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IL-6 stimulates STAT3 and Pim-1 kinase in pancreatic cancer cell lines

Katherine M. Block, Ph.D.^{*,†}, Neale T. Hanke, B.S.^{*,†}, Erin A. Maine, B.S.^{*}, and Amanda F. Baker, Pharm, D., Ph.D.^{*}

Amanda F. Baker: abaker@azcc.arizona.edu

^{*}College of Medicine, University of Arizona Cancer Center, 1515 N. Campbell Ave. Room 3977A, Tucson, AZ 85724, 520-626-6704 (fax), 520-626-0301 (phone)

Abstract

Objectives—We investigated the signaling pathways activated in response to Interleukin (IL-6) in pancreatic cell lines, with a focus on signal transducer and activator of transcription 3 (STAT3) and proto-oncogene serine/threonine-protein (Pim-1) kinase.

Methods—IL-6 receptor (IL-6R) expression and IL-6 induced cell signaling was measured by Western blotting in human pancreatic cell lines. Cucurbitacin I was used as a pharmacological tool to investigate the role of STAT3 in Pim-1 activation. Stably over-expressing Pim-1 kinase cell lines were characterized for their response to IL-6 *in vitro*, and for their growth rate as flank tumors in *scid* mice.

Results—IL-6R was expressed across multiple cancer cell lines. In Panc-1 cells, IL-6 treatment increased expression of P-STAT3 and Pim-1 kinase. Cucurbitacin I treatment alone increased pErk1/2 expression in wild-type and Pim-1 over-expressing cell lines and resulted in exaggerated Pim-1 kinase protein levels in control and IL-6 stimulated cells, suggesting upregulation of Pim-1 may be partially STAT3 independent. Pim-1 over-expression did not significantly impact growth rate *in vitro* or *in vivo* in Panc-1 or MiaPaCa2 cell lines.

Conclusions—IL-6 activates STAT3 and stimulates Pim-1 kinase in pancreatic cell line models. The regulation and consequence of Pim-1 expression appears to be highly context dependent.

Introduction

Pancreatic cancer remains one of the most devastating and elusive malignancies among neoplastic disease. In 2010, over 43,000 new cases of pancreatic cancer and 36,000 pancreatic cancer associated deaths were predicted¹. Due to its typically late presentation and refractory nature toward treatment, pancreatic cancer remains the fourth leading cause of cancer-related death in the United States. While pancreatic cancer involves deregulation across multiple cellular pathways, one common theme is the activation of v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS). More than 90% of pancreatic tumors carry an activating KRAS mutation, and this is believed to be an initiating event in oncogenesis². Over-activation of KRAS stimulates fibroblasts in the stroma and recruits inflammatory mediators to the tumor microenvironment, both of which exacerbate tumor growth and invasion by up-regulating interleukin-6 (IL-6) production^{3;4}.

[†]Both authors contributed equally to this work.

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IL-6 is a pleiotropic cytokine produced by a variety of cell types including macrophages, fibroblasts, endothelial cells, and myeloid cells. In the context of pancreatic cancer, it upregulates the expression of cytokines that enable proliferation, immune evasion, and resistance to apoptosis⁵. IL-6 binds to a composite receptor composed of a specific 80 kDa alpha subunit (IL-6R α , gp80) and two subunits of gp130 receptor which transduce a signal upon ligand binding⁶. Upon binding IL-6, gp130 activates the Janus kinase (Jak2)/signal transducer and activator of transcription 3 (STAT3) pathway by inducing cross-phosphorylation of the Jak2 proteins associated with the cytoplasmic side of the receptor⁷. Jak2 then phosphorylates key tyrosine residues on the gp130 receptor. These phosphotyrosine residues then serve as a docking site for the recruitment of STAT3 proteins, which act as cellular mediators of IL-6. STAT3 is an oncogene that is activated by tyrosine phosphorylation (P-STAT3) in response to extracellular signals and JAK pathway activation. Once phosphorylated through binding to activated JAK proteins, P-STAT3 forms dimers and translocates to the nucleus where it regulates the transcription of a key set of target genes involved in proliferation, survival, cell cycle progression, angiogenesis, and immunosuppression⁸. Several lines of evidence suggest that IL-6 stimulates cancer growth by activation of STAT3⁸. STAT3 is constitutively expressed in a multitude of human cancers, including pancreatic, and has a well-established role in neoplastic development⁹. Elevated levels of P-STAT3 correlate with increased IL-6 production in pancreatic cancer cell lines¹⁰. Moreover, activated STAT3 can also upregulate IL-6 expression¹¹, giving rise to a positive feedback loop that impinges on tumor progression, invasion, and chemoresistance¹².

