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ORIGINAL PAPERS

Dual effect of p53 on radiation sensitivity *in vivo*: p53 promotes hematopoietic injury, but protects from gastro-intestinal syndrome in mice

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Ionizing radiation (IR) induces p53-dependent apoptosis in radiosensitive tissues, suggesting that p53 is a determinant of radiation syndromes. In fact, p53-deficient mice survive doses of IR that cause lethal hematopoietic syndrome in wild-type animals. Surprisingly, p53 deficiency results in sensitization of mice to higher doses of IR, causing lethal gastro-intestinal (GI) syndrome. While cells in the crypts of p53-wild-type epithelium undergo prolonged growth arrest after irradiation, continuous cell proliferation ongoing in p53-deficient epithelium correlates with accelerated death of damaged cells followed by rapid destruction of villi and accelerated lethality. p21-deficient mice are also characterized by increased sensitivity to GI syndrome-inducing doses of IR. We conclude that p53/p21-mediated growth arrest plays a protective role in the epithelium of small intestine after severe doses of IR. Pharmacological inhibition of p53 by a small molecule that can rescue from lethal hematopoietic syndrome has no effect on the lethality from gastro-intestinal syndrome, presumably because of a temporary and reversible nature of its action.
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Introduction

The primary cause of death from ionizing radiation (IR) depends on the radiation dose. At doses of up to 9–10 Gy, mice die 12–20 days later, primarily from lethal bone marrow depletion known as hematopoietic (HP) syndrome. At this dose, irradiated mice can be rescued from lethality by bone marrow transplantation. Animals that received >15 Gy die between 7 and 12 days after treatment (before hematopoietic syndrome could kill them) from complications of damage to the small intestine – gastrointestinal (GI) syndrome (Gudkov and Komarova, 2003). In both cases of HP and GI syndromes, lethal damage of tissues starts from massive p53-dependent apoptosis (Potten, 1992; Merritt *et al*., 1994; Potten *et al*., 1994; Cui *et al*., 1995), suggesting that p53 could be a determinant of radiation-induced death. Consistently, p53-deficient mice are resistant to doses of radiation that kill through HP syndrome (Westphal *et al*., 1997; Komarov *et al*., 1999), and lethality of wild-type animals receiving 6–11 Gy of gamma radiation can be reduced by temporary pharmacological inhibition of p53 by small molecules (Komarov *et al*., 1999). Definition of p53 as a factor sensitizing tissues to genotoxic stress was further strengthened by demonstrating the p53 dependence of hair loss (alopecia) occurring as a result of experimental chemotherapy or radiation (Botchkarev *et al*., 2000). Hence, based on previous observations, one could expect that p53 continues to play an important role in the development of lethal GI syndrome after higher doses of IR.

The dynamics of cell population in small intestine has been analysed in great detail. Cell proliferation in epithelia of the guts is limited to the crypts where stem cells and early proliferating progenitors are located. After a couple of cell divisions, already differentiated descendants of crypt stem cells move up the villi to be shed at the villar tip. In the small intestine of the mouse, the entire 'trip' of the cell – from the proliferative compartment to the tip of the villus – normally takes between 3 and 5 days (Potten, 1992; Potten *et al*., 1997; Booth *et al*., 2002; Somosy *et al*., 2002). Although the reaction of small intestine to gamma radiation has been well examined at a pathomorphological level, it still remains unclear as to what is the exact cause of GI lethality, including the primary event. Death may occur as a direct consequence of the damage of epithelial crypt cells and followed denudation of villi leading to fluid and electrolyte imbalance, bacteremia and endotoxemia. Besides inflammation and stromal responses, endothelial dysfunctions seem to be the important factors contributing to lethality (Potten *et al*., 1997; Somosy *et al*., 2002).

