Real-time In vivo Dual-color Imaging of Intracapillary Cancer Cell and Nucleus Deformation and Migration

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Abstract
The mechanism of cancer cell deformation and migration in narrow vessels is incompletely understood. In order to visualize the cytoplasmic and nuclear dynamics of cells migrating in capillaries, red fluorescent protein was expressed in the cytoplasm, and green fluorescent protein, linked to histone H2B, was expressed in the nucleus of cancer cells. Immediately after the cells were injected in the heart of nude mice, a skin flap on the abdomen was made. With a color CCD camera, we could observe highly elongated cancer cells and nuclei in capillaries in the skin flap in living mice. The migration velocities of the cancer cells in the capillaries were measured by capturing images of the dual-color fluorescent cells over time. The cells and nuclei in the capillaries elongated to fit the width of these vessels. The average length of the major axis of the cancer cells in the capillaries increased to approximately four times their normal length. The nuclei increased their length 1.6 times in the capillaries. Cancer cells in capillaries over 8 μm in diameter could migrate up to 48.3 μm/hour. The data suggests that the minimum diameter of capillaries where cancer cells are able to migrate is approximately 8 μm. The use of the dual-color cancer cells differentially labeled in the cytoplasm and nucleus and associated fluorescent imaging provide a powerful tool to understand the mechanism of cancer cell migration and deformation in small vessels. (Cancer Res 2005; 65(10): 4246-52)

Introduction
Animal models of cancer that use the stable expression of green fluorescent protein (GFP) have made it possible to directly observe cell behavior in primary tumors in live animals (1, 2). The interaction of cells with the extracellular matrix and entry of tumor cells into the circulation in the primary tumor are important factors in metastasis (1).

Before the introduction of GFP and its derivatives, intravital-imaging studies were limited to the study of tumor cells that were transiently labeled with vital dyes. Stable fluorescent labeling via GFP expression vectors now allows direct imaging at the single-cell level in vivo (1–4).

Initial studies of tumor biology that used stable GFP expression focused on static images and examination of metastases (1, 2). The first use of stable GFP expression to characterize cancer cells in vivo was by Chishima et al. (5). The first use of GFP to observe motility and shape changes of carcinoma cells in live intact tumors in vivo was described by Farina et al. (6).

Cancer cells that escape from the primary site into the blood circulation eventually flow to the capillaries of the organs of the body (7). In vivo video microscopy has shown that both lung and liver capillaries are very efficient at arresting the flow of cancer cells (7). Most circulating cancer cells arrest by size restriction. Capillaries are small, typically 3 to 8 μm in diameter. Capillaries allow the passage of RBC which average 7 μm in diameter and are highly deformable (7). However, many cancer cells are large being 20 μm or more in diameter (7). Flow or arrest in capillaries is determined by physical factors, such as the relative sizes of the cells and the capillaries, the blood pressure in the organ and the deformability of the cell (7). Cancer cells can, under certain conditions, undergo adhesive arrest in the capillary vessels that are larger than the cell diameter.

Chishima et al. (5) and Huang et al. (8) showed that GFP-transduced cancer cells allowed the imaging of tumor cells in blood vessels. To examine cell behavior during intravasation, Wyckoff et al. (9) have used GFP imaging to view these cells in time-lapse images within a single optical section using a confocal microscope. In vivo imaging of the primary tumors indicated that both metastatic and nonmetastatic cells are motile and show protrusive activity. Metastatic cells show greater orientation toward blood vessels. Nonmetastatic cells fragment when interacting with vessels.

Naumov et al. (3), using GFP imaging, visualized fine cellular details such as pseudopodial projections, even after extended periods of in vivo growth.

Mook et al. (10) visualized initial arrest of GFP colon cancer cells in sinusoids of the liver due to size restriction after portal vein injection. Tumor cells divided exclusively intravascularly during the first 4 days.

Al-Mehdi et al. (11) observed the steps in early hematogenous metastasis of tumor cells expressing GFP in subpleural microvessels in intact, perfused mouse and rat lungs. Metastatic tumor cells attached to the endothelia of pulmonary precapillary arterioles and capillaries. Extravasation of tumor cells was rare. Early tumor colonies were observed growing entirely within the blood vessels.

