Inhibition of glycogen synthase kinase-3 activity triggers an apoptotic response in pancreatic cancer cells through JNK-dependent mechanisms

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Recent evidences suggest that the activity of glycogen synthase kinase-3 (GSK3) contributes to the tumorigenic potential of pancreatic cancer cells through modulation of cell proliferation and survival. However, further investigations are needed to identify GSK3-dependent mechanisms involved in the control of pancreatic cancer cell proliferation and survival. This study was undertaken to provide further support for a role of GSK3 in pancreatic cancer cell growth as well as to identify new cellular and molecular mechanisms involved. Herein, we demonstrate that prolonged inhibition of GSK3 triggers an apoptotic response only in human pancreatic cancer cells but not in human non-transformed pancreatic epithelial cells. We show that prolonged inhibition of GSK3 activity increases Bim messenger RNA and protein expressions. Moreover, we provide evidence that activation of the c-jun N-terminal kinase (JNK) pathway is necessary for the GSK3 inhibition-mediated increase in Bim expression and apoptotic response. Finally, we demonstrate that concomitant inhibition of GSK3 potentiates the death ligand-induced apoptotic response in pancreatic cancer cells but not in non-transformed pancreatic epithelial cells and that this effect also requires JNK activity. Considering that different approaches leading to stimulation of death receptor signaling are under clinical trials for treatment of unresectable or metastatic pancreatic cancer, inhibition of GSK3 could represent an attractive new avenue to improve their effectiveness.

Introduction

Pancreatic cancer has the highest fatality rate of all cancers with a 5 year survival rate <6% (1,2). In recent years, despite the identification of genetic lesions likely to be involved in the initiation and progression of pancreatic neoplasia (namely KRras, p16, p53 and SMAD4), this new knowledge has yet to lead to the elaboration of novel and/or effective strategies in the fight against pancreatic cancer. The late diagnosis of this disease as well as the chemoresistance of pancreatic cancer cells to current therapy still contribute to the dismal prognosis of pancreatic cancer. Hence, a better understanding of the biochemical and biological changes that occur during pancreatic epithelial cell transformation remains of major interest in order to develop early detection methods and new treatment strategies.

Recently, the glycogen synthase kinase-3 (GSK3) emerged as a potential therapeutic target in pancreatic cancer (3–6). GSK3 is an ubiquitously expressed serine/threonine kinase shown to be involved in multiple processes, including metabolism, cell fate determination, transcriptional and translation control, oncogenesis and neurological diseases (7). There are two mammalian isoforms of GSK3 encoded by different genes, GSK3α and GSK3β, which are highly homologous and share substrate specificity in vivo. Unlike most kinases, the GSK3 is constitutively active in resting cells and is inactivated by a variety of cellular stimuli, such as those leading to the activation of the Wnt and the PI3K/Akt pathways. When active, GSK3 phosphorylates a vast array of substrates, such as glycogen synthase, β-catenin, cyclin D1 and c-myc (7). Most substrates are inactivated and/or degraded upon GSK3 phosphorylation. Since several GSK3 targets are involved in cell proliferation and survival, it would be expected that inhibition of GSK3 would promote proliferation and survival. However, results from the literature do not support this straightforward hypothesis, especially with regard to pancreatic cancer cells. Indeed, inhibition of GSK3 activity has been shown to reduce pancreatic cancer cell viability in vivo (8–11) and arrest pancreatic tumor xenograft growth in vivo (12). Similar results have been obtained in glioma, medullary thyroid, prostate and colon cancer cells (13–17), suggesting that the positive contribution of GSK3 in cell proliferation and survival is not specific to pancreatic cancer cells. In addition, higher activity of GSK3 has been detected in pancreatic cancer cell lines comparatively to human embryonic kidney 293 (HEK293) cells considered non-neoplastic (18). More convincingly, in contrast to pancreatic normal tissues, pancreatic adenocarcinomas display higher expression of GSK3β messenger RNA and protein (12,19), lower immunoreactivity for the inactive form of GSK3 (20) and reduced expression of FRA1 and disheveled, two endogenous GSK3 inhibitors (19). Altogether, these results appear inconsistent with a tumor-suppressor function of GSK3 and rather suggest that GSK3 activity is upregulated and could participate in the tumorigenecity of pancreatic cancer cells (8–12). However, the mechanisms involved remain poorly understood.

