Tumor Susceptibility of *p21*^{Waf1/Cip1}-deficient Mice[1]

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ABSTRACT

The cell cycle regulator p21 mediates the ability of the tumor suppressor p53 to arrest cellular proliferation. We have examined the involvement of p21 in tumor suppression by following a large cohort of *p21*-deficient mice for an extended period of time. We report that *p21*-deficient mice develop spontaneous tumors at an average age of 16 months, whereas wild-type mice are tumor-free beyond 2 years of age. The tumors arising in *p21*-null mice derive from a variety of cell types and include hematopoietic (~65% of the tumors), endothelial (~20%), and epithelial (~10%) tumors. We have also studied radiation-induced carcinogenesis to test whether, in this setting, p53 exerts its tumor suppressor activity mainly through apoptosis, rather than through p21-mediated cell-cycle arrest. Concurring with this, *p21*-deficient mice did not show increased susceptibility to radiation-induced carcinogenesis. On the contrary, they were protected relative to wild-type mice. We conclude that p21, by mediating p53-dependent cell-cycle arrest, plays a significant role in tumor suppression.

INTRODUCTION

The protein p21^{Waf1/Cip1/Sdi1} (hereafter referred to as p21) was originally isolated as a general inhibitor of CDKs[3] (1–5). Subsequent studies (6) have provided a more complete and complex picture in which p21 plays a dual role. On one hand, it is an activator of the CDKs involved in early G_1 (CDK4 and CDK6 associated to D cyclins), whereas on the other hand, it is an inhibitor of the CDKs involved in the G_1-S transition (CDK2 associated to E and A cyclins). Indeed, when expressed at moderate levels, p21 preferentially binds to CDK4–6/cycD complexes, thus facilitating cell-cycle progression, whereas at high levels, p21 results in inhibition of CDK2/cycE-A complexes and, consequently, in a robust proliferation arrest (1–6). The main regulation of p21 protein levels occurs transcriptionally by p53-dependent and -independent mechanisms. Upon activation of p53, p21 is strongly up-regulated, reaching levels that completely arrest proliferation (1, 7). In support of the relevant role of p21 in mediating p53-dependent cell-cycle arrest, cells derived from *p21*-null mice arrest proliferation inefficiently after p53 activation (8–10). In addition to p53, there are other transcriptional activatory mechanisms that are not well understood for the most part and which operate in response to mitogenic stimulation (11, 12), transforming growth factor-β (13–15), and aberrant oncogenic signals (16, 17) or during a number of cell differentiation processes (18, 19).

p53 is a convergence point for various types of cellular stresses, notably DNA damage and oncogenic stress (20–22). Both types of stress are conveyed to p53 through separate routes. DNA damage is signaled by the related kinases ATM and ATR (20), whereas oncogenic stress is signaled by the tumor suppressor p19^{ARF} (21). p53 activation by any of these routes results in up-regulation of a large set of p53-transcriptional targets, many of which have been identified recently (23, 24) using DNA microarrays. Among the multiple p53 transcriptional targets, p21 stands out as the main, although not exclusive, effector of p53-mediated cell cycle arrest (8–10). Other p53 targets contribute to apoptosis induction, such as Bax, Noxa, and p53AIP1 (20, 22), or to the maintenance of genomic stability, such as GADD45a (25).

Inactivating mutations in either *p53* or in some of its upstream regulators, notably *ATM* and *p19*^{ARF}, are common in human cancers. In contrast, mutations in the various p53-transcriptional targets studied so far, including *p21*, are uncommon (26). However, it remains to be elucidated which is the relative contribution of the different p53-transcriptional targets to tumor suppression. Regarding *p21*-null mice, it was originally reported (9) that they are tumor-free at least until 7 months of age. Subsequently, the role of p21 in mediating tumor suppression has been examined in a number of murine models for tissue-specific tumorigenesis. The absence of *p21* accelerates the development of some tumors, particularly pituitary tumors in *Rb*-haploinsufficient mice and in *p18*^{INK4c}-deficient mice (27, 28), breast tumors in mouse mammary tumor virus/*ras* transgenic mice (29), and intestinal tumors in *Apc*-haploinsufficient mice (30). In contrast, the absence of *p21* has no effect on the tumor-prone phenotype of mice lacking the Werner syndrome gene (31), and, more surprisingly, it even delays the onset of thymic lymphomas in *ATM*-null mice (32). Finally, there are contradictory results concerning the susceptibility of *p21*-null mice to chemically induced carcinogenesis of the skin (33, 34). In this study, we have addressed the role of p21 in tumor suppression by directly assessing the susceptibility of *p21*-null mice to spontaneous tumors and also to radiation-induced tumors.

