Subcutaneously Inoculated Cells and Implanted Pancreatic Cancer Tissue Show Different Patterns of Metastases in Syrian Golden Hamsters

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ABSTRACT

Context We studied behavior of the subcutaneously implanted pancreatic tumors and the process of metastasis using syngeneic Syrian golden hamsters.

Design HaP-T1, a cell line derived from nitrosamine-induced pancreatic cancer in Syrian golden hamsters was used for this experiment. Thirty-five animals were divided into two groups: subcutaneous cell inoculation and subcutaneous tissue implantation. The tumor tissue was obtained from subcutaneously implanted cancer cells. One month after implantation, the tumors were resected and studied histopathologically. The animals were followed-up weekly by palpation of the peripheral lymph nodes in order to identify local recurrence. After death, necropsy was performed. Liver, lungs and pancreas specimens were taken for histopathogical study and detection of K-*ras* point mutation using the PCR/RFLP method.

Results The mean survival time in the subcutaneous cell inoculation group was 151±17.5 days, and in the subcutaneous tissue implantation group was 137±12.9 days. During the follow-up, 13 subcutaneously cell inoculated hamsters (86.7%) had right axillary lymph node metastasis while subcutaneously tissue implanted hamsters did not show any palpable lymph nodes. After necropsy, 10 of the 20 subcutaneously tissue implanted animals (50%) showed metastases in the lungs at the histopathological level. However, 16 of the 20 subcutaneously tissue implantated animals (80%) showed K-*ras* point mutation in the lung specimens. The lungs of the animals of the subcutaneous cell inoculation group did not show any metastases. No metastases were found in the liver or the pancreas in either group.

Conclusion This study suggests that homologous subcutaneous cell inoculation and subcutaneous tissue implantation models showed completely different patterns of metastasis. These models may aid further research to clarify the mechanisms of metastasis in pancreatic cancer.

INTRODUCTION

The process of metastasis in pancreatic cancer is not clear. Subcutaneously implanted nude mice models have been widely used in *in-vivo* research. However, the response to the therapy may sometimes be a false positive. The immunological rejection response to the graft may give a false impression that the drug works [1]. The homologous implantation model appears to be suitable for *in-vivo* experiments because of the low rejection rate.

Nitrosamine-induced pancreatic cancer in Syrian golden hamsters resembles that of humans immunologically, biologically and morphologically [2]. Thus, subcutaneously implanted tumor models in syngeneic golden hamsters appear suitable for use in *in-vivo* experiments for the biological study of pancreatic cancer. However, there have been no studies done to see if cell implantation and tissue implantation show different metastatic behaviors in this model.

The purpose of the present study was to clarify the behavioral differences of subcutaneously inoculated pancreatic cancer cells versus subcutaneously implanted pancreatic cancer tissue with respect to the process of metastasis depending on the method of tumor implantation.

MATERIALS AND METHODS

Cells

A cultured cell line derived from a pancreatic cancer induced by *N*-nitrosobis (2-hydroxypropyl) amine (BHP) in Syrian golden hamsters, HaP-T1, established by Saito *et al.* [3], was used in this study. The cell culture was maintained in Eagle's Minimum Essential Medium, containing glutamine, non-essential aminoacids and $NaHCO_3$, as described previously [3], through serial passages. This cell line shows a mutation from GGT to GAT in codon 12 of the K-*ras* gene [4].

Animals

Thirty-five Syrian golden hamsters of both sexes from 8 to 26 weeks of age were used.

Preparation of Suspension of Cell Lines

Subconfluent cultures were washed once with PBS, and harvested with trypsin 0.25% and EDTA 0.02%. After checking cell viability with the trypan blue-dye exclusion test, the cells were counted and adjusted to $2x10^6$ cells/mL, using cold serum-free culture medium. They were kept cold until use.

Preparation of Tumor Grafts

0.1 ml of tumor cell suspensions was injected into the subcutis of 4 animals. After one month, the tumors were resected aseptically, and cut into pieces of approximately 1 mm^3. They were maintained in a cold serum-free medium until use. Part of the resected tumors was taken for histopathological study.

