Subcellular Real-Time *In Vivo* Imaging of Intralymphatic and Intravascular Cancer-Cell Trafficking

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ABSTRACT

With the use of fluorescent cells labeled with green fluorescent protein (GFP) in the nucleus and red fluorescent protein (RFP) in the cytoplasm and a highly sensitive small animal imaging system with both macro-optics and micro-optics, we have developed subcellular real-time imaging of cancer cell trafficking in live mice. Dual-color cancer cells were injected by a vascular route in an abdominal skin flap in nude mice. The mice were imaged with an Olympus OV100 small animal imaging system with a sensitive CCD camera and four objective lenses, parcentered and parfocal, enabling imaging from macrocellular to subcellular. We observed the nuclear and cytoplasmic behavior of cancer cells in real time in blood vessels as they moved by various means or adhered to the vessel surface in the abdominal skin flap. During extravasation, real-time dual-color imaging showed that cytoplasmic processes of the cancer cells exited the vessels first, with nuclei following along the cytoplasmic projections. Both cytoplasm and nuclei underwent deformation during extravasation. Different cancer cell lines seemed to strongly vary in their ability to extravasate. We have also developed real-time imaging of cancer cell trafficking in lymphatic vessels. Cancer cells labeled with GFP and/or RFP were injected into the inguinal lymph node of nude mice. The labeled cancer cells trafficked through lymphatic vessels where they were imaged via a skin flap in real-time at the cellular level until they entered the axillary lymph node. The bright dual-color fluorescence of the cancer cells and the real-time microscopic imaging capability of the Olympus OV100 enabled imaging the trafficking cancer cells in both blood vessels and lymphatics. With the dual-color cancer cells and the highly sensitive imaging system described here, the subcellular dynamics of cancer metastasis can now be observed in live mice in real time.

Keywords: GFP, RFP, cancer cells, trafficking, blood vessels, lymphatic vessels, metastasis, real time imaging

1. INTRODUCTION

The development of fluorescent proteins to genetically label cells in vivo has greatly increased the possibility to observe cell behavior in vivo (1). Our laboratory initially developed the use of green fluorescent protein (GFP) to visualize cancer cells in live tissue (2) and in the intact animal by whole-body imaging (3).

With the use of dual-color fluorescent cells and a highly sensitive whole-mouse imaging system with both macro-optics and micro-optics, our laboratory developed subcellular real-time imaging of cancer cell trafficking in live mice. To observe cytoplasmic and nuclear dynamics in the living mouse, tumor cells were labeled in the nucleus with GFP and with RFP in the cytoplasm. Dual-color cancer cells were injected by a vascular route in an abdominal skin flap in nude mice. The mice were imaged with an Olympus OV100 small animal imaging system with a sensitive CCD camera and four objective lenses, parcentered and parfocal, enabling imaging from macrocellular to subcellular. We observed the nuclear and cytoplasmic behavior of cancer cells in real time in blood vessels (4).

The major pathways of cancer cell dissemination are the lymphatic system and the circulatory system. However, the role of the lymphatic system in cancer metastasis is less well understood compared with the circulatory system (5).

Small Animal Whole-Body Optical Imaging Based on Genetically Engineered Probes
edited by Alexander P. Savitsky, Robert E. Campbell, Robert M. Hoffman
Proc. of SPIE Vol. 6868, 68680J, (2008) · 1605-7422/08/$18 · doi: 10.1117/12.772120

Lymphangiogenesis is associated with an increased incidence of lymph node metastasis. Tumor-secreted cytokines, such as vascular endothelial growth factor (VEGF)-C and VEGF-D, bind to VEGF receptors on lymphatic endothelial cells and induce proliferation of new lymphatic capillaries (6, 7). However, despite its importance in lymphatic metastasis, cancer cell trafficking in lymphatic vessels, including entrance to the targeted lymph node, has been insufficiently investigated. Some studies suggest that the trafficking of tumor cells to lymph nodes resembles the normal migration of dendritic cells and that they follow a chemokine gradient (8). Tumor cells that express certain types of chemokine receptors are more likely to metastasize to the lymph node. A promising approach to the further understanding of trafficking of cancer cells in lymphatics is imaging (9).

