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Original Article

Quantification of metastatic load in a syngeneic murine model of metastasis

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ABSTRACT

Bioluminescence imaging (BLI) is an established method for evaluating metastatic load in preclinical cancer models; however, BLI can produce observational error due to differences in substrate concentration and signal depth. In our syngeneic murine model of metastasis (VM-M3), we used a quantitative polymerase chain reaction (qPCR) method of DNA quantification to bypass these limitations. Liver, spleen, and brain from VM/Dk (VM) mice bearing VM-M3 tumor cells were first imaged *ex vivo* with BLI. qPCR quantification of tumor cell DNA was then performed on DNA extracted from these organs. Linear regression indicated that qPCR data predicted BLI data in solid tissue. Furthermore, the tumor cell detection limit was lower for qPCR analysis than for BLI analysis. In order to validate qPCR for use in detecting blood metastases, qPCR quantification was performed on whole blood collected from mice whose global organ metastatic load (summation of liver, spleen, kidneys, lungs, and brain) was quantified through BLI. Linear regression indicated that qPCR data in blood predicted BLI data in solid tissue. The results demonstrate that qPCR is an accurate and sensitive method of metastatic quantification in syngeneic murine models.

Introduction

Metastasis involves the spread of cancer cells from the primary tumor to surrounding tissues and distant organs. The metastatic cascade is a series of sequential and interrelated steps that includes cancer cell detachment from the primary tumor, intravasation into the circulation, evasion of immune destruction, extravasation into a distant capillary bed, and invasion and proliferation in distant organs [1–6]. Metastatic cells also establish a microenvironment through the release of cytokines, lactic acid, and growth factors that facilitate angiogenesis and proliferation, which results in macroscopic, malignant secondary tumors. In addition, metastatic cells preferentially invade those organs (lymph nodes, lung, liver, brain, bone, pleura, and peritoneum) that promote tumor cell growth and survival consistent with the "seed and soil" hypothesis [3,7–9]. Most cancer therapies should be evaluated in terms of their anti-metastatic potential, as metastasis is responsible for greater than 90% of cancer deaths [10].

Quantification of systemic metastasis in preclinical murine models can facilitate the development of anti-metastatic therapies. However, current methods used to evaluate metastatic load have inherent limitations. The traditional method of histological examination, in which a few tissue slices are used to evaluate metastasis in a given organ, is outdated. No consensus has been reached regarding the distribution of metastases within organs (peripheral, central, or random sites) and recent studies suggest that the colonization of individual metastases is significantly variable [11]. Consequently, histological examination of whole organs for metastasis is extremely labor intensive and yields results that are largely qualitative [12].

Optical imaging methods of metastatic assessment have gained popularity due to their potential for rapid, noninvasive data collection throughout the course of treatment. Bioluminescence imaging (BLI) measures photon emission from cancer cells that are engineered to express the luciferase protein. The luciferase reaction is dependent on a number of variables including the presence of ATP, O_2, and the luciferin substrate. However, these conditions can vary

Abbreviations: BLI, bioluminescence imaging; BLT, bioluminescence tomography; CCD, charge-coupled device; CTCs, circulating tumor cells; ctDNA, circulating tumor DNA; Cq, quantification cycle; *in vivo* flow cytometry, IVFC; qPCR, quantitative polymerase chain reaction; s.c., subcutaneously; VM, VM/Dk; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element.

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between different tissue microenvironments, thus preventing absolute quantification between mice [13]. Further, BLI relies on planar image acquisition and thus does not provide information about the depth of the photon source. BLI is depth-dependent, such that the lack of spatial data precludes normalization between tumor cells in peripheral organ regions (with greater photon emission) and tumor cells in central organ regions (with less photon emission) [14,15]. Bioluminescence tomography (BLT), which can obtain a three-dimensional rendering of photon emission, could offset some limitations of BLI, but this technology is still under development [15]. *In vivo* fluorescence imaging can be used to offset issues with luciferase-based techniques. Fluorescent proteins emit light when excited by an external illuminator. This mechanism occurs independently of substrates and thus photon emission is not affected by the tissue microenvironment. *In vivo* fluorescence can also be used to identify cells with a high metastatic potential. Cells can be color-coded, which allows for the study of genetic exchange and its ability to convert cells from a low to high metastatic potential [16].

The amplification of genomic DNA using quantitative polymerase chain reaction (qPCR) can also be used as a quantitative procedure for evaluating metastatic load. Since qPCR is performed on DNA extracted from homogenized organ tissue, the data obtained are independent of the metastatic distribution within organs. Thus, qPCR can circumvent depth dependence issues associated with BLI. Also, since genetic markers are used to detect cancer cells, data are independent of the bioavailability of exogenously administered substrate. Thus, qPCR can be an accurate and sensitive method for quantification of metastatic load in preclinical models.

qPCR was first used to measure metastasis in xenograft models [17]. We adapted this method in order to evaluate systemic metastasis of VM-M3 cells grown in the syngeneic VM/Dk mouse strain [7]. The VM-M3 cells were transfected with a lentiviral vector containing a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) region that could be quantified through qPCR. Poeschinger et al. demonstrated that data from BLI imaging correlated with data from qPCR in a xenograft model of experimental metastasis [18]. However, this study did not compare sensitivity between the two methods. Thus, we chose to address sensitivity issues in our study.

