Time Course of Bioluminescent Signal in Orthotopic and Heterotopic Brain Tumors in Nude Mice

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ABSTRACT

In vivo bioluminescence imaging is becoming increasingly popular. Quantification of bioluminescent signals requires knowledge of the variability and reproducibility of this technique. The objective of this study was to analyze the time course of luminescent signal emitted from firefly luciferase-expressing tumors in two locations, following luciferin injection and at different times after tumor cell implantation. Knowledge of the kinetics of the bioluminescent signals is required for the reliable quantification and comparison of signal during longitudinal studies. The kinetics of bioluminescence was evaluated in orthotopic and heterotopic brain tumors in mice using a human brain tumor cell line constitutively expressing luciferase. Tumor cells were implanted in the brains and flanks of the animals, and whole-body images revealing tumor location were obtained. Tumor burden was monitored over time by the quantification of photon emission. The magnitude of bioluminescence measured in vivo varied with time after the injection of luciferin, as well as with dose, which necessitated that the comparison of the quantitative results take into consideration the time after injection. Heterotopic and orthotopic tumors exhibited significantly different time courses; however, time after implantation as characterized by kinetic studies performed on days 4 and 14 after cell implantation revealed no significant differences in orthotopic tumors. Future quantitative longitudinal studies must take into account the differences in the kinetics of different models.

INTRODUCTION

In vivo bioluminescence imaging is an ideal tool for evaluating tumor growth and effects of antineoplastic therapies (1). Advances in biotechnology have enabled the in vivo imaging of luciferase expression in living mice by the use of cooled charge-coupled device (CCD) cameras (2,3). In addition, because bioluminescence imaging, the imaging of light emitted by luciferase upon consumption of its substrate luciferin, has minimal background activity, it has the potential to be a highly sensitive technique. Firefly (Photinus pyralis) luciferase has been used in multiple studies of tumor growth evaluation (4–6). The substrate for firefly luciferase is D-Luciferin, which is known to cross the cell membrane and to penetrate the blood brain barrier after intraperitoneal (i.p.) or intravenous (i.v.) injection in mice, which allows it to be imaged in any organ. The optimal conditions and potential limitations of this novel technique have to be established to evaluate therapeutic response in preclinical studies.

The aim of this study was to analyze luciferase activity over time in orthotopic and heterotopic brain tumors using the region-of-interest (ROI) analysis of images to quantify light emission from tumor cells induced to express firefly luciferase. Orthotopic tumor models are arguably the most appropriate animal models of human cancer, but to date, in most studies, heterotopic tumors are generally used as cancer models (7). Orthotopic brain tumors are inaccessible for direct measurements, which makes the use of in vivo imaging techniques for different analyses particularly attractive. Our results indicate that the kinetics of luciferase is an important consideration for in vivo imaging. Using the ROI analysis of photon emission, we demonstrate that minimal differences in time following luciferin administration can give very different signals in bioluminescence imaging, which affects potential conclusions about the disease progression and response to therapy. We show that heterotopic and orthotopic tumors present different luciferase time courses over time after substrate injection, but the orthotopic brain tumors do not show significant differences of time courses at different time points after tumor cell injection.

MATERIALS AND METHODS

Animal Procedures

Glioblastoma cell line. The human brain tumor cell line U87MG (glioblastoma) was purchased from ATCC (Manassas, VA, USA) and grown in RPMI with 10% FBS in 5% CO₂ at 37°C.

