

Liver colonization competence governs colon cancer metastasis

(liver metastasis/nude mouse/orthotopic implantation/intrahepatic implantation)

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ABSTRACT Tumors that metastasize do so to preferred target organs. To explain this apparent specificity, Paget, >100 years ago, formulated his seed and soil hypothesis; i.e., the cells from a given tumor would "seed" only favorable "soil" offered by certain organs. The hypothesis implies that cancer cells must find a suitable "soil" in a target organ—i.e., one that supports colonization—for metastasis to occur. We demonstrate in this report that ability of human colon cancer cells to colonize liver tissue governs whether a particular colon cancer is metastatic. In the model used in this study, human colon tumors are transplanted into the nude mouse colon as intact tissue blocks by surgical orthotopic implantation. These implanted tumors closely simulate the metastatic behavior of the original human patient tumor and are clearly metastatic or nonmetastatic to the liver. Both classes of tumors were equally invasive locally into tissues and blood vessels. However, the cells from each class of tumor behave very differently when directly injected into nude mouse livers. Only cells from metastasizing tumors are competent to colonize after direct intrahepatic injection. Also, tissue blocks from metastatic tumors affixed directly to the liver resulted in colonization, whereas no colonization resulted from nonmetastatic tumor tissue blocks even though some growth occurred within the tissue block itself. Thus, local invasion (injection) and even adhesion to the metastatic target organ (blocks) are not sufficient for metastasis. The results suggest that the ability to colonize the liver is the governing step in the metastasis of human colon cancer.

A metastatic colony is the end result of a complex series of processes resulting from tumor–host interactions (1, 2). These include angiogenesis and intravasation of tumor cells (3), circulation and extravasation (4), and adhesive interactions with other cells, including endothelial and target cells (5–7), as well as colonization of the target organ. Adhesion molecules and proteolytic enzymes seem to play a role in this process (5–8). However, only some tumors metastasize and there is no clear understanding of the key properties and events that lead to metastatic colony formation.

Tumors can have enormously different characteristic metastatic rates. In contrast to the variability in rates, there is a surprising degree of specificity in the target organs colonized by metastases from a particular type of tumor (e.g., colon tumors most often target the liver). Over 100 years ago, Paget (9) noted the highly nonrandom spread of cancer to specific target organs. From these observations, he formulated the "seed" (primary cancer) and "soil" ("target organ") hypothesis of metastasis. A corollary of this hypothesis is that cancer cells must find a suitable "soil" in a target organ, one supporting colonization, for metastasis to occur. We demonstrate

in this report that ability of cells from a particular human colon cancer to colonize liver tissue (i.e., to use it as "soil") directly reflects whether that cancer is metastatic.

These experiments were made possible by the recently developed, "patient-like" models of human cancer in nude mice. These models are the first in which animal-implanted human tumors closely replicate the complex behaviors of the original tumor while still in the human host. The implanted tumors parallel the original tumor's characteristic growth and local invasiveness, its drug sensitivity and, most important, the tumor's rate of metastases to corresponding target tissues (10–35). These models are created in nude mice by surgical orthotopic implantation (SOI) (i.e., implantation to the organ or tissue corresponding to the original human tumor site) of histologically intact tumor blocks. Both the use of an orthotopic site of implantation and the use of tissue blocks rather than dispersed cells are critical to the accuracy of the model. Metastatic models of human colon cancer (10–16), stomach cancer (17–20), pancreatic cancer (21, 22), bladder cancer (23, 24) lung cancer (25–33), ovarian cancer (34), breast cancer (35), and prostate cancer (36) have been established with SOI. In every case, the implanted tumors show patterns of metastasis that closely resemble the patterns in the patient donors with regard to rates and the targeting of corresponding mouse tissue.

In this study we have utilized the realistic model of metastasis afforded by the SOI nude mouse model of colon cancer. The model accurately classifies human colon tumors as to whether they metastasize (M) or do not metastasize (non-M) to liver as the target tissue. Cells taken from M and non-M tumors were assayed for their ability to directly colonize the liver when implanted in liver tissue either by injection or by affixation of a block of tumor tissue. Cells from the M tumors colonized the liver when implanted by either method. In sharp contrast, cells derived from the non-M colon tumors were unable to colonize liver under either procedure. This radical difference suggests that the ability of the tumor cells to colonize the liver is critical for the metastasis of human colon cancer.

