Inhibition of p53 Response in Tumor Stroma Improves Efficacy of Anticancer Treatment by Increasing Antiangiogenic Effects of Chemotherapy and Radiotherapy in Mice

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Abstract

Inactivation of p53 function, which frequently occurs in tumors, can significantly modulate tumor cell sensitivity to radiation and chemotherapeutic drugs. However, in addition to acting on malignant cells, anticancer agents act on the cells of tumor stroma, causing activation of a p53 response. The effect of this response on treatment outcome has been the subject of the present study. Tumors with p53-deficient stroma were generated using mouse tumorigenic packaging cells that produce a p53 inhibitory retrovirus, encoding a dominant-negative p53 mutant. Tumors maintaining wild-type p53 in their stroma were formed by cells of similar origin but deficient in retroviral production due to the deletion of the packaging signal in the retroviral vector. Comparison of these tumor models, differing only in p53 status of their stromas, showed that tumors with p53-deficient stroma were significantly more sensitive to experimental chemotherapy and radiotherapy. A similar effect was achieved when anticancer treatment was combined with pharmacologic suppression of p53 by the cyclic form of pifithrin α, a small-molecule inhibitor of p53. Potentiation of the anticancer effect of chemotherapy and radiotherapy by p53 suppression in the tumor stroma is likely to be due to the increased sensitivity of p53-deficient endothelium to genotoxic stress as shown both in cell culture and in experimental tumors. Thus, reversible pharmacologic suppression of p53 may be a viable approach to improving anticancer treatment via an enhanced antiangiogenic effect of chemotherapy and radiotherapy. (Cancer Res 2006; 66(19): 9356-61)

Introduction

Stromal elements (fibroblasts, infiltrating immunocytes, and endothelium) play multiple roles in tumor growth and progression (1, 2) and may also significantly contribute to tumor response to anticancer treatment by radiation and anticancer drugs. Thus, tumors transplanted into acid sphingomyelinase-deficient or Bax-deficient mice, which have microvasculature resistant to apoptosis, are more resistant to radiation compared with those grown in wild-type (WT) mice (3). Damage to vascular endothelium was also shown to be the primary cause of radiosensitivity of small intestine in mice (4).

p53 is a key mediator of cell response to a variety of stresses that determines whether cells enter growth arrest or apoptosis after DNA damage caused by radiation or drugs. For a long time, p53 was predominantly considered as a treatment sensitivity factor. Indeed, loss of p53 in tumors is associated with an unfavorable prognosis in many forms of cancer (5–7). By promoting apoptosis, WT p53 can determine high sensitivity of experimental tumors to anticancer treatment, including antiangiogenic therapies (8, 9). However, if apoptosis is suppressed, which frequently occurs in tumor cells, the remaining functions of p53 (e.g., growth arrest at cell cycle checkpoints and modulation of DNA repair) can contribute to cell survival during anticancer treatment (10).

Browder et al. (11) reported that chemotherapy applied in the form of an “antiangiogenic schedule” was more effective in p53-null than in p53 WT tumor-bearing mice, suggesting that p53 can play a protective role in tumor endothelium under conditions of genotoxic stress. Although the exact mechanism of this phenomenon is not understood, it might be similar to the p53-mediated protection of the epithelium of the small intestine from γ irradiation (4), where p53 plays the role of a survival factor by allowing cells to reside in a growth arrested state, thereby reducing the risk of mitotic catastrophe (12). In the present work, we explored the therapeutic opportunities opened up by these observations and showed (a) the importance of the p53 status of tumor stroma in tumor response to radiation and chemotherapeutic drugs and (b) the feasibility of a new therapeutic approach that is based on the treatment of experimental mouse tumors with radiation or chemotherapy combined with pharmacologic inhibition of p53 by a small-molecule pifithrin α (PFTα; ref. 13).

Materials and Methods

Mice. Eight- to 10-week-old C57BL/6j mice and NIH Swiss mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and Harlan (Indianapolis, IN), respectively.