Recently, *in vivo* imaging has been adapted to visualize normal cell trafficking in lymph nodes (1, 10). von Andrian and Mempel (11) and Halin et al. (12) have developed fluorescence visualization of lymphocyte trafficking, cell migration, and cell-cell interaction in lymph nodes using intravital microscopy. Stoll et al. (13) visualized T cells interacting with dendritic cells in lymph nodes using multicolor fluorescence. Dadiani et al. (14) imaged breast cancer emboli clustering at a lymphatic vessel junction. However, trafficking of cancer cells in lymphatic vessels was not investigated in these studies.

With this new imaging technology, we studied cancer cell trafficking in blood vessels, extravasation and phenomena related to cancer cell trafficking in lymphatics (9).

2. MATERIALS AND METHODS

2.1 Production of RFP retroviral vector. For RFP retrovirus production, the HindIII/NotI fragment from pDsRed2 (Clontech Laboratories, Inc., Palo Alto, CA), containing the full-length RFP cDNA, was inserted into the HindIII/NotI site of pLNCX2 (Clontech Laboratories) containing the neomycin-resistance gene. PT67, an NIH3T3-derived packaging cell line (Clontech Laboratories) expressing the 10 μl viral envelope, was cultured in DMEM (Irvine Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bio-Products, Calabasas, CA). For vector production, PT67 packaging cells, at 70% confluence, were incubated with a precipitated mixture of LipofectAMINE reagent (Life Technologies, Inc., Grand Island, NY) and saturating amounts of pLNCX2-DsRed2 plasmid for 18 hours. Fresh medium was replenished at this time. The cells were examined by fluorescence microscopy 48 hours posttransduction. For selection of a clone producing high amounts of RFP retroviral vector (PT67-DsRed2), the cells were cultured in the presence of 200 to 1,000 μg/mL G418 (Life Technologies) for 7 days. The isolated packaging cell clone was termed PT67-DSRed2 (9).

2.2 Production of histone H2B-GFP vector. The histone H2B gene has no stop codon (15), which enables the ligation of the H2B gene to the coding region of the GFP gene (Clontech Laboratories). The histone H2B-GFP fusion gene was then inserted at the HindIII/CalI site of the pLHCX (Clontech Laboratories), which has the hygromycin resistance gene. To establish a packaging cell clone producing high amounts of histone H2B-GFP retroviral vector, the pLHCX histone H2B-GFP plasmid was transfected in PT67 packaging cells using the same methods described above for PT67-DsRed2. The transfected cells were cultured in the presence of 200 to 400 μg/mL hygromycin (Life Technologies) for 15 days to establish stable PT67 H2B-GFP packaging cells (9).

2.3 RFP and histone H2B-GFP gene transduction of cancer cells. Cancer cells were labeled with RFP and H2B-GFP. Clones expressing RFP in the cytoplasm were initially established. In brief, cancer cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67-RFP cells and RPMI 1640 (Irvine Scientific) containing 10% fetal bovine serum for 72 hours. Fresh medium was replenished at this time. Cells were harvested with trypsin/EDTA 72 hours post-transduction and subcultured at a ratio of 1:15 into selective medium, which contained 200 μg/mL G418. The level of G418 was increased stepwise up to 800 μg/mL. RFP-expressing cancer cells were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ) using trypsin/EDTA and amplified by conventional culture methods. For establishing dual-color HT-1080 cells, the cells were then incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 H2B-GFP cells and culture medium. To select the double transformants, cells were incubated with hygromycin 72 hours after transfection. The level of hygromycin was increased stepwise up to 400 μg/mL (9).

