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The effect of CT26 tumor-derived TGF-β on the balance of tumor growth and immunity

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ABSTRACT

Introduction: TGF-β is an important target for many cancer therapies under development. In addition to suppressing anti-tumor immunity, it has pleiotropic direct pro- and anti- tumor effects. The actions of increased endogenous TGF-β production remain unclear, and may affect the outcomes of anti-TGF-β cancer therapy. We hypothesize that tumor-derived TGF-β (td-TGF-β) plays an important role in maintaining tumor remission by controlling tumor proliferation in vivo, and that decreasing td-TGF-β in the tumor microenvironment will result in tumor progression. The aim of this study was to examine the effect of TGF-β in the tumor microenvironment on the balance between its anti-proliferative and immunosuppressive effects.

Methods: A murine BALB/c spontaneous colon adenocarcinoma cell line (CT26) was genetically engineered to produce increased active TGF-β (CT26-TGF-β), a dominant-negative soluble TGF-β receptor (CT26-TGF-β-R), or the empty neomycin cassette as control (CT26-neo). In vitro proliferation rates were measured. For in vivo studies, the three cell lines were injected into syngeneic BALB/c mice, and tumor growth was measured over time. Immunodeficient BALB/c nude mice were used to investigate the role of T and B cells.

Results: In vitro, CT26-TGF-β-R and CT26-TGF-β cells showed increased and suppressed proliferation, respectively, compared to control (CT26-neo), confirming TGF-β has direct anti-tumor effects. In vivo, we found that CT26-TGF-β-R cells displayed slower growth compared to control, likely secondary to reduced suppression of anti-tumor immunity, as this effect was ablated in immunodeficient BALB/c nude mice. However, CT26-TGF-β cells (excess TGF-β) exhibited rapid early growth compared to control, but later failed to progress. The same pattern was shown in immunodeficient BALB/c nude mice, suggesting the effect on tumor growth is direct, with minimal immune system involvement. There was minimal effect on systemic antitumor immunity as determined by peripheral antigen-specific splenocyte type 1 cytokine production and tumor growth rate of CT26-neo on the contralateral flank of the same mice.

Conclusion: Although TGF-β has opposing effects on tumor growth, this study showed that excessive td-TGF-β in the tumor microenvironment renders the tumor non-proliferative. Depleting excess td-TGF-β may release this endogenous tumor suppressive mechanism, thus triggering the progression of the tumor. Therefore, our findings support cautions against using anti-TGF-β strategies in treating cancer, as this may tip the balance of anti-immunity vs. anti-tumor effects of TGF-β, leading to tumor progression instead of remission.

1. Introduction

The mortality of advanced stage colon cancer remains high despite advances in chemotherapy. Clinical outcomes of patients with colon cancer depend on the balance of tumor proliferation and antitumor immunity; dominance of proliferation will lead to tumor progression, while dominance of anti-tumor immunity will result in tumor regression. Transforming growth factor-β (TGF-β) is produced by the majority of solid tumors, including colon adenocarcinoma, and has potent autocrine anti-proliferative and paracrine immunosuppressive effects. Thus, it plays a complex dual role in tumorigenesis, with both pro- and anti- tumor effects.

Studies on many epithelial cancers, including colorectal, breast, prostate, pancreatic, lung, skin, and ovarian carcinomas, have clearly shown that TGF-β has both tumor suppressor and tumor promoter effects [1]. The current paradigm is that td-TGF-β suppresses host innate

Abbreviations: td-TGF-β, tumor-derived TGF-β; EMT, epithelial-to-mesenchymal transformation

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and adaptive anti-tumor immunity [2,3], favoring tumor progression. TGF-β interferes with generation of tumor-specific cytotoxic T lymphocytes [2]. More specifically, TGF-β has been linked with CD4 + CD25+ Treg-mediated suppression of antigen-activated T cells [4]. Highly immunogenic tumor cells transfected with murine TGF-β cDNA did not stimulate CTL responses in vitro or in vivo, and thus evaded eradication in mice [5]. Through its modulation of the immune response, TGF-β facilitates tumor escape from immune surveillance. Thus, anti-TGF-β strategies are being developed to counter this effect [6]. However, various methods of TGF-β inhibition have shown mixed results (as reviewed by Neuzillet et al. [7]), suggesting that under certain pathological conditions, inhibition of TGF-β may promote tumor progression.

In addition, TGF-β has been shown to have a role in tumor promotion, with induction of epithelial-to-mesenchymal transformation (EMT) [8,9] as well as increased cell migration in vitro [10], which may cause increased tumor invasion and metastasis [11]. Overexpression of TGF-β in human cancers correlates with increased tumor angiogenesis, progression, metastases, and poor outcomes [12].

However, TGF-β is also known to act as a tumor suppressor via inhibition of cell growth and induction of apoptosis [13]. In one study, anti-TGF-β antibodies stimulated growth in human breast cancer cells [14]. In another study, over-expression of endogenous TGF-β in human malignant oral keratinocytes led to growth inhibition in vitro and in vivo; expression of a dominant-negative TGF-β type II receptor in these cells enhanced growth in vitro and diminished the tumor suppressor effect in vivo [15].

To further complicate matters, mutation of surface TGF-β receptors and signaling components within the TGF-β pathway, such as the SMAD family, aid further tumor progression by mitigating the autocrine antiproliferative effect of td-TGF-β [11]. Interestingly, in colon carcinomas, loss of the TGFBR2 receptor and therefore TGF-β signaling is associated with both suppressed growth of early stage tumors and dissemination of late stage tumors [16]. This mutation is associated with progression of late adenomas to colon carcinomas with MSI [17], but longer survival in patients with MSI colon carcinoma [18].

