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Modulation of the Constitutive Activated STAT3 Transcription Factor in Pancreatic Cancer Prevention: Effects of Indole-3-Carbinol (I3C) and Genistein

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Abstract. *Background: The signal transducer and activator of transcription 3 (STAT3) is a latent transcription factor required in proliferation and differentiation. STAT3 is activated constitutively in a number of cancers. Materials and Methods: This study was conducted to assess the possible involvement of STAT3 activation in pancreatic cancer and the potential for this pathway as a target in chemopreventive strategy. Results: STAT3 was shown for the first time to be constitutively activated in human pancreatic carcinoma specimens but not in normal pancreatic tissues. Constitutively activated STAT3 was also found in pancreatic tumor cell lines (Panc-1 and MIA PaCa-2) which could be modulated by indole-3-carbinol (I3C) and genistein. At concentrations higher than 10 μM, STAT3 constitutive activation is inhibited by both agents. Induction of apoptosis by I3C was also demonstrated. Conclusion: Given its critical role in tumorigenesis, our results suggest that STAT3 activation provides an important and appropriate target for chemoprevention in pancreatic cancer treatment.*

Pancreatic cancer has one of the highest mortality rates of any malignancy. Every year, over 28,000 patients are diagnosed with pancreatic cancer and most of them will die of the disease (1,2). Unfortunately, treatment for this cancer has not advanced much in recent years. This is mainly attributed to the lack of early diagnosis and effective chemotherapeutic treatments. To increase the survival rate of pancreatic cancer patients, better tumor markers for earlier diagnosis and new molecular targets for drug development and chemoprevention are essential for the treatment and prevention of this disease.

The signal transducer and activator of transcription factor 3 (STAT3) is a latent cytoplasmic transcription factor that functions as a downstream effector of cytokine and growth factor signaling (3,4). STAT3 has also been found to be embryonically lethal in development (5). STAT3 is activated by tyrosine phosphorylation, which in turn forms homodimers or heterodimers and is subsequently translocated into the nucleus where it binds to specific DNA elements, leading to the transcription activation (6,7). The activation of STAT3 is involved in a number of diverse biological processes that are regulated by extracellular ligands, including cell proliferation, differentiation, development and survival (5,8,9). Accumulating evidence has also suggested that abnormal activation of STAT3 signaling play a critical role in oncogenesis (10). For example, activation of STAT3 is able to transform fibroblasts from mouse and rat. Constitutive activation of STAT3 protein is found in a wide variety of malignancies such as breast cancer, head and neck cancer, prostate cancer, multiple myeloma and leukemias (11-14).

STAT3 involvement in oncogenesis may be through up-regulation of genes encoding apoptotic inhibitors and cell cycle proteins such as Bcl-x_L, Mcl-1, cyclins D1/D2, $p21^{WAF1/CIP}$ and c-Myc (10). In normal cells, STAT3 activation is temporary and usually last a few minutes to hours. Constitutive activation of STAT3 in cells is a consequence of persistent up-regulation of numerous genes encoding enzymes, ligand receptors, autocrine or paracrine cytokines and growth factors, which may result in genetic alterations that are found in tumors, but not in normal tissue (10). In view of this effect, modulation of constitutively activated STAT3 signaling would provide a novel approach for cancer therapeutic and chemopreventive strategies (15).

In the present study, normal and tumor pancreatic tissues from human donors were compared for constitutive

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activation of STAT3 protein, given the potential importance of this target in the etiology of pancreatic cancer and potential usefulness as a molecular target in chemoprevention. The activation status of STAT3 was also assessed in several human pancreatic tumor cell lines. Using an *in vitro* cell culture system, the dietary agents, indole-3-carbinol (I3C) and genistein, were examined for their effect on the modulation of constitutively activated STAT3. Both of these agents have shown chemopreventive potential against several cancers (16,17). Apoptosis induction by I3C was also explored. The results have permitted an assessment of STAT3 as a molecular target for these agents and provided insight into the potential mechanism of action of these agents in pancreatic cancer chemoprevention.

Materials and Methods

Tissue specimens and cancer cell lines. Frozen human pancreatic normal and cancerous tissues were purchased from the Southern Division of the Cooperative Human Tissue Network (Birmingham, AL, USA) and the International Institute for the Advancement of Medicine (Exton, PA, USA). Cell lines, PANC-1 (ATCC number: CRL-1469) and MIA PaCa-2 (ATCC Number: CRL-1420) were received from the American Type Culture Collection. These cells were grown in DMEM medium supplemented with 10% serum and antibiotic.

