p53 Induction and Apoptosis in Response to Radio- and Chemotherapy in Vivo Is Tumor-Type-dependent

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INTRODUCTION

A number of tissue culture experiments have demonstrated that cellular levels of the tumor suppressor protein p53 are normally low but rapidly accumulate in response to DNA damage, activated oncoproteins, or other physiological stressors (1, 2). The increased p53 levels lead to transcription of p53 target genes, cell cycle arrest, or apoptotic cell death depending on the cell type or context (3). Cells that lack functional p53 fail to undergo these responses, resulting in continued proliferation in the face of genetic damage, subsequent genetic instability, and tumor progression. These attributes of p53 are hypothesized to be central to its tumor-suppressing phenotype (4–7). Implicit in this idea is that the p53 pathway is operative in normal or developing tumor cells in the intact organism. However, with the exception of human skin (8), measurement of p53 accumulation in response to DNA damage in a wide range of human tissues in vivo is impractical and has, to our knowledge, not been done. Instead, the mouse has been used as a model to examine the p53 response pathway in vivo.

Adult mice exposed to whole body ionizing radiation show rapid (2–4 h) p53 accumulation and apoptosis in splenic and thymic lymphocytes, in intestinal crypt cells, in hair follicles, and ependyma (9–12). p53 accumulation, but no associated apoptosis, is observed in subpopulations of cells in the lung, salivary gland, choroid plexus, adrenal gland, kidney, epidermis, and myocardium. Little or no p53 protein is detected in liver, skeletal muscle, and brain. The increased apoptosis in lymphocytes and intestinal crypt cells in response to DNA damage is not seen in p53-deficient mice (9–11, 13). These studies contributed to the idea that DNA damage-induced apoptosis is mediated by p53 and that this property of p53 is central to its role as a tumor suppressor. However, it is also evident that most cells in the mouse accumulate little or no p53 and, further, that not all cells that accumulate p53 undergo apoptosis. The role of p53 as a tumor suppressor in these tissues is less obvious.

Tumor suppression by p53 is also tissue-type-dependent. For example, in mice, p53 has a prominent tumor-suppressing role during lymphomagenesis, sarcomagenesis, and skin carcinogenesis, but has shown little influence during liver or lung tumorigenesis (14–19). At present, the biological basis for this tissue specificity is not known but it may be related to differences in the activity of the p53 pathway.

Tumor cells have a number of genetic and phenotypic alterations, such as activated or overexpressed oncoproteins, abnormal proliferation, hypoxic growth conditions, and altered responses to growth factors, that may alter the activity of the p53 pathway relative to normal progenitor cells (3, 20). Also, p53 mutations are frequently observed at the later stages of tumor progression and, therefore, selective pressure in favor of cells with p53 mutations would be expected to occur within developing tumors rather than in normal tissue. Therefore, we examined one pathway involving p53, accumulation of p53, and apoptosis in response to DNA damage in developing autochthonous tumors.

Another line of investigation has shown that p53 is required for apoptosis and enhances tumor regression in transformed cells in response to radiation and chemotherapeutic agents (6, 21). From this and related studies a widely held view has emerged that cells in human tumors with wild-type p53, treated with anticancer agents, die by apoptosis leading to tumor regression (22, 23). Conversely, tumors that have lost the apoptotic response, for example because of p53 mutation, will be resistant to therapy. However, this assumption has been difficult to test in the clinic, and the role of p53 or apoptosis in the response of human tumors to therapy, especially those of nonhematological origin, is at present, not clear (24, 25).

In light of these issues, we examined p53 induction and apoptosis in response to commonly used genotoxic anticancer agents in six different murine tumor types. With the exception of lymphoid tumors, these were solid tumors of epithelial origin, typical of human malignancies. Importantly, these tumors were not manipulated as cell lines or with exogenous DNA but originated and clonally evolved in situ, and contained “physiological” levels of activated oncoproteins. This provides a realistic model for examining the short-term response of autochthonous tumors to therapeutic intervention.

