DNA Double-Strand Breaks, p53, and Apoptosis during Lymphomagenesis in scid/scid Mice

Kay E. Gurlay, Khoa Vo, and Christopher J. Kemp
Fred Hutchinson Cancer Research Center, Seattle, Washington 98109-1024

ABSTRACT

The tumor-suppressing phenotype of p53 is thought to be due to its accumulation in response to DNA damage and resultant cell cycle arrest or apoptosis. scid/scid mice are defective in DNA double-strand break repair due to a mutation in DNA-dependent protein kinase (DNAPK). Treatment of scid/scid mice with γ radiation or N-ethyl-N-nitrosourea resulted in ~86% incidence of T-cell lymphomas, compared with ~6% in wild-type mice. The incidence of other tumor types was not increased in scid/scid mice, suggesting that the types of DNA double-strand break that are unrepaired in these mice are not strongly carcinogenic. To determine whether mutations in DNAPK and p53 interact, we examined mice deficient in both genes. Both scid/scid p53−/− and scid/scid p53+/− mice spontaneously developed lymphomas at shorter latency than did mice with either defect alone. Loss of the wild-type p53 allele was observed in 100% of tumors from scid/scid p53+/− mice, indicating strong selection against p53. In contrast, p53 was not inactivated in lymphomas from scid/scid p53+/+ mice. Exposure of these tumor-bearing mice to γ radiation resulted in p53 protein accumulation and high levels of apoptosis in all tumors that were not observed in tumors from scid/scid p53+/− mice. Thus, there was a bifurcation of molecular pathways to tumorigenesis. When p53 was heterozygous in the germ line, loss of the wild-type allele occurred, and the tumors became apoptosis resistant. When p53 was wild type in the germ line, p53 was not inactivated, and the tumors remained highly apoptosis sensitive.

INTRODUCTION

The p53 tumor suppressor protein appears to be a central coordinator of the cellular response to DNA damage. The levels of p53 protein are normally very low, but they increase in some cell types following exposure to DNA-damaging agents (1). DNA dsbs1 constitute a major class of DNA lesions that lead to p53 accumulation (2). Some but not all cell types respond to these increased levels of p53 by arresting in G1 phase of the cell cycle or by undergoing apoptosis (1, 3, 4). Cells with nonfunctional p53 fail to undergo either response, indicating that p53 is required (3, 5–7). Increased genetic instability is also observed in cells lacking functional p53, and these properties are believed to be central to its role as a tumor suppressor gene (8–12).

In the majority of animal or human tumors that have sustained p53 mutations, the nature of the selective force against p53 function is not known. In mice, significant accumulation of p53 protein in response to whole-body γ radiation is limited to thymic and splenic lymphocytes, osteocytes, a subset of keratinocytes, epithelial cells in the bottom of the small intestinal crypts, and, to a lesser extent, some other tissues (1, 3, 4, 13). However, most cells in the intact animal do not accumulate immunohistochemically detectable p53, and they do not undergo G1 arrest or apoptosis in response to DNA damage. It is not known whether tissues that cannot induce p53 select against p53 during tumorigenesis or whether p53 is selected against only in those tissues that are capable of accumulating high levels of p53.

To address the roles of the DNA damage-p53 accumulation-apoptosis pathway in tumorigenesis, we elected to study the scid/scid mutant mouse. These mice are defective in DNA dsb repair, and as a consequence, their cells are radiosensitive and developing lymphocytes cannot complete V(D)J antigen receptor gene rearrangement. The latter leads to maturation arrest of developing lymphocytes and absence of functionally mature T and B lymphocytes (14). The genetic defect in these mice was recently identified as a mutation in the gene encoding the DNAPKcs (15). This large protein was originally identified biochemically as a serine-threonine kinase that required double-stranded DNA ends for its activity. Activated DNAPK phosphorylates many proteins in vitro, although the relevant in vivo targets have yet to be identified. It is not known precisely how DNAPK participates in dsb repair, but following the generation of free DNA ends during V(D)J recombination or following DNA damage, the proteins Ku70 and Ku80 bind to the ends and recruit DNAPKcs, leading to its activation and subsequent rejoining of the DNA ends (16). The DNA dsb repair defect appears to exist in all cells of scid/scid mice because myeloid cells, fibroblasts, intestinal crypt cells, epithelial cells, spermatogonial stem cells, and fibrosarcoma cells have all been shown to exhibit increased radiation sensitivity (17–20).

