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Models of human metastatic colon cancer in nude mice orthotopically constructed by using histologically intact patient specimens

(histologically intact colon tumor specimens/nude-mouse implant/orthotopic growth/carcinomatosis/metastasis)

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ABSTRACT **There is an important need for clinically relevant animal models for human cancers. Toward this goal, histologically intact human colon-cancer specimens derived surgically from patients were implanted orthotopically to the colon or cecum of nude mice. We have observed extensive orthotopic growth in 13 of 20 cases of implanted patient colon tumors. These showed various growth patterns with subsequent regional, lymph-node, and liver metastasis, as well as general abdominal carcinomatosis. Thus, models for human colon cancer have been developed that show (*i*) local growth, (*ii*) abdominal metastasis, (*iii*) general abdominal carcinomatosis with extensive peritoneal seeding, (*iv*) lymph-node metastasis, (*v*) liver metastasis, and (*vi*) colonic obstruction. These models permit the passage of the tumors to form large cohorts. They will facilitate research into the biology of colon cancer metastatic capability and the development of new drugs active against metastatic cancer. These models may also predict the clinical course and the *in vivo* response to drugs of the cancer of individual patients.**

There is a need for the development of better animal models for human cancer. Models based on athymic nude mice have been used for this purpose. However, metastatic rates from subcutaneous or intramuscular xenografts have been low or nonexistent even from tumors that were highly metastatic in the patient from whom the tissue was derived (1–5).

Recent work from a number of laboratories has indicated that implanting human tumor cells orthotopically in the corresponding organ of nude mice resulted in much higher metastatic rates. For example, a human renal-cell carcinoma obtained from a surgical specimen was dissociated by enzymatic treatment and subcutaneously injected into the renal capsule of nude mice as well as other sites. The injection of human renal-cell carcinoma cells into the kidney of nude mice produced the highest incidence of tumor establishment and of metastasis to the lungs and other peritoneal organs. The nude-mouse renal capsule appears to be a most advantageous site for implantation of human renal-cell carcinoma (6–8). However, the subrenal capsule may be an advantageous implant site for other tumor types also (9). Human colon-cancer cells were dissociated, grown in culture, and subsequently injected into the cecum of nude mice to produce tumors that eventually metastasized to the liver, demonstrating that orthotopic implantation can enhance the metastatic capability of human tumor cells in nude mice (5, 10–13). Similar results also have been achieved for orthotopic implantation of cell lines of human lung cancer (14), human pancreatic cancer (15), bladder cancer (16, 17), melanoma (18, 19), breast cancer (20–22), and head and neck cancer (23). It should be noted, however, that the effects of orthotopicity have not been fully evaluated in that, at least in some cases, metastasis may arise from nonorthotopic sites.

Our approach is to avoid disruption of tumor integrity and to orthotopically implant histologically intact tumor tissue directly. Such a model should better reflect the original properties of human cancer and could be of great value in development of new drugs and treatment strategies of cancer. With this overall strategy, we have constructed a model of human colon cancer in nude mice that can show the variety of clinical behaviors that occur in human subjects. These include (*i*) local growth, (*ii*) abdominal metastasis, (*iii*) general abdominal carcinomatosis with extensive peritoneal seeding, (*iv*) lymph-node metastasis, (*v*) liver metastasis, and (*vi*) colonic obstruction. A very high tumor-establishment rate of 13 cases of 20 attempts was observed.

MATERIALS AND METHODS

Mice. Four-week-old outbred *nu/nu* mice of both sexes were used for tumor implantation. All animals were maintained in a sterile environment. Cages, bedding, food, and water were all autoclaved. All animals were maintained on a daily 12-hr light/12-hr dark cycle. Bethaprim Pediatric Suspension (containing sulfamethoxazole and trimethoprim) was added to the drinking water.

Colon Cancer Specimens. Fresh surgical specimens were obtained as soon as possible, but no later than 24 hr after surgery, from local San Diego hospitals and kept in Earl's minimal essential medium (MEM) at 4°C. Before implantation, specimens were washed twice with antibiotic-containing Earl's MEM, at least 10 min each time, to prevent possible contamination and infection. The formulation, in 500 ml of Earl's MEM, was: 70 ml of fetal bovine serum, 75.2 mg of penicillin, 125 mg of streptomycin, 10 ml of fungizone, 5 mg of tetracycline, 50 mg of amikacin, 75 mg of chloramphenicol, and 50 mg of gentamycin.

