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# Nude Mouse Metastatic Models of Human Stomach Cancer Constructed Using Orthotopic Implantation of Histologically Intact Tissue

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## ABSTRACT

Nude mice have been used to develop s.c. growing human stomach tumors, but these rarely metastasize. Recently, I. J. Fidler and others have developed orthotopic implantation metastatic models using cell suspensions which are inoculated into the corresponding organ of nude mice from which the tumor cells were originally derived in the human. However, recent work has indicated that disaggregated cell suspensions may not always express their full metastatic potential. In this light, we have recently developed an orthotopic implant model utilizing intact tissue such as that obtained directly from surgery. This approach has yielded high take rates and frequent metastases in colon cancer, bladder cancer, lung cancer, pancreatic cancer, and prostate cancer. We report here the application of this intact tissue orthotopic implant technique to stomach cancer resulting in the formation of metastases in 100% of the mice with extensive primary growth to the regional lymph nodes, liver, and lung. In contrast, when cell suspensions were used to inject stomach cancer cells at the same site, metastases occurred in only 6.7% of the mice with local tumor formation, emphasizing the importance of using intact tissue to allow full expression of metastatic potential. Injuring the serosa similar to that occurring in intact tissue transplantation did not increase the metastatic rate after orthotopic injection of cell suspensions of stomach tumor cells. This intact tissue orthotopic implantation model should allow development of new treatment modalities and further study of the biology of human stomach cancer.

## INTRODUCTION

Stomach cancer is one of the two most prevalent cancers in the world. Despite this major problem, very few treatment strategies are effective for gastric cancers. Relevant animal models for human stomach cancer could possibly be highly important in the search for new therapeutics for stomach cancer.

With regard to models, human tumor xenografts grown s.c. in athymic nude mice closely resemble the original tumors morphologically, biologically, and biochemically (1-3). However, these tumors do not metastasize (4-6).

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, human colon cancer cells dissociated, grown in culture, and injected into the cecum of nude mice produce tumors which eventually metastasize to the liver, demonstrating that orthotopic implantation can enhance the metastatic capability of human tumor cells in nude mice (7-11). Similar results have also been achieved for orthotopic implantation of cell lines of human lung cancer (12), human pancreatic cancer (13), bladder cancer (14, 15), melanoma (16, 17), breast cancer (18-20), head and neck cancer (21), and stomach cancer (22).

However, recent work from our laboratory has indicated that cell suspensions used for orthotopic implantation may not express the full

metastatic potential of the original tumor compared with orthotopic implantation of histologically intact tissue (23).

Our approach is to avoid disruption of tumor integrity and to orthotopically implant histologically intact tumor tissue directly. With this overall strategy we have constructed models of human cancers in nude mice that can show the variety of clinical behaviors that occur in human patients. These models include colon cancer and have the following properties: (a) local growth; (b) abdominal metastasis; (c) general abdominal carcinomatosis with extensive peritoneal seeding; (d) lymph node metastasis; (e) liver metastasis; and (f) colonic obstruction. A very high take rate of 13 cases of 20 attempts was observed (24). We have also constructed models of human bladder cancer in nude mice that metastasize to the liver, spleen, lung, pancreas, and regional and distant lymph nodes (23). Additional models developed utilizing orthotopic transplantation of intact tumor tissue with resulting growth and patient-like metastases include lung cancer (25), pancreatic cancer (26), and prostate cancer (27). In this paper, we describe the application of this model to human stomach cancer where we find that orthotopic transplantation of histologically intact human stomach cancer to the subserosa of the stomach results in extensive orthotopic growth and metastases in 100% of the mice. In contrast, we observed metastases in only 6.7% of the mice with local growth resulting from inoculation of cell suspensions to the stomach wall. These results are similar to the results with bladder cancer where orthotopic implantation of intact tissue resulted in extensive metastases and that of a cell suspension did not (23).