Yamamoto et al. (12) reported the genetic engineering of dual-color fluorescent cells with one color in the nucleus and the other in the cytoplasm. The dual-color cells enable real-time nuclear-cytoplasmic dynamics to be visualized in living cells in vivo as well as in vitro. To obtain the dual-color cells, red fluorescent protein (RFP) was expressed in the cytoplasm of HT-1080 human fibrosarcoma cells, and GFP linked to histone H2B was expressed in the nucleus. Nuclear GFP expression enabled visualization of...
nuclear dynamics. Simultaneous cytoplasmic RFP expression enabled visualization of nuclear-cytoplasmic ratios as well as simultaneous cell and nuclear shape changes. Thus, total cellular dynamics can be visualized in the living dual-color cells in real time. Common carotid artery injection of dual-color cells and a reversible skin flap enabled the external visualization of the dual-color cells in microvessels in the mouse brain where extreme elongation of the cell body as well as the nucleus occurred.

In this report, we describe real-time imaging of the deformation of cancer cells and their nuclei in vivo. In addition to the deformability, we imaged migration of HT-1080-dual-color cells in microvessels and capillaries in real time. The capability to make such measurements in vivo should enable better understanding of the mechanism of metastasis.

Materials and Methods

Production of red fluorescent protein retroviral vector. For RFP retrovirus production (13, 14), 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 Al 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 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 post-transduction. 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 clone was termed PT67-DSRed2.

Production of histone H2B-green fluorescent protein vector. The histone H2B gene has no stop codon (15), thereby enabling the linkage of the H2B gene to the 5′-coding region of the GFP gene (Clontech Laboratories). The histone H2B-GFP fusion gene was then inserted at the HindIII/CoiI site of the plHXC (Clontech Laboratories) that has the hygromycin resistance gene. To establish a packaging cell clone producing high amounts of histone H2B-GFP retroviral vector, the plHXC histone H2B-GFP plasmid was transfected into PT67 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.

Red fluorescent protein and histone H2B-green fluorescent protein gene transduction of fibrosarcoma cells. For RFP and H2B-GFP gene transduction, 70% confluent human HT-1080 fibrosarcoma cells were used (12). To establish dual-color cells, clones of HT-1080 expressing RFP in the cytoplasm (HT-1080-RFP) were initially established. In brief, HT-1080 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 posttransduction 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. HT-1080-RFP cells were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ) using trypsin/EDTA and amplified by conventional culture methods.

For establishing dual-color cells, HT-1080-RFP 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 (Fig. 1).

Real-time visualization of deformation of HT-1080 dual-color cells in vessels in live mice. To visualize cell dynamics in vessels in live mice, cells were injected into the heart. Five 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. A total of 200 μL of medium containing 5 × 10^6 HT-1080-dual-color cells were injected into the heart. To observe the shapes of the HT-1080-dual-color cells within the microvessels before arrest, the epigastrica cranialis vein of the mouse was wired with a 6-0 suture (Ethicon Inc., Somerville, NJ) before cell injection. Immediately after injection, an arc-shaped incision was made in the abdominal skin, and then 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. The inside surface of the skin flap was directly observed under fluorescence microscopy. After making the skin flap, HT-1080-dual-color cells were imaged immediately and 2 hours later to determine the migration velocities of the cells. During the interval between imaging, PBS (Irvine Scientific) was occasionally sprayed on the inside of the skin flap to keep the surface wet. The skin flap could be completely reversed.

Morphologic analysis of cancer cell deformation. For morphologic analysis, we examined 30 cells in microvessels and 60 cells in capillaries in five mice. During the period of the measurement, the animal was kept under anesthesia. The animal was kept warm and the skin flap was kept hydrated with saline solution. Measurements were taken at the initial time and 2 hours later. Images were taken at the initial time. The image included the cell in its vessel as well as the surrounding vessels which were used as a map to relocate the cell in its vessel 2 hours later when the next images were captured. Usually, 10 to 20 cells were followed in a given experiment. The lengths of the major and minor axes of the whole cells and the nuclei were measured using Image ProPlus 3.1 software, where (A) was the length of the major axis and (B) was the length of the minor axis of the whole cell. For the nuclei, (a) is the length of the major axis and (b) is the length of the minor axis.

Motility analysis in vivo. For motility analysis, the epigastrica cranialis vein was not wired. All other procedures were the same as for morphologic analyses. Motility analysis was done using Image ProPlus 3.1 software. Images that were taken immediately after injection and 2 hours after taking the first images to determine migration velocities of 60 cells.