Interestingly, it appears that deregulated GSK3 activity is associated with several diseases. Accordingly, GSK3 inhibitors are used in clinical practice for bipolar disorder and are under development for other indications, including neurodegenerative diseases and diabetes (5,21). In addition, GSK3 has emerged as a potential therapeutic target for inflammation-based diseases and cancer including pancreatic cancer (3–6,21). To evaluate the future clinical potential of inhibiting GSK3 in the setting of pancreatic cancer patients, the current study was undertaken to evaluate whether the specific inhibition of GSK3 activity impacts pancreatic cancer cell growth and aimed to identify cellular and molecular mechanisms involved.

Herein, we show that prolonged inhibition of GSK3 activity promotes an apoptotic response specifically in pancreatic cancer cells but not in non-transformed pancreatic epithelial cells. We demonstrate that prolonged inhibition of GSK3 activity induces Bim expression, caspase-3 and -7 activation as well as poly adenosine diphosphate ribose polymerase (PARP) cleavage. Our data reveal that GSK3 inhibition leads to sustained c-jun N-terminal kinase (JNK)–cJun activation, which is necessary for the increased Bim expression and PARP cleavage. Finally, we provide the first evidence in pancreatic cancer cells that inhibition of GSK3 activity promotes the death ligand-induced apoptotic response through JNK-dependent mechanisms.

Materials and methods

Cell culture

The human pancreatic cancer cell lines PANC1, MIA PaCa-2 and BxPC-3 were obtained from ATCC and were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (PANC1 and MIA PaCa-2) or RPMI 1640 (BxPC3), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin in humidified 5% CO2 and 95% air at 37°C. The human non-transformed pancreatic epithelial cell line Human Pancreatic Ductal Epithelial (HPDE) was obtained from M.S.Tsao (Toronto) and cultured in...
keratinocytes-serum-free medium as described previously (22,23). HEK293T cells were cultured as described previously (24).

Materials

The specific GSK3 inhibitors, SB216763 (Sigma) and CHIR99021 (Selleck), were used. The JNK inhibitor SP600125 was purchased from Calbiochem. Tumor necrosis factor (TNFα) and TNF-related apoptosis-inducing ligand (TRAIL) were purchased from BioShop Canada. All other materials were from Sigma unless stated otherwise.

Antibodies

Antibody against GSK3β and caspase-8 were purchased from Transduction Laboratories (BD Biosciences Pharmingen). Antibodies for the detection of cJun and total extracellular signal-regulated kinase (Erk) were from Santa Cruz. Caspase-9 antibody was obtained from MBL International Corporation. All other antibodies were obtained from Cell Signaling.

Anchorage-independent growth

Concentrated DMEM-2X without phenol red was prepared from powder (Wisent). The medium was sterilized by 0.22 μm filtration and supplemented with 20% FBS. Prewarmed DMEM-2X was mixed 1:1 with autoclaved 1.4% agarose type VII (Sigma) kept at 42°C. Six-well dishes were precoated with 1.5 ml/well and plates were allowed to solidify under the hood. Then, 10,000 cells/ml were added to a DMEM-agarose mix and seeded at 2 ml/well on top of the precoated wells. Seded plates were allowed to solidify under the hood before placing them at 37°C and 5% CO₂. For assays with the PANCI-1 infected cells, fresh DMEM-1X without phenol red supplemented with 10% FBS was added on the surface of the agarose for every other day. For assays with the GSK3 inhibitor, fresh DMEM-1X without phenol red supplemented with 10% FBS containing or not the GSK3 inhibitor was added on the surface of the agarose and changed every day. After 2–3 weeks, colonies were stained by adding 500 μl of phosphate-buffered saline (PBS) containing 0.5 mg/ml of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (Calbiochem) on the surface of the agarose and incubated for 3 h at 37°C and 5% CO₂. Images were acquired and colonies were counted using the Image J 1.42 software.

Protein expression and immunoblotting

Cells were washed twice with ice-cold PBS, lysed in Triton buffer (1% Triton X-100, 50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 0.2 mM orthovanadate, 40 mM β-glycerophosphate, 50 mM NaF, 10% glycerol and 10% of protease inhibitor cocktail) and cleared of cellular debris by centrifugation (10,000 r.p.m., 10 min, 4°C). Protein concentrations were measured using the bicinchoninic acid reagent procedure from Pierce with bovine serum albumin as standard. Equal amounts of proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and proteins were detected individually after electrophoresis onto nitrocellulose or polyvinylidene difluoride membranes. Membranes were blocked in PBS containing 5% non-fat dry milk and 0.05% Tween-20 for 1 h at 25°C. Membranes were then incubated with appropriate primary antibodies in blocking solution, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. After extensive washing in PBS–0.05% Tween, blots were visualized with chemiluminiscence reagent.

