Trans-Lymphatic Metastasis in Peritoneal Dissemination

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Abstract
Mechanism of the formation of peritoneal metastasis (PM) through lymphatic vessels was studied.

Materials and methods: Parietal peritoneum was divided into 8 regions, and specimens of each zone were removed from patients with PM. The specimens were stained with enzyme histochemical staining for alkaline phosphatase (ALPase) and 5-Nase activity, and with immunohistochemical staining with D2-40. Surface of the peritoneum and subperitoneal tissue were observed by a scanning electron microscopy.

Results: Well-developed lymphatic lacunae were found in the shallow submesothelial layer of 7 regions except for the anterior abdominal wall. Lymphatic vessels were found in the deep submesothelial layer up to 200 micrometer from the peritoneal surface. The mesothelial stomata directly connect with the submesothelial lymphatic vessels through holes of the macula cribriformis. Migration of cancer cells through stoma was found, and cancer cells were detected in the submesothelial lymphatic lacunae. Lymphatic vessels were not found in the center of established PM, but were found in the adjacent normal tissue. In the subperitoneal tissue outside the PM, morphological findings suggesting lymphangiogenesis designated as cystic Lymphatic Island, ladder formation, budding, and extension of lymphatic vessels were found.

Conclusion: The triplet structure consisting of mesothelial stomata, holes on macula cribriformis and submesothelial lymphatic lacunae is essential for the migration of peritoneal free cancer cells into the submesothelial lymphatic lacunae. The route of the formation of PM through peritoneal lymphatic vessels was named as trans-lymphatic metastasis.

Keywords: Peritoneal metastasis; Trans-lymphatic metastasis; Angiogenesis; Peritoneal lymphatic vessels

Introduction
Traditionally, Peritoneal Metastasis (PM) has been considered to be established by a multi-step processes, consisting of the tumor cell detachment from the primary tumor, attachment on the peritoneum, invasion into the submesothelial tissue, and proliferation by the induction of angiogenesis and stromal tissue [1]. The concept was called as a trans-mesothelial metastasis to complete the process; however, concerted expression of metastasis-related genes must be required. In contrast, cancer cells having low biological behavior fail to establish peritoneal metastasis, because they cannot produce enough kinds of metastasis-related gene expression for the invasion and proliferation. Recently, Yonemura et al. reported that the peritoneal lymphatic network has an important role on the formation of peritoneal dissemination [1], and the concept was designated as trans-lymphatic metastasis. Milky spots on the greater omentum have been well known as a route of the trans-lymphatic metastasis [2]. However, the mechanisms of the trans-lymphatic metastasis except through milky spots have not been clarified. The present study demonstrated the novel findings of the trans-lymphatic metastasis in the human peritoneal tissue bearing peritoneal metastasis.

Materials and Methods
Human peritoneum
Parietal peritoneum was divided into 9 regions from region 0 to region 8 (Figure 1). Surgical specimens of the peritoneal tissues of each zone were removed from patients with PM from six and five patients

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with gastric cancer and pseudomyoma peritonei. Specimens were fixed with formalin, and embedded in paraffin.

**Staining of lymphatic vessels**

Peritoneum were removed and fixed with formaldehyde-CaCl₂ fixative (2% paraformaldehyde, 1% CaCl₂) in 0.1M cacodylate buffer containing 7% sucrose for 24 hours. Then, the tissues were embedded in OCT compound (Miles, Diagnostic Division, Elkhart, IN, USA), and the sections were cut at 15-μm thickness with a cryostat [3]. For the observation of whole mount specimens, 2 cm by 2 cm of peritoneum was removed. After complete removal of the fatty tissues from the sub peritoneal tissue, they were extended on the board and fixed with the same fixation methods. Staining of blood and lymphatic vessels was done by an enzyme - histochemical staining using alkaline phosphatase (ALPase) and 5'-Nase activity [3]. Lymphatic vessels showing positive staining for 5'-Nase activity revealed the lead sulfate reaction product, colored dark brown. In contrast, no 5'-Nase activity was recognized in the blood vessels [3]. After staining for alkaline phosphatase (ALPase), a blue reaction product was restricted to blood vessels. The whole mount specimens were observed by stereoscopic microscopy. D2-40 is a monoclonal antibody, which specifically reacts with O-linked sialoglycoprotein on the lymphatic endothelial cells. Immunohistochemistry was performed using deparafinized sections by D2-40 antibody (DAKO, Tokyo, Japan) [4].

