Published in final edited form as:
Mutat Res. 2009 November 2; 670(1-2): 103–106. doi:10.1016/j.mrfmmm.2009.09.001.

Mutagenesis in vivo in T cells of p21-deficient mice

Changshun Shao, **Li Liang**, **Xin Zhao**, **Yanping Chen**, **Betty Zheng**, **Jianmin Chen**, **Minjie Luo**, and **Jay A. Tischfield**
Department of Genetics, Rutgers University, 604 Allison Road, Piscataway, NJ 08854, USA

Abstract

Mice that are deficient in p53 exhibit an early onset of multiple types of tumors, especially thymic lymphoma. However, it remains unclear to what extent each of the p53-regulated pathways exerts its tumor suppressor activity. $p21^{Cip1/Waf1}$, acting down stream of p53, is a major G1/S checkpoint protein that restricts cell cycle progression into S phase in the presence of DNA damage. While at old ages *p21*−/− mice have a higher incidence of many types of tumors than *p21*+/+ mice, they are more resistant to thymic lymphomagenesis. In this study, we characterized mutagenesis *in vivo* in T cells of p21-deficient mice, using loss of heterozygosity (LOH) at *Aprt* locus as an indicator. We found that the spontaneous *Aprt* mutant frequency in T cells of *p21*−/− mice is lower than that in *p21* +/+ mice. The mutational spectra, however, are similar, with mitotic recombination being the predominant pathway. In contrast to the remarkable induction of LOH events in T cells of *p53*−/− mice exposed to x-rays, LOH in T cells of *p21*−/− mice is not significantly induced by x-rays. Correspondingly, lymphoid cells of *p21*−/− mice are more sensitive to IR-induced apoptosis than those of *p21*+/+ mice, in contrast to the radioresistance of p53-deficient lymphocytes. Reduction in mutation load in T cell lineages may contribute to the suppression of thymic lymphomagenesis in *p21*−/− mice.

Keywords

p21; mutagenesis; cell cycle checkpoint; apoptosis; lymphomagenesis

1. Introduction

The tumor suppressor p53 is a master regulator of the cellular responses to DNA damage. The activation of p53 up-regulates an array of genes that initiates cell cycle checkpoints, DNA repair and apoptosis[1]. While it has been firmly established that loss or mutation of p53 can lead to a broad spectrum of human malignancy, it remains controversial which of the p53-regulated pathways, or a combination of them, functions as a tumor-suppressing mechanism [2,3]. Whereas there is an early onset of many types of tumors, particularly thymic lymphomas, in mice that lack p53, disruption in each of the p53-regulated pathways only has limited tumor-enhancing effect [3–8]. For example, p53 mutant mice that are engineered to be deficient in

Correspondence should be addressed to C Shao, Department of Genetics, Rutgers University, 604 Allison Road, Piscataway, NJ 08854, USA. shao@biology.rutgers.edu; Tel: 1-732-445-5406; Fax: 1-732-445-1147.

Conflict of Interest
There is no conflict of interest.

induction of apoptosis, but not in other functions of p53, were found to escape the early onset of thymic lymphomas that characterize p53-null mice [3]. Moreover, the lymphomas and sarcomas that eventually developed in this particular strain of mice retained a diploid chromosome number, in sharp contrast to aneuploidy observed in tumors and cells from p53-null mice, suggesting that apoptosis is dispensable for the maintenance of chromosome euploidy in lymphoid cells. Also, knockout mice for *Puma*, a gene required for apoptosis, were shown to be relatively tumor free [7,8].

Studies involving mice that lack Cdkn1a (p21), a protein that arrests cell cycle progression at G1/S checkpoint in the presence of DNA damage, showed that p21 has tumor suppressor activity in a tissue-dependent manner. While lack of p21 predisposes mice to tumor development in skin, colon, intestine, pituitary, thyroid, mammary gland, salivary gland, connective tissue and histiocystic sarcomas, p21-deficient mice are more resistant to thymic lymphomas [9–11]. For example, *p21*−/− mice are more protected than wild type mice from irradiation-induced lymphomas. Absence of p21 also significantly reduces the incidence of spontaneous and radiation-induced thymic lymphomas in *p53*−/− and *p53*−/− mice. Increased rate of apoptosis in thymic cells has been proposed to be responsible for the decreased lymphomagenesis [11].

