Induction of Metastasis by Low-dose Gemcitabine in a Pancreatic Cancer Orthotopic Mouse Model: An Opposite Effect of Chemotherapy

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Abstract. *Background/Aim:* Gemcitabine is standard first-line treatment for patients with advanced pancreatic cancer, however the efficacy is limited. Although acquired drug resistance and side-effects are known to limit efficacy, opposite effects of a drug, which enhance the malignancy of treated cancer, have been observed but are not well understood. The aim of the present study was to determine whether gemcitabine has such opposite effects on the BxPC-3 human pancreatic cancer cell line expressing green fluorescent protein (BxPC-3-GFP) in an orthotopic mouse model. *Materials and Methods:* BxPC-3-GFP tumors grown subcutaneously in nude mice were harvested. Tumor fragments were orthotopically implanted in the tail of the pancreas of nude mice using the technique of surgical orthotopic implantation. The BxPC-3-GFP orthotopic models were divided randomly into three groups: Group 1: untreated control; Group 2: low-dose gemcitabine (weekly intraperitoneal injection at 25 mg/kg for 6 weeks); Group 3: high-dose gemcitabine (weekly intraperitoneal injection at 125

mg/kg for 6 weeks). Each group comprised eight mice. Tumor size, fluorescent area of metastases, and body weight were measured. *Results:* Low- and high-dose gemcitabine inhibited primary tumor growth in a dose-dependent manner, and to the greatest extent by high-dose gemcitabine compared to the untreated control ($p=0.0134$). In contrast, the extent of metastasis on the peritoneum was significantly increased by low-dose gemcitabine compared to the untreated control ($p=0.0112$). The extent of metastasis showed no significant difference between the untreated control and mice treated with high-dose gemcitabine. Body weight of the treated mice was not significantly different from that of the untreated mice. *Conclusion:* The use of very bright GFP expressing of BxPC-3 cells and the orthotopic model demonstrated an unexpected increase in metastasis by low-dose gemcitabine. Future experiments will investigate the mechanism of this phenomenon.

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Key Words: Pancreatic cancer, green fluorescent protein, gemcitabine, metastasis, opposite effect.

Pancreatic cancer accounts for approximately 3% of all cancer in the United States and about 7% of all cancer deaths (1). The 5-year survival rate of patients diagnosed with pancreatic cancer is still only 9%, despite improvements in the past decade. Gemcitabine has been standard first-line treatment for patients with advanced pancreatic cancer since 1997 (2). Although many clinical trials tested new chemotherapy drugs combined with gemcitabine for pancreatic cancer, only combination therapy with nab-paclitaxel has been shown to be superior to gemcitabine alone (3).

The efficacy of chemotherapy against pancreatic cancer is limited in most cases due to drug resistance. Chemotherapy itself may induce some changes in the tumor microenvironment which can promote cancer cell proliferation and metastasis (4). We previously showed that the chemotherapeutic cyclophosphamide actually enhanced the malignancy of implanted

cancer cells (5). Cyclophosphamide pretreatment enhanced intravascular proliferation, extravasation, and subsequent colony formation of HT1080 human fibrosarcoma cells. However, little is still known about the impact of the tumor microenvironment on chemotherapeutic outcome (6).

We have improved the utility of mouse models by transforming cancer cells with the green fluorescent protein (GFP) gene, from the jellyfish *Aequorea victoria*, to enable better detection of primary tumor growth and metastasis (7, 8). We also established an orthotopic model of metastatic pancreatic cancer where primary and metastatic sites can be imaged using GFP fluorescence (9). In particular, micro-metastases are easy to detect by GFP fluorescence.

In the preset study, we evaluated efficacy of gemcitabine on primary tumor and growth of metastases in the BxPC-3-expressing GFP orthotopic xenograft mouse model of pancreatic cancer. The aim of the present study was to identify whether gemcitabine can stimulate metastasis.

Materials and Methods

Mice. Athymic *nu/nu* nude mice (AntiCancer, Inc., San Diego, CA, USA), 4-6 weeks old, were used in this experiment. Animal housing and their diet were as previously described (10). Mice were observed on a daily basis and humanely sacrificed by CO₂ inhalation with humane-endpoint criteria described in previous publications (10, 11). All mice were handled according to the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals under Assurance Number A3873-1.

BxPC-3 human pancreatic cancer cells expressing green fluorescent protein (BxPC-3-GFP). The BxPC-3 human pancreatic cancer cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, and 1% penicillin-streptomycin at 37°C in 5% CO₂. GFP retroviral transduction and selection of high GFP-expressing BxPC-3 cells were established in our laboratory and previously reported (9, 11).

