In vivo Color-Coded Imaging of the Interaction of Colon Cancer Cells and Splenocytes in the Formation of Liver Metastases

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Abstract

The role of host cells in tumor progression and metastasis is critical. Intrasplenic injection of tumor cells has long been known as an effective method of developing liver metastases in nude mice, whereas portal vein (PV) injection of tumor cells can result in rapid death of the tumor cells. Host cells were thought to play a role in these phenomena. We report here that after splenic injection of tumor cells, splenocytes cotraffic with the tumor cells to the liver and facilitate metastatic colony formation. Human colon cancer cells that express green fluorescent protein (GFP) linked to histone H2B in the nucleus and red fluorescent protein (RFP) in the cytoplasm (HCT-116-GFP-RFP) were injected in either the PV or spleen of nude mice and imaged at the subcellular level *in vivo*. Extensive clasmocytosis (destruction of the cytoplasm) of the cancer cells occurred within 6 hours after PV injection and essentially all the cancer cells died. In contrast, splenic injection of these tumor cells resulted in the aggressive formation of liver and distant metastasis. GFP spleen cells were found in the liver metastases that resulted from intrasplenic injection of the tumor cells in transgenic nude mice ubiquitously expressing GFP. When GFP spleen cells and the RFP cancer cells were coinjected in the PV, liver metastasis resulted that contained GFP spleen cells. These results suggest a novel tumor-host interaction that enables efficient formation of liver metastasis via intrasplenic injection. (Cancer Res 2006; 66(23): 11293-7)

Introduction

The cellular mechanisms by which a cancer cell undergoes metastasis are largely unknown (1). Site-specific metastases are regulated by normal host cells as well as the cancer cells. This process has been poorly understood in the past due to lack of appropriate models and due to the inability to visualize tumor-host interaction on a cellular basis in real time in the live mouse.

To image tumor-host interactions that regulate metastasis, we developed previously a transgenic nude mouse ubiquitously expressing green fluorescent protein (GFP; ref. 2). With this mouse, it is possible to image the role of host cells in metastasis. The GFP nude mouse was obtained by crossing nontransgenic nude mice with the transgenic C57/B6 mouse, in which the β-actin promoter drives GFP expression in essentially all tissues. In the adult mice, the organs all brightly expressed GFP, including the heart, lungs, spleen, pancreas, esophagus, stomach, duodenum, skeleton, and spleen.

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Using the ubiquitously expressing GFP transgenic nude mouse, we have developed a simple yet powerful technique to visualize tumor-induced angiogenesis and other tumor-host interactions with dual-color fluorescence by transplanting red fluorescent protein (RFP)–expressing tumors in the model. This model clearly images interactions of the cancer cells and adjacent stroma, distinguishing unambiguously the host- and tumor-specific components of the malignancy (3).

Using the above *in vivo* dual-color fluorescent protein imaging technology, which was pioneered by our laboratory (2–5), Kaplan et al. (6) observed that GFP bone marrow–derived cells (BMDC), injected into mice prior to injection of cancer cells expressing RFP, resulted in the RFP-tagged tumor cells associating with the GFP-BMDC. The GFP-BMDC clustered at distant sites before the arrival of the metastatic cancer cells. The cancer cells then proceeded to form metastases at these sites. Kaplan et al. (6) showed that it was necessary for the BMDCs to express vascular endothelial growth factor receptor 1 in order for them to form cellular clusters that attracted the metastatic tumor cells.

We recently observed extensive clasmocytosis (destruction of the cytoplasm) and cell death of HCT-116-GFP-RFP human colon cancer cells within 6 hours after injection of the tumor cells in the portal vein (PV). However, when the host mice were pretreated with cyclophosphamide, the HCT-116-GFP-RFP cells survived and formed colonies in the liver after PV injection. These results suggested that a cyclophosphamide-sensitive host cellular system attacked the HCT-116-GFP-RFP cells in the PV area (7).

