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DNA Damage Responses and Chemosensitivity in the *E*μ-*myc* Mouse Lymphoma Model

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Delivering DNA damage is an established concept to treat cancer, but the precise mechanisms that determine whether or not this damage will kill tumor cells are poorly understood. Many anticancer agents are known to produce DNA damage upon interaction with specific intracellular targets (e.g., topoisomerases, DNA itself, and the replication machinery). This damage, in turn, can produce catastrophic cell death owing to gross metabolic dysfunction or initiate a programmed response (e.g., apoptosis) leading to the regulated death of the tumor cell. Resistance to catastrophic death presumably requires mutations that prevent the drug-target interaction, whereas resistance to programmed cell death can also involve effector defects that maintain viability despite substantial cellular damage. The relative impact of various resistance mechanisms to clinical drug resistance is controversial and few have been validated in naturally occurring tumors.

Genetic factors that influence treatment sensitivity are typically evaluated in established cancer cell lines treated in vitro or as xenografts in immunocompromised mice. However, cell culture studies fail to take into account the complexity of the tumor microenvironment (i.e., survival factors, hypoxia, normal cell interactions), which has been shown to play an important role in the treatment response (Graeber et al. 1996; Walker et al. 1997). Moreover, depending on the experimental readout for drug-induced cell kill—such as short-term viability or clonogenic survival—very different results may be obtained (Brown and Wouters 1999). Although ectopic or orthotopic tumor transplantation systems are certainly better approximations of the physiological state of bona fide human tumors, they still rely on cell lines that are necessarily selected for vigorous proliferation and survival in culture. To our knowledge, there has been no study demonstrating that cell lines recapitulate the treatment sensitivity of primary tumors.

Of course, patient material is the most relevant setting in which to study drug action. However, controlled human studies are often impractical and in general can only achieve correlative information between specific genetic changes to patient responses or overall survival. Moreover, many of these studies are inconclusive, perhaps because human tumors are highly heterogeneous or because it is difficult to correlate mutations in individual genes to treatment sensitivity in a tumor that may contain other mutations in the same genetic program (Schmitt and Lowe 1999). Indeed, different approaches have produced remarkably different perceptions of the relative impact of various resistance mechanisms on clinical outcome. For example, the impact of crucial genes such as *p53* and *bcl-2* on therapeutic outcome in patients is still under debate.

In principle, transgenic and "knockout" mouse models could be used to circumvent many of the problems associated with studies using cell lines or patient material. For example, specific genetic alterations can be introduced into the mouse germ line or into primary tumors to generate relatively defined tumors. A series of mice harboring spontaneous, but highly related, tumors can then be treated at their natural site with identical regimens to evaluate the impact of the genetic alteration on clinical outcome. Because experimental strategies are not limited by the same considerations applied to patients, studies using mouse models can achieve a much more direct and controlled analysis of treatment sensitivity. Yet, although transgenic mice have revolutionized our understanding of the molecular basis of tumor development, their contribution to our understanding of cancer therapy has been minimal (e.g., see references in Schmitt et al. 1999, 2000; Bearss et al. 2000; Omer et al. 2000). Our laboratory has made a concerted effort to investigate whether transgenic mouse models will be useful to identify determinants of treatment sensitivity. Here, we describe implications of a lymphoma model developed to study the genes and processes that affect anticancer therapy in vivo. Given the good approximation of corresponding human malignancies by transgenic tumor models, our studies suggest that these models will be useful in understanding the treatment sensitivity of human cancers.

EXPERIMENTAL PROCEDURES

Mice and tumor monitoring. *E*μ-*myc* transgenic mice and *INK4a/ARF*$^{-/-}$, *Rb*$^{+/-}$, and p53$^{-/-}$ mice were crossed and the offspring was genotyped by allele-specific polymerase chain reaction (PCR) (Jacks et al. 1992, 1994; Serrano et al. 1996). Transgenic mice of the F_1 generation (hereafter referred to as controls; 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 (LN), whereby "well palpable" lymphoma reflect LN enlargements of at least 5 mm in the longest diameter. Blood samples were obtained by tail artery bleeding. After red cell lysis, white blood cells were counted in a hemocytometer. Blood smears were fixed and stained according to a modified Wright's protocol using the Leukostat kit (Fisher Diagnostics). White blood cell counts >3 × 10^5/μl and de-

tectability of cells with lymphoblastic morphology in the peripheral blood smear were considered "leukemic."

Lymphoma processing and characterization. After CO_2 euthanasia, LN were resected and either fixed in 4% neutral-buffered formalin or minced in phosphate-buffered saline (PBS) and filtered through a 35-μm nylon mesh; 7-μm paraffin-embedded, formalin-fixed LN sections were stained with hematoxylin-eosin (HE) to evaluate apoptotic cell morphology. Single cell suspensions were plated on irradiated (30 Gy) feeder layer (2.5×10^5 NIH-3T3 cells/10-cm plate) in 45% Iscove's modified Eagle medium, 45% Dulbecco's minimal essential medium, 10% fetal bovine serum, 100 units/ml penicillin and streptomycin, 4 mM L-glutamine, and 25 μM 2-mercaptoethanol. Genomic DNA was isolated by proteinase K digest after short-term culturing to eliminate contaminating normal cells.

In vitro treatments and short-term viability assays. Viability of lymphoma cells was analyzed by Trypan Blue dye exclusion. For short-term in vitro assays, 10^6 viable lymphoma cells were plated in conditioned medium (i.e., supernatant from irradiated NIH-3T3 feeder layers); irradiation, adriamycin (ADR), or mafosphamide was administered at the indicated doses; and viability was measured relative to untreated controls 24 hours later.

Retroviral transduction of Eμ-myc lymphoma cells. Retroviral infections were performed using murine stem cell virus (MSCV)-based vectors that encode green fluorescent protein (GFP) 3′ of an internal ribosomal entry site of a bicistronic message. The murine *bcl-2* cDNA was cloned into MSCV-GFP. Retrovirus was produced using Phoenix packaging cells (G. Nolan, Stanford University, California) as described (Serrano et al. 1997) and collected in B-cell medium; 3 ml of high-titer viral supernatant containing 4 μg/ml polybrene (Sigma) was passed through a 0.45-μm filter and supplemented with 50 μg/ml lipopolysaccharide from *Salmonella typhimurium* (Sigma) to infect 5×10^6 exponentially growing lymphoma cells by spinoculation on feeder cells at 600 g for 10 minutes. The infection procedure was repeated three times every 8 hours. After 24 hours, GFP expression was assessed by flow cytometry (FACScalibur, Becton Dickinson) to estimate the percentage of infected cells. The transduced cell populations (typically >70% GFP-positive) were either propagated in recipient mice or used for subsequent in vitro assays.

Lymphoma reconstitution and in vivo treatment. Uncultured or retrovirally transduced lymphoma samples were transplanted into genetically matched, nontransgenic 6–10-week-old female mice by tail vein injection (10^6 viable lymphoma cells in PBS), and recipient mice were palpated for tumor formation twice a week. When tumors became well-palpable, γ-irradiation (IRR), ADR, or cyclophosphamide (CTX) was applied as a single 6 Gy total body, 10 mg/kg, or 300 mg/kg body weight intraperitoneal treatment, respectively, and mice were subsequently monitored for treatment response.

