Tumor-Derived Transforming Growth Factor-β is Critical for Tumor Progression and Evasion from Immune Surveillance

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Abstract

Tumors have evolved numerous mechanisms by which they can escape from immune surveillance. One of these is to produce immunosuppressive cytokines. Transforming growth factor-β(TGF-β) is a pleiotropic cytokine with a crucial function in mediating immune suppression, especially in the tumor microenvironment. TGF-β produced by T cells has been demonstrated as an important factor for suppressing antitumor immune responses, but the role of tumor-derived TGF-β in this process is poorly understood. In this study, we demonstrated that knockdown of tumor-derived TGF-β using shRNA resulted in dramatically reduced tumor size, slowing tumor formation, prolonging survival rate of tumor-bearing mice and inhibiting metastasis. We revealed possible underlying mechanisms as reducing the number of myeloid-derived suppressor cells (MDSC) and CD4⁺Foxp3⁺ Treg cells, and consequently enhanced IFN-γ production by CTLs. Knockdown of tumor-derived TGF-β also significantly reduced the conversion of naïve CD4⁺ T cells into Treg cells in vitro. Finally, we found that knockdown of TGF-β suppressed cell migration, but did not change the proliferation and apoptosis of tumor cells in vitro. In summary, our study provided evidence that tumor-derived TGF-β is a critical factor for tumor progression and evasion of immune surveillance, and blocking tumor-derived TGF-β may serve as a potential therapeutic approach for cancer.

Keywords: Tumor-derived TGF-β - shRNA - immune surveillance - Treg - MDSC

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Introduction

Intact immune responses, such as immune surveillance, are required for suppressing tumor development and progression, and tumors often create an immunosuppressive microenvironment that can facilitate tumor growth and block antitumor immune responses through producing soluble factors, such as cytokines, which in turn recruiting other regulatory cells. The promotion of MDSCs and Treg cells by tumor cells plays an crucial role in tumor immune evasion (Zou, 2006; Marigo et al., 2008). Blocking these negative pathways might serve as a potential therapeutic approach. Indeed, antibodies against T cell suppressing molecules, such as CTLA-4 and PD-1 have shown positive effects on tumor patients (Li et al., 2013), which is usually considered as a negative prognostic indicator (Buck et al., 2004; Tripsianis et al., 2013). Increased production of TGF-β occurs in both human cancer patients and animal models (Hu et al., 2013), which is identified as a potential therapeutic approach for cancer.

The TGF-β family are pleiotropic cytokines which play important roles in cancer and immunoregulation. The canonical TGF-β signaling pathways involves the binding of TGF-β ligands to the type I and type II TGF-β receptors, followed by the phosphorylation of Smad2 and Smad3, which then bind to Smad4 and enter the nucleus to regulate downstream gene transcriptions (Li et al., 2008). Three isoforms have been observed in mammalian animals, including TGF-β1, TGF-β2 and TGF-β3, and TGF-β1 is the most highly expressed by cells of the immune system. Alterations in TGF-β signaling have significant effects on tumor progression, and TGF-β is known to work as both a tumor suppressor and a tumor promoter (Bierie et al., 2006). TGF-β inhibits tumor progression through increased apoptosis, decreased cell cycle progression and reduced expression of growth factors. During the process of cancer progression, tumor cells frequently acquire the capability to resist the growth inhibitory action of TGF-β, and production of TGF-β often leads to tumor evasion from immunosurveillance (Akhurst et al., 2001; Siegel et al., 2003). Increased production of TGF-β occurs in both human cancer patients and animal models (Hu et al., 2013), which is usually considered as a negative prognostic indicator (Buck et al., 2004; Tripsianis et al., 2013).

TGF-β has an inhibitory effect on the immune system. TGF-β1 deficient mice manifest spontaneously activated T cells and severe autoimmunity pathology in vital organs (Kulkarni et al., 1993). Similarly, Smad3-deficient mice also present activated T cells that are resistant to the growth inhibitory action mediated by TGF-β (McKarns...
Studies with dn-TGF-βRII transgenic mice showed that abrogation of TGF-β signaling in T cells suppressed growth and metastasis when these mice were challenged with lymphoma and melanoma (Gorelik et al., 2001). A recent study suggested that TGF-β1 produced by activated CD4+ T cells antagonized T cell surveillance of tumor development (Donkor et al., 2012). Systemic neutralization of TGF-β in vivo enhances immune-mediated recognition and clearance of malignant cells (Thomas et al., 2005).

