NUCLEAR INSTRUMENTS & METHODS IN PHYSICS RESEARCH

Section A: accelerators, spectrometers, detectors and associated equipment

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Monitoring and quantitative assessment of tumor burden using in vivo bioluminescence imaging

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Abstract
In vivo bioluminescence imaging (BLI) is a sensitive imaging modality that is rapid and accessible, and may comprise an ideal tool for evaluating tumor growth. In this study, the kinetic of tumor growth has been assessed in C26 colon carcinoma bearing BALB/c mouse model. The ability of BLI to noninvasively quantitate the growth of subcutaneous tumors transplanted with C26 cells genetically engineered to stably express firefly luciferase and herpes simplex virus type-1 thymidine kinase (C26/tk-luc). A good correlation ($R^2 = 0.998$) of photon emission to the cell number was found in vitro. Tumor burden and tumor volume were monitored in vivo over time by quantitation of photon emission using Xenogen IVIS 50 and standard external caliper measurement, respectively. At various time intervals, tumor-bearing mice were imaged to determine the correlation of in vivo BLI to tumor volume. However, a correlation of BLI to tumor volume was observed when tumor volume was smaller than 1000 mm$^3$ ($R^2 = 0.907$). $\gamma$ Scintigraphy combined with [131I]FIAU was another imaging modality used for verifying the previous results. In conclusion, this study showed that bioluminescence imaging is a powerful and quantitative tool for the direct assay to monitor tumor growth in vivo. The dual reporter genes transfected tumor-bearing animal model can be applied in the evaluation of the efficacy of new developed anticancer drugs.

Keywords: Bioluminescence imaging; Murine colon carcinoma; Firefly luciferase; Mouse syngenic model; Xenogen IVIS 50; Gamma scintigraphy; [131I]FIAU

1. Introduction
Many preclinical in vivo studies of experimental therapeutics utilize rapidly growing transplantable mouse or human tumor cell lines injected subcutaneously in syngeneic or immunodeficient rodents, to facilitate quantification of tumor growth and treatment response using caliper measurements of tumor volume [1]. Preclinical neoplastic models predominantly use parameters such as tumor weight, or volume to assess disease burden.

Subsequent primary tumor growth is traditionally monitored by periodic caliper measurements if tumors are superficial or by single, end point measurements of weight or volume if tumors are internal and identifiable. Because caliper and weight measurements are relatively rapid and easy to perform and they have been a mainstay in the traditional assessment of animal tumor models. Nevertheless, there are recognized limitations associated with this approach. Experiments based on caliper data or weight measure total tissue mass including areas of necrosis and edema, and so do not necessarily assess the effect of a treatment on the number of viable cells in a tumor without additional processing and evaluation. Furthermore, when
animal data are attainable from primary tumors only after necropsy, experiments usually require groups of animals to be sacrificed at multiple and predetermined end points. This increases the number of animals per experiment, limits the number of time points for evaluation. These measurements do not allow for serial observation of neoplastic growth in the same animal in response to experimental interventions. To overcome such limitations, use of reporter genes to detect in vivo neoplastic processes has gained increasing popularity [2–6].

Reporters provide an elegant alternative strategy for the molecular and genetic studies that is easier, less expensive, more accurate and quantifiable than the standard techniques [7]. Protein or enzyme translated from the reporter gene, that is not normally found in the eukaryotic cells, can be detected by assaying their substrates or metabolites. Real-time imaging of these products could be accomplished by digital imaging microscopy, highly sensitive photon counting facilities, such as the CCD, optical imaging, or non-invasive radiological devices, such as MRI, SPECT and PET. Luciferase gene (luc) from firefly is one of the most widely used reporter genes for bioluminescence imaging (BLI). Animals carried luc gene offer the cell populations with sufficient stability of the reporter gene for various studies [8]. BLI could be used for tracing tumor growth and development in vivo, drug-treatment efficacy [9,10]. In vivo BLI has introduced a sensitive and rapid alternative to monitor and track tumor development from tumors cells implanted into mice [11–13], or tumors forming de novo in transgenic animals [14]. BLI has also been used to detect bacterial and viral infections [15–17] in various live animal models.

