Peritoneal myofibroblasts at metastatic foci promote dissemination of pancreatic cancer

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Abstract. Myofibroblasts in the stroma of pancreatic cancers promote tumor proliferation, invasion and metastasis by increasing extracellular matrix and secretion of several growth factors. In contrast, the role of myofibroblasts at peritoneally disseminated sites of pancreatic cancer has not yet been determined. This study was designed to assess the role of myofibroblasts at peritoneally disseminated sites of pancreatic cancer. Three primary cultures of human peritoneal myofibroblasts (hPMFs) were established from disseminated sites of pancreatic cancer and their interactions with the SUIT-2 and CAPAN-1 human pancreatic cancer cell lines were analyzed in vitro. Using a model in BALB/c nu/nu mice, we compared the dissemination ability of intraperitoneally implanted pancreatic cancer cells, with and without hPMFs, and examined the presence of green fluorescent protein (GFP)-labeled hPMFs at peritoneally disseminated sites in mice. hPMFs significantly promoted the migration and invasion of pancreatic cancer cells (P<0.05), while the cancer cells significantly promoted the migration and invasion of hPMFs (P<0.05). In vivo, the number of peritoneally disseminated nodules, more than 3 mm in size, was significantly greater in mice implanted with cancer cells plus hPMFs compared to mice implanted with cancer cells alone, with GFP-labeled hPMFs surviving in the peritoneal cavity of the former. hPMFs promote the peritoneal dissemination of pancreatic cancer. The cancer-stromal cell interaction in the peritoneal cavity may be a new therapeutic target to prevent the dissemination of pancreatic cancer.

Introduction

Pancreatic cancer is one of the most lethal cancers, with a 5-year overall survival (OS) rate of only 4% (1). Despite developments in diagnostic imaging, most patients with pancreatic cancer are diagnosed with an advanced stage of the disease. For example, almost 33% of 100,313 pancreatic cancer patients diagnosed from 1989 through 1995 had metastasis (2). Moreover, most patients who undergo curative resection develop incurable local relapses, liver metastases and/or peritoneal dissemination.

A prospective study of 100 patients with peritoneal carcinomatosis resulting from non-gynecological malignancies found that the second most common primary tumor was pancreatic cancer (20%) (3). A French multicentric prospective study of 370 peritoneal carcinomatosis patients with non-gynecological malignancies showed a median OS of 3.1 months, but was only 2.1 months in those with pancreatic cancer (4). These data suggest that the novel therapies to control the peritoneal dissemination of pancreatic cancer may improve patient survival.

Pancreatic cancer is one of the most stroma-rich cancers and characterized by excessive desmoplasia, which plays a crucial role in its aggressive behavior (5,6). The stroma in these tumors is very heterogeneous, consisting of cellular and acellular components, including fibroblasts, myofibroblasts, immune cells, blood vessels, extracellular matrix (ECM) and soluble proteins such as cytokines and growth factors (7). Stromal myofibroblasts derived from the primary site of pancreatic cancer were shown to enhance the progression of pancreatic cancer (8). Moreover, stromal components were shown to promote the malignant behavior of pancreatic cancer cells, with myofibroblasts being the especially associated with cancer-stromal cell interactions in primary tumors (7,9-12). Few studies, however, assessed cancer-stromal cell interactions at peritoneally disseminated sites (13-15).

Myofibroblasts are regarded as playing a central role in pathogenesis of peritoneal fibrosis, which provide a favorable environment for the dissemination of cancer cells (13). Although myofibroblasts have been reported to derive from resident peritoneal fibroblasts, human peritoneal mesothelial cells (hPMCs) (16,17), bone marrow progenitor cells or the primary tumor itself (18,19), the origin of these myofibroblasts has not been clearly established. Also, during the initial stages of peritoneal
metastasis, cancer cells have been reported to attach to areas of exposure of collagen-rich connective tissue matrices because of the lack of mesothelium (20-23). Thus, the interactions between cancer cells derived from the primary tumor and submesothelial layer components such as myofibroblasts may be a key to peritoneal dissemination (24). Hepatocyte growth factors (HGF) produced by human peritoneal myofibroblasts have been found to promote the peritoneal dissemination of gastric cancer (25), and myofibroblasts in omentum were shown to be activated by tumor cells and to promote the growth, adhesion and invasiveness of ovarian cancer (14). However, the roles of peritoneal myofibroblasts and their matrices in the peritoneal dissemination of pancreatic cancer remain unclear. The development of novel therapies to control the peritoneal dissemination of pancreatic cancer requires further understanding of the mechanisms of induction and the roles of peritoneal myofibroblasts in the process of dissemination.

In the present study, we established three primary cultures of human peritoneal myofibroblasts (hPMFs) isolated from peritoneally disseminated nodules of pancreatic cancer and investigated the interactions between primary cultures of hPMFs and pancreatic cancer cells in vitro and in vivo.

