Foxp3-Mediated Immunity of Human Pancreatic Cancer Cell Line PANC-1

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Abstract: Problem statement: More and more evidences have shown that human cancer cells can express Foxp3, the regulatory T cell-associated transcription factor. The role of Foxp3 in human cancer cells is still unclear. We detected Foxp3 expression in human pancreatic cancer cell line PANC-1. SiRNA assays showed that Foxp3 expression suppressed the expression of TGF-β and IL-10 which could induce immune tolerance. Approach: Thus, we proposed that Foxp3 expression in PANC-1 cells controlled the response but not tolerance characteristics of the cells. To prove the proposal, we first developed the PANC-1 cell-induced the immune response model. PANC-1 cells induced response in autoimmune Non-Obese Diabetes (NOD) mice aged of >12 weeks. In vitro cell co-cultured assay demonstrated that PANC-1 induced response in splenocytes from NOD mice aged of >12 weeks. Results: In the same time, we found that inflammatory cytokine such as INF-γ, IL-2 and IL-17 increased and anti-inflammatory cytokine such as IL-4, IL-10 and TGF-β decreased in the co-cultured supernatant. These results demonstrate that PANC-1 cells induced response in splenocytes from NOD mice aged of >12 weeks. SiRNA assays showed that Foxp3 in PANC-1 cells controlled the response in the co-cultured cells. Conclusion: The results suggested that Foxp3 control the immunological response characteristics of PANC-1 cells in autoimmune condition.

Key words: PANC-1, tolerance, response, nod mice, foxp3

INTRODUCTION

Foxp3 has been regarded as a key factor in regulatory T cell (Treg) [1]. Mutations in Foxp3 completely omit Treg in mouse and greatly reduce suppressive activity of Treg in human [2]. Moreover, ectopic expression of Foxp3 makes conventional murine T cells to gain the function of Treg [3], and human T cells to acquire many characteristics of Treg [4,5]. The molecular mechanisms through which Foxp3 determined the function and development of Treg are now studied by many groups. Foxp3 can form a complex with the nuclear factor of activated T (NF-AT) cells and regulates the transcription of several target genes including interleukin 2 [6]. The results are consent with the characteristics of Foxp3 as suppressive transcription factor.

Until recently, Foxp3 expression is found only in suppressive T cells including CD4+Foxp3+Treg, and CD8+Foxp3+Treg. Now, there are more and more evidences suggesting that human tumor cells also expressed Foxp3 [7-9]. The function of Foxp3 in human tumor cells is studied by many groups. In breast cancer, Foxp3 was regarded as an important suppressor for human breast cancer by suppressing HER-2/ErbB2 oncogene expression [10]. Another paper found that Foxp3-expressing pancreatic carcinoma cell line PANC-89 (also T3M4) suppressed T-cell proliferation, and knockdown of Foxp3 using RNA interference could effectively reduce the suppressive activity of these cells by up-regulation of IL-6 and IL-8 [7].

The role of Foxp3 in human tumor cells may be different in different tumor cells. This may be in fact that Foxp3 as suppressive transcription factor suppresses the different targeted molecular in different tumor cells. Previous study has found that PANC-89 expresses IL-6 but not TGF-β, whereas PANC-1 expresses TGF-β but not IL-6 [11]. Thus, we propose that if it was expressed in PANC-1 cells, Foxp3 may be an important factor inducing tumor immunity by suppressing TGF-β expression. We here study the proposal.
MATERIALS AND METHODS

Mice: NOD were obtained from the Jackson Laboratory and bred in our animal facilities under specific pathogen-free conditions. In female NOD mice, spontaneous diabetes began to appear by 12-weeks of age (increasing to 80-90% incidence at 30 weeks of age). Mice were screened for glucose levels every week and considered diabetic when glucose levels were ≥13.8 mMol L⁻¹. This study was approved by the Animal Care and Use Committee of the Beijing Institute of Basic Medical Science.