We developed a light-emitting cell line expressing firefly luciferase from the murine tumor cell line C26 (colon) and evaluated their bioluminescent stability over time. BLI monitoring of tumor growth was also compared to the traditional assays of tumor volume and established quantitative correlation between them. Our findings show that BLI improves upon and refines traditional animal cancer models by using fewer animals, offering a rapid, sensitive and less invasive monitoring of early neoplastic growth by providing an accurate, temporal assessment in the same animal over time.

2. Materials and methods

2.1. Cell culture and transfection

The C26 cells were grown in RPMI 1640 medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum at 37°C with 5% CO₂.

The plasmid was constructed from pGL3-basic and pEGFP-C1. Transfection of C26 tumor cells with tk, luc and an antibiotic resistance gene (neo) was carried out using LipofectamineTM 2000. Bioluminescent antibiotic resistant clones were amplified and characterized for stable in vitro and in vivo luciferase and tk activity. The clone, C26/tk-luc clone 1, was selected and used for further studies.

2.2. C26/tk-luc subcutaneous syngeneic tumor model

Male BALB/c mice at 6–7 weeks old were anesthetized by intraperitoneal injection of ketamine hydrochloride plus xylazine. Anesthetized animals received 2 × 10⁶ C26/tk-luc cells suspended in 200 μl sterile DPBS by subcutaneous injection below the dorsal flank. Perpendicular tumor diameters were measured 10 days after injection or until the bulge was observed using a Vernier scale caliper. Tumor volume was estimated by the formula: 1/2 × 4/3π × length/2 × width/2 × thickness = 0.523 × (length × width × thickness) [18]. Tumor growth was monitored weekly by in vivo bioluminescent imaging and by external caliper measurements for 4 weeks.

2.3. Bioluminescence imaging

In vitro and in vivo BLI was carried out with an IVIS50 imaging system. Images and measurements of bioluminescent signals were acquired and analyzed using living image software. D-luciferin was dissolved in sterile DPBS (0.015 g/ml). For in vitro imaging, bioluminescent cells were diluted from 5000,000 to 25 cells into appropriate cell culture media in black, clear bottom 96-well plates. D-luciferin at 150 μg/ml in media was added to each well, and the photon emission from each wells were acquired 1 min. For in vivo imaging, animals received D-luciferin at 150 mg/kg by intraperitoneal injection at 15 min before imaging. The animals were anesthetized using 1–3% isoflurane, and placed onto the warmed stage inside the camera box. The animals received continuous exposure to 1–2% isoflurane to sustain sedation during imaging. Image acquisition times were 1 min. Regions of interest (ROI) from displayed images were drawn around the tumor sites and quantified as photons/second (ph/s). Background bioluminescence in vivo was in the range 5 × 10⁴–1 × 10⁵ ph/s.

2.4. Correlation between BL and tumor volume

Following tumor cell injection, animals underwent BLI on postinoculation days 15, 21, 28 and 35 (n = 4). At each time point, D-Luciferin was then administered intraperitoneally (150 mg/kg) and the following steps were performed as the previously described.

2.5. Gamma image

Gamma scintigraphy of C26/tk-luc tumor-bearing mice after 100 μCi/0.1 ml [131I]FIAU by lateral caudal vein injection was obtained with eCam Multisite Cardiac (Siemens, Munich, Germany). Imaging was carried out at 24 h post injection. ROIs were drawn over the tumor area.
2.6. Whole-body autoradiography

Mice were injected with 100 μCi/0.1 ml [131I]FIAU via lateral caudal veins. Whole-body autoradiography was carried as previously described [19]. Animals were sacrificed with chloroform post-γ scintigraphy, and immediately dipped into isopentane which was pre-chilled with liquid nitrogen. The whole carcass was frozen for 1–2 min depending on body size. The frozen carcass was embedded on cryostat holder (7 × 5 cm) with 4% CMC. The embedded carcass was put on quick freezing stage (−40 °C) in cryostat for about 30 min. The frontal sections were carried out with a thickness of 30 μm at −20 °C. Pieces of pre-chilled adhesive tape were used for lifting the frozen sections. Sections mounted and placed on the imaging plate (IP) in the imaging plate cassette. The IP was assayed with FLA5000 reader (Fuji photo film) to acquire the phosphor image.

2.7. Statistical analysis

The mean bioluminescence ph/s, tumor volume and corresponding standard errors were determined for each experiment. Regression plots were used to describe the relationship between BLI and cell number, and tumor volume; $R^2$ values are reported to assess the quality of the regression model.