Generation of viruses and infection

Control (non-target), GSK3β- and c-Jun-specific targeting lentiviral short hairpin RNA (shRNA) expression vectors were purchased from Sigma. Lentiviruses were produced in HEK293T and used for cell infection according to Invitrogen recommendations (ViraPower Lentiviral Expression System, instructions manual). Briefly, subconfluent PANCI-1 cells were infected with the viral suspension containing 8 μg/ml of polybrene (Sigma) for 1 h. Complete medium supplemented with 8 μg/ml of polybrene was added, and cells were placed at 37°C and 5% CO₂. Forty-eight hours post-infection with shGSK3β-expressing lentiviruses, infected cells were trypsinized and seeded for anchorage-independent growth assays. For protein expression analysis, shGSK3β-infected cells were lysed 72 h post-transfection. For c-Jun expression using lentiviruses, stable PANCI-1-shno-target and PANCI-1-shcJun populations were obtained after 10 day selection with puromycin (2 μg/ml). For retroviral infections, the pBabeMEKK1:ER construct was kindly provided by Simon J. Cook (The Babraham Institute, UK) and has been production in HEK293T and retroviral infections of PANC1 were performed as described previously (24).

RNA extraction and real-time PCR

Total RNAs were extracted using the RNeasy plus kit (Qiagen) using gDNA eliminator spin columns. Reverse transcription reactions were performed using the Quantitect Reverse Transcription kit (Qiagen) according to the manufacturer’s instructions. Real-time polymerase chain reaction (PCR) analysis was performed using a LightCycler system (Roche Diagnostics). Experiments were run and analyzed with the LightCycler software 4.0 according to the manufacturer’s recommendations. Synthesis of double-stranded DNA during the PCR cycles was monitored with SYBR Green I (Quantitect SYBR Green PCR kit; Qiagen). All samples were run in duplicate. Target expression was quantitated relatively to beta-2-microglobuline expression. A standard calibration curve was prepared for each gene using serial dilutions of the calibrator sample, and crosspoint values were plotted versus the log of the relative concentration of each cDNA. This standard curve was used to correct for differences in PCR efficiencies. The primers used were the following: hnm 5’-TGAGAAGTCCTCCTCTG-3’ and 5’-CCCTGCTCATGATGAAGCC-3’, beta-2-microglobuline 5’-TGGCCTCTTCCTTG-3’ and 5’-GCTGCTTACATGTCTCGATCC-3’.

Transient transfections and luciferase assays

Experiments were performed as described previously (24). Briefly, cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. In luciferase assays, the nuclear factor-kappaB (NF-κB) (Clontech), TOPFlash and FOPFlash reporter constructs (Upstate) were used. Twenty-four hours following transfection, cells were treated with vehicle (dimethyl sulfoxide) or GSK3 inhibitor for 24 h. Cells were then harvested with passive lysis buffer (Promega), and luciferase activities were determined using the Dual Luciferase Assay kit (Promega) according to the manufacturer’s instructions. The data are expressed as firefly luciferase activity normalized to the activity of Renilla-luciferase (pRL-SV40; Promega) to control for transfection efficiency. For small interfering RNA (siRNA)-mediated silencing, the control siRNA and cJun siRNA used were from Santa Cruz. At 24 h post-transfection, cells were treated for 72 h with vehicle (dimethyl sulfoxide) or SB216763. Cells were then lysed and prepared for protein expression analysis.

Data presentation

All experiments were performed in at least three independent experiments. Typical western blots are shown. Densitometric analyses were performed using the Image J 1.42 software. Results are expressed as means ± SE and analyzed by Student’s t-test.

Inhibition of GSK3β expression or activity reduces pancreatic cell growth

Previous reports have shown that inhibition of GSK3 activity reduces pancreatic cancer cell proliferation and survival in vitro and arrests pancreatic tumor xenograft growth in vivo (8–12). To provide new data supporting these observations, we analyzed the impact of GSK3 activity inhibition on anchorage-independent growth, a hallmark of cancerous cells. We observed that reduction of GSK3β expression using a specific shRNA, which downregulated GSK3β protein expression by at least 70% as quantified by densitometry using the Image J software (see Figure 2A), strongly reduced the ability of PANCI-1 to form colonies in soft agarose (Figure 1A). These results were confirmed by treatment of the cells with a specific GSK3 inhibitor, SB216763 (28; Figure 1B). As suggested by others (8–12), these new data support a positive contribution of GSK3 activity in proliferation and/or survival of pancreatic cancer cells.

