—Original—

Reexpression of Reduced VEGF Activity in Liver Metastases of Experimental Pancreatic Cancer

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Abstract

Purpose: Vascular endothelial growth factor (VEGF) is thought to play a crucial role in the process of cancer growth and metastasis. In this study, the expression of VEGF in liver metastases of pancreatic cancer was investigated using an established hamster model.

Methods: Pancreatic cancer cells (PGHAM-1, 1×10^6) derived from N-nitrosobis (2-oxopropyl) amine (BOP)-induced pancreatic tumors in Syrian golden hamsters were transplanted into the pancreas of female hamsters. All hamsters were sacrificed at 21 days after transplantation and used for the histopathological examination of pancreatic and metastatic lesions (primary transplantation model). The metastatic liver tumors were minced with scissors and 1 mm^3 tumors were retransplanted into the pancreas of a second hamster. All hamsters were sacrificed 21 days after retransplantation, and the pancreatic tumors were removed (back transplantation model). Immunohistochemical analyses using antibody against VEGF were performed for all pancreatic and liver tumors. Reverse transcription-polymerase chain reaction (RT-PCR) was performed to examine the expression of VEGF mRNA in the tumors. In addition, we investigated the proliferation of each tumor using Ag-NOR staining.

Results: In the primary transplantation models, VEGF expression in the pancreatic tumors was positive, but that in the liver metastases was only weakly positive or negative. On the other hand, VEGF expression in the pancreatic tumors that had developed from the retransplantation of the liver tumors (back transplantation model) was strongly positive. VEGF mRNA was expressed in the pancreatic tumors of both primary and back transplantation models. In the metastatic liver tumors of the primary transplantation model, VEGF mRNA was expressed in all cases, although the immunohistochemical staining pattern was weakly positive or negative. Similarly, in the metastatic liver tumor of the back transplantation model, VEGF mRNA was expressed in all cases, although the immunohistochemical staining pattern was weakly positive or negative. No significant differences in Ag-NOR scores were found between the models.

Conclusion: Our results suggest that VEGF expression usually occurs in PGHAM-1 cells but that VEGF expression is reduced during the process of liver metastasis and revived by retransplantation. Thus, the interrelationship between cancer cells and the organ environment might play an important role in VEGF expression.

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Introduction

The incidence of carcinoma of the pancreas has steadily increased over the last 4 decades and now ranks as the fifth most common cause of cancer-related deaths in Japan[1,2]. The prognosis after operation remains poor because of the high incidence of recurrence and metastasis, despite the development of imaging diagnosis, surgical techniques and systemic chemotherapy[3]. The liver is the most common and critical site of distant metastasis, influencing the prognosis of patients with resected ductal pancreatic carcinoma[4].

The process of metastasis is thought to be extremely complicated: tumor cells at the primary site must invade newly formed vessels, circulate, proliferate at the secondary site, and neovascularize[5]. Among the diverse angiogenic molecules that have been described as direct-acting endothelial cell mitogens, vascular endothelial growth factor (VEGF) is thought to play a crucial role in the process of metastasis. Recent studies have demonstrated that VEGF expression at the primary site is correlated with metastatic ability in pancreatic cancer and colorectal cancer[6,7]. Furthermore, angiogenesis at the primary and metastatic sites is necessary for tumor progression and proliferation, and VEGF is thought to be play an important role in these processes. Some studies have shown that a high expression of VEGF at the primary site is correlated with a poor prognosis for various tumors[6,7]. However, only a few investigations regarding VEGF expression at the metastatic site have been reported, some of which found that VEGF expression was reduced in metastatic liver tumors, compared with that at the primary site[8,9].

We recently established a carcinoma cell line (PGHAM-1) originating in a transplantable subcutaneous tumor induced by N-nitrosobis (2-oxiopropyl) amine (BOP) in Syrian golden hamsters. The pancreatic tumors induced by PGHAM-1 consist of well -differentiated ductal adenocarcinomas that closely resemble human pancreatic carcinomas. Liver metastasis occurs at a high rate after the intrapancreatic transplantation of PGHAM-1[10–13]. The PGHAM-1 cell line is thus useful for studying both pancreatic cancer and liver metastasis.

Only a few studies have investigated the expression of VEGF in metastatic liver tumors of pancreatic cancer using experimental models. In the present study, we investigated the expression of VEGF in metastatic liver tumors of an experimental pancreatic cancer model induced by the intrapancreatic transplantation of PGHAM-1.

