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Award Number: DAMD17-99-1-9134

TITLE: p19 ARF-p53 Tumor Suppressor Pathway During Oncogene-Induced Apoptosis and Senescence

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REPORT DATE: June 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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The primary objective of this project is to provide new insights into the role of the p53 and ARF tumor suppressors in cancer development and therapy. During the first year of this study, we found that different oncogenes can promote different p53 post-translational modifications. Oncogene ras promotes phosphorylation of p53 on Serine 15, however, E1A does not. These data suggested that different oncogenes induce different phenotypes through different p53 post-translational modifications. It seems that p19ARF is required for p53 modifications, since p53 can not be phosphorylated on Ser 15 following Ras overexpression in ARF null cells. We also examined the impact of INK4a/ARF mutations on tumor development and therapy using the Eμ-myc transgenic mouse. Our results clearly showed that inactivation of the INK4a/ARF locus accelerated Myc-induced lymphomagenesis, leading to massively deseminated lymphomas that displayed markedly reduced apoptosis. In collaboration with C. Sherr, we tried to produce different monoclonal antibodies to p19ARF. Right now, we are doing the secondary screens to determine the antibodies specificity.
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Jianping Jin 06-12-00
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Introduction

Breast cancer is the second leading cause of cancer death in North American Women. Mutations in the p53 tumor suppressor gene are the most common genetic changes found in breast cancer so far. It has been suggested that p53 plays a critical role in the development of this disease, however, the signals triggering p53 in suppressing tumor growth remain poorly defined. We found that oncogenes induce p53 through a signal transduction pathway that requires the presence of another tumor suppressor, p19ARF. This pathway, when activated, directs the cells to apoptosis or senescence. The primary objective of this project is to provide new insights into the action of the tumor suppressor p53 and p19ARF in cancer development. Our original studies took advantage of mouse cells with a defined genetic background to analyze the relevance of single genes in the oncogene-p19ARF-p53 pathway. We propose to compare this highly controlled system to normal human breast cells in order to determine if the pathway is conserved in human cells and thus potentially relevant to breast cancer. Furthermore, by comparing and contrasting normal versus oncogene expressing cells, in these systems, we will be able to get insight to the mechanisms by which p19ARF is involved in p53 activation. Finally, a better understanding of the oncogene-p19ARF-p53-apoptosis pathway could allow us to exploit new strategies to enhance the chemo- and radio-sensitity of breast cancer cells. The research progress of the first year on this project is reported as follows.

Body

1. Oncogene signaling to p53 (Aims 2 and 3)

The primary objective of this study is to provide new insights into the role of the p53 and ARF tumor suppressors in breast cancer development and therapy.

p53 promotes cell cycle arrest or apoptosis in response to different stimuli, including DNA damage and mitogenic stimulation. Following DNA damage, signaling is mediated, at least in part, by a kinase that phosphorylates p53 on Serine 15, thereby stabilizing the protein by preventing its association with its negative regulator Mdm2.
In our original proposal, we presented data showing that the adenovirus E1A oncogene activates p53 through a mechanism distinct from DNA damage, involving cancellation of Rb function and activation of the ARF tumor suppressor, leading the cells to apoptosis. ARF-null MEFs expressing E1A are incapable of activating the p53 response and are more resistant to apoptosis following serum depletion or adriamycin treatment, compared to wild type MEFs. Reintroduction of ARF restores p53 accumulation and sensitizes cells to radiation and chemotherapy.

We decided to test whether ARF induction is a common event following oncogene overexpression. Our recent data indicated that the cellular ras oncogene, by activating MAP kinase pathway, also induces p19ARF and cooperates with p53 to promote premature senescence. (Lin, de Stanchina, Ferbeyre and Lowe unpublished). In this case, ARF null cells expressing ras can still induce p53 but exhibit an impaired p53 response and do not undergo premature senescence.

We have also been able to show that different oncogenes can promote different p53 post-translational modifications. In particular, we found that E1A does not promote phosphorylation of p53 on Serine 15, but Ras does it (de Stanchina and Lowe, unpublished). It seems that different oncogenic stimuli, which induce different outcomes, also are associated with different p53 modifications, raising the possibility that these differences in modifications alert p53 to the type of insult that has occurred and subtly influence the nature of the p53 response. It is interesting to note that ARF seems to be required for these functions, since in ARF null cells, p53 can not be phosphorylated on Ser 15 following Ras overexpression (de Stanchina and Lowe, unpublished).

Our current data imply that oncogenes (in part through ARF) and DNA damage cooperate to induce apoptosis or permanent cell cycle arrest and indicate that loss of ARF function may promote tumor progression and chemoresistance by disabling p53.

p53 mutations have been associated with aggressive cancers, poor prognosis and drug resistance in human patients. In principle, tumors with INK4a/ARF mutations might also display aggressive characteristics owing to extragenic defects in the p53 pathway. To test this, we examined the impact of INK4a/ARF mutations on tumor development and therapy using the Eμ-myc transgenic mouse. These mice constitutively express c-myc in the B cell lineage and develop B cell lymphoma and associated leukemia. The
Eμ-myc transgenic lymphoma model was used to generate genetically defined tumors with mutations in the INK4a/ARF or p53 genes. Thus it was possible to monitor the rate of formation of p53 or INK4a/ARF null lymphomas, their invasiveness and their resistance to chemotherapy in vitro and in vivo.