Materials and methods

Tissues, cells and culture conditions. Peritoneally disseminated tissues were obtained from 3 patients who underwent palliative (bypass) operations for unresectable pancreatic cancer at our institution under an Institutional Review Board-approved protocol following informed consent. Human peritoneal myofibroblasts (hPMFs) were isolated from these fresh surgical specimens using the outgrowth method in our laboratory (26,27), and the identity of these hPMFs was confirmed by morphology (spindle-shaped cells) and immunofluorescence staining for α-SMA, vimentin and cytokeratin 18 (CK18) (8,28). Cells at passage numbers 2-4 were used for all assays. Two pancreatic cancer cell lines, SUIT-2 (Japan Health Sciences Foundation) and CAPAN-1 (American Type Culture Collection, Manassas, VA, USA), were maintained as previously described (29).

Immunohistochemistry and histopathology. hPMFs isolated from peritoneally disseminated sites of pancreatic cancer were evaluated by H&E and α-SMA immunohistochemical staining. Immunohistochemical staining was performed using a Histofine SAB-PO kit (Nichirei, Tokyo, Japan). Tissues were sectioned to a thickness of 4 µm and were incubated with antibody overnight at 4˚C. Human tissues were incubated with mouse monoclonal anti-α-SMA antibody (1:50; Dako, Glostrup, Denmark) and mouse tissues were incubated with rabbit polyclonal anti-α-SMA antibody (1:50; Abcam, Cambridge, MA, USA) (30). Cells were considered positively stained when either the membrane or cytoplasm was stained. All slides were evaluated independently by two investigators blinded to the knowledge of the clinical features of each patient.

Immunofluorescence staining. hPMFs were incubated with rabbit polyclonal anti-vimentin antibody (1:100; Abcam), mouse monoclonal anti-CK18 antibody (1:100; sc-6259, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-α-SMA antibody (1:50; Dako). For comparison, human mesothelial cells from cancerous ascites of pancreatic cancer were incubated with antibodies to vimentin and CK18.

Matrigel invasion and migration assays (indirect co-culture). Cell invasion was measured by counting the number of cells that invaded Matrigel-coated Transwell chambers with 8-µm pores (BD Biosciences, Franklin Lakes, NJ, USA), as previously described (31). Briefly, Transwell inserts were coated with 20 µg/well Matrigel (BD Biosciences). Each lower well of a 24-well plate was seeded with myofibroblasts (2.0x10^5/well) in 750 µl of DMEM supplemented with 10% FBS or medium alone and incubated for 24 h. Cancer cells (4.0x10^5/well) in 250 µl of DMEM supplemented with 10% FBS were seeded into each upper well. After 69 h of incubation, cells on the lower surface of the Matrigel-coated membrane were fixed with 70% ethanol, stained with H&E, and counted in five randomly selected fields at a x100, magnification under a light microscope. The mobility of SUIT-2 and CAPAN-1 pancreatic cancer cells was assessed using uncoated Transwell inserts and incubation times of 24 and 48 h, respectively. To assess the invasiveness of myofibroblasts, each lower well was seeded with cancer cells (4.0x10^4/well) in 750 µl of DMEM supplemented with 10% FBS or medium alone and incubated for 24 h. Myofibroblasts (1.5x10^5/well) in 250 µl of DMEM supplemented with 10% FBS were seeded in each upper well and incubated for 20 h for the invasion assay and 14 h for the migration assay. The results were expressed as the mean number of invaded cells per field. Each experiment was carried out in triplicate wells and repeated at least 3 times.

Animal models. All experiments with mice were conducted with the approval of the Ethics Committee of Kyushu University. The model of intraperitoneal implantation of BALB/c nu/nu mice (5-6 weeks of age, Kyudo Co.) was used to analyze the dissemination activity of cancer cells alone and cancer cells plus hPMFs. All animals were bred in laminar-flow cabinets under specific pathogen-free conditions. Prior to implanting, the cells were briefly treated with trypsin/EDTA and washed twice with serum-free medium. The mice were anesthetized with ether and suspensions of 2x10^5 SUIT-2 or 1x10^6 CAPAN-1 cells in 200 µl PBS with or without 1x10^5 hPMFs in 200 µl PBS were transplanted into the peritoneal cavity of groups of 10 mice. All mice were sacrificed 28 days later, and disseminated nodules >3 mm in size were counted. Each experiment was repeated 3 times.

Green fluorescent protein (GFP)-labeled myofibroblasts in vivo. The GIPZ non-silencing control vector for shRNA (Thermo Fisher Scientific, Rockford, IL, USA) was used to express GFP in hPMFs. 2x10^5 SUIT-2 or 1x10^6 CAPAN-1 cells in 200 µl PBS with or without 1x10^5 hPMFs in 200 µl PBS were transplanted into the peritoneal cavity of groups of 10 mice. All mice were sacrificed 28 days later, and disseminated nodules >3 mm in size were counted. Each experiment was repeated 3 times.