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As in the case of the hematopoietic system, the proportion of apoptosis in crypts of small intestine was significantly higher in wild type than in p53-deficient mice during the first hours after IR (Merritt *et al*., 1994, 1997; Komarova *et al*., 2000). However, unlike what happens in bone marrow, this apoptosis does not correlate with the survival rate of clonogenic cells responsible for the recovery of epithelial cells of the intestine and was found to be independent of their p53 status (Hendry *et al*., 1997). Furthermore, delayed (more than 24 h after irradiation) apoptosis is observed in the crypts of p53-deficient mice, the scale of which is dramatically increased with the dose of IR and is presumably a consequence of aberrant mitosis (mitotic catastrophe) because of improper chromosome segregation (Watson and Pritchard, 2000). However, it remained unclear as to what is the exact impact of these processes in mouse lethality from IR-induced GI syndrome.

In this work, we have directly addressed the role of p53 in sensitivity of mice to high doses of radiation that kill through a GI syndrome. To our surprise, the p53 status of mice had the opposite effect on lethality from lower and higher doses of radiation, suggesting a new role for p53 in lethal IR-induced GI syndrome. The results we obtained suggest that, in p53-deficient mice, continuous proliferation of crypt cells after IR facilitates their entry into a mitotic catastrophe and fast death in contrast to wild-type mice, in which p53 prolongs survival of epithelium of the small intestine by arresting proliferation of crypt cells after severe IR. This indicates that two p53 functions – growth arrest and apoptosis – have the opposite effect in determining radiation-induced injury and their tissue-specific balance can be a determinant of a final outcome.

Results

p53 deficiency has an opposite effect on mouse sensitivity to low and high doses of IR

We compared the survival of p53-wild-type and p53-knockout mice (both on the C57Bl/6 background) after treatment with different doses of total body gamma radiation: 8, 10, 12.5, 15, and 25 Gy. In accordance with previous observations (Westphal *et al*., 1997; Komarov *et al*., 1999), p53-deficient mice showed a higher survival rate than wild-type mice at doses below 10 Gy that are known to cause death by inducing HP syndrome. However, at higher doses (above 12.5 Gy), p53-null mice became more sensitive to IR and died significantly earlier than wild-type animals (Figure 1a). Since it is known that lethality of mice at these doses of IR predominantly results from the injury to GI, these observations suggested that p53-deficient mice are more susceptible to the radiation-induced GI syndrome.

Development of radiation-induced GI syndrome is a complex process that involves the interaction of different cell types. Inflammation was previously suggested to play a role in the GI tract injury by radiation (Potten, 1990; Somosy *et al*., 2002), raising the possibility of involvement of cells of hematopoietic lineage in determining increased sensitivity of p53-deficient mice to GI syndrome. To estimate a role of inflammatory response in the development of different sensitivity in p53-wild-type and p53-null mice to high doses of IR, we created chimeric mice by eliminating the wild-type hematopoietic system with lethal gamma irradiation (11 Gy), followed by its reconstitution with transplanted p53-deficient bone marrow. Control group of lethally irradiated mice received wild-type bone

