Abstract. Background: The aim of this study was to evaluate whether apoptosis-resistant cancer cells have cancer stem cell (CSC)-like properties. Materials and Methods: Panc-1 pancreatic cancer cells were incubated in the presence of 5-fluorouracil (5-FU) for 24 h, and further incubated without 5-FU for 28 days. To assess the capacity of self-renewal, surviving cells were planted for sphere-forming assay. Epithelial-to-mesenchymal transition (EMT) was induced with TGF-β, then mRNA expression was evaluated by real-time PCR for E-cadherin, SNAIL, and vimentin. The E-Cadherin protein levels were also examined by immunoblot analysis. The Local invasion ability was analyzed by Matrigel invasion assay. Results: The frequency of cells that were capable of initiating spheres was higher in 5-FU-pre treated cells, which also overexpressed stem cell marker genes, OCT4 and NANOG. Matrigel invasion activity of apoptosis-resistant Panc-1 cells was greater than that of control Panc-1 cells. Conclusion: Apoptosis-resistant cancer cells have CSC-like properties, i.e., able to initiate sphere formation, express stem cell genes, and respond to EMT stimulation.

Pancreatic cancer is a highly aggressive malignant disease with notable resistance to chemotherapy and chemoradiotherapy (1, 2). Even when cases respond to chemotherapy, they gradually become resistant to treatment and the outcomes are quite disappointing.

Materials and Methods

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Emerging evidence suggests that malignant tumor is composed of a small population of undifferentiated- or poorly differentiated cells, the so-called cancer stem cells (CSCs) (3, 4). Recently, CSCs were reported to be enriched within chemoradiotherapy-resistant subpopulations in pancreatic cancer cell lines (5-7). Thus, selection of chemotherapy-resistant cells by using anticancer drugs such as 5-Fluorouracil (5FU) or gemcitabine would be an effective means to enrich for the CSC-like subpopulation.

There is also an increasing body of evidence to suggest that there is a molecular association between the epithelial-to-mesenchymal transition (EMT) phenotype and chemoresistance (5-9). Shah et al. (6) have demonstrated that induction of gemcitabine resistance in pancreatic cancer cells led to cells growing with an EMT-associated molecular status and mesenchymal-like morphology. More recently, Wang et al. (7) have suggested that gemcitabine-resistant pancreatic cancer cells exhibit a mesenchymal phenotype with enhanced NOTCH-associated molecules.

This report focuses on the CSCs-like properties of chemotherapy-resistant Panc-1 cells. First, we review apoptosis in Panc-1 cells caused by 5-FU exposure. Next, we compare the capability of initiating spheres and the expression of stemness genes, OCT4 and NANOG in surviving cells. We then review on EMT that is induced by TGF-β stimulation. Finally, we evaluate the EMT-associated molecular alterations. A better understanding of this drug-resistant population may lead to the development of more effective therapeutic interventions for this lethal disease.

Cells and cell culture and reagents. The Panc-1 cell line was purchased from the American Type Tissue Collection (Manassas, VA, USA) and grown in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum, 100,000 U/l penicillin, 100 mg/l streptomycin (Invitrogen, Carlsbad, CA, USA).
Eighty percent sub-confluent Panc-1 cells were cultured in DMEM containing 30 μg/ml 5-FU for 24 h. The medium was then changed to 5-FU-free medium. 5-FU and cisplatin were purchased from Wako (Osaka, Japan). Human TGF-β1 (R & D Systems, Minneapolis, MN, USA) was reconstituted according to the supplied instructions. The following antibodies were used for immunoblotting and immunocytochemistry: anti-E-cadherin (Cell Signaling Technology; Beverly, MA USA), anti-beta-actin (SIGMA; St. Louis, MO, USA).