Materials and Methods

1. Animals

Female 5-week-old Syrian golden hamsters were obtained from the Shizuoka Experimental Animal Center (Shizuoka, Japan). The animals were kept under standard laboratory conditions (temperature, 22±3℃; relative humidity, 40±5%; light/dark cycle, 12 h/12 h) and given a standard diet (MF-1, Oriental Yeast Co., Ltd., Tokyo Japan) and water *ad libitum*. The protocols of these animal experiments were approved by the Animal Experiment Committee of Nippon Medical School.

2. Hamster Pancreatic Cancer Cell Line

BOP was used to induce pancreatic cancer in hamsters using a previously reported method[10–13]. The pancreatic tumors were minced with scissors, and 1 mm^3 tumors were subcutaneously transplanted via a trocar into the interscapular area of hamsters. The recipient hamsters were sacrificed 6～8 weeks after transplantation, and a portion of the tumor tissue was serially transplanted. After 8 subcutaneous transplantations, the tumor was extracted under germ-free conditions, and minced in 0.05% trypsin and EDTA solution at 37℃ for 10 minutes, then centrifuged (10 minutes, 4,000 g). The cells were maintained in plastic culture flasks (Corning, NY, USA) containing Dulbecco's modified Eagle's medium (MEM: GIBCO, Gland Island, NY,

USA) supplemented with 10% fetal bovine serum (FBS), 100 units/m*l* of penicillin-streptomycin, 100 μg/m*l* of kanamycin, and 100 μg/m*l* of amphotericin B (GIBCO). The flasks were kept at 37℃ in a 5% CO_2 incubator. After maintaining the cultures for 60 passages from the first colony in 1996, the cells that had become established as a new cell line were named PGHAM-1. PGHAM-1 cells have been maintained for 8 years without remarkable biological and morphological changes.

3. Experimental Models

(1) Primary transplantation model

Under adequate anesthesia using diethyl ether, laparotomies were performed in 5-week-old Syrian golden hamsters (n = 15), in each animal, a suspension of PGHAM-1 cells (1×10^6 cells/0.1 m*l* in MEM) was injected into the splenic lobe of the pancreas via a 27 G tuberculin needle. Twenty-one days after transplantation, the hamsters were sacrificed. Pancreatic tumors and metastatic liver tumors were fixed in 10% formalin and embedded in paraffin for HE and immunohistochemical staining. At the same time, a portion of pancreatic tumor and metastatic liver tumor was preserved at −70℃ for RT-PCR.

(2) Back transplantation model

The metastatic liver tumor with negative VEGF expression was used for the back transplantation. The metastatic liver tumors obtained from the primary transplantation models were divided into two blocks: one for use in the pathological examination and the other for use in the back transplantation. The back transplantation was performed as follows: half of the tumor was minced with scissors, and a portion of the fragment was transplanted into the pancreas of a new hamster (n = 10). Twenty-one days after transplantation, the hamsters were sacrificed, and the pancreatic tumors and liver metastases were removed. The tumors were examined using a routine pathological study, and a portion of the tumors was preserved at −70℃ for RT-PCR.

4. Immunohistochemical Staining for VEGF Protein

Tissue blocks of the tumors removed from both models were sliced into 3-μm-thick sections. A standard immunoperoxidase method using a streptavidin-biotinylated-peroxidase complex was used to detect VEGF in deparaffinized tissue sections. Deparaffinized tissue sections were incubated with a mouse monoclonal antibody against VEGF (IBL Japan Co., Gunma, Japan) overnight at 4℃. The antibody dilution used to detect the antigen was 1 : 100. Subsequent steps were performed using a streptavidin-biotinylated-peroxidase (MULTI) kit (SAB-PO (M) kit); (Nichirei, Tokyo, Japan). The sections were counterstained using methyl green. The cross reactivity of the VEGF antibody was tested using pancreatic islet cells from a normal hamster as a positive control. Normal mouse serum was used as negative control. The extent of VEGF staining was graded on a scale of (−) to (3+), with (−) representing no detectable stain, (+) representing positive staining in less than 30% of the tumor cells, (2+) representing positive staining in 30%～80% of the positive tumor cells, and (3+) representing positive staining in more than 80% of the tumor cells.