Splenic injection has long been used as a successful route to obtain liver metastasis of colon cancer (8). The results in this report show that splenocytes remain associated with HCT-116-GFP-RFP, injected in the spleen during liver metastasis formation. Our hypothesis is that the splenocytes facilitate liver metastasis formation in an analogous manner as the bone marrow cells did in the experiments by Kaplan et al. (6) described above.

Materials and Methods

Cell culture. HCT-116 human colon cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in RPMI 1640 supplemented with 10% FCS, 2 mmol/L glutamine (Life Technologies, Inc., Grand Island, NY). The cell line was cultured at 37°C in a 5% incubator.

RFP vector production. The *RFP* (DsRed-2) gene (Clontech Laboratories, Mountain View, CA) was inserted in the retroviral-based mammalian expression vector pLNCX (Clontech) to form the pLNCX DsRed-2 vector (9). Production of retrovirus resulted from transfection of pLNCX DsRed-2 in PT67 packaging cells, which produced retroviral supernatants containing the *DSRed-2* gene. Briefly, PT67 cells were grown as monolayers in DMEM supplemented with 10% FCS (Gemini Biological Products, Calabasas, CA). Exponentially growing cells (in 10-cm dishes) were transfected with 10 μg expression vector using a LipofectAMINE Plus

(Life Technologies) protocol. Transfected cells were replated 48 hours after transfection and 100 μg/mL G418 were added 7 hours after transfection. Two days later, the amount of G418 was increased to 200 μg/mL G418. During the drug selection period, surviving colonies were visualized under fluorescence microscopy and RFP-positive colonies were isolated. Several clones were selected and expanded into cell lines after virus titering on the 3T3 cell line.

***RFP* gene transduction of cancer cells.** For *RFP* gene transduction, 20% confluent HCT-116 human colon cancer cells were incubated with a 1:1 precipitated mixture of retroviral-containing supernatants of PT67 cells and RPMI 1640 or other culture medium (Life Technologies) containing 10% fetal bovine serum (Gemini Biological Products) for 72 hours (9). Fresh medium was replenished at this time. Cancer cells were harvested with trypsin/EDTA and subcultured at a ratio of 1:15 into selective medium, which contained 50 μg/mL G418. To select brightly fluorescent cells, the level of G418 was increased to 800 μg/mL in a stepwise manner. Clones of cancer cells expressing RFP were isolated with cloning cylinders (Bel-Art Products) by trypsin/EDTA and amplified and transferred by conventional culture methods in the absence of selective agent.

Production of histone H2B-GFP vector. The histone *H2B* gene has no stop codon (7, 10), thereby enabling the ligation 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 *Hin*dIII/*Cal*I site of the pLHCX (Clontech Laboratories) that 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-DsRed-2. The transfected cells were cultured in the presence of 200 to 400 μg/mL hygromycin (Life Technologies), increased stepwise 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 (9). Clones expressing RFP in the cytoplasm were initially established as described above. 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, the RFP-expressing cancer cells were incubated with hygromycin 72 hours after transfection. The level of hygromycin was increased stepwise up to 400 μg/mL and selected in an analogous manner as described above for the RFP cancer cells.

Animals. Nude mice, either transgenic expressing GFP under control of the β-actin promoter (2) or nontransgenic between 5 and 6 weeks of age, were used in this study. The animals were bred and maintained in a HEPA-filtered environment at AntiCancer, Inc. (San Diego, CA) with cages, food, and bedding sterilized by autoclaving. 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 number A3873-1.

PV and spleen injection. 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) injected into the peritoneal cavity (7). Human HCT-116-RFP-GFP colon cancer cells (2.0×10^6/50 μL Matrigel) were injected in the PV or spleen of nontransgenic nude mice during open laparotomy for experiments described in Figs. 1 and 2. Human HCT-116-RFP colon cancer cells (2.0×10^6/50 μL Matrigel) were injected in the spleen of transgenic GFP nude mice during open laparotomy for experiments described in Fig. 3.