Inhibition of GSK3β expression or activity triggers an apoptotic response specifically in human pancreatic cancer cells and not in non-transformed pancreatic epithelial cells

Next, we specifically investigated whether GSK3 activity could mediate survival signals in pancreatic epithelial cells. GSK3 activity...
was downregulated using either a specific GSK3β shRNA or the GSK3 inhibitor, SB216763. As shown in Figure 2B, SB216763 was potent in significantly reducing GSK3 activity at all time points, as visualized by the increased mobility shift of the glycogen synthase corresponding to the reduced phosphorylation level of this well-characterized GSK3 target (7). As indicators of apoptosis, we assessed the cleavage of PARP and caspase-7 as well as caspase-3 activity. As shown in Figure 2A-C, prolonged inhibition of GSK3 expression or activity correlated with increased levels of cleaved PARP and caspase-7 as well as a 6-fold increase in caspase-3 activity. Similar results were obtained with another GSK3 inhibitor, CHIR99021 (Figure 2D) and in other pancreatic cancer cell lines, MIA PaCa-2 and BxPC-3 (data not shown).

To explore the mechanisms by which inhibition of GSK3 activity could promote caspases activation, expression of the BCL-2 protein family was analyzed. In pancreatic cancer cells, GSK3 inhibition consistently increased protein expression levels of BimEL, BimL and BimS and decreased protein expression levels of Bcl-2, whereas no modulation in Mcl-1, Bcl-XI, Bad, Bax and PUMA protein expression levels were detected (Figure 2A,B and D; data not shown). Following real-time PCR analysis, we observed a 2-fold increase in bim messenger RNA expression in SB216763-treated pancreatic cancer cells (Figure 2E), suggesting a transcriptional event by which GSK3 modulates Bim expression.

Interestingly, in non-transformed pancreatic epithelial cells, 72 h SB216763 treatment failed to modulate Bim and Bcl-2 protein expression levels or induce PARP cleavage as opposed to a well-known cytotoxic agent (doxorubicin) (Figure 3A). This was due to an inability for GSK3 to modulate its downstream targets as treatment of HPDE with SB216763 stimulated the TCF reporter gene, TOPFlash (Figure 3B). However, SB216763 was not equally potent to promote TOPFlash reporter activity in HPDE as in Panc1 suggesting that, as proposed by others (18), GSK3 activity is higher in pancreatic cancer cells comparatively with non-transformed cell lines. To estimate the relative activity of GSK3, the phosphorylation level of GSK3α/β was assessed on S21/9, which corresponds to the inactive form of GSK3. As depicted in Figure 3C, all three pancreatic cancer cell lines tested, namely Panc1, MIA PaCa-2 and BxPC-3, displayed lower phosphorylated GSK3/total GSK3 ratios, supporting the notion that GSK3 is more active in pancreatic cancer cells as compared with non-transformed cell lines, such as HPDE and HEK293T.

Taken together, as previously reported by others (8–12), our results support a positive role for GSK3 in cell survival of human pancreatic cancer cells. However, our results are the first (i) to identify Bim as a GSK3 downstream target in pancreatic cancer cells and (ii) to demonstrate that the prosurvival role of GSK3 is specific to pancreatic cancer cells since apoptotic markers were not induced in HPDE following SB216763 treatment.

Inhibition of GSK3 expression or activity leads to sustained JNK activation

Previous reports have shown that inhibition of GSK3 activity reduces NF-kB activity (8–10). However, in our hands, SB216763 had no significant effect on the activity of a NF-kB reporter gene (Figure 4A). We previously demonstrated that prolonged inhibition of the MeK/Erk pathway triggers apoptosis of pancreatic cancer cells (27). In addition, the PI3K/Akt pathway is well known for mediating survival signals in many cell types (29), although in our hands prolonged inhibition of the PI3K/Akt pathway with LY294002 in pancreatic cancer cells is not sufficient to induce DNA fragmentation or PARP cleavage (30). We thus verified whether GSK3 inhibition could impact the expression of apoptotic markers through the inhibition of Akt and Erk prosurvival pathways. Prolonged inhibition of GSK3 activity did not significantly reduced Akt or Erk activities (Figure 4B). However, prolonged treatment with GSK3 inhibitors consistently upregulated the activity of the JNK pathway as visualized by the increase in JNK and cJun phosphorylation as well as cJun expression (Figure 4B and data not shown).