5. RT-PCR for VEGF mRNA Expression in PGHAM-1 Cells and Tumors

Reverse transcription polymerase chain reaction (RT-PCR) analysis was performed to examine the expression of VEGF mRNA in the PGHAM-1 cells and tumors. Total cellular RNA from 1×10^6 PGHAM-1 cells was prepared using ISOGEN (Nippongene, Osaka, Japan) and the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method[14], according to the manufacturer's protocol. Total RNA (0.5 μg) extracted from the PGHAM-1 cell line was then used as a template for reverse transcription (RT) prior to the start of the PCR. PCR was performed at 94℃ for 1 minute, at 55℃ for 1 minute (annealing), at 72℃ for 1 minute (extension), for 25 cycles. The amplified products were then separated by electrophoresis on a 2% agarose gel and stained using 1 μg/m*l* ethidium bromide.

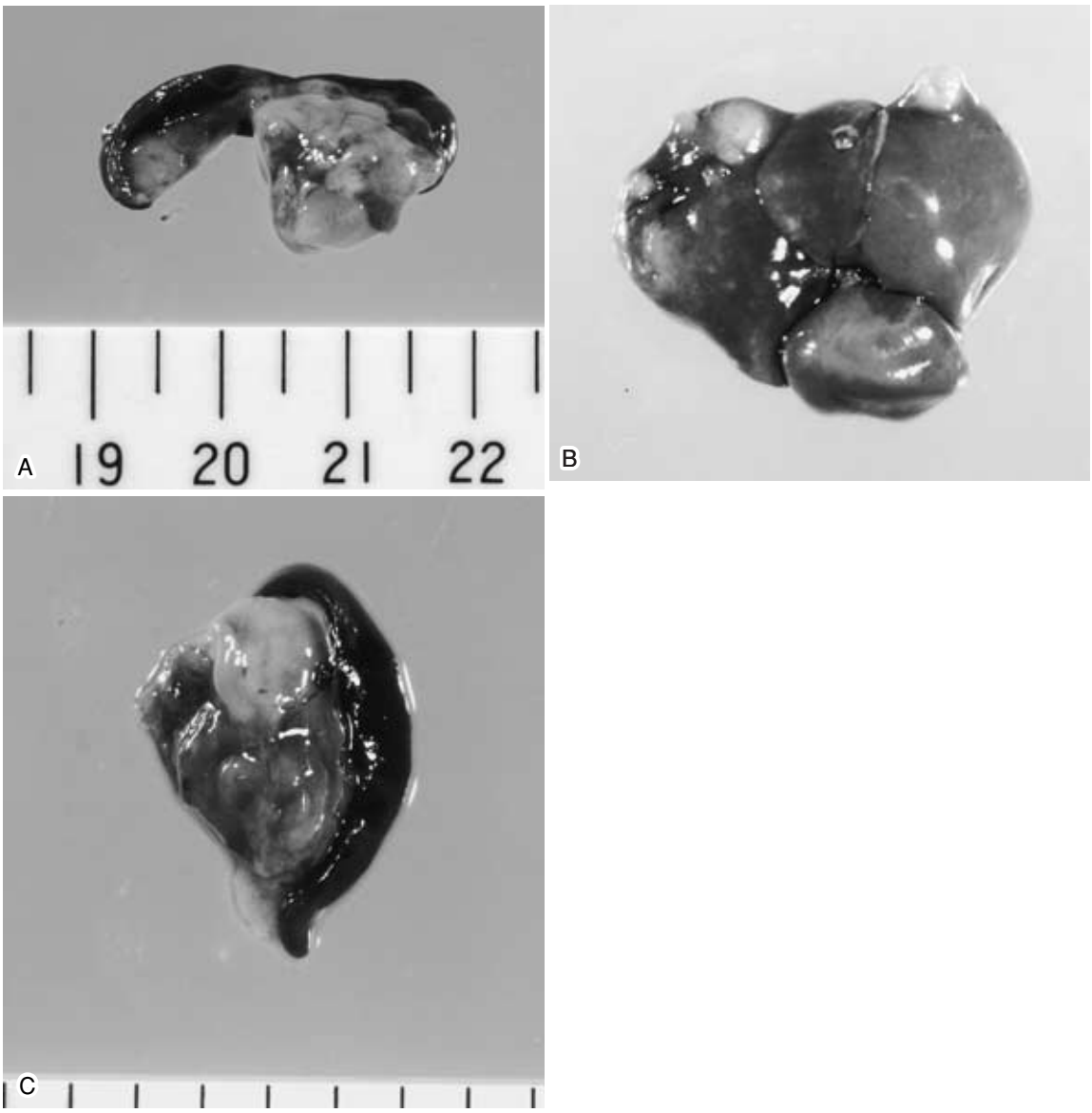

Fig. 1 Macroscopic appearance. **A**: Original pancreatic tumor in a primary transplantation model. **B**: Metastatic liver tumor in a primary transplantation model. **C**: Pancreatic tumor in a back transplantation model.

