Susceptibility to Tumors Induced in Mice by Ethynitrosourea Is Independent of Retinoblastoma Gene Dosage

Daniel J. Riley, Chen-Ching Lai, Chi-Yao Chang, Diane Jones, Eva Y-H. P. Lee, and Wen-Hwa Lee

Institute of Biotechnology, Center for Molecular Medicine, The University of Texas Health Science Center, San Antonio, Texas 78223-3207

Abstract

The retinoblastoma gene (RB) is a classical tumor suppressor. Several studies have shown that RB dosage is important in determining biological effects. To explore the effect of RB dosage on susceptibility to cancer, three groups of congenic C57Bl/6 mice, each of which expresses a different amount of Rb protein from one, two, or three alleles, were treated at postnatal day 12 with a single 60-kg/kg body weight i.p. dose of the DNA-alkylating agent N-ethyl-N'-nitrosourea (ENU). Mice heterozygous for the RB gene developed characteristic pituitary tumors with nearly complete penetrance, whether or not they were treated with ENU. Tumors initiated earlier or progressed more rapidly, however, in ENU-treated mice. Furthermore, although mice treated with ENU had a higher incidence of several nonpituitary tumors compared with untreated controls, no significant differences in the incidence of these tumors were found between wild-type mice (mRB−/−), mice carrying only one normal RB allele and deficient in RB protein expression (mRB+/−), and mice overexpressing Rb protein from two normal murine RB alleles and a human RB transgene (mRB++/−, hRB+/−). These studies underscore the tissue and mechanistic specificity of tumor predisposition caused by an inherited 50% reduction in RB dosage and indicate that most ENU-induced tumors occur independent of RB inactivation. Nonetheless, they suggest that certain point mutations induced by ENU may participate in the sequence of molecular steps involved in progression of tumor-prone, RB-deficient cells to the fully malignant state.

Introduction

One of the remarkable features of patients with a germ line mutation of RB is the limited spectrum of tumors that arise in them. Such patients develop retinoblastomas with 90% penetrance and soft tissue sarcomas with a much higher incidence than in the general population (1, 2), but few other tumor types with incidences higher than normal. Likewise, mice engineered to mimic humans with heterozygous inactivation of the RB gene also are susceptible to only a limited number of tumor types when tumors occur spontaneously. These mice develop specific pituitary intermediate lobe tumors with nearly 100% penetrance but few other spontaneous tumors (3–6). One report has suggested that one of three independently engineered lines also develops medullary carcinoma of the thyroid with high incidence and possibly some other neuroendocrine tumors (7). It is interesting to note that the tumor types in mice are different from those in humans and that they do not include retinoblastoma. It is also worth noting that the population of cells susceptible to spontaneous transformation in the setting of a germ line inactivation of one RB allele is so limited and specific when the RB gene is known to be expressed ubiquitously and to be fundamental to cell cycle machinery in many cells (8–10).

Since mice homozygous for mutant RB die in utero (3–5), RB++/− mice represent the low end of a spectrum of RB dosage in animal models available for study of the effects of RB on susceptibility to cancer. To investigate the roles of the retinoblastoma gene and protein in development and tumorigenesis, mice that overexpress Rb protein from a stable human transgene were also developed to represent the opposite end of the spectrum (11). One might expect Rb overexpression to provide extra protection against the development of tumors, if RB gene dosage is important in such protection. Recently, however, we showed that there is an important distinction, in determining biological effects, between RB gene dosage (i.e., the number of independent genetic loci from which functional Rb protein is expressed) and total Rb protein expression (12).

Studies of mice that express different amounts of Rb protein from one, two, or three genetic loci have shown different requirements for Rb in embryonic development and in susceptibility to spontaneous tumorigenesis. Normal mouse development seems to require a minimum amount of Rb protein expression (12), and supranormal amounts of Rb result in dose-dependent growth retardation (11). Expression of less than one-half the normal amount of Rb from any number of RB loci results in fetal death and characteristic defects in the differentiation of certain neuronal and hematopoietic cells. Susceptibility to spontaneous tumors of the pituitary neurointermediate lobe in adult mice, in contrast, depends not on the absolute amount of Rb protein but on the number of independent loci from which Rb is expressed. Mice rescued from embryonic demise by germ line transmission of a human RB transgene, even if they express greater-than-normal total amounts of functional Rb protein from a single genetic locus, are growth retarded but nonetheless susceptible to pituitary tumors with the same incidence and lag time as mice heterozygous for the wild-type murine RB gene (12).