The proto-oncogene Pim-1 is emerging as a target of STAT3-driven transcription that plays a key role in tumor progression and transformation. Pim-1 is a constitutively active serine/threonine kinase which has overlapping homology and function with Pim-2 and Pim-3 kinases¹³. Pim-1 kinase was identified as a gene induced by KRAS oncogenic activation in pancreatic carcinogenesis¹⁴, and is over-expressed in a wide range of cancers, including pancreatic tumors. Unlike other serine/threonine kinases, Pim-1 is regulated at the transcriptional and translational levels, with phosphorylation serving to stabilize the protein but not being required for activity (for a comprehensive review of Pim-1 kinase please see¹⁵). Pim-1 is associated with cell cycle progression, survival, and regulation of transcription factors^{16;17}. Because many targets of Pim-1 phosphorylation lie within the cell cycle, Pim-1 activity is closely linked to the G₁/S and G₂/M checkpoints and cellular proliferation status^{13;18;19}. While Pim-1 alone is only weakly oncogenic, it synergistically interacts with c-Myc to drive transformation by stabilizing the c-myc protein and enabling activation of c-Myc transcriptional targets^{20;21}. Pim-1 is also strongly associated with evasion of apoptosis and drug resistance^{22;23}. This has been observed extensively in prostate cancer, where IL-6 mediated Pim-1 over-expression has been correlated to resistance to docetaxel^{24,25}.

Given the critical role that IL-6 plays in pancreatic cancer development and progression, we believed that evaluating the effect of Pim-1 in the context of IL-6 signaling would be valuable in understanding how tumor/stromal interaction can drive tumor growth and survival. Our current studies found that IL-6 not only increased phosphorylation of STAT3 in pancreatic cell lines, but also resulted in increased Pim-1 kinase expression. In Pim-1 kinase over-expressing cell lines, IL-6 stimulation in cells pre-treated with the STAT3 inhibitor cucurbitacin I resulted in exaggerated Pim-1 kinase protein expression compared to IL-6 stimulation alone, implicating an additional STAT3 independent mechanism in Pim-1 regulation. However, tumor growth was not impacted by elevated Pim-1 expression in flank xenograft tumors. Together, these studies suggest that in pancreatic cancer, the impact of IL-6 and Pim-1 may be highly context dependent.

Materials and Methods

Materials

Antibodies against P-STAT3 (Tyr705) and total STAT3 were purchased from Cell Signaling Technology (Beverly, MA). Two Pim-1 antibodies were used, the first Pim-1 antibody was purchased from Invitrogen (Carlsbad, CA), previously Zymed Laboratories, and the second was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Pim-2, Pim-3, and IL-6 receptor α antibodies were also purchased from Santa Cruz. Total Erk1/2 and pErk1/2 antibodies were purchased from Cell Signaling Technologies (Danvers, MA). Actin antibody, and the secondary antibodies, peroxidase conjugated goat anti-rabbit and peroxidase conjugated goat anti-mouse were purchased from Jackson ImmunoResearch (West Grove, PA). Recombinant human IL-6 was purchased from PeproTech (Rocky Hill, NJ). Recombinant Human Epidermal Growth Factor (EGF) was purchased from Prospeco (Rehovot, Israel). Cucurbitacin I and antibody against α -Tubulin were obtained from Calbiochem (La Jolla, CA).

Cell Culture and Generation of Cell Lines

The MiaPaCa2, Panc-1, Su86.86., Capan-1, Capan-2, ASPC-1, BXPC-3, and HPAF-II cells were obtained from the ATCC and maintained in the recommended tissue culture media supplemented with 10% fetal bovine serum (FBS), 500 U/mL penicillin, and 5000 U/mL streptomycin. The HPDEV cells were a kind gift from Ming-Sound Tsao, University of Toronto, Toronto, Ontario, Canada and were grown in keratinocyte media from Invitrogen (Carlsbad, CA), supplemented with prequalified human recombinant Epidermal Growth Factor 1–53 (EGF 1–53) and Bovine Pituitary Extract (BPE). To verify cell line authenticity, genomic DNA was extracted (Sigma GIN70-KT), diluted appropriately in TE buffer, and submitted to the UA Genomics Core (Human Origins Genotyping Lab) for analysis. Autosomal STR typing was conducted across the 13 core STRs in CODIS and referenced against allelic peaks in cell lines of previously confirmed genotype. All cell lines were verified as authentic. Cells were tested for mycoplasma contamination on a quarterly basis using the MycoAlert mycoplasma detection assaykit from Lonza (Basel, Switzerland) and found to be negative. All cells were grown in 5% CO₂ at 37°C in a humidified tissue culture incubator. Over-expressing Pim-1 cells were created using a human Pim-1 plasmid purchased from GeneCopoeia (Germantown, MD). Negative control cells were created with a plasmid producing green fluorescent protein (GFP) also from GeneCopoeia. Both the Pim-1 and GFP plasmids utilize the same vector backbone containing a CMV promoter. These plasmids were transfected into MiaPaCa2 and Panc-1 cells using the transfection reagent FuGENE6 (Roche Molecular Biochemicals, Indianapolis, IN) at a 1:6 ratio. Sixteen hr later, cells were re-plated for clonal density and selected with 600 μ g/mL G418 sulfate (Fisher Scientific, Pittsburgh, PA). Colonies were isolated using cloning cylinders and expanded cell lines were screened by Western analyses and RT-PCR for expression of the human Pim-1 gene. Expression of the GFP plasmid was verified by fluorescent microscope. Established clonal colonies were subjected to continuous selection with 300 μ g/mL G418 sulfate.