Luciferase Construct and Transfection

SMPU-R-MND-Luc lentiviral vector. The SMPU-R-MND-Luc lentiviral vector was produced from the SMPU HIV-1-based lentiviral vector (a gift from Paula Cannon, Childrens Hospital Los Angeles). pSMPU was designed to minimize HIV-1 sequences, lacks long terminal repeat (LTR) enhancers and promoters [self-inactivating (SIN)], and contains only 80 bp from the HIV-1 gag region. SMPU has the cPPT/CTS sequences added back to increase the rate and extent of proviral integration and has the U3S sequences from simian virus 40 (SV40) to augment polyadenylation from the 3’ LTR. This improves gene expression and minimizes transcriptional read-through to downstream cellular sequences at the site of proviral integration. A minimal HIV-1 rev-responsive element (RRE) sequence (8) was added to increase the vector titer. The U3 region from the LTR of the MND retroviral vector (9) was inserted as an internal promoter, followed by the firefly luciferase gene (Promega, Madison WI, USA). The vector was packaged by the co-transfection of 293T cells with the vector plasmid, the 8.9 packaging plasmid, and pMD.G plasmid expressing the VSV-G protein, as described previously (10). The vector supernatant was concentrated 100-fold by ultracentrifugation at 100,000×g.

Transduction. The U87MG cells (5 × 10⁵) were twice exposed to 50 μL of the concentrated vector at 20-h intervals. After rinsing the culture with medium, the cells were grown for another 24 h and then cloned by limiting dilution, and a clone with high luciferase activity was used for in vivo experiments (11).
orthotopic xenotransplant model in nu/nu have been described previously (personal communication). Briefly, $1 \times 10^5$ tumor cells suspended in 1 $\mu$L serum-free RPMI medium were injected over 20 min into the forebrain at 2.0 mm lateral and 0.5 mm anterior to the bregma, at a depth of 3.3 mm. For heterotopic tumor growth, $1 \times 10^6$ tumor cells were injected subcutaneously into the right lower flanks of the mice. This higher cell number was necessary to warrant subcutaneous growth (12). The mice were kept under general anesthesia during these procedures as previously described (12). The injected animals were also examined with magnetic resonance imaging to demonstrate the presence of brain tumors (data not shown). All animal studies were done according to the National Institutes of Health guidelines (Bethesda, MD, USA) and approved by the Childrens Hospital Los Angeles Institutional Animal Care and Use Committee (IACUC; Los Angeles, CA, USA).

**In Vivo Bioluminescence Imaging**

In vivo bioluminescence imaging was performed with the cooled IVIS® animal imaging system (Xenogen, Alameda, CA, USA) linked to a PC running the Living Image™ software (Xenogen) along with IGOR (Wavemetrics, Seattle, WA, USA) under Microsoft® Windows® 2000. This system provides high signal-to-noise images of luciferase signals emerging from within living animals. Before imaging, 150 mg/mL luciferin (potassium salt; Promega) in normal saline was injected (i.p.) at a dose of 150 mg/kg body weight. During image acquisition, 2% isofluorane anesthesia in air was delivered via a nose cone system. An integration time of 1 min with binning of 5 min was used for luminescent image acquisition. The signal intensity was quantified as the flux of all detected photon counts within a ROI prescribed over the mouse head or flank using the LivingImage software package. The mice were imaged over 1 h at intervals of 6 min. Groups ($n = 3$) of animals (three per group) were evaluated, and a representative animal is shown in Figure 2. Group 1 represents mice at day 4 after orthotopic and heterotopic tumor cell implantation; group 2 represents mice at day 4 after orthotopic tumor cell implantation; and group 3 represents mice at day 14 after orthotopic tumor cell implantation.

**Quantification of Bioluminescence Data**

A grayscale photograph of the mice was first collected in the chamber under dim light-emitting diode illumination, followed by the acquisition and overlay of the pseudocolor luminescent image from violet (least intense) to red (most intense), representing the spatial distribution of detected photon counts emerging from active luciferase within the animal. The signal intensities from manually derived ROIs were obtained, and data were expressed as photon flux (photons/s/cm²/steradian), where steradian (sr) refers to the photons emitted from a solid angle of a sphere. The background photon flux was defined from a ROI of the same size placed in the same position before the injection of luciferin into each animal, and these data were subtracted from the signal intensities measured at the same site. Data were normalized to peak signal intensity of each time course and reported as mean values with standard deviations.

**Statistical Analysis**

Two-way analysis of variance (ANOVA) was used to test for the statistical significance of the results. A $P$ value of <0.05 was considered significant.