It is consistent therefore that mice carrying a human RB transgene and homozygous or heterozygous for the murine RB gene are no more or less prone to spontaneous tumors than are wild-type mice (11, 12). The question about whether RB gene or Rb protein dosage affects susceptibility to tumors induced by carcinogens, however, has not yet been examined. Here, we attempted to address this question by treating with a well characterized chemically carcinogenic, ENU, three groups of congenic C57BL/6 mice expressing different amounts of Rb protein from RB genes carried on one (RB++/−), two (RB+/+ or wild-type), or three loci (mRB++/−, hRB+/−, or transgenic). We then compared the incidence of all tumors in the three groups.

Materials and Methods

Experimental Animals. Mice heterozygous for a targeted disruption of exon 20 of the mRB gene (mRBΔ20) were generated by injection of strain 129/Sv embryonic stem cells into C57BL/6 blastocysts, as described previously (5). These chimeras were backcrossed with C57BL/6 mice to generate...
CS7BL/6 × 129/Sv mice with the genotype mRB\(^{+/+}\) (hereafter designated mRB\(^{+/+}\)) in the F1 generation. F1 mice were then cross bred with each other for two generations to produce the mRB\(^{+/+}\) and mRB\(^{-/-}\) mice used in this study.

Mice overexpressing Rb protein from a human Rb transgene (mRB\(^{+/+}\), hRB\(^{+/+}\)) were bred from crosses of a C57BL/6 founder mouse, RB.Rb3 (11), and wild-type CS7BL/6 females. Third and fourth generation mice were used in this study; therefore, the genetic background of the mice was equivalent to that of the RB heterozygote mice. Littermates of the transgenic mice were used as some of the wild-type controls. The RB.Rb3 strain harbors and stably transmits three tandem copies of a hybrid RB minigene consisting of a 1.6-kilobase human Rb promoter sequence, the 2.8-kilobase human Rb complementary DNA, and a 1.6-kilobase human β-globin gene polyadenylation sequence (11). RB.Rb3 mice express in all tissues tested about 200% of the amount of Rb protein expressed in wild-type mice.

**ENU Treatment.** N-Ethyl-N-nitrosourea was obtained from Sigma Chemical Corp., St. Louis, MO. A 10-mg/ml stock solution of ENU in phosphate-citrate buffer (3 mM sodium citrate in phosphate-buffered saline, pH 5.0) was prepared fresh for each new injection. Each treated animal was given at 12 days of age an injection of a single 60-mg/kg body weight i.p. dose of ENU. Males and females were housed in separate cages after they were weaned and genotyped at about 21 days of age. They were then provided with water carefully for tumors of all sorts. Gross tumors and surrounding normal tissues were frozen in liquid nitrogen and stored at −80°C for subsequent genomic DNA extraction.

**Genotyping by Southern Blotting.** Genomic DNA was isolated from biopsies of mouse tails or from frozen tumors and surrounding normal tissues dissected from the tumors, according to the method described by Laird et al. (13). Genomic Southern blotting was performed as described previously (12) except that autoradiographs were developed using a phosphorimager rather than film. A 370-base pair EcoRI/SacI fragment of mouse RB genomic DNA containing exon 19 was used to probe blots and to distinguish between the 9-kilobase wild-type BamHI mRB band and the 10.5-kilobase mutant mRB band (RB\(^{−/−}\), containing a neo insert at exon 20) (5). The presence of the human RB transgene in appropriate animals was detected by hybridization of the blots with the entire 6.0-kilobase minigene (11).

**Histology.** Excised tumors and surrounding normal tissues were fixed in phosphate-buffered 10% formalin, dehydrated with ethanol, cleared with xylene, and embedded in paraffin wax. Sections (7 μm thick) were stained with hematoxylin and eosin and examined with a light microscope.

**Statistical Analysis.** The small absolute numbers of individual tumor types induced by ENU required that tumors of different cell and tissue types be combined as total nonpituitary tumors for analyses and Fisher’s exact test.

