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Characterization of HCT116 Human Colon Cancer Cells in an Orthotopic Model[1]

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Background. **Colorectal cancer metastases result in a significant number of cancer related deaths. The molecular mechanisms underlying this complex, multi-step pathway are yet to be completely elucidated. In the absence of any transgenic models of colon cancer metastases, an *in vivo* model system that fulfills the rate limiting steps of metastasis (local invasion and distant colony formation) is needed. The purpose of this study was to characterize the behavior of a human colon cancer cell line, HCT116 in an orthotopic model.**

Materials and methods. **HCT116 cells were transfected with green fluorescence protein and subcutaneously injected into BALB/c nude male mice. Once xenografts were established, they were excised and orthotopically implanted into 32 other male BALB/c nude mice using microsurgical techniques. Animals were serially imaged and euthanized at 6–8 weeks post-implantation. Tissues were procured and processed for hematoxylin and eosin analysis.**

Results. **All 32 animals demonstrated primary tumor growth, invasion and peritoneal spread. Liver metastases were identified in 15/32 (47%), and lung metastases were confirmed in 13/32 (41%). In total, 19/32 (59%) animals demonstrated distant metastatic colony formation.**

Conclusions. **This orthotopic model of colon cancer fulfills the rate limiting steps of local invasion and distant colony formation in the process of metastases.**

HCT116 human colon cancer cell line in this *in vivo* model system provides a tool to dissect the molecular mechanism involved in the metastatic cascade. © 2008 Elsevier Inc. All rights reserved.

Key Words: **colon cancer; metastasis; orthotopic model.**

INTRODUCTION

Colorectal cancer is the second leading cause of cancer related deaths in the United States [1]. The mortality resulting from this disease, much like many other solid tumors, is not from the primary tumor itself, but from metastatic disease. Metastasis is a complex, multi-step process that requires changes in the extracellular matrix supporting invasion, increased cellular motility, cellular extravasation, and the ability of cells to initiate and maintain growth at a distant site [2]. The molecular mechanisms underlying this multi-step process are yet to be completely elucidated [3].

Numerous *in vitro* techniques have been used to study the processes of cancer cell motility, invasion, and growth. These studies, however, look at the steps of metastases in isolation with cell culture techniques that lack the complexity associated with the process in an *in vivo* setting. Studies using subcutaneous xenografts provide information in regards to tumor growth, but are limited in that xenografts rarely invade and metastasize. The well established technique of splenic injection that gives rise to liver metastases allows for the study of distant colony formation, but does not allow for the study of local invasion, which is a necessary first step in the metastatic cascade. Furthermore, in the absence of any transgenic models of colon cancer

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metastases, an *in vivo* model system that fulfills the rate limiting steps of metastasis involving local invasion and distant colony formation would allow for a working model to study the metastatic process in an *in vivo* setting.

Thus, the purpose of this study was to characterize the behavior of a human colon cancer cell line, HCT116, in an orthotopic model of colon cancer. HCT 116 is a human colon cancer cell line that is commonly used to study cancer biology. This is a growth factor-independent cell line that has been shown to be invasive and highly motile in *in vitro* studies [4–7]. Subcutaneous xenograft experiments have demonstrated it to be highly tumorigenic [8]. However, subcutaneous xenograft implants uniformly fail to show invasion and metastases.

MATERIALS AND METHODS

Cell Culture

HCT116 cells were maintained under serum-free conditions as previously described using McCoy's 5A medium supplemented with 4 ug/mL of transferrin, 5 ug/mL of insulin, and 10 ng/mL of EGF [9–10].

Green Fluorescence Protein (GFP) Transfection

Packaging cells, 293 GP (Clontech, Mountain View, CA), were co-transfected with a plasmid encoding VSVG envelope protein and a retroviral vector encoding GFP and the G418 resistance gene using FuGene (Invitrogen, Carlsbad, CA). The viruses were collected 48 h later and used to infect HCT116 cells. After 48 h, the infected HCT 116 cells were selected by treatment with G418 for 5 d. This resulted in a stable transfection.