The evidence described above, notably, the DNA dsb connection, predicts a functional relationship between DNAPK and p53. Although p53 protein is phosphorylated by DNAPK in vitro, p53 induction, G1 cell cycle arrest, and apoptosis in response to DNA damage all occur normally in SCID cells and scid/scid mice, indicating that DNAPK is not a required regulator of these responses (13, 21, 22).

In scid/scid mice, developing T lymphocytes are arrested at the double-negative CD4−CD8− stage due to the failure to complete T-cell receptor rearrangement (23). When scid/scid mice were crossed to p53 knockout mice to generate scid/scid p53−/− mice, CD4−CD8− cells were detected, indicating that p53 participates in the maturation arrest of scid T-lymphocyte precursors, and in the absence of p53, some cells are able to progress to the double-positive stage (23, 24). Additionally, mutations in p53 and DNAPKcs were shown to interact during tumorigenesis because scid/scid p53−/− mice developed lymphomas with reduced latency, as compared to either scid/scid or p53−/− mice alone (22, 23).

These results are consistent with a model in which p53 would respond to unrepairable dsbs in SCID lymphocytes and block maturation by cell cycle arrest or apoptosis (22, 23). In the absence of p53, these cells would survive and, thus, be at much greater risk for transformation. On the basis of the genetic interaction between DNAPK and p53 and current understanding of p53 function, one might expect a strong selective pressure to mutate p53 during tumorigenesis in scid/scid mice. Here, we show that spontaneous lymphomas rapidly developed in scid/scid p53+/− mice, and all these tumors had lost the remaining wild-type p53 allele, confirming this prediction. In contrast, all lymphomas examined from scid/scid p53+/+ mice retained the DNA damage-p53 accumulation-apoptosis pathway. Presumably, mutation or inactivation of a second, functionally unre-
lated pathway that did not involve loss of the DNA damage-apoptosis response was favored if p53 was wild type in the germ line.

MATERIALS AND METHODS

Tumor Induction. Balb/cByJSnn scid/J, C3HSmn.C-scid/J, Balb/cByJ, and C3H mice were purchased from The Jackson Laboratory and p53 deficient mice were obtained from Larry Donehower (25) and bred in-house. All scid/scid mice were maintained in germ-free microisolator cages and fed autoclaved food and water ad libitum. Balb/cByJSnn scid/J mice were crossed to C3HSmn.C-scid/J to generate experimental C3C F, scid/scid/scid mice. Similarly, Balb/cByJ mice were crossed to C3H mice to generate C3C F, wild-type controls. For the radiation experiment, mice were exposed between 24 and 48 h after birth to 1 Gy (100 rad) of whole-body γ radiation from a 137Cs source at a dose rate of 330 cGy/min. For the ENU experiment, mice were injected at 12–14 days of age with ENU i.p. (Sigma Chemical Co., St. Louis, MO; 0.5 µg/g body weight) dissolved in trioctanoin (Arcos). Mice were observed daily and sacrificed when they showed signs of tumor development. To generate double-deficient mice, C3HSmn.C-scid mice were crossed to C57BL/6J p53−/− mice to generate C3B6 F, scid/+ p53−/+ mice, which were intercrossed to generate F2 mice. The scid/scid p53−/+ mice from this cross were crossed again to generate scid/scid mice of all three p53 genotypes; thus, they are of mixed C3H and C57BL/6J background. The scid/scid mice were identified by examining peripheral blood smears for mature lymphocytes, and p53 genotype was determined by PCR analysis of toe DNA (26).