## MATERIALS AND METHODS

**Mice.** Male BALB/c-*nu/nu* mice, which originated from the Central Institute for Experimental Animals (Kawasaki), were obtained from CLEA Japan, Inc., Tokyo, Japan. Animals which were 6 to 8 wk old and weighed 20 to 22 g were used.

**Human Gastric Cancer Xenografts.** Four human gastric cancer xenografts were used in the study. H-111, a well-differentiated adenocarcinoma line, was kindly provided by Dr. M. Fujita, Osaka University. SC-1-NU, a poorly differentiated adenocarcinoma line, was established in Nagoya University and provided by the Central Institute for Experimental Animals (Kawasaki). St-4, a poorly-differentiated adenocarcinoma line, and St-40, a well-differentiated adenocarcinoma line, were established in the Pathology Division of the National Cancer Institute. All the xenografts were maintained by serial transplantation into nude mice at the Keio University School of Medicine.

**Orthotopic Tumor Tissue Implantations.** Tumors at the exponential growth phase in nude mice were resected aseptically, necrotic tissues were cut away, and the remaining healthy tumor tissues were scissor minced into pieces about 5 to 7 mm in diameter in Hanks' balanced salt solution. Each piece of tumor was weighed on a Mettler AM 100 balance (Mettler Toledo Ag, Switzerland) and adjusted to be 150 mg with scissors.

Mice were anesthetized with 2.5% Avertin, and an incision was made through the left upper abdominal pararectal line and peritoneum. The stomach wall was carefully exposed, and a part of the serosal membrane, about 3 mm in diameter, in the middle of the greater curvature of the glandular stomach was mechanically injured using scissors. A tumor piece of 150 mg was then fixed on each injured site of the serosal surface with a 4-0 Dexon (Davis-Geck,

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Inc., Manati, PR) transmural suture. The stomach was then returned to the peritoneal cavity, and the abdominal wall and skin were closed with 4-0 Dexon sutures.

In another approach, tumor tissue was cut into smaller pieces of about 1 mm<sup>3</sup>. Eight to 15 pieces of this 1-mm<sup>3</sup> tumor were implanted on the top of the nude mouse stomach where the serosa had been injured. An 8-0 surgical suture was used to penetrate these small tumor pieces and suture them on the wall of the stomach. Then the abdominal wall and the skin were closed as described above. Animals were kept in a sterile environment.

**Orthotopic Tumor Cell Suspension Implantation.** Parallel tumor pieces of 150 mg were further scissor minced as finely as possible and incubated at 37°C for 30 min with an enzyme cocktail containing 0.5 mg/ml of actinase E (Kaken Pharmaceutical Co., Ltd., Tokyo, Japan), 0.2 mg/ml of collagenase (type I; Sigma, St. Louis, MO), and 0.1 mg/ml of DNase I (type IV; Sigma). After incubation, homogenates were passed through a stainless steel mesh (200 m/s), resulting in only negligible amounts of nondisaggregated fibrous tissue remaining. The filtrates were then washed in RPMI-1640 followed by centrifugation for 10 min at 3000 rpm, and the tumor cells were then suspended in RPMI-1640 to a total volume of suspension of 0.1 ml per one original 150-mg tumor piece used. The cell concentration of viable cells in the suspension was determined by the trypan blue dye exclusion test. The total cell number included in the cell suspensions was found to range from 1 to 4 × 10<sup>7</sup> per 0.1 ml in four tumor lines. Almost 100% of tumor cells were shown to be viable by the trypan blue test in every preparation.

The tumor cell suspension was injected into the middle of the greater curvature of the carefully exposed stomach as described above at a volume of 0.1 ml per mouse, such that an equivalent amount of tumor cells was injected as was used for intact tissue implantation.