Cells and tumor cell models. The mouse Lewis lung carcinoma (LLC) cells and ectopic packaging cell line GP+E86-NIH-3T3 (further named as Eco-3T3) and C8 cells (mouse embryo fibroblasts transformed with E1a+ras) were cultured in DMEM with 10% fetal bovine serum (FBS). Eco-3T3 cells were transduced with the pLXSN-based vectors either with or without packaging signal (ΔΨ) that express GSE56 or mitochondria-targeted enhanced green fluorescent protein followed by G418 selection. Mouse aortic endothelial cells (MAEC) were isolated from WT C57BL/6j or p53-null mice as described previously (14) and maintained in MCDB/12 medium (Sigma, St. Louis, MO) containing 15% FBS, 0.009% heparin, and 0.015% endothelial cell growth supplement and used between passages 3 and 6.
Treatment of mouse tumors. NIH Swiss mice were injected s.c. with 3.5 × 10⁴ Eco-3T3 cells (virus producing, GSE56; nonproducing, ΔΨ-GSE56). After tumors reached 3 to 5 mm in diameter, animals were treated i.p. thrice, with 48-hour intervals, with 150 mg/kg cyclophosphamide or irradiated five times daily with 2 Gy (total, 10 Gy) using J. L. Shepherd (San Fernando, CA) irradiator (137Cs) at a dose rate of 2.5 Gy/min with or without cyclic form of PFTα (50 mg/kg, i.p.; Calbiochem, San Diego, CA) injected 1 hour before irradiation. LLC cells were injected s.c. in syngeneic C57Bl/6 mice. After tumors reached 3 to 5 mm in diameter, mice received cyclophosphamide (170 mg/kg, six i.p. injections with 48-hour intervals) with or without cyclic PFTα (100 mg/kg, i.p.). Cyclic form of PFTα is significantly less toxic, though less water soluble, than original PFTα. It was stored frozen as 10 mmol/L DMSO solution. So high dose was used to increase the duration of PFTα presence in circulation because after i.p. injection it formed a colloid mass in peritoneum that was gradually dissolved (up to 4 hours), thus creating the effect of a slow release. Tumor volumes were measured every 2nd day and calculated by the following formula: \( V = \frac{4}{3} \pi \times \left( r_1^3 - r_2^3 \right) \), where \( r_1 < r_2 \).

**Western blot analysis.** The levels of p53 and GSE56 expression in Eco-3T3 and C8 cells were verified by Western blot analysis with mouse monoclonal anti-p53 Ab-1 antibody (BD Biosciences Pharmingen, San Diego, CA).

**Cytotoxicity assays.** Cells seeded in 96-well plates (10⁴ per well) were treated with doxorubicin for 48 to 72 hours. MAECs from WT or p53 knockout mice were treated in suspension with indicated doses of irradiation and then plated. Forty-eight hours later, the cells were fixed by methanol and stained with 0.5% methylene blue.

**Immunohistochemistry.** The GSE56 and ΔΨ-GSE56 tumors (5 mm in diameter) were isolated and either frozen in OCT (Andwin Scientific, Addison, IL) or fixed in 10% zinc/formalin. Blood vessels in tumors were visualized by routine immunohistochemical staining using the rat anti-CD31 antibody (BD Pharmingen). Apoptotic cells in tumors were detected in paraffin-embedded sections with the ApopTag Plus Peroxidase In situ Apoptosis Detection kit according to the manufacturer’s protocol (Chemicon, Temecula, CA).

**Results**

PFTα potentiates the antitumor effect of cyclophosphamide. The effect of pharmacologic inhibition of p53 on tumor sensitivity to anticancer chemotherapy was tested in LLC cells. These cells were previously used to show the dependence of tumor sensitivity to anticancer chemotherapy was tested in LLC cells. These cells were previously used to show the dependence of tumor sensitivity on the p53 status of the host (11). C57Bl6 mice bearing s.c. growing syngeneic LLC cells were treated with cyclophosphamide with or without PFTα (Fig. 1). Although PFTα injection alone did not cause any effect on tumor growth, it greatly potentiated the antitumor effect of cyclophosphamide, resulting in complete regression of tumors. Because LLC cells have mutated p53 (15), the higher sensitivity of the tumors to drug treatment could not be explained by inhibition of p53 in the tumor cells themselves but is rather likely due to the effect of the p53 inhibitor on stromal cells.

However, the value of these encouraging results was limited due to hepatotoxicity observed in these mice (data not shown). The apparent synergistic hepatotoxicity of cyclophosphamide and PFTα was further complicated by the necessity to inject large doses of PFTα to ensure a sufficiently long-lasting p53 inhibitory effect that would be comparable with the pharmacokinetics of cyclophosphamide. We therefore switched to experimental radiotherapy, which requires significantly lower doses of PFTα with no associated hepatotoxicity. However, the high radio resistance of LLC tumors required the development of an alternative tumor model.

**Experimental tumor model capable of suppressing p53 in stroma.** To compare the treatment sensitivity of tumors differing in the p53 status of the stroma, we created a tumor cell model consisting of retrovirus-producing tumorigenic packaging cells (Eco-3T3) that could convert their own stroma into p53-deficient stroma by producing retrovirus capable of effectively transducing neighboring cells with the p53 inhibitor GSE56. GSE56 encodes the COOH-terminal fragment of rat p53, which has a strong dominant-negative activity caused by stabilization of WT p53 in an inactive conformation (Fig. 2; ref. 16). As a control, similar packaging cells were transduced with a modified retroviral construct that lack the packaging signal (ΔΨ) and therefore are incapable of virus production.