Actually almost all nucleated cells produce TGF-β, and the sources of TGF-β in tumors include cancer cells themselves as well as various stromal cells in the tumor microenvironment, such as leukocytes, macrophages, bone marrow-derived endothelial cells, and myeloid derived suppressor cells (Massague, 2008). However, the role of tumor cell-derived TGF-β in tumor immune responses as well as the tumor growth is unclear. In this study, we investigated the effect of tumor cell-derived TGF-β in antitumor immune responses by establishing a stable cell line of B16 transfected with TGF-β shRNA and studied the underlying mechanisms. Our study provide evidence that tumor-derived TGF-β is a critical factor for tumor progression and evasion of the immune system, and blocking tumor-derived TGF-β may serve as a potential therapeutic approach.

Materials and Methods

Mice and tumor cell line
C57BL/6 WT male mice were purchased from Beijing Huafukang Bioscience Co., Ltd. All experimental animals were used at 6-8 weeks of age, and maintained in specific pathogen-free conditions at the animal center of Nankai University, Tianjin, China. All animal study protocols were reviewed and approved by the animal care and use committee of Nankai University. The B16F0 cell line was cultured in DMEM-high glucose (Hyclone) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco) at 37°C under an atmosphere of 5% CO₂.

Establishment of TGF-β1 knockdown cell line
Four shRNA targeting TGF-β1 (NM 011577.1) were designed as follows: (1)5’-TAT CCT TGC TGT CAC AAG AGC-3’; (2) 5’-AAT AGT TGG TAT CCA GGG CTC-3’; (3)5’-ATT TCT GGT AGA GTT CCA CAT-3’; (4)5’-AGG GCA AGG ACC TTG CTG TAC-3’. These sequences were cloned into pcDNA™6.2-GW/EmGFP expression vector (Invitrogen). These four vectors and the negative control plasmid were transfected into B16 cells respectively by Lipofectamine 2000 (Invitrogen). The expression of Tgf-β1 mRNA was analyzed by real-time PCR and the most efficient sequence was selected. This sequence was cloned into pLenti6/6.2-GW/EmGFP lentiviral vector. The lentiviral vector was co-transfected into 293FT cells with the Packaging Mix, and the viral supernatant was harvested and used to transduce B16 cells. B16 cells were infected with the TGF-β1 specific or negative control lentivirus. After selection by blasticidin (5 μg/ml) for two weeks, the EmGFPhigh cells were sorted out by FACS.

Real-time PCR
Real-time PCR was performed by SYBR Premix Ex Taq™ Kit (TaKaRa). Specific primers for mouse TGF-β1 and GAPDH were as follows: TGF-β1 forward: 5’-GGC ACC ATC CAT GAC ATG AA-3’, reverse: 5’-CCA GAC AGA AGT TGG CAT GGT-3’; GAPDH forward: 5’-AAG GCT GTG GGC AAG GTC A-3’, reverse: 5’-TGCC TTC ACC ACC TTC TTG ATG T-3’. Data analysis was done by the ΔCt method for relative quantification.

ELISA assays
Cytokine assay for TGF-β production by B16 cells was done using a ELISA kit (R&D Systems). B16 cells were plated at a density of 1x10⁶/T-25 flask and cultured for 48 h in serum-free medium. The supernatant was collected and TGF-β level was measured according to the manufacturer’s instructions.

Tumor models
For subcutaneous tumor model, 2x10⁴ B16 cells were injected subcutaneously into the right flank of B6 WT mice and the tumor growth was measured with caliper every other day for up to 3 weeks. For metastasis assay, B6 WT mice were injected intravenously with 5x10⁴ B16 cells. Eighteen days later, mice were euthanized and lungs were excised, fixed with 2% Formaldehyde solution for gross inspection. To observe survival time, mice were inoculated in the same way as above and monitored every other day.

