—Originals—

Two types of peritoneal dissemination of pancreatic cancer cells in a hamster model

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Abstract

Peritoneal dissemination has an unfavorable impact on the prognosis of pancreatic cancer, and a peritoneal dissemination model was created in hamsters by using an experimental pancreatic cancer to clarify its pathological characteristics. PGHAM-1, a cancer cell line we established from BOP induced pancreatic cancer in Syrian golden hamsters, was inoculated into the abdominal cavity of Syrian golden hamsters. After inoculation, sequential changes in the diaphragm, omentum, and parietal peritoneum, and the metastatic patterns of the PGHAM-1 cells were morphologically investigated by macroscopical, microscopical, and ultrastructural observation. The cancer cells were easily absorbed at the stomata in the diaphragm and milky spots in the omentum, which were absorptive lymphatic structures, and lymphatic metastasis occurred 4 days after inoculation. In the parietal peritoneum, however, the cancer cells attached to and proliferated on the parietal peritoneum where mesothelial cells had exfoliated and the basement membrane was exposed. This process was comparatively time-consuming, and metastasis occurred in the parietal peritoneum at 7 days after inoculation. This study suggested that there might be two patterns of peritoneal dissemination of hamster pancreatic cancer. One route is lymphatic metastasis via stomata in the diaphragm and milky spots in the omentum, and the other is direct metastasis on the parietal peritoneum; each metastasis forms independently. (J Nippon Med Sch 1999; 66: 253—261)

Key words: Hamster pancreatic cancer, peritoneal dissemination, blood group-related antigen A

Introduction

Pancreatic cancer is difficult to detect in its early stage, and the prognosis is quite poor. The outlook remains miserable, even for patients with apparently localized stage I disease, with a 5-year survival rate of approximately 15% to 25%[1]. In certain cases, curative resection cannot improve the prognosis, because of liver metastasis, peritoneal dissemination[2,3], and local recurrence in the early stage[3,4]. Moreover, it has been reported that even in the "early" stage of the disease, cancer cells can be detected cytologically in peritoneal washing solution, with 20% to 30% positive results[5]. Under these circumstances, the possibility of peritoneal dissemination might be higher than expected. However, there are few effective methods of inhibiting peritoneal metastasis except in experimental research[6]. Thus, elucidating the mechanism of peritoneal dissemination of pancreatic cancer is very important for improving the prognosis of pancreatic cancer by preventing this process.

There have been some reports on experimental peritoneal dissemination models in nude mice[7,8]. Nude mice are special animals in cancer research, because they do not have an immune system, but there may be differences in the mechanism of peritoneal dissemination between humans and nude mice. Pancreatic

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cancer induced by N-nitrosobis (2-oxopropyl) amine (BOP) in Syrian golden hamsters morphologically, biologically, and immunologically resembles human pancreatic cancer[9]. Thus, theoretically, hamster pancreatic cancer might provide an ideal animal model of peritoneal dissemination of human pancreatic cancer. In this study, a newly established experimental peritoneal dissemination model with a hamster pancreatic cancer cell line (PGHAM-1) was used to observe the peritoneal dissemination patterns and the sequential changes of peritoneal metastasis in order to elucidate the mechanism of dissemination of pancreatic cancer.

Materials and Methods

Animals

Female 5-week-old Syrian golden hamsters were obtained from the Shizuoka Experimental Animal Center (Shizuoka, Japan). They were kept under standard laboratory conditions (temperature, 22 ± 3℃; relative humidity 40 ± 5%; light/dark cycle 12 hr/12 hr) and given a standard diet (MF-1, Oriental, Tokyo, Japan) and water *ad libitum*.

Hamster pancreatic cancer cell lines

BOP was used to induce pancreatic cancer in hamsters by the method we previously reported[10, 11]. The tumors were minced with scissors into 1 mm cubes, and then subcutaneously transplanted into the interscapular area of hamsters with a trocar. The recipient hamsters were sacrificed at 6-8 weeks after transplantation, and parts of the tumor tissues were serially transplanted. After subcutaneous transplantation 8 times, the tumor was extracted, germ-free, minced in 0.05% trypsin and EDTA solution at 37℃ for 10 min, and centrifuged (10 min, ×4,000). The precipitated cells were maintained in Dulbecco's modified Eagle medium (MEM: GIBCO, New York, USA) supplemented with 10% fetal bovine serum (FBS), 100 unit/m*l* penicillin-streptomycin, 100 $\mu g/ml$ kanamycin, and 100 $\mu g/ml$ amphotericin B (GIBCO, New York, USA) at 37℃ in a 5% CO_2 incubator in plastic culture flasks (Corning, New York, USA). Cells that had been maintained in culture for 60 passages, and established as a cell line were named PGHAM-1.