Fluorescent optical imaging and processing. 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 Corp.) was used for subcellular imaging in live mice (10). The optics of the OV100 fluorescence imaging system have been specially developed for macroimaging as well as microimaging with high light-gathering capacity and incorporate a unique combination of high numerical aperture and long working distance. Five 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 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; ref. 10).

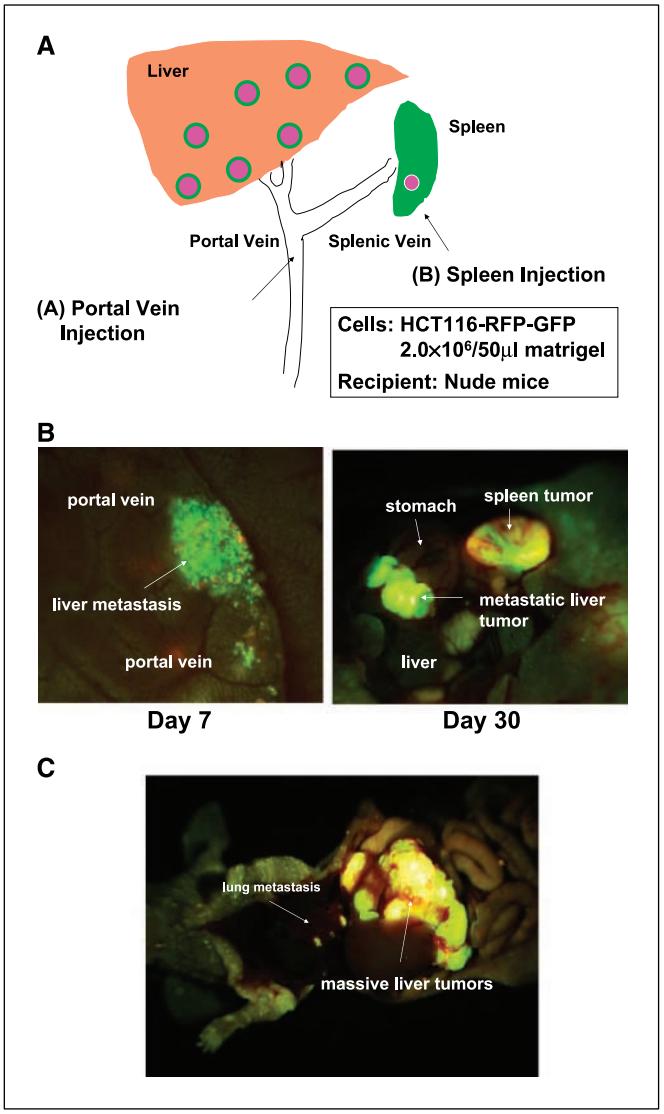

Figure 1. *A,* schematic representation of experimental protocol. Human HCT-116-RFP-GFP colon cancer cells (2.0×10^6/50 μL Matrigel) were injected in the PV or spleen of nude mice during open laparotomy. *B,* in contrast to the PV where the cells rapidly died (7), when the same cells were injected into the spleen, multiple metastatic tumors were imaged in the liver in 8 of 15 mice. Liver micrometastases were imaged by day 7. By day 30, large tumor colonies were imaged in the liver and spleen. *C,* by day 60, large tumors were imaged in the liver.

Visualization of cancer cells in the liver or spleen of living mice. At laparotomy, a glass slide was put on the exteriorized liver of the mice to regulate motion (10). Cancer cells in the liver or spleen of the mice were observed with the Olympus OV100 Small Animal Imaging System.

