Regulation of the Arf Tumor Suppressor in Eμ-Myc Transgenic Mice: Longitudinal Study of Myc-induced Lymphomagenesis

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Lymphomagenesis in E\(\mu\)-Myc mice is opposed by the Arf tumor suppressor, whose inactivation compromises p53 function and accelerates disease. Finding nascent E\(\mu\)-Myc-induced tumors in which p19\(^{Arf}\) causes cell cycle arrest or apoptosis is problematic, since such cells will be eliminated until Arf or p53 function is lost.

“Knock-in” mice expressing a green fluorescent protein (GFP) in lieu of Arf coding sequences allow analysis of Arf promoter regulation uncoupled from p19 Arf action. Prior to frank lymphoma development, unexpectedly low levels of E\(\mu\)-Myc-induced p19\(^{Arf}\) or GFP were expressed. However, as lymphomas arose in Arf\(^{+/GFP}\) heterozygotes, additional oncogenic events synergized with E\(\mu\)-Myc to further induce the functionally null Arf-GFP allele. Concomitant up-regulation of p19\(^{Arf}\) was not observed; instead, the wild type allele was inactivated. We infer that very low levels of Arf are tumor suppressive, and that further induction provides the selective pressure for the emergence of tumors that have inactivated the gene.

Introduction

The CDKN2A (INK4A/ARF) locus, which encodes two distinct tumor suppressors (p16\(^{INK4a}\) and p14\(^{ARF}\)) from partially overlapping reading frames, is commonly inactivated in human cancers.\(^1,2\) Mice homozygous or heterozygous for an Arf null allele are predisposed to cancer, and tumors arising in Arf\(^{+/}\) mice inactivate the wild type Arf allele, indicating that Arf behaves as a classical “two hit” tumor suppressor gene. Arf is induced by abnormally sustained, increased thresholds of mitogenic signals emanating from mutationally activated or dysregulated oncogenes. In turn, the Arf protein (p19\(^{Arf}\) in mice) antagonizes the activity of the p53 negative regulator Mdm2 to induce a p53 transcriptional response that leads to cell cycle arrest or apoptosis.\(^2,3\)

Consequences of Arf inactivation have been widely studied in E\(\mu\)-Myc transgenic mice\(^4-6\) in which Myc, regulated by the immunoglobulin heavy chain enhancer-promoter, is expressed in B lymphocytes.\(^7-9\) E\(\mu\)-Myc mice exhibit an initial pre-neoplastic phase
characterized by polyclonal expansion of pre-B cells already evident before birth.\(^8\) Within their first year of life, these mice develop malignant monoclonal lymphomas (mean latency = 25 weeks). Bi-allelic loss of \(Arf\) occurs in \(~25\%\) of these animals, whereas another 33% display \(p53\) mutations.\(^4\) \(Arf^{+/}\), E\(\mu\)-Myc mice more rapidly develop tumors (mean latency 7-10 weeks depending on strain background) in which the wild-type \(Arf\) allele is commonly inactivated, thus canceling selective pressure for \(p53\) mutations. \(Arf^{-}\), E\(\mu\)-Myc mice are even more prone to lymphoma development and die of aggressive lympholeukemias by only 4-7 weeks of age. Although the evidence that \(Arf\) acts as a tumor suppressor gene in this model is unequivocal, no longitudinal study of \(Arf\) regulation has yet been undertaken. Aided by use of a high-affinity monoclonal antibody to p19\(^{\text{Arf}}\)\(^{10}\) and a knock-in \(Arf\)-\(GFP\) reporter mouse,\(^{11}\) we have now evaluated the response of \(Arf\) throughout the course of lymphoma development in E\(\mu\)-Myc mice.

**Materials and methods**

Work with mice has been approved by the St. Jude Children's Research Hospital Animal Care and Use Committee under Animal Protocol #225 (Function of INK4a/ARF in pediatric neoplasia). A purified monoclonal antibody (5-C3-1) directed to mouse p19\(^{\text{Arf}}\)) which is 25-fold more sensitive than previously derived reagents\(^{10}\) was conjugated to AlexaFluor-647 and used to score Arf expression in fixed B lymphoid cells by fluorescence-activated flow cytometry (FACS). All other procedures for analysis of B cells from E\(\mu\)-Myc mice of various \(Arf\) genotypes have been described in detail.\(^4,11,12\)