Expression of blood group A antigen has been demonstrated immunohistochemically in a pancreatic cancer cell line established from a pancreatic ductal carcinoma induced by BOP in a hamster[12], and our newly established PGHAM-1 cell line also expressed human blood group A antigen[13]. Since blood group-related antigens, especially A antigen, are sensitive and specific for hamster pancreatic ductal cancer, blood group-related antigen (BGRA). A is regarded as a tumor marker for small hamster pancreatic cancerous lesions[12]. Accordingly, the immunohistochemical method can be used to detect small cancer foci.

Intraperitoneal inoculation of PGHAM-1 cells

PGHAM-1 was adjusted to 1×10^7 cells/m*l* in MEM. The cell suspension (total number: 2×10^6) was injected into the abdominal cavity throughe a 27 G tuberculin needle in the central abdomen in the experimental group, and MEM was injected into the abdominal cavity instead of PGHAM-1 cells in the control group. All of the intra-abdominal injections were performed without anesthesia.

Morphological observations

(1) Observation of normal peritoneum

Based on earlier studies, we selectively examined the morphology and structure of the peritoneum in three representative regions: the diaphragm, omentum, and parietal peritoneum. The first two have absorptive structures, but the last does not. Carbon ink (0.1 ml) was injected into the peritoneal cavity of the hamsters to identify the lymphatic absorptive structures closely related to lymphatic metastasis of cancer in the peritoneum. Two animals each were sacrificed under ether anesthesia 5,10,15 mins after the injection. The peritoneum was then removed, and the location and pattern of absorption were observed.

For light microscopical examination (LM), the samples were fixed in 10% formalin and processed routinely, and paraffin-embedded sections were processed for hematoxylin-eosin (H-E) staining.

For examination with a scanning electron microscope (SEM), the samples were rinsed in pH7.4 phosphate-buffered solution (PBS) and the blood elements were removed. The samples were then fixed in 2.5% glutaraldehyde in 0.1 M PBS, pH 7.4, for 2 hrs, postfixed in 1% osmium tetroxide for 1 hour at room

Table 1 Macroscopic evidence of cancer foci

	days after inoculation			
	4	7	14	21
diaphragm	−	1+	2+	3+
omentum	−	1+	2+	3+
parietal peritoneum	−	−	−	−

−：no foci, 1+：< 5 foci, < 1 mm in diameter, 2+：≧ 5 foci, ≧ 1 mm in diameter, 3+：clustered, large foci (> 3 mm in diameter)

Table 2 Microscopic evidence of cancer foci

	days after inoculation			
	4	7	14	21
diaphragm	1+	2+	3+	3+
omentum	1+	2+	3+	3+
parietal peritoneum	−	1+	2+	2+

−：no cancer cells, 1+：1—10 cancer cells/focus, 2+：10—100 cancer cells/focus, 3+：≧ 100 cancer cells/focus

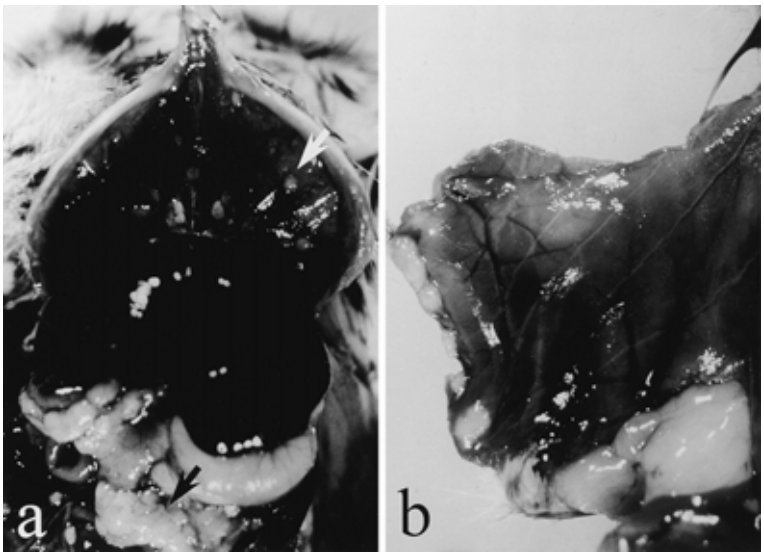

Fig. 1 Macroscopical evidence of peritoneal dissemination in the hamster. There were metastatic lesions in the diaphragm (white arrow) and in omentum (black arrow) (a). There were no obvious metastatic lesions in the parietal peritoneum (b).