RNA isolation and qRT-PCR

Total cellular RNA was extracted using NucleoSpin RNA II total RNA extraction kit (Macherey-Nagel, Duren, Germany). cDNA was generated from 1 μ g of DNase-free RNA using qScript cDNA Synthesis Kit (Quanta BioSciences, Gaithersburg, MD) per manufacturer's instructions. Reverse transcription (RT) was performed using a GeneAmp 7300 sequence detection system (PE Biosystems, Foster City, CA). Amplification reactions were set up in a reaction volume of 25 μ L by use of the Applied Biosystems TaqMan UniversalPCR Mastermix. PCR primers and TaqMan probes were synthesized and pre-

optimized by Applied Biosystems (Assays on Demand, Foster City, CA). Each reaction contained 1× PCR Mastermix, 900 nmol of each primer, and 250 nmol of the *Fam* probe. Reverse transcription was done at 48°C for 30 min, samples incubated for 10 min at 95°C and then amplification over 40 cycles at 15 sec at 95°C followed by 1 min at 60°C. Values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β 2 microglobulin (B2M), and Pim-1 message was quantitated using the delta cycle threshold (CT) method as described by Perkin-Elmer.

Western Blot Analysis

Cells were seeded at 6×10^5 per 100 mm plate in medium supplemented with FBS. After 24 hr, the medium containing serum was removed and replaced with medium without serum. The plates were incubated an additional 24 hr before treatment with 100 ng/mL IL-6, 50 μ g/mL EGF, and/or treatment with 10 μ M cucurbitacin I (STAT3 inhibitor). After various time points, the cells were rinsed with cold phosphate buffered saline (PBS) and harvested in a buffer containing 50 mM Tris HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 2mM EDTA, 5mM Na₃VO₄, 200 μ M NaF, 21 μ M leupeptin, 230 nM aprotinin, 1mM phenylmethylsulfonyl fluoride (PMSF). The cell lysate was centrifuged at $10,000 \times g$ for 10 min at 4°C. Protein concentration of the resulting supernate was determined using a 660 nm Protein Assay kit (Thermo Scientific, Rockford, IL). Twenty-five micrograms of total cell lysate was boiled for 5 min and resolved in 10% acrylamide/bisacrylamide gel by electrophoresis at 125 V for 105 min. Proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA). Membranes were blocked with 5% milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 30 min before overnight incubation at 4°C with various primary antibodies, typically at a 1:1000 dilution. Blots were rinsed with TBST and incubated for 2 hr at room temperature with secondary antibody at a 1:15,000 dilution. Reactive bands were visualized by exposure to film using HyGLO Chemiluminescent HRP Detection Reagent (Denville Scientific, Metuchen, NJ). Blots were stripped in 0.2 M NaOH with shaking for 10 min at room temperature and then re-probed for loading control. Densitometric quantitation of relative band intensity was performed using the NIH Image J program.

ELISA Analysis

Cells were grown in 6-well plates, seeded at 1×10^5 cells per well. After attaching overnight, media was replaced and cells were allowed to grow for an additional 24 or 48 hr before media was collected. IL-6 and VEGF in the media were measured using the Quantikine human IL-6 (sensitivity < 5 pg/mL) ELISA kit (R&D Systems, Inc., Minneapolis, MN) according to manufacturer's recommended conditions.

Cell Proliferation Assay (MTT)

Cells were seeded at a density of 3×10^3 cells/well in a 96-well plate with outer wells left empty for addition of water. After 24 hr of culture, the media was changed and vehicle, or IL-6, was added. The cells were incubated for the time specified; then 0.5 mg/mL MTT dye was added and the cells were incubated for an additional 4 hr. Formazan crystals were dissolved in dimethyl sulfoxide (DMSO) for 5 min and the plates were read spectrophotometrically at 590 nm with a reference of 650 nm. Each assay was performed at least three times.

BrdU Assay

Cell proliferation was also assessed by 5'-bromo-2'-deoxyuridine (BrdU) incorporation. The BrdU Assay kit (Millipore) was used according to the manufacturer's recommendations. Cells were seeded at a density of 3×10^3 cells/well in a 96-well plate.

