IL-6 Secreted from Pancreatic Stellate Cells Activates STAT3 and Promotes Cell Growth and Invasive Ability of Pancreatic Cancer

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BRIEF. PSCs promote cell growth and invasive ability of PDAC by activating STAT3 through IL-6.

ABSTRACT. Background: Pancreatic ductal adenocarcinoma (PDAC) is one of the major causes of cancer-related death worldwide and has the lowest 5-year survival rate of all cancers. The late diagnosis and the resistance to therapy make PDAC almost an incurable disease. A hallmark of therapy-resistant PDAC is the presence of reactive stroma. The aim of this study is to determine if pancreatic stellate cells (PSCs), a major component of pancreatic tumor stroma, affect the behaviors of PDAC cells. Methods: PSC-conditioned medium (PSC-CM) was collected from cultured PSCs. PDAC cells were treated with PSC-CM, and the levels of active STAT3 were determined by Western blot. Colony formation and cell invasion assays were performed using PDAC cells treated with serum free medium or PSC-CM, with or without IL-6 neutralizing antibody. Results: PSCs secreted IL-6, which activated STAT3, enhanced anchorage-independent growth, and increased invasive ability of PDAC cells. Neutralization of IL-6 blocked PSC-CM-induced activation of STAT3 and decreased colony growth and invasion of PDAC. Conclusion: This study demonstrates a novel role of PSCs in promoting PDAC progression and identifies IL-6/STAT3 pathway as an important mediator of the interactions between PSCs and PDAC cells.

INTRODUCTION.

Pancreatic cancer is one of the major causes of cancer-related death worldwide and has the lowest 5-year survival rate of all cancers(*1*). Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer and, due to the lack of an early detection marker, is often diagnosed at a late stage, a time when surgical removal of the tumor or the organ is often difficult, if not impossible(*1*). The first line of treatment for late stage PDAC is chemotherapy; however, responses are minimal. The reasons for treatment resistance include the highly heterogeneous nature of the pancreatic tumor, the high frequency of gene mutations, the existence of interdependent signaling pathways and feedback loops, and the presence of reactive stroma(*2, 3*).

Our laboratory's previous research has identified signal transducer and activator of transcription 3 (STAT3) as a biomarker of therapeutic resistance(*2*). Recent studies have shown that activation of the STAT3 signaling pathway is critical for the pathogenesis and chemo-resistance of PDAC(*4, 5*), indicating that active STAT3 signaling is not only associated with but also functionally involved in the initiation and progression of PDAC.

A major characteristic of chemo-resistant PDAC is the existence of dense, reactive stroma(*6*). Emerging evidence indicates that the extensive stroma of PDAC not only reduces drug delivery but may also play a role in promoting tumor growth and facilitating invasion and metastasis(*7, 8*). Pancreatic stellate cells (PSCs) are major components of pancreatic tumor-associated stroma(*9*) (*10*). Recent studies suggest that PSCs produce factors that modulate the synthesis and activation of extracellular matrix proteins and regulate the behaviors of pancreatic epithelial cells, resulting in an interference with drug delivery and promoting cancer progression(*9*) (*10*). However, although these studies on PSCs have revolutionized our understanding of pancreatic cancer progression, many things remain to be elucidated regarding the role of PSCs in regulating signaling in PDAC, the mechanisms through which PSCs communicate with PDAC tumor cells, and the factors that mediate the cross-talk between PSCs and PDAC. The purpose of this study was to determine if PSCs affect signaling in cancer cells and promote PDAC aggressiveness. This research also aimed to identify factors produced by PSCs that mediate the communication between stroma and PDAC cells.

This study found that PSCs secreted interleukin-6 (IL-6), which subsequently activated STAT3 and enhanced anchorage-independent growth and invasive ability of PDAC cells. This research revealed a novel role of PSCs in promoting PDAC progression and a potential therapeutic target for treating pancreatic cancer.

MATERIALS AND METHODS.

Cell Culture And Collection of Conditioned Medium.

RLT-PSC is a SV40 T-antigen immortalized human pancreatic stellate cell line(*11*). Human pancreatic cancer cell lines (MIAPaCa2 and PANC1) were purchased from ATCC (ATCC, Manassas, VA). The cells were cultured in Dulbecco's Modified Eagle Medium (with 10% fetal bovine serum) media. PSCs were grown on culture flasks until around 80% confluence and then were cultured in serum-free medium for 48 hours. PSC conditioned medium (PSC-CM) was collected and concentrated using Millipore Centriprep Centrifugal Filters (EMD Millipore, Billerica, MA).