temperature, and dehydrated in a graded ethanol series. They were subsequently dried by the butanol freeze drying method and mounted on aluminum stubs. After the specimens were sputter-coated, they were examined with a Hitachi S-570 type SEM.

(2) Observation of the peritoneum of inoculated hamsters

Five hamsters in each group were sacrificed under ether anesthesia at 4,7,14, and 21 days after intraabdominal inoculation of PGHAM-1 cells.

Peritoneum from the diaphragm, omentum, and the parietal peritoneum of each hamster were removed, and the number and the maximum size of cancer foci were measured. Depending on the combination of their numbers and sizes, peritoneal dissemination was divided macroscopically into 4 categories (−～3+, legend of **Table 1**). Microscopically, the number of cancer cells in the largest focus was also counted and divided into 4 categories (−～3+, legend of **Table 2**) using BGRA staining slides (×200). In the control group, pieces of peritoneum were examined in the same manner as normal peritoneum. Immunohistochemical staining with anti-BGRA-A (Dako, California, USA) was performed to detect the precise location of the peritoneal metastases with Histofine SAB-PO (M) kit (Nichirei, Tokyo, Japan). The antibody dilution for the antigen was 1: 100. The specimens in the control group were processed similarly, except that mouse IgM was used instead of primary antibody.

Results

Growth of PGHAM-1 cells in the peritoneal cavity

On day 21 after inoculation, the hamsters had severe carcinomatous peritonitis in the diaphragm and

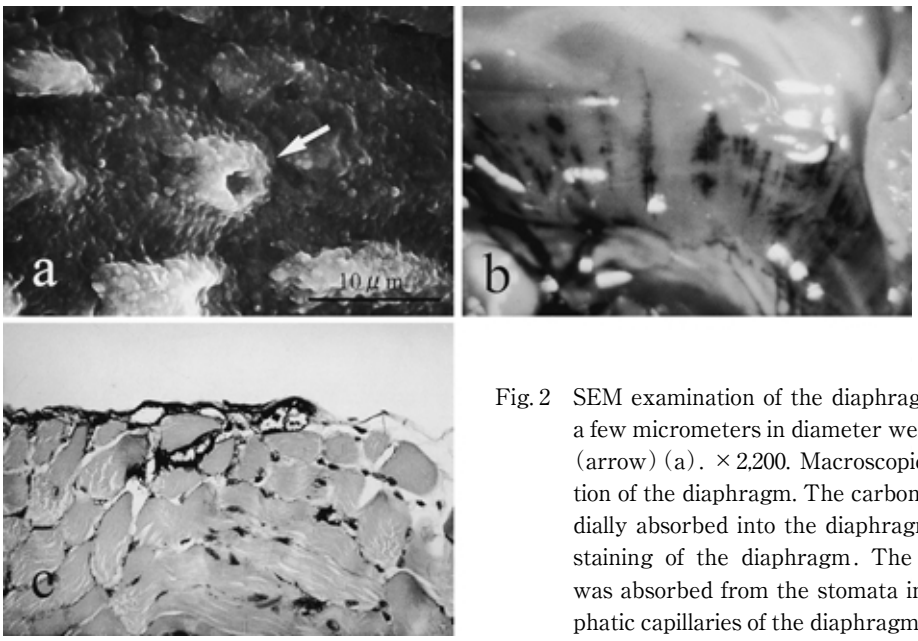

Fig. 2 SEM examination of the diaphragm. Stomata a few micrometers in diameter were observed (arrow) (a). ×2,200. Macroscopical observation of the diaphragm. The carbon ink was radially absorbed into the diaphragm (b). H-E staining of the diaphragm. The carbon ink was absorbed from the stomata into the lymphatic capillaries of the diaphragm (c). ×200.