Experimental Design

The animals were divided into two randomized groups, subcutaneous cell inoculation (SCI), and subcutaneous tissue implantation (STI). All implantations in both groups were performed on the back of the animal. The growth of the tumor and the body weight were monitored weekly. After one month, tumorectomy was performed to avoid death of the hamsters due to tumor necrosis or invasion of the deep tissues. Thus, we could study the process of metastasis during the early stage of tumor growth. The resected tumor specimens were studied histopathologically. The animals were followed-up until death. After death, necropsy was performed.

Subcutaneous Implantation

SCI (n=15) animals were anesthetized with diethyl-ether inhalation. After asepsy, 0.1 mL of cell suspension was inoculated once, subcutaneously, using a 29-Gauge needle.

STI (n=20) hamsters were anesthetized with diethyl-ether inhalation. After asepsy, a hole was opened in the cutis using a scalpel, and then one piece of the tumor was implanted in the subcutis. The hole was closed using a 4.0 nylon suture (Keisei Co., Tokyo, Japan).

Resection of the Implanted Tumor

After one month, hamsters were anesthetized with diethyl-ether inhalation and sodium pentobarbital 5 mg/kg body weight, intraperitoneally. They were placed in a sterile field. Asepsy was carried out. The skin was opened with a scalpel 2 mm from the developed tumor to avoid rupture of the

capsule, and possible spreading of the tumor cells. The dissection was made "circumferencially" around the tumor. Thick vessels were linked using 4.0 nylon sutures. After the linkage, the tumor was isolated in the lower part using Kelly, and was sectioned. Therefore, the resected specimen consisted of tumor and the upper skin. Next, the hole was closed with simple sutures using 4.0 nylon.

Follow-up of the Animals

The hamsters were followed-up weekly, in order to observe local recurrence of the tumor, palpable lymph nodes and general condition, i.e. if they become moribund or not. After death, the animals were necropsied in order to confirm the presence or absence of metastasis. The liver, lungs and pancreas were removed and fixed in formalin and some parts were frozen in liquid nitrogen for DNA analysis.

Histopathological Examinations

The resected tumor and necropsied tissues were stained with hematoxilin-eosin and alcian-blue/periodic acid Schiff.

Detection of K-*ras* Point Mutation at Codon 12

DNA extraction and detection of the K-*ras* gene were made according to the previous described PCR/RFLP method [4]. When mutation is present, the sample shows two bands, a mutant and a wild type. DNA extracted from HaP-T1, and from the liver of a 12-week-old hamster without tumor, were used as positive and negative controls, respectively.

ETHICS

The Syrian golden hamsters (GN strain), used in the present study, were purchased from the Nippon Institute for Biological Science (Oume, Japan), and they were maintained in the Laboratory Animal Center of our University, in a 12h/12h light/dark cycle, fed standard rations and water *ad libitum*. The use

of these animals was approved by the Animal Studies Committee of our University.

STATISTICAL ANALYSIS

Results were shown as mean values ±SD. Survival time was compared between the groups by means of the Mann-Whitney test, while the Fisher exact test was used to compare the incidence of metastasis. Histopathology and PCR/RFLP analysis for the detection of lung metastases were compared by means of the Mc-Nemar test. Statistical evaluations were performed by means of the SPSS/PC+ package running on a personal computer. A two-tailed P value less than 0.05 was accepted as statistically significant [5].

RESULTS

Success rate of Implantation, Appearance of Resected Tumors and Survival Time

All 35 animals developed tumors at the site of implantation, palpable after one week. At the time of resection, the diameter of the tumor ranged between 18 and 22 mm. Five hamsters in the STI group, but none in the SCI group, showed adhesion to deep tissue. The skin covering the tumor was adhered in all animals of both groups. All tumors showed a well-circumscribed surrounding capsule. Some necrosis was found in the center of all tumors in the SCI group, and in the center and periphery of all tumors in the STI group. All resected specimens were confirmed histologically as moderately differentiated adenocarcinoma. However, tumors of the STI group consisted of more connective tissue, as shown in Figure 1. Thus, the success rate of implantation was 100%. The mean survival time in the SCI group was 151±17.5 days (range: 120-156), and in the STI group was 137±12.9 days (range: 130-170); the survival time was not significantly different between the two groups (P=0.200) (Table 1).