Following 24 hr of culture, BrdU was added and allowed to accrue within proliferating cells for 2 hr. The cells were then fixed at room temperature for 30 min followed by incubation with an anti-BrdU monoclonal antibody for 1 hr. The cells were then incubated at room temperature with a secondary antibody conjugated with peroxidase. The substrate solution tetramethylbenzidine (TMB) was added with incubation for 30 min in the dark at room temperature. The staining reaction was terminated with a 2.5 N sulfuric acid solution and the plate read with a spectrophotometer at 450 nm.

Apoptosis (Annexin V/PI)

Apoptosis and necrosis were determined using an Annexin-V staining kit (Boehringer-Mannheim, Indianapolis, IN) according to the manufacturer's instructions. The cells were washed and resuspended in 50 μ L FITC-annexin-V/propidium iodide stain for 15 min at 22°, diluted with 500 μ L buffer, and the fluorescence of 10^4 cells was measured using a FACScan analyzer (Becton Dickinson) by the Arizona Cancer Center Flow Cytometry Core Service. Viable cells were determined by subtracting the apoptotic fraction (Annexin V-FITC positive) and necrotic fractions (PI positive).

Pim-1 Kinase Activity Assays

Panc-1 cells stably transfected to over-express Pim-1 (clone 14), Panc-1 cells stably transfected with an empty GFP vector (empty vector) and unmodified Panc-1 (wild-type) cells were used for this assay. All three cell lines were seeded in 100 mm dishes at a density of 4×10^6 cells per dish and incubated 24 hr. Once cells had reached 80% confluence, they were serum starved for 24 hr. Cells were treated with 100 ng/mL of IL-6 in serum free media for 1 hr, then lysed. Lysis buffer consisted of 50 mM Tris, 1% NP40 (v/v), 0.25% sodium deoxycholate (w/v), 150 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4 , 1 mM NaF, 50 μ M PMSF, 0.1 μ g/mL Leupeptin, and 0.1 μ g/mL Aprotinin. Cells were washed twice with ice-cold PBS, then 400 μ L of lysis buffer was added. Cells were scraped for 5 min on ice, then transferred to chilled microcentrifuge tubes and sonicated for 1 min. Cell lysates were incubated on ice for 15 min, then centrifuged at 12,000 rpm at 4°C for 15 min. Supernatant was removed and a BCA analysis was conducted to determine protein concentration. Samples were snap frozen in liquid nitrogen and shipped to SignalChem (Richmond, BC, Canada) for kinase activity testing. Immunoprecipitation was performed using the His-tag on stably transfected cell lines and immunoprecipitates were subjected to a radiometric kinase assay using ^{32}P labeled ATP (Amersham) to determine Pim-1 activity levels. Recombinant, active Pim-1 (SignalChem) was used as a positive control, and all kinase assays were conducted in triplicate.

Xenograft Growth Curves

Male *scid* mice were housed and maintained under specific pathogen-free conditions in accordance with the guidelines of the American Association for Laboratory Animal Care. All experiments met the current regulations and standards of the U.S. Department of Agriculture, the U.S. Department of Health and Human Services, the National Institutes of Health, and The University of Arizona Animal Care Facility. Experiments were conducted by the Arizona Cancer Center Experimental Mouse Shared Service. The growth rate for flank xenograft tumors were compared ($n=4$ mice/cell line). For each mouse, 10×10^6 cells were injected subcutaneously in 0.1 mL saline in the right flank. Mice were weighed and tumors were measured every 2–3 days using electronic calipers. Tumor volume was calculated using the formula: tumor volume = ($d_{\text{long}} \times d_{\text{short}}^2/2$). Mice were sacrificed when tumors reached 2000 mm^3 .

Statistical Analysis

To compare significance between MTT treatment groups, one-way analyses of variance (ANOVA) was performed. Statistical analysis of xenograft tumor proliferation rate was performed by the Arizona Cancer Center Biometry Shared Service. To compare *in vivo* growth rates between wild-type, negative control, and Pim-1 over-expressing Panc-1 and MiaPaCa2 cell lines, the least squares regression line of the cube root of the tumor volume by day was fit for each mouse. The cube root was used because it linearized the raw values. Statistical analysis was further limited for each mouse to tumor volumes falling within the linear portion of the curve. The slope of the regression line is interpreted as the tumor growth rate. The x-intercept is interpreted as the tumor latency. ANOVA using the General Linear Model were done to test for a treatment effect on the slope (growth) and the x-intercept (latency).