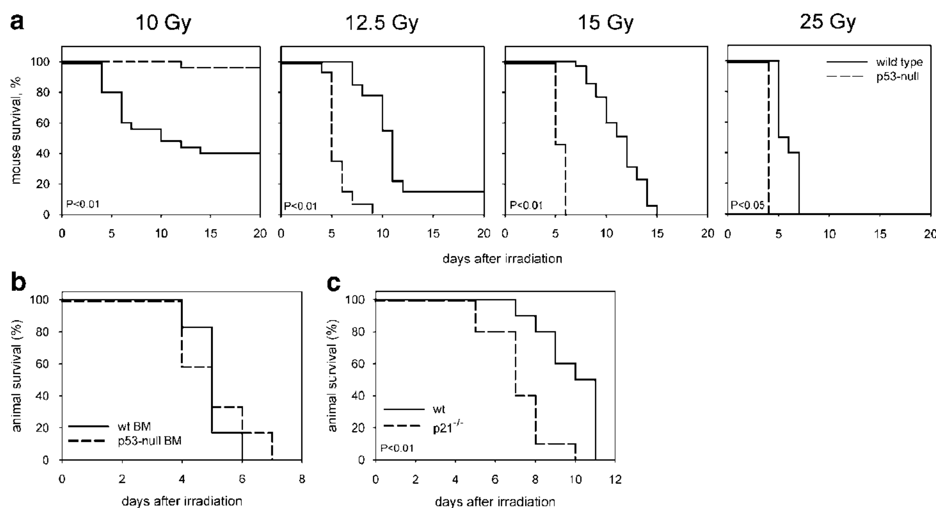

Figure 1 p53 deficiency has opposite effects on mouse survival after exposure to different doses of gamma radiation. (**a**) Mouse survival of wt p53 and p53-null mice (13–35 mice of wt p53 C57Bl/6 or p53-null C57Bl/6 background mice in each group) treated with different doses of IR. The effect of p53 gene on mouse survival after increasing the doses of IR was the opposite: wt p53 mice were more sensitive to IR with doses <12.5 Gy and more resistant to IR with doses >12.5 Gy compared to p53-null mice. *P*-values were calculated using the χ test (Microsoft Excel). (**b**) No difference in the survival of chimeric mice, created by eliminating the wild-type hematopoietic system with lethal IR followed by its reconstitution with transplanted p53-null bone marrow, and control group of lethally irradiated mice that received wild-type bone marrow was found after 15 Gy of IR. (**c**) Accelerated death of p21-null mice treated with 15 Gy of whole-body gamma irradiation

marrow and both groups were treated with 15 Gy of gamma radiation 1 month after bone marrow transplantation. The dynamics of death was similar in both groups of mice (Figure 1b), indicating no impact of cells of hematopoietic origin on the increased sensitivity of the guts of p53-deficient mice and their accelerated death from high doses of gamma radiation.

Cyclin-dependent kinase inhibitor p21, encoded by p53 responsive *p21/waf1/cip1* gene, is known to be a major mediator of p53-regulated checkpoint control. Induction of growth arrest by p53 in many types of cells, including the epithelium of small intestine, involves activation of p21. If the lack of growth arrest is indeed the cause of accelerated death of p53-null mice from IR-induced GI syndrome, one can expect a similar phenotype from *p21*-null mice. We compared the kinetics of death of *p21*-null mice after exposure to 15 and 11 Gy of gamma radiation inducing lethal GI and HP syndromes in wild-type mice, respectively. No differences were found between wild-type and *p21*-knockout mice after lower HP-inducing dose of IR; however, *p21*-null animals showed accelerated development of lethal GI syndrome after 15 Gy of IR (Figure 1c). This observation suggests that the protective role of p53 in IR-induced GI syndrome is determined by its growth arrest rather than apoptotic function.

Acceleration of radiation-induced injury in the small intestine of p53-deficient mice

We compared the morphology of epithelia of different divisions of the guts of p53-wild-type and p53-deficient mice at different time points (8, 24, 48, 56, 72, and 96 h) after 15 Gy of IR and found dramatic differences in the dynamic of injury in jejunum and ileum between the mice of two compared genotypes (Figure 2a). In accordance with previous observations (Merritt *et al*., 1994, 1997; Komarova *et al*., 2000), IR-induced injury was first detected in the crypts of wild-type mice, as judged by the appearance of a large number of apoptotic cells not seen in p53-null epithelium, at the first two post-radiation time points analysed (8, 24 h) (Figure 2b). However, 48–72 h after IR, the situation starts changing to the opposite with rapid accumulation of signs of damage in p53-deficient epithelia. Although we did not find any differences in the amount of villi in wild-type and p53-null mice at this time, a progressive reduction in the length of villi was registered in p53-null mice, accompanied by dramatic changes in the proportion of epithelial and connective tissue cells (Figure 2a).