Our results (Schmitt et al, 1999) clearly showed that inactivation of the INK4a/ARF locus accelerated Myc-induced lymphomagenesis, leading to massively deseminated lymphomas that displayed markedly reduced apoptosis. The latency, pathology and growth characteristics were indistinguishable from Myc-induced lymphomas lacking p53. Indeed, the p53 activity was compromised in INK4a/ARF null lymphoma cells despite the fact that these cells harbor wild-type p53 genes. These results demonstrated that genetic interactions between the p53 and INK4a/ARF locus are important during tumorigenesis and predict that disruption of the INK4a/ARF locus, like p53 loss, will be a negative prognostic marker in human tumors.

2. Production of highly specific monoclonal antibodies against the N-terminal portion of ARF(Aim 1)

In tumors, mutations affecting ARF occur primarily in the region coding for the C-terminus domain (exon 2), possibly leading to the formation of truncated isoforms. These putative forms would go undetected by the only currently available anti-ARF antibodies, which are directed against the C-terminus of the protein. For this reason we proposed to raise antibodies against the amino terminal portion of ARF, which has been shown to be necessary and sufficient to induce cell cycle arrest and to interact with p53 and/or Mdm2. In collaboration with C. Sherr, who provided us with the synthetic peptides (amino acids 1-64 of the mouse protein), we produced and analyzed different monoclonal antibodies. The CSHL Monoclonal Service Facility performed all the animal work, the fusions, single cell cloning and initial screens. I am currently doing the secondary screens to determine the antibodies specificity. I will initially use the antibodies for routine characterization studies, such as western blot analysis and immunoprecipitations, to detect ARF levels in different cell lines and to investigate the presence and abundance of the truncated isoforms in tumor derived cell lines and in clinical specimens.
3. ARF regulation by E1A in cancer chemotherapy

We have shown that ARF is involved in oncogene-induced apoptosis, by stabilizing p53 level. Next question we hope to address is how ARF is regulated by oncogene. We know E1A can induce ARF expression at messenger level and N-terminal function of E1A is highly relative to E1A-induced ARF expression. With the ARF promoter reporter gene construct in hand, we will be able to see how ARF is specially activated by oncogene. We also found that E1A can induce apoptosis in mouse skin fibroblasts (MSFs) as in MEFs. N-terminal deletion of E1A is defective in E1A-induced apoptosis in MSFs. Since MSFs cells have more homogeneous genetic background, with the new microarray technology, we will try to identify new genes that regulate ARF expression in oncogene-induced ARF-p53-apoptosis pathway.

Key Research Accomplishments

1. We found that different oncogenes can promote different p53 post-translational modifications. Oncogene Ras promotes phosphorylation of p53 on Serine 15, however, E1A does not. These data suggested that different oncogenes induce different phenotypes by different p53 post-translational modifications. It seems that p19ARF is required for p53 modifications.

2. We also examined the impact of INK4a/ARF mutations on tumor development and therapy using the Eμ-myc transgenic mouse. Our results clearly showed that inactivation of the INK4a/ARF locus accelerated Myc-induced lymphomagenesis, leading to massively deseminated lymphomas that displayed markedly reduced apoptosis.

3. In collaboration with C. Sherr, we tried to produce and analyze different monoclonal antibodies to p19ARF. Right now, we are doing the secondary screens to determine the antibodies specificity.
Reportable Outcomes


Conclusions

In conclusion, different oncogenes can promote different p53 post-translational modifications. It seems that p19\(^{ARF}\) is essential to these p53 modifications. Whether special p53 modifications are relative to different cancer development remains to be studied. In collaboration with Dr. Schmitt, Dr. de Stanchina published a paper about the mouse model of p19\(^{ARF}\)-p53-apoptosis pathway. Our results clearly showed that inactivation of the INK4a/ARF locus accelerated Myc-induced lymphomagenesis, leading to massively deseminated lymphomas that displayed markedly reduced apoptosis. We are continuing to work on this project, with a particular emphasis on how E1A induces ARF.

References


Appendices

INK4a/ARF mutations accelerate lymphomagenesis and promote chemoresistance by disabling p53

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INK4a/ARF mutations accelerate lymphomagenesis and promote chemoresistance by disabling p53

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Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724 USA

The INK4a/ARF locus encodes upstream regulators of the retinoblastoma and p53 tumor suppressor gene products. To compare the impact of these loci on tumor development and treatment response, the Ep-myc transgenic lymphoma model was used to generate genetically defined tumors with mutations in the INK4a/ARF, Rb, or p53 genes. Like p53 null lymphomas, INK4a/ARF null lymphomas formed rapidly, were highly invasive, displayed apoptotic defects, and were markedly resistant to chemotherapy in vitro and in vivo. Furthermore, INK4a/ARF-/- lymphomas displayed reduced p53 activity despite the presence of wild-type p53 genes. Consequently, INK4a/ARF and p53 mutations lead to aggressive tumors by disrupting overlapping tumor suppressor functions. These data have important implications for understanding the clinical behavior of human tumors.