The levels of GSE56 expression were found to be similar between virus-producing and nonproducing populations (GSE56 and ΔΨ-GSE56 cells, respectively; Fig. 2B). The ability of the created virus-producing cells to transmit GSE56 was confirmed by Western blot analysis of virus-infected C8 cells. These cells begin to express GSE56 only when treated with conditioned medium from Eco-3T3-GSE56 but not from Eco-3T3-ΔΨ-GSE56 cells. As expected, the expression of GSE56 in recipient C8 cells correlated with the stabilization of WT p53 in an inactive conformation (16).

A similar pair of virus-producing and nonproducing cells was created using constructs expressing mitochondria-targeted enhanced yellow fluorescent protein (EYFP; Fig. 2C, top). Virus produced by tumor cells growing in vivo effectively transduced the transgene into host stromal components, including tumor endothelium, as determined by coexpression of EYFP and the
Figure 2. A, experimental design and model. Tumorigenic variant of GP+E86-NIH-3T3-based ecotropic packaging cells (Eco-3T3 cells) was transfected with either of two versions of retroviral vector pL(GSE56)SN capable or incapable of packaging virus. After G418 selection, the cells were checked for GSE56 expression and drug sensitivity (doxorubicin) and injected s.c. to induce tumors. One kind of tumors had host stroma cells with inhibited p53 by virus transduction of GSE56 gene, and the other tumors contained WT stromal cells. After tumors reached ~5 mm in diameter, mice received cyclophosphamide or fractioned γ irradiation and the dynamics of tumor growth was monitored in comparison of these two kinds of tumors. B, characterization of the created cell lines for the expression of GSE56 in transfected Eco-3T3 cells and their capability (GSE56) or incapability (∆Ψ-GSE56) to transduce virus with conditioned medium to recipient C8 cells was determined in Western blot analysis using anti-p53 antibodies recognizing also GSE56 fragment. C, similar cells were created using constructs expressing mitochondria-targeted EYFP instead of GSE56. Fluorescent microscopy analysis of s.c. tumors formed by these cells revealed the difference in fluorescence of stromal components (middle, arrows), indicating that virus-producing tumor cells can effectively transduce the transgene into the host stroma cells. Bottom, immunofluorescent staining of tumor sections formed by EYFP-expressing tumor cells by antibodies against mouse endothelial marker CD31 confirmed effective transfer of the EYFP-expressing transgene into endothelial cells by virus-producing (EYFP) but not with non-virus-producing cells (∆Ψ-EYFP). Blue arrows, tumor cells; yellow arrows, endothelial cells. D, sensitivity of the original cells, cells transfected with empty vector (LXSN), GSE56, and ∆Ψ-GSE56 to doxorubicin in vitro was estimated by methylene blue staining of the cells survived after 48 hours of incubation with four different doses of doxorubicin.
CD31 endothelial marker (Fig. 2C, middle). There was no EYFP expression detected in the endothelium of tumors formed by the cells transduced with retroviral vector lacking the packaging signal as judged by immunofluorescent staining of tumor sections by antibodies against mouse endothelium marker CD31 (Fig. 2C, bottom). All created cell lines showed similar growth rates in vitro regardless of virus production or GSE56 expression (data not shown). Drug assays showed that both cells producing and nonproducing GSE56 virus were equally sensitive to treatment with several chemotherapeutic drugs in vitro (results with doxorubicin are shown in Fig. 2D).

Dependence of tumor chemosensitivity and radiosensitivity on the p53 status of stroma. There were no detectable differences in the growth rate of tumors formed by insert-free vector-transduced cells, GSE56, and ΔΨ-GSE56 cells regardless of whether they produced virus or not (Fig. 3A). However, tumors formed by the cells that released the p53-inhibiting GSE56 virus showed a much stronger response to cyclophosphamide treatment as shown by the complete disappearance of the majority of tumors (Fig. 3B). Thus, the created model, similar to LLC, showed elevated sensitivity of tumors with p53-deficient stroma to treatment with cyclophosphamide. Importantly, this phenomenon was shown in p53 WT mice and therefore cannot be attributed to systemic physiologic differences in WT and p53-null mice used in experiments with LLC cells (11).

