Foxp3 Expression in Pancreatic Carcinoma Cells as a Novel Mechanism of Immune Evasion in Cancer


Abstract

The forkhead transcription factor Foxp3 is highly expressed in CD4+CD25+ regulatory T cells (Treg) and was recently identified as a key player in mediating their inhibitory functions. Here, we describe for the first time the expression and function of Foxp3 in pancreatic ductal adenocarcinoma cells and tumors. Foxp3 expression was induced by transforming growth factor-β (TGF-β), but not TGF-β1 stimulation in these cells, and was partially suppressed following antibody-mediated neutralization of TGF-β. The TGF-β effect could be mimicked by ectopic expression of a constitutively active TGF-β type 1 receptor/ALK5 mutant. Down-regulation of Foxp3 with small interfering RNA (siRNA) in pancreatic carcinoma cells resulted in the up-regulation of interleukin 6 (IL-6) and IL-8 expression, providing evidence for a negative transcriptional activity of Foxp3 also in these epithelial cells. Coculture of Foxp3-expressing tumor cells with naive T cells completely inhibited T-cell proliferation, but not activation, and this antiproliferative effect was partially abrogated following specific inhibition of Foxp3 expression. These findings indicate that pancreatic carcinoma cells share growth-suppressive effects with Treg and suggest that mimicking Treg function may represent a new mechanism of immune evasion in pancreatic cancer. [Cancer Res 2007;67(17):8344–8350]

Introduction

The forkhead transcription factor Foxp3 has been identified as a key player in regulatory T cell (Treg) function and is, thus far, the only definitive marker of CD4+CD25+ Treg (1, 2). The importance of Tregs resides in their pivotal role in maintaining immunologic tolerance. Naturally occurring CD4+CD25+ Tregs as well as Foxp3-expressing CD4+ T cells inhibit naive T-cell proliferation, but not activation, and this antiproliferative effect was partially abrogated following specific inhibition of Foxp3 expression. This protein, Treg may enhance their metabolic fitness (11, 12). Down-regulation of Foxp3 with small interfering RNA (siRNA) in pancreatic carcinoma cells resulted in the up-regulation of interleukin 6 (IL-6) and IL-8 expression, providing evidence for a negative transcriptional activity of Foxp3 also in these epithelial cells. Coculture of Foxp3-expressing tumor cells with naive T cells completely inhibited T-cell proliferation, but not activation, and this antiproliferative effect was partially abrogated following specific inhibition of Foxp3 expression. These findings indicate that pancreatic carcinoma cells share growth-suppressive effects with Treg and suggest that mimicking Treg function may represent a new mechanism of immune evasion in pancreatic cancer.
support the concept that the tumor induces an increase of Tregs via TGF-β secretion, which results in an inhibition of the immune response against the tumor. Tumor cells have adopted inhibitory mechanisms originally thought to be specific for cells of the immune system, such as the ability to kill tumor-invading T cells via the expression of Fas ligand (24). In this report, we show another variation of this theme: the TGF-β2 and TGF-β type I receptor/ALK5-dependent autoinduction of FoxP3, which enables the tumor cells in vitro to suppress (potentially tumor-invading) T cells via direct inhibition of their proliferation rather than indirectly through Treg.

Materials and Methods

Cell lines and culture. Panc89 (also termed T3M4), Capan1, PancTu1, and Panc1 cells were cultured in RPMI 1640 supplemented with 10% FCS, 2 mmol/L glutamine, and 1 mmol/L sodium pyruvate (all from Invitrogen). Panc1 cells stably transduced with a kinase-active version of ALK5 (T204D mutation) have been shown previously to display high constitutive expression of various TGF-β target genes (25).

Samples and immunohistochemistry. Tissue samples were collected from patients with pancreatic adenocarcinoma. The present study was done with the approval of the local ethics committee, and informed consent was obtained according to the Declaration of Helsinki. Immunohistochemical staining of deparaffinized tumoral and peritumoral pancreatic tissue was done according to standard protocols using FoxP3 antibody (PCH101 or hFOXY, both from eBioscience). The staining intensities were graded as strong, moderate, and weak, respectively, by a pathologist specialized in pancreatic cancer.

Affymetrix chip analysis. Microdissected pancreatic carcinoma tissue and normal pancreas tissue were analyzed using Affymetrix U133 chip. Methods are described in detail in ref. 26.

