Abstract. Background: Patients diagnosed with pancreatic cancer have a high mortality rate relating to the highly malignant and refractory nature of their disease, and reputedly linked to the presence of can be cancerous pancreatic stem cells. These stem cells are believed to be deeply involved in distant metastasis. Therefore, the present study examined whether pancreatic cancer stem cells (CSCs) exhibit organ-specific migration patterns during metastasis. Materials and Methods: Pancreatic cancer cells derived from primary tumors isolated from a mouse model of pancreatic cancer were subcutaneously injected into wild-type mice to form tumor allografts. Allografts were isolated and dissociated into single cells prior to cell sorting using flow cytometry. Sorted cancer cells were injected into the tail vein or spleen of recipient wild-type mice, and analyzed for engraftment three weeks post-transplantation.

Results: Pancreatic cancer cells metastasized either to the liver or lungs. Furthermore, we compared the number and size of metastatic foci in the liver and lungs; metastatic liver foci were larger compared with those in the lungs. Conclusion: Our results showed that pancreatic CSCs metastasized to distinct organs with direct access to the transplantation site via the circulation. Clarifying the interaction between pancreatic CSCs and the liver microenvironment will lead to improved prognosis and treatments for pancreatic cancer.

Pancreatic cancer is the fifth leading cause of cancer-related deaths, with an approximate 5-year survival rate of 6% (1). The most common type of pancreatic cancer, accounting for 80-95% of tumors, is pancreatic ductal adenocarcinoma (PDAC) developing within the exocrine component of the pancreas. Over 80% of pancreatic carcinomas are therapy-resistant, locally advanced, or metastatic at the time of diagnosis, leading to a median survival of less than six months (2). The most important determinant in cancer prognosis is the incidence of metastasis. However, distinct tropisms of tumor metastases that vary according to cancer type have been reported. For example, gastric cancer favors migration to the liver and abdominal lymph nodes, while breast cancer metastases are typically observed in the bone, brain, and lungs. Similarly, colon cancer has been shown to prefer the liver, whereas lung cancer metastases are largely observed in the brain, bone, and liver. In particular, the major site of metastasis of pancreatic cancer is the liver, and to a lesser extent the lungs and lymph nodes (3).

Highly malignant and refractory cancer is considered to arise from cancer stem cells (CSCs). CSCs have been reported to be involved in carcinogenesis and are associated with metastasis, recurrence, and treatment resistance (4, 5). Thus, elucidation of metastatic mechanisms and tropisms of CSCs with metastatic potential is critical to improving prognosis. The interaction between organs that are targets for metastasis and CSCs has been reported in a breast cancer study. The report showed that the bone morphogenetic protein inhibitor COCO regulates the cycle of tumor dormancy and activity in the lungs and promotes metastasis of breast CSCs to the lungs but not to the bone or brain (6). The self-renewal ability and microenvironment of breast CSCs are closely associated (7). Although pancreatic CSCs are believed to be involved in pancreatic tumorigenesis and metastasis, the functional significance of the interactions between specific organs and CSCs from pancreatic tumors has not been completely elucidated. Liver metastases are most common in PDAC and profoundly affect patient prognosis. Thus, thorough investigation of the interaction between CSCs and distant metastatic tissues is important for elucidation of the metastatic mechanisms. In the present study, we analyzed whether pancreatic CSCs tropically engraft to the liver using a mouse model of pancreatic cancer.

Materials and Methods

All animal experiments were approved (11-61, F-A-14-079) by the Institutional Animal Care and Use Committee of Yokohama City University (Japan). Pancreatic cancer cells were derived from Pdx1-
Cre; LSL-KrasG12D; CDKN2aKO (8) mouse primary tumor tissues and tumor allografts formed by subcutaneous injection into wild-type (WT) C57BL/6 mice. Tumor tissues were isolated and dissociated into single cells. Non-blood cells were sorted using flow cytometry (MoFlo Legacy; Beckman Coulter, Koto-ku, Tokyo, Japan) using antibodies against Cluster of Differentiation 45 (CD45) (BD Biosciences, Minato-ku, Tokyo, Japan) and Ter119 (BioLegend, Bunkyo-ku, Tokyo, Japan). Sorted cancer cells were injected into the tail vein or spleen of recipient C57BL/6 mice and histogenetically analyzed for engraftment three weeks post-transplantation. Paraffin or frozen sections were subjected to hematoxylin and eosin, Alcian blue (for glycosaminoglycans), and histochemical and immunofluorescent staining for hyaluronan synthase 2 (HAS2) and α-smooth muscle actin for immunostaining analysis. Nine mice were used for the analysis of lung and liver metastatic potential, respectively. The same applies to tumor sections analysis.

**Results**

To analyze whether pancreatic CSCs possess tropism for the liver or lungs, we transplanted allograft-derived pancreatic cancer cells from mouse models of PDAC into the spleen or tail vein of WT mice, and evaluated lung and liver metastases three weeks after transplantation.

Metastatic foci were observed in the liver but not the lungs, when the pancreatic cancer cells were transplanted into the spleen (Figure 1A and B). When pancreatic cancer cells were transplanted into the tail vein, metastatic foci were observed in the lung (Figure 1A and B) and liver (data not shown). Comparison of liver and lung foci for examination of the interaction of CSCs with the organ microenvironment showed a greater number and larger-sized metastatic liver foci.

