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Preventing Liver Metastasis by Resecting the Primary Pancreatic Carcinoma at an Early Stage of Intrapancreatic Transplantation in Hamsters

Eiji Uchida, Matsuo Inoue, Takayuki Aimoto,
Yoshiharu Nakamura, Akira Katsuno and Takashi Tajiri

Department of Surgery, Division of Gastroenterology, General, Breast and Transplant, Nippon Medical School

Abstract

Purpose: To determine whether early primary pancreatic tumor resection can prevent liver metastases of intrapancreatic transplantation in a hamster model.

Methods: Cells from the PGHAM-1 cell line were transplanted into the pancreases of 30 Syrian golden hamsters. A suspension of 5×10^6 cells was injected into the splenic lobe of each pancreas. The primary pancreatic tumor was resected in 15 of the hamsters 10 days after transplantation (resection group). Fifteen other animals with transplantation but without resection served as controls (control group). All hamsters were killed 21 days after transplantation. The primary pancreatic tumors were measured for size and volume and examined histologically and immunohistologically for angiogenesis and tumor proliferation.

Results: In the resection group, small pancreatic tumors 4.7 ± 0.94 mm in diameter were found and resected 10 days after transplantation. Neither pancreatic tumors nor liver metastases were found in the resection group at the end of the experiment. All animals in the control group had pancreatic tumors 12.3 ± 3.29 mm in size, and 11 of 15 (73.3%) had liver metastases. The primary pancreatic tumors in the group with liver metastasis were significantly larger in diameter and volume than those in this group without liver metastasis ($p<0.01$). In the control group, proliferation of the primary pancreatic tumor, evaluated according to argyrophilic nucleolar organizer region, showed no differences within the pancreatic tumor group. On the other hand, the microvessel density of pancreatic tumors with liver metastases was significantly higher than that of tumors without liver metastases.

Conclusions: Our results suggest that 10 days after transplantation, the pancreatic tumors were small in size and volume and ready to proliferate but not yet ready to begin metastasizing through angiogenesis. This is one reason why early resection of the primary tumor prevents liver metastasis.

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Key words: pancreatic cancer, liver metastasis, prevention, angiogenesis, transplantation model

Correspondence to Eiji Uchida, Department of Surgery, Nippon Medical School, 1–1–5 Sendagi, Bunkyo-ku, Tokyo 113–8603, Japan
E-mail: uchida@nms.ac.jp
Journal Website (http://www.nms.ac.jp/jnms/)

Introduction

Cancer of the pancreas is the fifth leading cause of cancer death in Japan[1]. Pancreatic cancer is one of the most devastating diseases of the digestive organs. This poor prognosis for pancreatic cancer is due to 1) the lack of an effective method of early detection, and 2) its highly malignant characteristics, i.e., invasiveness and metastasis. Metastasis to the liver is thought to be a particularly significant problem in pancreatic cancer[2].

Published reports[3] raise doubts about the idea that patients with small tumors are more likely to have a better prognosis. Several reports indicate that even small pancreatic cancer tumors can metastasize to the liver[1,4,5]. Pancreatic cancer can metastasize to the lymph nodes and liver, even when the tumor is smaller than 2 cm in diameter[6]. In addition, clinical experience has shown frequent liver metastasis in patients that have undergone successful resection of the primary tumor at an early stage. However, surgery is now the only curative therapy in the management of pancreatic cancer, whether early resection of a small primary tumor is effective for preventing the induction of liver metastasis it is controversial, because the characteristics of pancreatic cancer may differ among patients because of differences in malignant potential.