MATERIALS AND METHODS

Transplantable Human Colon Tumors. Eight human colon cancer strains transplantable into BALB/cA *nu/nu* mice were used for the experiments described in this report. COL-2-JCK, COL-3-JCK, and COL-5-JCK were established at Tokai University (Atsugi, Japan) and supplied by T. Nomura (Central Institute for Experimental Animals, Kawasaki, Japan). Co-3 and Co-4 were established at the Pathology Division, National Cancer Center Research Institute (Tokyo). Co-6 was established at the Department of Surgery, Keio University School

Table 1. Human colon cancer xenografts

Name	Description
COL-2-JCK	Poorly differentiated adenocarcinoma
COL-3-JCK	Poorly differentiated adenocarcinoma
COL-5-JCK	Well-differentiated adenocarcinoma
Co-3	Well-differentiated adenocarcinoma
Co-4	Mucinous carcinoma
Co-6	Poorly differentiated adenocarcinoma
WiDr	Well-differentiated adenocarcinoma
COLO-205	Poorly differentiated adenocarcinoma

See text for derivation of the colon tumor xenografts.

of Medicine (37). COLO-205 was established as a cell line by Semple *et al.* (38), and was provided from the Pathology Division, National Cancer Research Institute (Tokyo). WiDr was provided by H. Ishitsuka (Nippon Roche Research Center, Tokyo). The histological types of these cancer lines are listed in Table 1. All strains were maintained by serial transplantation into nude mice (39, 40). Tumors in the exponential growth phase were used for the experiments.

Nude Mice. Male nude mice with a BALB/cA genetic background were purchased from CLEA Japan (Tokyo). They were maintained under specific pathogen-free conditions with an Isorack and were fed sterile food and water *ad libitum*. Six- to eight-week-old mice weighing 20–22 g were used for the experiments.

SOI. Human colon tumors growing subcutaneously in nude mice were harvested and transplanted as intact tissue by SOI onto the cecum of nude mice with microsurgical procedures (10–16). In brief, subcutaneously growing human colon tumors in nude mice were resected aseptically and the tumor tissues were minced with scissors into pieces about 4 mm in diameter, weighing about 75 mg each. Mice were anesthetized with a 2.5% solution of a 1:1 (vol/vol) mixture of 2,2,2-tribromoethanol (Aldrich) and *tert*-amyl alcohol (Wako Pure Chemical, Osaka).

An incision was made through the left lower abdominal pararectal line and peritoneum. The cecal wall was exposed and a part of the serosal membrane was scraped with a 27-gauge needle. Care was exercised to prevent the rupture of the cecal wall. One tumor piece was then fixed on each scraped site of the serosal surface with a 6-0 Dexon (Davis-Geck, Manatai, PR) transmural suture. The cecum was then returned into the peritoneal cavity, and the abdominal wall and the skin were closed with 6-0 Dexon sutures.

The mice were kept in a sterile environment and were sacrificed by cervical dislocation 12 weeks after SOI or earlier

if they developed signs of distress. Upon autopsy, all organs, including the cecum and liver, were processed for routine histological examination using hematoxylin and eosin (H/E) staining after careful macroscopic examination.

Intrahepatic Injection. For intrahepatic injection, dissociated tumor cells were obtained after harvest of human colon tumors growing subcutaneously in nude mice. Mice were anesthetized and a midline incision was made through the upper abdomen and peritoneum. A part of the liver was exposed and 50 μ l of the tumor-cell suspension per mouse, containing 5×10^6 viable tumor cells, was injected intrahepatically via a 27-gauge needle. Briefly, subcutaneous tumors in the exponential growth phase in nude mice were resected aseptically, necrotic tissues were cut away, and the remaining intact tumor tissues were minced with scissors as finely as possible in Hanks' balanced salt solution containing 100 units of penicillin and 100 μ g of streptomycin per ml (Hanks' solution). After incubation for 30 min at 37°C with a mixture of 0.02% collagenase (Boehringer Mannheim) and 0.02% DNase (Boehringer Mannheim), the homogenates were passed through a stainless steel mesh (200 pores per cm^2). The filtrates were washed once in RPMI-1640 medium (Nissui Seiyaku, Tokyo) containing 10% fetal bovine serum. The filtered homogenate was then centrifuged for 5 min at $516 \times g$.