TGF-β stimulation and Western blot analysis. Protein extracts were prepared from cells stimulated with 5 ng/mL of recombinant human TGF-β1, or TGF-β2, and 0.1 μg/mL anti-TGFβ1 antibody (all purchased from R&D Systems). Following the determination of protein concentrations with the bicinchoninic acid protein assay (Pierce), cellular lysates were fractionated by PAGE and transferred to polyvinylidene fluoride membranes. For FoxP3 detection, membranes were probed with hFOXY antibody (1:100 dilution) overnight at 4°C followed by a secondary horseradish peroxidase–conjugated antibody. Chemiluminescent detection was done with the enhanced chemiluminescence detection kit (GE Bioscience).

Flow-cytometric analysis. Cells were washed in PBS containing 1% bovine serum albumin (BSA) and 0.1% NaN3 before antibody staining, followed by fixation with 1% paraformaldehyde. All antibodies were purchased from BD Biosciences. A total of 10⁶ events, gated for lymphocytes, were collected using a FACSCalibur (BD Biosciences). Analysis was done using the Cell Quest program (BD Biosciences).

Isolation of T cells. Peripheral blood mononuclear cells (PBMC) were isolated from human peripheral blood from healthy donors by Ficoll-Hypaque gradient centrifugation. CD4+CD25− T cells were isolated using a
CD4+ T-cell isolation kit according to the manufacturer’s instructions (Miltenyi Biotec), followed by magnetic separation on MACS columns. TGF-β ELISA. TGF-β/2 in culture supernatants of pancreatic tumor cell lines was quantified with the TGF-β2 quantikine ELISA kit (R&D Systems) according to the manufacturer’s instructions. Briefly, tumor cells were seeded in six-well plates, and culture supernatants were harvested after 48 h. TGF-β2 concentrations were normalized to 1 x 10^6 cells/mL to account for differences in cell numbers.

**Small interfering RNA treatment.** Panc89 cells were seeded in six-well plates and treated with 300 nmol/L small interfering RNA (siRNA) targeting Foxp3 or control siRNA together with LipofectAMINE (Invitrogen) for 4 h in plates and treated with 300 nmol/L small interfering RNA (siRNA) targeting Foxp3 or control siRNA together with LipofectAMINE (Invitrogen) for 4 h in medium containing 0.5% FCS. Subsequently, cells were cultured in normal growth medium. The following siRNA sequences were used (5′ -> 3′): Foxp3 sense: GCACAUUCCAGAGUCC- UdTdT; Foxp3 antisense: AGGAAACUGUGGGAAUGUG-CdTcT; control sense: UUCUCCGAACGUUGUCACG- UdTdT; control antisense: ACAGUACAGUUCGGAGA-AdTdT.

**Reverse transcription-PCR.** Total RNA isolation from cell lines was done using the Micro-to-Midi total RNA purification system (Invitrogen) according to the manufacturers’ recommendations. Reverse transcription was carried out using Superscript II reverse transcriptase (Invitrogen) and random hexamers (Roche). Relative mRNA levels were determined by quantitative PCR (qPCR) done with IQ SYBR Green Supermix (Bio-Rad) and analyzed by the Opticon real-time PCR system using MJ Opticon Monitor Analysis Software (Bio-Rad). Semiquantitative PCR was carried out by applying standard conditions and including betaine (Sigma-Aldrich) in the reaction mixture. The following primers were used (5′ -> 3′): Foxp3 sense: CACAAATCGCCACCCTTTTCACC; Foxp3 antisense: AGGGTGTTGCGG- GATGGCGTTCTTC; IL-6 sense: TCCCAACGCGCTCTGCTGTT; IL-6 antisense: AGGTGAGTGGCTGTCTGTGT; glucose-6-phosphatedehydrogenase (G6PD) sense: AGGTGATGCGGACACACTACTG; G6PD antisense: ACACGCGCTG- CAAAATGGGC.

**Coculture experiments and carboxyfluorescein succinimidyl ester proliferation assays.** Three days after siRNA transfection, Panc89 cells were trypsinized and seeded into six-well plates at 2 x 10^5 cells per well. After cells had attached to the dishes, they were cocultured with 2 x 10^6 CD4+CD25− T cells for 3 days with Expander Beads (Miltenyi Biotec) using X-Vivo 15 medium. T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) before coculture by incubating them with 5 μmol/L CFSE for 10 min at 37°C in PBS, followed by addition of ice-cold complete medium and washing with PBS. Proliferation of T cells was measured by flow cytometry on the basis of CFSE dilution and calculated as the ratio of the number of proliferating over the total number of T cells.