Many TGF-β studies to date have used exogenous TGF-β [19]; these observations may not be applicable to tumor-derived TGF-β, which may act locally but not systemically. We hypothesize that td-TGF-β in the local microenvironment has dual effects on both suppression and promotion of tumor proliferation in addition to inhibition of anti-tumor immunity. The balance of these effects may depend on the amount of TGF-β secreted by tumor cells, thus affecting the outcome of anti-TGF-β therapies. Td-TGF-β may play an important role in maintaining tumor remission by controlling tumor progression in vivo, and decreasing TGF-β in the tumor microenvironment could potentially result in tumor progression. Therefore, we hope to clarify the consequences of over- and under-expression of TGF-β by tumor cells.

This study aims to further define and differentiate between direct/local and immune effects of TGF-β by comparing its effects in vitro and in vivo. To test our hypothesis, CT26, a BALB/c colon adenocarcinoma cell line, was transformed into three different cell lines: CT26-neo, CT26-TGF-β, and CT26-TGF-β-R, which expressed baseline, 10× baseline, and significantly reduced biologically active amounts of TGF-β, respectively. This allowed us to create tumors that expressed different amounts of TGF-β. The direct effect of TGF-β on tumor cells was evaluated by measuring cell proliferation in each of the cell lines in vitro. To examine the role of TGF-β on immune response and tumor growth, we compared the action of TGF-β in tumors injected into syngeneic BALB/c mice, BALB/c nude mice, and allogeneic C57Bl/6 mice. We also examined whether effects of td-TGF-β are predominantly local or systemic.

Our results demonstrated that endogenous td-TGF-β aids in suppressing local anti-tumor immunity, resulting in tumor growth, whereas excess td-TGF-β initially results in more rapid tumor proliferation and later growth suppression, independent of anti-tumor immunity. This supports cautions against using anti-TGF-β therapies, especially in tumors producing excess td-TGF-β, as depleting excess TGF-β may tip the balance towards suppression of anti-tumor immunity, leading to tumor progression.

2. Materials and methods

2.1. Cell culture and media

All cell lines were maintained in complete medium (CM), which consisted of RPMI 1640 with 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/mL streptomycin. CT26, a murine colon carcinoma cell line [20] from a BALB/c mouse, was propagated in CM and incubated at 37 °C. Cells were trypsinized when they reached confluency and were resuspended at half the original concentration in fresh CM approximately every other day. Cells were seeded at 10^5 cells/mL CM for all in vitro studies. Cells were stored at −80 °C in FBS with 5% DMSO.

2.2. Generation of a TGF-β-expressing CT26 cell line

A CT26 cell line stably transfected to express a constitutively active form of TGF-β1 (CT26-TGF-β) was generated using a retrovirus that expressed TGF-β and a neomycin resistance gene, kindly provided by Dr. G. Nabel (Vaccine Research Center, National Institutes of Health, Bethesda, MD) (28). A control CT26 cell line (CT26-neo) was generated similarly using the retrovirus backbone containing the neomycin resistance gene alone. An adenovirus encoding human TGF-β receptor type II fused to the Fc region of human IgM was used to express a soluble form of the neutralizing TGF-β receptor (TGF-β-R), which has been shown to cross-react with mouse TGF-β [21]. We have previously demonstrated that the soluble TGF-β-R neutralizes TGF-β produced by tumor cells [22]. A CT26 cell line that hence produces below physiologic levels of TGF-β (CT26-TGF-β-R) was generated using a retrovirus that expressed TGF-β-R and the neomycin resistance gene. The three CT26 cell lines were grown in a 12-well plates in 1 mL of CM per well. Cells were counted daily using a hemocytometer for 4 days.

2.3. TGF-β and TGF-β-R production by CT26 tumor cell lines

Tumor cell supernatant was collected and stored immediately at −20 °C until further use. Bioactive TGF-β secretion was determined by ELISA (R & D Systems). Supernatant was acidified according to the manufacturer's instructions: to activate TGF-β, 20 uL 1N HCl was added per 100 uL of sample, the samples were incubated for 10 min at room temperature, and then 20 uL 1.2N NaOH/0.5 M HEPES per 100 uL of sample were added to neutralize the pH.

To confirm the ability of TGF-β-R to capture TGF-β, we designed an in vitro assay that borrowed reagents from a TGF-β ELISA kit (R & D Systems). This modified ELISA estimates the TGF-β-R concentration by measuring the amount of TGF-β bound to TGF-β-R, and has previously been described in further detail [22].

2.4. Animal studies

Mice were housed at the Animal Mainte nance Facility at the University of Michigan Health System (Ann Arbor, MI). All animal experiments were reviewed and approved by the University Committee on Use and Care of Animals at the University of Michigan. All 3 CT26 cell lines were implanted into syngeneic BALB/c mice to examine the in vitro effects of endogenous td-TGF-β on tumor growth. A similar study was performed in BALB/c nude mice to isolate T and B cell-independent effects in vivo. To understand whether the effect of endogenous td-TGF-β is predominantly local or systemic, a dual tumor study was performed where control CT26 tumor cells were implanted into the mice in which each of the three CT26 cell lines had been injected into the contralateral