Antigen preparation: We predicted T-cell epitope of Glutamic Acid Decarboxylase 65 (GAD 65) and proved that GAD₅₀₀-₅₈₅ (representing the corresponding amino acids of GAD65) was a main T-cell epitope[24,25]. Antigen preparation has been described previously[24]. Briefly, the recombinant peptide GAD₅₀₀-₅₈₅ was produced from Escherichia coli BL-21 containing the bacterial expression vector pET28a⁺ vector. Recombinant GAD₅₀₀-₅₈₅ was purified using Co²⁺/TALONIMAC resin (Clontech) according to the manufacturer’s instructions. Eluted GAD₅₀₀-₅₈₅ fractions were dialyzed against PBS (pH7.2).

Cell preparation: Human pancreatic carcinoma cell lines PANC-1 (American Type Culture Collection, Manassas, VA) were grown in RPMI (Biochrom, Berlin, Germany) 10% FCS (Atlanta Biologicals, Atlanta, GA). Lymphocytes from the spleen were collected under sterile conditions, obtained with mouse Ficoll separation liquid, washed twice with an medium. 

In vitro proliferation: For analysis of cellular response, lymphocytes were collected and cultured (5×10⁶ cells/well) in triplicate in 0.1 ml of RPMI-1640 medium supplement with 1% horse serum and 1% beta-mercaptoethanol and restimulated with 30 µg mL⁻¹ GAD₅₀₀-₅₈₅ without or in the presence of PANC-1 or Foxp3⁺ PANC-1 cells (5×10⁵ cells/well). On day 3, cultures were pulsed with 1µCi/well of [³H] thymidine for the last 16h, and the cells harvested and counted by standard liquid scintillation. The results were expressed as the stimulation index (SI, c.p.m. with antigen divided by c.p.m. with medium alone) or CPM.

Determination of cytokines production: The splenocytes were collected and cultured as for lymphocyte proliferation assay. Supernatants of the cells were harvested 24h later for IL-2 and 48h later for IFN-γ, IL-17, IL-4, IL-10 and TGF-β1 assay using a sandwich ELISA. The ELISA kits used in this study were purchased from Biosource (IL-2), R and D (TGF-β1), eBioscience (IL-17A), and BD Pharmingen (IFN-γ, IL-4, IL-10), respectively.

Flow cytometry: Cells (1×10⁵ cells/sample) were washed with fluorescence-activated cell sorting staining buffer (phosphate-buffered saline, 2% fetal bovine serum or 1% bovine serum albumin, 0.1% sodium azide). All samples were incubated with the 2.4G2 anti-Fc receptors (BD Pharmingen), prior to incubation with other Abs diluted in fluorescence-activated cell sorting buffer supplemented with 2% anti-Fc receptor Ab. For the intracellular staining of Foxp3 protein, fixed cells were permeabilized with Perm Buffer (eBioscience) for 1 h at 4°C. Cells were incubated for 30 min at 4°C with phycoerythrin-conjugated anti-human Foxp3 Ab or isotype Ab (eBioscience, San Diego, CA, USA) at the concentration recommended by the manufacturer, washed, and analyzed by flow cytometry. Cells were washed and fixed with 1% paraformaldehyde prior to analysis in a FACSCalibur flow cytometer using CellQuest version 3.3 software (BD Biosciences, San Jose, CA).

Introduction of Foxp3-siRNA into PANC-1 cells: To introduce the human Foxp3 siRNAs (SC-43569, Santa Cruz Biotechnology, INC) into cultured PANC-1 cells, we used Lipofectamine Reagent and Plus Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s procedure. Briefly, siRNAs were incubated with the Plus Reagent in serum-free DMEM for 15min at room temperature, and then 350µl of the mixture was incubated with an equal volume of the Lipofectamine reagent (16.8 µg) for another 15min in order for them to form the complex of the siRNA with liposome. The PANC-1 cells on a 35×10 mm Petri dish (Becton Dickinson Labware, Franklin Lakes, NJ, USA) were then incubated in the mixture of siRNAs with the Lipofectamine reagents for 6h at 37°C, then added to 700 µL of DMEM containing 10% FBS and incubated for another 42 h. To confirm the introduction of Foxp3-siRNA into cultured PANC-1 cells, cells were stained with PE-conjugated anti-human Foxp3 and analyzed by flow cytometry.