Hematogenous tumor markers are an additional area of interest in metastatic assessment. *In vivo* flow cytometry (IVFC) can be used to detect fluorescently tagged circulating tumor cells (CTCs). This highly sensitive method is noninvasive and can detect CTCs in real time. Thus, IVFC allows the study of CTC kinetics over an experimental time course [19–21]. Previous studies have successfully implemented qPCR to detect human circulating tumor DNA (ctDNA) in xenograft models [22–24]. Our study extended this method for use in the syngeneic VM-M3 model of systemic metastasis; however, we performed qPCR on whole blood and thus quantified DNA from CTCs. We demonstrated that the abundance of CTCs as measured by qPCR could predict global organ metastatic load as measured by BLI. In summary, we adapted current qPCR protocols for evaluating metastatic load into a syngeneic model of metastasis. This immunocompetent model more closely mimics the tumor-host interactions that occur throughout the metastatic cascade than do xenograft models.

Materials and methods

Mice

Mice of the VM/Dk (VM) strain were obtained as previously described [7]. All VM mice used in this study were housed and bred in the Boston College Animal Care Facility using husbandry conditions as previously described [25]. Age- and sex-matched VM mice were used as tumor cell recipients. In the solid tissue study, mice were 3-month-old females. In the blood study, half of the mice were 3-month-old females and half were 3-month-old males. All animal procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care Committee.

Origin of VM-M3 tumor

The VM-M3 tumor arose spontaneously in the cerebrum of an adult inbred VM mouse, as we described previously [7]. The tumor was classified as a glioblastoma based on histological appearance and invasive growth behavior in the brain [26]. The tumor cells have several characteristics in common with microglia, which are neural mesenchymal cells. When given access to extra neural sites, the VM-M3 tumor cells display metastasis to multiple organ systems including liver, spleen, kidneys, lungs, and brain [27]. The extraneural metastasis seen for the VM-M3 tumor cells is similar to what has been documented for human glioblastoma cells that gain access to extraneural sites [28–32]. The VM-M3 cells therefore represent an ideal model system for measuring metastatic load in a syngeneic immunocompetent host.

Cell lines and culture conditions

The VM-M3 cell line was transduced with a lentiviral vector (CSCGW2-Fluc-IG; gift from Miguel Sena-Esteves) as previously described [7]. This vector contains a WPRE region and a Fluc region that encodes luciferase [33]. qPCR was used to quantify the WPRE region, while BLI was used to quantify the activity of the luciferase enzyme. VM-M3 cell lines were established and cultured as previously described [7].

Subcutaneous tumor implantation

For analysis of organ metastasis, approximately 3×10^6 VM-M3 cells were suspended in 0.5 mL of PBS and then injected subcutaneously (s.c.) into the left flank of VM mice. For analysis of tumor cell DNA in blood, approximately 1×10^6 VM-M3 cells were suspended in 0.5 mL of Corning® Matrigel® Matrix High Concentration and were then injected s.c. into the left flank of VM mice. Mice were sedated during injections through isoflurane that was delivered via a rodent nosecone. Mouse morbidity was the endpoint for terminating experiments.

BLI of solid organs

Mice were sacrificed via CO_2 euthanasia 25 days after tumor inoculation. Organs (liver, spleen, and brain) were resected and prepared for *ex vivo* BLI. Due to the time dependence of the luciferase reaction, all organs were prepared under an ordered time schedule. At 0 min, organs were immersed in 300 μg/mL of D-luciferin in PBS. At 10 min, organs were patted dry with absorbent paper and placed on petri dishes. At 15 min, organs underwent BLI. Photon emission was captured with an IVIS Lumina charge-coupled device (CCD) camera system (Parameters: exposure = 1 min, binning factor = 4, f/stop = 1) and analyzed with Living Image® software (Xenogen) as we previously described [27]. In order to minimize error caused by superficial depth penetrance, two BLI images were taken of each organ (ventral and dorsal). Total flux for each organ was expressed as an average of the values captured from these two images. In preparation for qPCR, organs were flash frozen on dry ice and stored at −80 °C.

BLI of blood

Blood was collected from mice via submandibular bleeding immediately before CO_2 euthanasia. Mice were sedated under isoflurane during blood collection. Mice were sacrificed via CO_2 euthanasia 25 days after tumor inoculation. In order to capture global organ metastasis, major organs (liver, spleen, kidneys, lung, and brain) were resected and prepared for *ex vivo* BLI following the previously mentioned protocol. Within each mouse, global organ metastasis was expressed as the sum of the average total flux collected from each major organ. Half of the whole blood collected from each mouse (~120 μL) was stored at −80 °C and saved for qPCR analysis. The other half collected was immediately imaged with BLI using the following protocol. Whole blood samples were prepared in a concentration of 300 μg D-luciferin/1.0 mL blood. A negative control was prepared in a concentration of 300 μg D-luciferin/1.0 mL PBS. These samples were then transferred into a Corning™ Costar™ 96-Well Black Clear-Bottom Plate. The plate was shaken at 200 rpm for 2 min. 15 min after the addition of D-luciferin, the samples underwent BLI (Parameters: exposure = 1 min, binning factor = 4, f/stop = 1).

As a positive control for this experiment, we prepared a standard curve of VM-M3/Fluc cells (1×10^2, 1×10^3, 5×10^3, 1×10^4, 5×10^4, 1×10^5, 5×10^5, 1×10^6) suspended in 200 μL PBS and spiked into 100 μL of control whole blood. D-Luciferin was added to all samples to achieve a concentration of 300 μg D-luciferin/1.0 mL reaction volume. Each standard was prepared in triplicate. These samples were then loaded into a 96-well plate and underwent BLI 15 min after the addition of luciferin. Imaging parameters were identical to those mentioned previously.

Tissue homogenization

Organs were thawed and a hand-held tissue homogenizer was used to prepare homogenates of the organs from tumor bearing mice ("experimental organs") and organs from non-tumor bearing mice ("control organs"). Homogenates were