MATERIALS AND METHODS

Mice and Animal Handling. Wild-type and *p21*-null (8) mice of the same genetic background (129Sv/C57BL6; 50:50) were housed at the National Center of Biotechnology (Madrid, Spain) in a pathogen-free barrier area. Moribund mice were killed humanely in accordance with the Guidelines for Humane End Points for Animals Used in Biomedical Research. For γ-radiation tumorigenesis, 1-month-old mice were irradiated weekly for 4 weeks with a 1.75-Gy dose from a ¹³⁷Cs source (MARK 1-30 Irradiator; Shepherd & Associates) at a rate of 1.14 Gy/min.

Histological Analysis. Tumors from nonautolyzed tissues were recovered from moribund or recently deceased mice. Tissue samples were fixed in 10% buffered formalin, embedded in paraffin wax, sectioned at 4 μm, and stained with H&E. Immunohistochemical staining was performed using the streptavidin-biotin-peroxidase complex method. Tissue sections were processed with 10 mM citrate buffer (pH 6.0) in a microwave (100°C; 15 min), although in some cases 10-min pre-trypsinization was also required. Endogenous peroxidase activity was inactivated by incubation with 3% hydrogen peroxide in methanol (15 min; room temperature).

A battery of immunochemical markers was used to confirm tumor histogenesis. Hematopoietic malignancies were diagnosed using the following markers: for B- and T-lymphocyte detection, rat antimouse CD45R/B220 mAb (Southern Biotechnology Associates, Birmingham, AL; dilution 1:100); for T-lymphocyte detection, rabbit antihuman CD3 polyclonal antibody (Dako, Glostrup, Denmark; 1:100); and for monocyte/macrophage detection, rat antimouse F4/80 mAb (BMA Biomedicals AG, Augst, Switzerland; 1:20) and rat antimouse CD11b mAb (Chemicon International, Temecula, CA; 1:20). Mes-

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enchymal tumors were diagnosed using goat antihuman vimentin polyclonal antibody (Sigma Chemical Co., St. Louis, MO; 1:40), rabbit antichicken desmin polyclonal antibody (Sigma Chemical Co.; 1:100), and rabbit antihuman factor VIII polyclonal antibody (Dako; 1:200). All of the antibodies were diluted in TBS. Primary antibodies were omitted for negative controls. Tissue sections were incubated in a humidity chamber (overnight; 4°C). After three rinses in TBS, 5 min each, samples were incubated with a secondary antibody. For detection of primary rabbit antibodies, we used biotinylated goat antirabbit IgG (Vector Laboratories, Burlingame, CA; 1:400). For primary rat antibodies, we used biotinylated rabbit antirat IgG (Vector Laboratories), and for primary mouse antibodies, we used biotinylated rabbit antimouse IgG (Vector Laboratories; 1:400). After 30-min incubation with the secondary antibody, tissue sections were incubated with streptavidin conjugated with peroxidase, diluted 1:20 in TBS (Zymed Laboratories, Inc.) for 30 min at room temperature. The chromogen was 3–3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.), diluted 0.002% in TBS, in the presence of 0.01% hydrogen peroxide. Nuclei were counterstained with Harris hematoxylin for 1 min.

RESULTS AND DISCUSSION

Spontaneous Tumor Development in *p21*-null Mice. We have studied the viability of a colony of 73 mice homozygously null for *p21* (8) over a 2-year period. For comparison, we have also followed in parallel a colony of 30 wild-type mice of the same genetic background as the *p21*-null mice (*i.e.,* C57BL6/129Sv 50:50). Only 6 of the 30 wild-type mice (20%) died during the course of this study because of fights or undetermined causes (five cases) and, in one case, because of a thymic lymphoma. In contrast, 100% of the *p21*-null mice died. When the survival curve of males and females were plotted independently, the reduction in viability of *p21*-null mice was more pronounced for females than for males (Fig. 1).

