Noninvasive Imaging of Spontaneous Retinoblastoma Pathway-dependent Tumors in Mice

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ABSTRACT

Identification of the critical pathways involved in tumorigenesis should ultimately lead to the design of better anticancer agents that target specific components of the disrupted pathways. Murine models of spontaneous cancer in which tumor formation is dependent on defined genetic alterations provide a powerful test system for evaluating the therapeutic efficacy of pathway-specific antineoplastics. We have generated a conditional mouse model for retinoblastoma-dependent sporadic cancer that permits noninvasive monitoring of pituitary tumor development in live animals via in vivo bioluminescence imaging of luciferase expression. We show that the high sensitivity of bioluminescence imaging can be used for noninvasive detection of luciferase expression in pituitary glands from tumor-free animals and for in vivo quantitation of tumor burden over a large dynamic range. This mouse model permits longitudinal monitoring of tumor onset, progression, and response to therapy and may be used effectively for testing cancer prevention and treatment strategies based on therapeutics that specifically target the retinoblastoma pathway.

INTRODUCTION

Tumor formation requires the accumulation of mutations in genes controlling cell proliferation, survival, angiogenesis, and metastasis, culminating in malignancies that can be life-threatening to man. Inactivation of the RB tumor suppressor gene is a common event in the development of human cancer (1–4). The RB protein has been recognized as a central component of the cellular machinery that controls passage from G1 into S phase of the cell cycle (5). The importance of this G1-S transition control is underscored by the fact that, besides RB1, genes for other pathway components such as cyclin D1, CDK4, and p16INK4A are frequently mutated in human cancer (6). In mice, RB also functions as a tumor suppressor, as exemplified by the finding that animals carrying one mutated RB allele are predisposed to the development of pituitary tumors originating from melanotrophs (7–9). In the resulting tumors, the wild-type RB allele is invariably lost, demonstrating the critical role of RB in preventing pituitary tumor formation. In line with this, somatic deletion of both RB alleles in the pituitary gland results in the rapid development of melanotroph tumors in mice homozygous for a conditional RB mutation (10). In principle, this conditional mouse model for RB-dependent sporadic cancer permits testing of anticancer therapeutics that specifically target the RB pathway. However, the stochastic nature of tumor onset in these mice complicates high-throughput testing of anticancer drugs because large numbers of animals are required to obtain statistically reliable results. Noninvasive methods that permit longitudinal monitoring of tumor growth and drug response would greatly reduce the number of mice required for these studies and broaden the scope of analyses that can be performed.

A number of in vivo imaging strategies have been developed over the past years that are applicable to mice. These include nuclear techniques such as MRI (11), single photon emission computed tomography or positron emission tomography (12), and optical techniques using green fluorescent protein (13) or near-IR fluorescence (14). In general, nuclear techniques offer good resolution at deep tissue sites, but imaging is usually time-consuming and requires expensive equipment and skilled personnel. Recently, bioluminescence imaging based on in vivo expression of luciferase, the light-emitting enzyme of the firefly Photinus pyralis, has been used successfully for the noninvasive detection of transplanted tumors in mice (15–18). This strategy relies on the ATP- and O2-dependent photochemical reaction between luciferin and luciferase, resulting in the release of photons from live cells only. Luciferase reporter gene expression in live animals can be measured with a cooled charge-coupled device camera minutes after the administration of luciferin. The low background of luminescence from normal tissue, the rapid turnover of luciferase enzyme, and the nonimmunogenic characteristics of luciferin make this method ideally suited for temporal in vivo imaging of gene expression.

Here we report the generation of a mouse model of spontaneous RB-dependent cancer that enables noninvasive bioluminescence imaging of pituitary tumor development. This model provides a powerful test system for cancer prevention and treatment protocols based on anticancer agents that interfere with the RB pathway and may be of particular value in view of the frequent disruption of the RB pathway in human cancer.

MATERIALS AND METHODS

Generation of POMCcre-POMCluc Transgenic Mice. A 1.84-kb fragment derived from pGL3 (Promega) containing the luciferase open reading frame was cloned into a vector containing 780 nucleotides of the 5′-flanking sequence of rat POMC followed by SV40 splice and polyadenylation signals (a gift from Dr. Malcolm Low). Construction of the POMCcre transgene has been described previously (19). To generate POMCcre-POMCluc transgenic mice, POMCcre and POMCluc fragments were isolated by agarose gel electrophoresis, mixed at a 1:1 molar ratio, and injected into fertilized FVB/N oocytes.