Immunohistochemical staining. Colocalization of GFP fluorescence and CD11c in the frozen tumor sections of liver metastases were detected

with the anti-rat immunoglobulin horseradish peroxidase detection kit (BD PharMingen, Franklin Lakes, NJ) following the manufacturer's instructions. The primary antibodies used were purified hamster anti-mouse CD11c (integrin α_x chain) monoclonal antibody (1:50; BD PharMingen). Substrate-chromogen 3,3′-diaminobenzidine staining was used for antigen staining.

Results

We used color-coded *in vivo* subcellular imaging (11) to visualize cancer cell trafficking and viability of cancer cells after their injection into the PV or spleen of nude mice (Fig. 1*A*). The cells were imaged intravitally in the liver or spleen at the single-cell level, using the Olympus OV100 Small Animal Imaging System. As shown previously, the HCT-116-GFP-RFP underwent rapid cell death after injection in the PV area (7).

In contrast to the PV injection, when the same tumor cells were injected into the spleen of non-GFP nude mice, multiple metastatic tumors were imaged in the liver in 8 of 15 mice compared with 0 of 15 after PV injection. Liver micrometastases were imaged by day 7 (Fig. 1*B*). By day 60, very large tumors were imaged in the liver (Fig. 1*C*).

By day 40 after splenic injection of the HCT-116-GFP-RFP cells, distant metastases were imaged in the lung and paraaortic lymph nodes (Fig. 2). The metastasis sites included liver, lung, lymph node, bone, adrenal gland, and brain. These distant metastases resulted at relatively late time points and frequently occurred. Apparently, the distant metastases were the result of remetastasis of the metastatic tumors growing in the liver, a process we and others have observed previously (12).

When HCT-116-RFP cells were injected into the spleen of transgenic GFP nude mice, multiple metastatic tumors were imaged in the liver, which contained not only HCT-116-RFP cells but also GFP-host spleen cells. By day 50, whole-liver images revealed the RFP-expressing liver metastasis surrounded by host GFP-splenocytes (Fig. 3*A*). In frozen sections of the liver metastasis, the GFP splenocytes surrounding the HCT-116-RFP colon tumor cells were distinguished by brilliant two-color fluorescence. These GFP-host cells stained positive with monoclonal antibodies to CD11c confirming that these cells were mouse splenocytes (Fig. 3*B*).

When the GFP-splenoctyes were cocultured with HCT-116-RFP cells and then injected in the PV, we observed liver metastases in 5 of 15 mice compared with 0 of 15 mice injected in the PV with the cancer cells only and in 8 of 15 mice after the cancer cells were injected in the spleen (Table 1). These results suggest that splenocytes are not only associated with the HCT-116-RFP cells during the formation of metastasis but also play a positive role in the formation of the metastatic colonies.

Discussion

The presence of GFP spleen cells in the liver metastases that resulted from intrasplenic injection of the tumor cells suggests a novel tumor-host interaction that enables efficient formation of liver metastasis via intrasplenic injection. The results of the present report and that of Kaplan et al. (6) described above show that host cells can have a strong promoting effect on the formation of liver metastasis. Our previous report (7) showed a cyclophosphamide-sensitive host system that resulted in the rapid cell death of HCT-116-RFP-GFP cells injected into the PV area. The spleen cells seem to overcome this host-cell killing system when coinjected with the tumor cells. These results indicate that distant organ colonization, which we identified previously as the governing step of metastasis (13), involves interaction of tumor cells with multiple types of promoting and inhibiting host cells. Future experiments will take advantage of this color-coded tumor-host model to further characterize tumor-host cell interactions and the role of host cells in promoting or inhibiting liver metastases.