Tumor Analysis. Tumor tissue was both frozen and fixed in formalin for routine processing and staining with H&E. In vivo apoptotic index was determined by irradiating tumor-bearing mice with 4 Gy of γ radiation, sacrificing the mice at several time points, and preparing tissues as above. The apoptotic index was the mean value of observed apoptotic bodies from three ×40 microscope fields. In most tumors, the number of apoptotic bodies was very similar throughout the entire tumor section. There were ~1000 cells per ×40 field, so an apoptotic index of 1000 indicates a virtually complete apoptotic response. p53 immunostaining was performed as described previously using anti-p53 CM-5 antibody (Novacastra Labs), visualized with 3,3′-diaminobenzidine (Sigma) and NiCl₂, and counterstained with methyl green (13). Lymphoid immunostaining was performed by cutting 6-µm frozen tumor sections, staining with primary antibody to CD3 (Serotec) for T-cell identification or CD45R (PharMingen) for B-cell identification, and visualizing with antirat FITC (Caltag). LOH of p53 in tumors was performed by Southern blot analysis as described (26), except that the probe was labeled with digoxigenin, followed by anti-digoxigenin alkaline phosphatase (Boehringer Mannheim).

RESULTS

scid/scid Mice Are Prone to Radiation-induced T-Cell Lymphomas but not Other Tumor Types. scid/scid mice have only been reported to show predisposition to T-cell lymphoma development (19, 23, 27, 28). This very narrow tissue specificity for a panorganismal DNA repair defect prompted us to examine radiation and carcinogen-induced tumor susceptibility. We argued that treating scid/scid mice with broad-range carcinogens such as radiation (29) and ENU (30) might reveal other tissues that were predisposed.

Sixty-four C3CF, scid/scid/J mice (Balb/cByJSnn scid/J × C3HSmn.C-scid/J F1, referred to as scid/scid mice) were exposed to a single dose of 1 Gy of γ radiation within 48 h of birth. Fifty-five of 64 (86%) of the mice developed thymic or disseminated lymphomas with a very short latency (Fig. 1). The tumor mass was most often within the thymus, with occasional spleen and lymph node involvement and metastasis to liver, kidneys, or lungs. Seven of seven tumors tested were of T-cell origin, as determined by anti-CD3 immunofluorescence (data not shown). No other tumors types were observed. For nine of the mice that became sick or died during the experiment, the cause of death could not be ascertained by necropsy. This exclusive T-cell lymphoma susceptibility is similar to published reports for scid/scid mice (19, 27, 28, 31).

The irradiated scid/scid mice may have been predisposed to tumorigenesis in other tissues, but this was not observed because they developed lymphomas at an early age. This predisposition might have been revealed had they had lived longer. Seven of 10 (70%) untreated C3CF, scid/scid mice, which were concurrently maintained in our colony, developed lymphoma. Full necropsy of those mice revealed only one liver tumor and one lung tumor, although some of these mice lived up to 20 months of age. Five of 23 untreated scid/+ control mice, also observed for 20 months, developed lung tumors, and 1 developed a liver tumor. Thus, scid/scid mice are only predisposed to spontaneous lymphomagenesis.

scid/scid Mice Are Prone to ENU-induced T-Cell Lymphomas but not Other Tumor Types. ENU is a broad-spectrum carcinogen, inducing tumors of the lung, liver, and elsewhere (30) and is thought to induce tumors by causing point mutations in target oncogenes (32). We wished to determine whether the scid defect would cooperate with ENU mutagenesis to increase these or other tumor types or their degree of malignant progression. We treated 64 C3CF, scid/scid/J and 51 wild-type control C3CF, (Balb/cByJ × C3H F1) mice with ENU at 12 days of age. Unexpectedly, 59 of 64 (92%) of the ENU-treated scid/scid mice developed lymphomas with a very short latency (Fig. 1). Significantly, the tumor induction kinetics were indistinguishable from the irradiated scid/scid mice. Upon gross and microscopic examination, the ENU-induced lymphomas were very similar in appearance to those from the irradiated mice, primarily occupying the thymus and occasionally involving the spleen and lymph nodes. Seven of seven of the tumors were CD3⁺, indicating that, as above, the tumors were of T-cell origin. Only 3 of 51 (6%) of the ENU-treated wild-type controls developed lymphomas during the course of the study. In the longer surviving ENU-treated scid/scid and wild-type animals, a number of lung and liver tumors were observed. scid/scid mice sacrificed between 20–30 weeks of age averaged 1 lung tumor per animal (52 tumors in 53 mice), whereas wild-type mice sacrificed between 20 and 36 weeks of age averaged 1.4 lung tumors per animal (32 tumors in 23 mice). This indicated that the SCID defect did not enhance lung tumor development. Because the liver tumor multiplicity increased sharply with age and the mice were sacrificed at different ages, it was not possible to directly compare between groups.