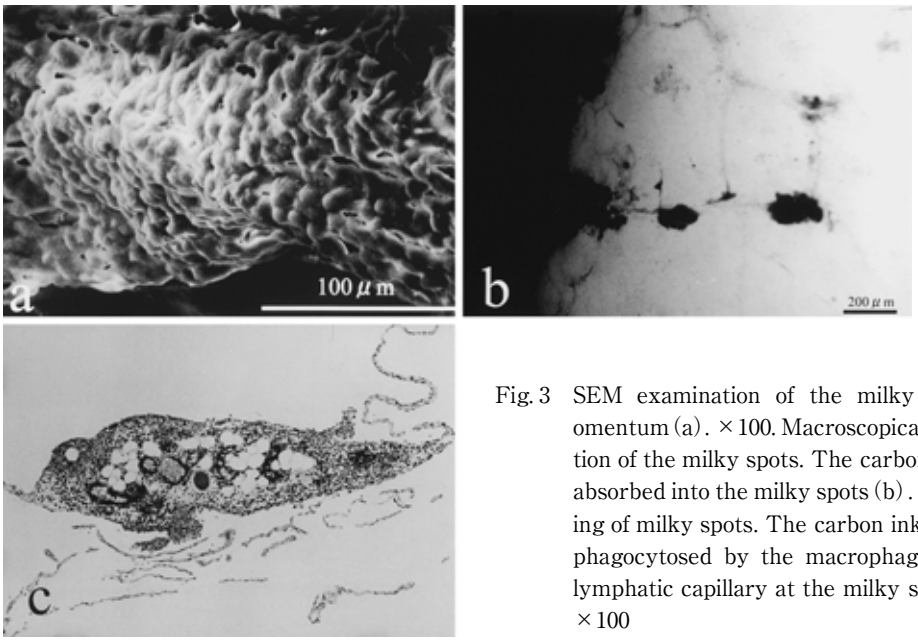

Fig. 3 SEM examination of the milky spots in omentum (a). ×100. Macroscopical observation of the milky spots. The carbon ink was absorbed into the milky spots (b). H-E staining of milky spots. The carbon ink was also phagocytosed by the macrophages in the lymphatic capillary at the milky spots (c). ×100

omentum, with bloody ascites, but there were no macroscopic metastatic lesions of the parietal peritoneum (**Fig. 1 a, b**). This alteration can cause hamster death. Our preliminary study confirmed that hamsters inoculated with PGHAM-1 cells always died of generalized carcinomatosis within 30 days.

Normal peritoneum and its absorptive system

Microscopically, the surface of diaphragm was observed to be lined by a flat, single layer of mesothelial cells, and there were lymphatic capillaries coursing beneath the mesothelial cells in some areas. SEM revealed large and small mesothelial cells arranged like paving stones. Beneath the small, flat mesothelial cells, there were clear upheavals that expanded from the center of the diaphragm to its edge, and stomata a few micrometers in diameter were observed between the mesothelial cells (**Fig. 2 a**). Carbon ink was radially absorbed from the stomata into the lymphatic capillaries of the diaphragm within 10 minutes after the injection (**Fig. 2 b, c**).

The milky spots were about 50 μm to 200 μm in size by SEM (**Fig. 3 a**). Macrophages and lymphocytes clustered around the microvessels were confirmed in

