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Noninvasive Fluorescent Imaging Reliably Estimates Biomass In Vivo

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ABSTRACT

Whole-body optical imaging of small animals has emerged as a powerful, user-friendly, and high-throughput tool for assaying molecular and cellular processes as they occur in vivo. As with any imaging method, the utility of such technology relies on its ability to provide quantitative, biologically meaningful information about the physiologic or pathologic process of interest. Here we used an animal tumor model to evaluate the extent of correlation between noninvasively measured fluorescence and more traditional measurements of biomass (tumor volume and tumor weight). C57/BL6 mice were injected subcutaneously with murine colon adenocarcinoma cells that were engineered to express GFP. Serial measurements of fluorescence intensities were performed with a macroscopic in vivo fluorescence system. The progressive increases in intensity correlated strongly with growth in tumor volume, as determined by caliper measurements ($R^2 = 0.99$). A more stringent correlation was found between fluorescence intensity and tumor weight ($R^2 = 0.97$) than between volume and weight ($R^2 = 0.89$). In a treatment experiment using tumor necrosis factor- α , fluorescence intensity (but not tumor volume) was able to differentiate between treated and control groups on day 1 post-treatment. These results validate the ability of noninvasive fluorescent imaging to quantify the number of viable, fluorescent cells in vivo.

INTRODUCTION

Advances in technology have made it possible to noninvasively image reporter gene products, such as luciferase and GFP, in the living animal (5,8). The emergence of such noninvasive imaging methods for small animals offers numerous advantages, including the ability to perform real-time imaging and the increased statistical power derived from studying individual animals repeatedly.

Since the first description of whole-body imaging of GFP-expressing tumors (16), a variety of biological processes has been studied in this manner. These include numerous tumor growth/metastasis/treatment models (1–4,7,10,12), organ-targeted transgene expression (15), and bacterial infection and treatment response (17). The imaging has been described as quantitative based on *relative* increases in signal intensity over time (16). In contrast, noninvasive bioluminescent imaging of murine tumor models has been shown to correlate well with tumor volume (13) and number of injected cells (14). Yet, bioluminescent imaging differs significantly from fluorescent imaging, and the results obtained using one method may not directly apply to those from the other. For the former, light is emitted from the animal after systemic administration of the substrate luciferin. The background signal is extremely low. In contrast, fluorescent noninvasive imaging employs an external light source of the appropriate wavelength to excite the fluorescent marker within the tissue of interest. Furthermore, the background signal can be relatively high because of tissue autofluorescence.

While the fluorescent signal from GFP-expressing cells has previously been shown to be strongly correlated to cell number in vitro (9), such experiments have not been described for in vivo imaging in small animals. Since the fluorescence intensity measured from a GFP-expressing tumor in theory should represent the population of viable tumor cells, we tested the hypothesis that this type of measurement is a more accurate estimate of biomass than tumor volume determination. Here we use a GFP-expressing murine tumor model to describe the relationships between fluorescence intensity, tumor volume, and tumor weight.

MATERIALS AND METHODS

Retroviral Transduction of MC38 Cells

The retroviral vector pCLNC-GFP, the vector pMD.G, and the cell line 293GP were obtained from P. Robbins (National Cancer Institute, Bethesda,

MD, USA). pCLNC-GFP contains the *gfp* gene and is based on the pCLNCX retroviral vector system (11). Pseudotyped retroviral particles were generated as previously described (6). Briefly, 293GP cells (stably transfected with the retroviral *gag* and *pol* elements) were cotransfected with pMD.G (containing the G protein gene from vesicular stomatitis virus) and pCLNC-GFP. Medium was changed after 24 h, and supernatant was collected and filtered at 48 h. MC38 murine colon adenocarcinoma cells (developed in the Surgery Branch, National Cancer Institute) were transduced with retroviral supernatant in the presence of hexadimethrine bromide (8 μ g/mL; Sigma-Aldrich, St. Louis, MO, USA) and selected in G418 (400 μ g/mL; Invitrogen, Carlsbad, CA, USA). All cells were maintained in DMEM with 10% FCS, 100 U/mL penicillin, 100 μ g/mL streptomycin, 50 μ g/mL gentamicin, 0.5 μ g/mL Fungizone, and 4 mM glutamine (Biofluids, Rockville, MD, USA). Expression of GFP was assessed by fluorescence microscopy. The in vitro growth rate of the MC38-GFP cell line was similar to that of the parental line, and the expression of GFP was stable in vitro even in the absence of selection agents (data not shown).