Protein sample preparation. Tissue samples were minced in liquid nitrogen and immediately lysed directly into lysis buffer containing 2 mM EDTA, 1 mM sodium pyrophosphate, 1 mM sodium fluoride, 0.2 mM sodium vanadate, 10% glycerol, 25 mM Tris-HCl (pH 6.8) and 1% SDS for protein analysis. The lysed tissue samples were immediately boiled for 7 minutes followed by centrifugation for 10 minutes at room temperature. The resultant supernatants were saved for protein analysis.

Treatment and cytotoxicity assays. Genistein and I3C were purchased from Sigma (St. Louis, MO, USA). Cells were treated for 18 h with 0.05, 0.2, 2, 10, 40, 100, or 200 μM of genistein or 0.5, 2, 10, 50, 200, or 400 μM of I3C. After incubation with the chemopreventive agents, the cells were washed 2x with PBS buffer (pH 7.4). The resultant supernatants were saved for protein analysis later. The cell toxicity of these chemopreventive agents was estimated by percentage of total proteins from the cells. Protein concentrations were measured using the Bio-Rad protein assay (Bio-Rad Laboratories; Richmond, CA, USA) according to the manufacturer's instructions. The rates of cell toxicity with the agents were calculated using the concentration of total proteins from untreated cells to normalize the data.

Immunoblot and SDS-PAGE. Aliquots of the supernatant from the tissue sample and cell lysates were analyzed further for specific STAT proteins. Proteins were separated on a 11% SDS gel and transferred to Immoblon-P memberane for immunoblot assay as described (18). The membrane was incubated with STAT3 or phospho-STAT3 (pSTAT3) antibodies. Antibodies to STAT3 and pSTAT3 were purchased from Cell Signaling (Beverly, MA, USA). Monoclonal antibody to actin was purchased from Sigma. The membrane was stripped with stripping buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM DTT, 1% SDS) when the same membrane were re-blotted with a different antibody.

Trevigen apoptotic cell system (TACS). To detect apoptosis, a TiterTACS™ colorimetric apoptosis detection kit purchased from Trevigen (Gaitherburg, MD, USA) was used. The TACS is a colormetric assay that allows quantitative detection of apoptosis using a 96-well plate. This method detects fragmented DNA. The labeling of the 3'-ends of DNA fragments provides an easy measure of cells undergoing apoptosis. Modified nucleotides solutions were made fresh prior to use. This assay also generated a positive control for normalization of data. Before labeling, cells were fixed with 3.7% buffered formaldehyde solution for 7 minutes; this solution was made fresh before use. The plate was centrifuged at 1000 g for 3 minutes and the fixative discarded. The plate was washed twice with 1x PBS at room temperature. The cells were post-fixed with 100% methanol for 20 minutes. The cells were washed twice before labeling. The remaining reagents were prepared from the kit following the manufacturer's instructions. Fifty μl of cytonin was added to each well for 15 minutes at room temperature. The plate was washed with 200 μl of dH_2O. A positive control was generated in 6 wells with treatment of TACS nuclease for 30 minutes at 37˚C. The remaining cells were incubated with 1x PBS for 30 minutes. After incubation the cells were washed for 2 minutes with PBS. To quench endogenous peroxidases, 50 ml/well of a freshly prepared 3% hydrogen peroxide solution was added for 5 minutes. The cells were washed once with 200 μl/well of dH_2O. One hundred and fifty μl /well of 1x TdT labeling buffer was added for 5 minutes. The plate was centrifuged and the buffer discarded. Fifty μl of labeling reaction mix was added to each well for 1 h at 37˚C. The reaction mixture was made according to the procedures in the kit. The reaction consisted of 1x labeling buffer, TdT dNTP mix, 50x Mn^{2+} and the TdT enzyme. The reaction was stopped after one hour with 150 μl of 1x TdT stop buffer. The plate was washed for 5 minutes with 1x PBS. After washing, 50 μl/well of Strep-HRP was added for 10 minutes at room temperature. The cells were washed with 200 μl/well of PBS with 0.1% Tween 20, followed by 100 μl/well of TACS-Sapphire at room temperature. The plate was incubated in the dark. The reaction was stopped with 100 μl of 2 N HCl per well and the absorbance was measured at 450 nm.

Results

Constitutively activated STAT3 was detected in pancreatic adenocarcinomas but not in normal tissues, as shown in Figure 1. STAT3 constitutive activation was also examined in two different pancreatic tumor cell lines. Figure 2 shows phosphorylated activated pSTAT3 in both Mia PaCa-2 (lanes 1-4) and the Panc-1 (lanes 5-8) tumor cell lines. Increased phosphorylation was noted in the Panc-1 cells. Cells were serum-starved to reduce the basal levels of pSTAT3 (lanes 1,3,5 and 7) for 24 h and stimulated for 1 h with serum (lanes 2,4,6,8).

Varying concentrations of the dietary agents (0.5 to 400 μM, I3C; 0.5 to 200 μM, genistein) were used to assess their ability to modulate pSTAT3 constitutive activation in cells.