**Evaluation of Growth and Metastasis of Orthotopically Implanted Tumors.** In the case of intact tissue implantation of 150-mg tumor pieces, mice were sacrificed 12 wk after implantations or earlier if they developed signs of distress. Since growth of the xenografts differed considerably, autopsies were performed from wk 2 to 4 for the SC-1-NU line, from wk 4 to 8 for the St-4 line, and from wk 8 to 12 for the St-40 and the H-111 lines after implantation. The implantation of multiple pieces of 1 mm<sup>3</sup> of intact tissue was used in addition for St-4, where the mice were allowed to grow for approximately 12 wk.

In the case of cell suspension implantation, mice were sacrificed 10 to 24 wk after implantations when they developed signs of distress. Mice in which tumors did not take were excluded from the study. Actual autopsies were performed from wk 10 to 16 for the SC-1-NU line and from wk 12 to 24 for the H-111, St-4, and St-40 lines. With longer growth time of the cell suspension implants, locally growing tumors attained weights equivalent to those in the case of intact tissue implantation. Autopsies were performed immediately, and

the tumors growing on the stomach wall were removed, weighed, and then examined histologically. The lungs, liver, and lymph nodes in the peritoneal cavity and other organs were processed for routine histological examination for metastases after careful macroscopic examination.

**Determination of Local Tumor Growth Rate.** For a particular study to compare local growth of orthotopically implanted intact tissue and cell suspension using the SC-1-NU line, mice were sacrificed at various times which were before they developed signs of distress. These mice were not evaluated for the formation of metastases. The growth rates of the locally growing tumors were determined by sacrificing 3 or 4 mice at various times and weighing the tumors that were removed.

**RESULTS**

**Growth Characteristics.** Fig. 1 shows the growth curve of the SC-1-NU line implanted orthotopically to nude mice as intact tissue or as a cell suspension. Tumor growth in orthotopically implanted intact

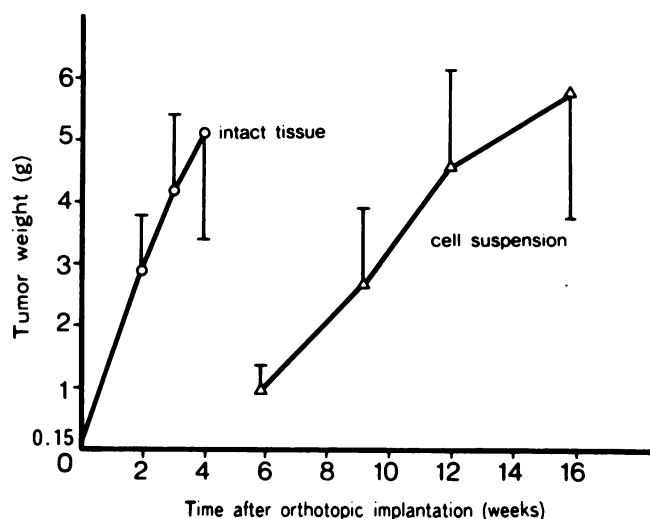


Fig. 1. Growth rate determined from the mean tumor weight of the SC-1-NU human stomach cancer line on the nude mouse stomach after orthotopic implantation as intact tissue or as cell suspension. Tumor growth was monitored at the various times indicated by sacrificing three or four mice whose tumors were removed and weighed. Points, mean weight of tumors from three or four mice; bars, SD. Please see "Materials and Methods" for procedures.

Table 1 Local tumor growth and metastases observed in orthotopic intact-tissue and cell suspension implantations

Local growth and metastatic capability after orthotopic implantation of tumor lines of human stomach cancer to the nude mouse stomach as intact tissue and cell suspensions are shown. Please see "Materials and Methods" for procedures.