**Results and Discussion**

Spleen and bone marrow cells were harvested from groups of overtly healthy E\(\mu\)-Myc and nontransgenic control mice of various ages, and B lineage cells, isolated using magnetic beads coated with the B220 antibody to CD45R, were lysed and immunoblotted using MAb 5-C3-1 to p19\(^{\text{Arf}}\). Low levels of p19\(^{\text{Arf}}\) were detected in B220+ cells from
Eµ-Myc (M) but not control (C) mice of two weeks of age and did not increase thereafter as long as disease was latent (Figure 1A). The p53 protein and p21$^\text{Cip1}$, a p53-responsive gene product, were also weakly induced, as compared to more robust p53 induction in Arf-null B cells exposed to ionizing radiation (lane 2 vs 1). The levels of p19$^\text{Arf}$ expressed were about 20-fold less than those observed in p53-null lymphomas, in which Arf is induced to high levels but is without effect, and at least 200-fold less than those in cultured, immortalized p53-null B cells (Figure 1A, lane 3; Figure 1B). Dual-color FACS analysis performed on gated B220+ cells using MAb 5-C3-1 conjugated to AlexaFluor-647 revealed that the entire population of pre-lymphomatous B cells exhibited modestly increased fluorescence (Figure 1C). Given that Arf inactivation greatly accelerates lymphomagenesis, these very low levels of p19$^\text{Arf}$ and p53 expressed throughout the B220+ population must contribute to tumor suppression. However, protection is by no means absolute because the proliferative fraction of B cells in the spleen and bone marrow is significantly increased in the presence of Eµ-Myc, and all such animals ultimately develop lymphomas.

We next performed longitudinal studies with Arf$^+/\text{GFP}$ heterozygotes, in which one Arf allele has been replaced by a cDNA cassette encoding GFP under the control of the Arf promoter. The Arf-GFP allele behaves as a null and accelerates Eµ-Myc-induced lymphomagenesis. Tumors arising in Arf$^+/\text{GFP}$ mice inactivate the wild type Arf allele, retain functional p53, and are brightly green fluorescent.

In Eµ–Myc mice, lymphoblastic lymphoma is accompanied by lympholeukemia, so that an increase in the peripheral blood white cell count precedes the appearance of clinically palpable tumors. Animals were considered to be in an intermediate phase of disease when their white blood cell counts, monitored weekly, rose to 30,000 per µl; moribund mice with frank tumor masses were classified as terminal. Expression of GFP and p19$^\text{Arf}$ in lymphoid organs was monitored throughout.
During the pre-lymphomatous latent phase, we detected low levels of p19\textsuperscript{Arf} in splenocytes from E\textsubscript{µ}-Myc, Arf \textsuperscript{+/GFP} mice, but not in nontransgenic control animals (Figure 2A, lanes 1-3). At this time, GFP expression could not be detected by FACS or immunoblotting in these same tissues, most likely due to the relatively reduced sensitivities of these assays. However, GFP-positive cells appeared in peripheral blood and accumulated as total white cell counts rose (Figure 2B), implying that at least one additional oncogenic event synergizes with E\textsubscript{µ}-Myc to further activate the Arf promoter.

In direct contrast to the Arf-GFP allele, expression of p19\textsuperscript{Arf} protein encoded by the wild type Arf allele was reduced (Figure 2A, lanes 5, 6, 8) or completely absent (lane 4) in splenocytes from Arf\textsuperscript{+/GFP} mice in the intermediate phase of disease and was almost invariably undetectable in 22 mice during their terminal phase (examples in lanes 9,10).

Thus, while further Arf induction might transiently enhance tumor suppression during the intermediate phase (as can only be inferred from the behavior of the Arf-GFP allele), this process must also provide a concomitant selective pressure favoring the outgrowth of lymphoma cells that no longer express the wild-type Arf allele.

In further support of the idea that Arf inactivation is itself insufficient to trigger lymphomagenesis in E\textsubscript{µ}-Myc transgenic mice, GFP was not detectably expressed in the lymphoid tissues of two week-old E\textsubscript{µ}-Myc, Arf\textsuperscript{GFP/GFP} homozygous mice that are even more highly tumor-prone. When GFP-positive cells later appeared in their peripheral blood, FACS analysis of gated B220+ spleen and bone marrow cells revealed the emergence of subpopulations exhibiting high GFP fluorescence, as would result from an expanding clonal malignancy (Figure 2C). Tumor masses and invasive malignant cells at terminal phase were uniformly and brightly fluorescent\textsuperscript{11}. In short, cryptic mutations other than Arf loss accompany the conversion of pre-malignant E\textsubscript{µ}-Myc B cells to tumor cells; this precedes robust Arf induction, but also precipitates Arf inactivation.