Specimens were then inspected, and grossly necrotic and suspected necrotic tissue was removed. Each specimen was equally divided into four to six separated parts, and each part was subsequently cut into small pieces of about 1 mm^3. Tumor pieces for each implantation were taken from each of the four to six parts of the specimen equally. In this way, the chance for viable tissue to be implanted was maximized.

Surgical Microprocedures. For direct implantation, nude mice were anesthetized, and the abdomen was sterilized with iodine and alcohol swabs. A small midline incision was made and the colocecal part of the intestine was exteriorized. Serosa of the site where tumor pieces were to be implanted was removed. Eight to 15 pieces of 1-mm^3 size tumor were implanted on the top of the animal intestine; an 8-0 surgical

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suture was used to penetrate these small tumor pieces and suture them on the wall of the intestine. The intestine was returned to the abdominal cavity, and the abdominal wall was closed with 7-0 surgical sutures. Animals were kept in a sterile environment.

For induction of a vascular bed prior to tumor implantation, gelfoam (Upjohn) was preimplanted, first being hydrated with Earle's MEM, cut into approximately $5 \times 5 \times 3$ mm^3 pieces, and stored in a CO_2 incubator. Mice were anesthetized with isoflurane inhalation, and the abdomen was sterilized with iodine and alcohol swabs. A small midline incision was made and the colocecal part of the intestine was exteriorized. Hydrated gelfoam was either implanted to the cecum or to the ascending colon 1 cm away from the cecum. Serosa was removed from the part where gelfoam was to be implanted. A $5 \times 5 \times 3$ mm^3 piece of hydrated gelfoam was implanted on the top of the colon or cecum. Two or three stitches of 8-0 surgical suture were applied to very small bits of the intestinal wall so as not to penetrate it. The intestine was returned to the abdominal cavity, and the wound was closed in one layer. Animals were maintained in a sterile environment. After 20 days, mice bearing the gelfoam preimplantation (now vascularized) were anesthetized and sterilized in exactly the same way as during the gelfoam implantation. The abdomen was reopened in the midline. The part of the intestine where the gelfoam had been implanted was exteriorized, and a small pocket was made into the vascularized gelfoam. About 8–15 pieces of the 1-mm^3 tumor, depending on the amount of tumor available, were implanted into the pocket. The pocket was closed with a stitch of 8-0 surgical suture. The intestine was then returned to the abdominal cavity. The wound was closed with 7-0 surgical sutures in one layer, and the animals were kept in a sterile environment.

A skin flap was induced into the abdominal cavity of nude mice to create a "sandwich-style" implantation. The main idea for doing so is because the tumor may have a better chance of growing in the subcutaneous environment. The nude mouse was anesthetized and the abdomen sterilized in the same way as in the gelfoam implantation. Tumor pieces were implanted between the skin flap and the cecum serosa.

For tumor and normal-surrounding-tissue coimplantation, the mice were anesthetized and the colocecal part of intestine was exposed in the same way as in direct implantation. After removing the serosa of the implantation site, 8–15 pieces of tumor together with 8–15 pieces of normal surrounding tissue were penetrated with 8-0 surgical sutures and sutured on the wall of the intestine. The intestine was returned to the abdominal cavity, and the abdominal wall was closed with 7-0 surgical sutures. Animals were kept in a sterile environment.

For tumor coimplantation with mouse embryonic tissue and gelfoam, anesthesia of the nude mice and the surgical approach to expose the colocecal part of the intestine were the same as in direct implantation. After removal of the serosa on the implantation site, a piece of $5 \times 5 \times 3$ mm^3 size gelfoam was implanted on the site. A pocket was made on the pieces of gelfoam, and 8–15 pieces of tumor and 8–15 pieces of mouse embryonic liver tissue were planted in the pocket; 8-0 surgical sutures were used to close the pocket. The intestine was then returned to the abdominal cavity, and the abdominal wall was closed with 7-0 surgical sutures. Animals were kept in a sterile environment.