### Results

**Incidence of ENU-induced, Nonpituitary Tumors Is Independent of RB Gene Dosage.** Neither the number of independent loci from which the RB gene is expressed nor the level of Rb protein expression seemed to have much bearing on the development of any tumors except those that arose from the intermediate lobe of the pituitary (Table 1). Only mice with the genotype mRB\(^{+/+}\) developed pituitary tumors, and they developed them with 96–98% incidence by 28 weeks of age. Furthermore, such tumors arose whether or not the animals were treated with ENU.

The absolute numbers of nonpituitary tumors detected were small, but the overall incidence of tumors was indeed higher in ENU-treated mice than in untreated controls (compare Parts A and B in Table 1). This finding strongly indicates that the carcinogenic effects of ENU were readily detectable in the experimental protocol. Studies done to establish the carcinogenic action of ENU in mice have generally found higher incidences than our study. For example, CS7BL/6J × C3HeB/FeJ F1, treated at the age of 15 days with equivalent i.p. doses of ENU developed liver and lung tumors with >90% incidence and malignant lymphomas with 5–24% incidence (14, 15). Similarly, CS7BL mice treated neonatally with ENU developed tumors of the lung, liver, or lymphoid system with a combined incidence approach 75% (16). In both these studies, however, the ENU-treated animals were allowed to live for >60 weeks; therefore the cumulative incidence of the respective tumors should indeed be higher than in our study, in which animals were sacrificed at 28 weeks of age. Because some mice with the genotype mRB\(^{−/−}\) die from spontaneous pituitary tumors by about 30 weeks of age, we chose to sacrifice the mice in our study prior to 30 weeks. The incidence of nonpituitary tumors may have been higher had we extended the study to later ages. In such a case, however, those mice that died only from spontaneous pituitary tumors would have skewed the results and increased the relative incidence of nonpituitary tumors in wild-type and Rb-overexpressing animals. Furthermore, the low incidence of nonpituitary tumors in wild-type control animals was necessary to provide a better opportunity to detect higher incidences of tumors in mRB\(^{+/+}\) mice, if there had been any. In the end, the numbers of tumors induced by 28 weeks of age, although small, were still adequate in aggregate for statistical comparisons.

In order to determine further whether RB loss was part of the process of tumorigenesis in spontaneous and ENU-induced tumors, we examined DNA from several pituitary and nonpituitary tumors harvested from mRB\(^{+/+}\) animals. Pituitary intermediate lobe tumors have previously been shown to lose heterozygosity for the wild-type RB allele in the vast majority of cases (4–7). We confirmed this finding in ten more tumors (those described in Fig. 1 and five others

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>RB-underexpressing (mRB(^{-/-}))</th>
<th>Wild-type (mRB(^{+/+}))</th>
<th>RB-overexpressing (mRB(^{+/+}), hRB(^{+/+}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. No. of mice</td>
<td>55:27 M, 28 F</td>
<td>43:25 M, 18 F</td>
<td>14:7 M, 7 F</td>
</tr>
<tr>
<td>Pituitary tumors</td>
<td>53 (96)%</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Other tumors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bronchogenic adenoma</td>
<td>1 (2)</td>
<td>2 (5)</td>
<td>1 (7)</td>
</tr>
<tr>
<td>Hepatocellular adenoma</td>
<td>3 (7)</td>
<td>3 (7)</td>
<td>1 (7)</td>
</tr>
<tr>
<td>Thymic lymphoma</td>
<td>2 (4)</td>
<td>1 (2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Splenic lymphoma</td>
<td>2 (4)</td>
<td>2 (5)</td>
<td>1 (7)</td>
</tr>
<tr>
<td>Uterine hemangiomia</td>
<td>1 (4)</td>
<td>1 (6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Soft tissue sarcoma</td>
<td>1 (2)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Skin papilloma</td>
<td>1 (2)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total nonpituitary tumors</td>
<td>11 (20)</td>
<td>9 (21)</td>
<td>3 (21)</td>
</tr>
<tr>
<td>Pituitary tumors</td>
<td>53 (98)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Other tumors</td>
<td></td>
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<tr>
<td>Skin papilloma</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total nonpituitary tumors</td>
<td>2 (4)</td>
<td>1 (4)</td>
<td>2 (9)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses, percentage.*
for which data are not shown). This loss of heterozygosity occurred in tumors from both ENU-treated animals (Fig. 1, tumors 18, 21, and 34) and from those whose tumors arose and progressed spontaneously (Fig. 1, tumors C55 and C88).