The causes of death for *p21*-null mice have been determined for a total of 151 animals (which include the original cohort of 73 *p21*-null mice, as well as others that were not part of the viability study group). The most significant cause of death among *p21*-null females (60%) was renal failure diagnosed by severe glomerulonephritis at an average age of 9.6 months (Table 1). In comparison, only 26% of males died of glomerulonephritis at an average age of 13.2 months (Table 1). The elevated incidence of glomerulonephritis in *p21*-deficient mice, with increased severity among females, has been reported previously (35) and is attributable to an autoimmune process produced by abnormal proliferation of memory T-cell lymphocytes.

A significant proportion of mice succumbed to tumors (55% of males and 27% of females; Table 1). The higher incidence of tumors among *p21*-null males, however, reflects the lower susceptibility to death by renal failure, and it is not indicative of a male-specific tumor susceptibility. For those *p21*-deficient mice (both males and females) that developed tumors, the average life span was 16 months (Table 1). The latency for spontaneous tumor development in *p21*-null mice is

longer than in *p53*-null mice (5 months; Refs. 36–38), and it is also longer than the latency in knockout mice for *ATM* (3 months; Refs. 39–41) or *p19^ARF* (10 months; Refs. 42, 43). In contrast, the pro-apoptotic protein and p53-transcriptional target, Bax, does not contribute to spontaneous tumor suppression, as deduced from the lack of tumors in *Bax*-deficient mice after a 2-year observation period (44). We conclude that p21 plays a significant role in tumor suppression, which is more important than that of Bax but weaker than that of p53 or its upstream regulators ATM and p19^ARF.

Tumor Spectrum in *p21*-null Mice. We have carried out a detailed histopathological analysis of all of the tumors spontaneously developed by *p21*-null mice ($n = 65$; Table 2). Approximately half of the spontaneous tumors were histiocytic sarcomas (52% of the tumors; Table 2). These tumors were characterized by the presence of macrophages infiltrating the affected organs. Macrophages were identified by their typical morphology, as well as by their reactivity to the cell surface marker F4/80 (Fig. 2A). The most commonly colonized organs were spleen and liver, and occasionally lymph nodes, bone marrow, and uterus. In the spleen, tumor cells grew in the red pulp, forming packed nodular groups of histiocytes. In the liver, tumor cells occupied the sinusoids and surrounded the central vein with a granulomatous pattern, often eroding the walls and invading the lumen (Fig. 2A).

The second most common tumor type in *p21*-null mice was of vascular origin (22%; Table 2). These were mainly hemangiomas characterized by abundant capillaries lined by a single layer of well-differentiated endothelial cells. A small proportion of vascular tumors were hemangiosarcomas (Fig. 2B). These malignant tumors were formed by poorly delineated vascular channels lined by pleomorphic endothelial cells forming solid areas and with frequent mitotic figures. Endothelial cells were further identified by their reactivity to vimentin and factor VIII (see Fig. 2B, *inset*). Both types of vascular tumors, hemangiomas and hemangiosarcomas, preferentially developed in the liver or the spleen.

Table 1 *Contributing causes of death in p21-null mice*

Cause of death	*p21*-null females ($n = 93$)		*p21*-null males ($n = 58$)		Total ($n = 151$)	
	Incidence (%)	Average latency (mo)	Incidence (%)	Average latency (mo)	Incidence (%)	Average latency (mo)
GN[a]	60%	9.6	26%	13.2	43%	11.4
Tumors	27%	13.8	55%	18.2	41%	16.0
Other[b]	13%		19%		16%	

[a] GN, glomerulonephritis.
[b] Fights and undetermined.