Analysis at shorter time points revealed a rapid phosphorylation of JNK and cJun as well as increased cJun expression upon SB216763 addition, which correlated with the SB216763-induced reduction in GSK3 activity, as visualized by the increased mobility shift of the glycogen synthase (Figure 4C). Moreover, reduction of GSK3β expression through shRNA silencing led to increased activity of the JNK pathway i.e. upregulation of JNK and cJun phosphorylation as well as increased cJun expression without altering Akt or Erk phosphorylation (Figure 4D). Altogether, these results suggest that reduction of GSK3 activity leads to a rapid and persistent activation of the JNK–cJun pathway in pancreatic cancer cells.
JNK–cJun activation is necessary but not sufficient per se for SB216763-induced expression of apoptotic markers

We then examined whether activation of the JNK pathway participated in the SB216763-induced expression of apoptotic markers by using the specific JNK inhibitor, SP600125. Addition of SP600125 efficiently blocked the SB216763-induced JNK-dependent phosphorylation of cJun (Figure 5A). Interestingly, inhibition of the JNK pathway strongly reduced the SB216763-induced PARP cleavage, caspase-7 and -3 activation as well as the SB216763-mediated increase in Bim protein expression levels (Figure 5A; data not shown). However, decrease in Bcl-2 protein expression level was still observed following SB216763+SP600125 treatment, suggesting that the SB216763-induced effect on Bcl-2 is not essentially JNK-dependent. To further characterize the link between the JNK pathway and the regulation of Bim expression, cells were transfected with a specific siRNA-targeting cJun. The siJUN was able to downregulate the basal and SB216763-induced cJun expression by >50% (Figure 5B). Interestingly, the siRNA-mediated downregulation of cJun did not modulate the basal protein expression level of Bim, whereas it strongly suppressed the SB216763-induced Bim expression. Comparable results were obtained in cells stably expressing a shcJun (Figure 5C). Alternatively, reduction in Bcl-2 protein expression was still observed in SB216763-treated si/shcJun cells (Figure 5B and C), again suggesting that SB216763-mediated Bcl-2 downregulation is essentially JNK–cJun independent. Although preventing the increase in Bim protein expression, the si/shRNA-mediated silencing of cJun expression was insufficient to completely prevent the SB216763-induced PARP cleavage suggesting that, in addition to its influence on the JNK–cJun pathway, inhibition of GSK3 activity might modulate other signaling pathways that together contribute to PARP cleavage. However, more vigorous cJun (or JNK) silencing will be required to entirely support this possibility.

To test whether the sole activation of the JNK pathway was sufficient to mimic the proapoptotic response of GSK3 inhibition, a stable population of PANC1 expressing an inducible MEKK1, ΔMEKK1:ER, an upstream activator of the JNK pathway, was generated. As expected, treatment of the cells with 4-HT led to activation of JNK, as shown by cJun phosphorylation, only in the PANC1–ΔMEKK1:ER population (Figure 5D). Despite strong activation of the JNK pathway after 4-HT addition, neither modulation of Bim protein expression levels nor PARP cleavage was observed following the sole activation of the ΔMEKK1–JNK–cJun cascade. In addition, the robust ΔMEKK1:ER-induced cJun activation did not potentiate SB216763-mediated PARP

Fig. 2. Inhibition of GSK3 activity in pancreatic cancer cells induces expression of apoptotic markers. (A) PANC1 cells were infected with lentiviruses encoding either for a non-targeting siRNA (shcontrol) or a shGSK3b. Cells were lysed 72 h post-infection. (B) PANC1 cells were treated with the GSK3 inhibitor, SB216763 (SB; 20 μM) for the indicated time period. (C) Caspase-3 activity was measured as described in experimental procedures. The relative caspase-3 activity in dimethyl sulfoxide (DMSO)-treated cells at each time point was set at 1. *P < 0.05, **P < 0.01. (D) PANC1 cells were treated with GSK3 inhibitors, SB216763 (SB 10 μM or SB 20 μM) or CHIR99021 (CHIR 5 μM) for 72 h. (A, B and D) Cells were lysed and equal amounts of proteins were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and submitted to western blot analysis using the indicated antibodies. GS, glycogen synthase. (E) Total RNA was isolated from DMSO- or SB216763-treated (SB; 20 μM) cells at the indicated time period. Bim messenger RNA (mRNA) expression was analyzed and quantified by real-time PCR as described in Materials and methods. The relative level of bim expression in DMSO-treated cells at each time point was set at 1. *P < 0.05, ***P < 0.001.
GSK3 inhibition triggers JNK-dependent apoptosis

 leads to embryonic lethality due to hypersensitivity of hepatocytes to et al. In 2000, Hoeflich and apoptotic response SB216763 treatment potentiates death ligand-induced JNK activity and cleavage in the PANCl–MEKK1 population, as compared with the pBabe control population. These results suggest that sustained activation of the JNK–cJun pathway is necessary but insufficient per se to trigger Bim expression and elicit efficient caspase activation in pancreatic cancer cells.