A decrease in the amount of crypts was observed in mice of both genotypes at the first 3 days after treatment. However, on day 4 after IR, the picture became very different in mice of two genotypes: the majority of crypts disappeared in p53-null mice, while regenerating crypts became detectable in wild-type mice (Figure 2a). Disappearance of crypts in p53-null mice was accompanied with the loss of integrity of the villus epithelium, which reached a complete

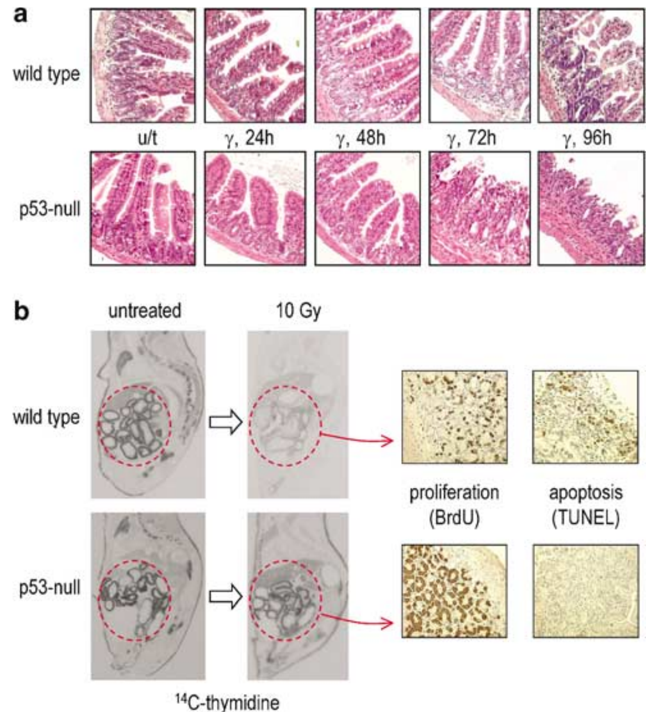

Figure 2 Differences in the radiation response of the epithelium of small intestine in wild-type and p53-null mice. (**a**) Comparison of the dynamics of injury in hematoxylin- and eosin-stained paraffin sections of wt p53 and p53-null small intestines after 15 Gy of IR (magnification: ×125). Acceleration of epithelial damage was observed in p53-null mice, reached at 96 h after IR a complete disappearance of crypts and dramatically shortening of villi. (**b**) Comparison of the levels of proliferation and apoptosis in the intestines of wt p53 and p53-null mice after IR. (Left) Autoradiographs of whole-body sections (×1.7 magnification) of 4-week-old wt p53 and p53-null mice injected intraperitoneally with C14-thymidine (10 μCi/animal) treated or untreated with 15 Gy of IR are shown. Arrows point to the mouse intestines. (Right) Higher level of proliferation (BrdU incorporation) and lower level of apoptosis (TUNEL staining) was found in the small intestine of p53-null compared with wt p53 mice 8–24 h after IR

destruction to the 96th hour time point followed by death within the next 24 h, with the signs of severe GI syndrome.

No visible IR-induced injury was detected in the large intestine of mice at the tested time points (data not shown), confirming previous observations of high radiation resistance of this compartment of the digestive tract (Cai *et al*., 1997).

Continuous proliferation correlates with rapid death of cells in the crypts of p53-deficient small intestine

There are dramatic differences observed in the proliferation rates of the epithelia of GI tract between wild-type and p53-null mice that are detected by monitoring DNA replication using ^{14}C-thymidine- and BrdU-incorporation assays (Figures 2b and 3a). While wild-type epithelium blocks proliferation as early as 8 h after radiation, in p53-null mice DNA replication continues at the same or even slightly higher rate. However, to the 96th hour, the situation changed