Western blotting analysis and assessment of apoptosis ex vivo. Western blotting analysis of whole-cell lymphoma cell lysates was carried out as described (Schmitt et al. 1999) using anti-p53 and antipoly(ADR-ribose) polymerase (PARP) polyclonal antibodies. Activation of caspases was detected by affinity labeling and Western blotting analysis of lymphoma cell lysates preincubated with the biotinylated tetrapeptide caspase inhibitors DEVD-chloromethyl ketone and YVAD-acyloxymethyl ketone, which mimic cleavage sites for active caspases. Binding of the biotinylated peptides was visualized by probing with a streptavidin-biotinylated horseradish peroxidase complex (Amersham) and a chemiluminescent substrate (Faleiro et al. 1997). Electrophoretic separation and visualization of internucleosomal DNA fragmentation (DNA laddering) of genomic lymphoma DNA samples were carried out as described previously (Lowe et al. 1993). Apoptotic DNA strand breaks of individual cells were detected in freshly isolated, single-cell lymphoma suspensions by a fluorescence-based TUNEL (terminal deoxynucleotidyl transferase dUTP nick end-labeling) assay, performed in accordance to the manufacturer's protocol (Boehringer Mannheim).

Statistical evaluation. Treatment response data reflect the time between remission, i.e., shrinkage of a palpable lymphoma to nonpalpability within 6 days after treatment, and recurrent palpability of a LN enlargement (relapse). Persistence of palpable LN within 6 days after treatment is considered "no response" and reflects either a "no change" (NC) situation or, in case of lymphomas growing under treatment, a "progressive disease" (PD). Individual time-to-relapse values were plotted in the Kaplan-Meier survival curve format, and the curves were compared using the log-rank (Mantel-Cox) test.

RESULTS

Eμ-myc Lymphomas Respond to Therapy

Eμ-myc transgenic mice overexpress the c-Myc oncoprotein in B cells and develop pre-B or B-cell lymphoma with associated leukemia by several months of age (Fig. 1A) (Adams et al. 1985; Harris et al. 1988). These mice provide several features that make them a potentially attractive system for studying drug-mediated cytotoxicity in vivo: (1) Tumor burden can be easily monitored by LN palpation or blood smears; (2) lymphomas are detectable long before the animals succumb to the malignancy; (3) high quantities of pure tumor cells can be extracted from lymphoma-bearing mice; (4) treatment responses are not biased by an immunocompromised environment; (5) lymphoma cells can be cultured and transplanted into numerous syngeneic (but nontransgenic) mice; and (6) the histopathological and genetic features of *Eμ-myc* lymphomas closely resemble human non-Hodgkin's lymphomas.

To test whether Myc-induced lymphomas respond to therapy, animals bearing well-palpable tumors (>50 mm^3) in prescapular or cervical LN were treated with various anticancer regimens. We have chosen total-body

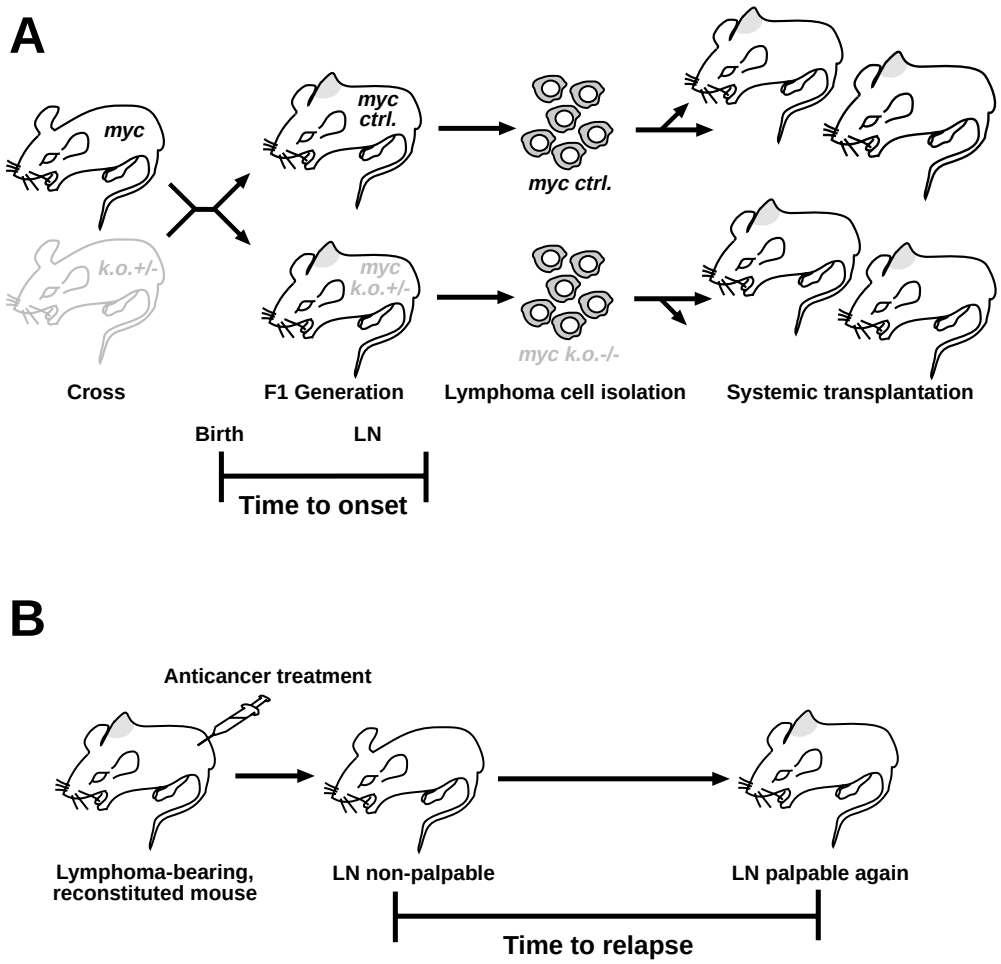

Figure 1. The *Eμ-myc* mouse as a tractable model of lymphomagenesis and anticancer treatment sensitivity. (*A*) Generation of transplantable, genetically controlled primary lymphomas. *Eμ-myc* transgenic mice develop B-cell lymphoma with palpable lymph-node (LN) enlargements by several months of age; for monitoring of lymphoma development, the time between birth and first-time palpability of peripheral lymphomas is considered the "time to onset." After CO_2 euthanasia, enlarged LN were resected, minced, and filtered in phosphate-buffered saline and subjected to further analysis or systemic transplantation by tail-vein injection into nontransgenic, genetically matched recipient mice. By crossing to mice harboring heterozygous disruptions of candidate collaborator genes (knockout mice, [k.o.]), offspring carrying the heterozygous constellation develop lymphomas that might have lost the remaining wild-type allele, making those lymphomas in fact null for the gene of interest. (*B*) Anticancer treatment of mice bearing reconstituted lymphoma. At the time of LN palpability, a single dose treatment is systemically applied. Failure to shrink the peripheral LN to a nonpalpable stage reflects "nonresponsiveness," whereas otherwise the time between non- to repalpability of the LN is considered the "time to relapse."

