V(D)J recombination activates a p53-dependent DNA damage checkpoint in scid lymphocyte precursors

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Double-stranded DNA breaks (DSBs) trigger p53-mediated cell cycle arrest or apoptosis pathways that limit the oncogenic consequences of exposure to genotoxic agents, but p53-mediated responses to DSB generated by normal physiologic events have not been documented. "Broken" V(D)J coding ends accumulate in scid lymphocyte precursors as a consequence of a mutation in DNA-dependent protein kinase (DNA-PK). The ensuing failure to rearrange efficiently antigen receptors arrests lymphoid development. Here we show that scid thymocytes express high levels of p53 protein, attributable to recombinase activating gene (RAG)-dependent generation of DSB adjacent to V, D, and J gene segments. To examine the functional importance of p53 expression in vivo, we bred p53-/− scid mice. The absence of p53 facilitated production of in-frame V(D)Jβ coding joints and developmental progression of scid thymocytes, in addition to a dramatic accumulation of pro-B cells. All mice developed disseminated pro-B or immature T cell lymphoma/leukemia by 7–12 weeks of age. We present evidence that p53 deficiency prolongs the survival of scid lymphocyte precursors harboring broken V(D)J coding ends, allowing the accumulation of aneuploid cells. These results demonstrate that a p53-mediated DNA damage checkpoint contributes to the immune deficiency characteristic of the scid mutation and limits the oncogenic potential of DSBs generated during V(D)J recombination.

[Key Words: DNA repair; scid; V(D)J recombination; protein kinase; p53 protein]

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The p53 tumor suppressor gene is a tetrameric transcription factor that plays a critical role in transducing a signal from damaged DNA to genes that control the cell cycle and apoptosis. Treatment of cells with DNA-damaging agents, such as ionizing or UV radiation, causes rapid accumulation of p53 by a post-transcriptional mechanism involving stabilization of p53 protein (for review, see Cox and Lane 1995). In addition to DNA damage, both types of radiation activate a wide spectrum of stress response pathways. However, recent studies show that small numbers of double-stranded DNA breaks (DSBs) provide a sufficient signal for p53 induction [Lu and Lane 1993; Nelson and Kastan 1994; Huang et al. 1996]. DNA damage-induced accumulation of p53 protein can cause cell-cycle arrest through p53-mediated transcriptional activation of the cyclin-dependent kinase inhibitor p21waf1/cip1 [for review, see Ko and Prives 1996]. In addition, DNA damage can induce p53-dependent apoptosis by transcriptional activation of death genes such as BAX, as well as by transcription-independent mechanisms (for review, see White 1996).

The importance of p53 functions in regulating the cell cycle and apoptosis in response to DNA damage arising from normal physiological processes, rather than from treatment with genotoxic mediators, has not been established. Embryonic and postnatal development proceeds normally in p53−/− mice [Donehower et al. 1992; Jacks et al. 1994], suggesting that p53 does not perform essential functions during DNA replication and cell differentiation. However, the crucial role of p53 in regulating DNA damage-induced cell cycle checkpoints and apoptosis is underscored by the observation that the p53 gene is mutated or deleted in the majority of spontaneous human malignancies, as well as in the inherited Li-Fraumeni cancer syndrome [Malkin et al. 1990]. In addition, mice lacking functional p53, because of overexpression of a dominant negative p53 transgene or to germ-line disruption of both p53 alleles, are highly tumor-prone.
V(D)J recombination activates a p53 checkpoint


A unique feature of lymphocyte development that may explain the sensitivity to p53 action is the site-specific DNA recombination process that assembles antigen receptor variable genes from dispersed coding segments. During this process, two recombinase-activating genes, RAG-1 and RAG-2, generate DSB at conserved recombination signal sequences (RSS) that flank V, D, or J coding segments (Mombaerts et al. 1992b; Shinkai et al. 1992, McBlane et al. 1995). Accumulating evidence suggests that lymphocyte precursors undergoing V(D)J recombination are arrested in the G0/G1 phase of the cell cycle (Lin and Desiderio 1995), presumably to minimize the chance of aberrant V(D)J joining events during the proliferative burst that occurs during lymphocyte development. It is not known, however, whether this cell cycle regulation occurs as a consequence of p53-mediated detection of V(D)J-specific DSB. Chromosomal translocations that juxtapose antigen receptor gene segments and proto-oncogenes are a common feature of lymphoid malignancies, attesting to the oncogenic potential of abnormal V(D)J recombination (Rabbitts 1994).

The DNA-dependent protein kinase (DNA-PK) regulates a DSB repair activity required for site-specific V(D)J recombination. Recent studies have shown that DNA-PK belongs to a subfamily of PI-3 kinase-related genes (Hartley et al. 1995) known to regulate DNA damage-induced cell cycle checkpoints (Keith and Schreiber 1995; Zakian 1995). These include RAD3 and MEC1 in yeast, mei-41 in Drosophila, and the ataxia telangiectasia (ATM) gene in humans. DNA-PK was first identified biochemically as a nuclear serine/threonine kinase with the unusual property of requiring the presence of DNA ends for catalytic activity (Lees-Miller and Anderson 1989; Carter et al. 1990; Lees-Miller et al. 1990). The large (≈460 kD) catalytic subunit, DNA-PKcs, is targeted to DNA ends by association with the Ku70/Ku80 heterodimer (Gottlieb and Jackson 1993). Recent studies have mapped the murine scid mutation (Bosma and Carroll 1991) to the DNA-PKcs gene (Blunt et al. 1995; Kirchgesner et al. 1995; Peterson et al. 1995). This mutation confers a generalized DSB repair defect that renders all cell lineages more sensitive to the effects of DSB-inducing agents (Fulop and Phillips 1990; Biedermann et al. 1991; Hendrickson et al. 1991) and also impairs V(D)J recombination (Lieber et al. 1988; Malynn et al. 1988).