Flow cytometry analysis. Cultured cells were harvested by exposure to trypsin/EDTA for 5 min at 37˚C, washed in 10% FBS/DMEM, suspended in ice-cold 1% FBS/PBS solution and analyzed using a flow cytometer (EC800 Cell Analyzer, Sony) equipped with a laser that provided an excita-
Expression of GFP in original hPMFs and GFP-labeled hPMFs was analyzed by flow cytometry.

Statistical analysis. For in vitro and in vivo experiments, all values were expressed as mean ± SD and compared using Student’s t-tests. All experiments were performed at least three times. Statistical significance was defined as P<0.05. All statistical analyses were performed using JMP 9 software (SAS Institute, Cary, NC, USA).

Results

Activated myofibroblasts are abundant in human peritoneally disseminated nodules. Specimens were obtained from peritoneally disseminated nodules of human pancreatic cancers. These nodules contained many activated hPMFs, as shown by their morphology (spindle-shaped cells; Fig. 1, left panel) and their positivity for α-SMA staining (Fig. 1, right panel). Primary cultures of these hPMFs were positive for α-SMA and vimentin and negative for CK18, suggesting that these cells were activated and were not contaminated by mesothelial cells (Fig. 2).

Figure 1. Immunohistochemical analysis of α-SMA expression at sites of peritoneal dissemination of human pancreatic cancer. The specimen was taken from a disseminated nodule of human pancreatic cancer. Many myofibroblasts were observed, as shown by H&E staining (left panel) and were immunohistochemically positive for α-SMA (right panel). Scale bars, 100 µm. Original magnification, x200.

Figure 2. Immunofluorescent staining of α-SMA, vimentin and cytokeratin 18 in hPMFs and human mesothelial cells. (A) All hPMFs expressed α-SMA and vimentin but not cytokeratin 18. (B) Mesothelial cells expressed cytokeratin 18 and vimentin. Original magnification, x200.
Mobility and invasiveness of hPMFs are enhanced when co-cultured with pancreatic cancer cells. To investigate the cancer-stromal cell interactions between hPMFs and pancreatic cancer cells, we evaluated the effect of pancreatic cancer cells on hPMFs migration and invasiveness using indirect co-cultures. We found that both SUIT-2 and CAPAN-1 pancreatic cancer cells markedly stimulated the migration (P<0.001) (Fig. 3A) and invasiveness (P<0.001) (Fig. 3B) of hPMFs.
Mobility and invasiveness of pancreatic cancer cells are enhanced when co-cultured with hPMFs. Similarly, we investigated the effect of co-cultured hPMFs on the mobility and invasiveness of pancreatic cancer cells. We found that hPMFs markedly stimulated the migration (P<0.05) (Fig. 4A and B) and invasiveness (P<0.01) (Fig. 4C and D) of SUIT-2 and CAPAN-1 cells.

hPMFs promote the peritoneal dissemination of pancreatic cancer cells in vivo. Intraperitoneal implantation of cells into BALB/c nu/nu mice, aged 5-6 weeks, was used to compare the dissemination over 28 days of pancreatic cancer cells alone and with hPMFs. Intraperitoneal injection of 2x10⁵ SUIT-2 cells plus 1x10⁶ hPMFs (Fig. 5A, right panel) yielded a mean 22.5±4.9 peritoneally disseminated nodules >3 mm, compared with 3.8±2.0 nodules in mice injected with SUIT-2 cells plus PBS (Fig. 5A, left panel), a difference that was statistically significant (P<0.0001) (Fig. 5B, left panel). Similarly, injection of 1x10⁶ CAPAN-1 cells plus 1x10⁶ hPMFs yielded a mean 12.8±1.5 peritoneally disseminated nodules, compared with 2.5±4.0 nodules in mice injected with CAPAN-1 cells plus PBS (P<0.0001) (Fig. 5B, right panel). H&E staining and immunohistochemical analyses of nodules in mice injected with SUIT-2 cells plus hPMFs showed the presence of α-SMA positive myofibroblasts (Fig. 6), similar to those observed human peritoneally disseminated nodules (Fig. 1).

hPMFs survive in peritoneally disseminated nodules of nude mice only when implanted together with pancreatic cancer cells. To investigate whether the hPMFs survive and exist in the peritoneally disseminated nodules after intraperitoneal implantation with or without pancreatic cancer cells, we used GFP-labeled hPMFs. Using flow cytometry, we evaluated the time-dependent changes in GFP expression of cultured hPMFs.
after labeling, finding that the expression of GFP in cultured hPMFs remained constant for 28 days after labeling (Fig. 7).