Surgical orthotopic implantation. BxPC-3-GFP cells (2×10⁶ cells, 0.2 ml) in fetal bovine serum-free RPMI 1640 were subcutaneously injected into the shoulder of nude mice. Growing tumors were harvested and cut into 5 mm³ fragments for surgical orthotopic implantation. A 10 mm transverse incision was made on the left flank of the mouse under anesthesia. The pancreatic tail was exposed, and a single tumor fragment was implanted orthotopically into the pancreatic tail with 8-0 nylon suture (Ethicon, Inc., Somerville, NJ, USA). The wound was closed with 6-0 nylon suture (Ethicon, Inc.) (10, 12).

Treatment of the BxPC-3-GFP orthotopic xenograft model. The BxPC-3-GFP orthotopic xenograft mice were randomized into three groups of eight mice each when the tumor volume reached 60 mm³ and treated for 6 weeks as follows (Figure 1A): Group 1: untreated control; Group 2: low-dose gemcitabine (weekly intraperitoneal (i.p.) injection at 25 mg/kg); Group 3: high-dose gemcitabine (weekly i.p. injection at 125 mg/kg). Mouse body weight was measured twice a

week. Tumor length and width were measured with calipers after opening the abdomen on day 0 and 42. Tumor volume was calculated based on a previously published formula (10, 11).

Imaging of the fluorescent BxPC-3-GFP tumors and metastases. The abdomen was opened on day 42 for fluorescence imaging of the macroscopic tumor performed with the UVP ChemoStudio (Analytik Jena, Upland, CA, USA). The fluorescent area of the images was measured.

Histological examination. Fresh tumor samples from the mice were fixed, sectioned and stained as previously described (10, 11). Images were acquired with AmScope software (AmScope, Inc., Irvine, CA, USA).

Statistical analysis. The data are presented as the mean±SD and one-way ANOVA was performed to evaluate the differences between the means. A value of $p<0.05$ was considered to be statistically significant. Statistical analyses were conducted with GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA, USA).

Results

Effect of treatment on primary tumor growth. Primary tumor volume in the low-dose gemcitabine-treated group did not significantly differ from that of the untreated control ($p=0.2620$). However, high-dose gemcitabine significantly inhibited primary tumor growth compared to the untreated control ($p=0.0134$) (Figure 1B).

Effect of treatment on metastasis. No obvious metastasis with GFP was detected on day 0 when the treatment was started. Figure 2A shows representative fluorescence images after primary tumor resection from each group on day 42. GFP-expressing peritoneal metastases in mice treated with low-dose gemcitabine had a significantly increased area compared to that of the untreated control group ($p=0.0112$). In contrast, there was no significant difference between the extent of GFP-expressing peritoneal metastasis in mice treated with high-dose gemcitabine and that of the untreated control ($p=0.7399$) (Figure 2B).

Effect of treatment on body weight. Body weight did not significantly differ between each group pre-treatment and post-treatment (Figure 3A).

Effect of treatment on tumor histology. Figure 3B shows representative images of hematoxylin and eosin staining of the primary pancreatic tumors from each group resected on day 42. Extensive proliferation of viable cancer cells in the primary tumors was observed in the untreated control. Low-dose gemcitabine caused some necrosis and fibrosis among many viable cancer cells. Furthermore high-dose gemcitabine caused extensive necrosis and fibrosis in the primary tumor and few viable cancer cells were present. Figure 3B also

Figure 1. A: Treatment protocol for the BxPC-3 expressing green fluorescent protein (GFP) orthotopic xenograft mouse model. The mice were randomized into three groups of eight mice each: G1: untreated control, G2: low-dose gemcitabine (GEM), and G3: high-dose GEM. GEM was injected in the intraperitoneal cavity once a week for 6 weeks. B: Efficacy of GEM treatment on primary tumor volume in the pancreas. Bar graphs show the tumor volume at pre-treatment and post-treatment. Data are the mean $\pm$ SD. *Significantly different at $p<0.05$.

Figure 2. Effect of gemcitabine (GEM) treatment on metastasis. A: Fluorescence imaging of green fluorescent protein (GFP)-expressing metastases. The primary tumor was removed before imaging. High fluorescence intensity represents metastases with GFP (arrows). B: Quantitation of area of fluorescence of metastases with GFP. Data are the mean $\pm$ SD. *Significantly different at $p<0.05$.

shows representative images of hematoxylin and eosin staining of the sites of peritoneal metastases resected from each group on day 42. Extensive proliferation of viable cancer cells in the peritoneal metastases was observed in all groups. There was no obvious difference in histology between these three groups at the metastatic sites.