In contrast to normal lymphocyte precursors, V(D)J coding ends accumulate in scid lymphocyte precursors (Roth et al. 1992a,b; Zhu and Roth 1995) and are rejoined inefficiently (Lieber et al. 1988; Malynn et al. 1988; Blackwell et al. 1989). The profound immunodeficiency in scid mice (Bosma and Carroll 1991) is thought to reflect the absence of immunoglobulin μ (Igμ) and T cell receptor β (TCRβ) containing pre-B and pre-T cell receptor complexes that transmit essential signals for developmental progression of B or T cell progenitors (von Boemer 1994). This is consistent with the demonstration that genetic disruption of either RAG-1 or RAG-2 arrests T and B cell development at the same stage as the arrest caused by the scid mutation (Mombaerts et al. 1992b, Shinkai et al. 1992). Introduction of functionally rearranged TCRβ transgenes, however, completely restores normal lymphocyte development in RAG-1- or RAG-2-deficient mice (Mombaerts et al. 1992a; Shinkai et al. 1993, Spanopoulou et al. 1994, Young et al. 1994), whereas introduction of receptor transgenes into scid mice only partially restores lymphocyte development (Reichman-Fried et al. 1990; Kishi et al. 1991; Shores et al. 1993). A recent study suggests that transgene-mediated rescue of lymphocyte development is incomplete in scid mice because of the potentially lethal effects of continuing attempts to rearrange endogenous receptor loci (Chang et al. 1995).

These considerations prompted us to investigate whether p53 might be operative in detection and management of DSB that arise during the process of V(D)J recombination, as this site-specific DNA damage potentially could contribute to oncogenesis. We have addressed the role of p53 in the context of V(D)J recombination in normal and scid lymphocyte precursors, which differ in their ability to resolve rapidly V(D)J-specific DSB into coding joints. We report that p53 protein is undetectable in normal thymocytes undergoing V(D)J recombination, and is dispensable for the cell cycle arrest that occurs in these cells. In contrast, we show that defective DNA-PK function in scid thymocytes results in high levels of p53 protein expression as a consequence of V(D)J-specific DSB. To examine the functional importance of p53 expression in scid lymphocyte precursors, we bred p53−/− scid mice. Analysis of these double-mutant mice indicates that a p53-mediated DNA damage checkpoint can be activated during site-specific V(D)J recombination to limit survival of precursors with unresolved V(D)J breaks, thus minimizing the oncogenic potential of abnormal V(D)J recombination.

Results

RSS-specific DSB induce p53 expression in scid lymphocyte precursors

We first sought to determine whether introduction of DSB adjacent to V(D)J coding segments causes accumulation of p53 protein in wild-type (WT) or scid thymocytes. Thymocyte nuclear extracts were evaluated for p53 protein levels by Western blotting. Freshly isolated, untreated scid thymocytes expressed substantial levels of p53 (Fig. 1). In contrast, p53 protein was not detected in RAG-2−/− thymocytes (Fig. 1). Failure to rearrange TCR genes causes similar developmental arrest at the CD25+ CD4/CD8 double-negative (DN) stage in both scid and RAG-2−/− mice, indicating that p53 up-regulation in scid thymocytes was not simply a consequence of developmental arrest. Freshly isolated scid
Figure 1. Western blot analysis of p53 protein expression in thymocyte nuclear extracts. Fifty micrograms of nuclear protein from each sample were resolved by SDS-PAGE, transferred to nitrocellulose filters and incubated with anti-p53 antibody, followed by anti-mouse horseradish peroxidase (HRP), and visualized by enhanced chemiluminescence (ECL). (A) Nuclear extracts prepared from fresh ex vivo thymocytes from the indicated mouse strains were analyzed for p53 protein expression by Western blotting. The last two lanes contain nuclear extract from each sample were resolved by SDS-PAGE, transferred to nitrocellulose filters and incubated with anti-p53 antibody, all membranes were stained with amido black to confirm equal protein loading (not shown).

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Absence of p53 partially restores TCRβ rearrangement and T cell development in scid mice