### Results and Discussion

**Detection of Foxp3 expression in pancreatic carcinoma tissue.** In an initial analysis of microdissected pancreatic carcinoma tissue using Affymetrix chip technology, Foxp3 was among the up-regulated genes in some tumor samples (26); (Fig. 1A). This was in contrast to other studies that failed to detect Foxp3 expression in nonhematopoietic cells and tissues (1). To further validate these results, semiquantitative reverse transcription-PCR (RT-PCR) analysis from 13 patients with pancreatic adenocarcinoma was conducted and revealed a significant overexpression of Foxp3 in the tumor specimens compared with seven cases of nonmalignant pancreatic tissues (Fig. 1B). Furthermore, by immunohistochemistry, we detected Foxp3 expression in tumor cells of 24/39 patients with pancreatic carcinoma (Table 1). Because Foxp3 expression in malignant epithelial cells was a novel and surprising observation, we confirmed the staining results in 12 of the above 39 cases with a different anti-Foxp3 antibody (hFOXY, data not shown). Foxp3 subcellular staining was mostly cytoplasmic in some patients (Fig. 1C, bottom left), whereas in others, it seemed predominantly nuclear (Fig. 1C, top left). Normal pancreatic duct cells were devoid of Foxp3 expression (Fig. 1C, bottom right). In all patients, Foxp3-positive, tumor-infiltrating lymphocytes (Fig. 1C, arrows), presumably representing Treg, were detectable as reported previously (4, 6). In this small series of patients with different clinicopathologic characteristics (including patients with curative tumor resection and palliative surgery), we were unable to find a correlation between Foxp3 expression or subcellular localization and tumor stage or survival. It has recently been shown that the activation of Treg with anti-CD3/anti-CD28 induces a change in the subcellular localization of Foxp3 from a more cytoplasmic/perinuclear to a nuclear expression pattern, which may result in...
from post-translational modifications (27). Therefore, the heterogeneous subcellular localization of Foxp3 in pancreatic carcinoma tissues may reflect the presence of different post-translationally modified forms of Foxp3. The functional relevance of this finding needs further investigation.

Foxp3 expression in pancreatic carcinoma cell lines and up-regulation by TGF-β2 but not TGF-β1. In accordance with the immunohistochemistry data pointing to FoxP3 expression in tumor cells (see above), Foxp3 expression was clearly detectable in several pancreatic carcinoma cell lines at both mRNA (Fig. 2A) and protein levels (Fig. 2B). It was recently described that pancreatic cancer cells can, via secretion of TGF-β, promote the prevalence of Treg (22), in part by converting CD4+CD25+ T cells to Treg (28). Treatment of Panc89, Capan1, and Panc1 cells with TGF-β2 led to an up-regulation of the Foxp3 protein, which was moderate for Panc89 and Capan1 and strong for Panc1 (Fig. 3A and D). Surprisingly, treatment of Panc89 cells with TGF-β1 did not change Foxp3 protein expression (Fig. 3A) despite the fact that TGF-β1 rather than TGF-β2 induces Foxp3 expression and converts CD4+CD25+ T cells into CD4+CD25+ Treg (13, 14, 16, 28). RT-PCR analysis detected a 13-fold (mean value of four independent experiments) increase of Foxp3 mRNA expression in Panc89 cells after a 48-h stimulation with TGF-β2, whereas no such effect was evident after stimulation with TGF-β1 (Fig. 3B).

Pancreatic cancer cells are known to secrete biologically active TGF-βs that might act in an autocrine fashion to enhance certain TGF-β responses. Because the induction of Foxp3 by exogenously applied TGF-β was comparatively weak, we tested the hypothesis that the Foxp3 protein levels detected might be actually induced by autocrine TGF-β(2) stimulation. This assumption was supported by the observation that the concentrations of TGF-β2 in the culture supernatants as measured by ELISA (Fig. 3C) correlated positively with the expression levels of Foxp3 quantified by densitometry (compared with Fig. 2B) in 3/4 cell lines analyzed (Panc89, Colo357, PancTu1). Moreover, the incubation of Panc89 and Capan1 cells with anti–TGF-β2 antibody for 48 h clearly suppressed the Foxp3 signal (Fig. 3A), suggesting that endogenously produced TGF-β2 maintains Foxp3 expression in pancreatic cancer cell cultures.