IL-6 Neutralization.

1:400 ratios of IL-6 antibody (abcam, Cambridge, MA) and CM were combined and incubated at 4°C overnight on a rocker. The IL-6 levels in PSC-CM were determined by ELISA according to manufacturer's instruction (R&D Systems, Minneapolis, MN).

Western Blot Assays.

Cells were collected by centrifuge and lysed in RIPA buffer. Cell lysates were sonicated and centrifuged, and the supernatants were quantified for the protein concentrations. The same quantities of protein from each sample were loaded onto a SDS-PAGE gel. After running the gel, the proteins were transferred to a PVDF membrane and probed for the proteins of interest. Antibody against total STAT3 and phospho-STAT3 were purchased from Cell Signaling Technology (Cell Signaling Technology, Danvers, MA) and aliquots were stored following manufacturer's instruction until use.

Cell Invasion Assay.

Cell invasion assays were performed using matrigel-coated 24- transwell plates with 8-μm pore (Corning, Tewksbury MA). GFP-labeled PANC1 cells were seeded in the upper chamber and PSC-CM with or without IL-6 antibody was added to the lower chamber of the transwells to serve as chemo attractants. Serum-free medium was used as a control. The transwell plates were then incubated at 37°C for 24 hours. GFP-labeled cells that invaded through the transwell were counted under fluorescent microscope. A t-test was performed to determine if the numbers of cells invaded were significantly different among the treatments.

Soft-Agar Colony Formation Assay.

Colony formation assays were conducted using MIAPaCa2 cells. The cells were cultured in regular medium for 2 weeks and then treated with serum-free medium or PSC-CM with or without IL-6 antibody for an additional 2 weeks. Representative pictures of colonies grown on soft agar were taken under a microscope, and the sizes of colonies were quantified and analyzed using the

Image J program. A t-test was performed to determine if the colony sizes were significantly different among the treatments.

RESULTS.

PSC-CM Treatment Activates STAT3 In PDAC Cells.

To study the role of PSCs in PDAC tumor progression, conditioned medium was collected from cultured RLT-PSCs. PDAC cells (PANC1 and MIAPaCa2) were treated with PSC-CM for 30 minutes up to 24 hours. Western blotting was used to analyze the molecular changes in these PDAC cells. As shown in Figure 1, MIAPaCa2 cells had higher baseline levels of STAT3 than PANC1, but PSC-CM activated STAT3 (pSTAT3) in both PANC1 and MIAPaCa2 cells in a time dependent manner. The experiments were repeated twice with similar results.

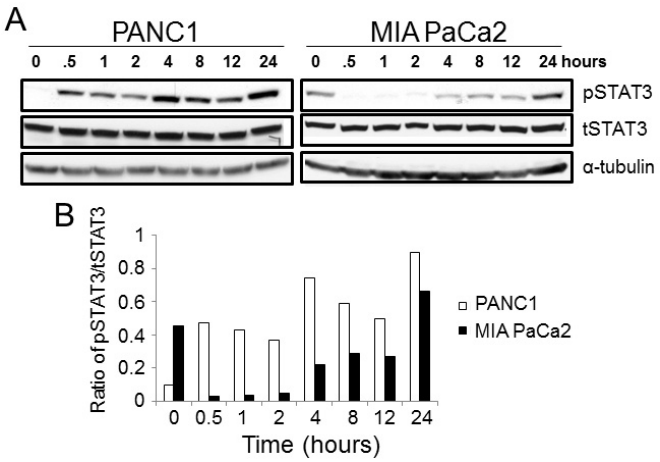

Figure 1. PSC-CM treatment activated STAT3 in PDAC cells. PANC1or MIAPaCa2 cells were treated with PSC-CM for 30 minutes up to 24 hours. A, western blot to analyze the levels of total and active STAT3 in PDAC cells. PSC-CM activated STAT3 in both PANC1and MIAPaCa2 cells in a time dependent manner. B, ratios of the quantified band intensity of pSTAT3 and tSTAT3 in A.

IL-6 Was Secreted From PSCs.

We further aimed to identify the factors that mediate the PSC-PDAC interactions. Given the connection of STAT3 with IL-6(*4*), it was hypothesized that PSCs secrete IL-6 to activate STAT3 signaling in PDAC cells. To test this hypothesis, the levels of IL-6 were measured by ELISA using serially diluted conditioned medium collected from RLT-PSCs culture. As shown in Table 1, RLT-PSCs secreted detectable concentrations of IL-6, and the levels of IL-6 increased as the concentration of conditioned media increased. The experiments were repeated twice with similar results.

