Dual-Color Imaging of Nuclear-Cytoplasmic Dynamics, Viability, and Proliferation of Cancer Cells in the Portal Vein Area

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Abstract
We used dual-color in vivo cellular imaging to visualize trafficking, nuclear-cytoplasmic dynamics, and the viability of cancer cells after their injection into the portal vein of mice. For these studies, we used dual-color fluorescent cancer cells that express green fluorescent protein (GFP) linked to histone H2B in the nucleus and retroviral red fluorescent protein (RFP) in the cytoplasm. Human HCT-116-GFP-RFP colon cancer and mouse mammary tumor (MMT) cells were HCT-116-GFP-RFP in the portal vein of nude mice. The cells were observed intravitally in the liver at the single-cell level using the Olympus OVIS100 whole-mouse imaging system. Most HCT-116-GFP-RFP cells remained in sinusoids near peripheral portal veins. Only a small fraction of the cancer cells invaded the lobular area. Extensive clasmocytosis (destruction of the cytoplasm) of the HCT-116-GFP-RFP cells occurred within 6 hours. The number of apoptotic cells rapidly increased within the portal vein within 12 hours of injection. Apoptosis was readily visualized in the dual-color cells by their altered nuclear morphology. The data suggest rapid death of HCT-116-GFP-RFP cells in the portal vein. In contrast, dual-color MMT-GFP-RFP cells injected into the portal vein mostly survived in the liver of nude mice 24 hours after injection. Many surviving MMT-GFP-RFP cells showed invasive figures with cytoplasmic protrusions. The cells grew aggressively and formed colonies in the liver. However, when the host mice were pretreated with cyclophosphamide, the HCT-116-GFP-RFP cells also survived and formed colonies in the liver after portal vein injection. These results suggest that a cyclophosphamide-sensitive host cellular system attacked the HCT-116-GFP-RFP cells but could not effectively kill the MMT-GFP-RFP cells. (Cancer Res 2006; 66(1): 303-6)

Introduction
The portal vein is a critical route for cancer cell metastasis to the liver. However, the early fate of cancer cells in the portal vein circulation is poorly understood because it has been difficult to visualize the behavior of single cancer cells and micrometastasis. Previously, cancer cells were transfected with the Escherichia coli β-galactosidase (lacZ) gene, which enables detection of micrometastases in tissue sections. However, lacZ does not allow direct visualization of cancer cells in live animals (1–5). We developed an approach to visualizing cancer cells in vivo through the use of green fluorescent protein (GFP; refs. 1–4).

Two processes have been proposed to explain the high incidence of colon cancer metastasis in liver. One explanation is based on the seed-and-soil theory of Paget, in which preferential growth of colon cancer cells to the liver forms the basis. Other studies have shown that cancer cells remain in the liver because they are arrested physically in the too-narrow sinusoids of the liver.

Mook et al. (6) used intravitreal videomicroscopy to visualize early events after injection of GFP-expressing colon cancer cells in the portal vein. Initial arrest of the colon cancer cells in sinusoids of the liver was due to size restriction. Adhesion of cancer cells to endothelial cells was never found. Instead, endothelial cells retracted rapidly and interactions were observed only between cancer cells and hepatocytes. Tumor cells divided exclusively intravascularly during the first 4 days.

Wang et al. (7) also visualized the trafficking of GFP-expressing metastatic cancer cells targeting the liver via the portal vein. Within 72 hours after transplantation of tumor cells on the ascending colon in nude mice, metastasis was visualized ex vivo on a single-cell basis around the portal vein by GFP imaging. At this early time point, a few cells were visualized trafficking to the liver via the portal vein. By post-implantation day 5, the caudate lobe of the liver was involved with trafficking metastatic cells, which subsequently formed colonies.

Real-time intravital videomicroscopy analysis of liver metastases after intraportal injection of GFP-expressing cells via a mesenteric vein revealed that both metastatic LM-EGFP and nonmetastatic E2-EGFP rat tongue tumor cells arrested similarly in sinusoidal vessels near terminal portal venules (8). The nonmetastatic E2-EGFP cells were completely eliminated from the liver sinusoids within 3 days, with no solitary dormant cells. However, a substantial number of LM-EGFP cells remained in the liver, possibly due to stable attachment to the sinusoidal wall. The LM-EGFP cells began to grow 3 to 4 days after inoculation (8).

In the current study, we visualized the early trafficking of dual-colored cancer cells, labeled with GFP in the nucleus and red fluorescent protein (RFP) in the cytoplasm (9), injected into the portal vein of nude mice. We report here the fate of different cancer cell types in the portal circulation. We also report the effect of cyclophosphamide pretreatment of the host mouse on the fate of the cancer cells in the portal circulation.