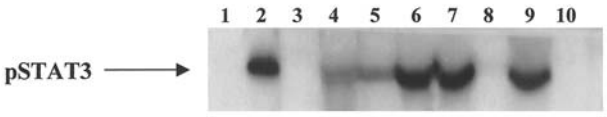

Figure 1. *STAT3 activation in normal pancreatic tissues, pancreatic adenocarcinomas and islet cell tumors. Lanes 1,3,10 - normal pancreatic tissue; lanes 2,4,6,7,9 - adenocarcinomas; lanes 5,8 - islet cell tumors.*

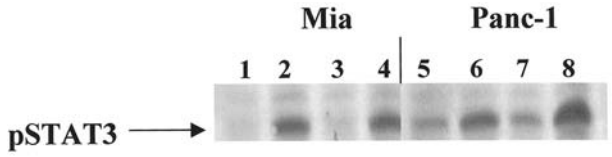

Figure 2. *STAT3 activation in pancreatic tumor cell lines. Mia, lanes 1-4 and Panc-1, lanes 5-8 (lanes 3,4 and 7,8 are replicates). Cells were serum-starved to reduce the basal levels of STAT3 for 24 h (lanes 1,3,5,7), stimulated for 1 h with serum (lanes 2,4,6,8).*

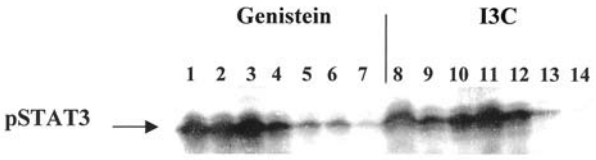

Figure 3. *Effect of genistein and I3C on STAT3 activation. Lanes 1 and 8, controls; lanes 2-7 represent 0.05, 0.2, 2, 10, 40 and 200 μM genistein; lanes 9-14 represent 0.5, 2, 10, 50, 200 and 400 μM I3C.*

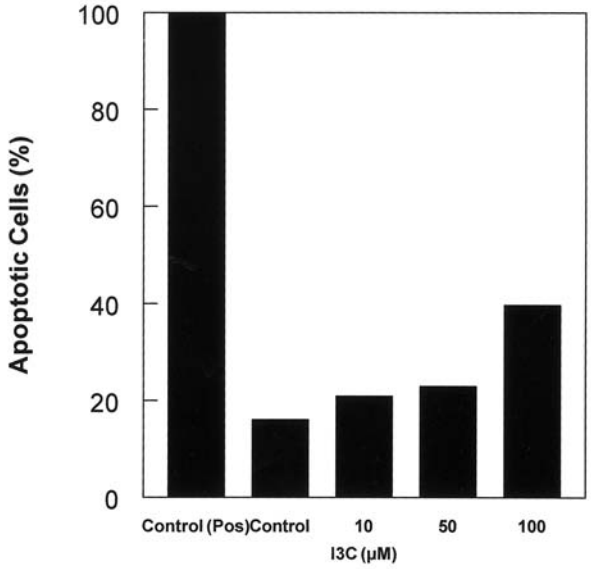

Figure 4. *Apoptosis induction by I3C. Using a TACS 96-well apoptosis assay, induction of apoptosis in pancreatic tumor cells was determined. "Control (positive)" was generated, as described in "Materials and Methods", for normalization of data.*

As shown in Figure 3, treatment of cells for 18 h with I3C or genistein suppressed pSTAT3 levels in a concentration-dependent manner. At the lower concentrations inhibition was not observed; however, at the highest concentrations pSTAT3 was reduced to barely detectable levels. Reblotting with anti-STAT3 antibody showed that treatment with these agents has no effect on protein levels of STAT3. Using a colorimetric apoptosis assay, I3C was also shown to induce apoptosis about 40% at a concentration of 100 μM (Figure 4). The higher concentrations of genistein and I3C used in this study were only slightly toxic to the cells, as shown in Figure 5. The effect of genistein was greater than that of I3C.

Discussion

Data from this study suggest that STAT3 signaling may serve as a potential target in chemopreventive strategies for pancreatic cancer. It provides the first evidence that STAT3 is constitutively activated in human pancreatic adenocarcinoma tissues. In contrast, activated pSTAT3 was not detected in normal pancreatic tissues. STAT3 activation was also demonstrated in pancreatic tumor cell lines, as has been recently reported by others (19). Activation of STAT3 is involved in a number of biological processes that play a major role in carcinogenesis such as cell proliferation, differentiation and survival (5,8-10). Compared with normal cells and tissues, constitutively activated STAT3 has been detected in a variety of other human cancer cell lines and primary tumors (11-14). Increasing evidence is accumulating also suggesting that STAT3 signaling can be an important intervention target for cancer prevention and treatment (15).