RESULTS AND DISCUSSION

Twenty different cases of colon cancer surgical specimens were implanted orthotopically directly or with the use of gelfoam, an internal skin flap or the coimplantation of human normal and tumor tissue or coimplantation of tumor with mouse embryonic tissue and gelfoam. Thirteen of 20 individual patient specimens showed local orthotopic growth, with different specimens showing subsequent regional, lymph-node, and liver metastasis. These can serve as models for human colon cancer, including a model for (*i*) local growth, (*ii*) abdominal metastasis, (*iii*) general abdominal carcinomatosis with extensive peritoneal seeding, (*iv*) lymph-node metastasis, (*v*) liver metastasis, and (*vi*) colonic obstruction.

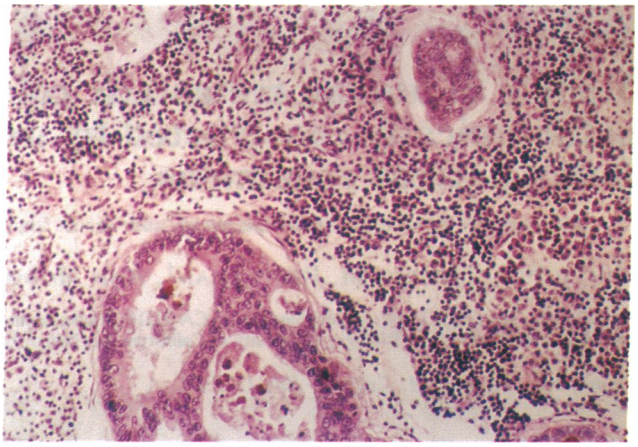

FIG. 1. Pathohistology of nude-mouse mesenteric lymph node involved with human-colon tumor metastases. (×130.)

Local Growth and Abdominal Metastasis. An example is specimen case 1701, an infiltrating mucinous adenocarcinoma of the right colon (modified Duke's classification C2). Two nude mice with preimplanted gelfoam were used for tumor implantation, two nude mice were used for tumor implantation with an internal skin flap, and two nude mice were used for direct implantation of tumor tissue to the cecum. Two of the six mice suffered early death (one with direct tumor implantation, one with gelfoam preimplantation) and were not available for assessment of tumor growth. All of the remaining mice demonstrated extensive primary growth ranging from 8 mm × 5.7 mm to 13 mm × 13 mm as well as abdominal-wall metastases ranging from 8 mm × 11 mm to 22 mm × 15 mm. All of the remaining mice showed visible tumor growth in the abdomen. Autopsies were performed 113–139 days after implantation.

Local Growth, Abdominal Metastasis, and Lymph-Node Metastasis. An example is specimen case 1707, an infiltrating adenocarcinoma of the right colon, moderately differentiated (modified Duke's classification D). Two nude mice were used

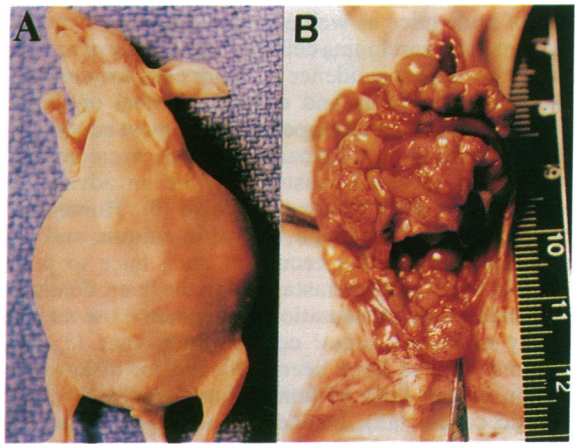

FIG. 2. (*A*) Nude mouse bearing orthotopically implanted human colon carcinoma. (*B*) Intraoperative view: Carcinomatosis growing extensively in nude-mouse abdominal organs and peritoneum after orthotopic implantation.