Lack of p53 is associated with increased chromosome instability *in vivo* [12,13]. Furthermore, chromosome instability is induced more prominently by IR in the absence of p53. For example, x-rays induce loss of heterozygosity (LOH) events more dramatically in T cells of *p53*−/− and *p53*−/− mice than in those of *p53*+/+ mice [14]. However, since p53 is involved in multiple cellular processes, it is unclear to what extent each of the p53-regulated pathways is responsible for the suppression of genetic instability.

In this study we addressed how a defective G1/S checkpoint contributes to mutagenesis *in vivo* by characterizing the somatic mutations that arise at *Aprt* locus in T cells of 129XC57F1 p21-null mice that are also *Aprt* heterozygous. In *Aprt* heterozygous mice, multiple pathways of LOH, including deletion, mitotic recombination and chromosome loss, can be evaluated [13,15,16]. The APRT-deficient mutant cells are recoverable by the virtue of their resistance to adenine analogs such as 2, 6-diaminopurine (DAP) and are designated as DAP^r.

2. Materials and methods

2.1. Mice

We obtained p21 knockout mice in a mixed 129/C57 background from the Jackson Laboratory [17]. The p21 mutant mice were backcrossed to 129S2 and C57 strains, respectively, for ten consecutive generations to reach (N10) C57 and (N10)129, respectively. The (N10)129 *p21*−/− mice were then crossed to 129*Aprt*−/− mice to generate 129 *p21*−/−*Aprt*−/−. 129 *p21*−/−*Aprt*−/− mice were then crossed to C57*p21*−/− mice to generate 129S2 X C57 F1 hybrids that are *p21*(+/+, −/−, −/−) and *Aprt* −/−. The purpose of the backcross is to obtain strain-specific chromosome 8, so that when they are introduced in hybrids, the intervals of LOH along chromosome 8, and indirectly the pathways of LOH, in DAP^r clones can be determined.

2.2. Ionizing radiation

Mice, about two months old, were subjected to whole-body x-irradiation using a Faxitron Cabinet x-ray System (Wheeling, IL) at the rate of 0.2 Gy/min using at 100 kVp. Irradiated mice were sacrificed for preparation of splenocytes two months after a single exposure. All mice, treated and untreated, were about 4 months old at the time of experiments.

2.3. Characterization of DAP^r mutant T cell clones

Splenocytes were prepared and cultured as described [18]. DAP^r mutant T cell clones were scored and analyzed as described [13]. The colony forming efficiency and mutant frequency were estimated for each mouse by assuming that the positive colonies are formed in the wells of a 96-well plate following a Poisson distribution.

2.4. Apoptosis assay

Apoptosis of splenic lymphocytes was estimated with the kit (Roche Applied Science, Indianapolis, IN) for detection and quantification of apoptosis based on terminal deoxyribonucleic transferase mediated dUTP nick end labeling (TUNEL) technology. Unirradiated mice and irradiated mice (6 hours following 1 Gy X-rays) were sacrificed and spleens were obtained and placed in ice-cold RPMI 1640 medium. Splenic lymphocytes were isolated as described [18] and were fixed in 2% paraformaldehyde (in 1× PBS) for 1 hour at room temperature. Cells were washed 3 times with PBS, resuspended in permeablization solution (0.1% triton X-100, 0.1% sodium citrate, freshly made), and incubated on ice for 2 minutes. Cells were washed twice with PBS, resuspended in TUNEL reaction mixture and incubated at 37°C for 1 hour. Samples were washed twice with PBS and analyzed under a fluorescence microscope.

3. Results and discussion

3.1. Spontaneous Aprt mutations in T cells of p21-deficient mice

We first compared the spontaneous frequency of DAP^r T cells *in vivo* between *p21*+/+ and *p21*−/− mice. In 129XC57F1 *p21*+/+ mice, the median frequency was 17.5×10^{-6} (N=15). In contrast, the median was 8.4×10^{-6} in *p21*−/− mice (N=12) (Table 1). The difference between the two groups is statistically significant (P=0.03, Mann-Whitney U test). Thus, in the absence of p21, the frequency of DAP^r mutant T cells, which reflect LOH at *Aprt* locus, is lower than when p21 is present.