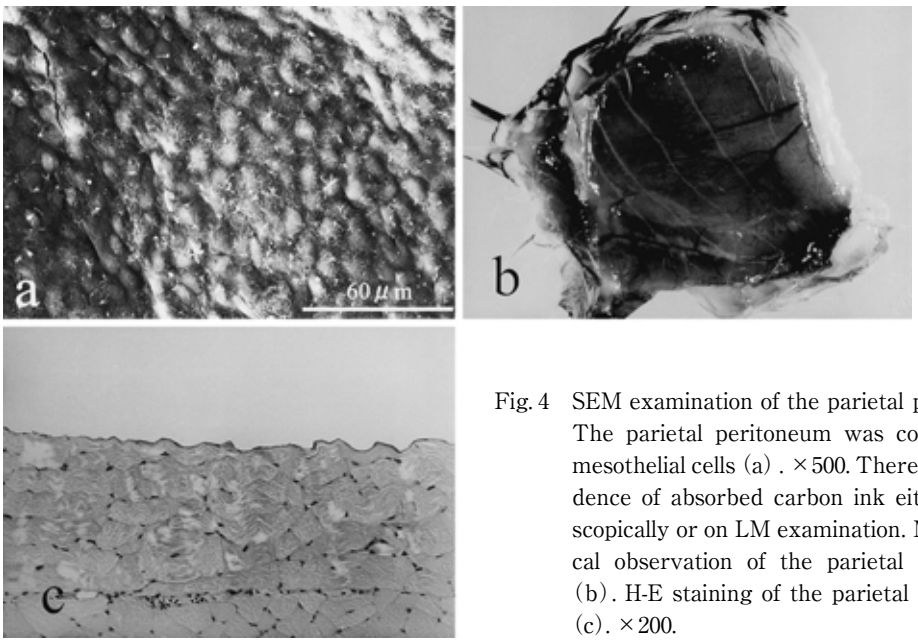

Fig. 4 SEM examination of the parietal peritoneum. The parietal peritoneum was covered with mesothelial cells (a). ×500. There was no evidence of absorbed carbon ink either macroscopically or on LM examination. Macroscopical observation of the parietal peritoneum (b). H-E staining of the parietal peritoneum (c). ×200.

the omentum by LM, and carbon ink was also phagocytosed by the macrophages in the lymphatic capillaries at the milky spots within 10 minutes of injection (**Fig. 3 b, c**).

On the other hand, the surface of the parietal peritoneum was covered with a flat, single layer of mesothelial cells, and there were no absorptive structures such as stomata or milky spots (**Fig. 4 a**). There was no evidence of absorbed carbon ink either macroscopically or by LM examination (**Fig. 4 b**, c).

Morphologic changes in the peritoneum after intraperitoneal inoculation of PGHAM-1 cells

The results of sequential morphological examination of different pieces of peritoneum after intraperitoneal inoculation of PGHAM-1 cells are summarized in **Tables 1 and 2.**

(1) Diaphragm

On day 4 after inoculation, there were no changes macroscopically, but metastatic micro-lesions were observed by LM, and PGHAM-1 cells had started to be absorbed into the stomata. The PGHAM-1 cells were especially detected by BGRA-A staining and cancer cells had clustered around the stomata (**Fig. 5 a**). On day 7, the metastatic lesions at the stomata in the diaphragm had tended to expand, and mesothelial cells had partly detached from the basement membrane where there were no stomata. On day 14, the metastatic lesions of the diaphragm had developed significantly, and they could be detected macroscopically as small foci (**Fig. 5 b**). On day 21, metastases were frequently observed as areas of proliferation on the basement membrane.

(2) Omentum

On day 4 after inoculation, there were still no macroscopical changes in the omentum, but there were micrometastases under the LM, and the PGHAM-1 cells had started to be absorbed into the milky spots, which were observed under the LM as carbon ink absorption routes in normal omentum. BGRA-A staining detected cancer cells in the milky spots (**Fig. 6 a**). On day 7, the metastatic lesions had become larger when examined under LM, and on day 21 after inoculation, the metastatic lesions had increased in size and could be easily detected grossly. The size of the milky spots had also expanded, and the lymphatic ducts had swelled compared to there normal size when examined with the SEM (**Fig. 6 b**).

(3) Parietal peritoneum

On day 4 after inoculation, the mesothelial cells of the parietal peritoneum seemed slightly hypertrophic and the gaps between the mesothelial cells had enlarged when examined with SEM (**Fig. 7 a**). However, there were no signs of attachment of PGHAM-1 cells to the intact surface of the parietal peritoneum. Both BGRA-A staining and SEM failed to detect any cancer cells. On day 7, the mesothelial cells on some parts of the parietal peritoneum had started to exfoliate, and