Genotyping of Mice. Transgenic POMCcre-POMCluc founders were identified by Southern blot analysis of tail-tip DNA using luc and cre probes, respectively. After founders were established, genotyping was performed by PCR amplification of cre using primers crel (5′-CGATGCACAGTGATGAGGTTCCTC-3′) and cre2 (5′-GCACCTCACCAGGCAATCAAC-3′), yielding a 345-bp product, and PCR detection of luc using primers pLuc5 (5′-AGAATCTCAGTCGTCCTGCAAGCT-3′) and pLuc3 (5′-CTATCGAAGGACTCTGGCCAC-3′), yielding a 335-bp product. Genotyping of RB1/1 mice was performed by PCR as described previously (20).

Histological Analysis. Pituitary tumors were isolated immediately after euthanasia of the animals, and tumor mass was determined as wet weight. For histological examination, tumors were fixed in 4% paraformaldehyde in PBS for at least 48 h, embedded in paraffin, cut into 5-μm sections, and stained with H&E. For whole mount β-galactosidase staining, tissues were fixed and stained as described previously (21).

In Vivo Bioluminescence Imaging. In vivo bioluminescence imaging was conducted on a cryogenically cooled IVIS system (Xenogen Corp., Alameda, California).
CA) using LivingImage acquisition and analysis software (Xenogen Corp.). n-Luciferin (potassium salt; Xenogen Corp.) was dissolved to 15 mg/ml in PBS, filter-sterilized, and stored at −20°C. Mice were anesthetized with hypnorm/dormicum or isoflurane and subsequently received i.p. injection with luciferin (225 µg/g body weight). Images were acquired 5–10 min after luciferin administration. A photographic image of the animal was taken in the chamber under dim illumination, followed by acquisition and overlay of the pseudocolor image representing the spatial distribution of photon counts produced by active luciferase within the animal. An integration time of 1 min with a binning of 100 pixels was used for luminescent image acquisition. Signal intensity was quantified as the sum of all detected photon counts within the region of interest after subtraction of background luminescence measured at the dorsal trunk.

**Doxorubicin Treatment of Mice.** All studies were conducted in accordance with the national guidelines for the care and use of animals and after protocol review by the institutional animal care and use committee. Doxorubicin hydrochloride (Adriblastina; purchased from Pharmacia & Upjohn) was dissolved in normal saline to a concentration of 2 mg/ml. Ten-week-old mice received i.v. injection of 5 µg doxorubicin/g body weight or vehicle once every week for 4 weeks. This dose level of doxorubicin has been reported to be well tolerated by mice (22). In line with this, a limited toxicity study showed that a similar treatment of wild-type animals did not result in lethality due to doxorubicin-induced cardiomyopathy.

**Statistical Analysis.** Statistical analysis was performed using the SPSS-10 statistical package for Windows. Pituitary tumor formation latency reflects the age of the animals with overt signs of disease (ataxic gait, tilted head, and/or protuberant cranium). For tumor-free survival curves, individual time values were plotted in the Kaplan-Meier survival curve format, and curves were compared using the log-rank test. Correlation coefficients ($r$) were calculated using Spearman ranking.

**RESULTS**

**Generation of POMCcre-POMCluc Transgenic Mice.** We exploited the strong tumor suppressor activity of Rb in the mouse pituitary gland to develop a model for Rb-dependent sporadic cancer that permits noninvasive imaging of tumor development and drug response (Fig. 1a). To simultaneously induce and monitor pituitary tumor development in conditional Rb mutant mice, we generated POMCcre-POMCluc transgenic mice that express both Cre recombinase and firefly luciferase under the control of the intermediate lobe-specific POMC promoter (Fig. 1b). We selected two of six independent transgenic founders to produce the POMCcre-POMCluc transgenic lines 05 and 06, which were subsequently crossed with mice carrying a conditional mutant Rb allele ($R^b_{19/19}$, Refs. 20 and 23) to obtain POMCcre-POMCluc; $R^b_{19/19}^{F19/+}$ mice. First, we determined whether both Cre and luciferase were functionally expressed in the pituitary gland of the POMCcre-POMCluc mice. To examine the efficiency and tissue specificity of Cre-mediated recombination, we crossed the POMCcre-POMCluc; $R^b_{19/19}^{F19/+}$ animals with ROSA26-lacZ Cre reporter mice (R26R) that permit in situ analysis of Cre activity via β-galactosidase staining (24). Whole mount β-galactosidase staining of pituitary glands from POMCcre-POMCluc;R26R or POMCcre-POMCluc;R26R; $R^b_{19/19}^{F19/+}$ animals showed specific staining of virtually all melanotrophs in the intermediate lobe and of a significant fraction of cells in the anterior lobe (Fig. 1c). Within the pituitary intermediate lobes from POMCcre-POMCluc;R26R; $R^b_{19/19}^{F19/+}$ mice, hyperplastic nodules were seen, consistent with a model in which