Orthotopic Implantation and Imaging

Mice were maintained in a HEPA-filtered environment. All *in vivo* studies were conducted in accordance with the principles and procedures outlined by federal, state, and institutional guidelines for the care and use of laboratory animals. Five $\times 10^6$ HCT116 GFP labeled cells were subcutaneously injected into BALB/c nude male mice. At 1 cm^3, the xenograft was excised and minced for implantation into other 4 to 6 wk old male BALB/c nude mice. The recipient animals were anesthetized with isoflurane inhalation and a 1 cm laparotomy was performed. Two 1 mm^3 pieces were subserosally implanted on to the ceca and ascending colons of 32 other BALB/c nude male mice as previously described [11–14]. Subsequently, animals were anesthetized with a 1:1 mixture of ketamine (10 mg/mL) and xylazine (1 mg/mL) with intraperitoneal injection (0.01 mL/mg) and weekly GFP fluorescence imaging was performed for up to 8 wk at which time all animals were euthanized and necropsied. Specifically, GFP fluorescence imaging was performed using a light box illuminated by fiberoptic lighting at 470 nm (Illumatool BLS; Lightools Research, Encinitas, CA). Emitted fluorescence was collected through a 515 nm long-pass filter (Lightools Research) using a Retiga EXi color CCD camera (QImaging, Burnaby BC, Canada). High-resolution images consisting of 1360 $\times$ 1036 pixels were captured directly using a MS-Windows based PC. Images were visually optimized for contrast and brightness using commercial software (Adobe Photoshop; CS2 Adobe, San Jose, CA). Excitation of GFP in the light box facilitated identification of primary and metastatic disease by direct near-real time visualization of fluorescence in live animals. Note that in some instances, real-time qualitative determination of tumor burden can be assessed by estimating fluorescent surface area of tumor as a
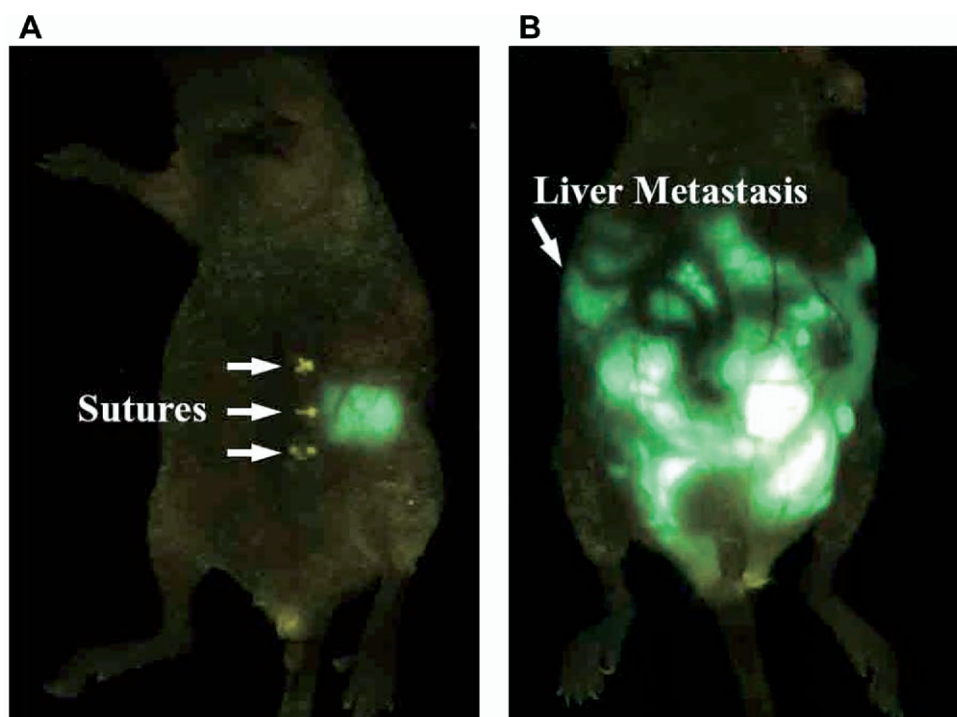