Table 1. Comparison between the subcutaneous cell inoculation (SCI) and subcutaneous tissue implantation (STI) groups.

|	SCI (n=15)	STI (n=20)	P values (SCI vs. STI)
Survival time (days)[a]	151±17.5 (range:120-156)	137±12.9 (range: 130-170)	P=0.200[b]
Success of implantation	15 (100.0%)	20 (100.0%)	ND
Local tumoral recurrence	0	0	ND
Regional metastasis	13 (86.7%)[c]	0	P<0.001[c]
Distant metastasis			
- Liver	0	0	ND
- Lung (Histopathological findings)	0	10 (50.0%)	P=0.002[c]
- Lung (K-*ras* point mutation by PCR/RFLP)	0	16 (80.0%)[d]	P<0.001[c]
- Pancreas	0	0	ND

ND: not done; NS: not significant

[a] Mean ± SD

[b] Mann-Whitney test

[c] Fisher Exact test

[d] P=0.031: histopathological findings vs. detection of K-*ras* point mutation (McNemar test; STI animals)
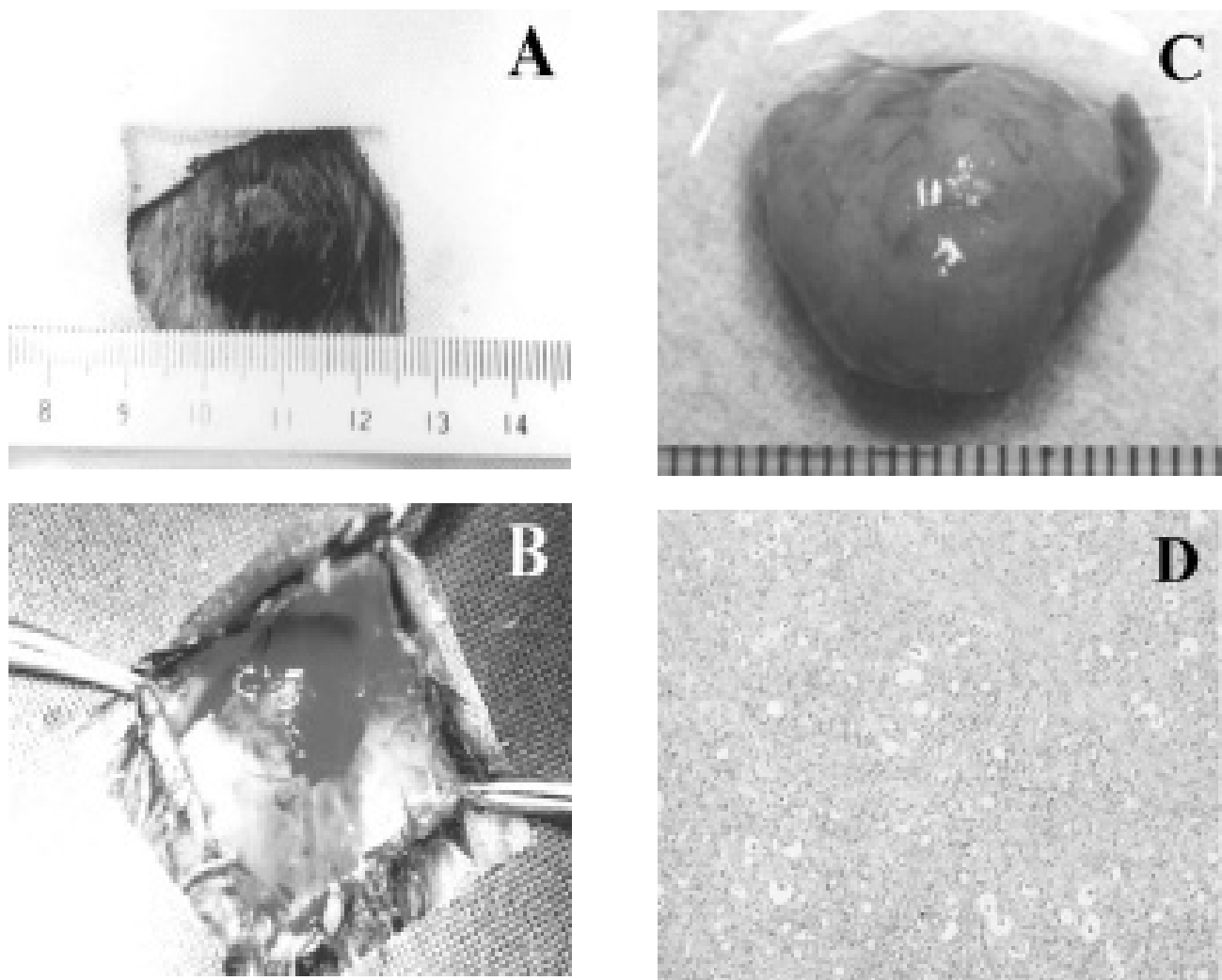