The experimental model of pancreatic cancer induced by N-nitrosobis (2-oxopropyl) amine in Syrian golden hamsters is useful for investigating the pathogenesis of pancreatic cancer owing to similarities in pathological features and biological characteristics to human counterpart[7]. However, the model rarely induces metastasis to the liver. A few reports have been published on experiments using this model. They describe liver metastasis of pancreatic cancer, but note that splenic or portal implantation of cancer cells did not yield pancreatic tumors[8]. A good model has been needed to study the liver metastasis of pancreatic cancer. Recently, we developed a pancreatic cancer model with a high incidence of liver metastasis in hamsters using intrapancreatic transplantation of an established pancreatic cancer cell line (PGHAM-1). This model produces primary pancreatic cancer and liver metastasis within 21 days of inoculation with pancreatic cancer cells[9–12]. In a previous study, we performed sequential analysis of intrapancreatic transplantation of PGHAM-1 and found that liver metastasis did not appear until 14 days after transplantation[9].

The aim of this study was to clarify whether such characteristics as the size and volume of primary pancreatic tumors influence the induction of liver metastasis. In addition, the study investigated the effect of early surgical resection of the pancreatic tumor on the induction of liver metastasis. We tried to determine whether early resection of the primary tumor prevents the induction of liver metastasis in our experimental model.

Materials and Methods

Animals

A total of 30 5-week-old female Syrian golden hamsters (Shizuoka Laboratory Animal Center, Shizuoka, Japan) were used. They were housed in plastic cages under standard conditions (temperature: 20 ± 5℃; humidity: 40 ± 10%; light/dark cycle: 12 hours/12 hours) and given a commercially available basal diet (Oriental MF, Oriental Yeast, Tokyo, Japan) and tap water *ad libitum*. This experiment followed the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996).

Hamster Pancreatic Cancer Cell Line (PGHAM-1)

The pancreatic cancer cell line (PGHAM-1) used in this study originated from Syrian golden hamsters with pancreatic cancer induced by N-nitrosobis (2-oxopropyl) amine (BOP). The development of the PGHAM-1 cell line was first reported in Japan[9], and several studies using this cell line have been published[10–14]. The PGHAM-1 cells were maintained in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum, penicillin (100 IU/m*l*), kanamycin (100 IU/m*l*), and amphotericin B (250 μg/m*l*).

Intrapancreatic Transplantation of PGHAM-1 Cells

PGHAM-1 cells in volumes of 5×10^6 in MEM medium were prepared for transplantation and injected into the splenic lobe of the untreated pancreases of 30 5-week-old female hamsters. The PGHAM-1 cells were injected through a 1-cm laparotomy in the left upper abdomen under sodium pentobarbital (10 mg/kg body weight) anesthesia. After the swelling of the splenic lobe of the pancreas was confirmed, the abdominal wound was closed with a 3-0 nylon uninterrupted suture. Twenty-one days after transplantation, the animals were examined at autopsy, and the sizes of the primary pancreatic tumors were measured. The volume of each pancreatic tumor was calculated from the formula: volume = height × width × depth (mm)/: 2.

Experiment for Resection of Primary Transplanted Tumor

The primary pancreatic tumor was resected in 15 of the 30 hamsters receiving transplants (resection group). We resected the primary pancreatic tumor on the tenth day after transplantation because previous studies had shown no liver metastasis by the tenth day[9]. The animals were anesthetized with sodium pentobarbital, and then a small midline incision was made. The splenic lobe of the pancreas was pulled out from the abdominal incision, ligated with the fatty tissue and the spleen, and then excised. After hemostasis was confirmed, the abdominal wound was closed with an uninterrupted suture. Another 15 animals receiving transplants underwent laparotomy, but the tumors were not resected. These animals served as the positive controls (control group). All animals were killed 21 days after transplantation.

Histology of Pancreatic and Liver Tumors

The pancreas, liver, lung, and other macroscopically abnormal organs were pathologically examined. The organs were immediately fixed in 10% neutral buffered formalin and processed for histologic examiantion according to conventional methods. The sections, 5 μm thick, were stained with hematoxylin and eosin (H and E).