Spontaneous B-Cell Lymphomagenesis in scid/scid p53−/− Mice Is Accelerated. Appropriate crosses were set up to generate scid/scid p53−/−, scid/scid p53+/−, and scid/scid p53+/+ mice on a mixed C3HSmn.C × C57BL/6J genetic background. Eleven of 11
LYMPHOMAGENESIS IN SCID MICE

(100%) of the scid/scid p53—/- mice developed lymphomas with very short latency (Table 1). The median age to tumor appearance was 8 weeks, and all of the mice had succumbed by 15 weeks of age. The tumor mass was located in the thymus, spleen, or lymph nodes with frequent metastasis to liver, kidneys, or lungs. Tumor latency was longer in the single mutants: only 9 of 18 (50%) of the scid/scid p53+/+ mice from the above cross developed lymphomas by 40 weeks of age. Single mutant p53—/- mice developed lymphomas and sarcomas, as well as a number of other tumor types with a median age of >20 weeks (25, 33). Although those p53—/- mice were not on the same genetic background as the scid/scid mice described here, the tumor induction kinetics of p53—/- mice has not significantly varied between different laboratories and on different genetic backgrounds (25, 33-36). Thus, tumor latency was decreased in the double-deficient scid/scid p53—/- mice relative to single-mutant scid/scid or p53—/- mice. These results are in close agreement with results from two other laboratories (22, 23), attesting to the robustness of the DNA-PK-p53 interaction.

In contrast to the almost exclusive T-cell origin of lymphomas from scid/scid or p53—/- mice (19, 22), five of six lymphomas examined from scid/scid p53—/- mice were of B-cell origin, as determined by anti-CD45R immunofluorescence (data not shown). Guidos et al. (23) also observed primarily pre-B cell lymphomas in scid/scid p53—/- mice.

Spontaneous T-Cell Lymphomagenesis in scid/scid p53+/- Mice Is Accelerated and There Is Selection for Loss of p53 Function. Seventeen of 51 (33%) of the scid/scid p53+/+ mice spontaneously developed lymphomas by 20 weeks of age versus only 2 of 18 (11%) of the scid/scid p53+/+ mice (Table 1). In total, 40 of 51 (78%) of the scid/scid p53+/+ mice developed lymphomas by 40 weeks of age, in contrast to 9 of 18 (50%) of the scid/scid p53+/+ mice. The median age for tumor development in p53+/- mice was >50 weeks of age (33, 37). Thus, a 50% reduction inp53 gene dosage also interacted with the scid defect to increase lymphomagenesis. Seven of eleven of these lymphomas were CD3+, indicating T-cell origin, similar to the scid/scid p53+/- tumors.

Tumor DNAs were analyzed by Southern blot to determine the fate of the wild-type p53 allele. Thirty of 30 (100%) of these tumors showed LOH of the wild-type allele (Fig. 2). Most tumors showed nearly complete LOH, whereas two showed partial LOH, indicating an evolutionary intermediate stage.