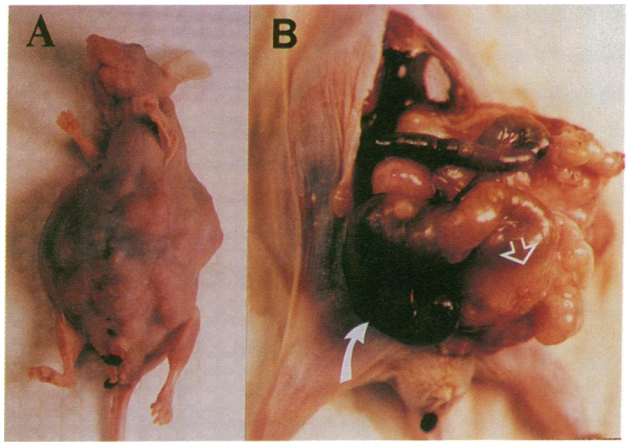

FIG. 3. (*A*) Nude mouse bearing orthotopically implanted human colon carcinoma. (*B*) Intraoperative view. Curved arrow indicates nude-mouse cecum. Hollow arrow indicates the site of implantation and tumor growth from there.

for tumor and normal-surrounding-tissue coimplantation to the cecum, and two nude mice were used for tumor direct implantation to the cecum. One mouse (direct implantation) was lost with no assessment of the tumor's growth. Orthotopic growth and abdominal metastasis occurred in the other three mice. A 10 × 10 mm primary tumor and 12 × 14 abdominal-wall metastasis were found at day 175 after implantation in one of the mice (tumor and normal surrounding tissue coimplanted). Lymph-node metastases were noted in this animal (Fig. 1). The histology of the original tumor and the orthotopically growing tumor both indicated adenocarcinoma. In the mouse with direct tumor implantation and in the other mouse with coimplantation of tumor and normal surrounding tissue, only local tumor growth occurred when observed at autopsy on days 159 and 230 after implantation, respectively.

General Abdominal Carcinomatosis with Extensive Peritoneal Seeding. An example is specimen case 1935, infiltrating mucinous adenocarcinoma moderately differentiated. Tumors were found to be growing in two mice after direct implantation. In one mouse 127 days after implantation, the primary tumor measured 19 × 16 mm. An abdominal mass measured 20 × 14 mm, and a mass on the pancreas, which was easily peeled off, measured 21 × 17 mm. In addition, extensive carcinomatosis was found with small tumors growing all over the peritoneum and abdominal organs (Figs. 2 *A* and *B*).

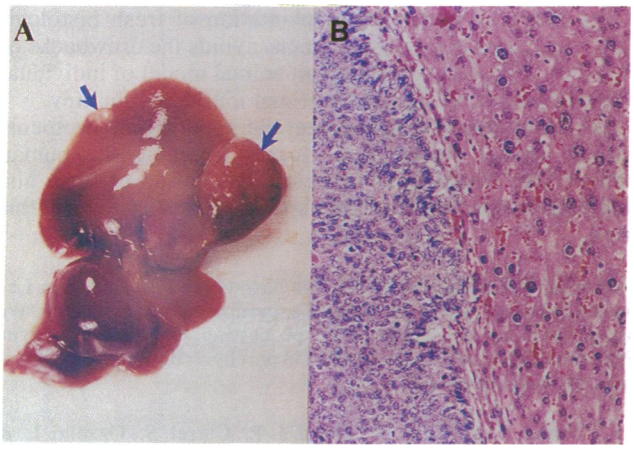

FIG. 4. (*A*) Nude-mouse liver involved with tumor metastases (arrows). (*B*) Pathohistology of tumor-involved nude-mouse liver as shown in *A*. (×130.)

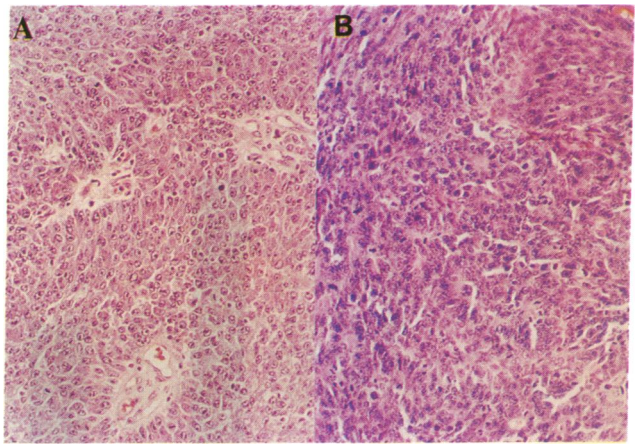

FIG. 5. (*A*) Pathohistology of original human-colon carcinoma prior to implantation. (×130.) (*B*) Pathohistology of primary tumor growth on nude-mouse colon as shown in Fig. 3*B*. (×130.)