Xenograft	Local tumor growth	Regional lymph node metastases	Distant metastases	
			Liver	Other organs
<i>Intact-tissue implantation</i>				
SC-1-NU	13/13 <sup>a</sup>	11/11 <sup>b</sup>	11/13	Lung, 3/9 <sup>c</sup>
H-111	4/4	4/4	1/4	None
St-4	5/5	5/5	2/5	Lung, pancreas, kidney, and adrenal gland, 1/5
St-40	4/4	3/3 <sup>b</sup>	4/4	None
Total <sup>d</sup>	26/26 (100) <sup>e</sup>	23/23 (100)	18/26 (69.2)	
<i>Cell suspension implantation</i>				
SC-1-NU	6/6 <sup>a</sup>	1/6	0/6	None
H-111	3/6	0/3	0/3	None
St-4	3/10	0/3	0/3	None
St-40	3/8	0/3	0/3	None
Total <sup>d</sup>	15/30 (50)	1/15 (6.7)	0/15 (0)	

<sup>a</sup> Data are shown as the number of mice in which local tumor growth or metastases were observed/number of mice evaluable.

<sup>b</sup> Lymph node metastases could not be evaluated because of massive i.p. growth of tumors in two mice in the SC-1-NU and one mouse in the St-40 xenografts.

<sup>c</sup> Lung metastases were not examined in four mice in the SC-1-NU xenograft.

<sup>d</sup> Total of four xenograft lines.

<sup>e</sup> Numbers in parentheses, percentage.