Tumor Formation and Treatment in Animals

Animal experiments were conducted according to protocols approved by the National Institutes of Health Animal Care and Use Committee (Bethesda, MD, USA). MC38 or MC38-GFP were maintained in culture as described above. Cells were resuspended in PBS, and 1×10^6 were inoculated in the right flank of six-week old female C57/BL6 mice. Tumors were measured at the indicated time points in two dimensions by the use of calipers, and tumor volumes were calculated according to the following formula: volume = $\text{Width}^2 \times \text{Length} \times 0.52$, where 0.52 is a constant to calculate the volume of an ellipsoid. For the treatment experiment, mice were injected with PBS, 2 μ g tumor necrosis factor- α (TNF- α), or 4 μ g TNF- α via the tail vein after the tumors had grown to an approximate volume of 160 mm³. Each group initially con-

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Table 1. Tumor Volume versus Fluorescence Intensity: Ability to Detect Statistically Significant Differences Between Control and High-Dose TNF- α Groups

Day	-1	1	2	3	4	5	6	7
Tumor Volume (mm ³)	0.432	0.106	0.018 ^a	0.013 ^a	0.030 ^a	0.033 ^a	0.028 ^a	0.026 ^a
Fluorescence Intensity (RFU)	0.334	0.004 ^a	0.004 ^a	0.011 ^a	0.009 ^a	0.010 ^a	0.016 ^a	0.012 ^a

Injections of TNF- α occurred at day 0.
^a $P < 0.05$ was considered statistically significant.

sisted of five mice, and three to four mice per group survived through the duration of the experiments.

Noninvasive Imaging

At each indicated time point, nonin-

vasive fluorescent imaging was performed. The equipment consisted of an ORCA-II CCD camera (Hamamatsu, Japan), a custom-designed light-tight imaging chamber (Microscope Services, Rockville, MD, USA), a GFP filter set [excitation filter (HQ470/40x),

barrier filter (OG515) (Chroma Technology, Brattleboro, VT, USA)], a 150-W light source (Schott-Fostec, New York, NY, USA), and a Power Mac G4. Exposure times were in the 0.1–0.2 ms range. Images were obtained and analyzed using Openlab software (Improvision, Lexington, MA, USA).

Animal Sacrifice

MC38-GFP tumors were harvested and weighed immediately after the mice were sacrificed at the conclusion of the experiments (15 days after the initial tumor injections).

Statistical Analysis

Statistical analysis was performed using Microsoft® Excel®. All data represent $\bar{x} \pm \text{SE}$. For Table 1, Student's t tests (one-tailed) were performed to calculate P values.

RESULTS AND DISCUSSION

A murine colon adenocarcinoma line (MC38) was engineered to stably express high levels of GFP by retroviral transduction. The MC38-GFP cell line was injected subcutaneously into mice, and resulting tumors were followed serially over time with both caliper-derived tumor volume measurements and noninvasive fluorescent imaging (Figure 1, A, B, and C). Figure 1D shows the correlation between these two measurements. On the last day of the experiment, additional correlations were performed between these two measurements and tumor weight (Figure 1, E and F).

To evaluate these relationships in a model of therapeutic response, animals bearing subcutaneous MC38-GFP tumors were injected with either PBS (negative control), low-dose TNF- α (2 $\mu\text{g}/\text{mouse}$), or high-dose TNF- α (4 $\mu\text{g}/\text{mouse}$). The growth of the tumors was again followed serially with both volume and fluorescence (Figure 2, A, B, and C). On the last day of the experiment, correlations were performed between these two measurements and tumor weight (Figure 2, D and E). In addition, the ability of either volume or fluorescence intensity to detect statistically significant differences between

Figure 1. Serial measurements of tumor volume and tumor fluorescence in vivo. MC38 or MC38-GFP tumor cells were injected subcutaneously into C57/BL6 mice. Noninvasive optical imaging was performed, and the tumors were measured with calipers on the days indicated. (A) Serial grayscale-fluorescent overlay images of a representative mouse bearing a MC38-GFP tumor. (B) Serial tumor volumes as measured by calipers and calculated using the formula $V=0.52 \times W^2 \times L$. (C) Quantification of tumor fluorescence intensity. (D) The serial tumor volume and fluorescence intensities from each experimental time point were correlated. (E and F) On day 15, mice were sacrificed after measurements were obtained, and the tumors were harvested and weighed. The tumor weights were correlated to tumor volume (E) and fluorescence intensity (F). $n = 4\text{--}5/\text{group}$.

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the PBS-treated and high-dose TNF- α -treated animals for each time point was also assessed (Table 1). While volume measurements could not detect statistically significant differences between the control and low-dose TNF- α groups on any day, fluorescence intensity did detect such differences on days 2 and 4 (data not shown).