MATERIALS AND METHODS

To induce tumors at different organ sites, it was necessary to use tissue-specific carcinogens and different inbred strains of mice. Lymphomas were induced in BALB/cJ × C57Bl/6 (CC3) F1 mice with a single treatment of 0.2 mmol of γ radiation (1 Gy, 137Cs). Spontaneous lymphomas from C57Bl/6 mice were also used. Squamous cell papillomas were induced in CC3 F1 mice with a single injection of N,N-diethylnitrosamine (0.2 mmol/kg body weight, i.p.) at 12 days of age. Lung tumors were induced in NIH/Ola F1 mice with a single injection of urethane (1.0 mg/g body weight, i.p.) given to...
Fig. 1. p53 induction and apoptosis in tumors treated with radio- or chemotherapy. Panel 1, lymphomas. A, untreated lymphoma from a wild-type mouse. B, p53 expression in lymphoma from a wild-type mouse 4 h after 4 Gy radiation. C, lymphoma from a wild-type mouse 4 h after 4 Gy radiation. Extensive apoptosis can be seen. D, lymphoma from a p53−/− mouse 4 h after 4 Gy radiation. There is lack of apoptosis. A, C, and D are stained with H&E and B is stained with p53 antibody. ×400. Panel 2, intestinal adenomas. A and B, untreated adenomas from the small intestine of Min/+ mice (no TX). C and D, adenomas from Min/+ mice treated with 4 Gy radiation at 4 h (TX). A and C, p53 staining; B and D, H&E staining. C, arrow, increase in nuclear p53 in tumor cells; arrowhead, no increase in surrounding normal cells. D, arrows, increase in apoptotic cells after radiation. ×400. Panel 3, breast tumors. A and B, untreated breast tumors (no TX). C and D, breast tumors from mice treated with 4 Gy radiation at 4 h (TX). A and C, p55 staining; B and D, H&E staining. C, arrow, increase in nuclear p53 in tumor cells after radiation; D, arrows, increase in apoptotic cells after radiation. ×400. Panel 4, squamous cell papillomas. A-D, serial
12-day-old mice. Mammary tumors were induced by six consecutive treatments of NIH/129 mice with DMBA (1 mg/week, by gavage). Intestinal adenomas arose spontaneously in C57BL/6J min/+ mice, which are predisposed to this tumor type because of a mutation in the Apc gene (27).

Mice that developed lymphomas, intestinal adenomas, mammary tumors, or papillomas were treated when they showed signs of tumor burden, between 12–40 weeks of age. Mice that developed lung or liver tumors were used between 30–50 weeks of age, based on historical data on the rate of tumor appearance at these sites. Two to 48 h prior to sacrifice, tumor-bearing mice were treated with one of the following: whole body γ radiation (4 or 8 Gy, using a 137Cs source at a dose rate of 0.7 Gy/min), etoposide (40 mg/kg, i.p.; Sigma Chemical Co.), doxorubicin (10 mg/kg, i.p.; Sigma Chemical Co.), cisplatin (20 mg/kg; “Platinol AQ”; Bristol Laboratories), or DMSO vehicle control. The dosages used were similar to or higher than those shown to be effective in inducing apoptosis or tumor regression using xenografted cell lines in nude mice (6, 28–30). All of the agents are known to induce DNA damage, p53 accumulation, and apoptosis and are widely used in cancer therapy (31–33).

At 2–48 h after treatment, tumor tissue was dissected free and fixed in 10% formalin for 4–6 h, transferred to 70% methanol, and then processed for paraaffin embedding and sectioning. Serial sections were cut from each sample. One section was stained with H&E for histopathological analysis and enumeration of apoptotic cells. Serial sections were stained with p53 antibody (CM5; Santa Cruz) and counter-stained with methyl green as described previously (34). TUNEL was performed on serial sections as previously described (34). There was a close correlation between the apoptotic index as determined by TUNEL and by morphological criteria in H&E-stained sections. The latter method is the most direct and was used for quantification. The percentage of cells positive for p53 nuclear staining and apoptotic index was determined by examining five 400× microscopic fields per tumor. The apoptotic index was expressed as the number of apoptotic cells per 400× field. All of the samples were examined without knowledge of tumor genotype or treatment. We confirmed the efficacy of agents administered to the tumor-bearing mice by examining intestinal crypt cells for p53 accumulation and apoptosis.