Preparation of tumor-infiltrating lymphocytes (TILs)
Freshly excised tumor tissues were minced and digested with Collagenase IV and DNase I for 1 hour at 37°C. Cell suspensions were filtered through a 70μm cell strainer. TILs were isolated by density-gradient centrifugation at 1500 rpm for 20 min at 25°C. TILs were collected from the interphase and washed twice in PBS.

Flow cytometry
APC-conjugated anti-mouse CD4 (clone GK1.5), PE-conjugated anti-mouse CD8α (clone 53-6.7), FITC-conjugated anti-mouse CD11b (clone M1/70), anti-mouse Gr-1 (clone RB6-8C5) and APC-conjugated anti-mouse CD4 (clone MF-14) were purchased from Biolegend. For analysis of surface markers, cells were incubated with antibodies for 20 min on ice. Foxp3 staining was performed using Foxp3 Staining Buffer Set (eBioscience). For IFN-γ staining, splenocytes or TILs were stimulated with PMA (50 ng/ml; Sigma, St. Louis, MO) and ionomycin (1 mg/ml; Sigma) in the presence of GolgiPlug (BD Biosciences) for 5.5 h. Data were acquired using BD FACSCalibur flow cytometer and analyzed with FlowJo software.

Culture of CD4+CD25+ T cells with conditioned medium (CM)
After surface staining with FITC-conjugated anti-mouse CD4 (clone GK1.5), PE-conjugated anti-mouse CD25 (clone PC-61.5.3) and APC-conjugated anti-mouse CD123, splenocytes or TILs were stimulated with PMA (50 ng/ml; Sigma, St. Louis, MO) and ionomycin (1 mg/ml; Sigma) in the presence of GolgiPlug (BD Biosciences) for 5.5 h. Data were acquired using BD FACSCalibur flow cytometer and analyzed with FlowJo software.
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CD44 (clone IM7), CD4+CD25−CD44low T cells were sorted from adult mice spleens on BD FACS Aria II. The purity of these T cells was found to be over 90%, as determined by FACS. CM were collected from a 3-day culture of B16 TGF-β knockdown cells and negative control cells respectively, and then diluted 1:1 with complete T cell medium. Isolated CD4+CD25−CD44low T cells (4×10⁵/well in a 48-well plate) were cultured in complete T cell medium or different CM in the presence of plate-bound anti-CD3, soluble anti-CD28 and IL-2 for 3 days. Three days later, cells were harvested and stained for CD4 and Foxp3. Data were acquired using BD FACSCalibur flow cytometer and analyzed with FlowJo software.

Wound healing assay

Confluent monolayer of B16 cells were scraped with pipette tips, washed with PBS, and incubated in DMEM supplemented with 2% FBS for 24 h.

MTT assay for cell growth inhibition

MTT (Sigma) was added to the culture medium 68 h post transfection. After incubation for 4 h, the culture medium was removed and 200 μL of DMSO was added to resolve the crystal. Absorbance was measured at 490 nm.

Detection of apoptotic cells by FACS

Cells were harvested by trypsinisation, washed in cold PBS and re-suspended in 1×Binding Buffer at a concentration of approximately 1×10⁶ cells/mL. 5μL of Annexin V- APC and 5μL of 7-AAD solution were added to each cell suspension and incubated at RT for 10 min in the dark. The cells were analyzed within 1 hour by FACS.

Statistics

Two-way analysis of variance test (ANOVA) was used for comparing and analyzing the tumor growth curve. The survival curve was compared and analyzed using the Log-rank test. Two-tailed unpaired Student’s t test was performed to analyze other data. All values were expressed as mean±SD. All analysis was performed using Graphpad Prism 5.01 for Windows. Throughout the text, figures, and legends, the following terminology was used to denote statistical significance: *p<0.05; **p<0.01; ***p<0.001.