SB216763 treatment potentiates death ligand-induced JNK activity and apoptotic response

In 2000, Hoeflich et al. (31) reported that targeted deletion of GSK3β leads to embryonic lethality due to hypersensitivity of hepatocytes to TNFα, supporting a physiological role for GSK3 in modulating death receptor-induced apoptosis. Interestingly because of their ability to induce apoptosis, activation of death receptors, including TNFα and TRAIL receptors, are currently under clinical trials for treatment of pancreatic cancer patients (32–34). Hence, we tested whether inhibition of GSK3 activity, through SB216763 treatment, promoted the TNFα-induced apoptotic response in pancreatic epithelial cells and evaluated the contribution of the JNK pathway in this process. In PANCl, SB216763 potentiated the TNFα-induced PARP cleavage, caspase-9 and caspase-7 activation as well as caspase-8 activation in a time frame (24 h) where SB216763 alone was insufficient to do so (Figure 6A). Moreover, combination of TNFα with SB216763 induced a strong cJun phosphorylation indicative of JNK pathway activation. Interestingly, prevention of JNK activity by addition of SP600125 diminished the intensity of cleaved caspases-9, -7, -8 and PARP to levels of TNFα-treated cells only. Similar results were obtained with another death ligand, namely TRAIL (Figure 6B) and in another pancreatic cancer cell line Mia PaCa-2 (Figure 6C), suggesting that GSK3 inhibition could universally promote death receptor-induced apoptosis in pancreatic cancer cells. Conversely, treatment of non-transformed pancreatic epithelial cells with similar concentration of TNFα, in combination or not with SB216763, had no significant effect on PARP cleavage neither on cJun phosphorylation/ JNK pathway activation (Figure 6D). These results suggest that the GSK3 inhibitor, SB216763, potentiates death ligand-induced apoptosis specifically in pancreatic cancer cells, leaving intact the non-transformed pancreatic epithelial cells.

Discussion

Previous studies have shown that inhibition of GSK3 activity reduces pancreatic cancer cell viability in vitro and arrests pancreatic tumor xenograft growth in vivo (8–12), suggesting that GSK3 activity contributes to the tumorigenicity of pancreatic cancer cells. However, the mechanisms involved remain poorly understood. Herein, we provide further support for a role of GSK3 in the maintenance of the transformed phenotype of pancreatic cancer cells by demonstrating that a reduction in GSK3 activity correlates with inhibition of anchorage-independent pancreatic cancer cell growth. For the first time, we demonstrate that treatment of pancreatic cancer cells with specific GSK3 inhibitors leads to sustained JNK–cJun activation and increased Bim expression of which are necessary but insufficient per se to trigger PARP cleavage. These results are corroborated by shRNA silencing of GSK3β, strongly supporting GSK3-dependent mechanisms of action of our GSK3 inhibitors. Interestingly, these GSK3-dependent mechanisms are exclusively occurring in pancreatic cancer cells and not in non-transformed pancreatic epithelial cells. We also provide evidence that the GSK3 inhibitor SB216763, in part by promoting JNK activation, potentiates death ligand-induced apoptotic response specifically in pancreatic cancer cells.