6. Analysis of Tumor Cell Proliferation

To evaluate tumor cell proliferation, argyrophilic nucleolar organizer region (Ag-NOR) was stained using the method described by Ploton et al[15]. The number of black dots in the nucleus was then counted in 200 nuclei using the method described by Howat et al. [16]under 1,000-fold magnification, and the mean number per nucleus was quantified as the Ag-NOR score[17,18]. The AgNOR score of the metastatic liver tumors was then compared with that of the primary pancreatic tumors.

7. Statistical Analysis

All data were analyzed for significance using unpaired *t*-tests. A P value of less than 0.05 was considered significant.

Results

1. Macroscopic and Microscopic Observations of the Tumors

In the primary transplantation models (n=15), pancreatic tumors were observed in all the hamsters (15/15, 100%); (**Fig. 1A**), and metastatic liver tumors were observed in 10 hamsters (10/15,

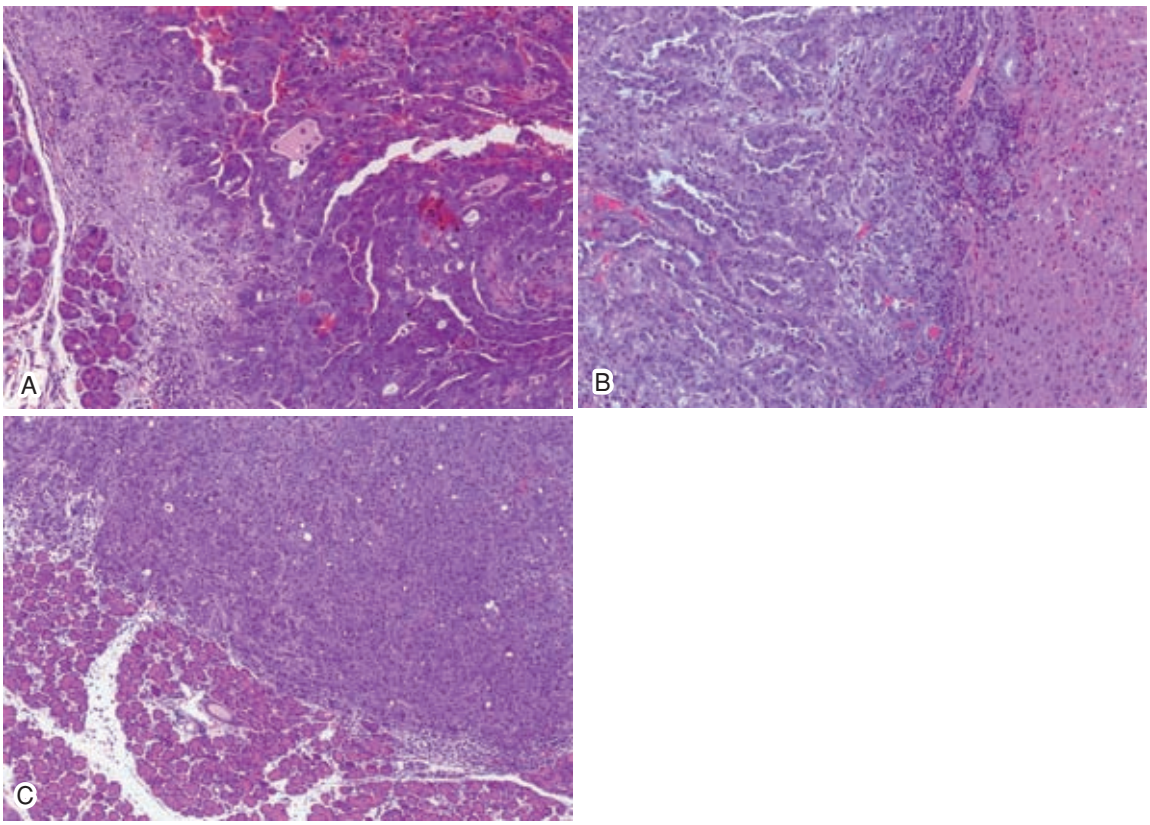

Fig. 2 Photomicrographs of the tumors (H.E staining). **A**: Original pancreatic tumor in a primary transplantation model. **B**: Metastatic liver tumor in a primary transplantation model. **C**: Pancreatic tumor in a back transplantation model.