Immunohistochemical Staining

To assess tumor angiogenesis, paraffin-embedded sections of the pancreatic tumor were stained for factor VIII-related antigen (von Willebrand factor) using the avidin-biotin-peroxidase complex-immunoperoxidase method previously described[15]. We used rabbit polyclonal antibody against factor VIII-related antigen (DAKO Japan Co., Kyoto, Japan) and an avidin-biotin-peroxidase complex kit (Nichirei Co. Tokyo, Japan). The polyclonal antibody was diluted 1 : 200 and allowed to react with the tissue specimens for 1 hour at room temperature. Positive staining was detected by means of substrate reaction with diaminobenzine. After locating the area of highest microvascular density in the tumor and identifying the highest number of microvessels at 200× magnification in a single field (0.78 mm^2 per field), we evaluated the microvessel density (MVD) (per square millimeter).

Analysis of Tumor Cell Proliferation

To evaluate tumor cell proliferation, argyrophilic nucleolar organizer region (AgNOR) was stained using the method described by Ploton et al.[16]. The number of black dots in the nucleus was counted in 200 nuclei under 1,000× magnification using the method described by Howat et al.[17]. The mean number per nucleus was quantified as the AgNOR score[10,11]. In the analysis of the AgNOR score, we compared the scores of primary pancreatic tumors with and without liver metastasis.

Statistics

The results are expressed as means ± standard deviation (SD). Statistical analysis was performed using the unpaired *t*-test. Differences with a p value less than 5% were considered significant.

Results

Intrapancreatic Transplantation and the Effect of Resection of the Primary Tumor

The results are shown in **Table 1**. Ten days after transplantation, pancreatic tumors about 5 mm in diameter were found in all hamsters. In the resection group, these tumors were resected. The

Table 1 Size and volume of pancreatic tumors in control and resection groups

	Liver Metastasis	Size of Pancreatic Tumor	Volume of Pancreatic Tumor
Control (n=15)	11 (73.3%)	12.3 ± 3.29 mm	549.2 ± 332.7 mm^3
	+ (n=11)	13.8 ± 2.29 mm *	688.1 ± 277.1 mm^3 *
	− (n=4)	8.0 ± 1.12 mm	167.4 ± 69.4 mm^3
Resection (n=15)	0 (0.0%)	4.7 ± 0.94 mm	41.9 ± 25.5 mm^3

* P<0.01

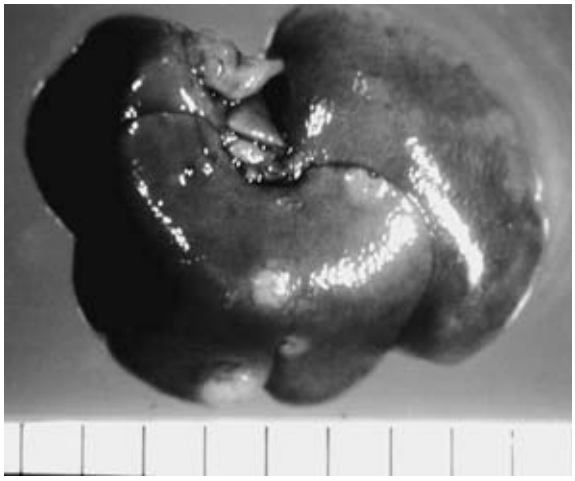

Fig. 1 Macroscopic appearance of liver metastases.

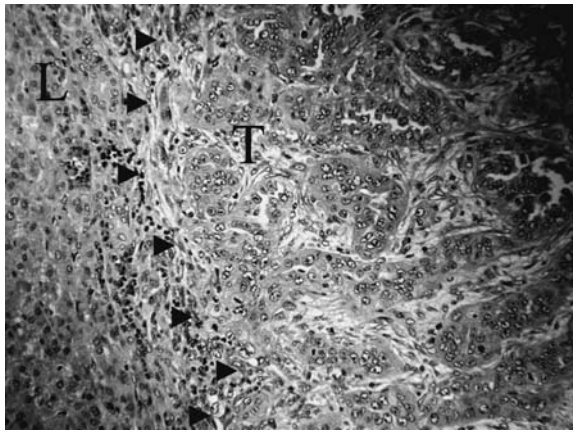

Fig. 2 HE staining of metastatic liver tumors. Tumors were moderately to well-differentiated adenocarcinomas. Original magnification, ×200

average diameter of the resected pancreatic tumors was 4.7 ± 0.94 mm, and the average volume was 41.9 ± 25.5 mm^3. When the hamsters were killed (11 days after resection), neither liver metastasis nor pancreatic tumors were observed in this group.