Liver Metastasis. Specimen case 1594 is a high-grade poorly differentiated adenocarcinoma of the colon (modified Duke's classification B_2). Tumor pieces were implanted in two mice with preimplanted gelfoam (20 days after gelfoam implantation), and two mice were implanted with the internal skin-flap technique. Forty-eight days after implantation, one mouse (with skin-flap implantation) was found to have a palpable mass in the abdomen. The animal was examined and extensive tumor growth measuring approximately 25 × 25 mm was found. There was no liver or other distal organ metastasis. The animal died during surgery. Another animal (with skin-flap implantation) was found moribund 79 days after implantation. Autopsy showed the implanted tumor grew extensively in the colorectal area. Three tumor nodules measuring 9 × 9 mm, 11 × 7.5 mm, and 13 × 9 mm respectively were found, but no distal organ metastasis was observed. The animals with the internalized skin flaps overlaying the tumor implanted on the colon grew more extensively than those with gelfoam implantations. One mouse with gelfoam implantation was examined 56 days after tumor implantation. Primary tumor growth of approximately 12 × 9 mm was found, but no regional or distal organ metastasis was observed. The animal was sacrificed for pathohistology study of the abdominal masses that indicated adenocarcinoma. The other nude mouse with gelfoam implantation was moribund 160 days after tumor implantation (Fig. 3*A*). Autopsy was

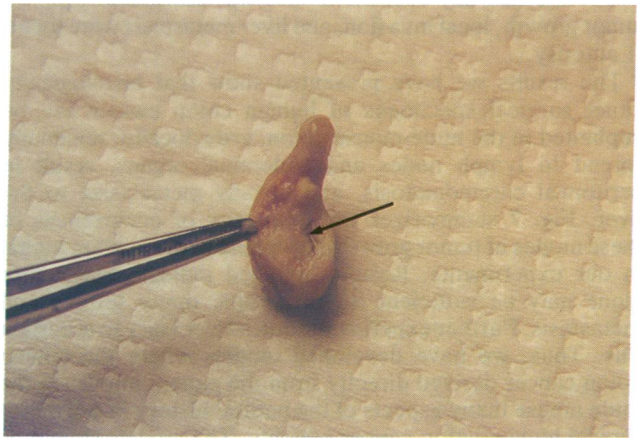

FIG. 6. Tumor-infiltrated nude-mouse cecum. Arrow indicates extremely narrowed lumen.

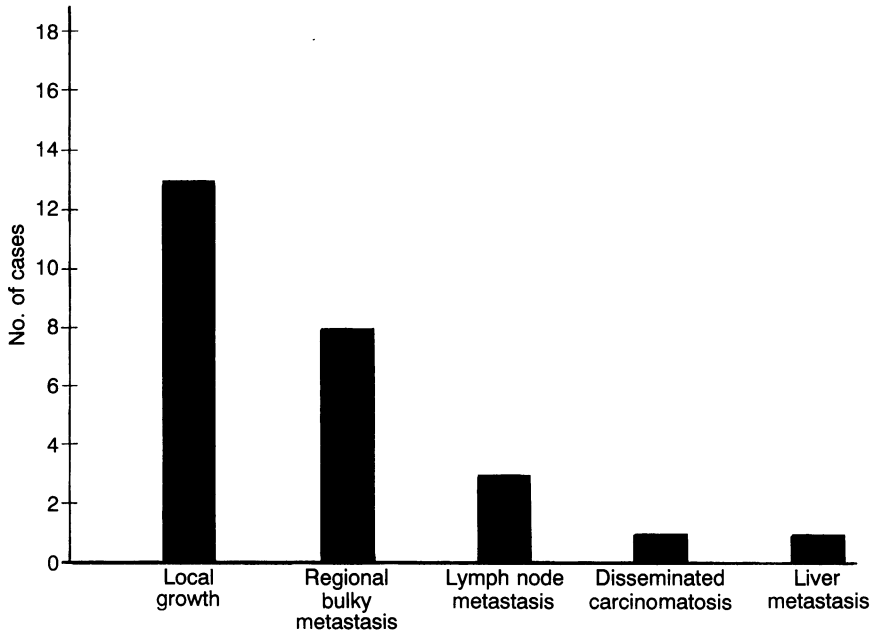

FIG. 7. Modes of growth and spread of human cancer specimens in nude mice after orthotopic implantation.