Materials and Methods
Production of RFP retroviral vector. For RFP retrovirus production, the HindIII/Not I fragment from pDsRed2 (Clontech Laboratories, Inc., Palo Alto, CA), containing the full-length RFP cDNA, was inserted into the HindIII/Not I site of pLNCX2 (Clontech Laboratories) containing the neomycin resistance gene (4). 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 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 of G418 (Life Technologies) for 7 days. The isolated clone was termed PT67-DSRed2.

Production of histone H2B-GFP vector. The histone H2B gene has no stop codon (10), thereby enabling the ligation of the H2B gene to the 5'-coding region of the GFP gene (Clontech Laboratories; ref. 4). 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 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.

RFP and histone H2B-GFP gene transduction of cancer cells. For RFP and H2B-GFP gene transduction, 70% confluent human cancer cells were used (4). To establish dual-color cells, 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 posttransduction and subcultured at a ratio of

Figure 1. A-F, fate of HCT-116-GFP-RFP human colon cancer cells after portal vein injection. A laparotomy was done on nude mice under ketamine anesthesia in order to inject HCT-116 GFP-RFP human colon cancer cells (0.25 x 10⁶) into the portal vein. The nuclei of the cancer cells express GFP linked to histone H2B and the cytoplasm expresses retroviral RFP. The liver was immobilized on a slide glass in order to limit movement. The liver surface was then imaged for dual-color fluorescence in the Olympus OX100 whole-mouse imaging system. The abdomen was opened and then closed for each imaging time point. Time course imaging after injection of HCT-116 RFP-GFP cells is described in (A-F). Destruction of RFP-expressing cytoplasm of the cancer cells began rapidly. The destruction of RFP-cytoplasm progressed with time. The striped nuclei seemed to remain intact within 6 hours after injection. The number of apoptotic cells with fragmented nuclei in the liver increased within 12 hours. Fragmentation of GFP nuclei is clearly seen. In A, groups of cells are visible. Individual cells can be visualized in B-F.

Figure 2. Fate of MMT GFP-RFP mouse mammary tumor cells after portal vein injection. A, MMT GFP-RFP mouse mammary tumor cells (0.25 x 10⁶) were injected into the portal vein and imaged as described in Materials and Methods. Most MMT-GFP-RFP cells maintained normal cell structure and survived >24 hours after injection. Some MMT-GFP-RFP cells produced cytoplasmic protrusions which seemed invasive. B and C, the time course behavior of MMT GFP-RFP cells was imaged after portal vein injection using the methods described above. Many MMT GFP-RFP cells, which impacted into sinusoids near the terminal portal vein after injection, survived and grew into colonies.
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 cancer 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.

**Animals.** Male athymic CD-1 nude mice, between 5 and 6 weeks of age, were used in this study. The animals were bred and maintained in a HEPA-filtered environment with cages, food, and bedding sterilized by autoclaving. The breeding pairs were obtained from Charles River Laboratories (Wilmington, MA). The animal diets were obtained from Harlan Teklad (Madison, WI). Ampicillin 5.0% (w/v; Sigma, St. Louis, MO) was added to the autoclaved drinking water. 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 no. A3873-1.

**Injection of cells: portal vein.** The nude mice were anesthetized with a ketamine mixture (10 μL ketamine HCl, 7.6 μL xylazine, 2.4 μL acepromazine maleate, 10 μL H2O) injected into the peritoneal cavity. Human HCT-116-GFP-RFP colon cancer cells and mouse mammary tumor (MMT)-GFP-RFP cells (0.125-1.0 × 10⁶ cells/50 μL) were injected in the portal vein of nude mice during open laparotomy.

**Pretreatment of animals with cyclophosphamide.** In some experiments, the host mice were pretreated by portal vein injection with cyclophosphamide (100 mg/kg) 6 days and 1 day before injection of HCT-116-GFP-RFP cells into the portal vein.

**Visualization of cell trafficking in liver of living mice.** At laparotomy, a glass slide was put on the exteriorized liver of the mice to regulate motion. The mice were observed with an Olympus OV100 whole-mouse imaging system. The images were taken immediately after cell injection, and 0.5, 1, 6, 12, and 24 hours, and daily thereafter.

**Results and Discussion**

**Entry of HCT-116-GFP-RFP cells in the portal vein circulation.** After the HCT-116-GFP-RFP human colon cancer cells were injected into the main portal vein, cells accumulated in the terminal portal veins. Initially, many HCT-116-GFP-RFP cells...
impacted into sinusoids just after injection (Fig. 1A). The diameter size of the sinusoids was ~5 μm. Most HCT-116-GFP-RFP cells remained in the sinusoids near the terminal portal vein.