The dissociated tumor cells were then suspended in Hanks' solution, and the concentration of viable cells in the suspension was determined by trypan blue dye exclusion. After centrifugation, the tumor cells were suspended at 10^8 viable cells per ml. Mice were anesthetized by the same method as mentioned previously. A scissors incision was made on the left lateral flank through the peritoneum. The liver was carefully exposed as mentioned above and 50 μ l of the tumor cell suspension per mouse, equivalent to 5×10^6 viable tumor cells, was carefully injected intrahepatically via a 27-gauge needle.

Hepatic Affixation of Colon Tumors. Two human colon carcinoma strains, COL-2-JCK (metastatic, M) and Co-4 (nonmetastatic, non-M) were used in this experiment. Mice were anesthetized and a midline incision was made through the upper abdomen and peritoneum. One tumor piece, about 6 mm, was then fixed on the surface of the liver with a 6-0 Dexon suture. The abdominal wall and the skin were closed with 6-0 Dexon sutures. The mice were kept in a sterile environment and were sacrificed 6 weeks after transplantation.

Suppression of Natural Killer (NK) Cells. Anti-asialo-GM1 antibody, an immunosuppressive agent against NK cell activity (41), was purchased from Wako Pure Chemical. The anti-asialo-GM1 antibody, containing 10 mg/ml per vial, was diluted

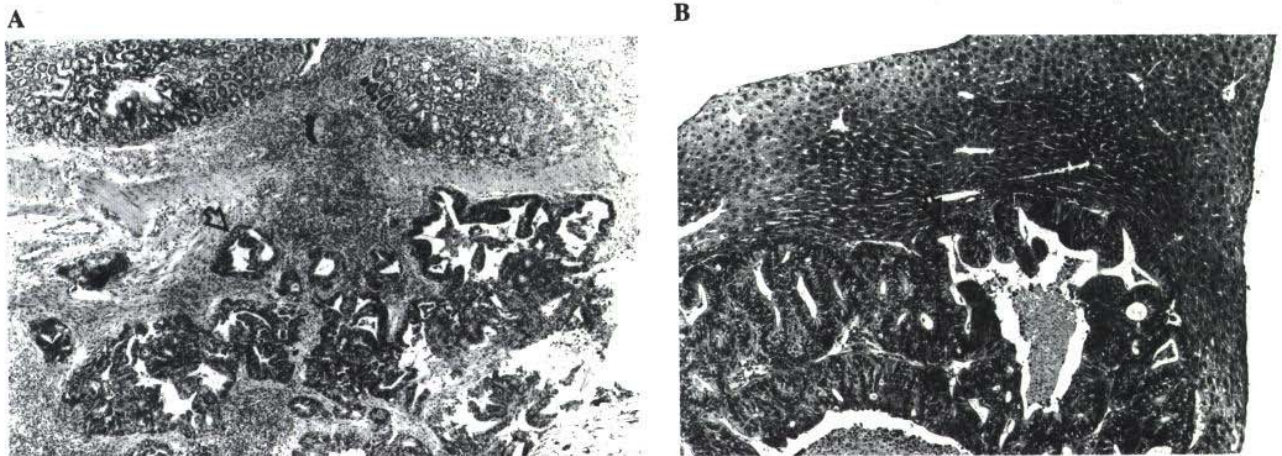


FIG. 1. (A) Local growth of human colon tumor Co-3, a well-differentiated adenocarcinoma, in the cecum of a nude mouse at 4 weeks after SOI. Arrows indicate the tumor cells. (H/E; $\times 100$.) (B) Liver metastasis of Co-3 at 4 weeks after SOI. (H/E; $\times 100$.)