Statistical analysis: Results of cytokine ELISA were analyzed by the Student’s t-test. Differences in diabetes incidence were analyzed by χ² quadrat test. The cumulative diabetes onset was compared by Kaplan-Meier analysis. All differences reported in the results were significant (p<0.05).
RESULTS

Foxp3 expression in PANC-1 cells: Despite many advances in understanding the mechanisms leading to tumor immunity, malignant tumors are tolerated, progress, and ultimately kill their host. Tumor cells employed different strategies to thwart immune responses: tumor-induced impairment of antigen presentation, the activation of negative co-stimulatory signals, and the elaboration of immunosuppressive factors\[^{12}\]. IL-10 and TGF-β synthesized by tumor which exerts suppressive effects on the immune system\[^{12-14}\]. We found PANC-1 cells also secreted high level of TGF-β and low level of IL-10 (Fig. 1A). In vitro studies have shown that TGF-β can impose a regulatory phenotype on CD4^+CD25^-T cells through the induction of Foxp3 expression\[^{15,16}\]. This makes us propose that PANC-1 expressed Foxp3. As expected, we first detect the expression of Foxp3 in PANC-1 cells by cell flow. We found that 11% of PANC-1 cells expressed Foxp3 (Fig. 1B).

Foxp3 suppressed IL-10 and TGF-β in PANC-1 cells: Whether does Foxp3 as a suppressive transcription factor suppress TGF-β expression? Firstly, we set up SiRNA assays to knock down Foxp3 expression. By flow cytometry, we found that the percentage of Foxp3-expressing PANC-1 cells decreased from 2-11% (Fig. 2A).

Next, we determined the effect of Foxp3 down-regulation on IL-10 and TGF-β synthesized by PANC-1. PANC-1 cells expressed much higher level of TGF-β and IL-10 when Foxp3 was knocked down by SiRNA assays (Fig. 2B). These results suggest that Foxp3 expression suppressed TGF-β and IL-10 expression in PANC-1 cells.

Fig. 1: Foxp3 expression in PANC-1 cells
Note: PANC-1 cells were cultured. After 48 h, cells and supernatant were collected. (A) The production of IL-10 and TGF-β was determined by ELISA. (B) Cells were stained for Foxp3 and analyzed by flow cytometry. The percentage of Foxp3^+ cells was showed. The results are representative of four separate experiments.

Fig. 2: Foxp3 suppressed TGF-β and IL-10 expression in PANC-1 cells
Note: Human Foxp3 siRNAs were used to transduce PANC-1 cells (called as Foxp3^-/^-PANC-1 cells). At 48 h after transduction, cells and supernatant were collected. (A) Cells were stained for Foxp3 and analyzed by flow cytometry. The percentage of Foxp3^+ cells was showed. (B) The production of IL-10 and TGF-β was determined by ELISA. The results are representative of three separate experiments (**: p < 0.01)
PANC-1 cells induced tolerance and response model:
Thus, we propose that in PANC-1 cells, Foxp3 may be an important factor inducing tumor immunity by suppressing TGF-β and IL-10 expression. To prove the proposal, we first develop PANC-1 cells induced tolerance and immunity model. The autoimmune Non-Obese Diabetic (NOD) mouse, characterized by T-cell mediated destruction of the insulin-producing β-cells in the pancreatic islets, were used[17]. About 1×10⁷ PANC-1 cells were injected into 7-weeks-old female NOD mice. As shown in Fig. 3A, the suppression of diabetes in PANC-1-treated group persisted for 18 weeks, compared to recipients of the control (p<0.05). After 24 weeks of age, PANC-1-treated NOD mice showed higher diabetes incidence than that in control groups (p<0.05). About 1×10⁷ PANC-1 cells were injected into 12 weeks-old female NOD mice. As shown in Fig. 3B, diabetes incidence was significantly higher in PANC-1-treated NOD mice than that in control groups (p<0.01).