Fluorescent optical imaging and data analysis. Images were captured directly with a Hamamatsu C5810 3CCD camera (Hamamatsu Photonics, Bridgewater, NJ). For microimaging, a Leica fluorescent stereo microscope (model MZ16) was coupled with the Hamamatsu camera. High-resolution images (1024 × 724 pixels) were captured directly on an IBM PC. Images
were processed for contrast and brightness and analyzed with the use of Image ProPlus 3.1 software.

All animal studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals under assurance number A3873-1. Animals were kept in a barrier facility under HEPA filtration. Mice were fed with autoclaved laboratory rodent diet (Teklad LM-485, Western Research Products, Orange, CA).

Results

Real-time observations were made of nuclear-cytoplasmic deformability in capillaries of HT-1080-dual-color cells. The cells were injected into the heart of mice whose epigastrica cranialis vein was wired to close it off (Fig. 1). The skin flap was made without injuring the epigastrica cranialis artery and vein, and then spread and fixed on a stand. Because of this stable fixation, thousands of cells could be easily visualized in capillaries and in microvessels in the skin in the live mouse under fluorescence microscopy (Fig. 2). The wired epigastrica cranialis vein was clogged with round cells. Some cells were trapped due to size restriction in capillaries, where the cells and nuclei were highly deformed.

We classified the shapes of the cells into four categories. One included the cells in microvessels, where the cells were round and the nuclei were oval (Fig. 3A). Another category included the cells that were elongated in capillaries, where the nuclei were also elongated (Fig. 3B). Another category was that of cells that were elongated and bifurcated at the corner of the capillaries (Fig. 3C). The other category was that of cells that were so elongated that the cytoplasm disconnected. The nuclei, however, remained intact although they were very elongated (Fig. 3D).

Quantitation of cancer cell and nuclear deformation. The lengths of the major and minor axes of the whole cells and the nuclei were measured (Fig. 4; Table 1). The average lengths of the axes of the whole cells in capillaries were 3.97 times as long as in microvessels. As for the nuclei, the average lengths were 1.64 times as long. The lengths of the minor axes of the whole cells in capillaries were equal to those of the nuclei.

Real-time observation of nuclear-cytoplasmic clasmatosis. Clasmatosis or cytoplasmic fragmentation was observed in the cells immediately after injection (13). The cytoplasm was removed from many cells leaving naked nuclei (Fig. 5C and D). Nuclei were also fragmented or stretched dramatically (Fig. 5A and B).

Motility analysis in capillaries. Two hours after taking the first images, the same cells were imaged again to determine the migration velocities of the cells. The bifurcated corners of the capillaries were used as markers to calculate the distance migrated. The distances from the marker to the centers of the nuclei were measured and subtracted (Fig. 6). Approximately 25% of the cells observed bifurcated at the corner of the capillaries and could not migrate and were therefore excluded from our calculations. Of the 45 cells that were not at the corner of the capillaries, 20 cells migrated and 25 cells did not migrate. The average migration velocity of the 20 cells that migrated was 13.2 μm/hour (1.1-48.3). A 1 μm difference in distance could be distinguished from the captured images of the dual-color fluorescent cells.

The diameters of the capillaries were noted when calculating migration velocity. The minimum diameter of the capillary that allowed cell migration was approximately 8 μm. Sixteen out of 20 cells in capillaries over 8 μm in diameter migrated up to 48.3 μm/hour. In contrast, 21 out of 25 cells in capillaries less than 8 μm in diameter could not migrate at all.

Discussion

Using the skin flap for observation of cells in capillaries has important advantages. The skin can be spread stably on a stand, such that motion from the mouse’s heartbeat or breathing has no
influence on imaging. Disturbance of the blood supply for the skin does not occur during the skin flap procedure, because the epigastrica cranialis artery is not injured during the procedure. In addition to these advantages, the skin flap could be completely reversed such that the mice need not be sacrificed. In our study, the skin flap was reversed after 24 hours.

To observe the shape of the HT-1080-dual-color cells within blood vessels, we wired the epigastrica cranialis vein closed before

Figure 3. Classification of the deformation of HT-1080-dual-color cells in the vessels in the skin. A, nondeformed cells are within a microvessel. The cells in the microvessel are round and the nuclei oval. The cells occupy the full diameter of the vessel. B, the cells and nuclei are elongated to fit a capillary. C, the cells are arrested at the capillary bifurcation. Because of the difference of the deformability between cytoplasm and nucleus, only the cytoplasm is bifurcated. The nucleus is also deformed but remains intact. D, cytoplasmic fragmentation in very thin capillary; bar, 50 µm.