2.4 Mouse model for imaging real-time nuclear-cytoplasmic dynamics of intravascular trafficking cancer cells. To image nuclear-cytoplasmic dynamics of trafficking cancer cells in live mice, the dual-color cancer cells were injected into the epigastric cranialis vein. Nude mice were anesthetized with a ketamine mixture (10 μL ketamine HCL, 7.6 μL xylazine, 2.4 μL acepromazine maleate, and 10 μL H_2O) via s.c. injection. An arc-shaped incision was made in the abdominal skin. The s.c. connective tissue was separated to free the skin flap without injuring the epigastrica cranialis artery and vein. The skin flap was spread and fixed on the flat stand. A total of 30 μL medium containing 5 x 10^5 LLC, MMT, or HT-1080 dual color cell were injected into the epigastrica cranialis vein. For imaging cancer-cell trafficking in blood vessels, images were acquired in real time. For extravasation, images were acquired every hour after injection with the skin flap open or every 12 hours by opening and closing the skin flap. The inside surface of the skin flap was directly imaged (16).

2.5 Mouse model for imaging real-time trafficking of cancer cells in lymphatic vessels in experimental metastasis. The cancer cells were injected into the inguinal lymph node. Nude mice were anesthetized with a ketamine mixture (10 μL ketamine HCL, 7.6 μL xylazine, 2.4 μL acepromazine maleate, and 10 μL H2O) via s.c. injection. An arc-shaped incision was made in the abdominal skin from the axillary to the inguinal region. The subcutaneous connective tissue was separated to free the skin flap without injuring the lymphatic vessel. Mice were laid flat and the skin flap was spread and fixed on the flat stand. The axillary lymph node was exposed from the inner side or the skin side. 5 x 10^4 HT-1080 dual-color cells in 10μL were injected into the center of the inguinal lymph node. In order to increase contrast, 5 μl PBS containing 5 x 10^4 RFP-expressing cancer cells were injected along with 5 μl FITC-dextran (molecular weight, 2 x 10^6; Sigma-Aldrich, Louis, MO). Images were acquired as described below with the Olympus OV100 small-animal imaging system (9).

2.6 Mouse model for imaging spontaneous shedding of tumor cells into lymphatic vessels. 2 × 10^6 HT-1080 dual-color cells in 20 μL were injected into the nude-mouse footpad. Four weeks later, mice were anesthetized with a ketamine mixture (10 μL ketamine HCL, 7.6 μL xylazine, 2.4 μL acepromazine maleate, and 10 μL H2O) via s.c. injection. Mice were laid in the prone position and the legs were fixed without stimulation of the tumor. A 2-cm straight incision was made in the ipsilateral popliteal region. The popliteal lymph node and lymphatic were exposed without injuring them. Shed tumor cells in the lymphatic vessel were imaged with the OV100 (9).

2.7 Effect of pressure on tumor cell shedding into lymphatic vessels. Pressure was generated by 25 and 250 gram-weights for 10 seconds each on the bottom surface of the tumor-bearing footpad to increase the internal pressure of the tumor. A cylindrical weight with a 10 mm diameter (78.5 mm^2) was used for the stimulation. After stimulation, cancer cell trafficking was imaged at video rate with the OV100 system at 100× magnification for 1 minute. The number of cell fragments, single cells and emboli were counted by reviewing the saved video file. The major axis length of the largest embolus in each experiment was also measured. A total of 10 mice were used in this study. Statistical analysis was done using the two-tailed paired Student's *t*-test (9).