Results

Establishment of TGF-β knockdown B16 cells

To knockdown tumor-derived TGF-β in B16 cells, four plasmids designed to express shRNA targeting mouse TGF-β1 and negative control plasmid coding for a scramble shRNA were transfected into B16 cells respectively, and the transfection efficiency was monitored by the Emerald green fluorescent protein (EmGFP) reporter within these plasmids (Figure 1a). Cells were collected 48 h post transfection and then analyzed for expression of Tgf-β1 by real-time PCR. Figure 1b showed that the shRNA4 was the most effective one among the four sequences. This sequence was cloned into pLenti6/V5-DEST lentiviral vector. B16 cells were infected with the TGF-β1 specific or negative control lentivirus and then cultured for 2 weeks in the presence of blasticidin. The EmGFPhigh cells were sorted out by FACS and over 99% of sorted B16 cells were EmGFP positive (data not shown). The TGF-β knockdown cell line that we finally obtained had a 79% reduction in the expression of Tgf-β1 mRNA when compared with negative control B16 cells (Figure 1c). The knockdown efficiency was further confirmed by ELISA (Figure 1d).

Tumor-derived TGF-β is required for tumor formation, progression and metastasis in vivo

To define the role of tumor-derived TGF-β in tumor growth in vivo, B6 WT mice were subcutaneously challenged with either negative control or TGF-β knockdown B16 cells, animals bearing TGF-β knockdown B16 cells showed a significant delay in tumor formation as well as a smaller tumor burden, when compared to those bearing negative control B16 cells (Figure 2a and 2b). To further study the effect on metastasis, B6 WT mice were intravenously injected with these two kinds of B16

Figure 1. Establishment of TGF-β Knockdown B16 Cell Line. A) B16 cells were transfected with negative control plasmid and four shRNA plasmids. The transfection efficiency was monitored by fluorescence microscopy (×100) and the expression of mRNA was examined by Real-time PCR. B) The effects of TGF-β knockdown in the finally obtained B16 TGF-β knockdown cells were determined by Real-time PCR C) and ELISA D). The data shown are representative of three independent experiments.
cells. A significantly increased survival rate was realized in the knockdown group (Figure 2c). It was obvious that the mice challenged with TGF-β knockdown B16 cells bore less pulmonary metastatic nodules than mice in the control group (Figure 2d). These observations indicated that tumor-derived TGF-β plays an important role in tumor formation, progression and metastasis in vivo.

Tumor-derived TGF-β regulates the number of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells and MDSCs in tumor microenvironment and CD8<sup>+</sup> T cell function in spleen and tumor

To define the underline mechanisms by which tumor-derived TGF-β mediates the immune suppression, tumor tissues as well as spleens from mice subcutaneously injected with two kinds of B16 cells were collected for the analysis of the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells and MDSCs. As shown in Figure 3a, about 24% of TILs were found to be MDSCs in the mice bearing negative control B16 cells, whereas only approximately 9% in the mice bearing TGF-β knockdown B16 cells. Similar reduction was also observed in the spleens. The proportion of Treg cells in CD4<sup>+</sup> T cells in the TILs was found to be 30% in the control group, but only 14% in the TGF-β knockdown group (Figure 3b). Interestingly, there was no significant difference of Treg cells in the spleen between the two groups. Adding to this, we also observed an increase in the CTL function in the mice challenged with TGF-β knockdown B16 cells. As indicated in Figure 3c, CD8<sup>+</sup> T cells in both the spleen and tumor tissue of mice challenged with TGF-β knockdown cells produced more IFN-γ than the negative control group. In our preliminary studies, inoculation of these two different B16 cells did not change the total number of TILs and splenocytes (data not shown).

Tumor-derived TGF-β mediates conversion of naïve CD4<sup>+</sup> T cells into Treg cells in vitro

To study the potential role of tumor-derived TGF-β in Treg conversion, CD4<sup>+</sup>CD25<sup>-</sup>CD44<sup>low</sup> T cells were sorted from the spleens of adult mice and then cultured in CM in the presence of plate-bound anti-CD3, soluble anti-CD28 and IL-2 for 3 days. Figure 4 shows that Foxp3 expression was significantly higher in naive T cells cultured with negative control B16 CM, as compared to those cultured with TGF-β knockdown B16 CM. Foxp3 expression was absent in T cells cultured in T cell medium. These results indicate that tumor-derived TGF-β is required in Treg conversion in vitro.