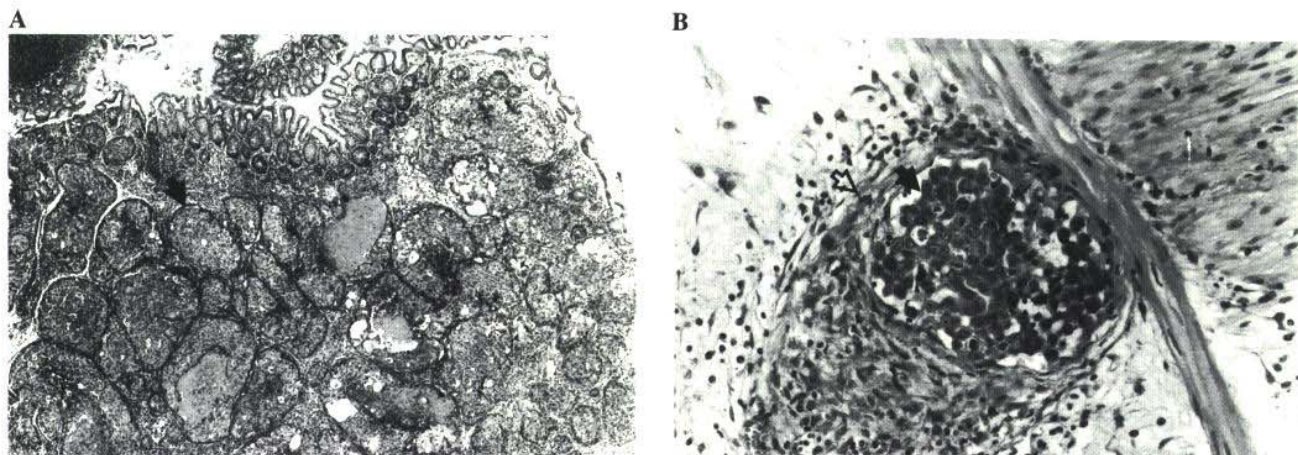


FIG. 2. (A) Extensive local growth of COLO-205, a non-M strain, in the cecum of a nude mouse at 7 weeks after SOI. Arrow indicates the tumor. (H/E; $\times 100$.) (B) Pathohistology of growing COLO 205, a poorly differentiated adenocarcinoma, in the nude mouse cecum at 7 weeks after SOI. Black arrow indicates invasive tumor cells and the open arrow identifies the blood vessel wall. (H/E; $\times 100$.)

in 0.9% NaCl to a total volume of 10 ml. To suppress NK cells the anti-asialo-GM1 antibody was administered intraperitoneally to the mice of the treated group on days -1, 0, 7, 14, and 21 at a dose of 200 μg per mouse, equivalent to 0.2 ml of the dissolved antibody. This was done to determine whether NK cells are a factor in liver colonization.

Analysis of Primary Tumor and Metastatic Growth. The liver and other organs were removed after sacrifice and processed for histological examination in H/E-stained sections by standard techniques.

RESULTS AND DISCUSSION

Metastatic and Nonmetastatic Tumor Strains. The data in Table 1 show the metastatic behavior of the two groups of tumors used here. Human colon tumors COL-2-JCK, COL-3-JCK, COL-5-JCK, and Co-3 gave rise to liver metastases in more than half of the nude mice after SOI of intact tumor tissue into the mouse cecum. These tumors were therefore classified as M (metastatic). The histological section in Fig. 1A shows the local growth of Co-3, a well-differentiated adeno-

carcinoma, at 4 weeks after implantation in the cecum. The histological section in Fig. 1B shows a typical metastasis in the liver arising from Co-3.

A second group of colon tumors, Co-4, Co-6, COLO-205, and WiDr, gave rise to no detectable liver metastases even 12 weeks after implantation and were classified as non-M (non-metastatic). Fig. 2A shows a histological section of COLO-205, a poorly differentiated adenocarcinoma, at 7 weeks after implantation in the mouse cecum. Fig. 2B is a higher-power view of COLO-205 showing tumor cells invading a blood vessel. Although non-M, this tumor is clearly highly invasive locally.

Apart from their different metastatic behavior, the M and non-M tumors appeared to be quite similar. Both M and non-M tumors showed similar extensive local growth and invasion of cancer cells into vessels of the cecal wall within 4-7 weeks after orthotopic transplantation. However, a major difference between the M and non-M tumors emerged when dissociated cells from the tumors were directly seeded on the liver.