TGF-β2 requires for high-affinity binding and signaling either the coreceptor betaglycan (type III receptor), together with TGF-β receptor type II (TβRII) and ALK5 (29), or an alternatively spliced TβRII-B; ref. 30). However, it was also reported that TGF-β2 can signal independently from TβRII or TβRII-B, provided that ALK5, TβRII, or TβRII-B is sufficiently expressed (31). To determine whether the activation of ALK5 alone can induce Foxp3 in pancreatic carcinoma cells, we employed Panc1 cells stably expressing a constitutively active mutant (T204D), named Panc1
ALK5-TD) of ALK5. Strikingly, Foxp3 expression was dramatically up-regulated in these cells and could not substantially be further increased by exogenous TGF-β2 (Fig. 3D). Moreover, Panc1 cells ectopically expressing a kinase-defective ALK5 mutant (K232R) that acts in a dominant-negative fashion failed to respond to TGF-β2 stimulation with the up-regulation of Foxp3 (data not shown). The expression level of ALK5 [low in Panc89 (32) and high in Panc1 (25)] might thus determine the strength of the regulatory effect of TGF-β2 on Foxp3.

TGF-β regulation of Foxp3 in Treg has been shown to involve Smad signaling (13). However, in pancreatic cancer, this signaling pathway is often nonfunctional due to mutations in Smad4 as exemplified by the Capan1 line (33, 34), whereas Panc1, Panc89, and Colo357 have retained a functional Smad pathway (32, 35). Interestingly, although Capan1 culture supernatants contained the highest amount of TGF-β2, they expressed only moderate amounts of Foxp3, which may reflect inefficient Smad-mediated transcriptional induction of the Foxp3 gene through TGF-β2, along with a compensatory overproduction of TGF-β2. However, the observation that Foxp3 expression was induced or suppressed in response to TGF-β2 ligand or neutralizing anti-TGF-β2 antibody, respectively, may be taken as evidence for Smad-independent regulation. Clearly, more extensive analysis is required to clarify the contribution of Smad and non-Smad signaling to TGF-β regulation of Foxp3 expression in pancreatic cancer cells.

Specific down-regulation of Foxp3 in pancreatic carcinoma cells results in up-regulation of IL-6 and IL-8. The control of inflammatory cytokine production is a key function of Foxp3 in Treg. To analyze whether Foxp3 modulates cytokine secretion in pancreatic carcinoma cells, Foxp3 expression was down-regulated by means of siRNA transfection in Panc89 cells. This cell line was chosen because both Capan1 and Colo357 cells, although displaying higher Foxp3 expression, were refractory to transfection with LipofectAMINE and other methods in our hands. Treatment of Panc89 cells with Foxp3 siRNA resulted in a strong down-regulation of both Foxp3 mRNA (Fig. 4A) and protein (Fig. 4B). A screen for changes in cytokine secretion in these cells using the Raybiotec Cytokine Array (Hoelzel Diagnostica) revealed an increase of IL-6 and IL-8 in the culture supernatant, whereas other cytokines remained unchanged (data not shown). Specific analysis of IL-6 and IL-8 expression by quantitative RT-PCR 48 h after transfection indicated 3.8-fold higher levels of IL-6 and 4.6-fold higher levels of IL-8 mRNA in Foxp3-siRNA-transfected cells relative to LipofectAMINE-treated control cells (Fig. 4C). Because IL-6 gene expression is activated by nuclear factor-κB (NF-κB; ref. 36) and Foxp3 interacts with NF-κB in Tregs and is able to repress cytokine gene expression (37) through the inhibition of NF-κB transcriptional activity, down-regulation of Foxp3 can promote IL-6 secretion via the relief of NF-κB inhibition. Our observation that siRNA-mediated suppression of Foxp3 resulted in the up-regulation of IL-6 suggests that in pancreatic cancer cells, Foxp3 operates in a similar way. IL-8 is an angiogenic factor whose expression is also dependent on NF-κB (38). IL-8 promotes growth of pancreatic tumors; however, the functional significance of Foxp3-mediated...
suppression of IL-8 secretion in these tumor cells is currently not known.