Table 2 *Spectrum of spontaneous tumors in p21-null mice*

Type of tumor	No. of tumors			Relative incidence (%)
	In females ($n = 28^a$)	In males ($n = 37^b$)	Total ($n = 65$)	
Histiocytic sarcomas	18	16	34	52.3%
Hemangiomas/h-sarcomas[c]	6	8	14	21.5%
B-cell lymphomas	3	6	9	13.9%
Lung carcinomas	0	3	3	4.6%
Skin tumors[d]	0	2	2	3.1%
Testis tumors[e]	0	2	2	3.1%
Urinary bladder tumor[f]	0	1	1	1.5%

[a] Of a total population of 93 female mice, 25 developed tumors (27%; see Table 1), including 3 that developed two independent tumors, yielding a total of 28 tumors.
[b] Of a total population of 58 male mice, 32 developed tumors (55%; see Table 1), including 5 that developed two independent tumors, yielding a total of 37 tumors.
[c] Eleven benign hemangiomas and three malignant hemangiosarcomas.
[d] One benign keratoachantoma (Fig. 2E) and one sebaceous gland adenoma.
[e] One Leydig cell adenoma and one malignant Leydig cell tumor with lung metastatic nodules (Fig. 2F).
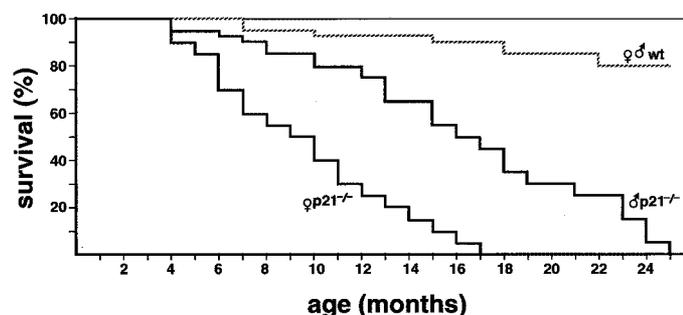[f] Further characterization was not possible because of autolysis.