STAT3 activation, as a target in pancreatic tumor cells for I3C and genistein, was explored. Epidemiological studies have suggested that consumption of cruciferous vegetables (20) and soy products (21) may decrease incidences of certain cancers. I3C is a naturally occurring hydrolysis product of an abundant glucobrassacin in cruciferous vegetables (22) and is also available as a dietary supplement. It has been documented to be chemopreventive against cancer in a number of animal models and is being investigated as a potential chemopreventive against breast cancer (16). Genistein, the predominant isoflavone in soy, has been widely shown to demonstrate chempreventive properties in some cancers (17). Both of these dietary agents were found to suppress STAT3 activation in pancreatic tumor cells in this study. Their mechanism of action in decreasing pSTAT3 levels remains to be elucidated. Reduction in pSTAT3 levels could be a result of

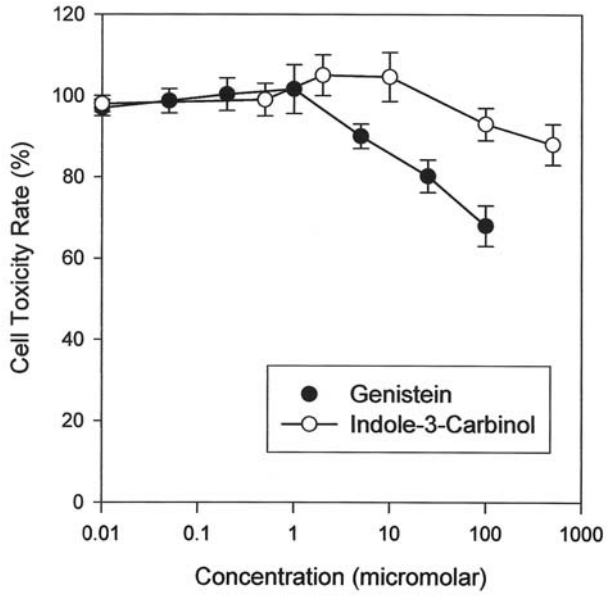

Figure 5. *Cell toxicity rate. The cell toxicity of genistein and I3C were estimated by percentage (%) of total proteins from cells. The total proteins were measured by Bio-Rad protein assay. The rates of cell toxicity were calculated using the concentration of total proteins from untreated cells to normalize the data.*

either inhibition of protein tyrosine kinases or activation of protein phosphotyrosine phoshatases. Several tyrosine family kinases, JAK and Src, are thought to be involved in STAT3 phosphorylation (10). In addition, other kinases may be involved (19). STAT3 is known to be phosphotyrosine dephosphorylated by two protein phosphotyrosine phosphatases, SHP-1 and SHP-2 (23,24). Genistein is a known inhibitor of tyrosine kinase activities (25) and could conceivably act by inhibiting the tyrosine kinase responsible for phosphorylating STAT3. I3C has also been shown to inhibit tyrosine kinase activity (26), but little is known about its effect on specific kinases. Alternatively, these agents could also activate physiologically inhibitors that are known to directly or indirectly down-regulate STAT3 activation. These include suppressors of cytokine signaling, STAT-induced STAT inhibitor, JAK-binding protein and STAT3-interacting protein (15).

Genistein and I3C are known to inhibit cell growth in tumor cells; however, the inhibitory effects can be concentration-dependent (27,28). Previous studies have shown that genistein at low concentrations actually stimulated growth in another pancreatic tumor cell line, HPAF-11 (29). The present study demonstrated that genistein at a concentration of 10 μM or less did not inhibit STAT3 activation in the two cell lines used, Panc-1 and Mia. At the higher concentrations (>10 μM), geinstein did block activation of STAT3. An important downstream consequence of STAT3 activation may be prevention of apoptosis induction thereby increasing cell survival (30). Inhibition of STAT signaling has been demonstrated to result in induction of apoptosis in tumor cells harboring constitutive activation of STAT3 (31). In the present study, I3C induced apoptosis in the pancreatic tumor cells. However, a causal relationship with suppression of STAT3 activation remains to be established. I3C has been shown to induce apoptosis in other cell lines, including breast cancer cells and prostate cancer cells (32,33). In the breast tumor cell line MDA MB468, I3C induced apoptosis through inhibition of phosphorylation and activation of protein kinase B (34).

In summary, constitutive activation of STAT3 has been demonstrated in human pancreatic adenocarcinoma tissues but not in normal pancreatic tissues. Blocking of STAT3 activation in pancreatic tumor cells by the dietary agents I3C and genistein suggests that STAT3 signaling may be a useful target in chemopreventive strategies against pancreatic cancer, as has been indicated for other cancers. Additionally, these results identify another molecular target for these chemopreventive agents.

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