To determine what types of mutation are reduced in the absence of p21, we characterized the mutational spectrum of DAP^r T cell clones. We found that the spectra are similar between *p21*+/+ and *p21*−/− mice, Table 2. As we reported earlier [19], mitotic recombination is the predominant mechanism. Thus, lack of p21 renders T cells less susceptible to all types of mutations leading to functional loss of *Aprt*.

3.2. IR-induced Aprt mutations in T cells of p21-deficient mice

In general, the frequency of LOH at *Aprt* locus in T cells is only slightly induced by a single dose of ionizing radiation and the IR-induced mutations are primarily of deletion type [20]. *p53*−/− and *p53*−/− mice, on the other hand, exhibit a much more pronounced induction of multiple types of mutations [14]. To test how *p21*−/− mice respond to IR, we characterized the mutation frequency and spectrum of DAP^r T cell clones in *p21*−/− mice exposed to x-rays. No significant induction in mutant frequency was detected (Table 1). In addition, the mutational spectrum of DAP^r clones recovered from irradiated mice was not affected by the lack or presence of p21 (Table 2). Especially, deletions, which are usually preferentially induced by x-rays[20], were not more commonly represented in *p21*−/− mice than in *p21*+/+ mice after x-ray exposure. Thus, T cells of *p21*−/− mice were not more predisposed to incur mutation in response to IR than those of *p21*+/+ cells.

While the frequency of *Aprt* LOH mutants was increased about eight fold in *p53*−/− mice after their exposure to 4 Gy of X-rays [14], the induction of LOH in T cells of *p21*−/− mice is minimal, if any. Thus, the remarkable induction of mutations in T cells of *p53*−/− mice is not caused by a compromised G1/S checkpoint.

3.3. Lymphoid cells of p21−/− mice are more sensitive to IR-induced apoptosis

We have previously shown that reduced induction of apoptosis in mice treated with repeated exposure to x-rays is correlated to an elevation of LOH at *Aprt* [20]. Considering that p21 has been proposed to be an anti-apoptotic factor in some cell lines [21] and in spleens of *p21*−/− mice [22], and that the expression level of p21 is correlated to the resistance to apoptosis [23], we speculated that the decreased mutant frequency of LOH at *Aprt* in T cells of *p21*−/− mice might be due to their increased sensitivity to DNA damage-induced apoptosis. It is possible that DNA damage is more tolerated in T cells (or their precursor cells) with functional *p21* so that p21-deficient T cells are more susceptible to DNA damage-induced apoptosis, thus less likely to harbor mutations. To gain further supporting evidence for this notion, we evaluated apoptosis in splenocytes *in vivo*. Mice were exposed in whole body to 1 Gy of x-rays and were sacrificed six hours later for the preparation of splenocytes. The splenic cells were then fixed and stained for scoring of apoptotic cells. As shown in Fig. 1, while apoptosis was induced two fold in splenocytes of *p21*+/+ mice, it was induced about five fold in those of *p21*−/− mice. The frequency of apoptotic splenocytes in *p21*−/− mice after x-ray exposure was also significantly higher than that in *p21*+/+ mice. This finding is consistent with previous reports [22,23] and adds further evidence for an inverse correlation between the induction of apoptosis and the level of LOH. However, while the splenocytes of *p21*−/− mice were observed to have a higher rate of spontaneous apoptosis than those of *p21*+/+ mice in spleen sections [22], such an enhancement in spontaneous apoptosis in *p21*−/− mice was not detected by our assay, it is therefore unclear whether the reduced spontaneous mutagenesis in T cells of *p21*−/− mice was related to apoptosis in T cells per se. If spontaneous mutations leading to LOH at *Aprt* primarily occur at earlier stages of hematopoietic development, but not in the mature T cells, an assessment of apoptosis in the mature T cells may not necessarily reflect the true nature of the target cells in which mutations arise. Indeed, while x-rays applied to adults preferentially induce deletions and point mutations, they primarily induce mitotic recombination when applied to fetus [24], suggesting that mitotic recombination, which accounts for the majority of the LOH events at *Aprt*, may primarily occur before birth.