Previous reports have shown that GSK3 participates in NF-κB-dependent transcription and pancreatic epithelial cell survival (8–12). One study has demonstrated that overexpression of NF-κB subunits, p65 and p50, was able to prevent the GSK3 inhibitor AR-A014418-induced decrease in NF-κB activity as well as apoptotic cell death (8). However, other studies have shown that p65 silencing or treatment with a specific IKKα/β inhibitor failed to affect pancreatic cancer cell viability or pancreatic tumor xenograft growth (35,36). These results hence suggested to us that NF-κB might be necessary for GSK3 inhibition-induced apoptosis but not sufficient in itself to promote pancreatic cancer cell death. Together with the present observations that SB216763 treatment did not affect NF-κB-dependent transcription but was sufficient to induce the activation of an apoptotic response, we postulated that GSK3 could act at the crossroad of multiple signaling pathways working together to control pancreatic cancer cell viability. Results herein demonstrated that the MEK/ERK and PI3K/Akt prosurvival signaling pathways remain intact in the presence of GSK3 inhibition, which suggests that these pathways are not involved in GSK3-mediated cell protection. Moreover, we
did not observe any effect on the p38 mitogen-activated protein kinase pathway (data not shown). However, increased JNK activity was detected as early as 1 h following SB216763 addition, and activation of the JNK–cJun cascade was maintained as long as GSK3 activity was inhibited. At this point, it is still not known as to how GSK3 regulates the JNK–cJun cascade in pancreatic cancer cells, although it has been documented that cJun phosphorylation on threonine 239 by GSK3 targets cJun to proteasome-dependent degradation (37). It is therefore possible that inhibition of GSK3 in pancreatic cancer cells prevents GSK3-dependent phosphorylation and degradation of cJun and thus explains the rapid increase in cJun expression in our SB216763-treated cells. However, it seems unlikely that direct regulation of cJun by GSK3 is the only mechanism involved in our system as GSK3 inhibition also led to increased JNK phosphorylation and increased JNK-dependent phosphorylation of cJun. In this regard, it was previously observed that GSK3 is able to bind MEKK4 and prevent downstream activation of the JNK cascade (38). Thus, it is possible that GSK3 also regulates JNK–cJun activity upstream of the cascade. Regulation of the JNK–cJun pathway both upstream of the cascade and at the level of cJun would provide an explanation for the present observations whereby the JNK inhibitor completely blocked the SB216763-induced JNK-dependent phosphorylation of cJun but partially prevented the SB216763-induced cJun expression. Further studies will be required to delineate the mechanisms by which GSK3 controls the JNK–cJun pathway in pancreatic cancer cells.

The role of JNK is complex since it has been implicated in promoting both apoptosis and cell survival. The physiological issue of JNK activation largely depends on the stimulus and the cell type involved, but it is generally accepted that sustained activation of JNK is associated with apoptosis, whereas acute and transient activation of JNK is involved in cell proliferation and survival (39). In the present study, we demonstrated that JNK activation is necessary for GSK3 inhibition-mediated Bim expression and PARP cleavage. Similar results were obtained in pancreatic cancer cells where JNK inhibition prevented cell death induced by various apoptotic stimuli (40–42), supporting a negative impact of sustained JNK activation in pancreatic cancer cell survival. However, the present results are the first to demonstrate that, although JNK activation is necessary to trigger expression of apoptotic markers, the sole persistent activation of the MEKK1–JNK–cJun pathway is not sufficient per se to elicit pancreatic cancer cell death. Studies in CC139

Fig. 4. Inhibition of GSK3 activity leads to JNK–cJun activation. (A) PANC1 cells were transfected with the NF-κB-luciferase and Renilla-luciferase reporter genes. The following day, transfected cells were treated with vehicle (dimethyl sulfoxide, DMSO) or SB216763 (SB; 20 μM) for 24 h. Cells were then lysed and luciferase activity was measured. The data are expressed as NF-κB-luciferase activity divided by Renilla-luciferase activity, where the relative activity in DMSO-treated cells was set at 1. (B and C) PANC1 cells were treated for the indicated time period with vehicle alone (DMSO) or SB216763 (SB; 20 μM). (D) PANC1 cells were infected with lentiviruses encoding either for a non-targeting shRNA (shcontrol) or a shGSK3β. Cells were lysed 72 h post-infection. (B–D) Equal amounts of cellular extracts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Akt, Erk and JNK activities were analyzed using phospho-specific antibodies. Total expression of glycogen synthase (GS), Akt, ERK, JNK as well as phospho-cJun, total cJun and GAPDH expressions are shown.
fibroblasts using the same MEKK1 construct have shown that selective and sustained JNK activation failed to initiate apoptosis unless a second pathway, namely the PI3K pathway, was also inhibited (25). However, in the present study, we show that the AKT pathway was not significantly modulated by GSK3 inhibition. Thus, it is likely that, in pancreatic cancer cells, GSK3 promotes cell survival at different levels i.e. by limiting JNK activity and by modulating other signaling pathways that act in concert to ensure cell survival.