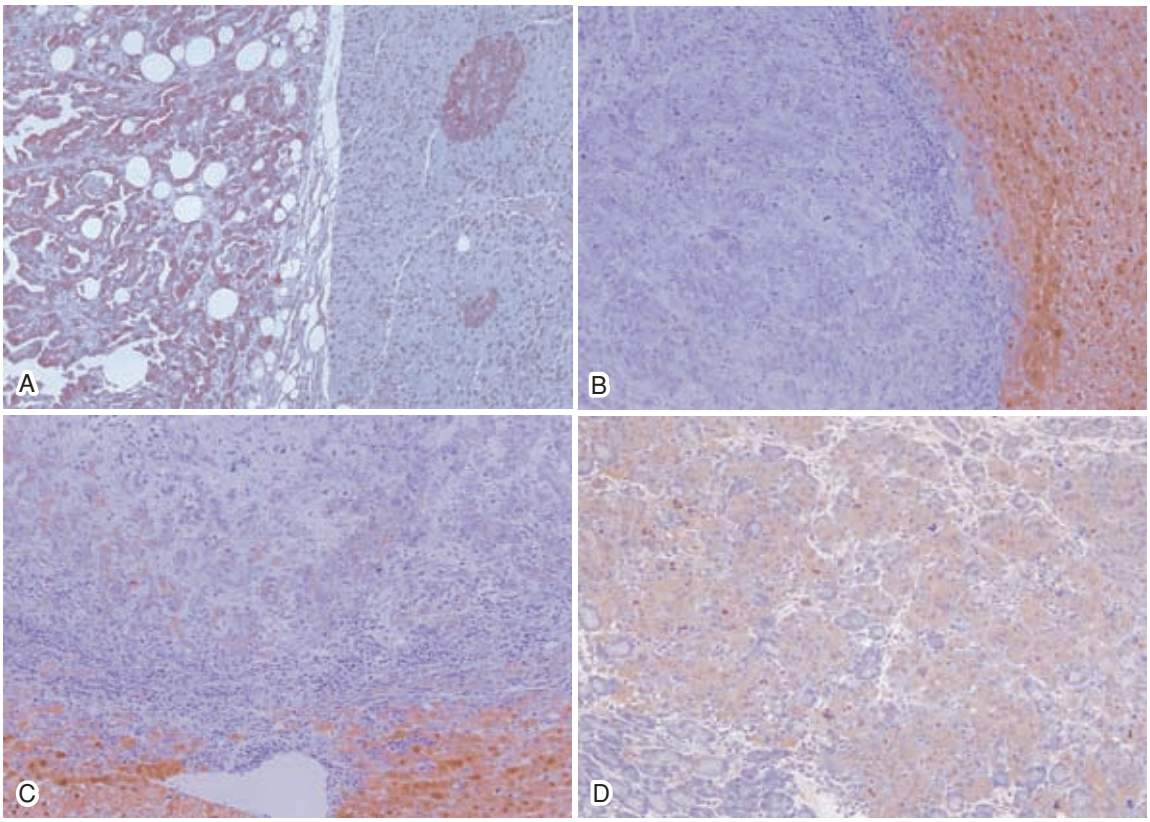

Fig. 3 Immunohistochemical staining for VEGF. **A**: Original pancreatic tumor in a primary transplantation model, VEGF (3+). **B**: Metastatic liver tumor in a primary transplantation model, VEGF (−). **C**: Metastatic liver tumor in a primary transplantation model, VEGF (1+). **D**: Pancreatic tumor in a back transplantation model, VEGF (3+).

Table 1

VEGF	Primary transplantation model		Back transplantation model	
	Original tumor of pancreas (n = 15)	Metastatic liver tumor (n = 10)	Pancreas tumor (n = 10)	Metastatic liver tumor (n = 4)
(−)	0	5 (50%)	0	2 (50%)
(1+)	0	5 (50%)	0	2 (50%)
(2+)	0	0	0	0
(3+)	15 (100%)	0	10 (100%)	0

66.7%); (**Fig. 1B**). In the back transplantation models (n=10), pancreatic tumors were observed in all the hamsters (10/10, 100%), and metastatic liver tumors were observed in 4 animals (4/10, 40%); (**Fig. 1C**). Examination of hematoxylin and eosin (H-E)-stained sections revealed that all the tumors were well-differentiated ductal adenocarcinomas (**Fig. 2A, B, C**). Necrosis and fibrosis was observed in center of tumors. But there were no differences in the macro or microscopic appearance of these tumors.