The nature of these additional mutations remains unknown. Various genetic alterations that attenuate apoptosis have been documented in cultured tumor cells and in
bone marrow recovered from E\(\mu\)-Myc mice,\(^{13-17}\) but this process is difficult to detect \textit{in vivo}, probably because dying B cells are very rapidly eliminated.\(^{18}\) Activating mutations in \textit{Myc} itself\(^{16}\) or those affecting other collaborating oncogenes\(^{15,17}\) could well exert similar inductive effects on the \textit{Arf} promoter.

Together, these findings argue that seemingly negligible levels of p19\(^\text{Arf}\) or p53 observed in incipient tumor cells sustaining initiating oncogenic mutations exert significant tumor suppressive effects. This may be particularly important in the analysis of human tumor material where antibodies capable of detecting equally low levels of p14\(^\text{ARF}\) are not yet available and where \textit{ARF} may therefore not be inferred to play any tumor suppressive role.
References


Acknowledgement

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Figure 1

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C

Relative Cell No.

Log Alexa-647 Fluorescence

10^1 10^2
Figure 1. *Arf* induction in pre-lymphomatous Eμ-Myc mice. (A) B220+ cells were purified from the bone marrow (BM) and spleens (Sp) of nontransgenic control C57BL/6 (C) mice or from syngeneic animals expressing the Eμ-Myc (M) transgene. Cells were harvested from healthy mice at bi-weekly (w) intervals after birth. Cell lysates [25 μg protein per lane from B220+ cells purified from bone marrow (BM) or spleen (Sp)] were separated on denaturing gels and proteins were immunoblotted with antibodies directed to p19Arf (5-C3-1)\(^{10}\), c-Myc (N-262, Santa Cruz), p53 (NCL-p53-505, Novocastra), p21 (F5, Santa Cruz), or beta-actin (A-5441, Sigma), as indicated at the left of the panels. Cultured *Arf*-null B cells were untreated (lane 1) or irradiated with 5 Gy 2 hours prior to lysis (lane 2) to induce higher levels of p53. Cultured *p53*-null B cells express very high levels of p19Arf (lane 3). (B) Lysates (μg total protein as indicated at the bottom of the panel) of B220+ bone marrow (BM) cells and splenocytes (Sp) purified from control (C) or Eμ-Myc (M) transgenic mice were immunoblotted with antibodies to p19Arf. The signals were compared with those generated with various quantities of lysate protein obtained from a representative *p53*-null lymphoma and from cultured *p53*-null B cells. (C) Cultured B cells from *p53*-null mice were stained with an isotype-matched control MAb (panel i, negative control) or with MAb 5-C3-1 to p19Arf (panel ii; *p53*-null cells as positive control) conjugated to AlexaFluor-647. *Arf*-null cells served as an additional negative control for MAb 5-C3-1 staining (panel iii). Bone marrow (BM) and spleen cells (Sp) from control [Myc (-)] or transgenic [Myc (+)] mice of 2 weeks of age were assayed by dual-color FACS using antibodies to B220 and to p19Arf (panels iv-ix). Gated B220+ cells in bone marrow (BM, panels iv-vi) and spleen (Sp, panels vii-ix) were analyzed for reactivity with AlexaFluor-647-conjugated MAb 5-C3-1 directed to p19Arf.
Figure 2

A

B

C

Relative Cell No.

BM

Sp

Log GFP Fluorescence

10^3 10^6 10^9

10^3 10^6 10^9
Figure 2. Analysis of p19Arf and Arf-GFP reporter expression in Eµ-Myc transgenic mice. (A) Levels of p19Arf were determined in splenocytes from control (lane 1) and Eµ-Myc transgenic animals at the designated stages of disease (lanes 2-10). Cultured Arf-null (lane 11) and p53-null B cells (lane 12) served as controls. Whereas splenocytes from mice in the latent phase do not express GFP, significant proportions of splenocytes (>20%) expressed the Arf-GFP allele at later stages of disease. The overall levels of p19Arf were diminished or absent in 4 of 5 intermediate phase mice (lanes 4-8) and almost invariably absent in terminal phase mice (two examples of 22 mice are shown in lanes 9 and 10). (B) High-level expression of the Arf-GFP allele was initially detected by FACS analysis in a subset of peripheral white blood cells (squares) just prior to the development of lympholeukemia, as manifested by white blood cell counts of >30,000 per µl (diamonds). Animals that became moribund were sacrificed ~2 weeks later (triangles; terminal phase). (C) B220+ bone marrow (BM) and spleen cells (Sp) taken during the intermediate phase of disease from Eµ-Myc, ArfGFP/GFP mice initially generated a subpopulation of cells that express high levels of the Arf-GFP allele.
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