Fig. 1. Survival of *p21*-null mice. A colony of *p21*-null mice composed of 43 females and 30 males and a colony of wild-type mice (20 females and 10 males), all with the same genetic background C57BL/6;129Sv (50:50), were observed for the indicated period of time. The percentage of live mice was recorded monthly for each group.
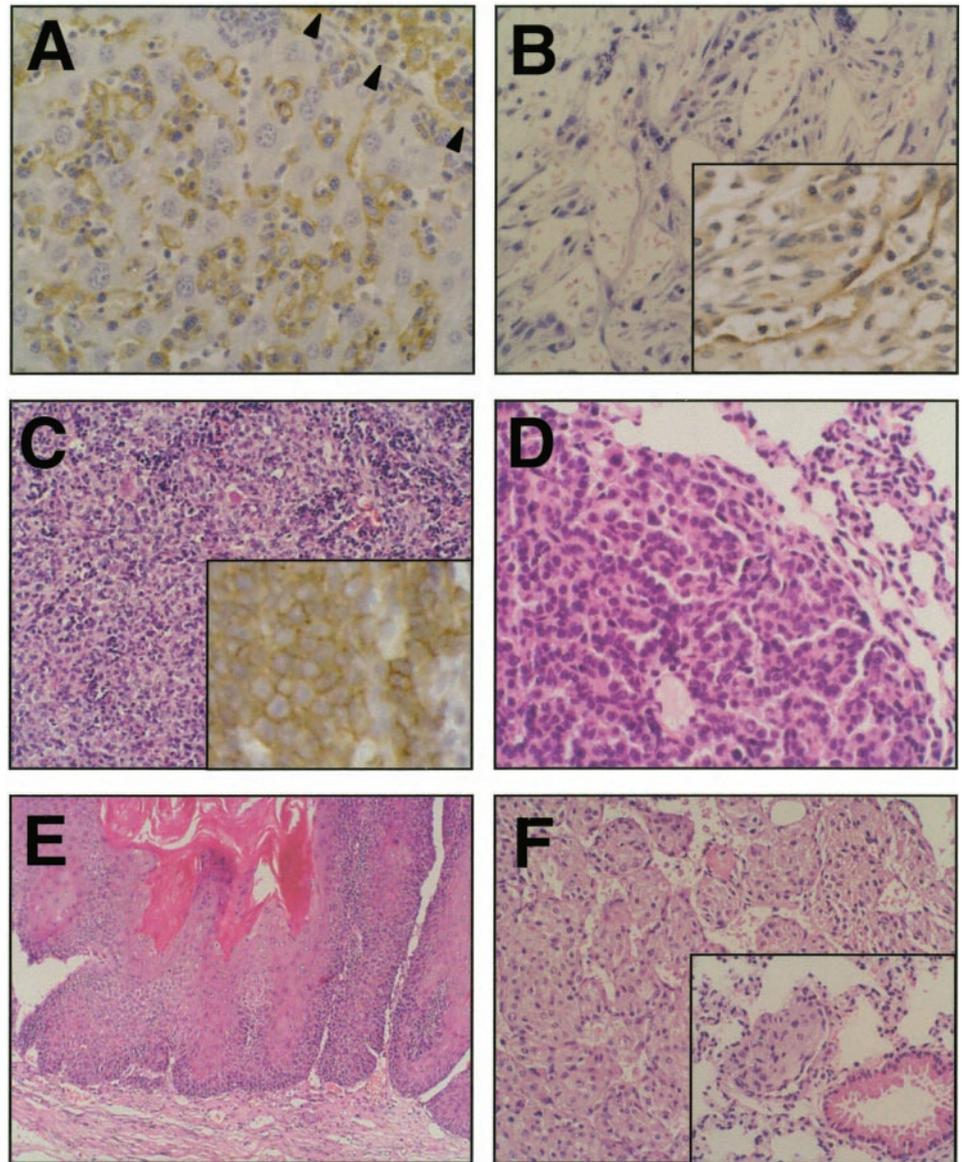
Fig. 2. Histology of spontaneous tumors developed by *p21*-null mice. *A*, histiocytic sarcoma in the liver. Macrophages were labeled with anti-F4/80 (*brown stain*); nuclei were counterstained with hematoxylin. A vein is shown at the *top right* corner with numerous monocytes (*arrowheads* mark the lumen). Original magnification, ×40. *B*, hemangiosarcoma in the spleen. H&E staining shows abundant vessels in the spleen. The inset shows positive immunostaining with anti-Factor VIII antibodies (*brown stain*); nuclei were hematoxylin counterstained. Original magnification, ×40. *C*, follicular B-cell lymphoma in the spleen. H&E staining shows a typical mixture of centroblasts, centrocytes, and small lymphocytes. The inset shows positive immunostaining with CD45R/B220 (*brown stain*); nuclei were counterstained with hematoxylin. Original magnification, ×20. *D*, alveolar/bronchiolar carcinoma. H&E staining. Original magnification, ×40. *E*, keratoacanthoma filled with mature keratin. H&E staining. Original magnification, ×10. *F*, Leydig cell carcinoma. The inset shows a metastatic nodule in the lung (adjacent to a bronchiole). H&E staining. Original magnification, ×20.

Approximately 14% of all of the tumors were lymphomas affecting the lymph nodes and/or spleen, usually with small metastatic foci in liver, lungs, or kidneys (Table 2). All of the lymphomas involved the B-cell lineage and were further classified in most cases as centroblastic-centrocytic or centroblastic follicular B-cell lymphomas (Fig. 2*C*).

The *p21*-null mice also developed epithelial tumors (Table 2). In particular, we observed three lung carcinomas (Fig. 2*D*), two benign skin tumors (Fig. 2*E*), one Leydig cell adenoma of the testis, and one malignant Leydig cell tumor with lung metastatic nodules (Fig. 2*F*). Overall, about 10% of tumors were of epithelial origin (Table 2).

In conclusion, *p21* deficiency results in the spontaneous development of a variety of tumors of hematopoietic, endothelial, and epithelial origin. This phenotype is reminiscent of the rich spectrum of spontaneous tumors developed by *p53*-null and by *p19^ARF*-null mice (36–38, 42, 43), albeit with a longer latency. It is interesting to note that *p21*-deficient mice did not develop T-cell lymphomas, which is one of the most common tumor types in p53-null and p19^ARF-null mice (36–38, 42, 43). Also, mice deficient in the p53-activating kinase ATM develop exclusively spontaneous T-cell lymphomas (39–41).

