no differences in kinetics. Another p53 transcriptional target, PUMA (p53 upregulated modulator of apoptosis), was induced more efficiently in Mel202 cells than in MCF7 cells (Fig. 3A). Thus, the ability of p53 to activate downstream targets transcriptionally appears to be intact in Mel202 cells.

Recently, deamidation of the antiapoptotic protein Bcl-x, at asparagines 52 and 66 has been shown to be an important marker of an intact DNA damage-induced apoptotic response in cancer cells.¹⁵,¹⁶ This deamidation can be identified by the appearance of more slowly migrating bands on Western blot analysis. We examined the Bcl-x, protein by Western blot analysis and found multiple migrating forms of Bcl-x, indicative of deamidation, in MCF7 cells but not in Mel202 cells (Fig. 3A). In light of this finding, we examined other cell lines for Bcl-x, deamidation. Appearance of a more slowly migrating band, consistent with deamidation, was present in NHDFs,
equivocal in Mel270 cells, and absent in MM28 or MM50 cells (Fig. 3B).

**DISCUSSION**

The weight of current evidence suggests that mutation of p53 is an uncommon event in primary, untreated uveal melanomas.11–13 In this study, we provided functional support for this idea by showing that p53 accumulates normally in response to DNA damage in uveal melanoma cell lines. This finding is consistent with our previous study of uveal melanomas that were analyzed shortly after plaque radiotherapy; p53 accumulated markedly and remained elevated for several months after plaque radiotherapy.23 We also show that ionizing radiation induces phosphorylation of p53 on Ser15 in all the uveal melanoma cell lines. Phosphorylation at Ser15, which serves as a marker for upstream signaling to p53, stabilizes p53 and allows it to accumulate by inhibiting its interaction with HDM2 (Fig. 4).8 Therefore, upstream signaling to p53 in response to ionizing radiation-induced DNA damage, which involves many proteins such as ATM, Mre11, Rad50, and nibrin,19,24 is likely to be intact in most uveal melanomas. In contrast, we found several downstream defects in the p53 pathway. Even though previous studies have shown that p21 is often expressed in uveal melanomas,25–27 two of our cell lines demonstrated faulty induction of p21 in response to ionizing radiation. Similar defects have been observed in cutaneous melanomas.28 As a potential mechanism for this functional deficit, p53 pathway inhibitors such as HDM2 and Bcl2 are frequently overexpressed in uveal melanomas and may blunt the normal p53 signaling to downstream targets.11,12,29

Deamidation of Bcl-xL recently has been recognized as a critical step in the DNA damage-induced apoptotic response.15 Bcl-xL deamidation can be detected as a change in the mobility of Bcl-xL by Western blot analysis. The precise mechanism of this biochemical event remains unclear, but it appears to involve a change in intracellular pH that occurs in response to DNA damage. Deamidation of Bcl-xL releases proapoptotic BH3 proteins such as Bax to activate the death machinery, and a block in Bcl-xL deamidation would be predicted to inhibit the
Bcl-xL deamidation was not as robust in normal uveal melanoma cell lines and equivocal deamidation in another line, suggesting that this may be a common abnormality in uveal melanomas. Bcl-xL deamidation was not as robust in normal uveal melanocytes as in MCF7 cells or normal fibroblasts (data not shown), suggesting that there may be an intrinsic resistance to apoptosis in uveal melanocytes.

Taken together, our findings suggest that the radioresistance of uveal melanomas is unlikely to be explained by a single genetic defect, but rather, these tumors appear to contain functional defects in various downstream components of the p53 pathway. Four of five uveal melanoma cell lines demonstrated at least one defect in the downstream p53 pathway (Table 1). Of note, we did not find any defects in one cell line (Mel270), indicating that other factors may also be involved in determining the radioresistance of these tumor cells. For example, radioresistant tumors often demonstrate increased efficiency in repair of DNA damage, allowing them to reenter the cell cycle and continue proliferating after a radiation-induced insult. Further work is needed to determine the contribution of this and other mechanisms to the radioresistance of uveal melanomas.

In summary, this study suggests that upstream signaling to p53 is intact in uveal melanomas but that a variety of functional defects in the p53 pathway occur downstream of p53, which may contribute to the radioresistance of these tumors. This information may provide helpful insights into the design of therapeutic approaches to increase the radiosensitivity of uveal melanomas. For example, pharmacologic blockade of p53 pathway inhibitors such as HDM2, Bcl2, and Bcl-xL may render cells more sensitive to apoptosis. Indeed, blocking the activity of these proteins using peptide inhibitors markedly increases the rate of apoptosis in uveal melanomas.

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References