Figure 1. A subcutaneously implanted hamster. **A.** Appearance before tumor resection. **B.** Panoramic view after resection. **C.** Resected specimen without the covering skin. **D.** Histopathologic view showing a moderately differentiated adenocarcinoma (H&E, 200x).

Follow-up, Local Recurrence and Findings in the Necropsy

In the follow-up, neither the SCI nor the STI group showed local recurrence at the site of implantation. Thirteen SCI hamsters (86.7%) developed right axillary lymph node metastasis (Table 1), which was palpable between 4 and 12 weeks after the resection. This lymph node in the early stage was movable. After approximately 2 weeks, it became adhered to deep tissue and grew to 6 cm in diameter. When the lymph node reached this size, the animals died. In the necropsy, there was no local recurrence, or macroscopic internal-organ metastases. The metastatic axillary lymph node showed central necrosis (Figure 2). All lymph nodes were confirmed as metastasis at the histopathological and molecular level. The lungs, liver and pancreas did not show any metastases. The remaining two animals of this group without axillary lymph node metastasis did not have any metastases in their internal organs. The cause of death was unknown.

The STI animals did not show any palpable lymph nodes. They became moribund about 17 to 21 weeks after tumorectomy, and quickly lost weight (about 10 g/day during the last 3 days of life). The occurrence of axillary lymph node metastases was significantly lower than that observed in the SCI group (P<0.001). After death, when necropsy was performed, 10 of 20 animals (50.0%) showed metastases in the lungs at the histopathological level, which was significantly higher when compared with SCI group (P=0.002). On the other hand, the incidence of lung metastases found by PCR/RFLP analysis (Figure 3) was 80.0%. This figure was significantly higher in comparison with the SCI group as well as in comparison with the histopathological findings observed in the same animals (P=0.031). No metastases were found in the liver and pancreas. The remaining 4 animals in this group did not show any macroscopic or microscopic metastatic sites.