In the control group, all hamsters had pancreatic tumors, and 11 of the 15 (73.3%) had liver metastasis on the day they were killed **(Fig. 1)**. The size and volume of the pancreatic tumors with liver metastasis in this group were 13.8 ± 2.29 mm and 688.1 ± 277.1 mm^3, respectively. The size and volume of the pancreatic tumors without liver metastasis in this group were 8.0 ± 1.12 mm and 167.4 ± 69.4 mm^3, respectively. The differences in the size and volume of the pancreatic tumors in these two groups were statistically significant ($p<0.01$).

Histology of Pancreatic Tumors and Liver Metastasis

The main structure of all pancreatic tumors was moderately to well-differentiated ductal adenocarcinoma. The liver metastasis was of the same histological type **(Fig. 2)**. Livers without metastasis had no microscopic metastatic foci.

Immunohistochemical Analysis

There were many positively stained cells that defined the vessels in the pancreatic tumors 21 days after transplantation **(Fig. 3a)**, whereas there were a few positively stained cells in the pancreatic tumors that had been resected 10 days after transplantation **(Fig. 3b)**.

The MVD of the pancreatic tumors 10 days after transplantation in the resection group was 16.58 ± 4.48/mm^2. In the control group, the MVD scores in the pancreatic tumors with and without liver metastasis were 41.14 ± 6.24 and 30.77 ± 4.68, respectively. The differences between the two groups were statistically significant ($p<0.01$) **(Table 2)**.

Tumor Cell Proliferation

Tumor cell proliferation was estimated by means of AgNOR staining **(Fig. 4)**. The Ag-NOR score for the resection group was 9.20 ± 1.08. In the control

Table 2 MVD and AgNOR scores of pancreatic tumor in control and resection groups

	Liver metastasis	MVD		AgNOR Scores	
Control (n=15)	11 (73.3%)	38.37 ± 7.42		9.67 ± 0.98	
	+ (n=11)	41.14 ± 6.24	*	9.72 ± 1.10	NS
	− (n=4)	30.77 ± 4.68		9.50 ± 0.58	
Resection (n=15)	0 (0.0%)	16.58 ± 4.48		9.20 ± 1.08	