[Key Words: INK4a/ARF locus; lymphomagenesis; chemoresistance; p53]

Received August 10, 1999; revised version accepted August 31, 1999.

Mutations in the p53 tumor suppressor gene and at the INK4a/ARF locus are the two most frequent genetic lesions identified in human tumors [for reviews, see Haber 1997; Ruas and Peters 1998]. p53 is a sequence-specific DNA-binding protein that can induce cell-cycle arrest or apoptosis in response to pathological insults such as DNA damage and expression of mitogenic oncogenes [Kastan et al. 1991, 1992; Lowe and Ruley 1993; Hermeking and Eick 1994; Serrano et al. 1997; for reviews, see Giaccia and Kastan 1998; Prives 1998]. As a consequence, inactivation of p53 can promote oncogenic transformation and resistance to many anticancer agents [for reviews, see Giaccia and Kastan 1998, Prives 1998, Wallace-Brodeur and Lowe 1999]. The INK4a/ARF locus encodes two tumor suppressors, designated p16INK4a and p19ARF. p16INK4a is a cyclin-dependent kinase inhibitor that acts upstream of the retinoblastoma (Rb) protein to promote cell-cycle arrest [Serrano et al. 1993; for reviews, see Haber 1997; Ruas and Peters 1998]. p19ARF is translated in an alternative reading frame from p16INK4a and activates p53 by interfering with its negative regulator, Mdm2 [Kamijo et al. 1997; Pomerantz et al. 1998; Stott et al. 1998; see also Tao and Levine 1999, Weber et al. 1999, Zhang and Xiong 1999; Zhang and Xiong 1999]. Consequently, INK4a/ARF mutations can disable both the Rb and p53 tumor suppressor pathways.

Recent studies indicate that p19ARF acts as an essential intermediate in oncogene signaling to p53 [Bates et al. 1998; de Stanchina et al. 1998; Palmero et al. 1998; Pomerantz et al. 1998; Zindy et al. 1998; for review, see Sherr 1998]. For example, oncogenes such as E1A or c-myc induce ARF message and protein in normal mouse embryo fibroblasts, which correlates with their ability to activate p53 and promote apoptosis. In contrast, these oncogenes fail to activate p53 in ARF-null cells, and promote proliferation without substantial apoptosis [de Stanchina et al. 1998; Zindy et al. 1998]. Together, these studies indicate that p19ARF acts as part of a p53-dependent fail-safe mechanism to counter hyperproliferative signals. Interestingly, p19ARF is not induced by DNA damage [Kamijo et al. 1997; Stott et al. 1998] but can cooperate with DNA damaging agents to induce apoptosis in oncogene expressing cells [de Stanchina et al. 1998]. These studies predict that disruption of ARF, or the INK4a/ARF locus, should cooperate with mitogenic oncogenes during tumor development, in part, by disabling p53.

p53 mutations have been associated with aggressive cancers, poor prognosis, and drug resistance in human patients [Schmitt and Lowe 1999, Wallace-Brodeur and Lowe 1999]. In principle, tumors with INK4a/ARF mutations might also display aggressive characteristics owing to extragenic defects in the p53 pathway. To test this, we examined the impact of INK4a/ARF mutations on tumor development and therapy using the Eμ-myc transgenic mouse. These mice constitutively express c-Myc...
in the B-cell lineage and develop B-cell lymphoma with associated leukemia (Adams et al. 1985; Adams and Cory 1991). This model was chosen for several reasons. First, because Myc induces p19ARF and activates p53 in cultured fibroblasts (Zindy et al. 1998), Ep-myc transgenic mice provide a relevant setting for comparing the impact of INK4a/ARF and p53 mutations on tumor behavior. Second, Ep-myc lymphomas/leukemias are easily monitored by lymph-node palpation or blood smears, a property that facilitates studies examining tumor responses to therapy. Finally, essentially pure tumor cells can be isolated from lymph nodes and studied ex vivo or expanded in genetically matched nontransgenic recipients. The tractable nature of this model is in stark contrast to human systems, which suffer from difficulties in obtaining well-characterized and comparable clinical material.