RESULTS

T-Cell Lymphomas. Previous studies showed that normal lymphocytes in mice underwent p53-dependent apoptosis in response to whole body γ irradiation (9, 10). Large-scale apoptosis was also seen in autotumorigenic T-lymphoma cells from irradiated scid/scid mice but not scid/scid p53−/− mice, which indicated that p53 mediated this response (35). However, scid/scid mice are radiosensitive, and this could influence the apoptotic response; therefore, here we examined 12 lymphomas from 12 mice that did not have the scid mutation. p53 was undetectable by immunohistochemistry in lymphoma cells from untreated mice. Nuclear p53 accumulation and apoptosis of the lymphoma cells in thymus, spleen, and lymph nodes was greatly increased at 4 h after 4 Gy (Fig. 1, panel 1; Fig. 2, panel 1). Treatment of lymphoma-bearing mice with etoposide and doxorubicin was also highly effective at inducing apoptosis. Lymphoma cells that were metastatic to the liver, lung, and kidney also underwent extensive apoptosis in response to the irradiation or etoposide treatments. In contrast, this extensive apoptosis was not observed in lymphomas from p53 null mice at 4 (n = 6), 24 (n = 2), or 48 (n = 3) h postradiation (4 Gy; Fig. 1, panel 1 and Fig. 2, panel 1). These results show that p53 is a critical mediator of genotoxic therapy-induced apoptosis in autotumorigenic and metastatic lymphoma cells in treated animals.

Intestinal Adenomas. To determine whether the p53 accumulation-apoptosis pathway was active in intestinal adenomas, we examined Min/+ mutant mice that spontaneously develop this tumor type (27). Fifteen adenomas of the small intestine from eight untreated Min/+ mice were examined. p53 staining was undetectable in five tumors, and, in the remaining tumors, weak nuclear p53 was detected in a significant proportion of tumor cells (Fig. 1, panel 2, and Fig. 2, panel 2). In all 15 adenomas examined from 10 irradiated mice (4 Gy 4 h), more intense nuclear p53 staining was seen, and a greater percentage of tumor cells stained positive (Fig. 1, panel 2, and Fig. 2, panel 2). Apoptotic index was low in untreated adenomas and was increased 4–5-fold by the radiation treatment (Fig. 1, panel 2, and Fig. 2, panel 2).

In a study of similar design, radiation-induced apoptosis was not seen in intestinal adenomas from Min/+ p53−/− mice, which indicated it was p53 dependent (36). In normal murine intestine, the activation of the DNA damage-p53 accumulation-apoptosis pathway is restricted to cells near the bottom of the crypt, which is where the stem and proliferating cells are located (11, 13). Thus, intestinal adenoma cells have a p53 response similar to these cells, but unlike differentiating cells in the upper crypt or villus in which p53 and apoptosis are not induced. This indicates that the adenomas originate from cells near the bottom of the crypt and retain the p53 response pathway. Alternatively, if the adenomas originate from cells in the upper region of the crypt or villus, then during their neoplastic evolution they must have acquired the ability to induce p53 and undergo apoptosis.

Mammary Tumors. Mammary carcinomas were induced in mice by treatment with DMBA. In all three tumors examined from untreated mice, p53 staining was undetectable in tumor cells, and apoptotic index was very low (Fig. 1, panel 3, and Fig. 2, panel 3). In all four tumors examined from irradiated mice, p53 was induced in the tumor cells and apoptotic index was significantly increased (Fig. 1, panel 3, and Fig. 2, panel 3). Thus, exposure of tumor bearing mice to DNA-damaging agents induces p53 and apoptosis in epithelial tumors of the small intestine and breast.