STAT3 Was Activated In PDAC Cells By IL-6 Secreted From PSCs.

In order to study whether IL-6 secreted from PSCs activates STAT3 in PDAC cells, PANC1 and MIAPaCa2 cells were treated with PSC-CM with increasing concentrations of total protein, which is correlated with IL-6 level. As shown in Figure 2A, treatment with PSC-CM of increasing total protein or IL-6 concentration resulted in an increase of pSTAT3 level in both PANC1 and MIAPaCa2 cells. In order to confirm the role of IL-6 in mediating PSCs-induced activation of STAT3 in PDAC cells, IL-6 in PSC-CM was neutralized by the IL-6 antibody and ELISA was conducted to determine the efficacy of IL-6 neutralization. The results showed that serum free medium (SFM) had a low level of IL-6 (16.2 pg/ml), whereas the IL-6 concentration in PSC-CM was 30.52 pg/ml before and 12.59 pg/ml after neutralization by IL-6 antibody, indicating that IL-6 neutralizing antibody effectively reduced IL-6 level in PSC-CM. As shown in Figure 2B, a reduction of IL-6 level in PSC-CM resulted in a decrease of the pSTAT3 level in both PANC1 and MIAPaCa2 cells, indicating that PSC-induced activation of STAT3 in PDAC cells was mediated by IL-6.

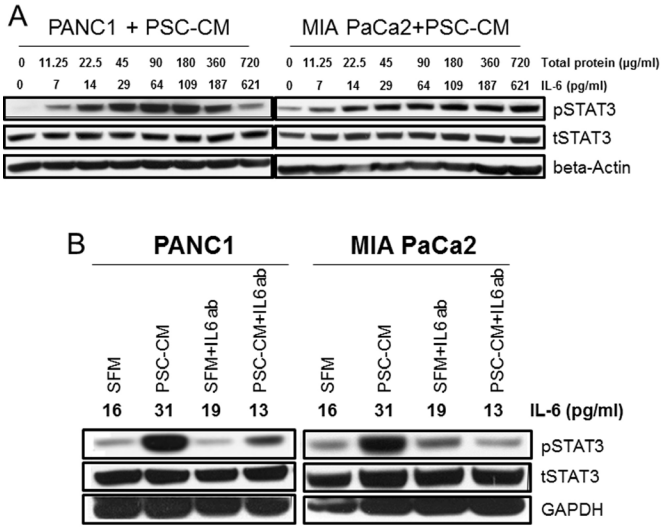

Figure 2. STAT3 was activated in PDAC cells by IL-6 secreted from PSCs. A, western blot analysis of STAT3 in PDAC cells. PANC1 or MIAPaCa2 cells were treated with PSC-CM at increasing concentration of total protein, which is correlated with increasing levels of IL-6. Increase in PSC-CM-protein or IL-6 concentration resulted in an increase of pSTAT3 level in both PANC1 and MIAPaCa2 cells. B, Neutralization of IL-6 blocks PSCs-induced activation of STAT3. The level of IL-6 in PSC-CM was reduced by IL-6 neutralizing antibody. The resulting PSC-CM with or without IL-6 antibody was used to treat PANC1 or MIAPaCa2 cells. Neutralization of IL-6 in PSC-CM resulted in a decrease of pSTAT3 in both PANC1 and MIAPaCa2 cells compared to PSC-CM treatment.

Neutralization of IL-6 Abrogated PSC-CM Enhanced Cell Growth And Invasion Of MIAPaCa2.

Anchorage-independent growth is one of the hallmarks of cancer cells. In order to assess whether PSC-CM affects anchorage-independent PDAC growth, soft agar colony formation assays were conducted using MIAPaCa2 cells treated with SFM or PSC-CM with or without the addition of IL-6 neutralizing antibody. The results showed that PSC-CM treatment significantly enhanced the sizes (p=0.049, t-test) of colonies formed by MIAPaCa2 cells (Figure 3A). Furthermore, the addition of IL-6 neutralizing antibody significantly blocked PSC-CM stimulated colony growth (Figure 3A, p=0.016, t-test). The experiments were done in triplicates.