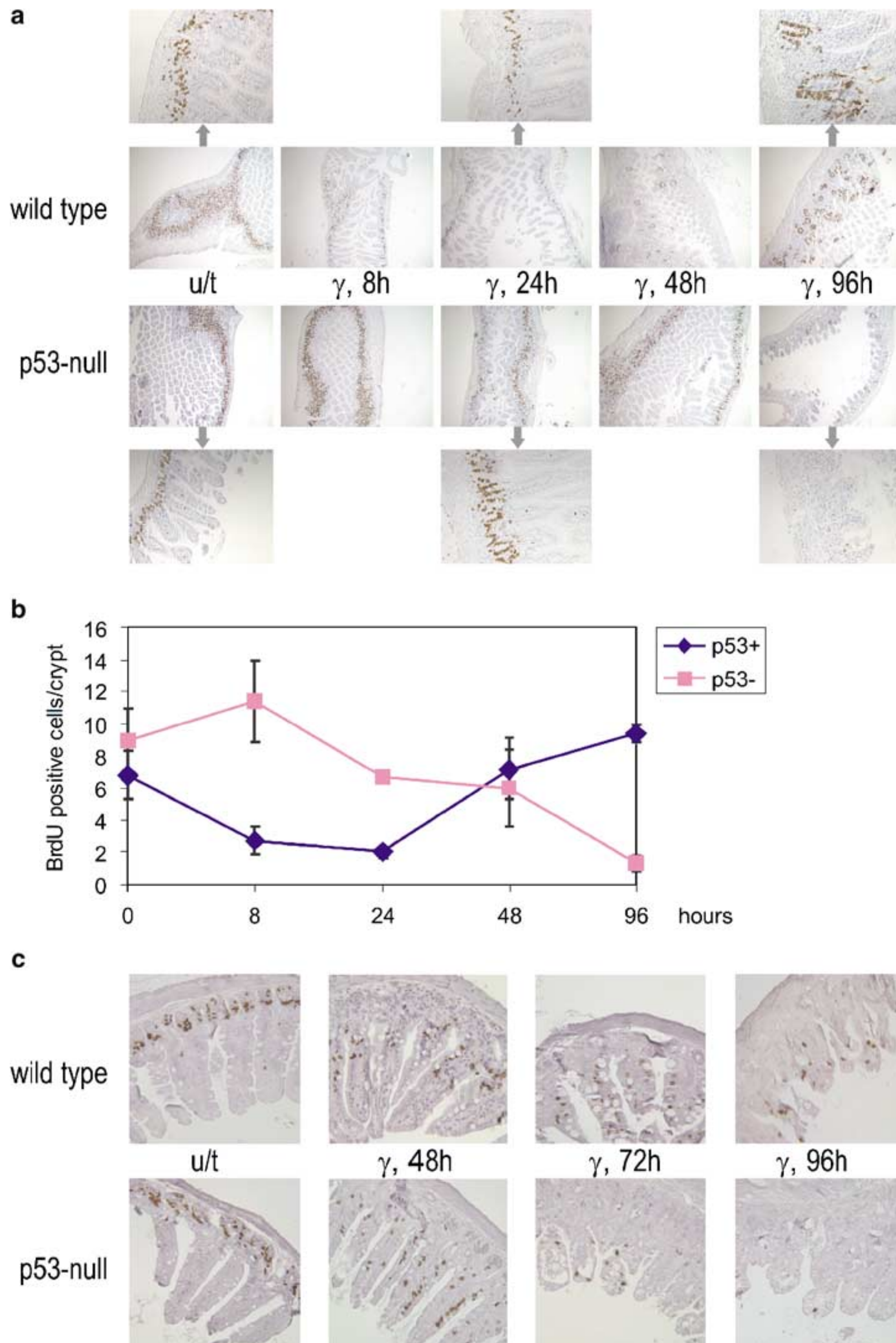

Figure 3 Cell proliferation in the epithelium of small intestine of wild-type and p53-null mice after 15 Gy of gamma radiation. (**a**) Comparison of BrdU incorporation in the small intestine of p53 wt and p53-null mice at different time points after 15 Gy of IR. BrdU injections (50 mg/kg) were done 2 h before killing mice at different time points. The increased level of proliferation observed at the first hours after IR in p53-null mice correlates with full disappearance of crypts and BrdU incorporation 96 h after IR in them. BrdU incorporation for 0, 24, and 96 h after IR is shown with smaller (×75) and larger (×125) magnifications. (**b**) Comparison of BrdU-positive cells/crypt (quantification) in the small intestine of wt p53 and p53-null mice at different time points after 15 Gy of IR. (**c**) BrdU incorporation (pulse-chase labeling was done immediately before IR) in the small intestine of wt p53 and p53-null mice after 15 Gy of IR. Accelerated migration of labeled cells from crypts to villi was observed in p53-null compared with wt p53 mice 48 h after IR

to the opposite in mice of two genotypes (Figure 3a). Almost no BrdU incorporation (because of complete death of crypt cells) was seen in the small intestine of p53-null mice, while in wild-type animals we could detect proliferation in distinct crypts with altered morphology. Quantitation of the changes in proportion of BrdU incorporating cells in wild-type and p53-null mice is shown in Figure 3b.