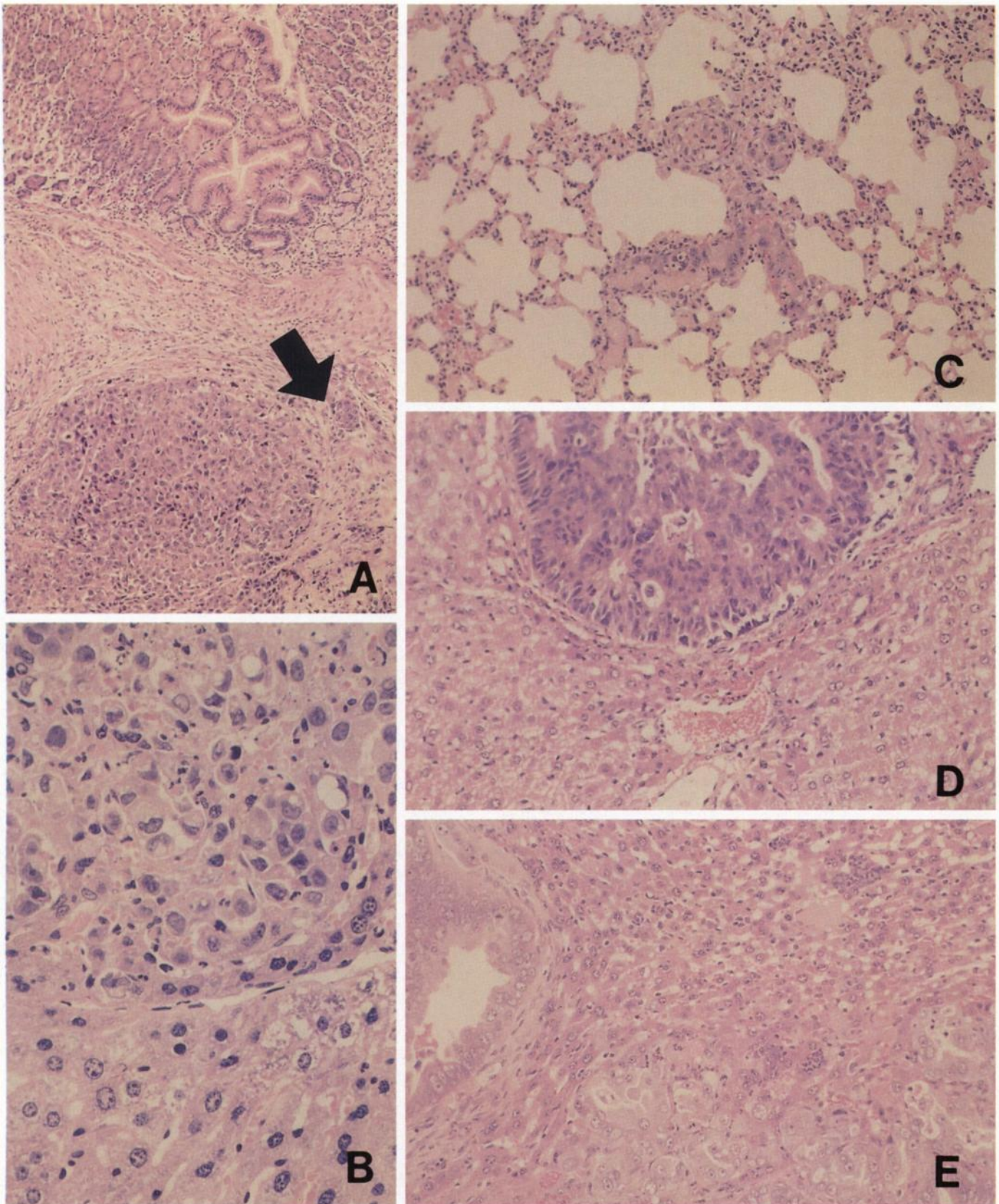


Fig. 2. Microscopic views of locally growing and metastatic tumors resulting from orthotopic implantation of intact tissue. *A*, locally growing SC-1-NU tumor (*arrow* shows the invasive growth of cancer cells into a lymph duct); *B*, liver metastases of the SC-1-NU tumor; *C*, lung metastases of the SC-1-NU tumor; *D*, liver metastases of the St-40 tumor; *E*, liver metastases of the H-111 tumor. Tissues were fixed, embedded, sectioned, and stained by H&E using standard procedures.

Table 2 Local tumor growth and metastases of human gastric cancer xenografts after orthotopic tumor cell suspension implantation on injured serosa

The stomach wall was carefully exposed, and a part of the serosal membrane, about 3 mm in diameter, in the middle of the greater curvature of the glandular stomach was mechanically injured using scissors. Tumor cell suspensions were then implanted into the muscular layer through the injured site of the serosal membrane, and the injured serosa was closed with a 4-0 Dexon suture. Mice were autopsied at 12 to 16 wk for the SC-1-NU line and at 12 to 18 wk for the St-40 line. The local tumors had grown over 1 cm in diameter.

Xenografts	Local tumor growth	Lymph node metastases	Liver metastases
SC-1-NU	5/5 <sup>a</sup>	0/5	0/5
St-40	3/4	0/3	0/3

<sup>a</sup> Data are shown as the number of mice with local tumor growth or metastases/number of mice evaluable.

tissue was more rapid compared with that of orthotopically implanted cell suspensions, despite the fact that almost 100% of the tumor cells were viable when implanted as cell suspensions.

**Local Tumor Growth and Metastases in Orthotopic Intact Tissue and Cell Suspension Implantations.** In the case of orthotopic implantation of cell suspensions of four tumor lines, although the take rates for local tumor growth were somewhat reduced from that of orthotopic implantation of intact tissue, 50% compared to 100% respectively, only one of 15 mice with local tumor growth had metastasis as opposed to 100% of the mice, after implantation of intact tissue, had metastases to local and distant regions (Table 1).

Invasive growth of cancer cells to lymph ducts was significant at the implantation site of intact tissue (Fig. 2A). Metastases observed after orthotopic transplantation of intact tissue included those to local and distant lymph nodes, the liver (Fig. 2, B, D, and E), the lung (Fig. 2C), the pancreas, the adrenal gland, and the kidney.

The general metastatic pattern after orthotopic implantation of intact tissue was the following (Table 1). For SC-1-NU, 11 of 11 mice had regional lymph node metastases, 11 of 13 had liver metastases, and 3 of 9 had lung metastases. For H-111, 4 of 4 mice had lymph node metastases, one of 4 had liver metastasis, and none had lung metastases. In the case of St-4, 5 of 5 mice had lymph node metastases, 2 of 5 had liver metastases, and one of 5 had lung, pancreas, kidney, and adrenal metastases. In the case of St-40, 3 of 3 mice had lymph node metastases, 4 of 4 had liver metastases, and none had lung metastases.