FIG. 1. Four to 6 wk old male BALB/c nude mice were orthotopically implanted with HCT116 tumor xenografts. Serial GFP imaging revealed primary tumor growth at week 1 (A), peritoneal dissemination and metastases by week 4 and a large tumor burden in a cachectic animal by week 6 (B).
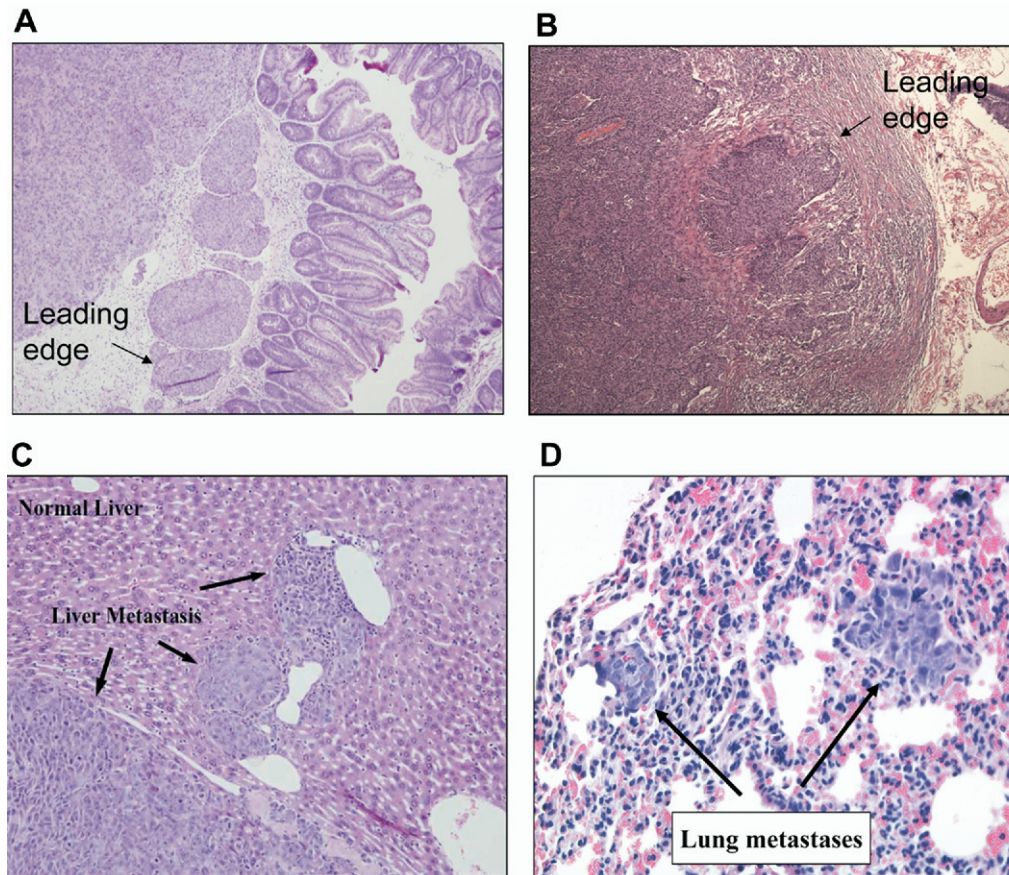
FIG. 2. (A) H&E section demonstrating primary tumor growth and the leading edge of tumor invasion into the mouse's colon (magnification 40×). (B) H&E section taken from a human colon cancer also demonstrating the leading edge of invasion (magnification 40×). (C) H&E section of the murine liver demonstrates tumor metastases from the orthotopic model (magnification 100×). (D) H&E section of murine lung depicts metastatic colonies (magnification 200×).

function of time and treatment [15–16]. However, this yields a two-dimensional estimate of a three-dimensional tumor. Therefore, the accuracy and nature of such correlative metrics of tumor burden are questionable and dependent on many parameters that are difficult to control experimentally. These include the depth and shape of tumor development from the surface of the animal, the strength, stability, attenuation, and nature of the GFP signal in time, positioning of user dependent instrumentation, etc. For example, a previous paper from our group demonstrated the utility of the method as a first approximation for estimating tumor burden using RFP in an orthotopic human pancreatic tumor model imaged in mice with tumors ~1500 to 2000 mm^3 [17]. However, one would suspect that significant errors in tumor volume would be obtained in this model, especially using GFP, which could confound interpretation of results. For this reason, quantifying fluorescent surface area of tumor was not herein performed.

Excised tissues were fixed in 10% buffered formalin and embedded in paraffin. Slides were cut and stained with hematoxylin and eosin (H&E) to evaluate local invasion and distant colony formation. Random single sections through the liver and lung parenchyma were taken to evaluate for metastases.

RESULTS

At 1 wk postimplantation, GFP imaging revealed primary tumor growth. Animals demonstrated palpable abdominal masses between 2 and 4 wk postimplan-

tation. By 4 wk postimplantation, peritoneal dissemination of tumor was evident. Six to 8 wk after implantation, animals were cachectic and were euthanized and necropsies were performed. All 32 animals demonstrated tumor growth and peritoneal dissemination of tumor as demonstrated by GFP imaging (Fig. 1). As the figure demonstrates, there is progressive growth of the primary tumor and peritoneal dissemination and ultimately by 4 wk, there is evidence of liver metastases.