2.8 Cellular and subcellular imaging in live mice. The Olympus OV100 Small-Animal Imaging System (Olympus Corp., Tokyo, Japan), containing an MT-20 light source (Olympus Biosystems, Planegg, Germany) and DP70 CCD camera (Olympus), was used for subcellular imaging in live mice. The optics of the OV100 fluorescence imaging system have been specially developed for macroimaging as well as microimaging with high light-gathering capacity. The instrument incorporates a unique combination of high numerical aperture and long working distance. Four individually optimized objective lenses, parcentered and parfocal, provide a 10^5-fold magnification range for seamless imaging of the entire body down to the subcellular level without disturbing the animal. The OV100 has the lenses mounted on an automated turret with a high magnification range of 1.6× to 16× and a field of view ranging from 6.9 to 0.69 mm. The optics and antireflective coatings ensure optimal imaging of multiplexed fluorescent reporters in small animals. High-resolution images were captured directly on a PC (Fujitsu Siemens, Munich, Germany). Images were processed for contrast and brightness and analyzed with the use of Paint Shop Pro 8 and Cell^R (Olympus Biosystems) (9).

2.9 Mice. Athymic *nu/nu* nude mice were kept in a barrier facility under HEPA filtration. Mice were fed with autoclaved laboratory rodent chow (Tecklad LM-485, Western Research Products, Orange, CA). All animal studies were conducted in accordance with the principals and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals under assurance no. A3873-1 (9).

3. RESULTS AND DISCUSSION

3.1 Dynamic subcellular imaging of intravascular trafficking of dual-color cancer cells in vessels in live mice. Interaction of cancer cells with blood vessels depends on the ability of the cytoplasm and nucleus to deform, interaction of the cancer cells with endothelial cells, and the diameter of the vessel. Real-time intravascular trafficking of HT1080-GFP-RFP cells was observed in the epigastric vein in a skin flap of live mice using the Olympus OV100 whole-mouse imaging system. The cytoplasm and nuclei were slightly elongated in normal size capillaries. In a larger vessel, the cells and nuclei remained spherical. In the larger vessels, some cells attached to the vessel walls. Some of the cells subsequently detached and began crawling on the vessel surface. When the cancer cells entered more narrow capillaries, both the whole cell and nucleus were observed to greatly deform by stretching (16). The cytoplasm seems more flexible than the nucleus. When the cancer cells move into capillaries where the diameter is smaller than the deformation limit of the cell, the cancer cell does not advance any further (4).

The cancer cell velocity depends in part on the size of the vessel. When the cancer cells are migrating in a large capillary, the velocity of the cells averaged 24.2 μm/s (16). When the cells went through a narrow capillary, the velocity slowed to an average of 6.1 μm/s (4, 16).

Intravascular aggregation of cancer cells is thought to be an important step in metastasis (17). One aggregate was visualized to collide with another aggregate that was already attached to the vessel wall. Aggregates sometimes attached to each other. Some aggregates become increasingly larger by repeated collisions. The cellular adhesion in the aggregates is not strong, and some cells escape in the bloodstream. The double labeling of the nucleus and cytoplasm allowed the distinction of the individual cells and nuclei in the emboli (4).

Cancer cell adhesion to the endothelium is also important in metastasis (18, 19). Not all cells, however, can attach to the other cancer cells or endothelial cells when they move. Cancer cell contact with other cancer cells or vessel walls was observed to be frequent. Extravasation was very rare among the HT-1080-GFP-RFP population (see below) (4).

3.2 Imaging the dynamics of cancer cell extravasation. MMT-GFP-RFP mouse mammary tumor cells, in contrast to HT-1080 cells, frequently extravasated. MMT-GFP-RFP cells extravasated by first extending cytoplasmic processes. After cytoplasmic processes were extended, the nuclei followed along the extension undergoing varying degrees of deformation to fit within the extended cytoplasmic protrusion as it left the blood vessel. The whole cell eventually extravasated. Extended cytoplasmic processes were frequently observed during extravasation of MMT-GFP-RFP cells. MMT-GFP-RFP cells that initially extravasated remained in proximity of vessels and seemed to surround the vessel by 24 hours after injection (4).