To examine the biological importance of p53 expression in scid lymphocyte precursors, we bred and analyzed p53\(^{-/-}\)scid mice. The phenotype of p53\(^{-/-}\)scid thymocytes was examined at multiple time points after birth. Three-week-old p53\(^{-/-}\)scid and p53\(^{+/+}\)scid (p53\(^{+/-}\)scid) thymocytes were homogeneously large (high forward scatter), CD25\(^+\)DN cells lacking intracellular TCRβ (iTCRβ) protein (Fig. 2A), an immature phenotype characteristic of arrested thymocyte development conferred by the scid mutation. In contrast, age-matched p53\(^{-/-}\)scid animals contained substantial populations of small DP thymocytes, a high frequency of which expressed iTCRβ protein. In six of seven 3-week-old p53\(^{-/-}\)scid mice, 10–60% of thymocytes expressed iTCRβ protein [Fig. 2B]. Maturation of p53\(^{+/+}\)scid DP thymocytes in the absence of iTCRβ protein also occurred, similar to observations illustrating a TCRβ-independent developmental pathway in TCRβ-deficient animals (Mombaerts et al. 1992a; Zumiga-Pflucker et al. 1994; Guidos et al. 1995). Between 10 days and 5 weeks of age, the maturation and expansion of DP cells accounted for progressive increases in thymus cellularity (Fig. 2B). Thus, p53 deficiency partially overcame developmental arrest conferred by the scid mutation, allowing TCRβ protein production along with development and expansion of DP thymocytes.
Figure 2. Kinetics of thymocyte development in p53⁻/⁻ scid mice. Thymocytes were obtained from mice of different ages and analyzed for forward scatter (FSC) and expression of CD4, CD8, and intracellular TCRβ chain expression by multiparameter flow cytometry. (A) Two-parameter contour plots (7% probability) display CD4 versus CD8 expression (far left). Single parameter histograms show the expression of intracellular TCRβ protein (clear histograms, middle) as compared with control scid intracellular TCRβ expression (shaded histograms). Surface TCRβ⁺ cells were found only in BALB/c mice (not shown). Also shown are single parameter histograms of the forward light scatter (an indication of cell size) of the total thymocyte population (shaded histogram, right) overlaid with the FSC of the DP thymocytes alone (dark outline). Control scid and BALB/c mice were 3 weeks old. Three- and 5-week-old p53⁻/⁻ scid mice are shown for comparison. (B) (top) Number of viable thymocytes recovered from individual p53⁻/⁻ scid mice at the indicated ages (open circles) is shown. The arrow on the y-axis indicates the average 3- to 6-week-old scid thymus value. The percentage of DP thymocytes (middle) or iTCRβ⁺ thymocytes (bottom) in individual mice was determined by flow cytometry. Note that two animals in the 5-week group had <1% DP or iTCRβ⁺ cells in their thymi.
TCRβ protein or mRNA is detected rarely in p53WT scid animals (Bosma and Carroll 1991; Danska et al. 1994), owing to impaired V(D)J recombination. Intracellular TCRβ protein expression in p53-/- scid thymocytes could result from diverse V(D)J rearrangements in many precursors, or the expansion of relatively few, oligoclonal precursors. To examine the heterogeneity of these rearrangements in individual animals, reverse transcriptase-coupled-polymerase chain reactions (RT-PCR) were performed with a series of seven TCRVβ-specific primers in conjunction with an antisense Cβ primer (Danska et al. 1994). In BALB/c thymocytes, each Vβ-specific reaction yielded TCRβ products, reflecting the use of multiple V-region gene segments in the WT thymocyte repertoire (Fig. 3A, top). At 3 weeks of age, transcripts containing all seven Vβ regions were detected in three of four p53-/- scid littermates, suggesting the heterogeneous use of Vβ gene segments. One 3-week-old p53-/- scid thymus lacked both Vβ7- and Vβ14-containing transcripts, suggesting more limited TCRβ diversity in this animal (Fig. 3A, second row). Using a Vα-consensus/Cα primer set for RT-PCR amplification (Danska et al. 1990), we found that two of four 3-week-old littermates contained low level TCRα transcripts of normal length (Fig. 3A, bottom). These results demonstrate that productive TCRβ rearrangement and DP thymocyte maturation in p53-/- scid animals was sometimes associated with progression to TCRα rearrangement and transcription. However, we did not detect surface TCRβ+ thymocytes in p53-/- scid mice (data not shown). Because efficient TCRβ transport to the cell surface is TCRα-dependent (Mombaerts et al. 1992a; Philpott et al. 1992), this observation suggests that few, if any, thymocytes harbored in-frame rearrangements at both TCRβ and TCRα loci. Accordingly, cloning and sequence analysis of TCRα transcripts from double mutant animals demonstrated a low frequency of normal, productive Vα coding joints (data not shown).

Previous studies have shown that rare V(D)J coding joints can be isolated from scid cells, often with characteristic structural features, including extensive P-nucleotide addition, homology-directed joining, and extensive coding sequence deletions (for review, see Lewis 1994). To examine the diversity and fidelity of individual V(D)Jβ coding joints from p53-/- scid mice, TCRβ transcripts from a 3-week-old p53-/- scid thymus were cloned by RT-PCR amplification (Danska et al. 1994). Only 3 of 21 sequences analyzed were unique, and the structure of some TCRβ coding joints revealed potential signatures of the scid V(D)J recombinase machinery (Fig. 3B and legend). Collectively, these phenotypic and molecular analyses suggest that p53 deficiency permits scid thymocytes to progress through multiple contingent steps characteristic of normal maturation: productive TCRβ rearrangement, expression of CD4 and CD8 coreceptors, and onset of TCRα rearrangement and transcription. However, the limited diversity and unusual structural features of TCR transcripts suggested that p53 deficiency did not elicit WT V(D)J recombination in scid thymocytes.

**Impact of p53 deficiency on B lineage development in scid mice**

In agreement with a previous report (Hardy et al. 1989), only 5–10% of p53WT scid bone marrow cells expressed the B lineage marker B220, about one-third the frequency in normal mice, and all scid B220+ cells were large, CD43+, and ILμ- (Fig. 4). In contrast, bone marrow from p53-/- scid mice was enriched in B220+ cells as early as ten days of age, and the B220+ frequency rose to 20–65% by 5 weeks of age (Fig. 4B). Bone marrow cellularity increased significantly during this time period, indicating an expansion of absolute numbers of B220+ cells (Fig. 4B). Despite the increase in the frequency and number of B220+ cells in the double mutant mice, p53 deficiency did not appear to cause significant developmental progression of pro-B cell blasts in scid mice. Although we sometimes observed slightly decreased expression of CD43, this was not accompanied by expression of detectable intracellular Igμ protein in B220+ cells (Fig. 4A). To examine Igμ transcription, we performed RT-PCR of bone marrow from 5- to 10-day-old p53-/- scid animals. Cμ germ-line transcripts characteristic of the pro-B cell stage were present, but we were unable to detect full-length Igμ mRNA in the p53-/- scid samples using a 5' VHα1 consensus primer paired with a 3' Cμ primer (data not shown). Thus, in contrast to the promotion of T cell development, p53 deficiency promoted accumulation of B-cell precursors but did not appear to circumvent the block in B-cell maturation conferred by the scid mutation.