Local invasion at the site of tumor implantation was confirmed in all 32 animals by histological evaluation using H&E staining (Fig. 2A). The H&E sections show that as the tumor grows, there is invasion of tumor into the bowel wall of the mouse. Fig. 3 demonstrates that as this invasion occurs, the tumor cells must change shape and elongate as they invade. Once the invasion occurs, they again change shape and continue to march forward and attack the host tissues. This process, in which a cancer cell loses membrane junctions, alters its adhesive properties and, therefore, changes shape has been extensively described in the literature as epithe-
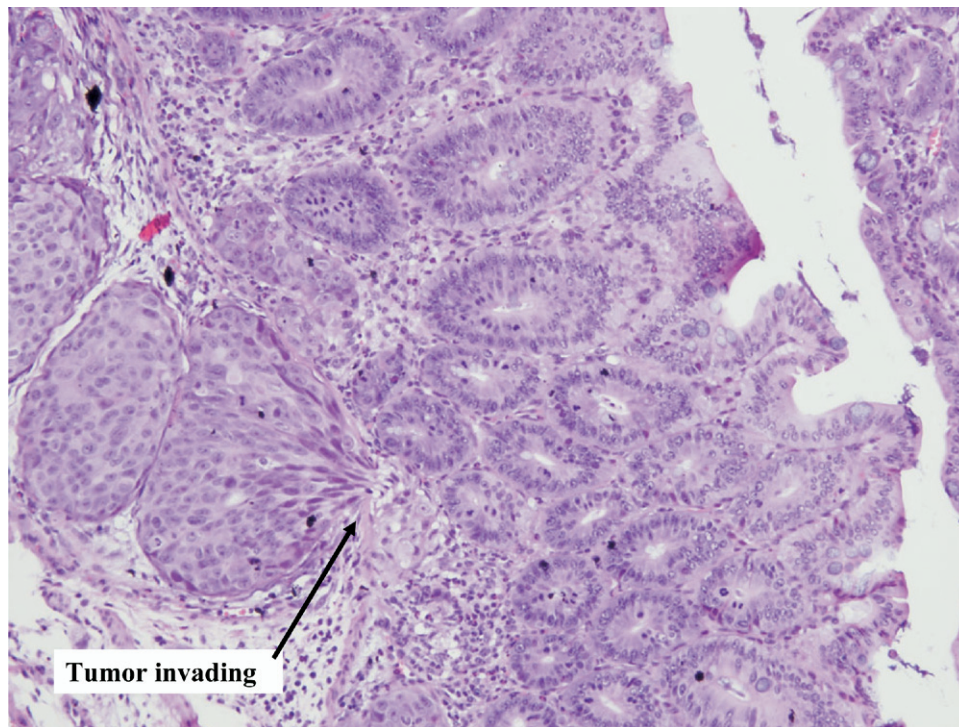
FIG. 3.   HCT116 tumor cells are seen changing shape and invading the host tissue documenting an epithelial to mesenchymal transition (magnification 200×).

lial to mesenchymal transition (EMT). The process of EMT is thought to be necessary for a tumor to progress and metastasize.

In 15/32 (47%) animals, liver metastases were identified and in 13/32 (41%) animals, lung metastases were histologically confirmed (Fig. 2D and E). In total, 19/32 (59%) animals demonstrated a distant metastatic colony (Tables 1 and 2). Not all metastases identified were macroscopically visible. It should be noted that these numbers probably underestimate the frequency of metastases as only a single section through the lung or liver was analyzed for each animal. A more thorough evaluation with increased numbers of histological sections would probably reveal a greater rate of metastases.

DISCUSSION

The burden of metastatic disease results in the vast majority of the morbidity and mortality associated with colorectal cancers. Despite developments in traditional toxic chemotherapeutics that have prolonged

TABLE 1

Results of Orthotopic HCT116 Implantation

Animals implanted	Local invasion	Liver mets	Lung mets	Liver and/or lung mets
32	32 (100%)	15 (47%)	13 (41%)	19 (59%)

disease-free survival, the overall survival in patients with metastatic disease remains poor. Furthermore, even with the addition of molecularly targeted therapies, the clinical response rates have been disappointingly low [18–20]. Thus, in the absence of transgenic metastatic models, the orthotopic model of colon cancer described in this study with HCT116 offers a more complete model for preclinical therapeutic evaluations and provides a powerful tool to dissect the molecular pathways involved with tumor metastasis. The ability to perform GFP imaging with the orthotopic model allows for the assessment of tumor burden and spread in real time [21]. This allows for the study of multiple time end points in each animal.