To our knowledge, the degree of correlation of the externally measured fluorescence intensity with more traditional measurements, such as tumor volume determined by calipers and tumor weight, has not been described. Here we use a murine GFP-expressing tumor model to analyze the quantitative characteristics of a macroscopic *in vivo* fluorescence system. In the serial tumor growth experiments (Figure 1), we observed an extremely strong correlation ($R^2 = 0.99$) between measurement of tumor fluorescence intensity and traditional calculation of tumor volume. In other words, the relative increases in fluorescence intensity in the growing subcutaneous tumor very closely match the relative increases in tumor volume. To determine how these measurements were related to a measurement of biomass, they were compared to the weight of the harvested tumors at the end of the experiment. Interestingly, a more linear correlation was found between fluorescence intensity and weight ($R^2 = 0.97$) than between volume and weight ($R^2 = 0.89$). If one assumes the weight to be an accurate estimate of biomass, then this would suggest that fluorescence intensity more closely approximates the true number of viable cells in the tumor than does volume. Tumor volume measurements will often overestimate the number of viable cells (e.g., when there are areas of necrosis or edema within a tumor). In addition, there is inherently larger variance in volume calculations because they assume an ellipsoid shape for the subcutaneous tumors. It has been suggested that *in vivo* bioluminescent measurement of luciferase expressing cells is more accurate than tumor volume in estimating the number of metabolically active tumor cells (13). The current study provides quantitative data to support this hypothesis for fluorescent imaging.

In mice treated with TNF- α (Figure

2), less robust correlation between volume and intensity was observed ($R^2 = 0.90$ for low dose, $R^2 = 0.59$ for high dose) than in the controls ($R^2 = 0.99$) (data not shown). These lower correlations are most likely due to the presence of edema and necrosis in the treated tumors (this was observed both by gross examination and histologically; data not shown). Tumor volume measurements as determined by calipers are likely overestimating the number of viable cells by inclusion of the necrotic/edematous areas. Also, at the end of the experiment, we again observed a stronger correlation between fluorescence intensity and tumor weight ($R^2 = 0.87$) than between tumor volume and tumor weight ($R^2 = 0.84$). While the magnitude of the difference is not striking, the finding is consistent with the results of the serial growth experiment. Moreover, the results highlight additional advantages of optical imaging. While both measurements detected sta-

tistically significant differences between the TNF- α -treated (high dose) and control groups on days 2 through 7, only fluorescence intensity was able to differentiate between the two groups on day 1 post-treatment (Table 1). For investigations in which the detailed kinetics of a biological process, such as tumor growth, are of interest, *in vivo* fluorescent imaging appears to be a more sensitive method than traditional tumor volume measurements. Using imaging, smaller relative changes in the number of viable cells can be observed. While this immediate loss of signal is most likely due to a decrease in the number of viable, GFP-expressing tumor cells, it should be noted that other processes, such as quenching of the GFP signal by edema or hemorrhage, may also be occurring. Distinguishing the relative contributions of such processes to a decrease in fluorescent signal is beyond the scope of this study. Future investigations should also exam-

Figure 2. Quantitative assessment of treatment responses. MC38-GFP tumor cells were injected subcutaneously into C57/BL6 mice. When tumors reached an approximate volume of 160 mm³, mice were systemically injected on day “0” with PBS (control, blue lines), 2 μ g TNF- α (pink lines), or 4 μ g TNF- α (yellow lines). Noninvasive optical imaging was performed, and the tumors were measured with calipers on the days indicated. (A) Serial grayscale-fluorescent overlay images of a representative mouse treated with 4 μ g TNF- α . (B) Serial tumor volumes as measured by calipers and calculated using the formula $V=0.52 \times W^2 \times L$. (C) Quantification of tumor fluorescence intensity. (D and E) On day 7 after treatment, mice were sacrificed after measurements were obtained, the tumors were harvested and weighed, and the tumor weights were correlated to tumor volume (D) and fluorescence intensity (E). $n = 3-5$ /group.

ine correlations between tumor volume/weight and fluorescence intensity in clinically relevant, orthotopic tumor models.

Using a subcutaneous murine tumor model, we have validated the quantitative capabilities of noninvasive *in vivo* fluorescent imaging. Compared to caliper-derived tumor volumes, the fluorescent signal more closely approximates the number of viable cells in a growing tumor. In doing so, it can give more reliable information about the early effects of a treatment, when the number of living cells is reduced by a relatively small amount.

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