**p53-deficiency results in rapid onset of lymphoma/leukemia in scid mice**

At 7–12 weeks of age, all p53-/- scid animals appeared moribund, and on necropsy, 16 of 16 animals were found to have variable degrees of thymic hyperplasia, lymphadenopathy, and splenomegaly, attributable to infiltration of large numbers of lymphoblastoid cells. Histological examination of infiltrated tissues revealed that normal architecture was replaced by densely packed lymphoblasts with abundant mitotic figures (data not shown). These findings are indicative of a significant lymphoma or leukemia burden. Tumors were observed in mice as young as 6 weeks of age, and the median age of onset was 8 weeks. In most animals, the lymphoma/leukemia was disseminated to multiple sites, including perithymic and inguinal lymph nodes, and spleen. Cell lines were readily derived from explanted thymic or lymph node tumor tissue of multiple animals.

Flow cytometric analyses of tumor phenotype demonstrated that both lymphoid lineages are highly prone to malignant transformation in p53-deficient scid mice. Fourteen of 16 tumors explanted from thymus and lymph nodes did not express T lineage markers (CD4, CD8, Thy-1; data not shown) but were comprised of uniformly large CD19+ B220+ CD43+ cells (Fig. 5A) that closely resembled normal pro-B cells (Hardy et al. 1991). In contrast, 2 of 16 animals had thymic tumors of an
A

BALB/c

p53⁻/⁻ scid 1

p53⁻/⁻ scid 2

p53⁻/⁻ scid 3

p53⁻/⁻ scid 4

B

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Figure 3. T-cell receptor repertoire and coding joint structure in p53⁻/⁻ scid thymocytes. (A) Thymus RNA was extracted from a BALB/c and four 3-week-old p53⁻/⁻ scid mice and reverse transcribed into cDNA. (Top) RT-PCR amplification was performed using seven different Vβ-specific forward primers in concert with a Cβ reverse primer, and the products were resolved by electrophoresis, and evaluated by Southern blotting with a Cβ probe. Note that TCRβ mRNA is rarely detected in p53⁺/⁺ scid mice using this technique (<1/30 animals) (Danska et al. 1994). (Bottom) cDNA from the same samples was RT-PCR amplified using a degenerate consensus Vα sense primer and a Cα antisense primer. The far right lane is a background control of BALB/c cDNA synthesized without reverse transcriptase. The PCR products were resolved by electrophoresis and evaluated by Southern blotting with a Cα probe. [B] Thymus cDNA from one 3-week-old p53⁻/⁻ scid mouse was amplified with a Vβ-consensus primer in conjunction with a Cβ antisense primer. The PCR products were cloned, selected by hybridization with a Cβ probe, and sequenced by dideoxy chain termination using a Cβ antisense primer. The nucleotides for each germ-line TCR V gene segment (underlined) are shown above the p53⁻/⁻ scid sequences. The lower sequence contained an in-frame sequence where the Vβ8.3 gene and an inverted repeat of the RSS heptamer were joined. Similar in-frame IgH signal-containing coding joints have been detected in scid bone marrow DNA [J. Pennycook and G. Wu, pers. comm.].

immature T-cell phenotype, as they expressed both CD4 and CD8, as well as intracellular TCRβ protein, and were negative for B-lineage markers (Fig. 5A; data not shown). In the example shown, both T- and B-lineage cells were detectable in the peripheral lymph nodes. Adoptive transfer of thymocytes from 3-week-old p53⁻/⁻ scid mice into p53WT scid recipients resulted in disseminated pro-B cell lymphoma within 6 weeks [data
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Figure 4. Analysis of B-cell development in p53−/− scid mice. (A) Bone marrow cell suspensions were obtained at the ages indicated and analyzed by flow cytometry for expression of B220 and intracellular Igλ protein. Shaded histograms in all plots represent staining with isotype-matched control antibodies. The percentage of B220+ cells in each bone marrow sample is indicated (clear histogram, far left). Both CD43 expression and FSC are displayed as single parameter histograms gated on B220+ cells. (B) (top) Bone marrow cellularity in individual p53−/− scid mice (dark bars) at the indicated ages. Age-matched p53WT scid animals (light bars) are shown for comparison. (Bottom) Bone marrow cells from p53−/− scid mice were obtained at 10 days, 3 weeks, and 5 weeks of age and stained for B220 expression as described in A. The bone marrow cellularity of p53WT scid age-matched controls are shown (light bars).

not shown), demonstrating that pre-malignant pro-B cells can be detected in the thymii of very young mice.

The Igλ locus was examined in pro-B lymphomas using a λH4 probe, which was chosen because it is the most 3′ JH gene segment and is retained on Igλ alleles after normal V(D)J rearrangements. These p53−/− scid tumors and lymphoma cell lines each contained apparently clonal Igλ rearrangements [Fig. 5B, top], but the TCRβ locus was in germ-line configuration [Fig. 5B, bottom], reinforcing the phenotypic evidence of their B-lineage derivation. Igλ protein was not detected, however, in any of the pro-B cell tumors [data not shown], suggesting that these rearrangements were incomplete [DJ only] or nonfunctional. Collectively, these data demonstrate that ablation of p53 activity in immature scid lymphocyte precursors universally results in extremely rapid progression to a highly invasive lymphoma or leukemia.