**Scanning electron microscopy**

Surface of peritoneum and the subperitoneal tissue after removal of mesothelial cells by a chemical digestion using 6N-KOH was observed by a scanning electron microscopy (Hitachi, S-4800, Tokyo, Japan) [3]. This study was approved by the ethical committee of Kishiwada Tokushukai Hospital and Kusatsu general hospital and signed written informed consent was obtained from all patients.

**Results**

**Lymphatic vessels in human peritoneum**

In human subperitoneal tissue, lymphatic vessels were found in the shallow subperitoneal tissues between one to 200 micrometer from the peritoneal surface (Figures 2 and 4). By the observation for the whole mount specimens, well-developed lymphatic vessels and lacunae were found in the Morrison's pouch, diaphragm, pelvic peritoneum, falciform ligament, lesser omentum, and greater omentum (Figure 3). As shown in figure 4, the lymphatic vessels in these regions locate in the submesothelial tissue shallower than the blood vessels. In contrast,
subperitoneal lymphatic vessels in the anterior abdominal wall locate (Figure 1, region 1) in the deep subperitoneal tissue in 200 micrometer from the peritoneal surface (Figure 5).

Figure 6 shows mesothelial stoma opened between mesothelial cells. Just below the mesothelial cells, basement membrane and macula cribriformis were found (Figure 7). The macula cribriformis is a plate consisted of collagen, and has holes in some area (Figure 8). Submesothelial lymphatic vessels locate just below the macula cribriformis and the mesothelial stomata directly connect with the submesothelial lymphatic vessels through holes of the macula cribriformis.

Figure 9 shows that a gastric cancer cell migrates into the

Figure 6: Mesothelial stomata on the diaphragm (SEM, right), and submesothelial lymphatic vessel, stained with D2-40 (left), PM peritoneal mesothelial cells. (From a 28 years-old male patient with pseudomyxoma peritonei).

Figure 7: SEM finding of mesothelial cells (PM), submesothelial basement membrane (BM) and holes on the macula cribriformis (from the same patient of Figure 6) (☆) on the diaphragm (Figure 1, region 3).

Figure 8: SEM finding of macula cribriformis on the Morrison’s pouch after chemical digestion by 6N-KOH. (From a 39 years-old patient with gastric cancer of stage IV and positive cytological study).

Figure 9: SEM finding of the macula cribriformis on the Morrison's pouch after chemical digestion by 6N-KOH. Gastric cancer cell migrates through a hole on the macula cribriformis. (From a 39 years-old patient with gastric cancer of stage IV and positive cytological study).

Figure 10: Gastric cancer cells proliferate in the submesothelial lymphatic lacunae on the pelvic peritoneum. (from the same patient of Figure 9).

Figure 11: 5’-Nase staining of peritoneal metastasis on the falciform ligament (left). D2-40 immunohistochemical staining (right). No lymphatic vessels were found in the tumor (from a 28 years-old male patient with pseudomyxoma peritonei with ascites).
submesothelial lymphatic vessels through a hole of macula cribriformis.

Figure 10 shows the micrometastasis in the submesothelial lymphatic vessel on the pelvic peritoneum, and clusters of gastric cancer cells were found in the lymphatic lacuna.

Figure 11 shows the established peritoneal metastasis with angiogenesis and stromal formation. The lymphatic vessels are not found in the center of the tumor, but were found in the adjacent normal tissue around the tumor nodule. In the subperitoneal tissue outside the peritoneal metastasis, several morphological findings suggesting lymphangiogenesis were observed. Cystic Lymphatic Island (Figure 12), ladder formation (Figure 13), budding from preexisting lymphatic vessels (Figure 14), and extension of the blind loop of lymphatic vessels were found (Figure 15). Furthermore, enlarged cystic lymphatic islands connected with fine lymphatic vessels (Figure 16). Figure 17 showed the cystic lymph sac which was separated by septum and showed lacework like structure, suggesting to form lymphatic plexus.

Discussion
The milky spots on the greater omentum are open lymphatic lacunae that absorb fluid and thereby bring tumor cells in large quantity
to the structure [2]. In contrast, peritoneal dissemination except on the greater omentum have been considered to be established through the trans-mesothelial metastasis, which consists of direct attachment of peritoneal free cancer cells (PFCCs) on the peritoneal surface, invasion into subperitoneal tissue and proliferation with stromal induction.

The present study first demonstrated the mechanisms of trans-lymphatic metastasis on the parietal peritoneum except on the greater omentum. Stereological analysis of lymphatic vessels cannot be done by the immunohistochemical study using thin section of paraffin-embedded specimens. This hampered the development of the study of the lymphatic distribution and lymphatic spread of animal and human cancer.