Results

Loss of the INK4a/ARF locus accelerates lymphomagenesis similarly to loss of p53

To generate lymphomas with defined alterations, we crossed the Ep-myc transgenic to mice heterozygous for germ-line deletions in the Rb (Rb+/−), INK4a/ARF (INK4a/ARF−/−), or p53 (p53−/−) genes (Jacks et al. 1992, 1994; Serrano et al. 1996). Of note, the INK4a/ARF−/− animals harbor deletions that disrupt both p19ARF and p16INK4a, thereby recapitulating the common gross deletions seen in human tumors (Haber 1997; Ruas and Peters 1998). The onset of Ep-myc lymphomas in Rb+/− animals was variable [Fig. 1A, green, b] and only slightly accelerated relative to that observed in the wild-type background [hereafter referred to as control] [Fig. 1A, black, a]. In contrast, the onset of Ep-myc lymphomas in INK4a/ARF−/− and p53−/− animals [Fig. 1A, blue, c, and red, d] was highly reproducible and greatly accelerated compared with controls (P < 0.0001 each); the timing of lymphoma development in INK4a/ARF−/− and p53−/− mice was virtually identical. Cell surface staining confirmed that all lymphomas were of the B-cell lineage (B220+, Thy1.2−), whereas the distribution of pre-B (IgM−) and B (IgM+) was similar between the genotypes. These data imply that p53 and products of the INK4a/ARF locus limit Myc-induced lymphomagenesis. Concordantly, Ep-myc lymphomas arising in the p53+/+ and INK4a/ARF+/+ animals invariably lost the wild-type p53 or INK4a/ARF allele (93.8% and 88.2%, respectively) [Fig. 1B]. Hence, these lymphomas were either p53-null [p53−/−] or INK4a/ARF-null [INK4a/ARF−/−]. Virtually all control [6 out of 7], Rb+/− [4 out of 4], and INK4a/ARF+/+ [9 out of 9] tumors retained wild-type p53 as indicated by RT–PCR and sequencing of p53 exons 4–8 [data not shown]. The one p53 mutation identified [H190R in mouse; H193R in human] corresponds to a mutation observed in B-cell leukemias and a Burkitt’s lymphoma (Beroud and Soussi 1998). Deletions of the INK4a/ARF locus were noted in 20% of control and Rb+/− tumors [4 out of 20] but never in p53−/− tumors [0 out of 10] [data not shown]. In no instance did lymphomas arising in Rb+/− animals lose the wild-type Rb allele [Fig. 1B], confirming that Rb loss has a minimal impact on Myc-induced lymphomagenesis. Because Rb and p16INK4a loss should produce similar phenotypes (Haber 1997; Ruas and Peters 1998), these data imply that p19ARF is crucial for suppressing Myc-induced lymphomagenesis. The onset of Ep-myc lymphomas is markedly accelerated in ARF-deficient mice (Eischen et al. 1999).

Loss of INK4a/ARF or p53 promotes lymphoma spreading into visceral organs

INK4a/ARF−/− and p53−/− lymphomas were highly invasive and infiltrated into various nonlymphoid organs. For example, in mice bearing INK4a/ARF−/− and p53−/− lymphomas, we observed extensive perportal invasion and spreading of lymphoma cell clusters throughout the liver parenchyma and massive malignant pulmonary infiltration as consolidated aggregation of large mononuclear cells and within the distended interstitial capillaries [Fig.
p53 mutations can decrease cell death, increase proliferation, and produce chromosomal instability depending on context (Schmitt and Lowe 1999; Wallace-Brodeur and Lowe 1999). To determine the impact of Ink4a/Arf and p53 mutations on these characteristics, we examined apoptosis, mitotic index, and DNA content in control, Ink4a/Arf-/- and p53-/- lymphomas. As revealed by histological staining and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling), control lymphomas contained large numbers of apoptotic cells that clustered (Fig. 3A). Apoptosis was much reduced in Ink4a/Arf-/- or p53-/- lymphomas, and the apoptotic cells that appeared were isolated. Furthermore, primary Ink4a/Arf-/- and p53-/- lymphoma cells explanted into culture survived much better than controls (Fig. 3B). The mitotic index (Fig. 3C) and S-phase fraction (Fig. 3D) of all lymphoma types analyzed were similar, implying that Ink4a/Arf or p53 mutations did not affect the proliferation rate. DNA content analysis revealed one notable difference: Whereas most of the p53-/- tumors were aneuploid (10 out of 12), most control and Ink4a/Arf-/- tumors remained diploid (13 out of 14 and 13 out of 14, respectively) (Fig. 3D). Together, these data demonstrate that highly aggressive lymphomas can occur in the absence of chromosomal instability and imply that the aggressive nature of Ink4a/Arf-/- and p53-/- lymphomas is due to an apoptotic defect.

INK4a/ARF-/- lymphomas show an apoptotic defect but no genomic instability

p53 mutations can decrease cell death, increase proliferation, and produce chromosomal instability depending on context (Schmitt and Lowe 1999; Wallace-Brodeur and Lowe 1999). To determine the impact of Ink4a/Arf and p53 mutations on these characteristics, we examined apoptosis, mitotic index, and DNA content in control, Ink4a/Arf-/- and p53-/- lymphomas. As revealed by histological staining and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling), control lymphomas contained large numbers of apoptotic cells that clustered (Fig. 3A). Apoptosis was much reduced in Ink4a/Arf-/- or p53-/- lymphomas, and the apoptotic cells that appeared were isolated. Furthermore, primary Ink4a/Arf-/- and p53-/- lymphoma cells explanted into culture survived much better than controls (Fig. 3B). The mitotic index (Fig. 3C) and S-phase fraction (Fig. 3D) of all lymphoma types analyzed were similar, implying that Ink4a/Arf or p53 mutations did not affect the proliferation rate. DNA content analysis revealed one notable difference: Whereas most of the p53-/- tumors were aneuploid (10 out of 12), most control and Ink4a/Arf-/- tumors remained diploid (13 out of 14 and 13 out of 14, respectively) (Fig. 3D). Together, these data demonstrate that highly aggressive lymphomas can occur in the absence of chromosomal instability and imply that the aggressive nature of Ink4a/Arf-/- and p53-/- lymphomas is due to an apoptotic defect.