Intrahepatic Injection of Colon Tumor Cells. Table 2 shows that dissociated cells from the M tumors were completely effective in originating tumors in the liver after intrahepatic injection of the dissociated tumor cells. These growths closely resembled spontaneously arising metastases from the same tumors (Fig. 3). In contrast, the cells from non-M tumors showed no detectable tumor growth in the liver after direct

Table 2. Liver colonization competence correlates with metastatic potential

Human colon carcinoma strain	Tumor growth in cecum*	Liver metastasis	Tumor growth after intrahepatic injection†
COL-2-JCK	12/12	9/12	4/4
COL-3-JCK	12/12	7/12	5/5
COL-5-JCK	15/15	8/15	4/4
Co-3	14/14	7/14	4/4
Co-4	8/8	0/8	0/6
Co-6	5/5	0/5	0/5
WiDr	8/8	0/8	0/6
COLO-205	8/8	0/8	0/6

Data are shown as no. of mice with tumor growth or liver metastasis after SOI and tumor growth after intrahepatic injection/no. of mice evaluated.

*Human colon tumors growing subcutaneously in nude mice were harvested and transplanted as intact tissue by SOI onto the cecum of nude mice. One tumor piece was then fixed on each scraped site of the serosal surface with a 6-0 Dexon transmural suture.

†For intrahepatic injection, dissociated tumor cells were obtained after harvesting human colon tumors growing subcutaneously in nude mice. A part of the liver was exposed and 50 μl of the tumor-cell suspension per mouse, containing 5×10^6 viable tumor cells, was injected intrahepatically via a 27-gauge needle.



FIG. 3. Pathohistology of a growing colony of COL-5-JCK, a well-differentiated adenocarcinoma, at 4 weeks after intrahepatic injection into a nude mouse liver. (H/E; $\times 100$.)

Table 3. Hepatic colonization of human colon carcinoma after liver affixation of tissue block

Strain	Tumor growth	Adhesion to liver	Liver colonization	Metastasis to liver
COL-2-JCK	5/5	5/5	5/5	9/12
Co-4	5/5	5/5	0/5	0/8

Two human colon carcinoma strains, COL-2-JCK (M strain) and Co-4 (non-M strain), were used in this experiment. Mice were anesthetized and a midline incision was made through the upper abdomen and peritoneum. One tumor piece, about 6 mm, was then fixed on the surface of the liver with a 6-0 Dexon suture. The abdominal wall and the skin were closed with 6-0 Dexon sutures. Mice were kept in a sterile environment and were sacrificed 6 weeks after transplantation. Data are shown as no. of mice with tumor growth, adhesion to liver, or liver colonization/no. of mice evaluated.

intrahepatic injection. As a further test, the cells from the non-M tumors showed no tumor growth in the liver even when NK cell activity of the mice was suppressed by anti-sialo-GM1 antibody (data not shown). Therefore, local immune mechanisms in the liver are not likely to be the cause of the marked difference between the M and non-M tumor cells.

Intrasplenic Injection of Colon Tumor Cells. A previous test for metastatic capability injected dissociated tumor cells into the mouse spleen (2). Intrasplenic injection of cells from the M tumors, COL-2-JCK, COL-3-JCK, COL-5-JCK, and Co-3, resulted in development of splenic tumors and metastases to the liver. In contrast, no tumor growth in the spleen was observed after intrasplenic injection of cells from non-M tumors Co-4, Co-6, COLO-205, and WiDr. Moreover, no mice developed liver metastases even 10 weeks after the intrasplenic injection (data not shown). Cells from non-M tumors did not develop tumor growth in the spleen or metastasize to the liver even when NK cell activity was suppressed by the anti-sialo-GM1 antibody.

Affixing Intact Colon Tumor Tissue Blocks to the Liver. The intrahepatic injection experiments necessarily employed dissociated cells. Cells from M and non-M tumors might have behaved differently under the stress due to the procedure. Colonization was therefore also measured by affixing tissue blocks from the tumors to the liver. Histologically intact tumor tissue from two of the human colon carcinoma strains, COL-2-JCK (M strain) and CO-4 (non-M strain), were affixed to the liver surface as a tissue block of ≈ 6 -mm diameter with a 6-0 Dexon suture. Table 3 shows that tumor growth adjacent to liver occurred for both strains. However, the M tumor, COL-2-JCK, showed extensive invasion and colonization of the liver