Since most of the nonpituitary tumors were taken in toto for fixation and histological studies, only a limited number were available for extraction of DNA from tumors and surrounding normal tissues. As shown in Fig. 1, however, none of the six nonpituitary tumors examined by genomic Southern blotting lost their remaining wild-type RB allele. Although it is possible that we could have missed inactivating RB point mutations by a method as crude as genomic Southern blotting, allelic loss seems by far to be the more common mechanism involved in loss of heterozygosity for RB in our model (4, 6, 7, 12). RB allelic loss, however, apparently is not crucial to the genesis of most ENU-induced, nonpituitary tumors.

Pituitary Neurointermediate Lobe Tumors Progress More Rapidly in ENU-Treated Mice Than in Untreated Controls. Mice with pituitary neurointermediate lobe tumors generally die when their tumors become large enough to encroach upon or crowd out other intracranial structures, to bleed into vital areas of the brain, or to prevent production of sufficient hormones from other parts of the pituitary gland. Mice with pituitary tumors typically become polyuric, anorexic, and emaciated as their pituitary tumors progress to end stage. They also and often develop central neurological deficits ante-mortem. The tumors therefore seem to be the proximate causes of death in the mice that bear them.

We sought to determine whether ENU treatment had any effect on the progression of pituitary tumors. Eighteen mice with the genotype mRB+/− were treated with ENU and followed without any other interventions until natural death. As experimental controls, 100 mRB+/− mice were likewise followed but not treated with ENU. All 18 ENU-treated mice had large pituitary tumors at the time of their deaths; 2 had metastases to cervical lymph nodes. One also had an hepatic adenoma and another had a splenic lymphoma. Sixteen had no visible tumors other than those in the pituitary. We concluded that these 16 mice died from their pituitary tumors, as did the 100 mRB+/− untreated mice followed as controls. Although 9 of the mice in the control group had metastatic tumor in anterior cervical lymph nodes, only one had a second primary tumor, a soft tissue sarcoma. Autolysis hampered examination of the small and large intestines in 3 animals treated with ENU and in 5 of the untreated animals; therefore some early intestinal tumors could have been missed in each group.

As shown in the life table in Fig. 2, the ENU-treated mRB+/− mice died significantly earlier (by a mean of 79 days or 23% of total life span) than untreated mRB+/− controls. This finding suggests that ENU treatment either caused earlier initiation of the tumors or hastened their progression. In any case, although RB gene dosage had little effect on the overall incidence of nonpituitary tumors, and although all mice with the genotype mRB+/− eventually developed and died from pituitary tumors, ENU treatment did have an apparent effect on accelerating progression of the pituitary tumors to end stages.

Discussion

Our results indicate that susceptibility to tumors induced in mice by ENU is independent of RB gene dosage but that ENU can accelerate progression of tumors in which RB inactivation is necessary. ENU was chosen to induce tumors because it induces a variety of predictable tumors and therefore is suitable for testing the hypothesis that RB dosage affects cancer susceptibility. The reason RB dosage had no effect on susceptibility to tumors induced by ENU is a matter of speculation. In retinoblastomas from patients with heterozygous germ line RB mutations, all manner of mutations have been observed (17–19). Gene deletion by chromosomal loss with reduplication, however, is quite common. This mechanism appears to be by far the
most common resulting in loss of heterozygosity in tumors of the murine pituitary neurointermediate lobe (4, 6, 7, 12). An alkylating agent like ENU, on the other hand, causes point mutations, not chromosomal losses (20, 21). Although specific point mutations induced by ENU are known to activate oncopgenes such as H-ras (22), they are not known to inactivate RB. Chromosomal mechanisms apparently are not initiating or rate-limiting events in the pathogenesis of most ENU-induced tumors. Perhaps a different carcinogen such as ionizing radiation might be more effective in causing chromosomal anomalies and thereby cooperating with or accelerating loss of the wild-type allele in RB""/>  