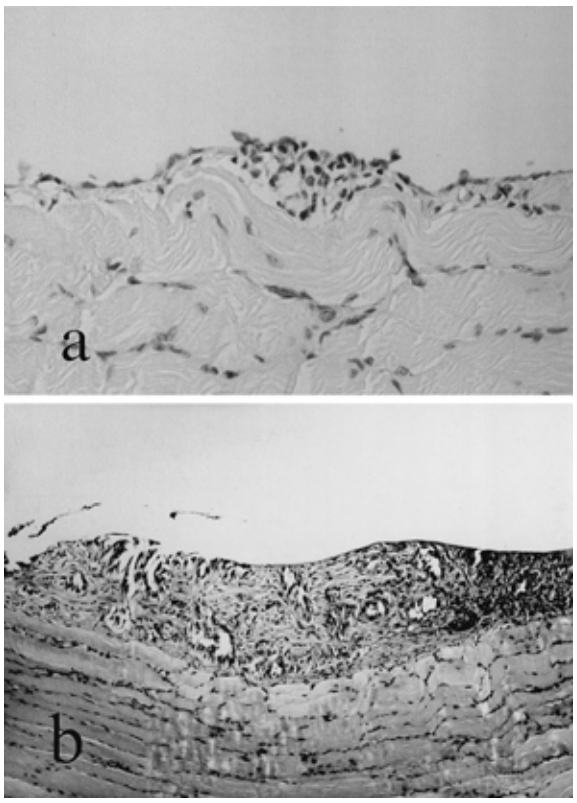

Fig. 5 BGRA-A staining of the diaphragm on day 4 after inoculation. The cancer cells clustered around the stomata (a). ×400. H-E staining of the diaphragm on day 14 after inoculation. There are huge metastatic lesions on the diaphragm (b). ×200.

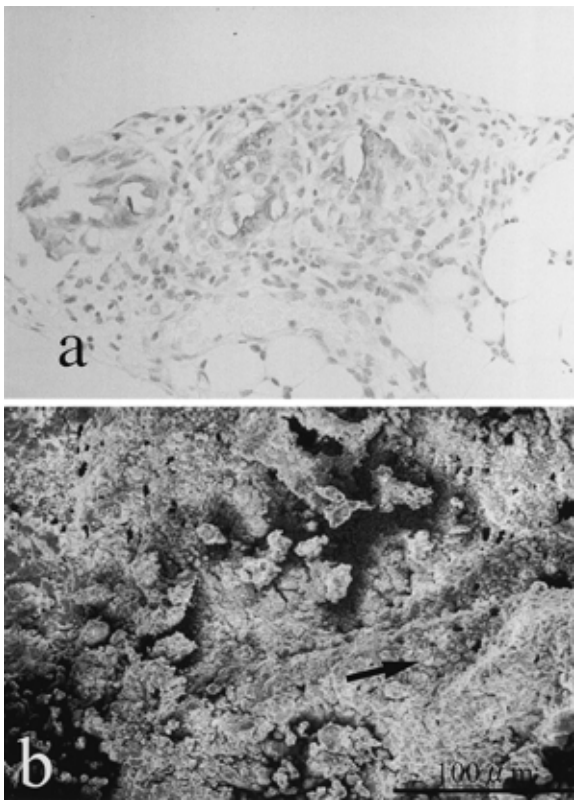

Fig. 6 BGRA-A staining of the omentum on day 4 after inoculation. The cancer cells have started to be absorbed into the milky spots (a). ×200. SEM examination of the omentum on day 21 after inoculation. The size of the milky spots has also increased, and the structure of the lymphatic ducts has been destroyed (arrow) (b). ×80

PGHAM-1 cells subsequently attached to the surface of the largely exposed basement membrane (**Fig. 7 b**, **c**). On day 14, there were no macroscopical metastatic lesions on the parietal peritoneum. LM and SEM examination showed PGHAM-1 cells superficially attached to the basement membrane, which was largely exposed due to exfoliation of mesothelial cells, but there was no invasion of the basement membrane by PGHAM-1 cells. In addition, it was noted that eminent neovascularization in the basement membrane appeared under the basement membrane. The distance between the neovascularized vessels and the cancer cells that had attached to the basement membrane and subsequently proliferated was about 100 μm (**Fig. 7 d**). On day 21, there were still no detectable metastatic lesions macroscopically. But microscopically, the metastatic lesions had clearly proliferated on the surface of the basement membrane alone, and the PGHAM-1 cells had not extensively invaded the basement membrane (**Fig. 7 e**).

Discussion

In this study, we examined the diaphragm and omentum, which are the most susceptible targets of the peritoneal dissemination; simultaneously we examined the parietal peritoneum, where dissemination occurrs less frequently in our experimental model. The stomata in the diaphragm and milky spots in the omentum are the absorptive structures in the human peritoneum[14-17].