IRR, the anthracycline ADR (a topoisomerase II inhibitor), and the oxazaphosphorin CTX (an alkylating agent), because these therapies are known to be effective against lymphoma/leukemia and are thought to have distinct resistance mechanisms. The tumor response is measured as time to relapse, the period from treatment-induced disappearance of the lymphomas to the re-appearance of a well-palpable tumor (Fig. 1B). A tumor is considered "no remission" if the LN enlargement persists despite treatment. In our scheme, mice are designated as in remission or "disease-free" if the tumor shrinks to a nonpalpable level within 6 days of treatment. Responses can be depicted as Kaplan-Meier curves showing the percentages of mice that are in remission after treatment at a given time. The vast majority of *Eμ-myc* lymphomas responded to therapy, albeit to a varying degree (see Fig. 2A for CTX). Furthermore, the disease-free time lasted in most cases longer than the observation period of 100 days after achieving initial remission, reflecting a cure from the malignancy.

A potential problem in studying lymphoma responses to therapy in transgenic mice is that all B cells express the *myc* transgene, which may account for unphysiological treatment effects on the normal hematopoietic compartment, and thereby could reduce the accuracy of the model. Moreover, it is difficult to distinguish tumor relapses from secondary malignancies. Therefore, we also studied tumor responses using transplanted lymphomas as outlined in Figure 1A. In principle, this strategy has the following advantages: (1) Recipient mice are the same age and sex, minimizing variation; (2) recipient mice are not transgenic; therefore, relapsed tumors are necessarily

derived from the original tumor; (3) material from the same tumor can be compared before and after treatment; (4) the same primary tumor can be assessed for response to different agents; (5) the same primary tumor can be assessed for reproducibility of treatment responses in several animals; and (6) the number of transgenic mice is reduced.

To determine the feasibility of this approach, lymphoma cells were harvested from transgenic mice harboring well-palpable LN, and 10^6 cells were immediately transplanted into 6–10-week-old female C57BL/6 mice by tail vein injection. Well-palpable lymphomas arose typically within 2–3 weeks. Primary and transplanted tumors appeared to be equally disseminated diseases and were histologically indistinguishable (Schmitt et al. 2000). Upon tumor manifestation, animals were treated in the same way as the transgenic mice. Primary and transplanted tumors treated with CTX responded similarly, whereby late relapses in the group of primary tumor-bearing mice were most likely due to secondary, transgene-driven malignancies (Fig. 2A). Furthermore, identical treatments of the same individual lymphoma reconstituted in several animals resulted in highly reproducible responses (data not shown). Therefore, reconstituted lymphomas can reliably recapitulate the responses of primary tumors.

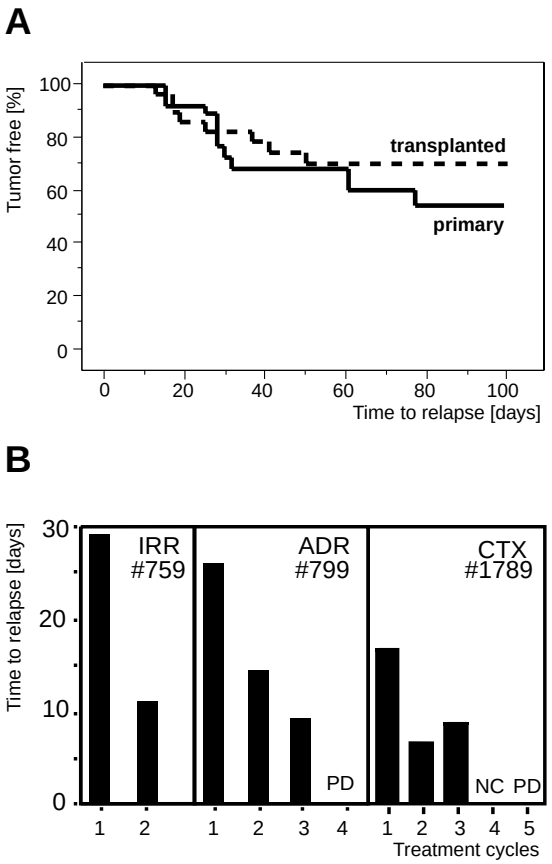

Figure 2. Transplanted *myc* lymphomas preserve their biological properties. (*A*) Mice harboring transplanted control lymphomas (*dashed line*) respond similarly to CTX compared to transgenic animals with primary control lymphomas (*solid line*). Plotted are the disease-free times (as time to relapse) in the Kaplan-Meier survival curve format after a single treatment with CTX ($n_{prim.} = 22$ and $n_{transpl.} = 60$, respectively). Note that late "relapses" of the primary lymphoma group might be in fact secondary transgene-driven malignancies. (*B*) Transplanted lymphomas recapitulate the progressive loss of chemosensitivity in cancer patients. Repetitive treatments (numbered as treatment cycles) with total body IRR, ADR, or CTX of mice (numbers indicate individual primary lymphomas) that relapsed to the initial treatment typically result in shortened remission periods (time to relapse) and finally in no response at all (as indicated as no change [NC] or tumor growth under treatment, which is progressive disease [PD]).

Eμ-myc Lymphomas Can Acquire Drug Resistance

In human patients, many initially responding tumors become progressively less responsive after the first relapse, thereby determining the ultimate fate of the patient. Similarly, in the subset of mice that did not achieve a long-term remission, repetitive treatments frequently shortened remission periods when compared to the first response and eventually resulted in resistance to any further treatment (Fig. 2B). Therefore, monitoring the "time to first relapse" in our system (Fig. 2A) seems to be a good prognosticator for terminal failure to repetitive treatments as well. Although drug-resistant tumors were produced by repetitive treatments with the same drug, they often became cross-resistant to other drugs with distinct primary mechanisms of action. For example, tumors selected for resistance to CTX were invariably cross-resistant to ADR (data not shown). We expect that the ability to model acquired drug resistance in vivo will facilitate a better understanding of this major clinical problem.

Anticancer Treatment Induces p53 and Promotes Apoptosis In Vivo

An advantage of the *Eμ-myc* model is that large numbers of lymphoma cells can be isolated from peripheral LN for biochemical analysis of therapy-induced death. Since well-palpable, malignant LN have at least 50–100 times the volume of normal LN, they predominantly consist of tumor cells, which minimizes effects of normal cell contamination. We have taken advantage of these properties to identify cellular changes that occur in lymphoma cells treated with DNA-damaging anticancer agents in vivo. For example, the p53 tumor suppressor can be induced by DNA damage leading to cell cycle arrest or apoptosis (Kastan et al. 1991; Lowe et al. 1993). To determine whether therapy activates p53 in *Eμ-myc* lymphomas in vivo, we injected a series of mice with 10^6 cells derived from the same lymphoma and allowed well-palpable lymphomas to form. Tumor-bearing mice received a single treatment, and LN were harvested 1, 2, or 4 hours later. Upon exposure to IRR or CTX, p53 protein levels were strongly induced in a time-dependent manner (Fig. 3A, top), whereby ADR activated p53 with a slower kinetic, achieving maximal induction at about 8 hours (data not shown).