Effects of p53 deficiency on cell cycle status, genomic instability, and survival of scid lymphocyte progenitors

The dramatic effects of p53 deficiency on lymphocyte development and neoplastic transformation in scid mice could be attributable to altered cell cycle regulation or to loss of an apoptotic pathway. CD25+ DN thymocytes and pro-B cells from WT mice have been demonstrated to undergo a G1 arrest coincident with TCRβ or Igλ rearrangement, respectively [Pearse et al. 1989; Godfrey et al. 1994] suggesting that V(D)J recombination, like other
forms of DSB repair, is tightly coordinated with the cell cycle (Lin and Desiderio 1995). Because we did not detect p53 up-regulation in purified CD25+ DN WT thymocytes [see Fig. 1A], we reasoned that the G1 arrest at this maturational stage in WT mice may be p53-independent. To examine this issue, we compared the cell cycle status of CD25+ DN thymocytes purified from WT B6 and p53−/− (WT at the scid locus) mice, using propidium iodide staining and flow cytometric analysis (Table 1). These data showed that >90% of both WT and p53-deficient CD25+ DN thymocytes were arrested in the G1 phase of the cell cycle. Similarly, CD25+ DN thymocytes from RAG-2−/− and scid mice consist primarily of cells in G0/G1 (Table 1). Loss of p53 did not decrease the proportion of scid CD25+ DN thymocytes [Table 1] or bone marrow pro-B cells [Table 2] in G0/G1. Thus, the G1 arrest accompanying V(D)J recombination in WT and scid lymphocyte precursors occurs independently of p53 accumulation.

Although p53 deficiency did not promote abnormal cell cycle progression of scid lymphocyte precursors, we noted that pro-B cells and thymocytes harvested from the double mutant animals during the premalignant phase displayed a significant degree of aneuploidy. Figure 6A shows that pro-B cells purified from p53−/− scid animals have ≤20% greater Gy/G1 DNA content than those isolated from p53+/− scid animals. This was best demonstrated when the two purified populations were mixed together and then stained for DNA content [Fig. 6A, bottom]. Hyperdiploidy was evident in purified pro-B cells from 75% of double mutant animals sacrificed at 5–6 weeks of age, but was not detected in bone marrow myeloid cells from the same animals (data not shown). We also examined primary tumor cell populations for evidence of aneuploidy. DNA content histograms showed bimodal G0/G1 peaks in bone marrow cells and thymocytes from a 10-week-old moribund p53−/− scid mouse, but not from an age-matched BALB/c mouse [Fig. 6B]. The B220 versus DNA content dot plot demonstrates that the B220+ cells are hyperdiploid in the double mutant, but not in BALB/c animals. However, in contrast to the premalignant aneuploid populations, the malignant pro-B cells were actively cycling (50% in S/G2/M). Similarly, we observed active cycling of hyperdiploid cells in CD4/CD8 DP thymic lymphoma [data not shown]. These data suggest that the absence of p53 promotes genomic instability in scid lymphocyte progenitors.

The accumulation of noncycling, hyperdiploid lymphoid progenitors in p53-deficient scid mice also suggests that p53 may limit the survival of cells harboring broken V(D)J molecules. To assess the role of p53 in the survival of scid cells with DSB, we compared the relative survival of thymocytes from RAG-2−/−, p53WTscid, p53+/- scid, and p53−/− scid mice after 1 or 2 days of culture. Multiple genetic studies have shown this assay to correlate well with survival of lymphocyte progenitors in vivo (Strasser et al. 1994b; Motoyama et al. 1995; Field et al. 1996). The surviving fraction [number of viable cells recovered/number of viable cells seeded] of p53WT scid and p53+/- scid thymocytes was <0.2 [Fig. 7A]. In contrast, the survival of RAG-2−/− or p53−/− scid thymocytes was respectively two- to fourfold greater or three- to sevenfold greater than scid thymocytes. p53-deficiency also enhanced dramatically the ability of scid lymphoma cell lines to survive DNA damage induced exogenously by low-dose γ-irradiation [Fig. 7B]. As expected, the lymphoma cell lines derived from p53WT scid mice were extremely radiosensitive and few cells survived for 24 hr after treatment with 200 and 500 cGy. Lymphoma lines adapted from p53−/− scid mice, however, were 4–14 times more radioresistant. Irradiated spleen cells from p53-deficient scid mice also showed substantially greater survival than those from p53WT scid mice, demonstrating that this effect was not limited to transformed cells, or to the lymphoid lineage [Fig. 7C]. In accord with previous studies on p53-deficient cells [Strasser et al. 1994a], p53-deficient scid cells also displayed some p53-independent, radiation-induced cell death. Collectively, these observations support the notion that loss of the p53-dependent DNA damage checkpoint in scid lymphocytes confers protection from cell death associated with both RSS-dependent and radiobiological checkpoints.

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Table 1. Effect of p53 deficiency on cell cycle status of CD25+ DN wild-type and scid thymocytes

Table 2. Effect of p53 deficiency on cell cycle status of scid pro-B cells

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1 x 10⁵ to 2 x 10⁶ CD19+ B220+ pro-B cells from individual mice (4–6 weeks old) of each genotype were purified by cell sorting, fixed, stained with propidium iodide, and analyzed by flow cytometry for DNA content. The double mutant mice used for this experiment appeared healthy and had only moderate accumulations of pro-B cells (33 ± 4%), and no lymphadenopathy, thymic hyperplasia, or splenomegaly. *Number of individual mice analyzed.
Figure 5. (See facing page for legend.)
V(D)J recombination activates a p53 checkpoint

Numerous studies have demonstrated that imposition of DNA breaks by exogenous agents are sufficient to initiate a p53-mediated cell cycle arrest or apoptosis, but normal physiological conditions that activate p53 pathways have not been identified previously. Here we provide the first demonstration that this DNA damage checkpoint is activated physiologically by V(D)J recombination in scid mice. Examination of p53 protein levels in scid versus RAG-2-deficient scid thymocytes demonstrated that RAG-mediated introduction of DSB in scid thymocytes provides a necessary signal to cause p53 accumulation [Fig. 1A]. Given that (1) TCRβ, TCRγ, and TCRδ are all recombinationally active in this cell population, (2) that V to D joining is not usually attempted before successful D to J joining, and (3) rearrangement of both alleles can be attempted simultaneously, the number of broken V(D)J ends present in an individual CD25+ DN thymocyte is likely between 2 and 16. This implies that the DNA damage-sensing mechanism is highly sensitive, in accord with a recent study suggesting that a single DSB can activate the p53 checkpoint [Huang et al. 1996]. We could not detect p53 accumulation, however, in recombinationally active WT lymphocyte precursors. We suggest that the differential accumulation of p53 in WT versus scid lymphocyte progenitors reflects the abnormal persistence of V(D)J coding ends caused by the scid mutation in DNA-PK [Roth et al. 1992a,b; Zhu and Roth 1995]. Nonetheless, rare WT precursors in which V(D)J coding ends persist may also activate the p53-dependent checkpoint.