**Reverse transcription-PCR analysis of stem cell marker genes.** Total RNA was extracted from Panc-1 cells with and without 5-FU treatment using the TRIzol reagent (Invitrogen), followed by cleanup with the RNeasy Mini Kit (Qiagen, Venlo, Netherlands), according to the manufacturer’s instruction. The RNA samples were treated with deoxyribonucleases I (Invitrogen) and reverse transcription polymerase chain reaction (RT-PCR) was performed using the Super Script First-Strand Synthesis System for RT-PCR (Invitrogen), Real-time PCR technology (StepOne; Applied Biosystems, Foster City, CA, USA), and SYBR green system (Applied Biosystems). The comparative Ct method was used to determine relative gene expression levels for each target gene and 18S RNA was used as an internal control. The PCR primers determine relative gene expression levels for each target gene and 18S RNA was used as an internal control. The PCR primers included *E-cadherin*: forward: GGT TCT TGA GTG TTT TTG TAA, reverse: GGAGGCTTATGGAGGA, *SNAIL*: forward: GCT CCT TCG TCC TTC TCC TC, reverse: TGA CAT CTG AGT GGG TCT GG, *OCT4*: forward: AGT GAG AGG CAA CCT GGA GA, reverse: AGA GTG GTG ACG GAG ACA GG, and *NANOG*: forward: CAA AGG CAA ACA ACC CAC TT, reverse: CTG GGG TAG GTA GGT GCT GA.

**Immunoblot analysis.** Cells were lysed by incubation on ice for 30 min in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% TritonX-100, 150 mM NaCl, 10% glycerol, 1 mM Na3VO4, 50 mM NaF, 100 mM phenylmethylsulfonyl fluoride, 1 mM PMSF, and a protease inhibitor mixture (Complete Protease Inhibitor Cocktail; Roche, Basel, Switzerland). After centrifugation at 14,000 x g for 15 min at 4°C, the supernatants were collected. Samples were resolved by 12.5% SDS-PAGE, transferred to nitrocellulose membranes, and blotted with primary antibodies. Horseradish peroxidase-conjugated secondary antibodies (BioSource International, Camarillo, CA, USA) were used at appropriate concentrations. The bound antibodies were visualized using a chemiluminescent substrate (ECL; GE Health Care Sciences, Little Chalfont, UK) and exposed to Kodak X-Omat film (Eastman Kodak, Rochester, NY, USA).

**Cell viability assay.** To assess chemosensitivity of 5-FU-resistant cells, cisplatin, and gemcitabine, cells were seeded at 1.000 per well (96-well Coster) and treated for 5 days with 30 μg/ml 5-FU, 5 μg/ml cisplatin, and 7.5 mM gemcitabine. Relative cell numbers were determined by MTT assays.

**Invasion assay.** Invasion assay was performed using Biocoat Cellwre (Becton Dickinson, Franklin Lakes, NJ, USA). Cells were pre-treated with 7.5 ng/ml TGF-β for 72 h. Five hundred microliters of cell suspension containing 2×10⁴ of TGF-β-pretreated cells were added to the matrigel-coated inserts, and 750 μl of culture containing FBS were added to the lower chamber. The assay chambers were incubated at 37°C with humidified 5% CO₂ for 22 h. The invaded cells were stained with a Calcein-AM solution (Dojinkagaku, Kumamoto, Japan).

**Statistical analysis.** Data are presented as the mean±SD. To assess statistical significance of differences, the t-test was performed. p-Values <0.05 were considered significant.

**Results**

5-FU-pretreated cells exhibit re-growing capability for regrowth. We initially exposed pancreatic cancer cells to 5-FU (30 μg/ml) to eliminate 5-FU-sensitive cells. This concentration was approximately 300-fold higher than the IC₅₀ value (≈100 ng/ml). On short time exposure (24 hr) to 5-FU, the majority of Panc-1 cells underwent apoptosis within a week, and a very small subpopulation (fewer than 1%) of cells had survived at 14 days (Figure 1A). The surviving cells started to proliferate in the same culture dish by additional culturing for 1 to 2 weeks. Finally, these cells formed a large colony composed of proliferating cancer cells (Figure 1A). In addition, when these cells were kept in culture by re-plating onto another dish, the cells grew to exhibit a similar appearance to that of the untreated, control Panc-1 cells.