We also monitored these lymphomas for characteristic features of apoptosis, including caspase activation, DNA fragmentation, and morphological changes. Anticancer treatment led to cleavage of the caspase substrate PARP

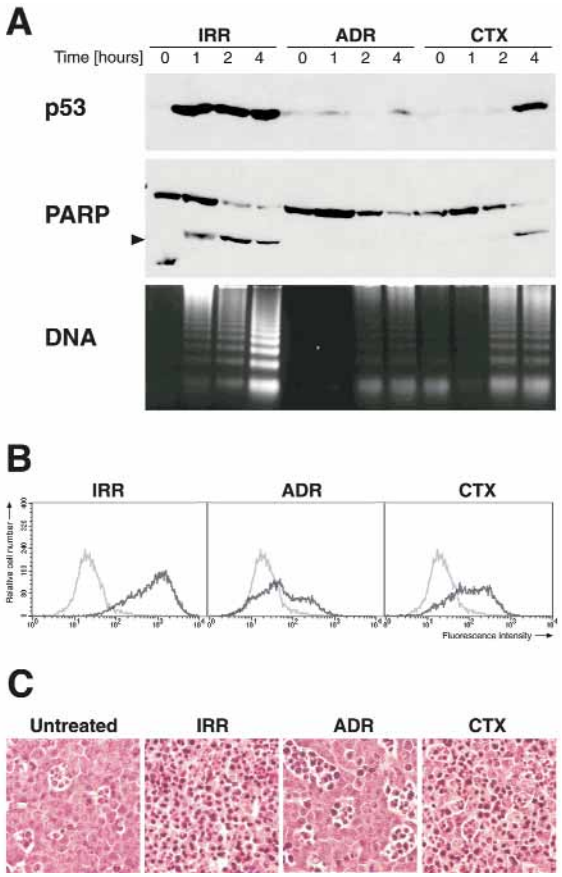

Figure 3. Anticancer treatment activates the apoptotic program in vivo. (*A*) Time-course analysis of the apoptotic response in mice reconstituted with a control lymphoma after either IRR (6 Gy total body dose), ADR (10 mg/kg body weight intraperitoneally), or CTX (300 mg/kg body weight intraperitoneally). Lymphoma cells were isolated at the indicated time points and subjected to Western blot analysis for p53 expression and poly(ADP-ribose) polymerase (PARP) cleavage (arrow indicates cleavage product) or to agarose gel electrophoresis of genomic DNA to visualize DNA laddering. (*B*) FITC-fluorescence-based TUNEL reaction to detect DNA strand breaks, another hallmark of apoptosis, by flow cytometry of single-cell suspensions derived from the same reconstituted control lymphoma untreated or 4 hr after treatment with IRR, ADR, or CTX. Untreated cells as negative control (*gray curve*), treated samples (*black curve*) as indicated. (*C*) Hematoxylin-eosin (HE)-stained LN sections of lymphoma samples as in *B*. Note the extent of chromatin condensation and nuclear morphology as the typical hallmarks of apoptotic cell deaths in islands of spontaneous cell death in the untreated sample and as a consequence of anticancer treatment.

(Kaufmann et al. 1993; Lazebnik et al. 1994), with kinetics closely following that observed for p53 accumulation (Fig. 3A, middle). Moreover, active caspases were detected in treated lymphomas using biotinylated tetrapeptide inhibitors such as DEVD and YVAD (data not shown). All treatments resulted in internucleosomal fragmentation of genomic DNA, a late event in apoptotic cell death known as "DNA laddering" (Fig. 3A, bottom) (Wyllie et al. 1992). Cells with fragmented DNA can be quantified using a modified version of the TUNEL assay that labels DNA strand breaks with fluorescein. Note that the TUNEL reaction preferentially labels apoptotic cells instead of cells undergoing necrosis or containing primary strand breaks directly caused by radiation or anticancer drugs (Gorczyca et al. 1993). Using this assay, we observed a 10–100-fold signal induction relative to the untreated control in isolated lymphoma cells 4 hours after treatment (Fig. 3B).

To complement this analysis, cell death was examined in situ using standard histological staining. Untreated LN had small islands of cells with morpholgical features of apoptosis, suggesting a relatively high rate of spontaneous apoptosis (Fig. 3C) (see Harris et al. 1988; Hsu et al. 1995; Schmitt et al. 1999). However, IRR and CTX treatment induced massive apoptosis throughout the LN within 4 hours of treatment. As for p53 induction, the amount of apoptosis was less dramatic for ADR (Fig. 3B,C). Hence, anticancer agents can induce p53 and promote apoptosis in *Eμ-myc* lymphomas in vivo.

The p53 Pathway and Treatment Sensitivity

Although p53 loss can impair treatment sensitivity in many test systems, the extent to which p53 contributes to treatment outcome of human tumors is not well established. Several explanations may account for these discrepancies, including the possibility that the role of p53 in therapy depends on the cellular context, as well as technical difficulties to correctly assess p53 status or to uncover such a relationship in heterogeneous patient material (for a detailed review, see Schmitt and Lowe 1999). If p53 is in fact a key regulator of therapy-induced cell death in vivo, then lymphomas lacking functional p53 should display a cell death defect upon exposure to treatment. To test this hypothesis, we crossed *Eμ-myc* transgenic mice to mice harboring heterozygous targeted disruptions of either the *p53*, the *INK4a/ARF,* or the retinoblastoma protein (*Rb*) locus (Fig. 1A) (Jacks et al. 1992, 1994; Serrano et al. 1996). Of note, the *INK4a/ARF* mutation used here disrupts both ARF function, an upstream regulator of p53, and $p16^{INK4a}$, which disables the Rb tumor suppressor pathway (for review, see Sherr 1998), and recapitulates the gross deletions of the *INK4a/ARF* locus frequently found in human cancer entities. Lymphomas arising in $p53^{+/-}$ or $INK4a/ARF^{+/-}$ mice invariably lost the wild-type *p53* or *INK4a/ARF* allele, respectively, whereas $Rb^{+/-}$-derived lymphomas retained the wild-type *Rb* allele. This approach of generating "loss-of-function" mutants intrinsically allows one to monitor the impact of a disrupted gene on lymphoma onset, measured as time between birth and first-time palpability of enlarged LN (Fig. 1A). Recent studies have shown that activated oncogenes signal to p53 via ARF (de Stanchina et al. 1998; Zindy et al. 1998). Indeed, disruption of either *p53* or the *INK4a/ARF* locus dramatically accelerated *myc*-induced lymphomagenesis, whereas $Rb^{+/-}$ status did not, implying that the ARF-p53 pathway, rather than the $p16^{INK4a}$-Rb axis, acts as a failsafe mechanism for *myc*-induced lymphomagenesis (for detailed discussion, see Schmitt et al. 1999).