For comparison of radiation response of tumors differing in the p53 status of their stroma, we used fractionated total body irradiation (2 Gy, five times daily) of mice with ΔΨ-GSE56 as well as GSE56 tumors producing GSE56-expressing virus, with or without injection of PFTα (Fig. 3C). Again, there were no differences in the growth rates of virus-producing and non-virus-producing tumors, and injections of PFTα had no effect on tumor growth (Fig. 3C, shown only for untreated group of ΔΨ-GSE56 tumors). In irradiated animals, however, GSE56 virus-producing tumors showed significant size reduction or even complete disappearance with no relapse within the period of observation (3–4 weeks), whereas ΔΨ-GSE56 tumors responded only with a slight decline in growth rate (Fig. 3C and D). Importantly, PFTα in combination with radiation strongly sensitized ΔΨ-GSE56 tumors to radiation, making them as responsive as virus-producing tumors.

Antiangiogenic effect of pharmacologic and genetic suppression of p53 in stroma of irradiated tumors. Analysis of tumors 3 days after 10 Gy of total body irradiation revealed significantly more apoptotic cells (both tumor and stromal cell subpopulations) in both GSE56 and ΔΨ-GSE56 tumors treated with PFTα compared with the tumors with intact p53 in the stroma (Fig. 4A). This observation was consistent with the stronger response of these tumors to treatment with radiation or cyclophosphamide (Fig. 3). Because both virus-producing (GSE56) and nonproducing (ΔΨ-GSE56) tumor cells were equally sensitive to DNA-damaging treatment in vitro (Fig. 2D), we assumed that higher sensitivity of p53 inhibitory virus-producing tumors in vivo could be due to p53 inhibition of their stromal components presumably in endothelial cells.

To directly test the role of p53 in endothelial cell sensitivity to genotoxic stress, we established primary cultures of mouse endothelial cells from aortas of WT and p53-null mice and tested their radiosensitivity in culture by comparing their growth
following 10 to 25 Gy of γ radiation. Whereas p53 WT cultures underwent growth arrest with no traces of cell loss within the period of observation (72 hours), p53-null cells showed significant dose-dependent cell death within the range of radiation doses applied (Fig. 4B). Consistently, tumor vascularization was strongly affected in tumors with pharmacologic or genetic inhibition of p53 in contrast to control tumors. Dramatic differences were observed in both size (Fig. 4C) and number (Fig. 4D) of blood vessels. All the above observations are consistent with the hypothesis that the strong sensitization of p53-deficient endothelial cells to genotoxic stress results in the stronger antitumor effect of radiotherapy and chemotherapy.

**Discussion**

p53 alterations have been linked to tumor cell resistance to radiotherapy and chemotherapy in a variety of cancers (ref. 17, reviewed in ref. 5). In hematologic malignancies, p53 mutations are associated with resistance to chemotherapy (5, 18). In breast cancer, p53 mutations have been linked to resistance to tamoxifen, doxorubicin, and radiotherapy (5, 7), whereas similar mutations in ovarian cancer were associated with resistance to platinum-based chemotherapy (19). However, clinical data are also emerging to support a reverse correlation between p53 mutations and drug resistance of some tumors (5). Our recent work has shown that WT p53 function can be associated with drug resistance in melanomas.\(^5\) This apparent controversy reflects the multifunctional nature of p53 (e.g., p53-dependent apoptosis, p53-induced senescence, p53-dependent temporary growth arrest, and p53-mediated DNA repair). The overall effect of treatment will be determined by the prevailing function of p53 in a particular tumor. Thus, in the absence of apoptosis, p53 frequently becomes a survival factor causing growth arrest at cell cycle checkpoints that are essential for completion of repair and safe entrance of mitosis (12). All this explains why p53 does not have one defined function in cancer susceptibility to treatment and indicates that the view on p53 as an anticancer treatment target ultimately depends on the tumor type.

In the present work, we have added one more degree of complexity to understanding the role of p53 in cancer treatment by showing that both genetic (retroviral transduction of a dominant-negative mutant) and pharmacologic (PFTα) repression of p53 in the stroma strongly sensitizes p53-deficient tumors to experimental radiotherapy and chemotherapy. Although we have not analyzed the exact nature of tumor cell death in vivo, this effect is most likely attributed to the high sensitivity of tumor vascular endothelium to

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\(^5\) Khanduri and Gudkov, unpublished observation.
genotoxic stress under conditions of p53 suppression followed by dramatic reduction of vital blood supply for the tumor. The mechanism of this sensitization remains to be characterized. It may involve mitotic catastrophe (20), which affects only proliferating cell populations and may explain why inhibition of p53 does not cause collapse of vascular endothelia in normal mouse tissues to the same extent as it does in the tumor. The tumor specificity of the chemosensitizing and radiosensitizing effects of PFTα shown in this study suggests that p53 inhibition is a plausible approach for improving the efficacy of anticancer chemotherapy and radiotherapy in the clinic. Such a strategy is applicable to those tumors that would not benefit from p53 suppression, including tumors with mutant p53, a p53 pathway suppressed by other mechanisms and tumors in which p53 acts as a treatment resistance factor.

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