Results

IL-6 R alpha is expressed in pancreatic cell lines

In order to explore the impact of IL-6 signaling in pancreatic cancer, we evaluated a panel of pancreatic cell lines for their IL-6 α receptor expression (Figure 1A). All the lines examined expressed the IL-6 α receptor. MiaPaCa2 and Panc-1 cells were selected for use in future experiments due to their differential expression of IL-6 α receptor levels, with MiaPaCa2 cells having the lowest expression while Panc-1 cells exhibited the highest expression. HPD-EV cells, which are normal, transformed ductal cells, were chosen as a control for purposes of comparison.

Secreted IL-6 is not detected in pancreatic cell line conditioned media

Having confirmed the presence of IL-6 receptors in pancreatic cells, we next sought to determine if these cells actively secrete IL-6 and could therefore participate in autocrine signaling mechanisms. Using an ELISA for soluble IL-6, we analyzed conditioned media from MiaPaCa2 and Panc-1 cells grown for 24 and 48 hr in both normoxia and 1% oxygen (24 hours). IL-6 levels were below the limit of detection in all of the samples we analyzed (data not shown). It should be noted that Feurino et al²⁶ reported detectable IL-6 levels in media from the Panc-1 cell line, but used a different assay than our group that may have been more sensitive. The absence or low level of IL-6 secretion in these cell lines suggests that in pancreatic cancer, a paracrine IL-6 signaling pathway driven by the tumor stroma may be more dominant than an autocrine signaling loop.

IL-6 stimulation activates STAT3

We treated the HPD-EV, MiaPaCa2 and Panc-1 cell lines with 100 ng/mL IL-6 and observed an early (10 min) increase in P-STAT3 that was sustained for up to 8 hr (Figure 1B). The stimulation of P-STAT3 was greater in Panc-1 cells, consistent with higher IL-6 receptor expression. No increase in the P-AKT pathway was observed in the Panc-1 cell line in response to IL-6 treatment (data not shown). Although not investigated in our study, Feurino et al²⁷ reported IL-6 also activates the ERK2 signaling pathway in Panc-1 cells.

Pim kinases are broadly expressed across pancreatic cell lines grown in vitro

We hypothesized that one consequence of IL-6 induced STAT3 activation in pancreatic cell lines may be expression of Pim-1 kinase. We first surveyed the expression of Pim-1, Pim-2, and Pim-3 in a panel of pancreatic cell lines (Figure 2A). All three Pim kinase family members were expressed in the cell lines investigated. Pim-1 kinase has previously been reported to have two isoforms including a 33 kDa form that is present in both the cytosol and nucleus and a 44 kDa primarily found in the plasma membrane. We observed both

Pim-1 kinase isoforms in total cell lysates, with the most prominent being the 33 kDa isoform. One exception was the MiaPaCa2 cell line which showed increased 44 kDa isoform expression compared to the 33 kDa form. We compared the expression of Pim-1 protein to Pim-1 message (total RNA) (Figure 2B) by qRT-PCR analysis. The Panc-1 cell line demonstrated the highest levels of both Pim-1 message and protein compared to the other cell lines investigated.

IL-6 stimulates Pim-1 kinase in a STAT5 independent manner

IL-6 treatment resulted in an increase in Pim-1 kinase protein expression in the Panc-1, but not the MiaPaCa2 cell line (Figure 3A). This Pim-1 increase specific to the Panc-1 cell line is consistent with the higher level of IL-6 α receptor observed in these cells. IL-6 did not result in an increase in STAT5 phosphorylation, indicating that the IL-6 stimulated increase in Pim-1 is not mediated through the STAT5 pathway. As a positive control, cells were also stimulated with epidermal growth factor (EGF) which did result in both STAT5 phosphorylation and an increase in Pim-1 protein expression.

Cucurbitacin I has minimal impact on IL-6 induced Pim-1 expression

To investigate the dependency of Pim-1 stimulation on STAT3, we used the pharmacological agent cucurbitacin I which has been previously reported to specifically inhibit activation of STAT3 and to have anti-proliferative effects in pancreatic cancer models²⁸. In Panc-1 cells, a 4 hr pre-incubation with 1, 5, and 10 μ M cucurbitacin I, followed by a 1 hr IL-6 treatment, resulted in a dose-dependent inhibition of STAT3 phosphorylation with optimal (but not complete) inhibition observed with 10 μ M (Figure 3B). In contrast, the IL-6 induced expression of Pim-1 was only minimally inhibited in response to cucurbitacin I.