2. Immunohistological Staining for VEGF Protein

All of the original pancreatic tumors in the primary transplantation models were (3+) for VEGF. In the metastatic liver tumor cells of the primary transplantation models, however, 5 of the animals (50%) were (−) for VEGF and 5 (50%) were (1+). In the back transplantation model, all 10 animals were (3+) for VEGF in the pancreatic tumors, and 2 out of 4 animals with liver metastases were weakly (1+) positive for VEGF in their liver tumors. In the tumor cells expressing VEGF, reactivity was observed mostly in the cytoplasm. (**Table 1**, **Fig. 3**). Liver tissues surrounding the metastases were also positive for VEGF. There was no differences between negative staining and weakly positive staining in microscopic appearance, tumor size, or location of staining.

3. RT-PCR Analysis of VEGF in PGHAM-1 Cells and Tumors

Expression of VEGF mRNA was analyzed by RT-PCR in PGHAM-1 cells and tumors from the primary and back transplantation models. A 208-bp band corresponding to VEGF m-RNA was found in PGHAM-1 and all the tumors, including the liver metastases in the primary and back transplantation models that did not show any or only weak immunohistochemical staining for VEGF (**Fig. 4**).

4. Tumor Cell Proliferation

Tumor cell proliferation was estimated using Ag-NOR staining. The Ag-NOR scores (mean±SD) of the original pancreatic tumors and metastatic liver tumors in the primary transplantation models were 9.60±1.40 and 9.60±1.78, respectively, while the scores of the pancreatic tumors and metastatic liver tumors in the back transplantation models were 9.20±1.75 and 9.70±2.15, respectively (**Fig. 5**). No significant differences were observed among the groups (**Fig. 6**).

Discussion

VEGF, which has a molecular weight of 45 kDa, is thought to be an angiogenic factor that also acts as a selective mitogen for endothelial cells. VEGF is an important prognostic marker and has been demonstrated to induce endothelial cell proliferation, migration, and invasion[19] and may also have a direct stimulatory effect on the growth of new blood vessels[20,21].

Solid tumors require angiogenesis for growth and metastasis[22]. Tumor angiogenesis may be regulated by angiogenic factors that are secreted by tumor cells. VEGF is thought to be one such factor. Correlations between the expression of VEGF and either metastasis or a poor prognosis have been reported for various cancers. For example, Seo et al. analyzed the correlations between VEGF expression

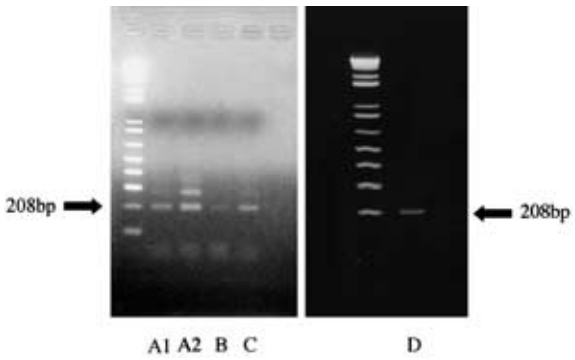

Fig. 4 Expression of VEGF mRNA as analyzed by RT-PCR. A1: Metastatic liver tumor in a back transplantation model. A2: Metastatic liver tumor in a primary transplantation model. B: Pancreatic tumor in a back transplantation model. C: Pancreatic tumor in a primary transplantation model. D: PGHAM-1 cells.

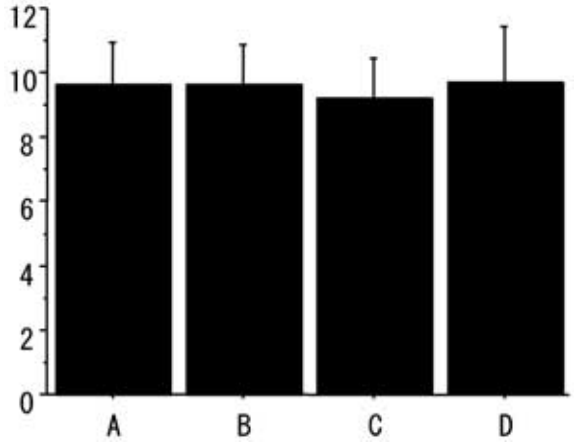

Fig. 5 Quantitative assessment of proliferative indices using AgNOR scoring. Original pancreatic tumor in a primary transplantation model (A), metastatic liver tumor in a primary transplantation model (B), pancreatic tumor in a back transplantation model (C), and metastatic liver tumor in a back transplantation model (D). No significant differences in the proliferative indices were observed.