3.4. Mutational response at Hprt is unaffected by the status of p21

While *Aprt* can detect a broad spectrum of mutations as a reporter, the X-linked *Hprt* gene as a reporter primarily detects more local genomic alterations such as point mutations and small deletions [20]. Our previous studies showed that *Hprt* and *Aprt* respond differently to x-rays [20]. In mice that were exposed to x-rays in their adulthood, there was a clear dose-dependent increase in the *Hprt* mutant frequency, but the increase in the *Aprt* mutant frequency was very modest. On the other hand, when x-rays was delivered to fetus in uteri, it had no effect on the *Hprt* mutant frequency, while *Aprt* mutant frequency was increased several fold, primarily due to an elevated mitotic recombination [24]. Interestingly, for reason(s) unknown, the mutational response to x-rays at *Hprt* is not affected by the status of apoptosis. We first estimated the spontaneous *Hprt* mutant frequency in T cells of *p21*−/− mice. We found that there was virtually no difference in this frequency between *p21*−/− and *p21*+/+ mice (Table 3). This result indicates that reduced spontaneous mutagenesis in the absence of p21 is locus-specific.

We further tested how *Hprt* respond to x-rays in the absence of p21 *in vivo* and found that *p21*−/− mice appeared to respond to IR similarly as *p21*+/+ mice. Both types of mice showed a dose dependent increase in the mutant frequency (Table 3). Thus, neither the spontaneous nor induced *Hprt* mutant frequency in T cells is affected by p21 status. Clearly, a defective G1/S checkpoint does not predispose the lymphoid cells of *p21*−/− to mutational accumulations at *Hprt* locus. Thus, the increased propensity to undergo apoptosis that have probably precluded the accumulation of mutation at *Aprt* had little effect on mutations at *Hprt*.

Our data showed that the reduced spontaneous mutagenesis in *p21*−/− mice does not apply to the X-linked *Hprt*. The mutagenic effect of x-rays is also locus-specific in *p21*−/− mice. It appears that the degree to which *Aprt* mutations, but not *Hprt* mutations, are induced is more indicative of the induction of lymphomagenesis in mice exposed to x-rays [20]. With the same total dose, fractionation at weekly intervals is more efficient than a single dose in the induction of lymphomas in mouse models. In corresponding to the more efficient induction of lymphomagenesis, x-rays fractionated at weekly intervals (4 x 1 Gy) is more potent in inducing LOH mutations at *Aprt*, but less so in inducing *Hprt* mutations. Also, a decrease in the induction of apoptosis accompanied the elevated *Aprt* mutant frequency.

In conclusion, lack of p21 does not cause an increased accumulation of spontaneous or induced mutations in T cells. Rather, the spontaneous mutagenesis measured by LOH at *Aprt* in T cells is reduced in *p21*−/− mice. Such a reduction in mutation load probably contributes to the reduced lymphomagenesis in those mice. The observed increase in mutagenesis in T cells of *p53*−/− mice in response to x-rays is therefore independent of the p21-mediated pathway.

Acknowledgments

This work was supported by grants from the National Institutes of Health (R01ES011633) and New Jersey Stem Cell Research grant (SNJ-CST-07-2042-014-90).