Figure 1. Pancreatic cancer stem cells have a high directivity for liver into wild-type mice. A: Difference of metastatic sites by difference in site of cancer cell transplantation. Left, tail vein transplantation; right, splenic transplantation. B: Comparisons of lung metastatic foci and liver metastatic foci in mice transplanted with tumor. Top, Liver metastatic region of pancreatic cancer cells; bottom, lung metastatic region of pancreatic cancer cells. C: Comparison of the number and size of lung metastatic foci and liver metastatic foci (n=3). Bar shows the average of the size of metastatic foci.
Figure 2. Histological analysis of tumor sections in Pdx1-Cre; LSL-KrasG12D; CDKN2aKO mouse. Hematoxylin and eosin staining for normal pancreas (A) and primary cancer lesion, allograft, metastatic lesion (B-E, respectively) in Pdx1-Cre; LSL-KrasG12D; CDKN2aKO mouse. Alcian blue staining for normal pancreas (F) and primary cancer lesion, xenograft, metastatic lesion (G-J respectively) in Pdx1-Cre; LSL-KrasG12D; CDKN2aKO mouse. hp, Hepatic parenchyma cells; lp, lung parenchyma cells; *metastatic cells. Scale bars, 100 μm.

Figure 3. Activation of tumor-associated fibroblasts during the progression of murine pancreatic ductal adenocarcinoma (PDAC). A-C: Co-immunofluorescence staining with 4',6-diamidino-2-phenylindole (DAPI), hyaluronan synthase (HAS2) and α-smooth muscle actin (α-SMA) in the normal pancreas, liver, and lung. D-H: Activated tumor-associated fibroblasts are expressed and are abundant in murine pancreatic ductal adenocarcinoma (PDAC). (D), xenograft (E), large metastatic foci, about 2.0 cm³ (F), but not in the small metastatic foci, about 0.5 cm³ (G, H). Scale bars, 100 μm.
compared to those in the lungs (Figure 1C and D). Furthermore, histochemistry and immunofluorescence of extracellular matrix components was used to evaluate the potential cause of size differences in metastatic foci between these tissues. Expression of glycosaminoglycans was elevated in proliferating CSCs within liver and lung metastases (Figure 2). Qualitative alterations in the extracellular matrix were more frequent in cancerous tissues. Hyaluronic acid is a major carbohydrate component of the extracellular matrix. High levels of hyaluronic acid are often associated with metastasis in several types of cancer (9, 10). Expression of HAS2, an enzyme that synthesizes hyaluronic acid, was analyzed in metastatic lesions of the lungs and liver. HAS2 expression was confirmed in PDAC cells, allografts, and large liver and lung metastatic foci (Figure 3A-F) but not in small metastatic lesions (Figure 3G and H).

Discussion

Distant metastasis has considerable effects on the prognosis of patients with pancreatic cancer. Pancreatic cancer metastasizes mainly to the liver, and to a lesser extent to the lungs and lymph nodes. However, metastatic tropism to specific distant organs is not well-understood. Some cancer cells will be shed into the vasculature from the primary tumor and escape from the immune system, flowing through the bloodstream as circulating tumor cells (CTCs) that form metastatic foci. In a breast cancer study, it was reported that cells expressing aldehyde dehydrogenase (ALDH) and CD44 were detected in the blood of patients with metastatic breast cancer (11, 12). It was also observed that CTCs were present in the blood of patients with pancreatic cancer (13). When these cells were transplanted into the femurs of immunodeficient mice, metastases were observed in the lungs, brain and bone, indicating that breast CSCs with metastatic potential were present in the blood (14). However, in our study, pancreatic cancer cells most frequently metastasized to the lungs when transplanted into the tail vein. This suggests that pancreatic CSCs tropically metastasize to distant organs with direct circulatory access to the transplantation site. In contrast, liver metastatic foci were larger compared to those in the lungs, suggesting that the specific organ microenvironments of the lung and liver influence the rate of CSC growth. It has been suggested that interaction with the liver microenvironment stimulates considerable proliferation of CSCs. However, in the current study, we were unable to conduct a long-term analysis of circulating cells because mice died from early metastasis. It would be interesting to analyze circulating CSCs by transfusion of blood from mouse model of pancreatic cancer into WT mice.

In our study, normal lung and liver tissue exhibited peripheral fibrosis around large metastatic foci but not small foci. Activation of tumor-associated fibroblasts has been suggested to play a minor role in the early stages of enhanced proliferation of metastatic CSCs. It has been reported that metastasis of CSCs into organs is important for initial expansion of cancer cells in metastatic lesions. Periostin (POSTN), a protein that mediates the interaction between breast cancer cells and their niche, was identified as a factor expressed by stromal cells. This factor is required for CSC maintenance and metastatic colonization (15). It may be possible that certain factors derived from normal cells located around CSCs in the early stages of metastatic colonization play important roles in CSC colonization in distant organs, particularly the liver. Taken together, it is necessary to uncover not only the role of CTCs in the blood but also interactions between CSCs and distant organs (in this case, the liver) after engraftment. From our study, it is clear that elucidating the mechanism of interaction between CSCs and the local organ microenvironment is critical for eradication of metastasis and will lead to better prognosis of patients with pancreatic cancer.

References


Received March 23, 2014
Revised April 15, 2014
Accepted April 16, 2014