Several properties of DNA-PK suggest that it may play a direct role in activation of the p53 checkpoint. It belongs to a subfamily of PI-3 kinase-related genes implicated in yeast, Drosophila, and humans that regulate DNA damage checkpoints [Zakian 1995], and defects in the human ATM family member cause abnormalities in DNA damage-induced p53 accumulation, trans-activation function, and cell cycle arrest [Kastan et al. 1992; Khanna and Lavin 1993; Lu and Lane 1993; Khanna et al. 1995]. Intriguingly, the enzymatic activity of DNA-PK requires association with DNA breaks, and scir residues in the p53 amino-terminal trans-activation domain are targets of phosphorylation by human and rodent DNA-PK in vitro [Lees-Miller et al. 1992; Finnie et al. 1995]. Although scid DNA-PK fails to phosphorylate this site in vitro [Blunt et al. 1995], the biological significance of phosphorylation at this site is unclear [Ko and Prives 1996]. We show here that p53 protein is up-regulated in scid thymocytes in vivo as a consequence of attempted V(D)J recombination. Moreover, scid and WT thymocytes accumulated similar levels of p53 in response to ionizing radiation. We also observed radiation-induced p21waf1/cip1 mRNA expression in scid cells (data not shown), indicative of normal p53-mediated trans-activation. Thus, in contrast to mutations in ATM, the scid mutation does not compromise the DNA damage-induced accumulation and function of p53.

Effects of p53 deficiency on survival, V(D)J recombination, and developmental progression of scid lymphocyte precursors

Our results show that p53 deficiency enhances survival of T and B cell precursors in scid mice, as evidenced by prolonged in vitro survival of thymocytes, and by accumulation of noncycling pro-B cells in vivo. We observed both productive TCRβ rearrangements and progression to the DP stage, in thymocytes, whereas productive Igα rearrangements and developmental progression of pro-B cells were not seen. Previously, we observed a similar lineage dichotomy in restoration of V(D)J recombination and development in newborn scid mice given low doses of ionizing radiation [Danska et al. 1994]. Productive TCRβ rearrangements were also documented in the latter system [Bogue et al. 1996; Livak et al. 1996], which can facilitate development of DP thymocytes in the absence of TCRβ [Mombaerts et al. 1992a; Kersh et al.].
Figure 6. (See facing page for legend.)
Thus, we suggest that productive TCRβ or TCRδ rearrangements promoted DP development in p53⁻/⁻-scid mice, and that the absence of pro-B cell maturation in these animals reflects the failure to generate productive Igμ rearrangements. It remains unclear whether the differential effects of p53 deficiency and irradiation on promoting successful TCR versus Ig rearrangements in scid pro-B cells reflect unique, Igμ-specific constraints on V(D)J recombination, or owe to different properties of the thymus versus bone marrow microenvironments.

We observed oligoclonal, in-frame TCRβ transcripts, suggesting expansion of a limited cohort of p53⁻/⁻-scid precursors with productive coding joints (Fig. 3). An interesting implication of these results is that scid DNA-PK has a higher capacity to mediate coding joint formation than has been appreciated previously. The frequency of coding joint formation in scid cells has been measured as 10⁻¹⁻¹⁰⁰-fold below WT levels (Lieber et al. 1988; Malynn et al. 1988; Pennycook et al. 1993), and normal V(D)J rearrangements have been seen in rare lymphocytes in aged scid mice (Carroll and Bosma 1988; Blackwell et al. 1989; Carroll et al. 1989; Hendrickson et al. 1990; Kotloff et al. 1993; Young and Kearney 1995). Our results help to rationalize this “leakiness” by suggesting that the scid mutation causes an inefficiency in the V(D)J recombination process that limits cell survival. However, the limited diversity and “scid-like” TCR coding joints we observed suggest that scid DNA-PK still functions relatively ineffectively to coordinate DSB repair when thymocyte survival is enhanced by p53 ablation.

Independent support for the notion that attempted V(D)J recombination is deleterious for scid lymphocyte precursors has been reported. Transgenic expression of Bcl-2, which can inhibit DNA damage/p53-mediated cell death (for review, see White 1996), improved survival and developmental progression of scid pro-B cells, but this study did not evaluate whether this was associated with productive Igμ recombination (Strasser et al. 1994b). In another study, restoration of B-cell production by transgenic expression of Igμ and Ig light chains in scid mice was dependent on the capacity of different transgenes to inhibit endogeneous light chain rearrangement (Chang et al. 1995). We posit that p53-mediated apoptosis eliminated pre-B cells attempting light chain gene rearrangement, but this pathway was not invoked in precursors bearing a transgene that suppressed endogenous rearrangements. From this perspective, the profound immune deficiency conferred by the scid mutation does not result exclusively from failure to generate positive selection signals through pre-T and pre-B cell surface antigen receptors. Rather, scid lymphocyte survival is limited by V(D)J coding breaks that trigger p53-mediated apoptosis.