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**Fig. 1.** Bioluminescence imaging of spontaneous Rb-deficient tumors. **a,** POMCcre-POMCluc transgenic mice expressing a luciferase reporter and Cre recombinase in POMC-expressing cells of the pituitary gland were crossed with mice carrying conditional Rb alleles ($R^b_{19/19}^{F19/F19}$). Expression of Cre recombinase leads to melanotroph tumors that have lost Rb ($R^b_{19/19}^{F19}$). Longitudinal tumor growth kinetics were determined by bioluminescence imaging of luciferase activity. **b,** structure of the POMCcre-POMCluc transgenics. **c,** measurement of Cre recombinase activity via lacZ reporter gene switching in POMCcre-POMCluc; R26R; $R^b_{19/19}^{F19/+}$ mice. β-Galactosidase activity is seen throughout the intermediate lobe (IL) but not the neural lobe (NL) of the pituitary gland. Hyperplastic areas (arrows) within the intermediate lobe are clonal expansions from cells that have undergone Cre-mediated recombination of the $R^b_{19/19}^{F19}$ allele followed by spontaneous loss of the wild-type Rb allele. **d,** bioluminescence imaging of luciferase activity in disease-free POMCcre-POMCluc transgenic mice. Left panels show photographic images; right panels show pseudocolor images of bioluminescent signal. External monitoring shows bioluminescent signal from the head (top right panel) and the pituitary region after removal of the brain (bottom right panel). Acquisition times were 1 min at a binning of 100 pixels. Relative light units/pixel are indicated in the color scale bar.

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stochastic loss of the wild-type Rb allele follows Cre-mediated deletion of the Rb\(^{F19}\) allele (10). Next, we determined whether we could detect luciferase activity in tumor-free POMC\(\text{cre}^{-}\)POMC\(\text{luc}\);Rb \(^{F19/F19}\)/H11001 animals. Anesthetized mice were depilated around the head, luciferin was injected i.p., and a 1-min whole-body bioluminescence measurement was taken between 5 and 10 min after injection. In mice from both POMC\(\text{cre}^{-}\)POMC\(\text{luc}\) lines, we detected a luminescent signal emanating from the head (Fig. 1d, top panels). To confirm that the signal was indeed produced by the pituitary gland, we performed direct measurements on exposed pituitaries (Fig. 1d, bottom panels). Bioluminescence was restricted to a region where the pituitary gland is located. Thus, we concluded that POMC\(\text{cre}^{-}\)POMC\(\text{luc}\) transgenic mice display pituitary-specific Cre activity and that the high sensitivity of bioluminescence imaging allows for the noninvasive quantification of luciferase expression in the normal pituitary glands of POMC\(\text{cre}^{-}\)POMC\(\text{luc}\) mice.

**Bioluminescence Imaging of Pituitary Tumor Development in POMC\(\text{cre}^{-}\)POMC\(\text{luc}\);Rb \(^{F19/F19}\) Mice.** Bioluminescence imaging of orthotopic xenografts in mice has demonstrated a linear correlation between tumor bioluminescence and tumor volume as measured by MRI (17). To investigate whether such a linear correlation was also maintained during spontaneous Rb-dependent tumor development in our model, we crossed POMC\(\text{cre}^{-}\)POMC\(\text{luc}\);Rb \(^{F19/F19}\) transgenic mice with Rb \(^{F19/F19}\) conditional mutant mice to obtain POMC\(\text{cre}^{-}\)POMC\(\text{luc}\);Rb \(^{F19/F19}\) animals that develop pituitary tumors with 100% incidence after a relatively short median latency (Fig. 3c). We measured the luciferase activity of pituitary glands in POMC\(\text{cre}^{-}\)POMC\(\text{luc}\);Rb \(^{F19/F19}\) mice at various stages of tumor development. The exposed pituitary glands were imaged between 5 and 10 min after i.p. injection of luciferin into the anesthetized animals, and the signals were plotted against the wet tumor weight (Fig. 2a). We observed a good correlation between detected photons and tumor weight. Outlying data points with a low signal:mass ratio represent, in most cases, end-stage tumors in which hemorrhaging caused quenching of the emitted photons or in which necrotic regions or accumulated fluid contributed to tumor mass, but not to the signal.