By determining the gene expression profiles during these processes by DNA microarray analysis, we found that expression levels of certain stemness-genes such as *OCT4* and *NANOG* were enhanced in surviving cells (Figure 1B). These results lead us to believe that cells which are capable of expressing OCT4 and/or NANOG selectively survive after exposure to 5-FU. These observations appear to be consistent with the concept that so-called CSCs expressing such proteins are chemotherapy-resistant (3).

The frequency of cells that were capable of initiating spheres was higher in 5-FU-pretreated cells. Since we determined that both OCT4 and NANOG levels were up-regulated in 5-FU-pretreated Panc-1 cells, we next evaluated if these alterations were associated with CSCs. Anchorage-independent growth, one of the characteristics of the CSC-like phenotype (10), was then evaluated by performing a sphere-forming assay using an ultra-low attachment culture dish in serum-free conditions. The number of spheres which arose from control cells was approximately 50 per 104 cells, while 5-FU-pretreated cells formed approximately 350 spheres per 104 cells (Figure 2A). These results suggest that sphere-forming activity is greater in the 5-FU-pretreated Panc-1 cell population. To evaluate if expression of either *OCT4* mRNA or *NANOG* mRNA expression was concordant with sphere formation, we
Figure 1. 5-FU-pretreated small subpopulation of Panc-1 pancreatic cancer cells can re-grow with expression of stemness genes such as OCT4 and NANOG. Panc-1 cells were cultured in the presence or absence of 5-FU (30 μg/ml) for 24 hours and subsequent cultured in 5-FU-free conditions was performed. A: Representative images of the cultured cells before (day 0) and after 5-FU treatment (day 7, 14, 28, after re-plating). Cells gradually decreased by apoptosis and a small number of cells still survived at day 14. Surviving cells re-grow by continuous culturing under 5-FU-free conditions. The appearance of re-growing cells and the cells after re-plating was similar to that of control (day 0) cells exhibiting epithelial-like morphology. B: Cells were lysed and total RNA samples were extracted at the indicated time period before and after 5-FU treatment. Real-time PCR was performed to analyze mRNA levels of both OCT4 and NANOG. Data are expressed as the mean±SD from three independent experiments. \( p <0.05 \) for 5-FU treatment (day 7, day 14) vs. control.
performed real-time PCR of these molecules from the Panc-1-derived spheres (Figure 2B). As expected, spheres arising from Panc-1 cells expressed greater amounts of OCT4 mRNA and NANOG mRNA, while non-sphere-derived Panc-1 cells expressed relatively small amount of these mRNAs.

The 5-FU-pretreated subpopulation was responsive to TGF-β-mediated EMT and invasion. Since EMT has been identified as a key phenomenon which is tightly linked to CSC-like properties (9), we next recorded the responsiveness to an EMT-inducing stimulus such as TGF-β.