Reference List

1. Levine AJ, Hu W, Feng Z. The P53 pathway: what questions remain to be explored? Cell Death. Differ 2006;13:1027–1036. [PubMed: 16557269]
2. Schmit CA, Fridman JS, Yang M, Baranov E, Hoffman RM, Lowe SW. Dissecting p53 tumor suppressor functions in vivo. Cancer Cell 2002;1:289–298. [PubMed: 12086865]
3. Liu G, Parant JM, Lang G, Chau P, Chavez-Reyes A, El Naggar AK, Multani A, Chang S, Lozano G. Chromosome stability, in the absence of apoptosis, is critical for suppression of tumorigenesis in Trp53 mutant mice. Nat. Genet 2004;36:63–68. [PubMed: 14702042]
4. Brugarolas J, Chandrasekaran C, Gordon JI, Beach D, Jacks T, Hannon GJ. Radiation-induced cell cycle arrest compromised by p21 deficiency. Nature 1995;377:552–557. [PubMed: 7566157]
5. Deng C, Zhang P, Harper JW, Elledge SJ, Leder P. Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. Cell 1995;82:675–684. [PubMed: 7664346]
6. Lindsten T, Ross AJ, King A, Zong WX, Rathmell JC, Shiels HA, Ulrich E, Waymire KG, Mahar P, Frauwirth K, Chen Y, Wei M, Eng VM, Adelman DM, Simon MC, Ma A, Golden JA, Evan G, Korsmeyer SJ, MacGregor GR, Thompson CB. The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol. Cell 2000;6:1389–1399. [PubMed: 11163212]
7. Jeffers JR, Parganas E, Lee Y, Yang C, Wang J, Brennan J, MacLean KH, Han J, Chittenden T, Ihle JN, McKinnon PJ, Cleveland JL, Zambetti GP. Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. Cancer Cell 2003;4:321–328. [PubMed: 14585359]
8. Villunger A, Michalak EM, Coultas L, Mullauer F, Bock G, Ausserlechner MJ, Adams JM, Strasser A. p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. Science 2003;302:1036–1038. [PubMed: 14500851]
9. Wang YA, Elson A, Leder P. Loss of p21 increases sensitivity to ionizing radiation and delays the onset of lymphoma in atm-deficient mice. Proc. Natl. Acad. Sci. U. S. A 1997;94:14590–14595. [PubMed: 9405657]
10. Martin-Caballero J, Flores JM, Garcia-Palencia P, Serrano M. Tumor susceptibility of p21(Waf1/Cip1)-deficient mice. Cancer Res 2001;61:6234–6238. [PubMed: 11507077]
11. De la CE, Garcia-Cao I, Herranz M, Lopez P, Garcia-Palencia P, Flores JM, Serrano M, Fernandez-Piqueras J, Martin-Caballero J. Tumorigenic activity of p21Waf1/Cip1 in thymic lymphoma. Oncogene 2006;25:4128–4132. [PubMed: 16462758]

12. Bouffler SD, Kemp CJ, Balmain A, Cox R. Spontaneous and ionizing radiation-induced chromosomal abnormalities in p53-deficient mice. Cancer Res 1995;55:3883–3889. [PubMed: 7641208]

13. Shao C, Deng L, Henegariu O, Liang L, Stambrook PJ, Tischfield JA. Chromosome instability contributes to loss of heterozygosity in mice lacking p53. Proceedings of the National Academy of Sciences of the United States of America 2000;97:7405–7410. [PubMed: 10861008]

14. Liang L, Shao C, Deng L, Mendonca MS, Stambrook PJ, Tischfield JA. Radiation-induced genetic instability in vivo depends on p53 status. Mutat. Res 2002;502:69–80. [PubMed: 11996974]

15. Shao C, Yin M, Deng L, Stambrook PJ, Doetschman T, Tischfield JA. Loss of heterozygosity and point mutation at Aprt locus in T cells and fibroblasts of Pms2−/− mice. Oncogene 2002;21:2840–2845. [PubMed: 11973643]

16. Shao C, Deng L, Chen Y, Kucherlapati R, Stambrook PJ, Tischfield JA. Mlh1 mediates tissue-specific regulation of mitotic recombination. Oncogene 2004;23:9017–9024. [PubMed: 15480418]

17. Jacks T, Remington L, Williams BO, Schmitt EM, Halachmi S, Bronson RT, Weinberg RA. Tumor spectrum analysis in p53-mutant mice. Current Biology 1994;4:1–7. [PubMed: 7922305]

18. Liang L, Deng L, Shao C, Stambrook PJ, Tischfield JA. In vivo loss of heterozygosity in T-cells of B6C3F1 Aprt(+/−) mice. Environmental & Molecular Mutagenesis 2000;35:150–157. [PubMed: 10712749]

19. Shao C, Deng L, Chen Y, Kucherlapati R, Stambrook PJ, Tischfield JA. Mlh1 mediates tissue-specific regulation of mitotic recombination. Oncogene 2004;23:9017–9024. [PubMed: 15480418]