Spontaneous or Induced T-Cell Lymphomagenesis in scid/scid p53+/- Mice Does Not Select for Loss of p53 Function. A prediction from the above results was that, during tumorigenesis in scid/scid p53+/- mice, there would also be strong selective pressure in favor of mutant p53, leading to functional inactivation of p53 in tumors. We examined p53 function in lymphomas from scid/scid mice of all three p53 genotypes by measuring γ radiation-induced p53 accumulation and apoptosis. Tumor-bearing mice were exposed to 4 Gy of γ radiation and sacrificed 4 h later, and apoptotic index was determined. Apoptotic index is the number of apoptotic bodies per 400 microscope field. Data points, individual tumors from individual mice. Apoptotic index was very low in all but two untreated tumors from scid/scid mice, regardless of p53 genotype (A). Radiation increased apoptosis only in scid/scid p53+/- tumors; little or no increase was seen in scid/scid p53-deficient tumors (B).

Table 1 Spontaneous lymphoma incidence in scid/scid p53-deficient mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age (weeks)</th>
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<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>scid/scid p53—/-</td>
<td>9/11 (82%)</td>
</tr>
<tr>
<td>scid/scid p53+/-</td>
<td>1/51 (2%)</td>
</tr>
<tr>
<td>scid/scid p53+/+</td>
<td>9/18 (9%)</td>
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that were recorded as unirradiated showed a virtually complete apoptotic response, similar to those in the irradiated groups, which could be a physiological reaction in these particular animals, due to, for example, tumor hypoxia. At 1, 2, or 4 h postirradiation, lymphoma cells from all 23 scid/scid p53+/- mice examined showed nuclear p53 protein accumulation (Fig. 4D). By 4 h, 22 of 22 lymphomas from irradiated scid/scid p53+/- mice showed very high levels of apoptosis, as compared to lymphomas from unirradiated mice (Figs. 3 and 4C).

In sharp contrast, all 10 irradiated tumors examined from scid/scid p53—/- or scid/scid p53+/- mice showed very little or no induction of apoptosis over background levels (Figs. 3 and 4D). p53 protein was largely undetectable in irradiated scid/scid p53+/- tumors, which is consistent with the results above showing loss of the wild-type p53 allele. Two tumors showed a mixed population, with distinct, focal areas of high apoptosis and others of low apoptosis. In serial sections of these tumors, focal areas of p53 protein expression (Fig. 4H) or no
cells with complete loss of p53, which is supported by the partial LOH expression (Fig. 4F) were observed that spatially coincided with areas showing little apoptosis and mitotic recovery.

**DISCUSSION**

**DNA dsbs and Tumorigenesis.** Indirect evidence indicates that the mutagenic and carcinogenic effects of γ radiation are mediated by DNA dsbs. Because scid/scid mice have a panorganismal defect in DNA dsb repair, they provide a useful model to test the role of radiation, dsbs, and cancer. Cumulative observations from two laboratories and this study indicate that, of >130 irradiated scid/scid mice, none developed malignancies other than lymphomas, and all of those examined had a T-cell origin. Thus immature T lymphocytes are uniquely sensitive to spontaneous or radiation-induced tumorigenesis due to DNA PK deficiency, whereas other cell types are not.

To further address this very narrow tissue predisposition, we treated scid/scid mice with ENU, an alkylating agent and broad-range carcinogen. As with radiation, nearly 100% of these mice developed lymphomas, and again, other tumor types were not increased. Thus, the SCID dsb defect does not cooperate with ENU-mediated tumorigenesis in most tissues, with the marked exception of lymphomagenesis. The almost identical tumor induction kinetics with radiation and ENU (Fig. 1) was remarkable and implies that both treatments, despite their different mechanisms of mutagenesis, induced similar events with similar frequency.

The methylating agent MNU has a slightly different mutagenic spectrum than does ENU and also induces lymphomas at high frequency in scid/scid mice. Why are immature T lymphocytes, which lack functional DNA PK, uniquely predisposed to transformation by these diverse agents?

Lymphocyte precursors are the only somatic cell type to undergo large-scale programmed genetic rearrangements, which are initiated by the Rag-1 and Rag-2 gene activities. These free DNA ends, normally rejoined by DNA PK, would remain unjoined in scid/scid mice and, perhaps, increase illegitimate recombination with oncogenic targets. In fact, chromosomal translocations linking antigen receptor genes to proto-oncogenes are frequently observed in lymphoid malignancies.