* P<0.01, NS: not significant

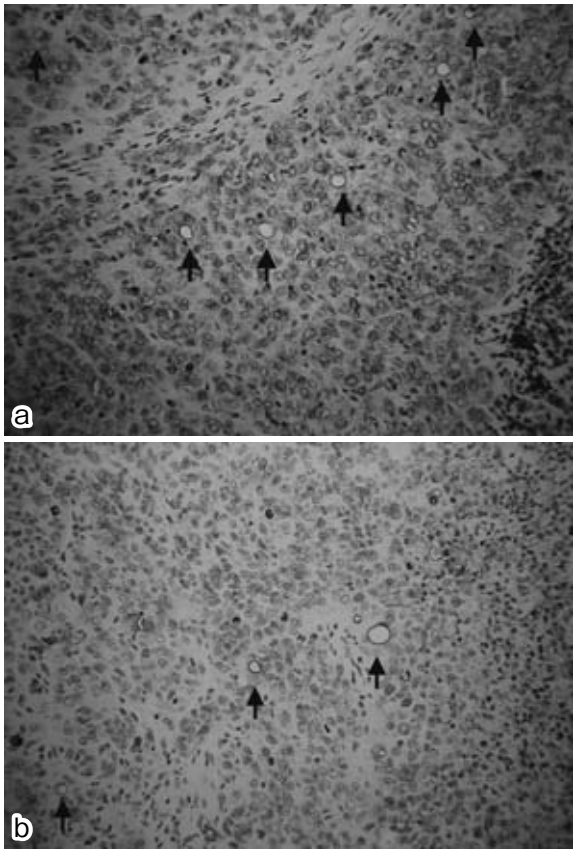

Fig. 3 Immunohistochemical staining of pancreatic tumors for factor VIII-related antigen in the control and resection groups. a) Many positively stained cells **(arrows)** define the tumor vessels within the pancreatic tumors of the hamsters in the control group. b) A few cells **(arrows)** were stained within the pancreatic tumors resected 10 days after inoculation. Original magnification, ×200.

group, the AgNOR scores of pancreatic tumors with and without liver metastasis were 9.72 ± 1.10 and 9.50 ± 0.58, respectively. The AgNOR score for the pancreatic tumors in the control group was not related to the induction of liver metastasis (**Table 2**).

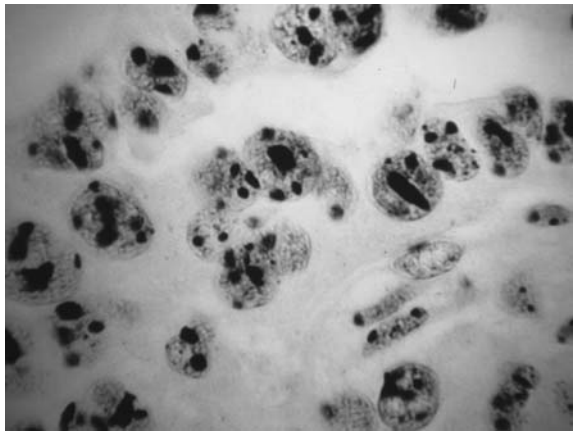

Fig. 4 Proliferation of pancreatic tumors was analyzed with AgNOR staining in the control group. The number of black dots in the nucleus indicates the level of proliferative activity in the tumor cells. Original magnification, ×1,000.

Discussion

Pancreatic cancer is the fifth leading cause of death in Japan and is known for its extremely poor prognosis due to liver metastasis, peritoneal dissemination, and local invasion[1]. The liver is the most common site of metastasis in pancreatic cancer, and until now there have been no effective treatment[18]. Moreover, small pancreatic cancer is difficult to diagnose at an early stage. Because some small pancreatic cancers can metastasize to the liver, surgery has little effect on survival in these cases. However, surgeons play an integral role in the management of patients with pancreatic cancer, with surgery providing the only potentially curative treatment for small pancreatic cancers.

The reasons for the high incidence of liver metastasis may be the aggressive biological characteristics of the pancreatic cancer cell itself

and the progression of the primary tumor. Inoue et al.[19], have reported that liver micrometastases might be present in patients with pancreatic cancer at the time of surgery despite the absence of macroscopic findings. However, if pancreatic cancer can metastasize to the liver due solely to its specific biological characteristics, then all cases of pancreatic cancer would show liver metastasis at an early stage. In fact, not all cases of pancreatic cancer clinically show liver metastasis[6]. This means that not every pancreatic cancer has the aggressive characteristics for liver metastasis at an early stage. Moreover, some pancreatic cancers might later gain a metastatic potential as a result of tumor progression.

There have been some reports on an experimental liver metastasis model of pancreatic cancer using a human cancer cell line and nude mice[8,20]. However, there may be many differences between humans and the nude mice model. Although the cancer cells used in these experiments originated in humans, the circumstances concerning cancer cells in nude mice are completely different from those in humans. The heterotrophic transplantation model used in previous studies ignores the histological and anatomic specificity of the primary organs and the distant metastatic organs[8]. On the other hand, hamster pancreatic cancer induced by N-nitrosobis (2-oxopropyl) amine resembles its human counterpart morphologically, biologically, and immunologically. In this experiment, intrapancreatic transplantation of PGHAM-1 could induce both primary pancreatic tumors and liver metastasis in a very short time while preserving the biological and immunological circumstances. The incidence of liver metastasis associated with primary tumors in this experiment was higher than has ever been reported for any other transplantation model[21]. This model was thought to be suitable for studying the mechanism of liver metastasis. Yokoyama et al.[9], have reported that liver metastasis was detected from day 14, whereas primary pancreatic tumors were found macroscopically on day 7 after intrapancreatic transplantation. Thus, we decided to resect the primary pancreatic tumor on the tenth day.