Primary *myc*-driven *p53* null or *INK4a/ARF* null lymphomas provide a useful system to examine the impact of these loci on treatment response. Since these tumors arise

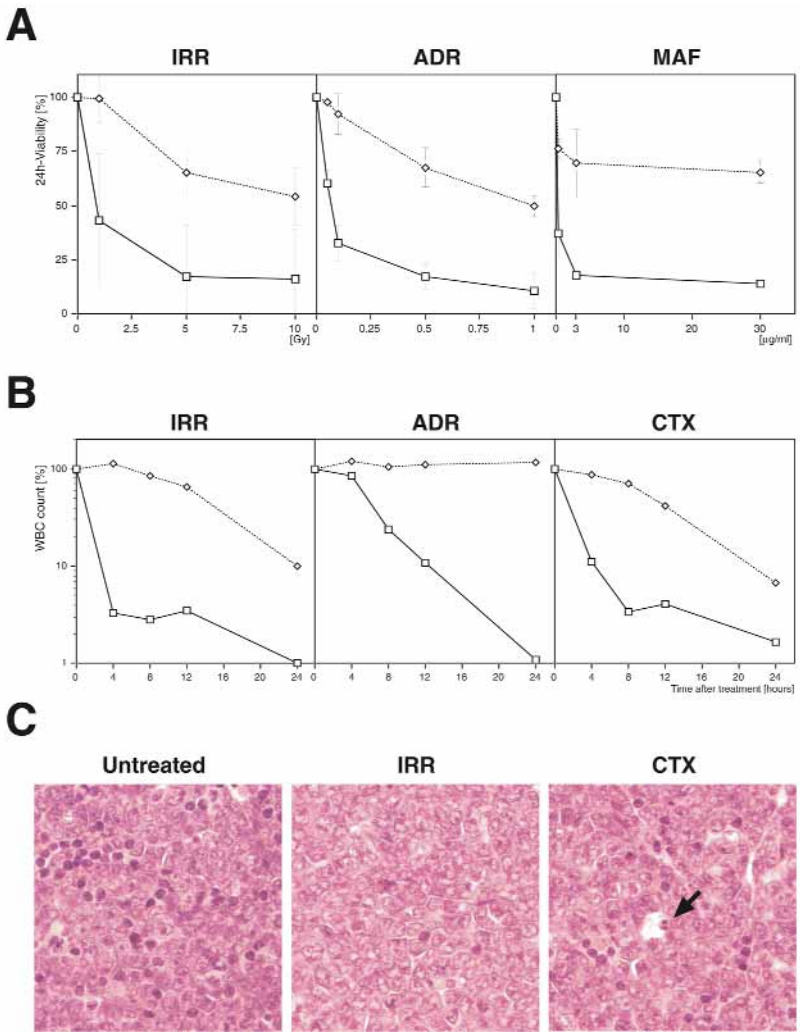

Figure 4. Short-term response to anticancer treatment dependent on the *p53* status. (*A*) 24-hr in vitro viability assay of freshly extracted control (*solid line, squares*) and *p53* null (*dotted line, diamonds*) lymphoma cells treated with IRR, ADR, or mafosphamide (MAF, a CTX analog active in vitro) at the indicated doses relative to untreated samples. (*B*) 24-hr in vivo time course of the white blood cell (WBC) count in leukemic mice harboring either control (*solid line, squares*) or *p53* null (*dotted line, diamonds*) lymphomas after a single treatment as indicated relative to the initial WBC count. (*C*) HE-stained LN sections of a reconstituted *p53* null lymphoma untreated or 4 hr after treatment with IRR or CTX (arrow points to a single dying cell with apoptotic morphology); compare to Fig. 3C.

spontaneously and can only be considered "isogenic" for the depleted loci, analysis of multiple individual tumors controls for the unknown genetic alterations arising in these cells. Therefore, numerous control (i.e., Eμ-*myc* lymphomas arising in a control genetic background), *INK4a/ARF* null, and *p53* null lymphoma cells were either placed in short-term cultures in vitro or injected directly into genetically matched recipients where lymphomas were allowed to form. In short-term viability assays, *INK4a/ARF* null and *p53* null lymphomas were highly resistant to various anticancer treatments. Whereas very low doses of each treatment achieved nearly quantitative killing of control lymphomas, more than 50% of the *INK4a/ARF* null (Schmitt et al. 1999) or *p53* null lymphomas remained viable even at very high doses (Fig. 4A).

To address short-term responsiveness in vivo, we examined white blood cell (WBC) counts in leukemic animals or evaluated LN several hours after systemic treatment of mice bearing either control, *INK4a/ARF* or *p53* null lymphomas with IRR, ADR, or CTX. Measuring WBC counts in a time-dependent manner is a powerful tool to assess short-term drug-induced effects in our system (Fig. 4B). Again, control leukemias responded well, resulting in a substantial drop of the relative WBC count already after 4 hours (IRR and CTX; ADR delayed) and virtually a clearing of the circulation from leukemic blasts after 24 hours for all treatment types. In contrast, *p53* null leukemias disappeared with much slower kinetics; 12 hours after treatment application, the relative WBC count was at least tenfold higher compared to control leukemias at that time. However, *p53* null leukemias were not completely refractory to therapy, since we typically failed to detect leukemias at later time points (data not shown). Similarly, in LN sections from mice harboring *p53* null lymphomas, spontaneous cell death was ab-