Figure 4. Shapes of HT-1080-dual-color cells in different size vessels. (Top) cells in microvessels; (bottom) cells in capillaries.
injection of the cells into the heart. We observed many round cancer cells in the microvessels in the skin. As for the cells in capillaries, almost all the cells and their nuclei deformed in order to conform to the diameter of the capillaries. The cancer cells and their nuclei deformed into three-pronged forks when the cells were arrested at capillary bifurcations.

There is an apparent limitation to cell and nuclear deformation when the cells are arrested in capillaries. We found many cancer cells whose cytoplasms seemed to fragment and separate from nuclei, a process called clasmatosis. We also found that nuclei could fragment. When the cells arrested in very narrow capillaries, the cytoplasm of many cells as well as their nuclei were destroyed.

Morris et al. (13) examined clasmatosis of melanoma and mammary carcinoma cells in vivō. Morris suggested that the cells undergoing clasmatosis were damaged and would ultimately die. Clasmatosis may account in part for the inefficiency of metastasis.

The nucleus seems to be less deformable than the cytoplasm. This may be due to the difference between the cytoskeleton in the cytoplasm and the nucleus (16, 17). In our study, the lengths of the major and minor axes of the nuclei and the whole cells deformed to fit the width of the capillaries.

Bioluminescence imaging, magnetic resonance imaging, positron emission tomography, and single photon emission

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<th>Table 1. Imaging cellular and nuclear morphometry in microvessels and capillaries in living mice</th>
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NOTE: HT1080 human fibrosarcoma cells were labeled with GFP in nuclei and RFP in cytoplasm. The cells were injected in the mice as described in Materials and Methods. The dual-color cells were observed in both capillaries and microvessels in a skin flap in live mice under fluorescent microscopy (as described in Materials and Methods). Morphometric measurements described in the table were made from digitally captured images of the dual-color cells.

Figure 5. Clasmatosis of HT-1080-dual-color cells in vivō. A, cells with nuclei and no cytoplasm remain in microvessels except for one intact cell. One of the nuclei is deformed like a spoon and is fragmented in two (arrow). B, a nucleus with the cytoplasm removed is deformed like a comet. C, fragmented cytoplasm remains around a nucleus. The remaining cytoplasm is stretched out dramatically. The nucleus is also deformed in a capillary. D, the cell is bifurcated and the cytoplasm is fragmented into two (arrow). Another cell has only a part of its cytoplasm but the nucleus is intact (arrowhead); bar, 50 µm.
computed tomography have allowed detailed and dynamic views of tissues. However, these methods lack the spatial and/or temporal resolution to visualize single-cell dynamics in situ. Such information can be generated with fluorescence imaging. Newer techniques such as multiphoton microscopy can be combined with second harmonic generation imaging to visualize cellular behavior in the interstitium of solid organs (18). The dual-colored cells described in this report should be a powerful tool when used with multiphoton microscopy in future experiments.

Acquisition of protease-independent amoeboid dissemination was visualized for HT-1080 cells injected into the mouse dermis monitored by intravital multiphoton time-lapse microscopy (19). The transition from proteolytic mesenchymal toward nonproteolytic ameboid movement shows a plasticity mechanism in cell migration (19). Such transitions in movement can now be imaged with the dual-colored cells described in the present report in order to visualize the role of the nucleus as well as the cytoplasm.

The development of transgenic mice expressing GFP in specific cells will also be important to visualize single-cell behavior in vivo. For example, T cells with a CD2 promoter driving GFP expression allowed visualization of T cell rolling and adhesion in postcapillary venules during inflammation in vivo (20). Transgenic GFP-

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**Figure 6.** Migration velocity of HT-1080-dual-color cell in capillaries. **A,** cell immediately after injection. **B,** the same cell as in **A** imaged 2 hours later. The corner of the capillary was regarded at the starting point of migration. **C–F,** migration of cells in capillaries from 2 to 14 hours; bar, 50 μm.
expressing mice can be developed to image many aspects of cell biology, including cancer.

The present study shows that HT-1080-dual-color cells are useful for visualization of cellular and nuclear dynamics in vivo. After injection of the cells into the heart, we could observe cancer cell and nuclear deformation as well as clasmatosis in real time. Furthermore, we could calculate the migration velocities of tumor cells in capillaries.

The dual-color cells, with GFP in the nucleus and RFP in the cytoplasm, along with new approaches to in vivo imaging in appropriate hosts provide a powerful tool to understand the mechanism of cancer cell migration and deformation in very small vessels in vivo. With this new information, we can better understand the mechanism of metastasis.

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