20. Liang L, Mendonca MS, Deng L, Nguyen SC, Shao C, Tischfield JA. Reduced Apoptosis and Increased Deletion Mutations at Aprt Locus In vivo in Mice Exposed to Repeated Ionizing Radiation. Cancer Res 2007;67:1910–1917. [PubMed: 17332317]

21. Gartel AL, Tyner AL. The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. Mol. Cancer Ther 2002;1:639–649. [PubMed: 12479224]

22. Komarova EA, Christov K, Faerman AI, Gudkov AV. Different impact of p53 and p21 on the radiation response of mouse tissues. Oncogene 2000;19:3791–3798. [PubMed: 10949934]

23. Wallace M, Coates PJ, Wright EG, Ball KL. Differential post-translational modification of the tumour suppressor proteins Rb and p53 modulate the rates of radiation- induced apoptosis in vivo. Oncogene 2001;20:3597–3608. [PubMed: 11439323]

24. Liang L, Deng L, Mendonca MS, Chen Y, Zheng B, Stambrook PJ, Shao C, Tischfield JA. X-rays induce distinct patterns of somatic mutation in fetal versus adult hematopoietic cells. Repair (Amst). 2007

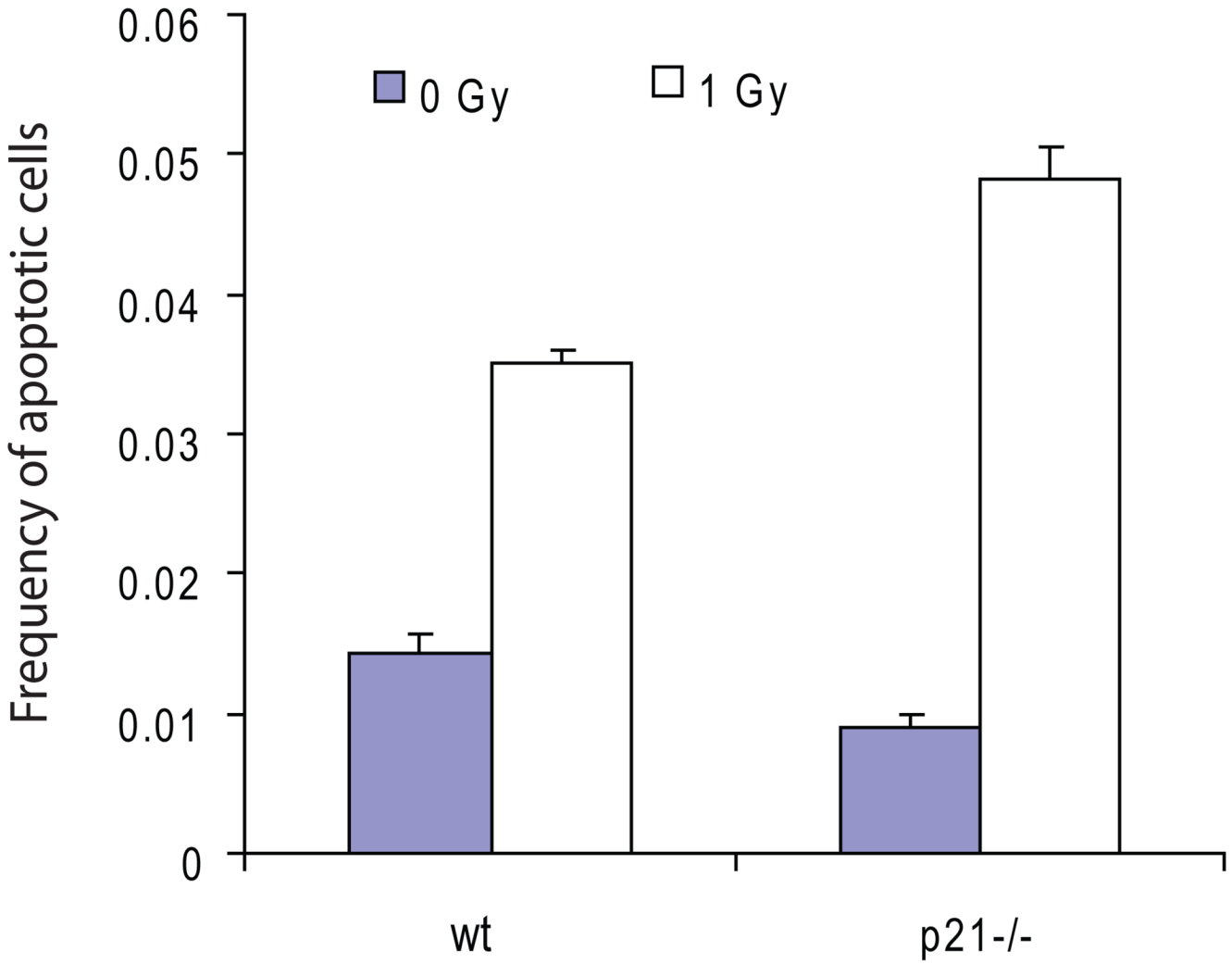

Figure 1.
p21-deficient splenocytes were more susceptible to x-ray-induced apoptosis. Mice were exposed to 1 Gy of x-rays and were sacrificed 6 hours later for the collection of splenocytes. Terminal deoxyribonucleic transferase mediated dUTP nick end labeling (TUNEL) assay was performed to determine the level of apoptosis (N= 3 mice /group).

Table 1

Mutant frequency of DAP^r T cell variants

Genotype	Strain	X-rays (Gy)	No. mice	CFE ± SE (%)	Median MF ($\times 10^{-6}$)
p21+/+ (group 1)	129 × C57	0	15	8.0 ± 2.2	17.5