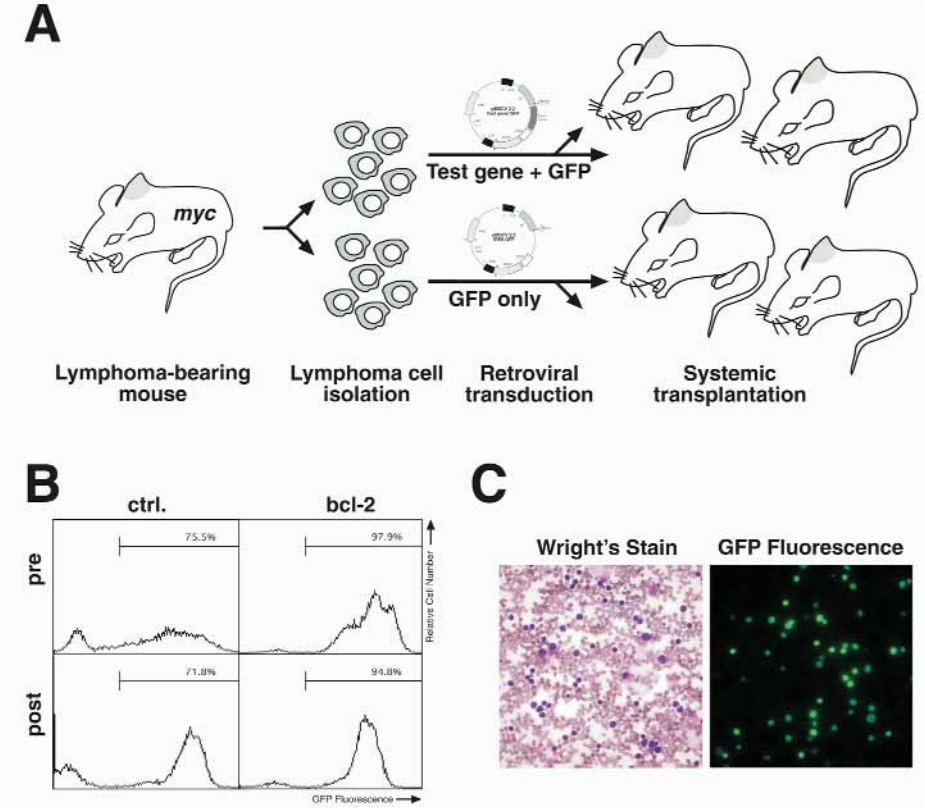

Figure 5. Retroviral gene transfer into primary *myc* lymphomas. (*A*) Technical outline. Freshly harvested primary lymphomas are subjected to short-term culture in two identical aliquots. Using the murine stem cell virus (MSCV) optimized to drive long-term gene expression in B cells (Prince and Rigby 1991), a test gene in conjunction with GFP on a bicistronic message or GFP alone can be efficiently introduced into lymphoma cells within 4 days. Upon infection, lymphoma cells can be transplanted into recipient animals. (*B*) Infection efficacy (here with MSCV-GFP as control [ctrl.] and MSCV-bcl-2-GFP [bcl-2]) can be monitored by flow cytometric assessment of green fluorescence before (pre) transplantation. After tumor formation (post), similar percentages of GFP-positive lymphoma cells can be isolated from the animals, underlining that there is no in vivo selection against GFP. (*C*) Visualization of GFP-expressing lymphoma cells in vivo. Blood smear (Wright's stain, *left*) of a leukemic recipient of MSCV-GFP infected lymphoma cells using fluorescence microscopy (*right*).

rogated, and virtually no cell death was detectable 4 hours after treatment with IRR or CTX (Fig. 4C; compare to Fig. 3C). The short-term in vivo response curves of mice harboring *INK4a/ARF* null leukemias and the extent of cell death in their LN sections several hours after treatment with CTX were found to be similar to *p53* null lymphomas (Schmitt et al. 1999).

Candidate Genes Can Be Introduced into Primary Lymphomas and Studied In Vivo

Due to the possibility that loss of the remaining wild-type allele in *INK4a/ARF*$^{+/-}$ or *p53*$^{+/-}$ lymphomas may occur at a relatively early stage in lymphomagenesis, the established malignancies might biologically differ more substantially from controls than only in their *INK4a/ARF* or *p53* status, respectively. Therefore, we developed a protocol to rapidly and efficiently infect primary lymphomas with MSCV-based retroviral vectors encoding a test gene in conjunction with GFP on a bicistronic message or GFP alone. Using this approach, gain-of-function activities—or dominant-negative derivatives—can be introduced along with empty vector controls into a series of primary lymphomas to generate "matched pairs" differing only in the status of the test gene (Fig. 5A). Lymphomas infected with the empty vector or a candidate resistance gene—here, the proto-oncogene *bcl-2*–can be monitored for infection efficacy by flow cytometric GFP expression analysis. Moreover, in vivo formation, maintenance, or selection of a (partially) GFP-positive (and simulta-neously test gene expressing) transplant malignancy can be addressed by flow cytometric analysis of re-extracted lymphoma cells (Fig. 5B) or by fluorescence microscopy of leukemic blood smears (Fig. 5C).

Cell Culture Establishment Alters Treatment Sensitivity In Vitro

Using the retroviral gene-transfer strategy outlined in Figure 5A, we generated a series of primary lymphomas expressing GFP alone or together with Bcl-2 and confirmed in this "matched pair" setting that overexpression of Bcl-2 could protect from drug-induced cell death in short-term viability assays (Schmitt et al. 2000). However, when these freshly harvested lymphomas were subjected to continuous culturing for 4 weeks, the relative

impact of Bcl-2 on drug sensitivity was dramatically reduced. This effect was due to the fact that control lymphomas rapidly acquired resistance to anticancer treatment simply by serial passaging in culture (Schmitt et al. 2000). The dramatic change in treatment sensitivity produced by cell culture establishment of lymphomas raises general concerns about the use of cell lines to study drug action.

Classic Clonogenic Survival Assays Fail to Detect Apoptosis-related Chemoresistance

Since short-term viability assays do not measure overall survival but examine a "snapshot" in time, it is formally possible that alterations that score in these assays may only delay cell death. In contrast, clonogenic survival assays measure long-term proliferative capacity of treated cells and, hence, have often been considered the "gold standard" for in vitro assessment of chemosensitivity (Brown and Wouters 1999). For example, although Bcl-2 can suppress cell death in short-term assays, several groups have shown that Bcl-2 does not reduce drug-induced cell kill in clonogenic survival assays (Yin and Schimke 1995; Lock and Stribinskiene 1996; Kyprianou et al. 1997; Schmitt et al. 2000). Indeed, these observations have questioned the relative importance of apoptosis on treatment sensitivity.

Using matched pairs of primary lymphoma cells differing only in their Bcl-2 status, we have provided evidence that the failure of Bcl-2 to enhance cell "survival" may result from several technical caveats of the assay. First, we demonstrated that Bcl-2 did not enhance clonogenic survival following drug treatment of primary lymphomas under standard conditions, but revealed modest protective function when the assay was performed in the presence of interleukin-7 (Schmitt et al. 2000). This is consistent with previous reports that survival factors provided by the natural tumor microenvironment can influence the ability of Bcl-2 to block cell death (Walker et al. 1997). Those factors are probably insufficient or absent at the very low cell densities and the single cell type setting of a standard clonogenic assay. Second, we noticed that clonal outgrowth of primary tumor cells seeded at a clonogenic density is already extremely poor in the absence of any drug (Schmitt et al. 2000). This suggested that colonies might be escape mutants that already possessed an apoptotic defect with activities similar to those of Bcl-2, making comparisons between expressing and nonexpressing samples noninformative. In fact, reverse transcriptase-PCR (RT-PCR) sequencing detected *p53* mutations in more than half of the clones tested (Schmitt et al. 2000). Finally, our in vivo analyses clearly showed that Bcl-2 can promote drug resistance and tumor cell survival following drug treatment (see below).