INK4a/ARF mutations compromise p53 function in vivo

The remarkable similarities between Ink4a/Arf-/- and p53-/- lymphomas suggest that these mutations disrupt...
overlapping tumor suppressor functions. In agreement, Es-myc lymphomas arising in mice heterozygous for both genes [INK4a/ARF-/-, p53-/-] were detected at the same time as lymphomas in the INK4a/ARF-/- and p53-/- animals (50th percentile = 38 vs. 38 vs. 36 days to onset, respectively) [Fig. 1A, orange, e] and never displayed coincident loss of both wild-type INK4a/ARF and p53 alleles [data not shown]. Therefore, inactivation of both genes is pathologically identical to their respective primary tumors (data not shown). In control lymphomas, p53 and p21 levels were dramatically increased 4 hr after treatment with cyclophosphamide (CTX), an alkylating agent used to treat human leukemia and lymphoma [Fig. 4B].

INK4a/ARF mutations affect the short-term response to anticancer treatment

Loss of either INK4a/ARF or p53 had a profound effect on drug-induced cell death in vitro and in vivo. In short-term cultures, INK4a/ARF-/- or p53-/- lymphomas displayed a marked resistance to mafosphamide [a CTX analog active in vitro] [Fig. 5A]. In peripheral blood, control animals harboring associated leukemias displayed a nearly 100-fold reduction in the white blood cell count (WBC) within 4 hr of CTX therapy, which coincided with a 6- to 10-fold accumulation of apoptotic cells [Fig. 5B, C]. In contrast, INK4a/ARF-/- and p53-/- leukemias took 12–24 hr to achieve a similar reduction. Apoptosis was not detectable, perhaps because the slow death rate allowed clearance of apoptotic cells before they could accumulate. In lymph nodes, control lymphomas displayed massive apoptosis 5 hr after CTX therapy, whereas the INK4a/ARF-/- and p53-/- lymphomas displayed substantially fewer dying cells [Fig. 5D].

INK4a/ARF mutations impair the long-term response to anticancer treatment

The ultimate determinant of drug-induced cell kill is tumor regression and the duration of remission. To assess long-term responses, animals harboring control, INK4a/ARF-/-, or p53-/- lymphomas were treated with CTX and monitored for remission and relapse by lymph node palpation and WBC. Control lymphomas responded extremely well to CTX treatment, and >70% remained in remission during the 100-day observation period [Fig. 6; black, a]. In stark contrast, INK4a/ARF-/- and p53-/- null tumors displayed an extremely poor response to CTX therapy: Despite initial responses, only 1 out of 14 p53-/- and 4 out of 35 INK4a/ARF-/- lymphomas remained in remission. p53-/- tumors [Fig. 6; red, c] were the most relapse prone [50th percentile = 20 days in remission, P < 0.0001 compared with control], although the defect in the INK4a/ARF-/- response [Fig. 6 blue, b] was also highly significant [50th percentile = 28 days in remission].

INK4a/ARF mutations reduce p53 activation following chemotherapy

The fact that p19ARF can cooperate with DNA-damaging agents to induce p53 and apoptosis raises the possibility that INK4a/ARF mutations might compromise cancer therapy [de Stanchina et al. 1998]. To test this, we examined the impact of INK4a/ARF or p53 mutations on drug-induced responses in reconstituted lymphomas or following short-term culture. Reconstituted lymphomas were produced following intravenous injection of primary lymphoma cells into syngenic (nontransgenic) recipients, thereby eliminating the possibility that secondary malignancies might complicate scoring tumor responses. Importantly, these lymphomas were histopathologically identical to their respective primary tumors (data not shown). In control lymphomas, p53 and p21 levels were dramatically increased 4 hr after treatment with cyclophosphamide (CTX), an alkylating agent used to treat human leukemia and lymphoma [Fig. 4B]. p53 and p21 levels also increased in INK4a/ARF-/- lymphomas, although this response was consistently reduced compared with controls. Therefore, in Es-myc lymphoma cells, INK4a/ARF mutations can reduce p53 activation by a DNA damaging agent.

Figure 4. p53 levels and activity in untreated and CTX-treated Ep-myc lymphomas. [A] Control [three independent tumors], INK4a/ARF-/- [four independent tumors], and p53-/- lymphoma lysates were probed against p53 and the p53 downstream target p21, reflecting p53's activity. Normal splenocytes [N] from nontransgenic mice were used for comparison. Tubulin [Tub] was used to verify protein loading. [B] Control, INK4a/ARF-/-, and p53-/- lymphoma cells were isolated from lymph nodes of untreated animals [NT] or 4 hr after CTX treatment [T1 and T2] and analyzed as above. For each tumor type, T1 and T2 were derived from separate primary tumors, whereas NT and T1 represent reconstituted lymphomas derived from the same primary tumor.
remission, $P = 0.0053$ compared with control). The response of INK4a/ARF$^{-/}$-p53$^{-/}$ double-mutant lymphomas ([Fig. 6; orange, d] was virtually identical to the p53$^{-/}$ tumors [50th percentile = 20 days in remission], and the relapsed tumors never displayed loss of the wild-type INK4a/ARF allele [data not shown]. Therefore, although INK4a/ARF mutations promote chemoresistance in the presence of wild-type p53 genes, they confer no additional survival advantage once p53 is mutated. These data demonstrate that INK4a/ARF mutations can compromise therapy, at least in part, by disabling p53.