Radiation-induced Carcinogenesis in *p21*-null Mice. Ionizing radiation is a well-known tumorigenic agent, which preferentially induces thymic lymphomas in mice. The tumor suppressor p53 is critical in the protection of cells against radiation-induced carcinogenesis, most likely through its ability to trigger apoptosis of radiation-damaged cells (45). This is particularly evident in the case of the thymus, which undergoes massive p53-dependent apoptosis after radiation (46, 47). p21 is highly induced by radiation in the thymus in a p53-dependent manner (48), but it is not required for thymocyte apoptosis (9). On the basis of this, we have hypothesized that *p21*-deficient mice should not have an increased susceptibility to radiation-induced tumorigenesis. We have evaluated the susceptibility of *p21*-deficient mice to γ-radiation carcinogenesis. As a control, irradiation of wild-type mice resulted in 100% mortality after a period of 9 months after irradiation, when the mice were 11 months old (Fig. 3 shows the survival curve of the females alone for the purpose of simplifying the discussion below); and mortality was mainly attributable to the development of T-cell lymphomas (Table 3). In contrast, irradiated *p21*-deficient mice survived longer than irradiated wild-type mice, and only one-third (32%) of them developed tumors (Table 3; Fig. 3). As it was the case among irradiated wild-type mice, the predominant tumors induced by radiation in *p21*-null mice were T-cell lymphomas (Table 3). These results clearly indicate that *p21* deficiency does not increase the susceptibility to radiation-induced carci-

nogenesis, but, on the contrary, it provides a certain degree of protection delaying the appearance of thymic lymphomas. This protective effect could be attributable to an enhanced apoptotic response of the *p21*-null cells. Indeed, other investigators have reported previously (32) that the radiation-toxicity syndrome is more severe in *p21*-deficient mice than in wild-type mice, and it is associated to an enhanced apoptotic response of the intestine in *p21*-null mice. We have considered the possibility that radiation could directly result in more apoptosis in the thymus of *p21*-null mice; however, quantitation of sub-(G_0/G_1) thymocytes after acute irradiation (10 Gy; and analysis 3 h after irradiation) has not supported this idea (data not shown). We have also examined the constitutive levels of apoptosis in the thymic lymphomas developed in *p21*-null mice and in wild-type mice after irradiation (Fig. 4). Interestingly, *p21*-null lymphomas exhibited a high level of apoptosis with tangible apoptotic bodies, often surrounded by a narrow halo indicative of phagocytosis (Fig. 4*B*). In contrast, lymphomas from wild-type mice had a uniform morphology with scarce evidence of apoptosis (Fig. 4*A*). We conclude that thymic lymphomas in *p21*-null mice have a significantly higher level of apoptosis compared with that in wild-type mice. Similar conclusions have been reached by other investigators (32) after comparing the levels of constitutive apoptosis in thymic lymphomas of *ATM*-deficient mice *versus ATM/p21*-deficient mice. Also, lymphocytes in the spleen of *p21*-deficient mice have a high level of constitutive apoptosis in comparison with wild-type mice (49). All together, it appears that the absence of p21 entails a higher degree of apoptosis and, consequently, a growth disadvantage that can explain the delayed incidence of radiation-induced thymic lymphomas in *p21*-deficient mice.

It is worth pointing out that irradiated *p21*-deficient mice survived even longer than nonirradiated *p21*-deficient mice (Fig. 3). This can
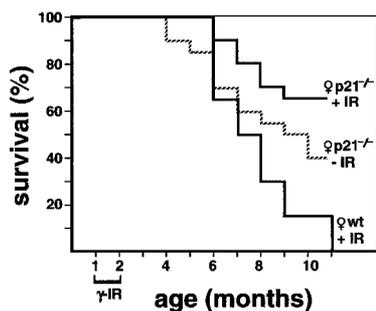