**Time course of HCT-116-GFP-RFP cell death in the portal vein area.** Thirty minutes after injection of HCT116-GFP-RFP in the portal vein, 70% of the cells were viable (Fig. 1B). However, by 2 hours (Fig. 1C), only 50% of the cells were viable, and by 6 hours, only 10% of the cells were viable (Fig. 1D). Viability was readily observed as the dead cells were stripped of their RFP-expressing cytoplasm leaving only the GFP nucleus (Fig. 1E). The number of apoptotic cells rapidly increased within the portal vein area. fragmentation of GFP nuclei could be clearly visualized (Fig. 1F).

**Fate of MMT-GFP-RFP cells in the portal vein.** MMT-GFP-RFP mouse mammary tumor cells injected into the portal vein had a very different fate in the liver compared with HCT-116-GFP-RFP cells. Although there was rapid cell destruction of some MMT-GFP-RFP cells similar to HCT-116-GFP-RFP cells, many MMT-GFP-RFP cells survived to 24 hours and beyond after injection. Many MMT cells became invasive with cytoplasmic protrusion within 48 hours after injection (Fig. 2). MMT-GFP-RFP cells subsequently formed micrometastases in the liver from days 2 to 5 (Fig. 2).

**Efficacy of cyclophosphamide pretreatment on portal vein viability of HCT-116-GFP-RFP cells.** When the host mice were pretreated with cyclophosphamide, the HCT-116-GFP-RFP cells also survived and formed colonies in the liver after portal vein injection, in striking contrast to their rapid cell death in the untreated animals (Fig. 3). These results suggest that a host cellular system attacked the HCT-116-GFP-RFP cells. Most HCT116-GFP-RFP cells survived 24 hours after injection in the cyclophosphamide-treated mice and subsequently formed metastasis in the liver (Fig. 3) in three of five mice compared with none of the non-cyclophosphamide-treated mice. Some of the HCT116-GFP-RFP micrometastases became vascularized by day 14 (Fig. 3).

Morris et al. (8) previously described clasmatosis of cells in the liver. We could visualize this phenomenon more clearly by using bright dual-color fluorescent cancer cells with the cytoplasm labeled with RFP and the nucleus labeled with GFP. HCT-116-GFP-RFP cells injected into the portal vein were all dead within 12 hours. Rapid cell death was also reported by Morris et al. (8). The liver may contain an innate rapid local defense for cells entering the liver portal veins. Our results suggest that such a host defense is sensitive to cyclophosphamide. However, MMT-GFP-RFP cells had a very different fate compared with HCT-116-GFP-RFP cells. The MMT-GFP-RFP cells survived and formed metastases in the liver of untreated nude mice.

The fate of injected malignant cells in the portal vein seems dependent on the species of origin. Mouse cells go on to form metastatic colonies whereas human colon cancer cells die. The difference is clearly due to the host response because prior treatment of the mice with cyclophosphamide obliterates the inhibitory response to human cells. This finding should be studied further.

GFP techniques have also shown that the CT-26 (mouse colon carcinoma) primary tumor inhibits the development of liver metastasis in BALB/c mice that receive intraportal injections of GFP-expressing tumor cells (11). Intravital microscopy of the livers of these mice showed that the growth of primary tumors promoted dormancy of single tumor cells for up to 7 days. Immunohistologic staining for Ki-67 confirmed that these solitary cells were indeed dormant. By contrast, in the absence of a primary tumor, GFP-expressing tumor cells quickly developed into micrometastases. Thus, primary CT-26 tumor implants seem to inhibit tumor metastasis by the promotion of a state of single-cell dormancy. Thus, both primary tumor and host may have defense mechanisms against tumor cells in the portal vein circulation.

Cyclophosphamide is a widely used cancer chemotherapy drug, including low-dose therapy (12). The results of the present study show that cyclophosphamide might, in certain instances, promote cancer cell growth as shown for the HCT-116 human colorectal cancer cells in the portal vein. Further studies into the sensitivity of host antitumor mechanisms to cyclophosphamide are indicated. In conclusion, some cancer cells may be very sensitive to host mechanisms or the primary tumor when in the portal circulation, whereas other cancer cells seem resistant to this host mechanism. The host defense mechanism in the portal vein area is sensitive to cyclophosphamide. The implication of these results are important for understanding the tumor-host interaction of liver metastasis.

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