Squamous Cell Papillomas. In 31 of 31 untreated papillomas, p53 protein was constitutively expressed and highly localized to the keratinocyes in the basal layer of the tumor (Fig. 1, panel 4). p53 expression decreased in the suprabasal and terminally differentiating cells of the tumor and was not observed in stomal cells. This highly localized region of p53 expression spatially coincided with Ki67 positive cells, which were also confined to the basal proliferating tumor cells (data not shown). Despite these high levels of p53, apoptotic cells were very infrequently observed (Fig. 1, panel 4, and Fig. 2, panel 4). At 4 (n = 8), 24 (n = 6), or 48 (n = 4) h after 4 Gy irradiation of papilloma-bearing mice, the intensity of p53 staining and the percentage of positive cells increased in the same highly restricted cell layer but was again absent from suprabasal cells (Fig. 1, panel 4). This pattern was also observed in papillomas from etoposide- and doxorubicin-treated mice. Altogether, 38 treated papillomas were examined. In contrast to tumors described above, the apoptotic index was not increased in papillomas from radiation-, etoposide-, or doxorubicin-treated tumors.
DISCUSSION

Tumors could be divided into three classes with respect to their response to DNA-damaging agents. In the first class, cells from T-cell lymphomas and, to a lesser extent, tumors of the mammary gland and small intestine accumulated p53 and underwent apoptosis in response to genotoxic treatments. In the second class, squamous cell papillomas constitutively expressed p53, which was further induced by the treatments but this did not lead to apoptosis. Adenomas of the liver and lung were in a third class that were highly resistant to either p53 induction or apoptosis, which indicated that the p53 pathway was inactive or dormant in these tumor cells. In general, the response of tumor cells closely mirrored that of the normal tissue from which they were derived.

An unresolved issue has been the biological basis for the tissue specificity of tumor suppression by p53, as measured by the tumor spectrum of p53 null mice and the frequency of somatic mutations in p53 in different tumor types. Although we measured only one aspect of p53 function, DNA-damage-induced accumulation of p53 and...
apoptosis, there was a good correlation between this measure of activity and tumor suppression by p53.

p53-/− knockout mice are highly prone to spontaneous lymphoma development, with >70% developing lethal lymphomas by 6 months of age (14, 37, 38), p53 +/− heterozygous mice are also predisposed and frequent loss of the wild-type p53 allele in these tumors indicates there is a strong selective pressure in favor of cells with nonfunctional p53 (15, 35, 39). Somatic mutations in p53 were detected in ~15% of T-cell lymphomas, which were induced by similar agents as in the present study (40). Thus, lymphocytes and lymphoma cells are the most sensitive to p53-mediated apoptosis, and p53 plays a prominent role in lymphoma suppression.

In mice bearing intestinal adenomas, whole body radiation led to the induction of p53 expression and increased apoptosis. Does p53 play a role in intestinal tumorigenesis in mice? Although p53−/− mice are not prone to intestinal adenomas, recent studies, using a controlled genetic background, have revealed that adenomas from Min/+ p53−/− mice were slightly more numerous and tended to be more malignant as compared with those from Min/+ mice (41). Mutations in the p53 gene were detected in 11% of chemically induced colon tumors in mice (42). Therefore, loss of p53 provides a weak but measurable selective advantage during murine intestinal adenoma development.

p53 induction and apoptosis in response to whole body γ irradiation was also observed in mammary tumors. p53-deficient mice have not shown a strong predisposition to spontaneous breast tumor development, but if combined with MMTV-Wnt1 or MMTV-ras transgenic models of mammary cancer, they did show significantly decreased mammary tumor latency and/or increased tumor progression (43, 44). Using a protocol similar to that used in our study, p53 mutations were not observed in DMBA-induced primary mouse mammary tumors (45), but mutations did accrue in preneoplastic outgrowths derived from mammary epithelial cells cultured in vitro. Finally, mammary epithelium transplanted from p53 null mice into wild-type recipients formed tumors at high frequency (46). Thus, p53 does play a tumor-suppressing role in murine breast cancer, but this is contingent on modifying genetic or environmental factors.