Discussion

In the present study, high-dose (125 mg/kg, weekly) gemcitabine inhibited primary tumor growth in the BxPC-3-GFP pancreatic cancer orthotopic xenograft mouse model. However, low-dose (25 mg/kg, weekly) gemcitabine had an opposite effect, increasing the extent of peritoneal

metastases, without significant inhibition of primary tumor growth compared to the untreated control. In our previous study, weekly dosing with 100 mg/kg gemcitabine inhibited tumor growth in a PDOX mouse model of pancreatic cancer (13, 14). In another study, gemcitabine inhibited tumor growth in a pancreatic cancer xenograft mouse model in a dose-dependent manner (from 40 to 120 mg/kg, weekly) (15). In the present study, weekly dosing of 125 mg/kg gemcitabine was considered a high dose and weekly dosing of 25 mg/kg gemcitabine was considered low dose. In histological examination, gemcitabine caused necrosis and fibrosis in the primary tumor, which was developed more strongly using high-dose than with low-dose gemcitabine.

Figure 3. Body weight and tumor histology. A: Effect of gemcitabine (GEM) treatment on mouse body weight. Bar graphs show the tumor volume pre- and post-treatment. Data are the mean $\pm$ SD B: Effect of GEM treatment on tumor histology, hematoxylin and eosin staining. Scale bars: 100 μ m.

The mechanisms underlying gemcitabine-induced metastasis remain unclear. Gemcitabine pharmacologically prevents the synthesis of deoxyribonucleotides required for DNA replication and repair (16). Gilbert *et al.* showed DNA damage creates a chemo-resistant niche that promotes the survival of cancer cells (6). According to the present histological findings, gemcitabine inhibited BxPC-3-GFP cells in the primary tumor but not on the peritoneum. It is possible that BxPC-3-GFP cells that become metastatic also become relatively chemoresistant.

Cytotoxic chemotherapy drugs may change the tumor microenvironment (17), which might cause a negative effect of chemotherapy. In our previous studies, cyclophosphamide pretreatment enhanced intravascular proliferation, extravasation, and subsequent colony formation (5, 18). In other studies, cisplatin and paclitaxel induced expression of vascular endothelial growth factor receptor 1 on endothelial cells, creating an environment favorable for cancer cell homing (19), and paclitaxel was

shown to promote liver metastasis of breast cancer cells in a mouse model (20). Furthermore, neoadjuvant chemotherapy of paclitaxel in a clinical trial may have induced expression breast cancer metastasis (21). D'Costa *et al.* reported that gemcitabine induced expression of tissue inhibitor of metalloproteinases 1, which may promote tumor growth and liver metastasis in pancreatic cancer (22). Kerbel *et al.* investigated the relationship of drug resistance and metastasis (23, 24). They found that drug-resistant cancer cells clustered very tightly and that highly clustered cancer cells may possess an enhanced metastatic capability (25, 26). The p27 gene appears to regulate cancer-cell clustering. We will investigate this mechanism on GEM-induced metastasis in future studies. Our previous studies also implicated natural killer cells in preventing metastasis (27). It is possible that low-dose GEM may inhibit natural killer cells and induce metastasis. Kerbel *et al.* also showed that inhibiting angiogenesis increased metastasis by inducing hypoxia (28). We showed gemcitabine inhibited angiogenesis (29). Future studies will be needed to dissect the mechanism of gemcitabine-induced increase of metastasis.

In conclusion, the use of very bright GFP expression of BxPC-3 cells and the orthotopic model enabled us to observe an unexpected increase in metastasis induced by low-dose gemcitabine therapy. Future experiments will investigate the mechanism of this phenomenon and its implication for treating pancreatic cancer in the clinic with GEM (30).

Conflicts of Interest

The Authors declare that there are no potential conflicts of interest. AntiCancer, Inc. uses pancreatic cancer orthotopic xenograft mouse models for contract research. NS, KM, TH, HO, ZZ, JP, and RMH are or were unsalaried associates of AntiCancer Inc.

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Authors' Contributions

Conception and design: NS and RMH. Acquisition of data: NS, KM, TH, HO, JP, and KK. Analysis and interpretation of data: NS, KM, TH, HO, JP, KK, SPC, MB, SRS, MU, and RMH. Writing, review, and/or revision of the manuscript: NS, RMH, MU, and SRS.

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