The stomata have been reported to be the absorptive structures for ascites and an extravascular fluid pathway. Once inflammation has occurred in the abdominal cavity, to absorb the fluid and foreign bodies, the stomata, which are 1–3 μm in diameter between the mesothelial cells, become larger and increase in number[18-20]. Stomata are shut by the membrane of the

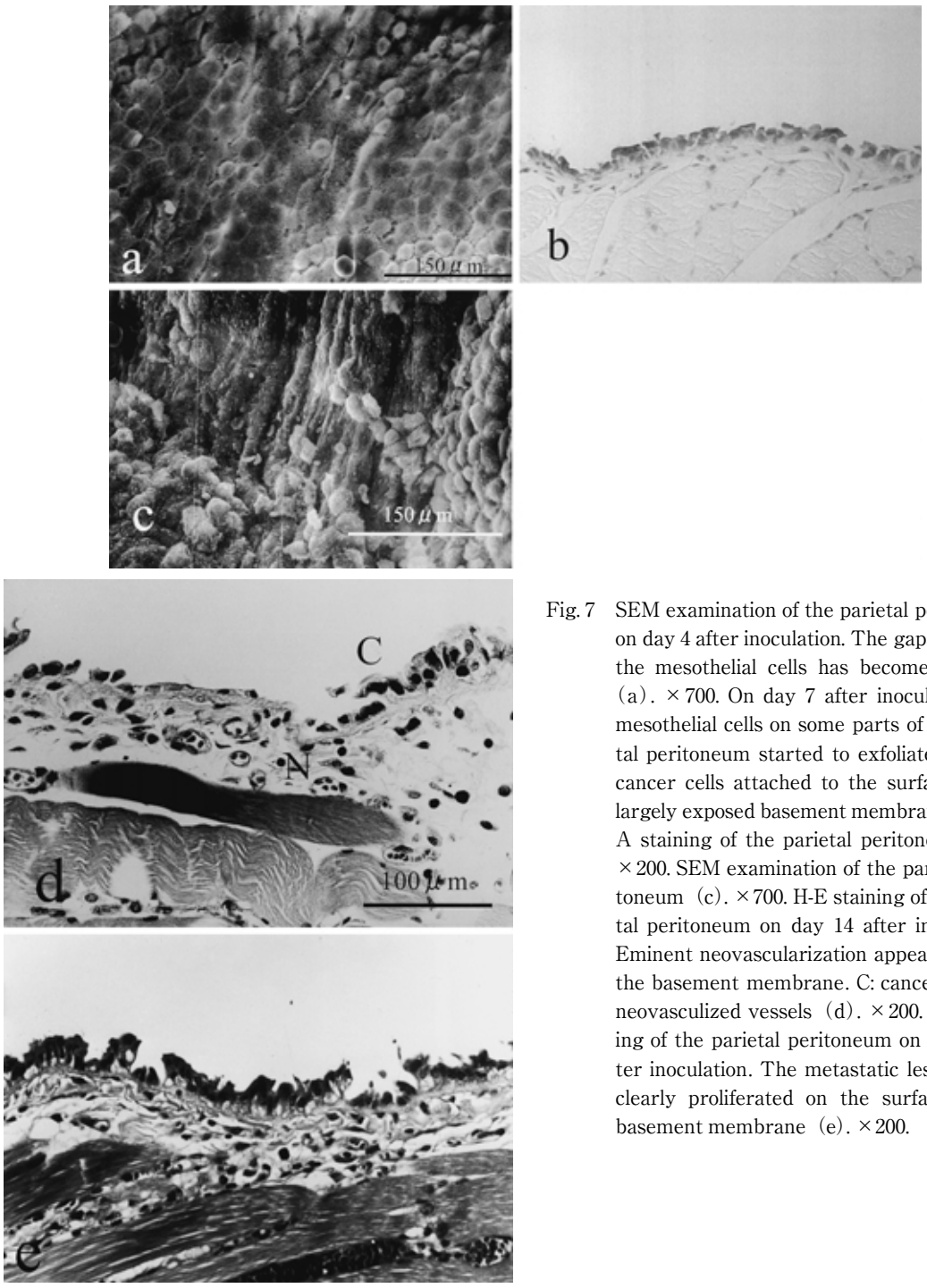

Fig. 7 SEM examination of the parietal peritoneum on day 4 after inoculation. The gaps between the mesothelial cells has become enlarged (a). ×700. On day 7 after inoculation, the mesothelial cells on some parts of the parietal peritoneum started to exfoliate, and the cancer cells attached to the surface of the largely exposed basement membrane. BGRA-A staining of the parietal peritoneum (b). ×200. SEM examination of the parietal peritoneum (c). ×700. H-E staining of the parietal peritoneum on day 14 after inoculation. Eminent neovascularization appeared under the basement membrane. C: cancer cells, N: neovasculized vessels (d). ×200. H-E staining of the parietal peritoneum on day 21 after inoculation. The metastatic lesions have clearly proliferated on the surface of the basement membrane (e). ×200.