done, and the mouse was found to have extensive primary tumor growth measuring approximately 37 × 30 mm (Fig. 3*B*) and multiple liver metastases ranging from 1.5 × 1.5 mm to 9 × 9 mm (Fig. 4*A*). Pathohistology studies on the original tumor tissue (Fig. 5*A*), abdominal masses (Fig. 5*B*), and multiple liver lesions (Fig. 4*B*) indicated adenocarcinoma.

Colonic Obstruction. An example is specimen case 1701, infiltrating mucinous adenocarcinoma of the right colon. Fig. 6 shows the cecum to be infiltrated and obstructed 127 days after direct implantation of the specimen.

Cohort Construction. To utilize the models for testing treatment modalities, it is necessary to construct cohorts. We have now orthotopically passaged tumor from mouse 1594 six times and have developed a cohort of 12–20 animals with growing tumors, some of which have metastasized to the liver as occurred in the first group of animals implanted.

Sequential Appearance of Primary Tumor and Metastasis. Laparotomy was performed on day 26 on the nude mouse implanted with patient tumor 1594. Primary tumor growth was observed. No local or distal organ metastases were observed. The animal was returned for further observation on day 78 when the second laparotomy was performed. Primary growth was found. No liver or other distal organ metastasis was found. On day 160, the mouse was sacrificed. Primary tumor growth, local invasion, and liver metastasis were found at autopsy.

The results we have presented show that histologically intact surgical specimens of human colon cancer can be implanted in the nude-mouse colon, grow locally, regionally spread to lymph nodes and abdominal organs, result in peritoneal carcinomatosis, and distally metastasize to the liver. Fig. 7 summarizes the frequency of occurrences of these modes of tumor growth and spread in the implant model in our experiments. Results obtained indicate that tumor alone may be sufficient for orthotopic tumor growth and metastasis. Further experimentation is necessary to ascertain this. Thus, we have developed models where the human colon cancer derived directly from the patient mimics in the nude mouse many aspects of the natural history in a series of typical human colon-cancer patients. The models should be superior to existing animal models, which use highly deviated tumors to study new treatment modalities. The model may also be useful in predicting drug response or clinical course for individual patients (24).

Significance of These Findings. Subcutaneous tumor implantation had been a standard methodology for establishing animal models for human cancer research for years (4, 5). Although such a model has helped us to understand the nature and therapeutic treatment for human cancer, major problems still remain unresolved. One of them is that the tumor that is derived from a patient and subsequently put into immunodeficient animals subcutaneously no longer behaves as it did in the human patient—i.e., although the tumor can grow subcutaneously, the tumor is encapsulated and fails to metastasize either regionally or distally.

Recently a new strategy of what is called "orthotopic implantation" had been used for developing human tumor animal models (4, 5). Cell lines or disaggregated cells are injected to the corresponding organ of the mouse where the human tumor was derived. It was shown that this method of implantation allows metastasis to occur. However, the cell lines and disaggregated cells used for orthotopic implantation were obtained from breaking the original structure of human tumor tissue, which may lead to a change in the nature and the biological behavior of the tumor (25).

The model of orthotopic implantation of fresh histologically intact human tumor specimens avoids the drawbacks of previous animal models. Such an animal model of individual human tumors can facilitate optimal individual therapy.

The development of new cancer therapeutics and protocols require animal models that closely resemble the human patient. The model of orthotopic implantation of fresh histologically-intact human-tumor specimens seems to meet this need.

This paper is dedicated to the 70th birthday of Professor Sun Lee for his founding role in the field of experimental microsurgery. We are grateful for the expert word processing of Ms. Polly Jayne Pomeroy. This study was supported in part by a contract from Glaxo, Inc., to AntiCancer, Inc.

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