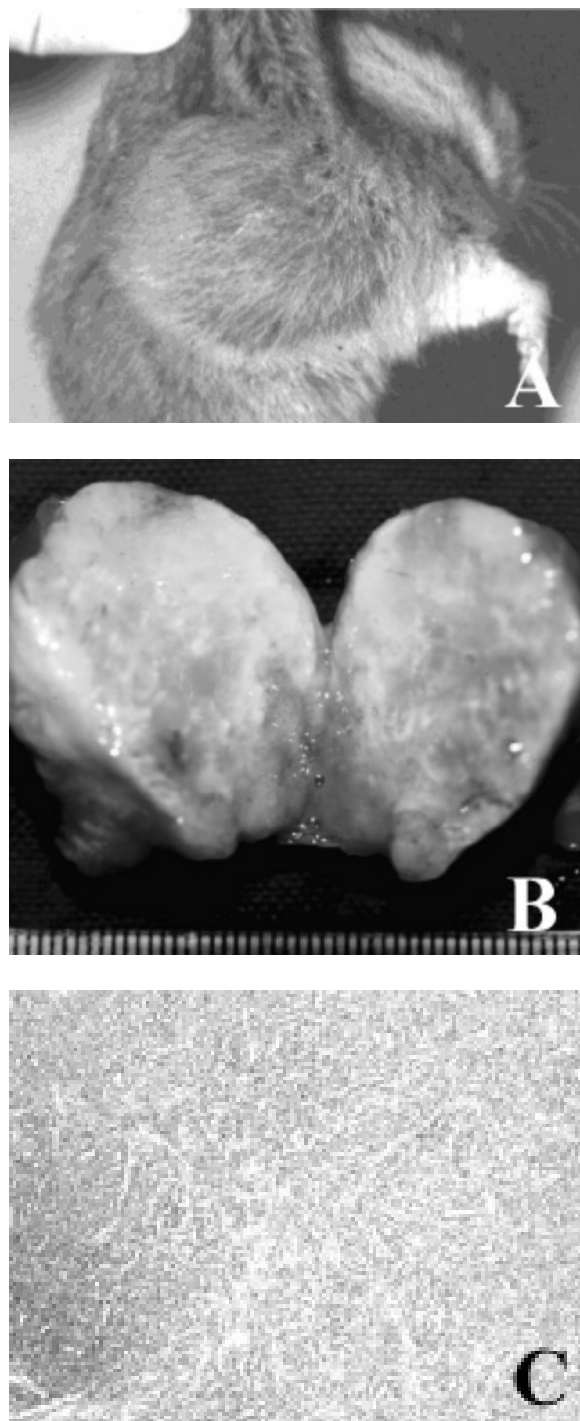
Figure 2. A case from the SCI group. **A.** Appearance of the right lymph node. **B.** Necropsied lymph node specimen cut sagitally. **C.** Histopathologic view of the lymph node (H&E, 100x).
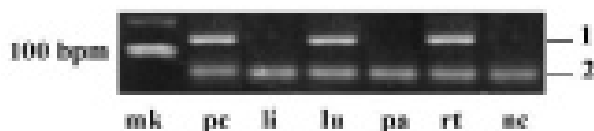
Figure 3. An ethidium-bromide stained agarose gel electrophoresis of PCR/RFLP analysis. (1: mutant band; 2: wild band; mk: marker; pc: positive control (HaP-T1 cell line); li: liver; lu: lung; pa: pancreas; rt: subcutaneously-resected tumor (STI); nc: normal liver tissue.

DISCUSSION

With the purpose of studying the response to the drugs *in-vivo*, many studies prefer to use the subcutaneous transplantable tumor models because the method is simple and monitoring of the results is easy. Since the majority of these experiments use human cell lines, nude mice have been widely used. However, in most of these studies, the animals have not been studied over a long period because of the immunological response which increases as the animal gets older [6]. Klein and Bevan [7] stated that it could be due to age-related reconstitution of the nude mouse immune system by endogenous production of interleukin-2. Thus, monitoring of the local response to the therapy obscures the validity of long-term treatment studies since tumor reduction may be due to histocompatibility of the tumor rejection rather than specific anti-tumor activity. Therefore, we used a homologous model of subcutaneous implantation.

Kyriaziz et al. [8] subcutaneously injected human carcinoma of the larynx and human colon carcinoma cell lines into nude mice. After observing for 6 months, they noted regional lymph node metastases, capsule infiltration and invasion of lymphatic vessels. However, they did not resect the local tumor, as was done in the present study. In our preliminary findings, when the local tumor was resected within 21 days, no metastases were found in either group at 6 months follow-up (data not shown).