Figure 2. 5-FU-pretreated Panc-1 cells exhibit so-called CSC-like characteristics. Panc-1 cells exposed to short-term 5-FU-treatment. Control (without 5-FU treatment) and 5-FU-treated (21 days after the 5-FU exposure) cell were collected. A: The cells were transferred into anchorage-independent ultra-low attachment conditions as described in the Materials and Methods. The number of spheres formed from 104 cells were counted. Data are expressed as mean±SD from three independent experiments. P <0.05 for 5-FU (day 7, day 14) vs. control. B: RNA samples were obtained from non-sphere forming Panc-1 cells (control) and Panc-1-derived spheres. Real-time PCR was performed to analyze the mRNA levels of both OCT4 and NANOG. Data are expressed as the mean±SD from three independent experiments. P <0.05 for sphere vs. control.
Figure 3. 5-FU-pretreated Panc-1 cells are highly responsive to TGF-β-mediated EMT-like alterations and invasion. Panc-1 cells exposed to short-term 5-FU treatment as described in the Materials and Methods. Control and 5-FU-pretreated cells were incubated in the presence or absence of TGF-β (7.5 ng/ml). A: RNA samples were extracted from control and 5-FU-pretreated cells. Real-time PCR was performed for E-cadherin and SNAIL mRNA. B: Protein samples were obtained from control and 5-FU-pretreated Panc-1 cells on 14 and 28 days after the treatment. Immunoblot analysis was performed using anti-E-cadherin antibodies as a primary antibody. C: Control cells and 5-FU-pretreated cells (21 days after 5-FU removal) were transferred into an invasion assay system as described in the Materials and Methods. Data are expressed as the mean±SD from three independent experiments. P <0.05 for 5-FU vs. control.
Panc-1 cells are known as a TGF-β-responsive cell line, which undergoes EMT-like alterations, spindle-like appearance, rapid decrease in E-cadherin level, and enhanced expression of EMT-associated transcription factors, such as SNAIL (11). To evaluate the EMT-associated molecular alterations, we quantified the mRNA levels of both E-cadherin and SNAIL. Surprisingly, E-cadherin mRNA levels were greatly enhanced in 5-FU-pretreated Panc-1 cells without TGF-β treatment as compared to Panc-1 control cells (Figure 3A). However, when these cells were treated with TGF-β, a reduction of E-cadherin mRNA levels was clearly identified in 5-FU-pretreated cells (~13-fold decrease), as compared to untreated control cells (~2-fold decrease). Because E-cadherin is a well-known cell surface marker of epithelial phenotype, these results suggest that 5-FU enhances epithelial-like characteristics of Panc-1 cells. In addition, SNAIL mRNA levels greatly responded to TGF-β in 5-FU-pretreated cells (~35-fold increase) as compared to untreated control cells (~2-fold increase), consistent with E-cadherin mRNA responses to TGF-β. Finally, we determined, E-cadherin protein levels by immunoblot analysis (Figure 3B). Panc-1 cells before 5-FU exposure expressed modest levels of E-cadherin protein, while protein levels gradually increased (day14-21) after 5-FU treatment. As enhanced E-cadherin levels were strongly suppressed by TGF-β in 5-FU-pretreated cells, these results were consistent with the E-cadherin mRNA alterations observed by real-time PCR.

Increased invasion activity is one of the important characteristics of the EMT. Thus, we next determined the invasion activity by using a matrigel invasion assay system (Figure 3C). Interestingly, TGF-β modestly enhanced matrigel invasion activity in control Panc-1 cells, while TGF-β-associated invasion was strongly enhanced only in 5-FU-pretreated Panc-1 cells. Taken together, these results suggest that 5-FU-pretreated Panc-1 cells were more responsive to TGF-β-induced EMT-associated alterations.

Discussion

The principal findings of this study relate to the characteristics of chemotherapy-resistant pancreatic cancer cells. The results demonstrate that: i) a small population of Panc-1 cells is resistant to short time exposure to anticancer drugs such as 5-FU; ii) the resistant cells are able to regrow after removal of 5-FU; iii) the 5-FU-resistant cells exhibit CSC-like properties, sphere-forming growth in anchorage-independent conditions; iv) the resistant cells do not exhibit EMT-like alterations during their re-growing period, however, they are highly responsive to TGF-β-mediated EMT. These results are consistent with previous reports regarding the chemo/radioresistance and CSC-like phenotypes (3, 9).