peritoneal mesothelial cells like valves, and these valves are opened and closed by respiratory cycle[22]. The fluid in the peritoneal cavity can be absorbed into the lymphatic system from the stomata[19,21]. As the foreign bodies in the stomata are assumed to flow into the micro-lymph duct through the small foramina[22,23], the spaces between the stomata and the diaphragmatic lymph ducts must be the routes in the extravascular fluid pathway[24,25]. A similar finding was reported based on examination of the diaphragm in the hamster: Fukuo and colleagues[26] described the stomata as small round holes, several micrometers in diameter in the inferior diaphragm. In our experiments, the carbon ink absorbed by the stomata located around the edge zone flowed centripetally in the lymph ducts in the diaphragm. These findings suggest that the stomata play an important role in abdominal fluid drainage.

The milky spots in the omentum are lymphatic structures composed of macrophages, lymphocytes, and lymph ducts surrounding glomerular capillaries. There are orifices several micrometers in diameter in the surface of milky spots, which fulfill the same function as the stomata in the diaphragm, absorbing ascitic fluid and foreign bodies[16,27,28]. Certain types of foreign bodies in the abdominal cavity are absorbed by milky spots and phagocytosed by macrophages, but others flow into the lymph ducts without phagocytosis[27]. The structure of mouse milky spots anatomically resembles that of humans[16]. Although hamster pancreatic cancer frequently invades the omentum, the role of milky spots has never been reported. The structure of the milky spots in hamsters also resembles that in humans, and the finding that the carbon ink was phagocytosed by the macrophages in the milky spots and collected by the lymph ducts clearly confirms that hamster milky spots have the same function as those of other species.

In our study, cancer cells were found in the stomata and the milky spots on day 4 after inoculation. These cells flowed into the lymphatic structure. Therefore, it is clear that the final metastatic pattern in the diaphragm and omentum is lymphatic metastasis because of the above described structures; it is also clear that the cancer cells can be absorbed at an early stage and flow into the lymph ducts.

Birbech and Wheatley reported in experiments using Ehrlich ascites tumor cells that metastasis in the parietal peritoneum of nude mice occurred after detachment of the mesothelial cells and exposure of the basement membrane[8]. The course that they found was very similar to ours. From the results of sequential observation of the parietal peritoneum, it is supposed that increases in tumor size and the formation of metastatic lesions may take much longer than those in the diaphragm and omentum. In our study, the cancer cells attached to and spread on the basement membrane of the parietal peritoneum, where the mesothelial cells had become deformed and exfoliated. The parietal peritoneum did not have the absorptive devices which existed in the diaphragm and omentum; therefore, the metastatic pattern in the parietal peritoneum is not lymphatic but might have direct involvement with the basement membrane.

It has reported that a blood supply from microvessels within 150 μm of a tumor is necessary for cancer cells to survive experimentally[29]. In our experiments, the distance from the border of the cancer foci on the peritoneum to the new vessels was about 100 μm or less. This reflected the fact that neovascularization beneath the cancer was an essential pathological alteration of the parietal peritoneum. Thus, marked cancer cell proliferation could only occur after the process of neovascularization, which is also a time-consuming step, and that may be one reason for the delayed occurrence of metastasis in the parietal peritoneum.

In summary, lymphatic metastasis occurs in a much earlier phase, because the cancer cells are absorbed by its absorptive structures. By contrast, metastasis of the parietal peritoneum occurs much later than metastasis of the diaphragm and omentum, and metastasis of the parietal peritoneum is accompanied by neovascularization, which occurs in response to cancer cell attachment to the basement membrane. Therefore, there are at least two patterns of peritoneal dissemination in the hamster pancreatic cancer model created with PGHAM-1 cells. One is lymphatic metastasis of the diaphragm and omentum, and the other is direct attachment to the basement membrane of the parietal peritoneum.

The hamster pancreatic cancer cell line, PGHAM-1, organized morphologically, resembles human pancreatic cancer cells. Since the hamster has a well developed immune system, it is a satisfactory experimental model of human pancreatic cancer that can be widely used for research on the mechanism and prevention of peritoneal dissemination.

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