Table 4. Liver colonization competence is essential for the metastatic process

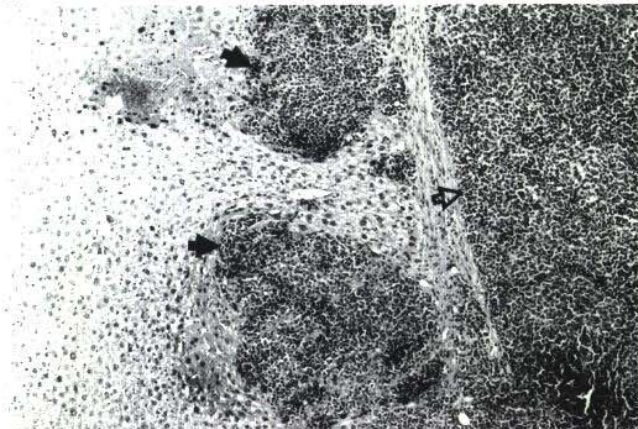
Process	Presence in M strains	Presence in non-M strains
Growth	+	+
Invasion	+	+
Lymphatic/hematogenous spread	+	+
Adhesion to distant organ	+	+
Liver colonization capability	+	-

(Fig. 4A). In contrast, the tissue from the non-M tumor, Co-4, did not colonize the liver by invasion even after adhesion to liver for 6 weeks (Fig. 4B). In the latter case, the border delimiting liver tissue from the affixed tumor remained remarkably regular and sharply defined despite extensive external growth by the tumor cells. The results support those of the injection experiments.

Colonization Competence Governs Metastasis. The experimental results show that although non-M tumors can grow locally on the colon and invade surrounding tissue, including blood vessels, their cells cannot colonize the metastatic target organ even when they are injected directly or are affixed to the liver as a tissue block. Only cells from tumors that can actually metastasize from the primary site can also colonize the liver after direct implantation (Table 4). The inability of cells from non-M tumors to grow in the liver when directly injected or affixed to the liver as a tissue block suggests that an essential event in metastasis is the successful seeding and growth of colon cells in the liver. Simple adhesion of the tumor cells to the liver is clearly insufficient for colonization. Since both non-M and M tumors are locally invasive at the primary site, such local invasion is clearly insufficient to result in distant metastasis to the liver. In this realistic model of tumor metastasis the ability of the colon cancer cells to colonize the liver appears to govern the metastatic process.

The animal models of human cancer described here make possible further study of metastatic process of colon cancer in general and, in particular, the critical step of liver colonization. Most important, the SOI model avoids trivial obstacles to metastasis such as the encapsulation characteristic of ectopic implantation (e.g., subcutaneous tumors). The models can be used for evaluating drugs and biologicals which may directly prevent colon tumors from colonizing the liver. Also, study of the basic mechanisms underlying metastasis will be greatly facilitated by selecting metastatic variants (2) on the basis of their ability to directly colonize the liver.

A



B

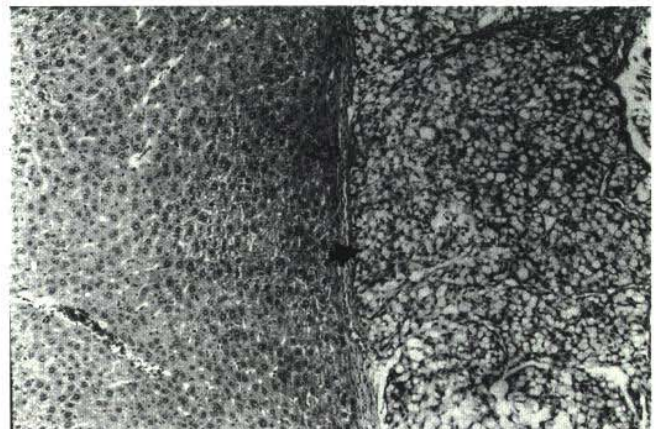


FIG. 4. (A) Junction of normal liver and a tissue block from the M tumor COL-2-JCK. Note the extensive invasion and colonization of the liver. (H/E; $\times 100$.) (B) Junction of normal liver and a tissue block from the non-M tumor Co-4. (H/E; $\times 100$.)

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