Tumor-associated microenvironment has been suggested to promote cell invasion, a critical step for cancer cells to invade into surrounding tissues and metastasize to distant organs(*9*). In order to test whether PSCs affect the invasive ability of pancreatic epithelial cells and to assess the role of IL-6 in this process, cell invasion assays were conducted. As shown in Figure 3B, PSC-CM significantly enhanced the invasion of PANC1 cells (p=0.029, t-test); and IL-6 neutralizing antibody blocked PSC-CM-induced invasion of PANC1 cells (p=0.018, t-test). The experiments were done in triplicates.

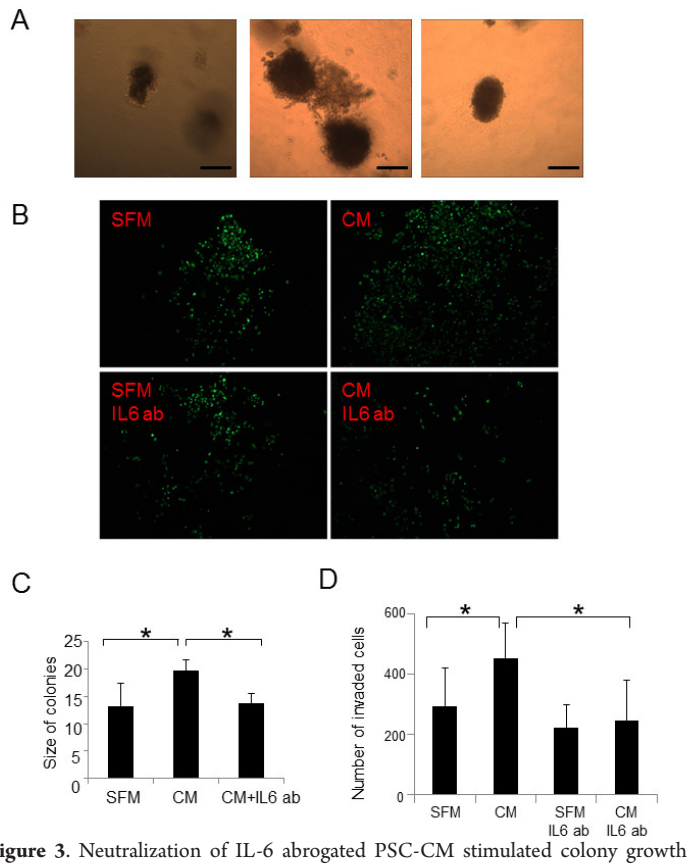

Figure 3. Neutralization of IL-6 abrogated PSC-CM stimulated colony growth and cell invasion of PDAC. A, representative microscopic images showing colonies formed by MIAPaCa2 cells cultured in (from left to right) serum free medium (SFM), PSC-CM, or PSC-CM with IL-6 antibody. B, representative fluorescent microscopic images showing invaded PANC1 cells. C, quantification of the size of colonies. PSC-CM significantly increased the size of colonies formed by MIAPaCa2 cells; and neutralizing antibody of IL-6 blocked PSC-CM mediated increase of colony size of MIAPaCa2 cells (*p<0.05, t-test). D, quantification of invaded cells. PSC-CM significantly enhanced PANC1 invasion and neutralizing antibody of IL-6 blocked PSC-CM induced invasion of PANC1 cells (*: p<0.05, t-test).

DISCUSSION.

Due to the lack of a diagnostic marker, most pancreatic cancer patients are at an advanced stage of the disease at the time of diagnosis and are thus not suitable for surgery. What is even worse is that these late stage PDACs are often resistant to chemotherapy. In an effort to study the mechanisms of resistance to therapy, previous research has shown that active STAT3 is a biomarker and a key mediator of therapeutic resistance in PDAC(*2, 3*). In order to further study how STAT3 was activated in PDAC, it was hypothesized that interactions between pancreatic tumor-associated stroma, specifically PSCs, and PDAC play a role in the activation of STAT3 in PDAC cells thus contributing to the development of therapy resistance in pancreatic cancer. Although PDAC originates from transformed epithelial cells in the pancreas(*12*), a hallmark of therapy resistant PDAC is the presence of dense stroma(*6, 8, 10*). As a major component of pancreatic tumor stroma, PSCs regulate ECM deposition and tumor behaviors(*7, 9, 13*). Accumulating evidence suggest that cytokines produced by PSCs are capable of modulating the differentiation, proliferation, and migration of adjacent cells thus promoting PDAC progression(*9, 14*). The data presented here showed that conditioned medium collected from cultures of PSCs activated STAT3, stimulated colony growth, and enhanced invasion of PDAC cells (Figures 1-3), demonstrating a critical role of PSCs in promoting PDAC progression.