The results of the present article differ from those of Kaplan et al. (6). In our report, we show that direct interaction between tumor cells and splenocytes results in the tumor cell survival in the

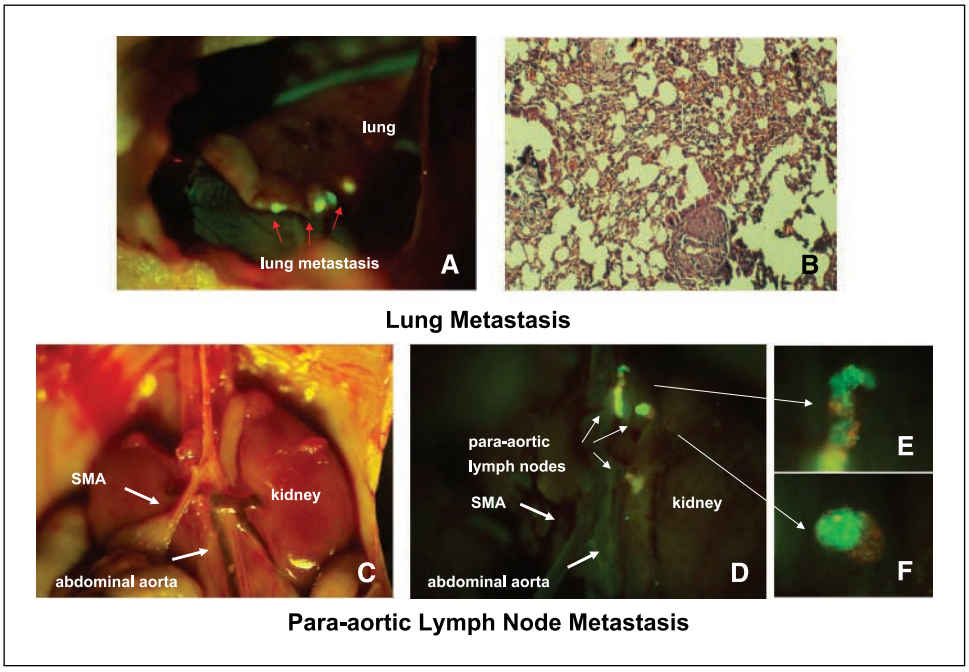

Figure 2. By day 40, after splenic injection of HCT-116-RFP-GFP cells, distant metastases were imaged in the lung (*A*, fluorescent image; *B*, H&E stain) and paraaortic lymph nodes (*C*, bright field; *D*, fluorescent image; *E* and *F*, magnified views of paraaortic lymph nodes). *SMA,* supermesentaric artery.