**Inhibition of T-cell proliferation in a coculture system is dependent on Foxp3 expression in Panc89 cells.** The results presented thus far raised the possibility that not only is antitumor immunity influenced by tumor-infiltrating Treg (3, 4, 22), but also that pancreatic carcinoma cells themselves might modulate T-cell function via expression of Foxp3. To evaluate the effect of (Foxp3-expressing) pancreatic carcinoma cells on T-cell activation and proliferation, Panc89 cells were cocultured with naive CD4+/CD25− T cells. After 3 days of coculture with anti-CD3 and anti-CD28 stimulus, proliferation of T cells was measured using CFSE-labeling and flow cytometry analysis. In the cocultures, Colo357, Panc89, and PancTu1 tumor cells strongly inhibited the proliferation of anti-CD3/anti-CD28–stimulated T cells (Fig. 4D). This effect could not be overcome by exogenous IL2 (data not shown). In contrast, no antiproliferative effect was observed when tumor cells and T cells were separated by a membrane using Transwell inserts (data not shown), suggesting that cell-cell contact was important. To explore whether the proliferation arrest in the coculture system was associated with a lack of T-cell activation, we monitored the expression of various activation antigens on the cell surface of activated T cells cultured in the absence or presence of Panc89 cells. As shown in Fig. 5B, CD25 GITR and CD69 were up-regulated after anti-CD3/anti-CD28 stimulation, irrespective of the presence or absence of tumor cells. These data clearly show that tumor cells inhibit proliferation rather than the activation of T cells.

To verify the presumed suppressive role of Foxp3 expression in pancreatic tumor cells on T-cell proliferation, we repeated the coculture experiments with Panc89 cells in which Foxp3 expression had been silenced by siRNA. When using these cells, we observed an ~45% recovery of T-cell proliferation in the coculture system (Fig. 5A). The percentage of proliferating T cells in the coculture was increased from 3% to 28% as measured by CFSE staining. Together, these results show that Panc89 cells inhibit, in a Foxp3-dependent manner, the proliferation of naive T cells without generally impeding their activation. Recent reports have shown that tumor cells can induce Treg via secretion of TGF-β (22, 28, 39). Blocking TGF-β in the coculture with anti–TGF-β antibody did not reverse the antiproliferative effect on the T cells (data not shown). In addition, in preliminary experiments, activated T cells recovered from the coculture with the tumor cells did not suppress proliferation of freshly isolated CD4+/CD25− T cells (data not shown). Therefore, we assume that the inhibition of T cell proliferation in the coculture system was independent of the induction of Tregs.

In summary, we show for the first time the ectopic expression of Foxp3 in pancreatic adenocarcinoma tissue and pancreatic carcinoma cell lines. Our observations are novel in that Foxp3 expression was thought to be restricted to hematopoietic cells and tissues. Its expression in epithelial cancer cells may represent an example of molecular mimicry and reveals another mechanism of immune evasion in pancreatic cancer. However, the function of Foxp3 in tumor cells may be much more subtle because it has been reported that it transcriptionally down-regulates intracellular PDE3B in Treg, which is associated with increased expression of metabolic fitness genes, better survival, and reduced apoptosis (after IL2–driven expansion; ref. 11). Because apoptosis resistance is a hallmark of pancreatic tumors, it may be speculated that Foxp3 confers a survival advantage on tumor cells in normal tumor physiology and under chemotherapeutic stress. Given the crucial role of TGF-β2 in maintaining Foxp3 expression, our results are of immediate relevance in view of an ongoing phase I/II study with a TGF-β2 antisense compound in pancreatic carcinoma (40). Furthermore, these findings might open new perspectives for immunotherapeutic strategies against this tumor.
type. Intriguingly, Foxp3 expression was detected in 60% of all pancreatic carcinoma patients and in 4/4 pancreatic cancer cell lines analyzed. Although the number of patients was too small to establish a meaningful correlation between Foxp3 expression and patient survival, the issue of whether Foxp3 might serve as a prognostic marker has to be evaluated in a larger series of patients.

Acknowledgments

Received 9/6/2006; revised 4/30/2007; accepted 6/19/2007.

Grant support: Deutsche Krebshilfe (70-2979-Sel); L. Pagrols-Raluy received a graduate fellowship from 70-2988-Schm3 and the Deutsche Forschungsgemeinschaft (SFB 415 A3 and A15 and a Gerok-Fellowship grant to S. Hinz).

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