Intraperitoneal transplantation of $2 \times 10^5$ SUIT-2 cells and $1 \times 10^6$ GFP-labeled hPMFs resulted in peritoneal dissemination in mice (Fig. 8A). Moreover, GFP-labeled hPMFs were observed in these peritoneally disseminated nodules 28 days after transplantation with pancreatic cancer cells (Fig. 8B).

**Discussion**

In the present study, we successfully isolated hPMFs by outgrowth from peritoneally disseminated nodules of pancreatic cancer and investigated the interaction between these primary cultured hPMFs and pancreatic cancer cells. We found that myofibroblasts were abundant around cancer cells at peritone-
ally disseminated sites, similar to primary sites of pancreatic cancer, and that hPMFs promoted the migration and invasiveness of pancreatic cancer cells. Conversely, pancreatic cancer cells also promoted the migration and invasiveness of hPMFs. Our in vivo results revealed that number of peritoneally disseminated nodules was significantly greater when cancer cells were transplanted with hPMFs than when cancer cells were transplanted alone. In vivo experiments using GFP-labeled hPMFs showed that peritoneally disseminated nodules contained transplanted human myofibroblasts. Others also reported that myofibroblasts from human omentum were shown to promote the peritoneal dissemination of ovarian cancer cells in nude mice (14), and peritoneal myofibroblasts promoted the mobility and invasiveness of gastric cancer cells (13,32). These findings suggest that hPMFs accelerate the malignant behavior of cancer cells through cancer-stromal cell interactions during the formation of peritoneally disseminated nodules.

Although several reports exist on methods of isolation of peritoneal myofibroblasts or mesothelial cells (14,28), there is no difference between the two methods with the exception of the digestion time by trypsin/EDTA. These reports suggested that primary cultures of human peritoneal myofibroblasts and mesothelial cells could be contaminated with other cell types. In contrast, we established hPMFs from peritoneally disseminated nodules using an outgrowth method, similar to the method used to isolate human pancreatic stellate cells (hPSCs) from primary pancreatic cancers (26,27). Microscopic observation of the primary cultured cells and in vitro immunofluorescence staining revealed that the isolated cells were spindle-shaped, positive for α-SMA and vimentin, and negative for CK18, indicating that these isolated cells are activated hPMFs and that there was no contamination with hPSCs (28).

To confirm whether hPMFs, which were co-implanted with cancer cells, were present in the peritoneally disseminated nodules in mice, we used GFP-labeled hPMFs and found GFP positive cells in some disseminated nodules. Similar results were obtained using GFP-labeled hPSCs derived from primary pancreatic cancers. hPSCs transplanted into mouse peritoneum with pancreatic cancer cells were shown to be present at peritoneally disseminated sites of pancreatic cancer (19). In addition, evaluation of the disseminated nodules derived from cancer cells co-transplanted with hPSCs in the peritoneal cavity showed that these hPSCs promoted the dissemination of pancreatic cancer cells in vivo, with no significant differences between hPMFs and hPSCs (data not shown). We also found that both hPMFs and hPSCs were not engrafted in the peritoneal cavity of nude mice if transplanted without cancer cells (data not shown). These results suggest that myofibroblasts liberated from their original tissues could engraft into the peritoneal cavities of mice when transplanted along with pancreatic cancer cells.

The peritoneum consists of a monolayer of mesothelial cells supported by a basement membrane that rests on a layer of connective tissue (24). Transforming growth factor-β1 (TGF-β1) derived from cancer cells in the peritoneal microenvironment was shown to activate hPMCs and transform these cells to myofibroblast-like cells (17). These findings focused on the role of hPMCs in the early phases of formation of peritoneally disseminated nodules of cancer cells. We found that hPMFs were abundant around cancer cells at peritoneally disseminated sites of pancreatic cancer, but we could not distinguish hPMC-derived myofibroblasts from other myofibroblasts.

hPMFs may derive from normal fibroblasts in the sub-mesothelial layer of the abdominal wall or from the transformation of mesothelial cells by TGF-β1 or HGF (16,17,25,33). Alternatively, hPMFs may be hPSCs liberated from the pancreas (19) or mesenchymal stem cells from the bone marrow (10). Further efforts are needed to identify the origin of these cells and the mechanism inducing their activation. These findings may contribute to the identification of new therapeutic targets to prevent the peritoneal dissemination of pancreatic cancer.

In conclusion, hPMFs can be isolated as primary cultures without contamination by hPSCs from disseminated nodules of pancreatic cancer. The cancer-stromal cell interactions between pancreatic cancer cells and hPMFs are important in the peritoneal dissemination of pancreatic cancer cells. Therapy targeting this interaction may improve the prognosis of patients with pancreatic cancer.

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