Kyriaziz et al. [9] implanted pieces of various human tumors, including pancreatic cancer, in the anterior part of the lateral thoracic region in nude mice. Most of the implanted tumors metastasized to regional and mediastinal lymph nodes and the lungs. The authors stated that the lung metastases occurred through lymphatic and hematogenous routes.

There appear to be two patterns of arrest of tumor cells at target sites; one is based on anatomic and physiologic factors, and the other is based on selectivity [1]. In the pathogenesis of the metastasis cascade, the process consists of a long series of sequential interrelated steps. Thin-walled venules, like lymphatic channels, offer very little resistance to penetration by tumor cells and provide the most common pathways for tumor cell entrance into circulation [1, 10]. Thus, cells entering the lymphatics could have been sequestered via the draining lymph node in the SCI group. Metastatic cells of STI group could have entered the venous circulation, via the metastatic cascade, and encountered their first barrier in the lung, as Nicholson and Poste reported [1]. This could be explained by the non-specific trapping of metastatic tumor cells, which often occurs in the first organ encountered by the circulating cells. Greene and Harvey [11] stated that the localization of a metastatic colony to a particular organ might depend on the formation of an initial bond between the tumor cells and the adhesive molecules on the luminal side of the vascular endothelium of that organ. However, the reasons why lymphatic dissemination was absent in the STI group should be clarified in the future.

Growth rates, invasiveness and metastatic behavior of the transplanted tumors differ depending on the route of implantation [2, 12]. Although the subcutaneous route of implantation was similar in the present experiments, the histology of the STI resected tumors showed more developed architecture of the interstitial tissue. Therefore, the tumoral architecture, including vascularization, may be an important factor in the process of the spread of metastatic cells. Moreover, in the STI model, it should be taken into account that the implanted tumor tissue was derived from the tumor cell line inoculated subcutaneously in Syrian golden hamsters. The different pattern of metastasis might be due to the fact that a second step of

implantation could modify the cell biology, thus favoring hematogenous spread.

In human pancreatic cancer, the first target organ of hematogeneous metastases is the liver because the tumoral cells usually spread via the portal vein [13]. We reported a high liver metastatic rate when orthotopic implantation of pancreatic tumor tissue was performed [4]. Thus, other routes of tumor implantation, i.e. intrapancreatic or intraperitoneal, could favor tumor spread to the liver, more closely resembling to the human pattern. Lung metastases found in the present experiments may be a consequence of non-specific trapping of metastatic tumor cells, as stated before.

Ductal adenocarcinoma, the most common form of pancreatic cancer in humans, is associated with activation of the K-*ras* oncogene in approximately 90% of cases [14]. This has been shown in other nitrosamine pancreatic cancer induced models to take place an elevated high rate [9, 15, 16, 17]. The present cell line has had the point mutation confirmed [4]. Moreover, the early diagnosis of pancreatic cancer in humans may be carried out by the detection of the K-*ras* point mutation. In the present study, the incidence of lung metastases found by PCR/RFLP analysis was greater than the histopathological findings (80% vs. 50%) and was statistically significant as well. Thus, detection of the K-*ras* point mutation could identify the micrometastasis, which could not be found using histopathologic examinations alone. Therefore, the sensitivity of the detection of the K-*ras* point mutation might be greater than that of histology.

The cause of death of the animals not showing any metastases was unknown. However, we believe that it was related to the metastasis phenomena, considering that healthy animals have a two to three year life-span. We think that these animals could have had metastases in the brain, bone or bone marrow, sites, which were not observed at necropsy.

In conclusion, homologous SCI and STI models showed different patterns of metastasis. The event of metastasis appeared to occur between 21 and 30 days after subcutaneous implantation. The SCI model combined with tumorectomy may be used as a lymphatic metastasis model, (simple to monitor) and the STI model combined with tumorectomy may be used as a hematogenic metastatic model of the lung. However, these findings are preliminary and further studies are required to clarify the mechanisms of metastasis. In the future, the different patterns of metastases after implantation of tumor cells and tumor tissue should be evaluated using other cell lines.

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