Discussion

By comparing the properties of Myc-induced lymphomas in several genetic backgrounds, we provide compelling evidence that INK4a/ARF deletions can impact tumor development and anticancer therapy by compromising p53 function. Like p53$^{-/}$ tumors, INK4a/ARF$^{-/}$ lymphomas formed rapidly, were highly invasive, displayed apoptotic defects, and were markedly resistant to chemotherapy. Furthermore, INK4a/ARF$^{-/}$ lymphomas displayed attenuated p53 activity despite the presence of wild-type p53 genes. The profound impact of INK4a/ARF and p53 mutations on Myc-induced lymphomagenesis indicates that the ARF-p53 pathway contributes to oncogene-induced cell death in developing tumors and underscores the importance of this fail-safe mechanism in tumor suppression [also see Eischen et al. 1999; Jacobs et al. 1999]. Furthermore, that INK4a/ARF mutations can compromise drug-induced cell death in Ep-myc lymphomas implies that cooperative effects between oncogenes [in part via ARF] and DNA damage contribute to the remarkable drug sensitivity of some tumors.

The only substantial difference between INK4a/ARF$^{-/}$ and p53$^{-/}$ lymphomas was that the INK4a/ARF$^{-/}$ lymphomas remained diploid, whereas the p53$^{-/}$ lymphomas were aneuploid. This pattern is reminiscent of ARF$^{-/}$ and p53$^{-/}$ fibroblasts [Kamijo et al. 1997] and implies that p19ARF does not control the p53 functions involved in maintaining chromosome stability. Although we have not analyzed the secondary changes arising in INK4a/ARF$^{-/}$ tumors in detail, these data argue that invasive, chemoresistant lymphomas can arise in the absence of substantial chromosomal insta-

Figure 5. INK4a/ARF, p53, and short-term response to chemotherapy. (A) Explanted control ( ), INK4a/ARF$^{-/-}$ ( ), and p53$^{-/-}$ ( ) lymphoma cells were treated with mafosfamide [MAF]. Viability was analyzed after 24 hr by trypan blue exclusion; each value was normalized to untreated controls and represents the mean ± S.D. of two independently derived tumors reproduced in duplicate. (B) Leukemic mice were treated with CXT, and blood samples were taken at the indicated times. Each WBC is relative to its pretreatment value and represents the mean ± S.D. of three independent leukemias. Symbols are as in A. (C) Same as in B, except that blood samples were ethanol-fixed and stained with the DNA fluorochrome DAPI to visualize the chromatin condensation characteristic of apoptotic cells. Each value reflects the percentage of cells with apoptotic morphology of 200 cells counted and represents the mean ± S.D. of three independent leukemias. Symbols are as in A. (D) HE staining and TUNEL of lymph nodes harboring control, INK4a/ARF$^{-/-}$, and p53$^{-/-}$ lymphomas 5 hr after CXT treatment.

Figure 6. INK4a/ARF, p53, and long-term response to chemotherapy. Nontransgenic mice harboring reconstituted control ( , black, a), INK4a/ARF$^{-/-}$ ( , n = 35; blue, b), p53$^{-/-}$ ( , n = 14; red, c), and INK4a/ARF$^{-/-}$, p53$^{-/-}$ ( , n = 15; orange, d) lymphomas were treated with CXT and monitored for tumor regression and relapse. Importantly, CXT is not affected by classic multidrug resistance mechanisms that might complicate drug delivery. Tumor shrinkage to nonpalpability within 6 days after treatment is considered 'remission' and creates the tumor-free population at time 0. Relapse was defined by recurrent palpable lymph node enlargement. Values were plotted in Kaplan-Meier survival curve format and presented as percentage of mice in remission over the time post-therapy. Note that the overall rate of treatment failure in control lymphomas (~25%–30%) is consistent with the combined frequency of INK4a/ARF and p53 mutations we observe in these tumors.
bility. In turn, because INK4a/ARF mutations disable p53, the chromosomal instability observed in p53-/- lymphomas appears dispensable for the aggressive behavior of these tumors. More likely, the increased invasiveness and drug resistance of INK4a/ARF-/- and p53-/- lymphomas arises from an apoptotic defect. INK4a/ARF-/- and p53-/- lymphomas displayed decreased apoptosis in situ and ex vivo (see Figs. 3 and 5), whereas there was no obvious relationship between tumor-cell genotype and proliferation, as measured by mitotic index in vivo, DNA content analysis ex vivo, and proliferation properties in vitro (see Fig. 3, data not shown). These results stand in contrast to an earlier report indicating that p53-null Ep-myc lymphomas do not display an apoptotic defect [Hsu et al. 1995]. Although we cannot explain this discrepancy, it is worth noting that disruption of apoptosis using a bcl-2 transgene is sufficient to accelerate Myc-induced tumorigenesis [Strasser et al. 1990]. Moreover, ectopic expression of bcl-2 in the control lymphoma cells used in this study has no effect on proliferation but renders these cells highly invasive and chemoresistant following transplantation into syngenic mice [C.A. Schmitt and S.W. Lowe, unpubl.].