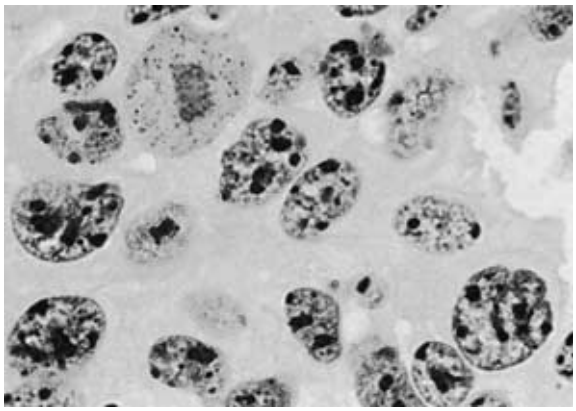

Fig. 6 AgNOR staining. The number of black dots in the nucleus indicates the proliferative ability of the tumor cells.

and microvessel density (MVD), clinicopathologic factors, and clinical outcome in 142 cases of ductal pancreatic adenocarcinoma[6]. They conclude that VEGF expression was closely correlated with MVD and seemed to be an important predictor for both liver metastasis and a poor prognosis in patients with ductal pancreatic carcinoma. VEGF has also been reported to be associated with the progression, invasion and metastasis of colorectal carcinoma, and overexpression of VEGF mRNA in the primary tumor appears to be closely correlated with a poor prognosis[7]. Furthermore, Maeda et al. showed that the expression of VEGF might be a good prognostic indicator and predictor of the mode of recurrence in patients with gastric carcinoma[23].

VEGF is involved in the process of metastasis through its effects on angiogenesis. Recent studies have demonstrated that VEGF expression can be found in primary tumor sites in the colon, pancreas, and stomach and that its expression is correlated with liver metastasis[6,7,23]. Warren et al. reported that the administration of VEGF monoclonal antibodies in tumor-bearing mice led to a marked reduction in the number and size of experimental liver metastasis[24]. Bruns et al. reported that therapy with VEGF receptor-2 (VEGFR-2/flk-1/KDR) antibodies inhibited tumor growth in a murine model of liver metastasis from colon carcinoma[25]. These reports suggest that VEGF plays an important role in the process of liver metastasis. Angiogenesis is also important for tumor growth at the metastatic site. VEGF is thought to be closely related to angiogenesis at the metastatic site, but few studies have investigated the expression of VEGF in metastatic liver tumors.

Syrian golden hamsters develop ductal adenocarcinomas of the pancreas in response to BOP; these adenocarcinomas resemble human pancreatic cancers morphologically and biologically, and hamster pancreatic cancer may serve as a good animal model for human pancreatic cancer[26,27]. The

hamster pancreatic cancer cell line PGHAM-1 was derived from BOP-induced hamster pancreatic cancer cells through repeated cell passages and subcutaneous implantations. PGHAM-1 cells produce a ductal adenocarcinoma that expresses VEGF mRNA *in vitro* and *in vivo* and often leads to liver metastasis shortly after implantation in the pancreas[10–13]. Moreover, in metastatic liver tumors arising from the allogeneic intrapancreatic transplantation of PGHAM-1 cells, angiostatin therapy, a potent anti-angiogenesis treatment, has been reported to be effective against liver metastasis[12].