Cucurbitacin I inhibits pancreatic cancer cell proliferation

In order to assure the studies presented in Figure 3B were conducted with pharmacologically relevant concentrations, we compared the *in vitro* sensitivity of the MiaPaCa2 and Panc-1 cells following 72 hr treatment with cucurbitacin I using an MTT assay. The MiaPaCa2 cell 72 hr IC₅₀ value was 49 nM in contrast to 208 nM in the Panc-1 cell line (Figure 3C). Because STAT mediates cell survival through factors such as BCL-2, this data suggests that the Panc-1 cell line is more resistant to cucurbitacin I due to its increased level of STAT-3 expression. These results are consistent with our observation that IL-6 results in a greater fold increase in STAT3 in the Panc-1 compared to the MiaPaCa2 cell line. These data also correlate with the higher levels of IL-6 receptor present in Panc-1 cells, suggesting that IL-6 activation results in the observed increase in phosphorylated STAT3.

Cucurbitacin I treatment results in increased Pim-1 protein in cell lines over-expressing Pim-1

Pim-1 kinase over-expressing Panc-1 and MiaPaCa2 stably transfected cell lines were generated and characterized by qRT-PCR and Western blot analysis (Figure 4A). Both wild-type and Panc-1 Pim-1 clone 14 cells were treated with IL-6 for up to 6 hr and Pim-1 kinase protein expression was measured by Western blot analysis. Pim-1 induction occurred as early as 10 minutes and was sustained for up to 6 hr in wild-type and transfected cell lines (Figure 4B). To ensure that the Pim-1 kinase activity was also increased, the Pim-1 kinase activity was measured as described in the methods section in serum starved vs IL-6 (1 hr) stimulated Panc-1 negative control cells and Pim-1 over-expressing cells (clone 14). The Pim-1 over-expressing cells (clone 14) demonstrated 61.2 $\pm$ 9.8% greater activity compared to the negative control cells. With regard to Pim-1 kinase mRNA levels, the over-expressing Panc-1 cells exhibit a 15 – 20 fold increase in Pim-1 message expression. IL-6 treatment did

not result in a significant increase in Pim-1 kinase message expression in wild-type Panc-1 cells or in Pim-1 stably transfected cells (clone 14) (Figure 4C) suggesting that IL-6 mediated increase in Pim-1 protein expression may be controlled by enhanced protein stabilization. When Pim-1 over-expressing cells were pre-treated with cucurbitacin I for 4 hr prior to IL-6 stimulation for 1 hr, cucurbitacin I treatment resulted in enhanced Pim-1 expression in Panc-1 and MiaPaCa2 Pim-1 over-expressing cell lines, but not in the wild type cells (Figure 4D). Cucurbitacin I treatment alone, in serum starved cells, also increased Pim-1 expression in Pim-1 transfected cells but not wild-type cells (Figure 4E). This observation suggests that Pim-1 kinase regulation occurs via both STAT3 dependent and independent pathways. Because cucurbitacin I has previously been shown to increase Erk1/2 phosphorylation²⁹, and the Erk1/2 pathway has been associated with Pim-1 kinase regulation, we measure phosphorylated and total Erk1/2 expression in the same serum starved cells treated with cucurbitacin and found increased pErk1/2 in all cell lines treated with cucurbitacin (Figure 4E).

Pim-1 kinase over-expression did not result in increased proliferation in vitro or in vivo

To further investigate the biological impact of Pim-1 over-expression we studied the basal apoptotic rate of the Panc-1 stably transfected cell lines clone 14 compared to the wild-type and negative control cell lines (Figure 5A). No significant differences in apoptosis or necrosis were noted. Using the same cell lines, we investigated basal proliferation rate using the BrdU assay (Figure 5B) and compared cell doubling times *in vitro* (Figure 5C). No differences in proliferation rates were noted by either method. To determine if proliferation rate may be impacted by host factors and microenvironmental effects, including hypoxia³⁰, we investigated tumor growth in a flank xenograft model in *scid* mice (Figure 5D) in a small pilot study (N=4). No significant differences in growth rate or tumor latency between wild-type and transfected cell lines were observed (P<0.05). In a similar pilot study, we also investigated the *in vivo* growth rate of the MiaPaCa2 Pim-1 Clone 4 and observed no significant differences in growth rate compared to wild-type or negative control cell lines (data not shown). Because IL-6 treatment results in enhanced secretion of VEGF⁵, we investigated the impact of Pim-1 over-expression on VEGF secretion in the wild-type Panc-1 and negative control cells compared to the Pim-1 clone 14 (Figure 5E). Pim-1 over-expression did not result in increased secretion of VEGF compared to wild-type or negative control cell lines. We also investigated whether IL-6 treatment would result in enhanced proliferation in the Panc-1 Pim-1 over-expressing cells compared to control or negative control cells. No significant difference in proliferation was observed across a broad concentration range of IL-6 (Figure 5F).