3.3 Imaging the fate of post-extravasation cancer cells. Previous studies with the LLC have indicated that these cancer cells adhered to the basement membrane of the lung tissue 24 hours after injection in the tail vein of mice (20). Another study suggested that the time of extravasation of the LLC cells varied in different organs (21). For example, the time of extravasation was 6 hours in the liver and adrenal gland, 16 hours in the lung, and 48 hours in the brain (16). The time of extravasation varied, depending on the structural complexity of the microcirculation in each organ.

In the skin vessels in the present experiments, the extravasated LLC-GFP-RFP cells seemed to stay very closely associated with the vessel. Extravasated LLC-GFP-RFP cells initially migrated along the vessels, a mechanism termed extravascular migratory metastasis. Lugassy et al. (22) suggested that some melanoma cells were able to migrate along the outside of vessels in a pericyte-like location, forming angiotumoral complexes. We also observed cancer cell division outside of the vessels (4).

By 120 hours after injection, LLC-GFP-RFP cells were observed to surround a large vessel. Most of the cells were highly elongated, and the major axes of the cells stretched to ~100 μm. The cells and nuclei elongated to occupy as much area as possible on the vessel surface. Rounded premitotic cancer cells on the vessel were observed. The cancer cells, including their nuclei, elongated and extended their cytoplasmic processes to surround the vessel. The elongated cells seem to round up before cell division, similar to attached cells in culture (4).

In contrast to the LLC-GFP-RFP and MMT-GFP-RFP carcinoma cells, HT-1080 cells extravasated at a low frequency. HT-1080-GFP-RFP cells did not extend cytoplasmic processes into the host tissue. Occasionally, a cell was observed to divide in a vessel (data not shown), as also observed by Al-Mehdi et al. (23) in static imaging studies. In contrast to the LLC carcinoma and MMT cells, HT-1080-GFP-RFP cells were also observed dying in vessels. Many cells were observed to be dying within vessels by 8 hours after injection. The cells remained round, and cytoplasmic fragmentation was observed in the vessels (data not shown). Even at 24 hours after injection, the majority of the cells remained in vessels without extravasation (data not shown). However, vessels with cancer cells seemed to expand perhaps due to an increase of intravascular pressure (4).

3.4 Dynamic subcellular imaging of trafficking of cancer cells in lymphatic vessels in live mice (experimental model). We observed cell trafficking in lymphatics after injection of the tumor cells in the inguinal lymph node. With FITC-dextran labeling of the lymphatics, we could see both lymphatic structure and the cancer cells trafficking**.** Cancer cells were imaged flowing in the center of the duct. At the junction of afferent lymph duct and subcapsular sinus of the lymph node, the cancer cells disseminated by avoiding attachment to the lymphatic vessel wall. However, 7 days after injection, a tumor mass was occasionally observed at the junction. The junction seemed to be a focal point in lymph node metastasis (9).

3.5 Dynamic subcellular imaging of trafficking of cancer cells in lymphatic in live mice (spontaneous model). Real-time trafficking of HT-1080 dual-color cell was observed in the popliteal lymphatic. Most of the cells were fragmented and seemed single color in the lymphatic, suggesting that most of the cancer cells already had fragmented before entering the peritumoral lymphatic and lymph flow. There were few living cells in the lymph duct (9).

3.6 Shedding of trafficking cancer cells in lymphatic as function of pressure. Cell fragments, single cells and emboli were easily distinguished with the dual-colored HT- 1080 cell line and the OV100 imaging system. With 25 g stimulation of the footpad tumor, the number of single cells shed increased compared to no stimulation. With 250 g stimulation, the total cell number shed increased further and large emboli were seen. The number of cell fragments, cells, and emboli significantly increased with heavier stimulation. The maximum major axis of the trafficking emboli also increased with increased pressure. These data suggest that pressure in the tumor is important for not only the number of cells shed but also the size of emboli shedding into lymphatics around the tumor. The larger emboli could have more possibility to survive and form a metastasis in the lymph node (9).

The imaging technology described here can image in real time cancer-cell trafficking in blood vessels and lymphatics, the critical routes of cancer metastasis.

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