In this study, we compared VEGF expression in the original pancreatic tumors and metastatic liver tumors that were induced using PGHAM-1 cells. Using immunohistochemical staining, we found that VEGF was minimally expressed (− or 1+) in the metastatic liver tumors although the original pancreatic tumors was strongly stained (3+). Two possible hypotheses may be considered to explain the reduction in VEGF expression in the liver metastases. First, the PGHAM-1 cell line may have contained both VEGF-positive and VEGF-negative clones. During the process of metastasis, the VEGF-negative clones may have been selectively transported to the liver. In other words, the metastatic liver tumors consisted of VEGF-negative clones. However, the presence of a VEGF-negative clone in the original tumor seems unlikely, since all of the pancreatic tumors, including the back transplanted ones were positive for VEGF when examined using immunohistochemical staining. The second possibility is that the mechanism of VEGF expression in the tumor cells was altered at the liver of site. Accordingly, we examined VEGF expression in the pancreatic tumors and liver metastases using a back transplantation model. In the pancreatic tumors in the back transplantation models, VEGF expression was once again positive (3+) in the pancreatic tumors and (−) or. (1+) in the subsequent liver metastases. This observation shows that PGHAM-1 usually has the ability to express VEGF, but that expression is reduced during the process of liver metastasis and revived in the pancreas after retransplantation. VEGF may be necessary for tumor growth and metastasis in the pancreas but not in the liver, which already has a large quantity of the blood flow needed for tumor growth. Since the liver tissues surrounding the metastases exhibited VEGF positivity, the tumor cells in the liver might be supplied with VEGF produced by peripheral liver cells. In RT-PCR analysis, VEGF mRNA was found to be expressed in PGHAM-1 cells and all induced tumors. This finding was interpreted as indicated that VEGF expression at the protein level is suppressed by micro-environmental factors in the liver. A. similar phenomenon was reported by Naito et al.[28], in which lumican, a member of a small, leucine-rich proteoglycan family, was not detected in non-cancerous squamous epithelial cells located close to cancers of the uterine cervix despite the abundant transcription of its mRNA. In the report by Naito et al., cytokine and hormones in the microenvironment were thought to have induced an instability in the translational (post-transcriptional) stage of the cells[29].

Berney et al. undertook a retrospective study to investigate VEGF expression in colorectal cancer (including adenomas) and liver metastasis[9]. The authors reported that the mean VEGF expression was similar in the primary tumors but not liver metastases , where VEGF expression was significantly reduced. Hypoxia has been suggested to act as a stimulus for the up-regulation of VEGF transcription in malignant tumors[30]. Since metastatic tumor cells receive a sufficient blood supply from the highly vascularized liver parenchyma, to ensuring proliferation, the absence of a hypoxic stimulus may influence VEGF expression. According to the idea, the necrosis and fibrosis frequently found in human liver metastasis might be the result of hypoxia without sufficient induction of angiogenesis factors like VEGF. Ishigami et al. investigate a VEGF expression in surgical specimens of primary tumors and metastatic liver tumors from 74 patient with colorectal cancer using Northern hybridization and immunohistochemistry[8]. The authors reported that the expression of VEGF may have been influenced by the enviroment of the target organ. We examined tumor proliferation using the Ag-NOR score. No significant differences were

observed among the original tumors and the metastatic liver tumors of the primary transplantation model and the pancreatic tumors of the back transplantation model. Therefore, the proliferative ability of the tumor is thought to remain constant and to not be affected by the reduction in VEGF protein expression in the metastatitc liver tumors. Some reports have discussed differences in the biological properties of primary and metastatic tumors. Ohta et al.[31] investigated differences in the expression of carcinoembryonic antigen (CEA) and carbohydrate antigen (CA19-9) using a mouse model with subcutaneous implantations of a human colon cancer cell line. The expression of CEA in the metastatic liver tumor was higher than that in the primary tumor, while the expression of CA19-9 was significantly lower in the metastatic liver tumor than in the primary tumor. Takahashi et al[32]. investigated the differences in CEA and CA19-9 staining in primary gastric cancers and lymph node metastases. No significant differences in CEA staining were found, but CA19-9 staining was reduced in many of the lymph node metastasis specimens, compared with that in the primary tumors. These reports suggest that the biological properties of the primary and metastatic sites of cancer may differ. The results of the present study are thought to reflect this phenomenon.

The re-expression of VEGF in the back transplantation model suggests that selective metastasis of VEGF-negative clone did not occur, but that the biological properties of PGHAM-1 were altered by the environment of the liver. In the present study, minimal VEGF staining (1+) was observed in 50% of the animals with metastatic liver tumors. This result implies that the majority of the tumor cells are adjacent to a different environment (the liver parenchyma), and that the tumor cells may be easily influenced by the peripheral environment. Therefore, VEGF expression in the metastatic lesions may be in flux, since the environment of the majority of the tumor cells is always changing.

In this study, we found that PGHAM-1 typically has the ability to express VEGF, but that this property appears to be altered during the process of liver metastasis. The metastatic tumor cells are thought to be affected by their peripheral environment, and the interrelationship between cancer cells and the organ environment may play an important role in VEGF expression.

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