Fig. 3. Survival of *p21*-null female mice after γ-radiation. One-month-old wild-type (*n* = 6) and *p21*-null (*n* = 18) female mice were irradiated (+IR) weekly for 4 weeks with 1.75 Gy. All of the mice were on a similar mixed genetic background, C57BL/6;129Sv (50:50). After the first radiation dose, the percentage of live mice was recorded monthly for each group. As control, the survival of nonirradiated (−IR) *p21*-null female mice is also shown.
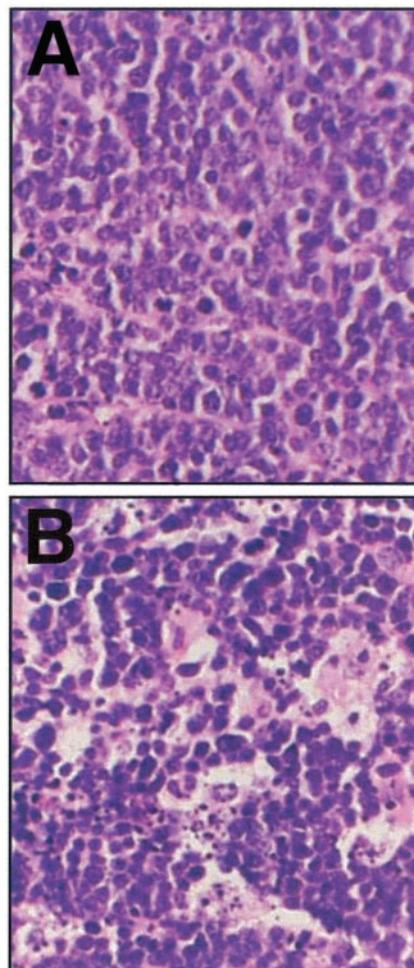

Fig. 4. Radiation-induced thymic lymphomas in *p21*-null mice exhibit increased levels of constitutive apoptosis. Radiation-induced thymic lymphomas from a wild-type mouse (*A*) and from a *p21*-deficient mouse (*B*). *p21*-deficient lymphomas (*n* = 2) presented a high level of apoptosis, compared with wild-type lymphomas (*n* = 3). H&E staining. Original magnification, ×20.

be attributed to the complete absence of deaths by autoimmunity-related glomerulonephritis among the irradiated *p21*-null mice, both males and females (Table 3). This phenomenon is in agreement with the well-established observation that radiation, by eliminating autoreactive memory lymphocytes, delays the progression of autoimmune processes (50, 51).

Concluding Remarks. In this study, we have performed a detailed analysis of the survival and tumor susceptibility of *p21*-deficient mice. Our observations demonstrate that p21 contributes to tumor suppression, albeit to a lesser extent than p53. Notably, *p21*-null mice develop a variety of neoplasias, which include tumors of hematological, endothelial, and epithelial origin. Interestingly, *p21*-deficient mice did not develop T-cell lymphomas, which is one of the most common tumor types in *p53*-null mice (36–38). The development of spontaneous tumors in *p21*-null mice is in contrast to *Bax*-deficient mice, which do not develop tumors even after 2 years of age (44). We also show that in the specific case of radiation-induced carcinogenesis, *p21* deficiency does not accelerate tumor development. On the contrary, *p21* deficiency delays the onset of radiation-induced T-cell lymphomas, which is in line with the delay in the appearance of spontaneous T-cell lymphomas produced by *p21* deficiency in *ATM*-null mice (32). It can be proposed that p21 contributes to mediate p53-dependent tumor suppression in some tumor types, which would include histiocytic sarcomas, hemangiomas, B-cell lymphomas, and lung carcino-

Table 3 *Spectrum of γ-radiation-induced tumors in p21-null mice*

Mortality (9 months after irradiation)	Irradiated wild type (*n* = 16[a])	Irradiated *p21*-null (*n* = 22[b])
Dead with tumors	13 (81%)[c]	7 (32%)
	7 T-cell lymphomas[d]	5 T-cell lymphomas[d]
	3 hemangiomas/h-sarc	1 hemangioma/h-sarc
	2 ovarian tumors	1 pituitary carcinoma
	1 fibrosarcoma	
	1 rhabdomyosarcoma	
	1 lung carcinoma	
Cause of death unknown	3 (19%)	0 (0%)
Alive after 10 months	0 (0%)	15 (68%)

[a] 6 females and 10 males.
[b] 18 females and 4 males.
[c] Two mice had two independent tumors.
[d] Thymic or multicentric.

mas, but not in other tumor types, particularly spontaneous or radiation-induced T-cell lymphomas.

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