Above results makes us propose that PANC-1 cells induce immune tolerance of splenocytes from 7-week-old female NOD mice and response of splenocytes from 12-week-old female NOD mice to autoantigen Glutamic Acid Decarboxylase 65 (GAD65)[18]. We used splenocytes from 7 or 12-week-old female NOD mice and PANC-1 cells to co-culture in vitro. As expected, PANC-1 induced immune tolerance of splenocytes from 7 week-old female NOD mice and response of splenocytes from 12 week-old female NOD mice to GAD₅₀₀₋₅₈₅ (Fig. 4A). In the parallel with cell proliferation, PANC-1 cells induced anti-inflammatory cytokines IL-10, TGF-β and suppressed inflammatory
Fig. 5: Foxp3 determined PANC-1 cells-inducing immunity

Note: PANC-1 cells were transduced with human Foxp3 SiRNA, after 48 h, Foxp3 SiRNA-transduced PANC-1 cells (Foxp3−/−PANC-1) were used to co-culture for 48 h with splenocytes from 7 or 12-week-old female NOD mice in the presence of GAD500-585. We found that Foxp3−/−PANC-1 cells induced tolerance in GAD500-585-stimulated splenocytes from 7 or 12-week-old female NOD mice (Fig. 5A). In addition, Foxp3−/−PANC-1 cells upregulated anti-inflammatory cytokines IL-10, TGF-β and downregulated inflammatory cytokine IL-2, IFN-γ in GAD500-585-stimulated splenocytes from 7 or 12-week-old female NOD mice (Fig. 5B). These results suggest that Foxp3 is an important factor for PANC-1 cells-induced immune response.

DISCUSSION

Foxp3 expression has been thought to be restricted to the T-cell lineage. Until recently, several reports were published about Foxp3 expression in human cancer cells[7-9]. In these studies, they showed Foxp3 expression in many tumor cell lines of different tissues origins by RT-PCR and Western blot assay. We also detected Foxp3 in human pancreatic cancer cell line PANC-1 by cell flow (Fig. 1B). Except for in tumor cells, Foxp3 is expressed in normal breast epithelium[10].

TGF-β and IL-10 were factors most frequently released by pancreatic ductal carcinoma cell line[11]. We also found that PANC-1 expressed TGF-β and IL-10 (Fig. 1A). TGF-β can converse peripheral CD4+CD25− naive T cells to CD4+CD25− regulatory T cells by inducing Foxp3 expression[16]. These results suggest that auto-secreted TGF-β may induce Foxp3 expression in PANC-1 cells. A report found that Foxp3 expression was induced by TGF-β2, but not TGF-β1 stimulation in pancreatic ductal adenocarcinoma cells[7].

The role of Foxp3 in TGF-β expression is still unclear. We have known that Foxp3 is a suppressive transcription factor[19]. Foxp3 suppress the transcription of several target genes including IL-2, IFN-γ, IL-4, and IL-10[1,6] in Treg, HER-2/ErbB2 oncogene expression in breast cancer[16], IL-6 and IL-8 in pancreatic carcinoma cell line PANC-89[7]. Can Foxp3 as suppressive transcription factor suppress TGF-β expression? Foxp3-expressing CD4+CD25+ Treg cells-mediated tolerance is dependent on TGF-β-dependent[16] suggesting that Foxp3 may promote TGF-β expression. Foxp3 expression was knocked down in PANC-1 cells by SiRNA assays to study the role of Foxp3 in TGF-β secreted by PANC-1. TGF-β and IL-10 were up-regulated in PANC-1 cells when Foxp3 was knocked
down (Fig. 2B). These results suggest that Foxp3 expression suppressed TGF-β and IL-10 expression in PAN-C1 cells.