We also followed pituitary tumor development in POMC\(\text{cre}^{-}\)POMC\(\text{luc}\);Rb \(^{F19/F19}\) mice in time by measuring bioluminescence with 2-week intervals starting at week 4 until the mice became moribund (Fig. 2, b–d). Whereas control nontransgenic and POMC\(\text{cre}^{-}\)POMC\(\text{luc}\);Rb \(^{F19/F19}\) mice showed no change in signal over time, POMC\(\text{cre}^{-}\)POMC\(\text{luc}\);Rb \(^{F19/F19}\) mice showed an almost doubling of the signal every 2 weeks, indicating exponential tumor growth. Variation of bioluminescent signals detected between different mice of the same age may reflect the stochastic onset of tumor development in these animals, although we have also noted quenching of emitted photons in pigmented animals.

**Bioluminescence Imaging of Response to Chemotherapy in Vivo.** Subsequently, we investigated whether bioluminescence could be used to measure the antitumoral efficacy of chemotherapeutic agents in our model. For this purpose, we used doxorubicin (Adriamycin), an S-phase-specific drug that is used to treat a diverse range of human tumor types (25). To determine whether doxorubicin treatment could affect POMC-driven luciferase expression, we compared...
luminescence from POMCcre-POMCluc mice before and 36 h after receiving a single i.v. doxorubicin (dox) injection. We found that signals between untreated mice show no significant variation and that doxorubicin treatment does not affect luciferase expression in the pituitary gland of these mice. Furthermore, long-term effects of doxorubicin on luciferase expression are unlikely because plasma levels of doxorubicin decrease rapidly to <10% after 40 h.

Based on our previous histological examination of tumor progression in pituitary gland-specific Rb knockout mice, we initiated treatment of POMCcre-POMCluc;RbF10/F10 mice at the age of 10 weeks, when most mice show substantial hyperplasia of the intermediate lobes. Mice were given doxorubicin (i.v., 5 μg/g body weight) or vehicle once every week for 4 consecutive weeks, and bioluminescence imaging of tumor burden was performed every 2 weeks. Measurements of mice between 4 and 8 weeks of age showed, in most instances, steady-state bioluminescence levels, indicating the absence of neoplastic growth. From 8 weeks onward, mice that received no treatment showed approximately exponential tumor growth until moribund. In contrast, mice that received doxorubicin (n = 7) showed stasis of tumor development during the period of intervention. However, between 2 and 4 weeks after the last doxorubicin injection, all tumors resumed exponential growth until the mice became moribund. In line with the observed stasis of tumor development, we found a significantly increased survival in doxorubicin-treated mice compared with non-treated mice.

**DISCUSSION**

Now that the critical pathways involved in tumorigenesis are beginning to emerge, a major challenge lies ahead in translating this knowledge into improved therapeutic intervention strategies. Tumor-prone mice with genetically defined alterations are potentially of great value for gaining insight into the etiology and treatment of cancer. However, the stochastic nature of tumor onset in mouse models of sporadic cancer complicates efficient testing of anticancer drugs because large numbers of animals are required to obtain statistically reliable results. Noninvasive methods that permit longitudinal monitoring of tumor growth and drug response would greatly reduce the number of mice required for these studies and broaden the scope of analyses that can be performed.

Bioluminescence imaging allows a rapid and noninvasive measurement of tumor growth before, during, and after treatments in animals. Consecutive images acquired from the same animals permit temporal and spatial information throughout an entire experiment instead of only the end point data of more conventional approaches. Although the spatial resolution of optical imaging is limited when compared with MRI or positron emission tomography, bioluminescence imaging is uniquely suited for high-throughput imaging because of the ease of operation, the short acquisition times (typically 10–60 s), and the possibility of simultaneous measurement of six or more animals. In combination with inhalant anesthesia, mice can be repetitively imaged with very short time intervals without any adverse effects of the procedure. This is in contrast to nuclear imaging.
...techniques, where repeated exposure to ionizing radiation may lead to unwanted secondary effects. Importantly, bioluminescence imaging will only measure live cells because luciferase requires O₂ and ATP to catalyze light from its substrate, luciferin. This feature renders bioluminescence imaging a particularly attractive method to measure tumor cell kill as opposed to other techniques, such as MRI, that measure total tumor volume including necrotic areas. One current drawback of bioluminescence imaging when compared with MRI or nuclear techniques is that it cannot be used for three-dimensional reconstruction. However, it is expected that bioluminescence image acquisition using rotating charge-coupled device cameras will permit volumetric acquisition of luciferase expression patterns in mice, particularly when combined with novel red-shifted luciferases that show better tissue penetration than the currently available luciferases.