Our results showed that liver metastasis was induced in animals in the control group that had larger primary tumors. Moreover, resection of the primary tumor (about 5 mm in diameter) on the tenth day after transplantation completely inhibited liver metastasis until the end of experiment. Therefore, the primary pancreatic tumors in the resection group may have been unable to progress on the tenth day.

Nucleolar organizer regions (NORs) are defined as nucleolar components containing a set of argyrophilic proteins, and AgNOR protein expression is closely related to the cell duplication rate in cancer tissues[16]. The proliferation of pancreatic tumors, as estimated with AgNOR, which we have routinely used in studying PGHAM-1, was not related to the presence of liver metastasis in the two groups. This finding suggests that the primary tumors in both groups already showed the same proliferating activity and could gain proliferative ability on the tenth day, which was required for accommodation to the circumstances.

As a tumor grows, the primary tumor releases cancer cells, which ender the blood stream via the portal vein, resulting in the development of liver metastasis. The growth of solid tumors is generally dependent on angiogenesis, as is the process of metastasis[22]. The degree of angiogenesis assessed with MVD has been reported to be predictive for metastatic disease in breast cancer[15]. Seo et al.[23] have reported that vascular endothelial growth factor (VEGF) correlates closely with MVD and seems to be an important predictor of both liver metastasis and poor outcome in ductal pancreatic adenocarcinoma. Folkman et al.[24] have reported that cortisone and heparin are potent inhibitors of new vessel formation and that this inhibition is accompanied by reductions in the size of the primary tumor mass and in the incidence of metastasis. In fact, in this hamster model, a new selective matrix metalloproteinase inhibitor called MMI-166 proved to have antitumor activity through the mechanism of angiogenesis[11]. Yanagi et al. have reported that angiostatin inhibits the liver metastasis of PGHAM-1 by inhibiting angiogenesis and apoptosis[10]. In our experiment, factor VIII was used to evaluate MVD because of its usefulness

reported in previous studies[10–12], and high MVD scores in the tumors of the control group were associated with liver metastasis. In contrast, the MVD scores in the tumors resected on the tenth day after transplantation in the resection group were significantly lower than those in the control group, in which tumors were resected 21 days after transplantation, suggesting that primary tumors are not yet ready to initiate the metastasis on the tenth day. Moreover, the primary tumors may not have yet achieved the ability to progress on the tenth day, so surgical treatment before tumor progression might lead to curative resection.

In our present study, angiogenesis, as represented by the MVD score, was influenced by the size of the tumor. Because the risk of metastasis seems to increase with the size of the primary tumor, angiogenesis may play an important role in determining the spread of cancer. Thus, the status of the primary tumor is closely related to liver metastasis through angiogenesis. Human tumors are comprised of many different tumor populations, whereas all tumors in this experiment originated from a single established cell line. Therefore, theoretically, there may be a relationship between the size of pancreatic tumors and liver metastases. An analysis by Fortner et al.[25] has found that the size of the primary cancer is the single most important determinant of surgical cure. Other reports, however, suggest that tumor size itself has no effect on either the early or late course in patients with resectable exocrine pancreatic cancer[26]. In our experiment, resection of the primary pancreatic tumor in the early stage (5 mm in diameter on tenth day) prevented liver metastasis. Small tumors may have theoretically better prognosis through this mechanism, excluding individual biological characteristics.

Because the prognosis of pancreatic cancer may depend on its biological characteristics and on tumor progression, improving the survival rate for pancreatic cancer requires early detection and subsequent resection of small pancreatic tumors.

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