To further explore the role of Foxp3 in the immunity of PAN-C1 cells, we develop the PAN-C1 induced tolerance and immunity model. The NOD strain of mouse is an increasingly useful and important model of autoimmune type 1 diabetes[17]. The autoimmune disease characterized by progressive destruction of pancreatic beta cells we found that PAN-C1 cells could control diabetes until 13 weeks after therapy when injected into 7-week-old female NOD mice (Fig. 3A). In vitro experiment further proved that PAN-C1 cells induced tolerance (Fig. 4A) and immunoregulatory cytokines IL-10 and TGF-β, and suppressed inflammatory cytokine IL-2, IFN-γ and IL-17 in splenocytes from 7 week-old female NOD mice (Fig. 4A and B). These results show that PAN-C1 cells induced tolerance in splenocyte from 7 weeks-old female NOD mice. Tumor cells employed different strategies to thwart immune responses; tumor-induced impairment of antigen presentation, the activation of immunosuppressive factors[13]. IL-10 and TGF-β are immunoregulatory cytokines that are involved in induction and maintenance of peripheral tolerance by affecting autoreactive T-cell-mediated response[21-23]. The studies suggest that PAN-C1 cells induce immune tolerance partly by auto-secreted TGF-β and IL-10.

When PAN-C1 cells were injected into 12-weeks-old female NOD mice, diabetes incidence was significantly higher in PAN-C1-treated NOD mice (Fig. 3B). In vitro experiment further proved that PAN-C1 cells induced response (Fig. 4A) and inflammatory cytokine IL-2, IFN-γ and IL-17, and suppressed immunoregulatory cytokines IL-10 and TGF-β in splenocytes from 12 week-old female NOD mice (Fig. 4B). The fact that most mice demonstrate severe insulitis by 10 weeks of age explained that inflammatory response is very strong in 12-week-old female NOD mice. These results suggest that PAN-C1 cells induced immune response in splenocytes from 12-week-old female NOD mice. We did not know the reason for inflammatory response induced by PAN-C1. The PAN-C1 cells were injected into NOD mice and they maybe difficulty form tolerance microenvironment in autoimmune condition. Thus, they may induce response over tolerance in autoimmune condition.

PAN-C1-induced immune response and tolerance model were very useful in studying the role of Foxp3 in immunity of PAN-C1 cells. In the different condition, the response of cells and the molecular expression and function may vary. There are PAN-C1-induced immune response and tolerance model make us gain more information in the function of Foxp3. We found that Foxp3+/PANC-1 cells significantly induced tolerance in GAD65-stimulated splenocytes from 12-week-old and 7-week-old female NOD mice (Fig. 5A). In addition, Foxp3+/PANC-1 cells induced anti-inflammatory cytokines IL-10, TGF-β and suppressed inflammatory cytokine such as IL-2, IFN-γ and IL-17A in GAD65-stimulated splenocytes from 12-week-old and 7-week-old female NOD mice (Fig. 5B). These results suggest that Foxp3 control the capacity of PAN-C1 cells in inducing immune response. In addition, the extent to reducing response in PAN-C1-induced response model was obviously bigger than that in PAN-C1-induced tolerance model. This may explain for the necessary of building PAN-C1-induced response model.

CONCLUSION

In conclusion, human pancreatic cancer cell line PANC-1 exhibits immunological response characteristics by expressing Foxp3. The effect may act partly by suppressing TGF-β expression. Thus, we proved our proposal that Foxp3 may be an important factor inducing tumor immunity by suppressing TGF-β expression in PAN-C1 cells. It is under study that immunogenic response of PAN-C1 cells is enhanced by Foxp3 over-expression.

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