Tumor-derived TGF-β promotes cell migration, but does not change the proliferation and apoptosis of tumor cells in vitro

To evaluate whether the knockdown of tumor-derived TGF-β has changed the cell biology characteristics effect of B16 cells, we did the following experiments. Figure 5a showed that knockdown of TGF-β in B16 cells inhibited cell migration in vitro. MTT analysis result indicated that there was no significant difference between negative control and TGF-β knockdown B16 cells, suggesting that tumor-derived TGF-β has no inhibitory action on cell migration.
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Figure 4. Tumor-Derived TGF-β Mediates the conversion of naïve CD4+ T cells into Treg Cells. CD4+CD25−CD44− T cells were sorted from spleen of adult mouse and cultured in different CM. After culture for 3 days, cells were harvested and stained for CD4 and Foxp3 expression. Data shown are represent of three independent experiments.

Figure 5. Knockdown of Tumor-Derived TGF-β Leads to Inhibited Cell Migration but not Decreased Cell Proliferation and Increased Apoptosis. Wound-healing assay A) and MTT assay B) were performed. C) Cell apoptosis was evaluated by FACS. Data shown are represent of three independent experiments.

Discussion

The role of TGF-β in tumor progression and inducing immunosuppression has been extensively studied. TGF-β produced by T cells has been demonstrated as an important factor for suppressing antitumor immune response. Many types of tumors are rich sources of TGF-β; however, the effects of tumor cell-derived TGF-β on the tumor immune suppression and the underlying mechanisms were not fully elucidated. Melanoma is a type of highly immunogenic cancer that causes almost 75% of deaths related to skin cancer (Jerant et al., 2000). B16 cells produce substantial TGF-β, which to some extent was responsible for the decreased metastases. Next proliferation (Figure 5b). The apoptosis rates between the two groups also had no significant difference (Figure 5c).

MDSCs also play an essential role in the immunosuppressive networks that lead to tumor immune evasion (Zou, 2006; Marigo et al., 2008). They expand significantly in the bone marrow, spleen, blood, liver and tumor sites of numerous patients and experimental mice with cancers (Liu et al., 2010). MDSCs can be recruited into mammary carcinomas with TGF-β type II receptor gene deletion and directly promote tumor metastasis (Yang et al., 2008). Our results showed a
reduction in the percentage of MDCs both in spleen and tumor sites in tumor-bearing mice challenged by TGF-β knockdown B16 cells. Publications from many groups have shown that MDCs are also one of the major sources of TGF-β in the tumor-bearing host (Ghiringhelli et al., 2005; Yang et al., 2008). So we speculate that reduction of MDCs in the tumor sites was a possible consequence of the knockdown of tumor-derived TGF-β, and the reduction of MDCs further resulted in a lower level of TGF-β in the tumor microenvironment. The conversion from naive CD4+ T cells into Treg cells was therefore partially blocked. It is necessary to point out that the interaction between these two immunosuppressive cell populations was a bit complicated and the papers on this point of view are sometimes contradictory to each other. Some reports indicated that immature DCs, phenotypically and functionally impaired by the immunosuppressive environment induced by cancer, may be able to induce Treg cell expansion (Chung et al., 2009). Others suggested that MDCs expanded in 4T1 or LLC tumor bearing mice are capable of suppressing TGF-β-induced polarization of naive T cells into Treg cells (Centuori et al., 2012). This question could not be addressed in our recent study; therefore, we will set up experiments in the future to make it clear which kind of roles tumor-derived TGF-β played in the relationship between MDCs and Treg cells in the tumor microenvironment.

Current therapeutic inhibitors that block TGF-β signaling via antisense molecules, neutralizing antibodies and small molecule inhibitors often lack specificity and consequently target systemic TGF-β signaling. So elucidating the role of tumor-derived TGF-β is important, not only for a better understanding of the mechanisms of TGF-β-mediated tumor progression and immune suppression, but also for the design of more effective and specific TGF-β-targeting cancer immunotherapy.

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