Discussion

The significance of IL-6 in pancreatic tumorigenesis and tumor progression is not fully understood. The present study supports the importance of an IL-6 → STAT3 survival pathway in pancreatic cancer, showing that IL-6R α receptors are broadly expressed in pancreatic cancer cell lines and respond to IL-6 by an immediate and prolonged stimulation of P-STAT3. Although our studies did not show any increased proliferative effect in response to IL-6 stimulation, we did observe an increase in VEGF in response to IL-6 treatment, consistent with previously published studies³¹. Inhibition of STAT3 with cucurbitacin I resulted in anti-proliferative action at low nM concentrations, which is consistent with previously published studies.

The current study also demonstrated that in addition to activating P-STAT3, IL-6 increases Pim-1 kinase protein expression in pancreatic cell lines. The increase in IL-6 stimulated Pim-1 appears to be partially independent from activation of P-STAT3. Our observations are consistent with studies by Aksoy et al 2007³² which demonstrated that activation of Pim-1

by G-CSF in murine embryonic stem cells occurred by both STAT-3 and STAT-3-independent mechanisms. In the context of Pim-1 over-expression, inhibition of STAT3 with cucurbitacin I resulted in an exaggerated Pim-1 induction, suggesting the STAT3 blockade and/or the presence of cucurbitacin I results in activation of alternate signaling mechanisms involving Pim-1.

Regulation of Pim-1 is complex and includes transcriptional, translational, and protein stabilization mechanisms. Regulation of Pim-1 has been reported to occur by activation by protein kinase C (PKC) and mitogen-activated kinase 1/2 (MAPK).³³ Our observation that cucurbitacin I treatment increased pErk1/2 expression (Figure 4E) is consistent with recent studies in B-Leukemic cells show that cucurbitacin I increases p38 MAPK and ERK 1/2²⁹. Therefore, it is possible that in control, non-IL-6 stimulated Pim-1 over-expressing cells, that cucurbitacin I may be increasing Pim-1 via MAPK activation. The exaggerated induction of Pim-1 observed in response to IL-6 in Pim-1 over-expressing cell lines may also be through activation of MAPK signaling. While cucurbitacin I inhibits signaling through STAT-3, it does not impact JAK2 activity³⁴, and consequently, gp130 continues to be phosphorylated by JAK2 on key tyrosine residues. This phosphorylation recruits adapter proteins such as Grb2 and SOS which in turn recruit and activates Ras. Signaling then proceeds via a Ras/Raf/Mek/MAPK cascade³⁵ with MAPK upregulating the level of Pim-1 transcription and resulting in increased Pim-1 protein levels^{33;36;37}. Cucurbitacin I was not associated with measurable increases Pim-1 protein expression in wild-type Panc-1 or MiaPaCa2 cell lines. However, this could be due to the limited sensitivity of Western blotting to detect an increase. Alternatively, this observation may suggest that an excess level of intracellular Pim-1 kinase is necessary for cucurbitacin I induction of Pim-1 protein, implicating a role for protein-protein interactions in mediating the pathways involved.

Cucurbitacin I is frequently utilized as a specific inhibitor of STAT-3 activity, however, our results indicate that use of this inhibitor may result in potential artifacts due to its ability to activate other signaling pathways. Because all of the secondary, or off-target mechanism of action of cucurbitacin are currently unknown, caution must be used when selecting this agent as a pharmacological inhibitor and further characterization studies are needed in this area.

Our study is consistent with prior reports that Pim-1 kinase is constitutively expressed in pancreatic cell lines and is modulated by K-Ras signaling³⁸. Interestingly, Pim-1 over-expression in the Panc-1 and MiaPaCa2 cell lines was not associated with changes in basal apoptosis rates, cell cycle changes, or proliferation. In contrast, molecular inhibition of Pim-1 kinase by shRNA in MiaPaCa2 cells has previously been shown to promote apoptosis, decrease anchorage-dependent, and independent cell growth, and invasion through matrigel and decrease radioresistance³⁸. Together, these observations suggest that malignant pancreatic cell lines express sufficient basal rates of Pim-1 to mediate many of its biological functions. Future studies should investigate the consequence on Pim-1 over-expression on non-malignant pancreatic models.

Given the many biological functions of Pim-1 combined with multiple mechanisms of regulation and the impact of paracrine effectors, the consequence of Pim-1 over-expression may be highly context dependent. Particularly in the case of IL-6, paracrine activity of this cytokine from the tumor stroma has been directly implicated in carcinogenesis in multiple studies. This indicates that the tumor microenvironment plays a paramount role in cellular transformation and tumorigenesis^{8;39;39}. Although xenograft models in *scid* mice can provide important insights into some microenvironmental stresses including hypoxic stress signaling, the lack of a fully functional host immune system limits activation of some cytokine signaling pathways. Furthermore, cells implanted in the mouse flank would not

have been exposed to the abnormally high levels of IL-6 typically produced by the tumor stroma in human pancreatic cancer. Future studies characterizing the impact of Pim-1 over-expression in pancreatic cancer will seek to address stromal interaction through the use of fibroblast and pancreatic cell co-cultures, as well as through genetically engineered mouse models of pancreatic cancer to more closely mimic the tumor microenvironment.