Genetic Defects of the Apoptotic Machinery Reduce Disease-free Survival upon Anticancer Treatment

Using different approaches—crosses to knockout mice generating loss-of-function mutants as well as retroviral gene transfer producing gain-of-function mutants—to evaluate the contribution of p53-mediated apoptosis to treatment outcome, we established a profound relationship between apoptotic function and disease-free survival in vivo. To assess long-term responses, animals harboring control, *INK4a/ARF,* or *p53* null lymphomas were treated with CTX and monitored for remission and relapse by peripheral LN palpation (Fig. 1B). Control lymphomas responded extremely well to CTX and about 70% remained in remission during the 100-day observation period (Fig. 6, top). In statistically highly significant contrast, *INK4a/ARF* and *p53* null tumors displayed an extremely poor long-term result to CTX. Despite initial responses, only 1/14 *p53* null and 4/35 *INK4a/ARF* null lymphomas remained in remission, and most of the relapses occurred already within 40 days after drug administration (Fig. 6, top). We never detected loss of the remaining wild-type *INK4a/ARF* allele in relapsed "double knockout" *p53* null/*INK4a/ARF*$^{+/-}$ lymphomas (C. Schmitt and S. Lowe, unpubl.), implying that *INK4a/ARF* mutations can compromise therapy outcome, in part, by disabling p53 (Schmitt et al. 1999).

Infection with an "empty vector" control did not interfere with the excellent response of control lymphomas to CTX, here achieving a long-term remission in about 65% of the mice (Fig. 6, bottom). However, about 80% of the mice harboring Bcl-2 overexpressing lymphomas failed to respond initially to CTX, and the small fraction of re-

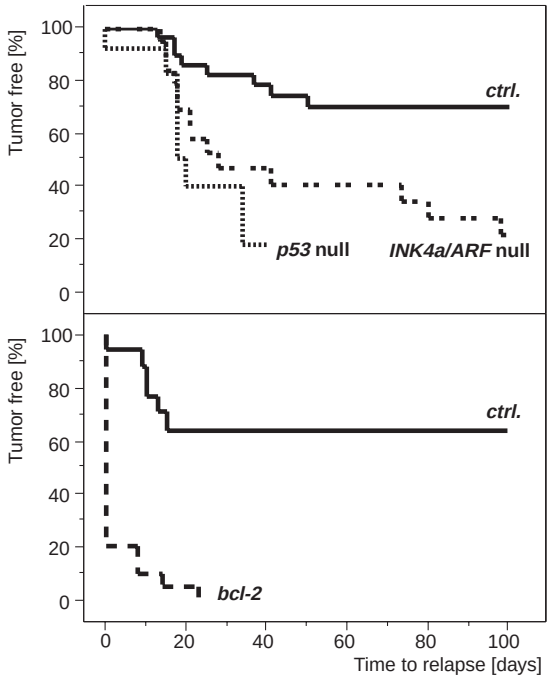

Figure 6. Long-term response to anticancer treatment of mice harboring genetically controlled lymphomas. Kaplan-Meier curves for disease-free survival (time to relapse) of mice being transplated with ctrl. (*solid line*), *INK4a/ARF* null (*dots*), or *p53* null (*dashes*) lymphomas (*top*; $n = 60$, 35, and 14, respectively) or harboring either MSCV-GFP (ctrl.; *solid line*) or MSCV-bcl-2-GFP (bcl-2; *dashed line*) infected control lymphomas (*bottom*; $n = 17$ and 20, respectively) after a single treatment with CTX. (Reprinted, with permission, from Schmitt et al. 1999 [copyright Cold Spring Harbor Laboratory Press] and from Schmitt et al. 2000 [copyright Nature Publishing Group.] http://www.nature.com.)

sponding animals had only very short disease-free survival periods (Fig. 6, bottom) (Schmitt et al. 2000). Therefore, Bcl-2 seems to act as a potent drug resistance gene if assessed in a model capable of reflecting the complexity of a whole organism and the interactions of a tumor with environmental factors at its natural site.

DISCUSSION

The *Eμ-myc* Model Is a Physiological Test System to Study the Genetics of Chemoresistance

Although the biological and genetic determinants of chemosensitivity are typically studied in established cell lines, it is not obvious whether these systems accurately reflect or predict the treatment response of bona fide tumors. In this paper, we show that aspects of chemosensitivity relevant in vivo can be missed using cell lines or certain in vitro assays, and we present an in vivo model to study responses of spontaneous tumors treated at their natural site. Our system is based on the *Eμ-myc* transgenic mouse, which develops B-cell lymphomas that resemble closely human non-Hodgkin's lymphomas with respect to their histopathological properties and clinical behavior in response to treatment. For example, treatment of mice harboring control lymphomas with various antineoplastic treatments produces in most cases long-lasting remissions. As in patients, tumors that relapse typically display a progressive shortening of subsequent remission periods under repetitive treatments. Moreover, in *Eμ-myc* lymphomas, disruption of p53 function severely reduced treatment sensitivity and long-term responses (Schmitt et al. 1999). This is in good agreement with clinical findings relating *p53* mutations to drug resistance in human hematologic malignancies (El-Rouby et al. 1993; Wattel et al. 1994; Ichikawa et al. 1997; Wilson et al. 1997; Navaratnam et al. 1998; Moller et al. 1999). Hence, the information we obtain from this model should be applicable to human cancer.

The extent to which findings from animal models can be extrapolated to the human condition has been the subject of much debate, although most of these concerns relate to technical limitations of a specific model (Berger 1999; Bibby 1999; Fiebig et al. 1999; Rudmann and Durham 1999). The major power of our system is the ability to use transgenic mouse methods and gene transfer protocols to engineer tumors with specific genetic lesions, and then study the treatment sensitivity of a series of these tumors in vivo. This approach achieves a general homogeneity of clinical material that vastly exceeds that possible in human clinical trials. At the same time, the ability to treat a series of mice harboring independent but related tumors recapitulates the unique character of individual tumors in a patient population and presents a more realistic picture of treatment sensitivity than repetitive treatments of the same tumor cell line in xeno- or allograft settings. In contrast to other new models using surgical orthotopic implantation of primary tumor material (for review, see Hoffman 1999), the *Eμ-myc* model is an easy to handle system and allows statistically meaningful data to be generated in a relatively cost-effective way.

Treatment Sensitivity Relies on Intact Apoptotic Pathways

By studying the impact of genes on treatment sensitivity in the *Eμ-myc* model, we provide important insights into the genetic mechanisms of chemoresistance. First, our data underscore the notion that apoptosis is important for treatment sensitivity and that disruption of apoptosis is an important mechanism of chemoresistance. Second, we demonstrate that disruption of apoptosis is a true multidrug resistance mechanism, since lesions in the apoptotic program produced similar reductions in sensitivity to different treatments (e.g., alkylating agent vs. irradiation). Third, we have shown that mutations that disable apoptosis during tumor development can simultaneously coselect for drug resistance, even in the absence of any prior treatment. For example, lymphomas arising as *p53* null tumors not only formed much faster than their $p53^{+/+}$ counterparts, but also responded much worse to a single dose treatment. Finally, we have established the importance of p53 for treatment sensitivity in a physiological setting and shown that disruption of components of the *p53* pathway can produce treatment resistance even in the presence of wild-type *p53* genes. This highlights the problem that clinical studies classifying tumors strictly on *p53* status might overlook *p53* pathway defects as a potential cause of drug resistance despite the absence of *p53* mutations.