By monitoring the number, localization, and morphology of BrdU-labeled cells during different times after treatment, we traced the fate of cells that were in the S phase of cell cycle at the moment of radiation (BrdU was injected immediately before irradiation, Figure 3c). We detected accelerated migration of labeled cells in p53 null from crypts to villi during the first 48 h after IR, which was consistent with the lack of growth arrest in the crypts of the small intestine of p53-null animals. Movement of BrdU-labeled cells towards villi was followed by their rapid disappearance occurring within 72–96 h after radiation. These processes are delayed in wild-type epithelium: BrdU-labeled cells remain alive in the crypts and start their movement towards the villi when the majority of BrdU-labeled cells in p53-null mice are already dead. Hence, the lack of growth arrest and continuous proliferation seem to be the main causes of accelerated death of epithelial cells in small intestine in p53-deficient mice after 15 Gy of gamma radiation.

p53 inhibitor pifithrin-alpha does not affect lethality from GI syndrome

To compare the effect of temporary inhibition of p53 (by chemical compounds) with a constant p53 deficiency (p53 knockout mice) on mice survival after various doses of IR, we have tested the effect of the chemical p53 inhibitor PFTα (Komarov *et al*., 1999) on survival of C57Bl/6 mice after single (9, 12.5, 25 Gy) and fractioned (5×2.5 Gy) doses of radiation. PFTα (a single i.p. injection, 5 mg/kg) protected mice from a single 9 Gy dose of IR and from a fractioned cumulative radiation dose of 12.5 Gy (2.5Gy $\times$ 5, daily), which were relevant to anticancer treatment doses. PFTα had no effect on mouse sensitivity to higher killing doses of IR (12.5–25 Gy) leading to lethality from GI syndrome (Figure 4).

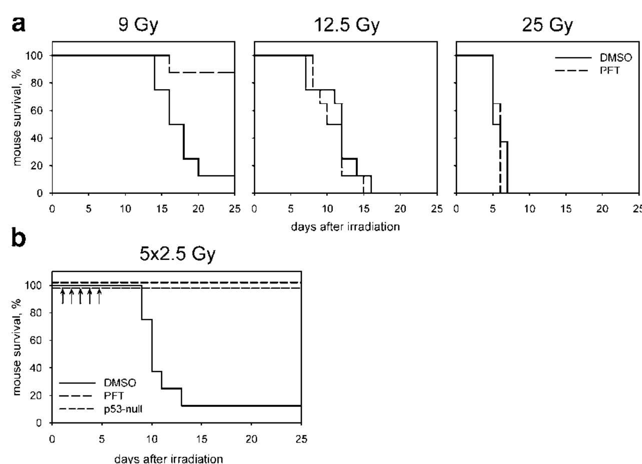

Figure 4 Effect of PFTα in C57Bl/6 mice, treated with single and fractionated whole-body doses of IR (results of representative experiments are shown). (**a**) A single i.p. injection of PFTα (10 mg/kg) had a strong rescuing effect after 9 Gy but not after 12.5 or 25 Gy. (**b**) PFTα had no effect on mouse survival after single 12.5 and 25 Gy of IR