Herein, we showed that, as a consequence of GSK3 inhibition, the JNK–cJun cascade promotes Bim expression in pancreatic cancer cells. In other systems, studies have suggested that GSK3 can regulate Bim function (43,44). Moreover, there is evidence that JNK–cJun signaling contributes to Bim induction as well as neuron death evoked by normal growth factor deprivation (45–48), supporting a relationship between JNK–cJun signaling and Bim expression. Potential mechanisms of action rely on JNK-dependent phosphorylation of Bim, favoring its proapoptotic function (48,49) and transcriptional regulation of bim via an AP-1 site (47). However, in the latter case, it was proposed that induction of bim expression occurs only if the AP-1-, FoxO- and Myb-binding sites of the promoter are simultaneously occupied, providing a fail-safe mechanism to prevent accidental cell death following single pathway activation. Observations in the present study fit well with such a model i.e. JNK–cJun activation is necessary but not sufficient for Bim expression. Such model would also provide an explanation why inhibition of JNK activity prevented the SB216763-induced Bim expression and PARP cleavage, whereas the sole activation of this pathway was unable to induce Bim expression and initiate PARP cleavage. Again, our results support a model where GSK3 acts at the crossroad of multiple signaling pathways that work together to promote cell survival. Noteworthy, inhibition of JNK–cJun activity was unable to efficiently prevent the SB216763-induced Bcl-2 downregulation, implying that GSK3 could also operate by other means than the JNK–cJun pathway to influence cell survival.

One of the first evidences that inhibition of GSK3 activity favors apoptotic cell death emerged in 2000 when it was observed that GSK3β knockout mice died in utero due to massive TNFα-dependent apoptosis (31). As early as 1989, the GSK3 inhibitor lithium (50) had been identified as a factor, which increases TNFα-mediated cytotoxicity in several cell types in vitro and in mouse tumors in vivo (51), supporting the notion that low GSK3 activity sensitizes cells to TNFα-induced apoptosis. To date, reduced GSK3 activity has been shown to potentiate death receptor-induced apoptosis in hepatoma, prostate, kidney, breast and colon cancer cells (52–56). Our studies are the first to extend this observation to pancreatic cancer cells. In addition, we provide evidence that this potentiation effect requires JNK activation.

The significance of these data is considerable since the effectiveness of ‘TNFerade’, a replication-deficient adenovirus vector containing the TNFα controlled by a chemoradiation-inducible promoter (57), was under investigation in phase III clinical trial for treatment of unresectable locally pancreatic cancer (32). During the redaction of this manuscript, the study was stopped after the two-third interim
analysis, which showed only 8% lower risk of death in the TNFerade-treated patients as compared with standard-of-care-treated patients. Nevertheless, a humanized monoclonal antibody targeting and activating the TRAIL receptor, DR5, is under phase II clinical trial for treatment of unresectable or metastatic pancreatic cancer (33,34). TRAIL is considered a better candidate for cancer therapeutics than TNF-α as it was shown to trigger apoptosis preferentially in cancer cells with no or minimal toxicity to non-malignant human cells (58). Since SB216763 treatment does only affect pancreatic cancer cells, inhibition of GSK3 could represent an attractive new avenue to improve the effectiveness of therapies targeting death ligand/receptor in pancreatic cancer cells while leaving intact normal pancreatic epithelial cells. The applicability of such a combination to preclinical or even clinical settings is greatly simplified by the fact that small GSK3 inhibitors are used in clinical practice for bipolar disorder and are under development for other indications, including neurodegenerative diseases and diabetes (5,21). It is noteworthy that long-term use of the GSK3 inhibitor, lithium, for patients with bipolar disorder has not been associated with increased risk of cancer (59). Collectively, our data support a therapeutic use for GSK3 inhibitors in the treatment of pancreatic cancer, a cancer known for its dismal mortality rate. A better understanding of the signaling downstream of GSK3 is however needed to further recognize the central role of these kinases in the regulation of pancreatic cancer cell growth. Our data contribute to build up a better picture of the signaling pathways under the regulation of GSK3 activity in pancreatic cancer cells.

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**References**


**Fig. 6.** Inhibition of GSK3 activity potentiates death ligand-induced expression of apoptotic markers in pancreatic cancer cells. PANC1 cells were treated for 24 h with SB216763 (20 μM) ± TNFα 10 ng/ml (A) or TRAIL 10 ng/ml (B) in presence or absence of the JNK inhibitor, SP600125 (SP; 25 μM), as indicated. MIA PaCa-2 (C) or HPDE (D) were treated for 24 h with SB216763 (20 μM) ± TNFα 10 ng/ml as indicated. (A–D) Equal amounts of cellular extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and submitted to western blot analysis using the indicated antibodies.

![Figure 6](https://example.com/figure6.png)
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