The process of metastasis has been divided into relatively efficient and inefficient or rate-limiting steps [22–24]. Cancer-cell invasion and subsequent shedding of cancer cells into the circulation is rate limiting. Survival in circulation, arrest in a distant organ, and extravasation are regarded as efficient or non-rate-limiting processes. Initial and persistent growth of cancer cells at a distant site is the second rate-limiting step in the metastatic process. Therefore, the rates of invasion and metastatic colonization predict overall metastatic ability. For a cancer cell to achieve the changes necessary to overcome the rate-limiting steps of invasion and colonization, there are a number of genes and subsequently a number of signal transduction pathways that must be dysregulated.

TABLE 2

Results of Individual Animal Orthotopic Implants

Animal	Local invasion	Liver metastasis	Lung metastasis
1	yes	yes	yes
2	yes	yes	no
3	yes	no	yes
4	yes	yes	yes
5	yes	yes	yes
6	yes	yes	no
7	yes	yes	no
8	yes	no	no
9	yes	no	no
10	yes	no	no
11	yes	no	no
12	yes	yes	yes
13	yes	no	no
14	yes	no	no
15	yes	no	no
16	yes	no	no
17	yes	yes	no
18	yes	yes	yes
19	yes	no	no
20	yes	no	yes
21	yes	yes	yes
22	yes	no	yes
23	yes	yes	yes
24	yes	no	yes
25	yes	yes	yes
26	yes	yes	no
27	yes	no	no
28	yes	yes	yes
29	yes	yes	no
30	yes	no	no
31	yes	no	no
32	yes	no	no

In vitro studies have shown HCT116 to be invasive and highly motile and subcutaneous xenograft experiments have demonstrated it to be highly tumorigenic [6, 8]. *In vitro* studies are limited in regard to the study of metastasis as tumor cell motility and invasion assays focus on the premise of single cell locomotion and penetration of an extracellular matrix coating [25]. Furthermore, *in vivo* subcutaneous xenografts are limited, as they do not metastasize. Clinically, however, when pathologic specimens of colorectal cancer are examined, there is no evidence for single cell locomotion [26, 27]. What is actually seen is a front or a cohort of cancer cells on the leading edge that is invading the host tissues. Figure 2A demonstrates this type of cohort of cells that is invading the mouse's normal colon. In Fig. 2B, an H&E section of a colon adenocarcinoma resected from a patient shows a similar type of invasive pattern in the bowel wall. Historical models of splenic injection do not allow for the study of invasion at the primary site of tumor growth. Cecal injections of cell suspensions offer an improvement over subcutaneous xenograft studies and splenic injections as invasion at the site of injection can be obtained. The cell suspen-

sion injection model is limited, however, as it results in relatively low rates of metastases compared with orthotopic implantation [28].

For invasion and progression of a cancer to occur, it is hypothesized that there is an EMT. EMT results in the loss of epithelial features with a down-regulation of epithelial markers that result in the loss of cell polarity and intercellular junctions [29]. The loss of these features is accompanied by increased cellular motility and the expression of mesenchymal genes. All in all, the end result of EMT is what contributes to making a cell malignant, i.e., the loss of contact inhibition, loss of growth control, and increased invasion [30]. This process of EMT has been debated in the literature as a recent review questioned the importance of EMT in the role of carcinoma, citing a lack of evidence that it occurs *in vivo* [31]. Figure 3 demonstrates EMT in the *in vivo* orthotopic model and thus may provide a means of further studying the complex molecular changes involved with EMT.

In conclusion, this orthotopic model of colon cancer fulfills the rate-limiting steps of local invasion and distant colony formation in the process of metastasis. The HCT116 human colon cancer cell line in this *in vivo* model system provides a tool to dissect the molecular mechanisms involved in the metastatic cascade. Further knowledge of invasion and successful growth of tumor cells at distant sites may allow for the development of novel therapeutic agents to impact the morbidity and mortality attributed to colorectal cancer.

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