**Figure 6.** Presence of hyperdiploid premalignant and malignant pro-B cells in p53-deficient scid mice. (A) Bone marrow-derived CD19⁺ B220⁺ pro-B cells were purified by cell sorting from 6-week-old p53⁻/⁻-, p53⁻/-, and p53⁻/⁻-scid mice. The latter appeared healthy and tumor free (no thymic hyperplasia or lymphadenopathy) at the time of sacrifice. Cells were then fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry. The G0/G1 DNA content of pro-B cells from p53⁻/⁻-scid and p53⁻/- scid mice was identical, therefore only the former cells are shown. To ensure that the apparent hyperdiploid shift of the p53⁻/- scid cells was not attributable to minor differences in dye or cell concentration, approximately equal numbers of pro-B cells from p53⁻/⁻-scid and p53⁻/- scid were mixed before propidium iodide staining. The bimodal G0/G1 peak of the mixture confirms the unequal DNA content of the two populations. (B) Bone marrow cells and thymocytes from the indicated mice were stained for surface expression of B220 as described for Fig. 3, followed by fixation and propidium iodide staining for DNA content. The B220 versus DNA content dot plot demonstrates a hyperdiploid shift of the B220⁺ cells from both tissues of double mutant mice. Numbers on each plot refer to the percentage of B220⁺ cells in G0 or G1/S/G2, or M phases. Note that in normal thymocytes, a significant B220⁺ population is not detectable (ND), and that the majority of B220⁺ bone marrow cells in normal mice are in G0/G1.
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**Figure 7.** Effect of p53-deficiency on in vitro survival of scid thymocytes and radiosensitivity of scid lymphoma cell lines and spleen cells. [A] The surviving fraction (number viable irradiated/number viable control) of p53 +/- DP thymocyte development in the double mutant animals. First, similar to the data presented here, Bogue and colleagues showed that nontreated p53-/- scid mice have an elevated frequency of DP thymocytes compared with age-matched p53 +/- scid animals. Thus, a radiation-dependent effect on DP thymocyte expansion would have to be detectable above the background of “spontaneous” DP thymocyte development in the double mutant animals. Second, this study examined a single 2-week time point, which may have obscured detection of a similar developmental process that occurs with altered kinetics in irradiated p53 +/- versus p53-/- scid mice. Finally, we show here that loss of DP thymocytes in nontreated p53 +/- scid mice occurs coincident with immigration of growth dysregulated pro-B cells as early as 3 weeks of age. Others have shown that irradiation of p53-deficient (but otherwise WT) mice greatly accelerates lymphoma onset [Kemp et al. 1994]. Thus, the kinetics of lymphomagenesis in irradiated p53-deficient scid is predicted to be extremely rapid, potentially confounding distinctions between effects on “normal” DP thymocyte maturation and the consequences of rapid lymphomagenesis. Thus, the role of p53 in radiation-induced rescue of TCR rearrangement and lymphocyte maturation in scid mice may not be readily resolved in these double mutant mice.

The oncogenic potential of V(D)J recombination

On a p53WT background, scid mice develop thymic lymphoma with low incidence [15%] and long latency, but susceptibility to other tumors is not significantly elevated over that seen in BALB/c mice [Custer et al. 1985]. p53-deficient mice with WT DNA-PK function also develop primarily thymic lymphoma [50% incidence at 16–20 weeks] [Donehower et al. 1992; Harvey et al. 1994, 1995; Jacks et al. 1994]. Thus, lymphoma is the predominant tumor caused by two different genetic lesions that affect DNA repair and damage detection in all tissues. Here we show that loss of p53 synergizes with the scid mutation to promote extremely rapid development of thymic lymphoma or lymphocytic leukemia by 6-12 weeks after birth.

Precipitous lymphoma onset may indicate that broken V(D)J molecules in p53-deficient scid lymphocyte progenitors frequently participate in aberrant rejoining events initiating an oncogenic process. In support of this notion, we show that loss of p53 in the context of the scid mutation permits the rapid accumulation of aneuploid pro-B cells, but not myeloid cells, in very young mice. Thus, recombinationally active lymphocyte progenitors appear to be highly dependent on p53-mediated checkpoints to maintain genomic integrity. Several classes of aberrant V(D)J joining events have been observed in human lymphocytic leukemias and lymphomas. These include translocation of antigen receptor regulatory elements to proto-oncogenes [for review, see Rabbitts 1994], and recombination mediated through “cryptic” RSS [for review, see Lewis 1994]. These abnormal joining events highlight the opportunities for RAG-1/2-mediated generation of DSB at inappropriate sites in immature lymphocytes, underscoring the importance of the p53 DNA damage checkpoint during lymphocyte development.

Materials and methods

**Mice**

All scid and RAG-2 +/- animals were maintained under SPF conditions, and WT strains (BALB/c, B6) were maintained under conventional conditions at the Hospital for Sick Children. C.B-17 scid mice were maintained as homozygotes. TCRβ-transgenic mice heterozygous for the scid mutation were obtained from E. Shores and A. Singer [Shores et al. 1993] and bred to C.B-17 scid mice. TCRβ* scid/scid mice were detected by PCR amplification of tail DNA using the Vβ8incl/Cβuni primer pair [Danska et al. 1994]. p53 +/- scid mice (Donehower et al. 1992) were bred to ICR.scid homozygotes (scid), and F1 offspring were intercrossed to derive p53 +/- and p53 +/- scid animals. Homozy-
peripheral blood cells stained with monoclonal anti-Ig~ and gous. p53 homozygotes were identified by PCR of tail DNA with two primer pairs: p53WT: sense 5’-GTG GGA GGG ACA AAA GTT CGA GGC C-3’, antisense 5’-TTT ACG GAG CCC TGG CGC TCG ATG T3’, yields a 170-bp fragment. PCR conditions were 30 cycles of 30 sec at 94°C, 60 sec at 55°C, and 40 sec at 72°C. The mice used in this study were the progeny of p53+/+ scid parents, or p53+/− scid females and p53−/− scid males. p53 genotypes were determined retrospectively. RAG-2−/− scid double mutants were generated by backcrossing (RAG-2−/− × CB-17.scid) F1 progeny to CB-17.scid. The RAG-2−/− line has been described previously (Shinkai et al. 1992). Peripheral blood from backcross progeny was screened for the presence of T- and B-cells by flow cytometry to identify scid homozygotes. WT and KO RAG-2 alleles were then identified in scid homozygotes by PCR amplification of tail DNA using a primer trio of RAG 2-3 (5’-GCC TGC TTA TTG TCT CCT GGT ATG-3’), Neo-3 (5’-GCC TGC TTA TTG TCT CCT GGT ATG-3’), and RAG 2-1 (5’-TAA ATT CAA CCA GGC TTC TCA CTT-3’). Thermal cycling conditions for this trio were 30 cycles of 30 sec at 94°C, 1.5 min at 60°C, and 2 min at 72°C. Under these conditions, RAG 2-3 and Neo-3 amplify a 937-bp KO fragment, and RAG 2-3 and RAG 2-1 amplify a 973-bp WT fragment that were resolved by 2% agarose gel electrophoresis. RAG-2−/− scid mice were maintained as double homozygotes.

