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

Michael Bouvet, Kazuhiko Tsuji, Meng Yang, Ping Jiang, Abdool R. Moossa, and Robert M. Hoffman

**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.

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

**Requests for reprints:** Robert M. Hoffman, AntiCancer, Inc., 7917 Ostrow Street, San Diego, CA 92111. Phone: 858-654-2555; Fax: 858-268-4175; E-mail: all@anticancer.com.

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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.

**Figure 1.** A, schematic representation of experimental protocol. Human HCT-116-RFP-GFP colon cancer cells (2.0 × 10^7/50 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.

**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 αx 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. 1A). 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. 1B). By day 60, very large tumors were imaged in the liver (Fig. 1C).

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. 3A). 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. 3B).

When the GFP-splenocytes 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 cojected 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
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

<table>
<thead>
<tr>
<th>Cells</th>
<th>PV injection</th>
<th>PV injection</th>
<th>Spleen injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT-116</td>
<td>HCT-116</td>
<td>HCT-116</td>
<td>HCT-116 + splenocytes</td>
</tr>
<tr>
<td>Frequency of liver metastasis</td>
<td>0/15</td>
<td>5/15</td>
<td>8/15</td>
</tr>
</tbody>
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NOTE: When the GFP-splenocytes 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.

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Host Splenocytes Enhance Liver Metastasis

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