In contrast, Table 1 shows that, of all the mice implanted orthotopically with tumor cell suspensions, only one mouse had a metastasis, which was to a regional lymph node. The metastatic rate for cell suspension implantations did not increase when the serosa was first injured as in intact tissue transplantations described above (Table 2).

## DISCUSSION

Tumor implantation s.c. has been a standard methodology for many years for establishing animal models for human cancer research (6, 7). Although such a model has helped us to understand the nature and therapeutic treatment of human cancer, major problems still remain unresolved. One such problem is that the tumor that is derived from a patient and subsequently put into immunodeficient animals s.c. no longer behaves as it did in the human patient; *i.e.*, although the tumor can sometimes grow s.c., the tumor is encapsulated and usually fails to metastasize either regionally or distantly.

Recently a new strategy of what is called "orthotopic implantation" has been used for developing rodent models of metastatic human cancer (6, 7). In the first generation of these models, cell lines or disaggregated cells are injected into the organ of the mouse that corresponds with the organ from which the human tumor was derived. It was shown that this method of implantation allows metastasis to

occur in at least certain cases such as in colon cancers (7-11), pancreatic cancer (13), and stomach cancer (one case) (22). However, the cell lines and disaggregated cells used for orthotopic implantation were obtained from disrupting the original structure of human tumor tissue, which may lead to a change in the nature and the biological behavior of the tumor (28) and could be the basis of the greatly reduced metastatic rate observed in the present study and in a previous study with a bladder tumor (23), compared with orthotopic implantation of intact tissue. In our study, 100% of the mice orthotopically implanted with intact tissue formed metastases as opposed to only 6.7% of the mice implanted with cell suspensions forming metastases.

It should be emphasized that the cell suspensions utilized in the present study were almost 100% viable as determined by the trypan blue assay. It should be also noted that the cell number inoculated orthotopically in the cell suspensions was similar to that of the intact tissue, since a piece of tumor which had the same weight as that used for intact tissue implantation was disaggregated into a cell suspension. Although a 100% take rate is one advantage of intact tissue implantation, local tumor growth was also observed in 50% of the mice using cell suspension implantation. The growth rate of the locally growing tumor that formed after orthotopic implantation of the SC-1-NU cell suspension was less than that of the tumor that formed after orthotopic implantation of intact tissue. However, the locally growing tumors of all tumor lines that formed after orthotopic implantation of cell suspensions were allowed to attain, before sacrifice, a weight equivalent to those tumors that formed after orthotopic implantation of intact tissue. Therefore, cell viability of the inoculum of the cell suspension and resultant local tumor size do not seem to be the basis for the greatly reduced metastatic capability observed in our study.

Even first injuring the serosa, as in intact tissue transplantation, did not increase the metastatic rate after orthotopic transplantation of cell suspensions of stomach tumors.

Therefore, the very low metastatic rate observed after orthotopic implantation of cell suspensions may be due to the disruption of native cell-to-cell interactions necessary for full expression of the malignant characteristics of human cancer cells (28, 29). Thus, the tumors that formed after the orthotopic inoculation of cell suspensions may not be reformed natively, thereby greatly reducing their metastatic capability. It is possible in the cases where metastases were observed after orthotopic implantation of cell suspensions, such as in some colon cancer lines (8-11) and one stomach cancer line (22), that they were exceptionally metastatic and that the metastatic potential had progressed to the point of becoming independent of normal cell-to-cell interactions possibly required for tumors with lesser metastatic capability.

Thus, the model of orthotopic implantation of histologically intact human tumor specimens avoids the drawbacks of previous animal models. The development of new cancer therapeutics and protocols requires animal models that closely resemble the human patient. The model of orthotopic implantation of histologically intact human tumor specimens seems to meet this need.

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