3. Results

3.1. In vitro imaging

In vitro BLI correlated with an increase in cell number as depicted in Fig. 1. Linear regression analysis revealed an excellent correlation ($R^2 = 0.998$) between the cell number and BLI. The minimum number of detectable cells was between 2000 and 5000 cells per well.

3.2. In vivo imaging

3.2.1. Correlation between BLI and tumor volume

The ability of BLI to quantify in vivo tumor burden in a subcutaneous animal model was investigated through in vivo measurements of both the tumor volume and photon emission from the same group of animals over time. Linear regression analysis between overall BLI and tumor volume yielded a bad correlation coefficient of $R^2 = 0.256$ (data not shown). If the animals with tumor volumes >1000 mm$^3$ were excluded, the correlation coefficient increased to $R^2 = 0.907$ (Fig. 2).

3.2.2. γ Scintigraphy

Fig. 3 depicts mice bearing C26/tk-luc tumors imaged 15 min after administration of [131I]FIAU. The tumors had great radiolabel uptake. The tumor-to-muscle (T/M) ratios were 30.78 for tumor size 775 mm$^3$ and 42.08 for tumor size 2668.9 mm$^3$.

3.2.3. Whole-body autoradiography (WBAR)

The WBAR showed high levels of radioactivity accumulation in the tumor, thyroid and GI (Fig. 4). Comparison with γ scintigraphy the images were identical. In general, high uptake of radioactivity can be identified clearly in tumor by WBAR.
4. Discussion

BLI in preclinical cancer models has brought about unique advantages such as temporal monitoring of tumors and response to interventions without the need for animal sacrifice [2]. Reports have validated BLI in preclinical models by correlating BLI with tumor burden as measured by currently available methods [1,20]. In this setting, we sought to establish BLI correlation with tumor burden, explore technique sensitivity by subcutaneous tumor inoculation, and discuss limitations for future use.

In this study, caliper-based measurements were used to validate BLI-based measurements of subcutaneous tumor growth in animals. There appears to be a moderate linear relationship between caliper-determined tumor volume and tumor light output as measured through BLI. Both living and dead tumor cells and peritumoral edema may all contribute to the caliper-determined tumor volume. In contrast, tumor light output is presumably derived solely from metabolically active transformed tumor cells. This may result in less robust correlation between caliper-determined tumor volume and photon counts in large tumors in which regions of the tumor may be necrotic. In support of this hypothesis, we have observed less significant correlations between tumor volume larger than 1000 mm$^3$ and photon counts. Decreasing BLI over time, despite growing tumor burden in certain models, has been attributed to necrotic and hypoxic regions within the neoplasm not amenable to detection [21]. In a murine intrahepatic tumor model, animals deviating from regression analysis of BLI versus tumor volume were those with predominantly fibrotic tumors [20]. The phenomenon might be influenced the correlation between BLI and tumor volume. However, this may represent an advantage of BLI over direct measurements of tumor volume using calipers as it provides a quantitative surrogate measure of the number metabolically active tumor cells.

Other advantages of the BLI approach for preclinical evaluation of therapeutic interventions include reasonable equipment costs, short scanning times of only 1–5 min, simultaneous scanning of multiple animals in a single image acquisition, and minimal post-processing requirements. However, BLI is currently a two-dimensional imaging modality with only 1 cm spatial resolution due to photon scattering, which impairs detailed assessment of tumor anatomy and invasion [2,21].

Monitoring primary tumor growth, the status of residual disease, and the development of metastatic disease are currently possible in vivo with the use of small animal imaging technologies including BLI. The development of bioluminescent tumor models, including the murine C26/tk-luc cell line reported in this study, improves upon traditional methodologies to provide early and sensitive assessment of tumor growth or advanced treatment regimens.

5. Conclusions

We have successfully established a non-invasive optical imaging system for the evaluation of tumor growth. Correlation with BLI and tumor volume study carried on BALB/c mice bearing C26/tk-luc tumors indicated that BLI was rapid and quantitative assessment of tumor growth while tumor < 1000 mm$^3$. This study demonstrates the benefit and potential application of a multimodality imaging system not only in temporal monitoring of tumor growth but also in therapeutic and diagnostic strategies to evaluate drug or treatment efficacy, and may also apply to other fields of biomedical research as well.

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