Flow cytometry

Staining of thymocyte, bone marrow, and lymph node cell suspensions or lymphoma cell lines for surface or intracellular expression of various lineage and developmental markers was performed as previously described (Guidos et al. 1990, Danska et al. 1994) using a FACScan flow cytometer with Lysis II software (Becton Dickinson & Co., Mountain View, CA). The following monoclonal antibodies were affinity purified from hybridoma culture supernatants and conjugated to various fluorochromes using standard techniques, or were purchased from Pharmingen (San Diego, CA): TCRβ [H57], CD4 [YTS 191.1], CD8 [YTS 169.4], B220 [6B2 or 14.8], CD43 [anti-S7], Igα [R40-97], CD19 [1D3], and Thy-1.2 [53-2.1]. Purification of DN thymocyte subsets was performed by magnetic bead depletion of CD4, CD8, CD43-expressing thymocytes, followed by staining with anti-CD25 and and fluorescence-activated cell sorting. Both DN populations were reanalyzed and found to be >98% pure (data not shown). Purification of bone marrow pro-B cells was done by sorting cells staining positive with fluorescein isothiocyanate (FITC)-anti-B220 and biotin-anti-CD19/avidin-phycocerythrin. Reanalysis of these populations showed them to be >98% pure. Cell cycle analysis of both CD25+ DN thymocytes, and B220+ CD19+ pro-B cells was done by staining ethanol-fixed cells with propidium iodide as previously described (Groves et al. 1995).

Cell lines and survival assays

p53−/− scid cell lines were derived by explanting tumors from lymph nodes and thymus into Iscove’s medium supplemented with 10% fetal bovine serum, 10 mM HEPES [pH 7.0], 50 μM 2-mercaptoethanol, 2 mM glutamine, and 1% penicillin/streptomycin using standard techniques. Derivation of lymphoma cell lines from p53WT scid mice was described previously (Danska et al. 1994). These cell lines display radiation-induced p53 protein expression, p21WAF1/CIP1 mRNA expression, and apoptosis, and thus appear to have normal p53 function (data not shown). For determination of sensitivity to ionizing radiation, 2×10⁶ cells were irradiated with a 137Cs source, and cultured overnight in 4 ml of RPMI supplemented as described above. The number of viable cells remaining was determined by counting trypan blue excluding cells 24 hr later. For thymocyte survival assays, 1–2×10⁶ cells in 2 ml of supplemented RPMI were seeded into 24-well plates and cultured for 1 or 2 days. The number of surviving cells was determined by counting with trypan blue.

Molecular analysis of antigen receptors

Reverse-transcriptase coupled PCR amplification was performed as previously described (Danska et al. 1994). Seven TCR Vβ-specific primers were used in separate reactions with a TCR CB antisense primer (Danska et al. 1994, Galley and Danska 1995). RT–PCR products were cloned into the pTA vector [Invitrogen Corp., San Diego, CA], screened by hybridization with a CB probe, and sequenced as previously described (Galley and Danska 1995). For genomic Southern blot analyses, DNA was prepared from the thymus, bone marrow, or kidney of WT or p53−/− scid mice at the indicated ages and from cell lines. DNA samples were digested with EcoRI or PvuII, and electrophoresed on 0.8% agarose gels. Southern blot filters were hybridized to Igλ,α4 (Atkinson et al. 1991), HPRT, or TCRβ3 probes. RT–PCR analysis of Iga transcripts was performed with a pair of Cμ primers (forward, 5’-CTA CGG AGG CAA AAA CAA AGA-3’ and reverse, 5’-TAG CCA CAC CCT TAG CAC TGA-3’), and with a degenerate VHI forward primer (VHI; see Guidos et al. 1995) paired with the reverse Cμ primer. PCR products were resolved by electrophoresis, transferred to Southern blots and hybridized to a radiolabeled Cμ cDNA probe. PhosphorImages were obtained with a Molecular Dynamics imaging system.

Western blot analysis

Monoclonal antibody Pab 242 [ATCCG, Rockville, MD] was used to detect p53 expression in nuclear extracts. Briefly, cells were swelled on ice in 10 mM HEPES [pH 7.9], 10 mM KCl, 1 mM DTT, and protease inhibitors, lysed by addition of NP-40 to 0.6% and microcentrifuged for 30 min at 4°C. Nuclear proteins were eluted from the pellet in 20 mM HEPES [pH 7.9], 0.4 mM KCl, 1 mM EDTA, 1 mM DTT, and protease inhibitors by agitation at 4°C. The supernatant was recovered after microcentrifugation, and adjusted to 120 mM KCl by addition of 10 mM HEPES [pH 7.9]. Protein concentrations were measured [Bio-Rad protein assay] and 50–100 μg of each sample was subjected to SDS-PAGE [reducing conditions], Western blotting, and enhanced chemiluminescence detection [ECL, Amersham] using standard methods.

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