Although Ep-myc lymphomas harboring INK4a/ARF or p53 mutations are defective in CTX-induced cell death, CTX therapy induces complete remissions irrespective of p53 status. We suggest that the p53-independent death is due to apoptosis, because Ep-myc lymphomas expressing Bcl-2 are completely nonresponsive to CTX therapy at the maximally tolerated dose [C.A. Schmitt and S.W. Lowe, unpubl.]. In contrast to CTX, doxorubicin fails to induce remissions in p53-/- lymphomas, although high doses induce p53-independent apoptosis in vitro [R.R. Wallace-Brodeur, M.E. McCurrach, and S.W. Lowe, unpubl.]. Thus, the ability to achieve p53-independent killing depends on agent and dose. These data are consistent with the view that p53 is not an essential component of the apoptotic machinery but, rather, increases the probability that these agents trigger cell death [Lowe et al. 1993]. In Ep-myc lymphomas, this increased propensity for apoptosis can determine tumor cure or relapse.

Our data have important implications for the understanding of the clinical behavior of human tumors. First, they provide compelling evidence that disruption of apoptosis during tumor development can simultaneously select for chemoresistant cells. This pattern of coselection may explain why some tumors are de novo ‘resistant’ despite having no prior exposure to drug and why it is difficult to separate the impact of p53 mutations on treatment sensitivity from its contribution to overall patient prognosis. Second, our results demonstrate that tumors with extragenic mutations in the p53 pathway can display properties of p53 mutant tumors. This fundamental point is crucial for interpreting studies relating p53 mutations to clinical parameters in human patients, which typically classify tumors strictly by p53 gene or protein status. Our data imply that a substantial number of p53 ‘normal’ tumors would be misclassified by this approach (e.g., those harboring ARF mutations) and may explain why some studies fail to correlate p53 mutations with adverse clinical features [for review, see Brown and Wouters 1999].

This study provides the first evidence that INK4a/ARF mutations can have a negative impact on the outcome of cancer therapy and suggests that this defect arises from the failure of drugs to appropriately activate p53. Consequently, these data predict that disruption of the INK4a/ARF locus will contribute to chemoresistance in human tumors. As for p53 mutations, it seems likely that the overall impact of INK4a/ARF disruption on chemoresistance will depend on additional factors, such as tissue type, agent, and the mutational background of the tumor [for review, see Wallace-Brodeur and Lowe 1999; also see Bunz et al. 1999]. However, it is noteworthy that p53 mutations are strongly associated with highly aggressive tumors and chemoresistance in human hematologic malignancies (e.g., see Elrouby et al. 1993; Diccianni et al. 1994; Fan et al. 1994; Wattel et al. 1994; Wilson et al. 1997), indicating that Ep-myc lymphomas can recapitulate the behavior of human tumors. Therefore, we anticipate that this model will be useful for testing strategies to counter p53 and INK4a/ARF mutations in hematologic malignancies and other cancers.

Materials and methods

Mouse strains and tumor monitoring

All animal protocols used in this study were approved by the Cold Spring Harbor Laboratory Animal Care and Use Committee. Ep-myc transgenic mice [C57BL/6 inbred strain] and Rb-/-, INK4a/ARF-/-, and p53-/- mice [C57BL/6 x 129/sv] were crossed, and the offspring was genotyped by allele-specific PCR (Jacks et al. 1992, 1994; Serrano et al. 1996). Transgenic mice of the F1 generation [pooled from the different crosses] or transgenics being heterozygous for the named loci were monitored twice a week by palpation of the prescapular and cervical lymph nodes. Enlargements of at least 5 mm in the longest diameter were considered ‘well palpable’ and reflect malignant disease. For determining white blood cell status, blood smears and 20 µl of peripheral blood were obtained by tail artery bleeding. After ammoniumchloride hemolysis of the PBS-diluted and K3-EDTA-anticoagulized blood sample, white blood cells were counted in a hemocytometer. Blood smears were fixed and stained using the Leukostat kit (Fisher Diagnostics). Mice having WBC > 3 × 10⁹/µl and being positive for lymphoblastic cells in the blood stream were considered ‘leukemic’.

Histopathology

Animals harboring control, Rb-/-, INK4a/ARF-/-, and p53-/- lymphomas were sacrificed when prescapular lymph nodes reached a well-palpable size. Paraffin-embedded (7 µm), 4% neutral-buffered formalin-fixed tissue sections derived from lymph nodes and lung and liver specimens were stained with hematoxylin-eosin (HE) to evaluate apoptotic nuclear morphology and invasiveness of lymphoma cells into visceral organs.