Using a conditional mouse model of Rb-dependent pituitary cancer, we show that bioluminescence imaging of spontaneous tumor formation permits a sensitive and quantitative assessment of the effects of therapeutic intervention. The fact that luciferase expression in tumor-free pituitary glands can readily be detected via bioluminescence imaging of live animals renders the POMCcre-POMCLuc;RbFlox/Flox mouse strain an ideal model for chemoprevention studies. Importantly, noninvasive imaging of the normal pituitary gland through the skull underscores the sensitivity of bioluminescence imaging because skull bones and brain tissue reduce the signal 100-fold when compared with direct measurements on exposed pituitary glands. In addition to signal location, signal strength is another parameter that determines sensitivity, which is determined by many factors including the number of luciferase-expressing cells, the promoter used to drive luciferase expression, the transgene copy number, and the transgene integration site. We have successfully used two independent transgenic lines that differ by approximately 1 order of magnitude in luciferase expression for imaging of the normal pituitary gland in mice (data not shown). Currently, we do not know what the lower detection limit is for measuring normal or tumor cells in deep tissue sites such as the pituitary gland. Others have reported detection of as few as 3000 cells in vivo (18). Together, our results strongly support the notion that bioluminescence imaging has sufficient sensitivity to monitor spontaneous tumor growth in most mouse tissues. The correlation between detected photons and tumor weight over a large dynamic range enables a sensitive detection of tumor growth, stasis, regression, or relapse. In addition, it permits matching of animals on the basis of tumor burden, thus circumventing the interanimal variation caused by the stochastic nature of spontaneous tumor formation. It remains to be established whether the POMCcre-POMCLuc;RbFlox/Flox mouse strain permits discrimination between a normal pituitary gland and early hyperplasia. Therefore, future work will be directed toward establishing a relationship between histological stage and luciferase expression.

The Rb pathway is found disrupted or deregulated in virtually all human cancers (29). The affected gene products include, in addition to Rb, upstream and downstream components such as p16INK4A, cyclin D1, CDK4, and cyclin E. Therefore, therapeutics that target this pathway could be of profound importance in the treatment of cancer. The POMCcre-POMCLuc;RbFlox/Flox model is particularly suited for testing such therapeutics because pituitary tumor development in these mice is highly dependent on cell cycle checkpoint inactivation through Rb loss. Using this model, the antitumoral efficacy of therapeutic intervention or chemoprevention protocols can be evaluated at distinct stages of tumor development. Furthermore, because Rb inactivation in these mice is restricted to POMC-expressing cells in the pituitary gland, tumorigenesis can be studied in the context of a normal microenvironment. These aspects constitute a significant improvement over rodent xenograft models that are currently used as preclinical models to evaluate anticancer therapies. Therefore, we expect the present mouse model to be the first example of a new generation of spontaneous tumor models suitable for efficient screening and preclinical testing of pathway-specific anticancer drugs for a range of clinical end points.

ACKNOWLEDGMENTS

We thank D. Jenkins, M. Cable, and B. Nelson of Xenogen Corporation (Alameda, CA) for providing the IVIS imaging system and LivingImage acquisition and analysis software and for helpful suggestions on the bioluminescence imaging of mice; H. van der Gulden for generation and purification of the POMC luc transgenic construct; P. Krimpenfort for zygote injections; O. van Tellingen for providing doxorubicin and helpful suggestions on the doxorubicin treatment of mice; N. Bosnie for the doxorubicin injections; members of the animal facility for animal care; F. Mathiesen for isolation of mouse tail-tip DNA; members of the histology core facility for histotechnical assistance; and P. Borst, A. Schinkel, H. van der Poel, and M. van Lohuizen for critical reading of the manuscript.

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A PRECLINICAL MOUSE MODEL FOR Rb-DEPENDENT TUMOR FORMATION


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