The role of Pim-1 kinase in pancreatic cancer continues to be an active area of investigation. Studies by Reiser-Erkan C et al, 2008³⁰ showed that Pim-1 kinase is a positive prognostic factor in pancreatic cancer. Studies by Chen J, et al 2009²³ demonstrate a role for Pim-1 kinase in tumorigenesis that is facilitated by hypoxia. A better understanding of the regulatory pathways controlling Pim-1 kinase and the consequence of its expression are needed to guide the translation of Pim-1 kinase targeted therapies in the clinic.

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Figure 1.

A. Western blot analysis of total cell lysate prepared from pancreatic cancer cell lines. The blot was probed for IL-6R α and stripped and re-probed for actin as a loading control. Densitometry analysis was performed and quantitation displayed as fold change from the HPD-EV cells. **B.** Western blot analysis of total cell lysates prepared from pancreatic cell lines which were serum starved for 24 hr prior to treatment with IL-6 for the times indicated. Blots were probed using an antibody specific for STAT3 phosphorylated at tyrosine 705. Blots were stripped and re-probed for total STAT3 as a loading control. Bands were subjected to densitometric analysis and quantitation displayed as fold change from the mock treated cells.

Figure 2.

A. Western blot analysis of whole cell lysates prepared from pancreatic cell lines. Blots were probed for Pim-1, Pim-2, Pim-3 and actin as a loading control. **B.** Pim-1 message expression in a panel of pancreatic cell lines measured by quantitative RT-PCR and normalized to β 2 Microglobulin (β 2M). Cells were not treated with IL-6. These results are the mean value of samples run in duplicate. Similar results were obtained in three separate studies.

Figure 3.

A. Western blot analysis of total cell lysates from cells that were serum starved and treated with either IL-6 or EGF for the times indicated. Blots were probed for Pim-1 with tubulin used as a loading control. A separate blot was also probed for phosphorylated STAT5 A/B with total STAT5 used as a loading control. **B.** Western blot analysis of total cell lysates from Panc-1 cells which were serum starved for 24 hr followed by a 4 hr pre-incubation with cucurbitacin I prior to a 1 hr incubation with IL-6. Blots were probed for Pim-1 and P-STAT3 with tubulin and total STAT3, respectively, for loading controls. **C.** Analysis of cell proliferation measured using an MTT assay. Panc-1 (■) and MiaPaCa2 (▲) cells were grown for 72 hr and exposed to increasing levels of cucurbitacin I (N=6).

Figure 4.

A. Western blot analysis of whole cell lysates prepared from Panc-1 and MiaPaCa2 cells including wild-type, negative control, and Pim-1 stably transfected cells. The blot was probed for Pim-1 and actin as a loading control. **B.** Western blot analysis of whole cell lysate prepared from wild-type and Pim-1 transfected Panc-1 cells which were serum starved for 24 hr and then treated with IL-6 for the times indicated. **C.** Relative Pim-1 message expression measured by qRT-PCR in wild-type Panc-1 cells that were serum starved 24 hr prior to treatment with IL-6 (100ng/mL) for the times indicated. Data shown is representative of the observed trend in three separate experiments. **D.** Western blot analysis of total cell lysates from wild type, negative control, and stably over-expressing Pim-1 kinase MiaPaCa2 and Panc-1 cells. Cells were serum starved 24 hr followed by a 4 hr pre-incubation with cucurbitacin I (5 μ M) prior to a 1 hr incubation with IL-6. Blots were probed for Pim-1 and P-STAT3 with tubulin and total STAT3, respectively, for loading controls. **E.** Western blot analysis of total cell lysates from cells serum starved for 24 hours and treated with vehicle alone, or cucurbitacin (5 μ M) for 4 hours. Blots were probed for Pim-1, tubulin as a loading control, and pErk1/2, and total Erk1/2.

Figure 5.

A. AnnexinV/PI staining in the Panc-1 panel (wild-type, negative control, and Pim-1 over-expressing Pim-1 kinase cells) following 24 hr of serum starvation. Data shown is from one experimental replicate that represents the trend observed in three separate experiments. **B.** BrdU staining in the Panc-1 panel following 24 hr of serum starvation. **C.** Cell number counted every 24 hr for 4 days (96 hr) in the Panc-1 panel. **D.** Growth of the Panc-1 panel as flank xenografts in *scid* mice (N=4 mice/group). **E.** VEGF-A concentration in cell culture media from the Panc-1 panel of cells collected 48 hr following IL-6 treatment (N=3). **F.** MTT analysis of Panc-1 cells which were treated with IL-6 for 72 hours (following serum starvation for 24 hours). Each assay represents an N=6.