To identify the factor that activates STAT3 and mediates the cross-talk between PSCs and PDAC cells, it was found that IL-6 was secreted from PSCs (Table 1). IL-6 is a multifunctional cytokine implicated in many physiological and pathological processes such as inducing differentiation of immune-cells, promoting inflammation, stimulating cell proliferation, as well as facilitating cancer progression(*15*). It has been shown that an elevated serum IL-6 level was correlated with significantly worse survival in patients with pancreatic cancer (16, 17), and that IL-6 promotes the development of pancreatic cancer in a mouse model(*4*). The findings here show that PSCs secrete IL-6, which subsequently activates STAT3 and promotes aggressiveness of pancreatic cancer cells. Neutralization of IL-6, which reduced IL-6 levels in PSC-CM, blocked PSCs-induced activation of STAT3 (Figure 2) and reduced PSCs-stimulated growth and invasion of PDAC cells (Figure 3). These data indicate that IL-6 plays an important role in mediating PSC/PDAC interactions and promoting PDAC progression, which is an important consideration in designing new therapies.

Table 1. Measurement of IL-6 concentration in PSC-CM. Conditioned media collected from RLT-PSCs culture were serially diluted to obtain PSC-CM with increasing total protein concentration. The levels of IL-6 in PSC-CM were measured by ELISA.

	Increasing concentration of protein (μg/ml)							
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Protein Conc of PSC-CM (μg/ml)	0	11.25	22.5	45	90	180	360	720
IL-6 in PSC-CM (pg/ml)	0	7.33	14.03	28.83	63.53	109.41	187.07	621.18
IL-6/protein in PSC-CM (pg/μg)	0	0.65	0.62	0.64	0.71	0.61	0.52	0.86

Our data (Figure 3) showing that neutralizing IL-6 significantly reduced colony growth and cell invasive ability of PDAC suggest that targeting IL-6 may delay PDAC tumor progression. In agreement with our findings here, clinical studies have shown that elevated serum IL-6 level was correlated with poor outcomes of pancreatic cancer patients(*16, 17*), and targeting IL-6 signaling by a neutralizing antibody has shown promising results in a clinical trial for treating ovarian cancer(*15*). More IL-6 targeting reagents are under-going clinical trials for the treatment of renal cell cancer, prostate cancer, lymphoma, and multiple myeloma(*15*). The research here provides important pre-clinical information for targeting IL-6 in pancreatic cancer.

In Figure 1, the levels of active STAT3 displayed a clear trend of time-dependency after the treatment with PSC-CM. It is noteworthy that the PANC1 cells showed higher levels of pSTAT3 after 4 hour and 24 hour treatments. The laboratory's recent data suggest that activation of STAT3 has a reciprocal inhibitory effect on the activation of MAPK, and the active MAPK pathway modulates the activation of STAT3 in turn. The jump of pSTAT3 level at 4 hours may reflect the existence of this regulatory loop between STAT3/MAPK in PDAC1 cells. Further experiments are needed to clarify the mechanism.

Taken together, this research revealed a mechanism through which PSCs regulate the growth and invasive ability of PDAC, identified IL-6/STAT3 signaling as a key mediator of pancreatic stroma/PDAC interactions, and demonstrated a novel role of PSCs in promoting PDAC progression. These results suggest that targeting PSCs and specifically IL-6 secreted by PSCs could potentially break off the communication between the tumor and the stroma and lead to a delay of PDAC progression.

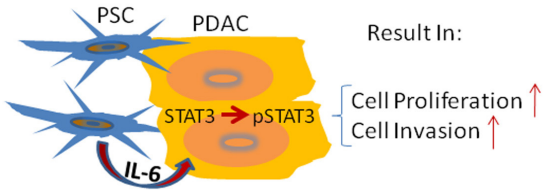

Figure 4. Activated PSCs produce cytokines (such as IL-6 identified in this research) to modulate signaling pathways (such as STAT3) in PDAC cells and stimulate cell proliferation and invasion of PDAC.

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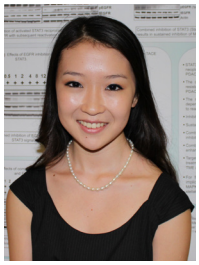

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