We used a method to stain the peritoneal lymphatic and blood vessels in the human peritoneum using the double-enzyme staining method (5’-nucleotidase [5’-Nase] and alkaline phosphatase [ALPase]) [3]. Since lymphatic endothelial cells express 5’-Nase, the lymphatic vessels are stained brown. In contrast, the arterial blood capillaries are stained blue by the ALPase reaction. Accordingly, the lymphatic and arterial blood capillaries can be visualized using this method. This method is also useful to investigate stereological network of peritoneal lymphatic system, when the whole mount specimens are stained and observed by a stereoscopic microscopy.

The present study demonstrated that PFCCs migrated into the submesothelial lymphatic lacunae through mesothelial gaps (stoma) and holes on the macula cribiformis. In the omental milky spots, stomata can be detected on the surfaces of such milky spots, and these stomata connect with holes on the macula cribiformis beneath the mesothelial basement membrane. The lymphatic lacunae attach to the holes on the cribiform plate. Many arterial blood capillaries are distributed around the lymphatic lacuna at milky spots [5]. PFCCs can easily migrate into the lymphatic lacunae through the stomata and then proliferate in the lymphatic lacunae.

The triplet structure consisting of mesothelial stoma, holes on macula cribiformis and submesothelial lymphatic lacunae is essential for the migration of PFCCs into the lymphatic lacunae (Figure 18). The present study demonstrated that these structures are detected not only in the milky spots but also in the parietal peritoneum except anterior parietal peritoneum (Figure 1, region 0).

The peritoneum of diaphragm, pelvis, paracolic gutter, and Morrison’s pouch and perihepatic ligaments does not have any milky spots, but it does have the triplet structure. Under normal conditions, the mean diameter of stoma is 1.2 mm (ranging from 1 – 30 mm) [6]. The mean stomata density of the rabbit diaphragm is 250/mm² [6-8]. The size of the stomata changes by the contraction of mesothelial cells. In the normal condition, mesothelial cells show a characteristic rounded morphology, and several cytokines produced from cancer cells cause the separation of cell-to-cell contacts of mesothelial cells, resulting in the exposure of the macula cribiformis [9-11]. The macula cribiformis is a collagen plate with numerous holes. The surface is covered with basement membrane, to which the mesothelial cells attach. Beneath the macula cribiformis, a rich network of diaphragmatic submesothelial lymphatic vessels exists. The PFCCs move from the peritoneal cavity to the subperitoneal lymphatic vessels through the stomata and holes in the macula cribiformis (Figure 18). In an experimental study, intraperitoneal inoculation of a highly metastatic cell line induces mesothelial cell contraction, and cancer cells were detected in the submesothelial lymphatic vessels on day 3 [11].

PFCCs migrated in the lymphatic lacunae proliferate by supplying the oxygen and nutrients from the arterial capillary. Rapid tumor growth generates intratumoral stromal pressure and may induce the collapse of lymphatic vessels. Because the lumen of lymphatic vessels is maintained by the traction of fine anchoring filament due to the low intraluminal pressure of the lymphatic vessels [3], the intratumoral pressure may collapse the lumen of lymphatic vessels, resulting in the dysfunction and destruction of intratumoral lymphatic vessels. The present study demonstrated the lymphangiogenesis in the subperitoneal tissue adjacent to the established PM or in the peritoneum without PM. Two types of lymphangiogenesis were found. One is the budding from preexisting lymphatic vessels, which were designated as budding, ladder formation and extension. Another type was the lymphatic vessel neoformation from lymphatic islands, which were detected as cystic structure in the the stroma. Asahara have proved that human peripheral blood contains endothelial (EC) progenitors, which can differentiate endothelial cells [7]. These progenitor cells may be incorporated in the sites of active lymphangiogenesis. Lee reported a contribution of podoplanin positive bone marrow cells to lymphatic vessel formation [8]. These podoplanin positive cells highly express markers for lymphatic endothelial cells, hematopoietic lineages, and stem/progenitor cells, and on further cultivation, they generate lymphatic endothelial cells. In addition, these cells exist in small numbers in peripheral blood of normal mice, but are significantly augmented after tumor inoculation. After injection of these cells, they were incorporated into the lymphatic vasculature and increased lymphatic vascular density in tissue [8]. Lymphatic Island is isolated from the preexisting lymphatic vessels, and may be derived from lymphatic endothelial progenitor cells in bone marrow or macrophages [10,12]. VEGF-C produced from tumor cells may induce lymphangiogenesis in the subperitoneal tissue [13-20].

References
Isolation of putative progenitor endothelial cells for angiogenesis. Science 275: 964-967.