Squamous cell papillomas were in a class by themselves, in that p53 was expressed at fairly high levels in untreated tumors and was further induced by DNA-damaging treatments, but apoptosis was not induced. The prominent expression of p53 protein in DMBA/TPA-induced papillomas predicts a tumor-suppressing role for p53 in this tumor model. Mutations in p53 are not observed in DMBA/TPA-induced papillomas, but 20–30% of carcinomas, which develop from papillomas, do have somatic biallelic mutations in p53 (47). Also, in DMBA/TPA-treated p53-deficient mice, the rate and frequency of conversion of papillomas to carcinomas were greatly increased (16). This indicates that p53 expression in papillomas has a potent tumor-suppressing phenotype, which is to inhibit malignant progression. However, the lack of a p53-dependent apoptotic response in papillomas suggests that other functions of p53 besides apoptosis, such as a role in G1 cell cycle arrest or differentiation, may be more critical for the suppression of malignant progression by p53.

Adenomas of the lung or liver were markedly resistant to both the induction of p53 and apoptosis by DNA-damaging treatments, which mirrors that of normal parenchymal cells from these tissues (12, 48). Thus, increased proliferation per se or oncogenic mutations associated with lung and liver adenoma formation are not sufficient to de-repress the p53 induction pathway. Adenomas of the lung and liver are among the most frequently observed tumor type in mice and, despite extensive analysis by several groups, mutations in p53 have rarely been found (18, 19, 49). Furthermore, p53-deficient mice have not shown predisposition to either tumor type, even after tumor initiation with chemicals or radiation (15, 17, 39). We suggest that the apparent lack of a tumor-suppressing role for p53 in these tumor types may be attributable to an inherent inability to induce p53 in the developing tumor cells, rendering p53 phenotypically silent. In support of this idea, when murine liver tumor cells were placed in tissue culture, they regained the ability to induce p53 expression, and cell lines established from these cultures frequently developed p53 mutations (18, 50).

In summary, the activity of the p53 induction-apoptosis pathway varies widely between tissues and tumor types when assayed in vivo. Variation in this activity roughly correlates with the known tissue specificity of tumor suppression by p53. In tissues or developing tumors in which p53 is inducible, such as lymphoid or skin, there is significant selective pressure in favor of cells with mutant p53. For tumors such as those of the mammary gland or intestine, tumor suppression by p53 may be more context-dependent, e.g., it may be latent under normal conditions but revealed under different genetic backgrounds or environmental conditions. In tissues or developing tumors in which the p53 pathway is less active or inactive, such as lung or liver tumors, there may be less selective pressure in favor of cells with mutant p53. Although the generality of this model remains to be demonstrated, a prediction is that tumors developing from tissues in which p53 is not inducible would rarely select for cells with mutant p53. Conversely, activation of the p53 pathway, which might occur because of genetic or physiological changes during tumor progression or because of environmental factors, would result in increased selective pressure in favor of cells with mutant p53.

With regard to the apoptotic response of tumor cells to genotoxic therapy, our results indicate striking variation between different tumor types. Lymphomas showed the strongest apoptotic response, which was p53 dependent. Mammary and intestinal tumors had an intermediate apoptotic response; the latter was also shown to be p53 dependent (36). However, tumors of the skin, lung, and liver showed little or no therapy-induced apoptosis. Thus, the tenet that p53 controls the apoptotic sensitivity of tumors to chemotherapy is not universally applicable to all tumor types. We did not measure the long-term response of tumors to therapy. Given our results, it is likely that the role of p53 and apoptosis in tumor regression may also be tumor-type-dependent: prominent in lymphomas but less so in other tumor types. It remains to be shown whether therapy-induced p53 activation and apoptosis vary between autochthonous human tumor types as well, and if this has bearing on the response of these tumors to therapy.

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