Discussion

Our perspective on p53 as a determinant of radiation-induced injury was based on the assumption that p53-dependent apoptosis is the result of damage to radiosensitive tissues. In fact, p53-dependent apoptosis is limited to those organs that are most sensitive to IR: hematopoietic system, epithelium of small intestine, and cells of hair follicles (Gudkov and Komarova, 2003). Moreover, knockout of p53 gene protects hematopoietic cells and hair follicles from genotoxic stress, making p53-deficient mice insensitive to hematopoietic syndrome and alopecia associated with radiation and chemotherapy (Westphal *et al*., 1997; Komarov *et al*., 1999; Botchkarev *et al*., 2000). Therefore, we automatically extended this paradigm to the gastrointestinal syndrome, presuming that it will also result from p53-dependent apoptosis that is known to occur in the crypts shortly after irradiation (Merritt *et al*., 1994, 1997; Komarova *et al*., 2000). To our surprise, the experiments showed quite the opposite: GI syndrome developed faster in p53-deficient animals and epithelial damage was more severe, resulting in the disappearance of crypts, dramatic shortening of villi, followed by accelerated GI lethality. Hence, p53 was found to act as a death-delaying factor under conditions of a severe radiation damage of the small intestine, regardless of the earlier pronounced p53-dependent apoptosis occurring in the guts of p53-wild-type but not in p53-deficient mice.

What is the cause of the accelerated GI syndrome in p53-deficient mice? In a recent paper from Richard Kolesnik's group (Paris *et al*., 2001), radiation-induced damage of intestinal endothelium, occurring through ceramide-mediated mechanism, was defined as a major cause of GI syndrome in mice that received high (>15 Gy) doses of gamma radiation. However, this mechanism cannot be attributed to the observed p53-specific phenomenon, since endothelial injury was found to be p53-independent. Therefore, we focused on the most striking difference in behavior of intestinal cells in mice of different p53 status: while proliferating cells in wild-type animals underwent fast and prolonged growth arrest after exposure to IR, cells in the crypts of p53-deficient small intestine continued to proliferate (Komarova *et al*., 2000). Tracing the fate of such proliferating cells brought us to a conclusion that they rapidly disappear after they have moved up towards villi. At the same time, similar cells in wild-type p53 crypts remain alive but growth arrested and, therefore, do not change their position inside the irradiated crypts as fast as p53-deficient cells do. Radiation-damaged cells in p53-null animals, which do not undergo growth arrest, enter into cell cycle and subsequently die, presumably of mitotic catastrophe (Watson and Pritchard, 2000), thus further complicating the recovery of villi. In fact, we did not see new proliferating crypts in p53-null mice, while in p53 mice with wild-type p53 they started appearing 3 days after 15 Gy of IR. These new proliferating crypts in wild-type mice presumably originate from those growth-arrested cells that could

successfully repair the damage caused by IR (what was impossible to be done for p53-deficient cells that were forced to continue proliferation). This model fits well the opinion that death of villi occurs as a consequence of depletion of epithelial clonogenic cell population (Potten, 1990; Potten *et al*., 1997).

Our interpretation of these observations is that p53 in small intestine plays the role of an inhibitor of mitotic catastrophe, inducing growth arrest. This conclusion means that p53-mediated growth arrest has a much more significant impact on the radiation-induced injury of digestive tract than p53-dependent apoptosis, thus reverting the image of p53 from a pro-death to a pro-survival factor. Accelerated death of p21-null mice after high doses of IR confirms this conclusion.

The fact that p53 can be an inhibitor of mitotic death in the small intestine after IR indicates that it is important to review and possibly revise the role of p53 in radiation- and chemotherapy-induced injury not only of normal tissues, but also of those tumors that maintain functional p53. In fact, there is an accumulating body of evidence that p53 in such cells can play the role of a treatment resistance factor (as shown for small intestine cells) that increases the survival of cells after genotoxic stress, presumably providing them a safe 'repair dock' of a reversible growth arrest, thus preventing cells from mitotic catastrophe (Roninson *et al*., 2001).

This view seems to contradict the well-accepted notion that p53-mediated apoptosis is a major factor in determining tumor's responsiveness to treatment. However, this view on p53 as a treatment-sensitivity factor was predominantly based on the data obtained in lympho- and hematopoietic tumors and artificial tumor cells that preserved p53-mediated apoptosis (Lowe *et al*., 1994). However, under conditions of predominant growth arrest response or the lack of apoptotic response that is an acquired property of the majority of natural tumors, p53 turns from a killing factor to a survival- or life-prolonging factor.