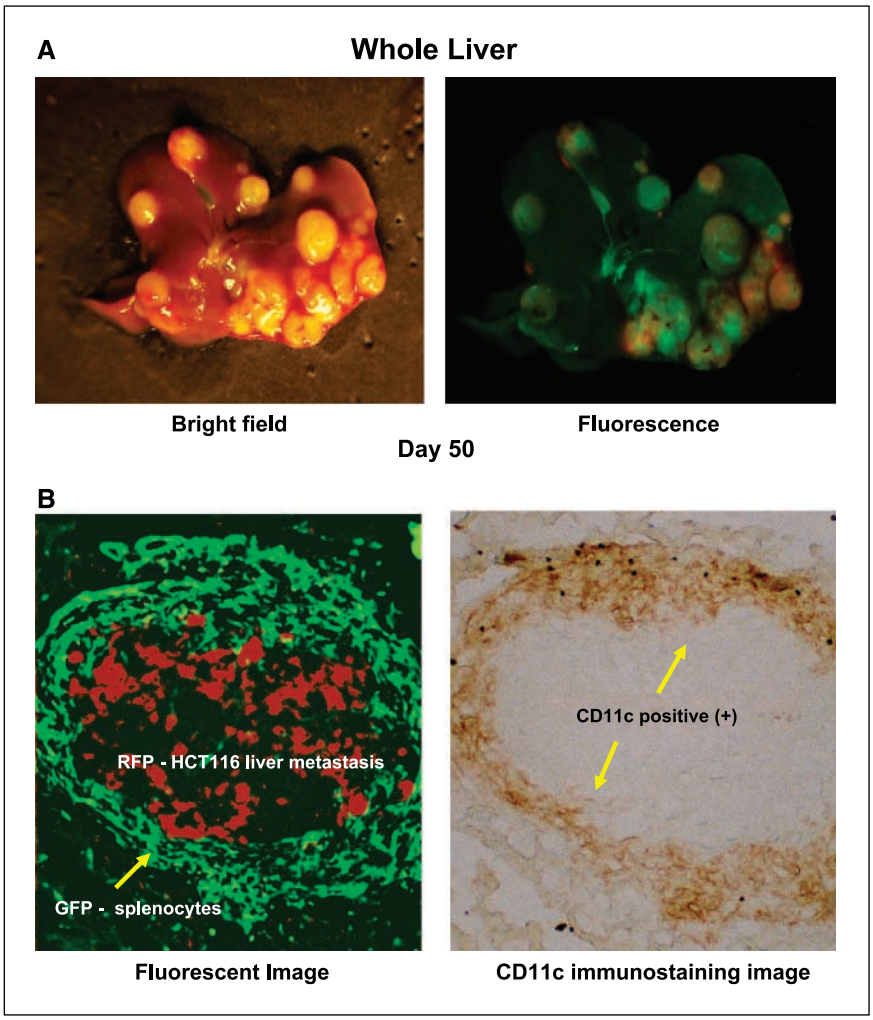

Figure 3. HCT-116-RFP cells were injected into the spleen of transgenic nude mice expressing GFP in all cells. Resulting liver metastases in the liver were found to be surrounded by bright green cells thought to be splenocytes. *A,* by day 50, the GFP cells were very prominently surrounding the tumor cells in the liver. *Left,* bright field image of whole liver; *right,* fluorescent image of same liver. *B,* sister slides analyzed by fluorescence and immunohistochemistry. *Left,* frozen section from a liver metastasis showing by fluorescence microscopy GFP splenocytes surrounding the HCT-116-RFP colon cancer cells; *right,* immunohistochemical analysis shows that the GFP-host cells stained positive (+) with monoclonal antibodies to CD11c confirming that these cells were mouse splenocytes.

PV area and enables the cancer cells to form subsequent liver metastasis and, at later times, distant metastasis, such as in the lung. Kaplan et al. (6) hypothesized that tumor and normal cells interacted at a distance through possible soluble factors. The "seed and soil" hypothesis of Paget (1) assumes direct interaction of tumors and normal cells (i.e., "the seeds growing in the soil"), the analogy of tumor cells growing in a distant organ. This hypothesis was confirmed by us in 1995 when we showed that colon tumors would metastasize to the liver only if the tumor tissue were able to grow when implanted directly on the liver (13). If colon tumor tissues were unable to grow when implanted directly on the liver, the tumors were also unable to metastasize. These results indicated that the governing step of metastasis is the ability of the tumor cells to grow in the distant organ. Our current results show that direct interaction between tumor and normal cells, such as the colon tumor cells and the splenocytes, facilitate the growth in distant organs. In summary, unlike Kaplan, our results indicate the importance of direct interaction of the tumor and normal cells in the process of formation of metastasis. These results suggest novel targets for antimetastatic drugs.

Table 1. Role of spleen cells in promoting liver metastasis

	PV injection	PV injection	Spleen injection
Cells	HCT-116	HCT-116 + splenocytes	HCT-116
Frequency of liver metastasis	0/15	5/15	8/15

NOTE: When the GFP-splenoctyes were cocultured with HCT-116-RFP cells and then injected in the PV, we observed liver metastases in 5 of 15 mice compared with 0 of 15 mice injected in the PV with the cancer cells only and in 8 of 15 mice after the cancer cells were injected in the spleen. These results suggest that splenocytes are not only associated with the HCT-116-RFP cells during the formation of metastasis but also play a positive role in the formation of the metastatic colonies. See Materials and Methods for experimental details.

Acknowledgments

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