Our results also suggest that the enhanced potential to undergo EMT is linked to chemoresistance. Our results may suggest that the chemotherapy-resistant subpopulation can regrow, expressing an epithelial phenotype and possessing an enhanced capability to undergo EMT by certain stimuli such as TGF-β. Thus, ideally, our results caution that the cell population surviving after chemotherapy may have a more aggressive invasion/metastatic potential, as well as chemoresistance.

Identifying CSCs from pancreatic cancer cells has been successfully carried out through some methods based on the properties of CSCs. Firstly, isolating the population by flow cytometry according to cell surface markers such as CD44, CD24, or CD133 (12, 13). Secondly, the side population (SP) cells has been characterized as CSCs, as we and others have reported previously (14-16). Thirdly, selective elimination of chemotherapy- and/or chemoradiotherapy-resistant cells enriches CSC-like cells within malignant cells, including pancreatic cancer cells (5-7). In the present study, we chose the chemotherapy-resistant population, as the major purpose was the phenotypic analysis of drug-resistant cancer cells. In addition, we chose short-term exposure (~24 h) to a relatively high concentration of 5-FU, and the subsequent long-term observation was performed under 5-FU-free conditions. We believe that our experimental conditions may mimic an in vivo tumor exposed to 5-FU, as this drug exhibits a relatively short half life in vivo (17).

We found that 5-FU-pretreated Panc-1 cells expressed enhanced mRNA levels of the so-called stemness genes OCT4 and NANOG (18-20). In addition, we identified that 5-FU-pretreated cells exhibited a so-called CSC-like phenotype, including a sphere-forming capability. Our results support the concept that chemotherapy may enrich the population of drug-resistant CSC-like cells.

There have been some reports suggesting that chemotherapy, and/or chemoradiotherapy-resistant pancreatic cancer cells exhibit a mesenchymal phenotype (6, 7). In contrast, our results demonstrate that the levels of E-cadherin gradually increased after 5-FU treatment, suggesting an enhanced epithelial phenotype. This discrepancy would be explained if drug-resistant CSCs did not always exhibit a mesenchymal phenotype but they rapidly underwent the converse phenomenon, MET, after drug removal. Indeed, our previous study demonstrated that the CSC-enriched SP of pancreatic cancer cells exhibited enhanced E-cadherin expression during their proliferative phase (14). These SP cells were responsive to TGF-β-induced EMT and underwent rapid MET after TGF-β removal. In addition, the SP cells which underwent MET were still highly responsive to TGF-mediated EMT (14). Thus, taken together with the present results, CSC-like cells may have exquisite potential to change their phenotype between epithelial and mesenchymal. These characteristics likely contribute to their metastatic potential, as well as their drug-resistance.
EMT-inducing stimuli, e.g., exposure to TGF-β and induction of transcription factors such as SNAIL, have been reported as they also contribute to EMT-associated chemoresistance and stemness (8, 9). Thus, we tested if TGF-β enhanced sphere formation in 5-FU-pretreated Panc-1 cells. As expected, TGF-β dramatically increased the number of spheres formed by 5-FU-pretreated cells. These results may support the concept that chemotherapy-resistant cells are responsive to an EMT-associated phenomenon such as enhanced CSC-like properties, although those subpopulations do not always exhibit a mesenchymal phenotype.

In conclusion, our results demonstrate that pancreatic cancer cells surviving after 5-FU exposure exhibit re-growth, with enhanced expression of the so-called stemness genes OCT4 and NANOG. The surviving cells were enriched with the cells which have the capability to form spheres under anchorage-independent conditions, an important characteristic of CSCs. The 5-FU-pretreated cells were highly responsive to TGF-β-mediated EMT and invasion. Thus, our results are consistent with the concept that incomplete chemotheraphy may occasionally enhance the malignant potential of cancer. Our results directly demonstrate that chemoresistance correlates with the enhanced potential to undergo EMT, as well as invasion. Targeting the chemoresistance-resistant subpopulation and CSCs would be attractive strategies for the treatment of pancreatic cancer.

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References


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