**RESULTS**

Bioluminescent images of a representative animal from group 1, displayed as a pseudocolor image overlaid on a grayscale reference image of the head and flank of the mouse, revealed intense signals arising from the tumor. Figure 1 shows the bioluminescent images of a representative animal with heterotopic and orthotopic tumors from this experiment.

Serial images were obtained from all animals, and the mean photon flux relative to peak signal was determined. A variation in bioluminescent signal was observed over time following luciferin injection. The maximum photon flux occurred some minutes after substrate administration and subsequently declined. To ease visualization, a polynomial regression of order 3 was used to fit the time courses. The corresponding bioluminescent images and time courses revealed similar trends in detected photon emission over time. Figure 2 shows the plots of time courses for each group of animals. The kinetics of luciferase in mice with orthotopic brain tumors only were not significant.

![Figure 1: Representative animal with orthotopic and heterotopic tumors at different time points following luciferin administration.](image)

The signal gradually increased in the brain and flank up to a peak at approximately 20 min, after which time, the signal slowly decayed.

![Figure 2: Time course of luciferase signal following the intraperitoneal injection of luciferin.](image)

Graph A represents the group of mice with heterotopic and orthotopic tumors, while graphs B and C were generated from mice with only orthotopic tumors. Data are plotted as the mean percentage with standard deviations of photon counts over time from three animals per group.
ly different from animals that had both brain and flank tumors \((P = 0.64)\). The kinetics of luciferase tumors on day 4 compared to day 14 were not significantly different \((P = 0.19)\). In contrast, time courses of orthotopic and heterotopic tumors were significantly different \((P < 0.01)\).

Table 1 shows a summary of several parameters that were evaluated. The maximum signal in the brain was similar at day 4, independent of the presence of flank tumor, which indicated that the head signal did not vary in the presence of these flank tumors. The signal in the heterotopic flank tumors was considerably higher than in the brain tumors because of the superficial localization and the 10-fold higher cell concentration that was injected. We also determined the error in signal intensity quantification that would be obtained if the analyses were performed at time points similar to those reported previously in the literature. The time points of 4 min revealed errors of up to 83\% in some of the conditions, while this error dropped to 12.6\% at 28 min (Table 1). These data indicate that the detection time is crucial, and the intensity of the signal can vary dramatically if studies are not done at the maximum peak time point of the curve.

We also investigated the bioluminescence dynamics using 1/10th and 1/30th the dose typically used in the literature and found that the curves were progressively and significantly \((P < 0.01)\) shifted toward longer time values (Figure 3). The maximum photon flux decreased with dose reduction but linearity is indeterminate with only three points. The mean peak signal at typical, 1/10th and 1/30th luciferin doses were \(3 \times 10^7\), \(9.2 \times 10^5\), and \(3.8 \times 10^5\) (photons/s/cm\(^2\)/steradian).

**DISCUSSION**

In this study, bioluminescence-based measurements were used to evaluate the kinetics of luciferase activity in orthotopic and heterotopic brain tumors. The luciferase enzyme produces light in the presence of the substrate luciferin, oxygen, and ATP (13); the light produced penetrates mammalian tissues and can be externally detected and quantified using sensitive light-imaging systems (14). Because tumor cell growth in individual animals can be quantitatively assessed, the time and number of animals required can be greatly reduced, but accurate quantification requires detailed knowledge of the kinetics of the model.

We determined the optimal time points for quantification after luciferin injection in our murine tumor model. We observed that there were differences in time courses, and these can affect the quantification and estimates of tumor mass and the assessment of tumor development. Many studies have reported using between 5 and 20 min after injection of luciferin for image acquisition (4–6), and, considering this report, the potential differences using disparate time points could alter the conclusions in such studies. We note that heterotopic flank tumors and orthotopic brain tumors presented significantly different time courses, emphasizing the need to do a complete curve or at least several time points with each experimental condition. We did not investigate the nature of this difference. However, the vessels in these brain tumors have been found to be leaky to contrast agents at this stage (unpublished data). The remaining blood brain barrier may be the cause of differences seen in the dynamics of bioluminescence, but these dynamics may also be affected by the tumor microenvironment. Whether similar results will be observed with different cell lines remains to be determined.