Additionally, the lymphocyte maturation arrest or other unknown phenotypes of DNA PK deficiency may play a role. For example, in addition to inducing dsbs, treatment of scid/scid mice with radiation or MNU partially rescued lymphocyte maturation, resulting in the rapid appearance of CD4+CD8+ thymocytes and increased thymic cellularity (24, 28). Interestingly, this effect was specific to T cells because B cell maturation was not affected. If ENU induced a similar response, this maturation rescue might contribute to the unique T-cell predisposition in irradiated, ENU- and MNU-treated scid/scid mice.

p53 might also contribute to the lack of a generalized tumor predisposition in scid/scid mice. However, the only tumor type observed in scid/scid p53−/− mice was lymphoma, mostly pre-B cell lymphoma (Refs. 22 and 23 and this study). Thus, generalized tumor suppression by p53 does not explain the narrow tumor spectrum of scid/scid mice.

Finally, we have shown that scid/scid mice are not predisposed to two stage chemical carcinogenesis of the skin or to chemically induced liver tumors. Taken together, these results indicate that unrepaired dsbs due to DNA PK deficiency in scid/scid mice is not a major predisposing factor for tumorigenesis in most tissues. SCID cells are able to repair a subset of radiation-induced dsbs, perhaps due to homologous recombination or other repair pathways (16). Perhaps these alternative repair pathways are critical for dsb-mediated carcinogenesis.

**Bifurcation of Molecular Pathways to Tumorigenesis Depending on p53 Germ-Line Status.** We and others (22, 23) observed a decrease in tumor latency in scid/scid p53−/− mice. We also observed a very high frequency of LOH of p53 in tumors from scid/scid p53−/− mice and consequent loss of DNA damage-induced apoptosis. The 100% frequency of LOH is higher than that seen in spontaneous lymphomas from p53−/− mice, in which the frequency is only 55–70% (33, 37). Thus, the DNA PK defect increases the frequency of LOH of p53 in tumors, and this proves that there is very strong...
selective pressure against p53 function during lymphomagenesis on a scid/scid mutant background. This also implies that loss of the DNA damage-p53-apoptosis pathway might be a critical rate-limiting step for lymphomagenesis in these mice.

However, in scid/scid p53+/+ mice, this pathway was not inactivated. Despite the fact that these tumors arose very rapidly and were highly malignant, they remained exceedingly radiation sensitive, with many showing a virtually complete apoptotic response. Apparently, in these mice, tumors evolved down an entirely different molecular pathway, which did not involve p53 or other components of the DNA damage-apoptosis response. Future studies will be directed toward identifying components of this alternate rate-limiting pathway, which may involve other mechanisms of apoptosis, for example, those related to c-myc or bcl-2, or may not involve deregulated apoptosis.

The difference in frequency of inactivating p53 in tumors from scid/scid p53+/+ versus scid/scid p53+/+ mice could be trivially explained by the fact that one p53 allele was already lost in all somatic cells in the former mice. Complete loss of p53 in tumors would occur at a frequency related to the spontaneous rate of loss of the second allele and subsequent clonal selection. In the scid/scid p53+/+ mice, both alleles would have to be inactivated within the same cell lineage, which would occur with much lower probability. In this model, loss of either allele would occur at equal probability. However, the very large difference in the frequency of p53 loss suggests another model in which loss of the second p53 allele occurs at higher probability than loss of the first allele, due to p53 haploinsufficiency. We previously showed by karyotype analysis of bone marrow cells taken directly from untreated mice that only 2% of the cells from wild-type mice were aneuploid, compared to 26% from p53+/− mice and 52% from p53−/− mice (11). These aberrations were not clonal, in that each karyotype differed between cells examined, indicating that a 50% reduction in p53 gene dosage resulted in significantly increased karyotypic instability. This haploinsufficient phenotype provides a plausible mechanism whereby a mutation in one p53 allele would increase the probability of loss of the remaining wild-type allele during tumor cell evolution.

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