It is important to determine what would be the impact of these new findings on the perspectives of therapeutic applications of p53 inhibitors that, as we suggested earlier, could be useful to reduce cancer treatment side effects (Komarova and Gudkov, 1998). The first p53 inhibitor, pifithrin-alpha, was isolated using p53-dependent transactivation as a readout. It could also block apoptosis (Komarov *et al*., 1999; Culmsee *et al*., 2001; Duan *et al*., 2002; Zhu *et al*., 2002) and was effective as a radioprotector from doses of radiation that induced HP syndrome (<11 Gy) (Komarov *et al*., 1999). The opposite role of p53 in HP and GI syndromes made us worry if p53 inhibitor would promote development of a GI syndrome that could limit its clinical applications. Fortunately, direct experimental testing overcame this concern: PFTα treatment did not accelerate GI lethality of mice. Why does the temporary inhibition of p53 affect the GI syndrome differently from gene knockout, while both have a similar effect on HP syndrome? Although we do not know the exact answer, a possible explanation is that a short-term inhibition of p53 is enough to change the cell's intention to kill itself, but is not enough to block the growth-arrest program, the real basis for the protective role of p53 from GI syndrome.

There are some other p53-related factors that might be involved in determination of the observed phenomenon. Although our experiments with bone marrow reconstitution have shown that systemic immunity was not a dominating factor in the development of accelerated IR lethality of p53-null mice, local immunity might be involved in this effect (i.e., by modulating intestinal bacteria colonization), the possibility that remains to be tested. The increased stromal response such as fibrogenesis, which we observed in p53-null mice, could also contribute to their high radiation sensitivity (Ohkusu-Tsukada *et al*., 1999; Yamanishi *et al*., 2002). Muc-2 gene, one of the major components of mucins that provides a protective barrier between epithelial surfaces and the gut lumen, it was found, can be directly activated by p53 in different cell lines, including colon cancer (Ookawa *et al*., 2002).

Materials and methods

Gamma irradiation of mice

C57Bl/6 wild-type and p53-null C57Bl/6 mice, C57Bl/6x129S2 and p21-null C57Bl/6x129S2 mice purchased from Jackson lab, were irradiated using the Shepherd 4000 Ci Cesium 137 source at a dose rate of 4 Gy/min.

Bone marrow transplantation

For bone marrow reconstitution experiments, 8-week-old C57Bl/6J mice received a single 11 Gy dose of total body γ-irradiation. Bone marrow cell suspensions were isolated by flushing the femurs and tibias from male p53-null or wild-type mice with PBS. Single-cell suspensions were prepared by passing the cells through a 30-μm nylon gauze. Irradiated recipients were reconstituted 12 h later with approximately 1.5×10^7 bone marrow cells by intravenous injection into the tail vein. Mice were housed on autoclaved bedding in air-filtered cages and were injected 2 days with gentamycin (7 mg/kg). Non-reconstituted lethally irradiated mice were included as controls in experiment, and typically had to be killed between days 8 and 14 post irradiation, due to bone marrow aplasia.

Detection of proliferation and apoptosis in tissues in situ

BrdU and TUNEL assays were done according to previously described protocols (Botchkarev *et al*., 2000; Komarova *et al*., 2000). BrdU (50 mg/kg) was injected intraperitoneally into mice at different time points before or after irradiation.

In vivo *^{14}C-thymidine incorporation assay*

^{14}C-thymidine (10 μCi per animal) was injected intraperitoneally into untreated or irradiated mice 8 h after 15 Gy of whole-body irradiation. Mice were killed 24 h after ^{14}C-thymidine injection, embedded into OCT solution and frozen. Whole-body sections (25 μm) were prepared using cryostatic microtome, and exposed to X-ray film to determine the distribution of incorporated ^{14}C-thymidine.

Quantitation of villi, crypts, and BRDU positive cells in the small intestine in situ

Five ileum cross sections per animal were prepared, stained with hematoxylin–eosin (three animals were analysed for each time point after 15 Gy of IR) and analysed microscopically to estimate the number of crypts and villi. The amount of BrdU-positive cells in the crypts was counted in five random fields under ×200 magnification (100–30 crypts) and the average number of BrdU-positive cells per crypt was determined.

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