It is interesting to compare our study with similar studies done in mice deficient for another tumor suppressor, p53. All p53-deficient mice, both heterozygotes and homozygotes, are susceptible to spontaneous tumors comparable to those seen in humans with the Li-Fraumeni syndrome (23). p53""/> mice spontaneously develop osteosarcomas and soft tissue sarcomas; p53""/> mice develop predominantly malignant lymphomas and hemangiosarcomas (24, 25). The effects of a chemical carcinogen on tumor progression in p53""/> mice have also been reported (25). Dimethylnitrosamine, a carcinogen that acts by a mechanism similar to that of ENU but which requires metabolic activation in the liver (26), was used to induce hepatic and bronchogenic tumors in these mice. p53""/> mice developed the same spectrum of tumors (primarily hepatic hemangiosarcomas) with the same incidence as wild-type mice treated with equivalent doses of dimethylnitrosamine, but their tumors progressed more rapidly to end stages. The conclusion was made therefore that a 50% reduction in p53 dosage cooperatively enhanced progression of tumors induced by a DNA-alkylating agent.

In another study, p53 gene dosage had little effect on the incidence of initiation or promotion of chemically induced skin tumors but did enhance the rate of their malignant progression (27). A well characterized system for inducing and promoting murine skin tumors was used in studies with p53 null, heterozygous, and wild-type mice. This system was particularly appropriate because p53 mutations are often associated with progression from skin papillomas to carcinomas (28). Papillomas were initiated with dimethylbenzanthracene and promoted with 12-O-tetradecanoylphorbol-13-acetate. p53""/>, p53""/>, and p53""/> mice developed papillomas with similar incidences, but the tumors progressed more rapidly in the p53""/> mice and more rapidly still to invasive stages in p53""/> mice.

Our study, in contrast to the studies with varying p53 dosage, shows that varying RB dosage in mice does not affect the incidences of most alkylating agent-induced tumors. The alkylating agent, however, can cooperatively enhance progression of tumors that arise because of RB inactivation. The differences between the effects of RB and p53 dosage in mice may reflect the different actions of these two tumor suppressors. Although p53 and RB protein both suppress tumor formation and negatively regulate progression through G1 (8–10, 29), they do not function through precisely the same mechanisms and pathways. p53 has been shown to have important roles “guarding the genome” from propagation of detrimental mutations and in mediating apoptosis (30, 31). Because of the role of p53 in repairing DNA point mutations and because p53 alterations leading to cellular transformation are predominantly point mutations (Refs. 29, 30, and references therein), varying p53 dosage should be expected to affect carcinogenesis induced by alkylating agents.

The molecular roles of Rb protein, however, are more obscure. Rb may function in a “corral” to compartmentalize and inactivate transcription factors and other proteins that normally promote commitment to proliferation when not sequestered (32). How Rb inactivation results ultimately in defects in chromosomal segregation during mitosis (and only in certain cells) is unknown. Perhaps chromosomal loss is an epiphenomenon of deregulated proliferation and unrelated directly and specifically to loss of Rb expression. Alternatively, cells susceptible to transformation when RB is inactivated may have unique Rb-associated proteins that are integral parts of chromosome structure. Whereas these proteins might normally be subject to regulation in complexes with Rb, without Rb the regulation might be lost and chromosome structure thus altered (32). Much remains to be known about the molecular mechanisms by which Rb functions.

The results presented here do not answer questions about molecular mechanisms directly. Rather, they lead to two interesting conclusions. First, normal and extra doses of the RB gene and RB protein do not protect animals from all types of cancer. This result is expected, given the limited spectrum of tumors in which RB inactivation is fundamental and the still more limited spectrum of tumors that arise spontaneously in germ line RB heterozygote humans and mice. Second, somewhat unexpected, ENU-induced mutations apparently can be involved, in certain murine pituitary neurointermediate cells, in the same multistep carcinogenesis pathways as RB inactivation. This fact suggests one of two possibilities: (a) either ENU treatment provides the mutational events required for initiation of pituitary carcinogenesis in mRB""/> mice; or (b) ENU may cause mutations in certain genes that regulate progression of cells that have already lost Rb expression to more fully transformed states. Studies to determine some of the steps other than RB inactivation will be important, as will determination of whether RB allelic loss occurs early or late in the process of tumor initiation or progression. Although mutations in multistep tumorigenesis are not always sequential, RB loss may occur early and be rate limiting in some tumors.

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