P21+/+ (group 2)	129 × C57	0	14	7.9 ± 1.2	20.3
p21+/+ (group 3)	129 × C57	0	5	6.7 ± 1.1	19.5
p21−/−	129 × C57	0	12	8.5 ± 0.8	8.4*
p21 wt	129 × C57	1	2	5.4 ± 0.7	18.1
p21−/−	129 × C57	1	12	4.2 ± 0.4	13.9
p21 wt	129 × C57	4	13	5.1 ± 0.7	17.1
P21+/−	129 × C57	4	4	3.2 ± 0.3	30.8
p21−/−	129 × C57	4	11	5.5 ± 0.5	10.0

CFE, colony-forming efficiency; MF, mutant frequency

*$P = 0.03$, compared to *p21*+/+ group 1.

Table 2

Spectrum of mutational pathways to APRT-deficiency

Genotype	X-rays	N	No. DAP^r clones	No. PM/EI (%)	No. MR (%)	No. del (%)	Average ratio of MR
p21+/+	0 Gy (Group 1)	13	60	29 (48)	31 (52)	0 (0)	0.64
	0 Gy (Group 2)	14	69	24 (35)	43 (62)	2 (3)	0.65
	0 Gy (Group 3)	5	18	5 (28)	13 (72)	0 (0)	0.72
	4 Gy	10	16	4 (25)	10 (63)	2 (12)	0.65
p21+/−	4 Gy	4	18	4 (22)	14 (78)	0 (0)	0.74
p21−/−	0 Gy	12	60	18 (30)	38 (63)	4 (7)	0.63
	1 Gy	8	23	12 (52)	10 (43)	1 (5)	0.33
	4 Gy	11	29	11 (38)	17 (59)	1 (3)	0.47

PM/EI, putative point mutation/epigenetic inactivation; MR, mitotic recombination; del, deletion. Ratio of MR in each mouse was as (number of MR clones)/(number of DAP^r clones analyzed). The average ratio is more representative of the distribution of MR in a group.

Table 3

Spontaneous and IR-induced *Hprt* mutations in T cells of *p21* null mice

Genotype	Strain	x-rays (Gy)	No. mice	Median MF ($\times 10^{-6}$)
p21+/+	129 × C57	0	17	2.7
p21−/−	129 × C57	0	13	2.9
p21+/+	129 × C57	1	9	21.5
p21−/−	129 × C57	1	6	37.5
p21+/+	129 × C57	4	5	52.8
p21+/−	129 × C57	4	5	60.1
p21−/−	129 × C57	4	9	48.6