Lymphoma characterization, LOH analysis, and RT-PCR sequencing

After CO₂ euthanasia, lymph nodes were dissected, minced in
PBS, and filtered through a 35-μm nylon mesh. Single cell suspensions of freshly harvested lymphomas were immunophenotyped by flow cytometry using antibodies directed against Thy-1.2, B220, and IgM [Pharmingen]. Pre-B-cell lymphomas are Thy-1.2+, B220+, and IgM-, whereas mature B-cell lymphomas are Thy-1.2+, B220+, and IgM+. To determine the mutational status of various genes, primary lymphoma cells were subjected to short-term culturing to eliminate normal cell contamination. Loss of the remaining wild-type allele [loss of heterozygosity (LOH)] in tumors arising in mice being heterozygous for an indicated tumor suppressor locus was detected by allele-specific PCR [Jacks et al. 1992, 1994; Serrano et al. 1996]. Exons 4-8 of the p53 gene were sequenced by dye termination in an automated sequencer (Perkin-Elmer) after reverse transcription (SuperScript, Gibco BRL) and PCR amplification of lymphoma cell total RNA. Finally, the gross integrity of the INK4a/ARF locus was assessed using PCR of exons 16 and exon 2 in a multiplex PCR reaction harboring primers to a positive control.

**Lymphoma cell culture and in vitro treatment**

Single cell suspensions of freshly extracted lymphoma cells (see above) were plated on irradiated [30 Gy] feeder layer [10⁶ NIH-3T3 cells/2.4-cm plate] in 45% Iscove's modified Eagle medium, 45% Dulbecco's minimal essential medium, 10% fetal bovine serum, 100 U/ml penicillin and streptomycin, 4 mM l-glutamine, and 25 μM 2-mercaptoethanol. For in vitro drug assays, mafosphonide (cyclohexylammonium salt, a CTX analog active in vitro; a generous gift from Asta Medica, Germany) was added at 0, 0.3, 3, and 30 μg/ml, and viability was measured (see below) 24 hr later.

**Assessment of viability, cell-cycle parameters, and apoptosis**

Viability of short-term cultured lymphoma cells was analyzed by trypan blue dye exclusion. For analysis of ploidy, apoptosis (as percentage of cells in sub-G₁ peak), and proliferation (as percentage of viable cells in S phase), 10⁶ ethanol-fixed lymphoma cells were incubated for 30 min at room temperature in 1 ml of DNA staining solution (200 μg of propidium iodide and 2 μg of RNase in 10 ml of PBS), and DNA content was measured at 488 nm in a flow cytometer (FACScalibur, Becton Dickinson). In situ proliferation was estimated by counting of mitotic figures (cells in anaphase or telophase) relative to cell number in HE-stained lymphoma sections (four samples each genotype, seven different fields, 200 cells each). In situ apoptosis was visualized in lymphoma sections by HE staining and a fluorescence-based TUNEL assay. TUNEL assays were performed in accordance to the manufacturer's protocol (Boehringer Mannheim). Leukemias were analyzed for apoptotic nuclear morphology by fluorescence microscopy after ethanol fixation and DAPI (4',6-Diamidino-2-phenylindole) staining of peripheral blood samples.

**Western blotting analysis**

Whole-cell lymphoma cell or normal splenocyte lysates were generated by lysing of extracted cells in SDS sample buffer (60 mM Tris-HCl at pH 6.8, 10% glycerol, 2% SDS, and 5% 2-mercaptoethanol). Samples corresponding to 60 μg of protein [Bio-Rad Bradford protein assay] were separated on a SDS-polyacrylamide gel and transferred to Immobilon-P membranes (Millipore). p53 was detected using the polyclonal antibody CM5 [Novocastra, 1:2000 dilution], p21 using the polyclonal antibody C-19 [Santa Cruz, 1:500 dilution], and α-tubulin using the monoclonal antibody B-5-1-2 [Sigma, 1:2000 dilution]. Protein detection was visualized by ECL (Amersham) or Supersignal (Pierce).

**Lymphoma reconstitution and in vivo treatment**

Immediately after extraction, 10⁶ lymphoma cells in 100 μl of PBS were reconstituted by tail vein injection into genetically matched, nontransgenic recipient mice (two mice per individual lymphoma sample) to monitor response to treatment. Tumors derived from the INK4a/ARF¹⁻ and p53¹⁻ backgrounds were reconstituted in C57BL/6 x 129/sv mice (Jackson Laboratories). CTX was applied as a single 300-mg/kg dose i.p. when arising tumors became well palpable.

**Statistical evaluation**

Tumor onset data reflect the time between birth and first-time palpability of enlarged lymph nodes; treatment response data reflect the time between remission and relapse as first-time palpability of a recurrent lymph node enlargement. Individual time values were plotted in the Kaplan-Meier population-event-time course format and compared using the log-rank (Mantel-Cox) test. Comparisons of means and standard deviations (S.D.) were performed using the unpaired t-test. Ploidy, cell cycle distribution, and sub-G₁ content were analyzed using the ModFit LT 2.0 software.

**Acknowledgments**

We thank T. Jacks for the Rb¹⁻ and p53¹⁻ mice, M. Serrano and D. Beach for the INK4a/ARF¹⁻ mice, A. Harris for the Eµ-myc transgenic mice, K. Sokol for histopathology, L. Bianco and the CSHL animal facility for technical assistance; M. Ockler and M. Roussel, C. Sherr, and J. Cleveland for discussion of unpublished data. This work was supported by a Dr. Mildred Scheel Cancer Foundation fellowship (C.A.S.), a DOD Breast Cancer Research fellowship (E.d.S.), a Kimmel Scholar Award (S.W.L.), and a grant (CA13106) from the National Cancer Institute (S.W.L.).

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**References**


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