We observed that the time courses in brain tumors between different days after the implantation of the cells were not significantly different, and that the presence of the flank tumor did not significantly affect the time course of the signal in the head.

We used the amount of luciferin per

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**Table 1. Assessment of Bioluminescent Signal over Time in Nude Mice with Orthotopic and Heterotopic Tumors**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Orthotopic and Heterotopic</th>
<th>Orthotopic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Head Flank Head Head</td>
<td>Head Head</td>
</tr>
<tr>
<td>Days after cell implantation</td>
<td>4 4 4 14</td>
<td>4 14</td>
</tr>
<tr>
<td>Maximum signal (photons/s)</td>
<td>(3 \times 10^7) (3.9 \times 10^8) (2.6 \times 10^7) (9.8 \times 10^6)</td>
<td>(2.6 \times 10^7) (9.8 \times 10^6)</td>
</tr>
<tr>
<td>Error at 4 min (%)</td>
<td>40.8 53.4 68.7 83.2</td>
<td>68.7 83.2</td>
</tr>
<tr>
<td>Error at 28 min (%)</td>
<td>37.1 2.7 12.6 1.6</td>
<td>12.6 1.6</td>
</tr>
</tbody>
</table>

ROI were drawn over tumors, and signals were determined over the course of approximately 1 h, until signals had decayed considerably. Mean maximum signals are shown for days 4 and 14, as well as the error in signal (relative to peak) that would be obtained if quantification were done at 4 and 28 min after the injection of luciferin. This demonstrates the effect on quantification if one of these times were chosen rather than the peak time.

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**Figure 3.** Time course of luciferase signal in orthotopic model at the typical dose and at two dilutions, 1/10th and 1/30th the dose, respectively: 150, 15, and 5 mg/kg. Data were obtained from mice \(n = 3\) at day 4. Data are plotted as the mean percentage with standard deviations of photon counts over time from three animals per group. The mean peak signal at 1/10th and 1/30th luciferin dose relative to the typical dose were 1/33rd to 1/30th, respectively (data not shown).
animal that is typically reported in the literature, which shows little evidence of saturation. Relatively large decreases in the doses of luciferin that were used significantly (but not to a large degree) affected both the time course and the signal intensity. This adds another factor that needs to be controlled in longitudinal studies.

In summary, this study shows that tumor detection and quantification varies considerably depending on the time point of detection, and this fact will need to be taken into account for reproducible quantification in comparative studies.

REFERENCES


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Two-Color Image Analysis Discriminates between Mineralized and Unmineralized Bone Nodules In Vitro

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ABSTRACT

Functional assays of progenitor cell capacity for colony formation in vitro typically depend on the investigator’s expertise with quantification. The ability to enumerate and analyze colony types with standardized criteria with no bias would be a useful tool for research and drug development. We report the development of a two-color automated analysis system for colony-forming unit-osteoblasts that is capable of reporting progenitor frequency and bone nodule number, size, and type (mineralized or unmineralized). Our image analysis system was validated using the rat calvaria cell model to measure in vitro bone nodule development. With computer-aided image analysis, data on nodules can be rapidly generated with a minimum of user bias and fatigue. This novel tool will distinguish mineralized and unmineralized bone nodules, facilitate quantification, enable large-scale experimental design, allow for long-term data storage and tracking, and lead to the identification of new parameters that impact bone development.

INTRODUCTION

With the advance of imaging technology, there are many fields that will benefit from the development and application of new methods. To illustrate the scope, consider a few of the biomedical advances in imaging: automated diagnosis of cancers (1–3); additional prognostic information (4); high-throughput drug screening (5–7); in vitro colony evaluation (8,9); and quantification of bone tissue (10,11).

Bone development in vitro is now routinely measured via the formation of bone nodules (12–17). This assay facilitates the assessment of pharmacological agents with potential positive or