Genes Contribute to the Probability of a Given Cell to Undergo Drug-mediated Cell Death

Although tumors overexpressing Bcl-2 or lacking the *INK4a/ARF* or *p53* genes displayed very poor long-term responses to therapy, only Bcl-2 overexpression rendered lymphomas nonresponsive to treatment. Particularly when treated with CTX, most *INK4a/ARF* or *p53* null lymphomas ultimately achieved substantial tumor reductions that could even be classified as a "complete response," since the enlarged LN and associated leukemias often completely disappeared, albeit with delayed kinetics compared to control tumors. Nevertheless, almost all of these tumors relapsed, indicating the presence of residual disease. Of note, we ruled out the possibility that these relapses could originate from lymphoma cells that survived in compartments not accessible for CTX, since total body IRR produced treatment failures of *p53* null and *INK4a/ARF* null lymphoma-bearing animals as well (C. Schmitt and S. Lowe, unpubl.).

How can we explain that a mutation accounts for shortened remission periods, although a treatment can achieve a complete remission irrespective of this mutation in vivo? Drug-induced cell death is a dynamic phenomenon occurring at a stochastic likelihood in a cell population: A susceptible fraction of the tumor will eventually die based on (1) the locally effective drug dose (after drug delivery and pretumoral metabolism); (2) the ability of the treatment to kill in a specific (apoptosis) and nonspecific manner (necrosis, catastrophic death) or to initiate other specific cellular response programs (e.g., long-term arrest); and (3) the condition of any given cell (cellular fitness) depending on previously influencing factors (hypoxic

conditions, survival factors provided by surrounding cells, etc.). In other words, the key question is: When, if at all, will each individual cell of a tumor exposed to treatment die? Following up on a model previously proposed based on observations in *p53*-deficient mouse embryo fibroblasts (Lowe et al. 1993), we understand the role of p53 in anticancer treatment response as a regulator to facilitate cell death upon sensitization by DNA damage. In this—necessarily oversimplified—"probability-to-die" model, the efficiency of a drug to kill at a given time is plotted as a dose-dependent probability to die (Fig. 7). This correlation between dose and cell death follows a sigmoid-shaped distribution function. Although functional p53 promotes drug-induced death, loss of p53 shifts this curve to the right, making it less likely for a cell to die from the same dose. Importantly, by controlling the death probability in any individual cell in response to a given amount of damage, genes control the rate of cell death in the entire tumor cell population. Reflecting the same stochastic model, the efficiency of a treatment to destroy a tumor mass facing a constant amount of damage follows a sigmoid-shaped time-dependent distribution function. Thus, the reduced likelihood of a *p53* null cell to die results in a slower tumor reduction in a *p53*-deficient malignancy or, in turn, requires higher doses to achieve death rates comparable to *p53* wild-type tumors. Since dose escalation is limited by general anticancer drug-related toxicity as well as drug-specific side effects, the administered dose cannot exceed the therapeutic window, which is the dose range between a tumor-effective and an intolerably toxic drug dose.

As seen in several examples in this paper, different treatments work in different therapeutic windows with respect to the *p53* status: IRR or CTX substantially decreased the number of *p53* null lymphoma cells in circulation, whereas no reduction was observed in the same period for treatment with ADR (see Fig. 4B). Therefore, different treatments kill in their specific therapeutic windows in a mostly p53-dependent manner, a partially p53-independent or even an almost p53-independent manner (Fig. 7). According to this model, a more p53-independent treatment like CTX can even kill log-ranks of cells in a *p53*-deficient tumor but cannot prevent the very small fraction of remaining cells to cause the clinical relapse.

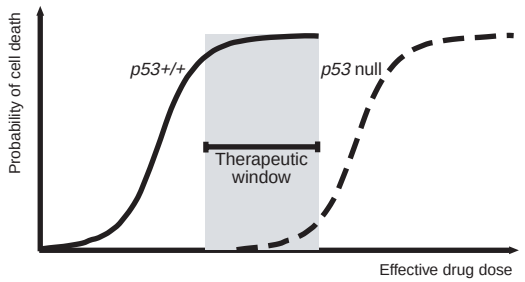

Figure 7. Probability-to-die model of chemosensitivity and *p53* status. Given a normally distributed likelihood for any individual cell exposed to the same local dose of an anticancer treatment to die from that treatment within a certain time, a dose increment will theoretically result in a higher fraction of killed cells in the observed population. As discussed in the text, cells lacking functional p53 (*dashed line*) have a decreased probability of dying from the same local dose, but ideally still maintain the dose-response characteristics of their *p53* wild-type (*solid line*) counterparts. A treatment applied within the therapeutic window, the dose range between objective anticancer effects, and the limitations due to toxic side effects in nonmalignant compartments will therefore preferentially kill *p53* wild-type cells but, to a lesser extent, *p53* null cells as well. Note that the therapeutic window is a treatment-specific constant, making different treatments more or less p53-dependent.

If genetic lesions within a tumor affect the probability that a tumor cell will finally die within a certain exposure time or dose of damage, then three other parameters become important: drug half-life, tumor regrowth rate, and alternative cellular response programs. Although drug half-life and tumor regrowth are exactly the factors allowing a *p53*-deficient tumor to die slowly enough to emerge from the remaining viable population when no drug is effective anymore, alternative response programs add more complexity to the model. For example, it is known that tumor cells can undergo a long-term arrest in response to DNA damage but may still be able to re-enter the cell cycle (Di Leonardo et al. 1994). In fact, we observed that Bcl-2 overexpressing lymphomas, which typically failed to regress upon treatment, progressed very slowly until they eventually killed the tumor-hosting animal (C. Schmitt and S. Lowe, unpubl.). Furthermore, this behavior seems to be due to a p53-dependent cell cycle arrest, since mice harboring *p53* null Bcl-2 overexpressing lymphomas rapidly reached a terminal stage after unsuccessful treatment (C. Schmitt and S. Lowe, unpubl.). Although their functions only partially overlap—Bcl-2 strictly antiapoptotic, p53 controlling apoptosis and arrest—the effect on the probability-to-die model is synergistic for Bcl-2 overexpression and loss of p53 function, since both promote a slower death rate in the tumor population, which, in turn, is the basis for relapses. Although this model certainly highlights only selected aspects of drug sensitivity and resistance, it is in good agreement with our in vivo observations and typical clinical courses of patients under chemotherapy.

PERSPECTIVE: MOUSE MODELS AND CANCER THERAPY

We believe that physiological mouse model systems such as the *Eμ-myc* transgenic mouse provide a useful "bridge" between cell-line-based systems and clinical trials. Like cell line systems, these models are highly manipulatable. At the same time, they allow analysis of drug action in realistic settings without the cost and ethical considerations involved in patient trials. Mouse systems can be used to study the impact of specific genes on treatment sensitivity, and, indeed, we have validated this approach using *Eμ-myc* lymphomas harboring mutations that are extremely common in human malignancies.In addition, despite inevitable species-specific differences between mice and humans, we expect that analysis of treatment sensitivity in mice will establish general principles that will apply to the human condition. Finally, the unique ability to genetically engineer highly defined tumors in mice will facilitate preclinical studies to evaluate the action of novel compounds directed against specific genetic lesions.

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