Suppression of Tumor Metastasis by Blockade of Transforming Growth Factor β Signaling in Bone Marrow Cells through a Retroviral-mediated Gene Therapy in Mice


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Abstract

Transforming growth factor β (TGF-β) is a potent immunosuppressive cytokine that is frequently associated with mechanisms of tumor escape from immunosurveillance. We report that transplantation of murine bone marrow (BM) expressing a dominant-negative TGF-β type II receptor (TβRIIDN) leads to the generation of mature leukocytes capable of a potent antitumor response in vivo. Hematopoietic precursors in murine BM from donor mice were rendered insensitive to TGF-β via retroviral expression of the TβRIIDN construct and were transplanted in C57BL/6 mice before tumor challenge. After i.v. administration of 5 × 10^6 B16-F10 murine melanoma cells into TβRIIDN-BM transplanted recipients, survival of challenged mice at 45 days was 70% (7 of 10) versus 0% (0 of 10) for vector-control treated mice, and surviving TβRIIDN-BM mice showed a virtual absence of metastatic lesions in the lung. We also investigated the utility of the TGF-β-targeted approach in a mouse metastatic model of prostate cancer, TRAMP-C2. Treatment of male C57BL/6 mice with TβRIIDN-BM resulted in the survival of 80% (4 of 5) of recipients versus 0% (0 of 5) in green fluorescent protein-BM recipients or wild-type controls. Cytolytic T-cell assays indicate that a specific T-cell response against B16-F10 cells was generated in the TβRIIDN-BM-treated mice, suggesting that a gene therapy approach to inducing TGF-β insensitivity in transplanted BM cells may be a potent anticancer therapy.

Introduction

Tumor immunotherapies to date have focused largely on the priming of immune responses to fight cancer, with mixed results and generally poor efficacy. In addition to immune stimulation, the issue of overcoming active immune suppression must also be considered when developing an immune-based strategy for cancer therapy (1, 2), particularly with regard to secreted soluble factors that are known to down-regulate immune function and antitumor response. Most significant of these is the pleiotropic cytokine TGF-β. TGF-β signaling in hematopoietic stem cells in the BM, TRAMP-C2. Treatment of male C57BL/6 mice with TβRIIDN-BM resulted in the survival of 80% (4 of 5) of recipients versus 0% (0 of 5) in green fluorescent protein-BM recipients or wild-type controls. Cytolytic T-cell assays indicate that a specific T-cell response against B16-F10 cells was generated in the TβRIIDN-BM-treated mice, suggesting that a gene therapy approach to inducing TGF-β insensitivity in transplanted BM cells may be a potent anticancer therapy.

Materials and Methods

Mice. Male C57BL/6 mice, 6–8 weeks of age, were obtained from Jackson Labs (Bar Harbor, ME) and maintained in pathogen-free facilities at the Center for Comparative Medicine at Northwestern University Feinberg School of Medicine in accordance with established guidelines. Male C57BL/6 mice were used for all experiments. A total of 10^6 cells in collagen-I-coated T-25 flasks (BD Biosciences, San Diego, CA) were seeded at a density of 2.5 × 10^5 cells in collagen-I-coated T-25 flasks (BD Biosciences, San Diego, CA) 24 h before plasmid transfection in antibiotic-free DMEM-10, such that the cells were ~70–90% confluent at the time of transfection.
transfection, at which point the cells were rinsed with PBS to remove residual serum. A mixture of 2 μg of retroviral plasmid and 2 μg of VSV-G envelope plasmid were cotransfected in serum-free DMEM using LipofectAMINE-Plus (Invitrogen, Gaithersburg, MD) according to the manufacturer’s protocols with the following modifications. Cells were transfected for 12 h followed by the addition of an equivalent volume of DMEM-20 and reincubation for an additional 12 h. After 24 h of total transfection time, the supernatant was aspirated, the cells were rinsed gently in PBS, and 3 ml of fresh DMEM-10 was added to each flask. After 24 h, virus-containing supernatant was collected and used to infect target cells.

Western Blotting for SMAD-2 Phosphorylation. The infected primary mouse BM cells were treated with or without 10 ng/ml TGF-β1 for 30 min in culture to test the functionality of the TGFβ signaling pathway (12). Proteins in the cell lysate were subjected to electrophoresis (Novex/10% acrylamide gel) and blotted onto a polyvinylidene difluoride membrane. Blots were probed using monoclonal antibody against phosphorylated SMAD-2. Blots were stripped and re probed with antibodies against SMAD-2 and then glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Retroviral Infection and Transplantation of Murine BM. Cultured murine BM cells were infected on days 2 and 3 postisolation via spin infection as follows: an aliquot of 1 ml of viral supernatant was added to each well of a 96-well tissue culture plate. The infected primary BM cells were challenged i.v. with 5 × 10^5 B16-F10 cells (n = 10 mice/group) or TRAMP-C2 cells (n = 5 animals/group) 2 months after transplantation. The B16-F10-challenged mice were monitored for morbidity and mortality for 6 weeks, and the TRAMP-C2-challenged mice were monitored for 8 weeks. At the conclusion of each experiment, all of the animals were inspected for the presence of metastases. Statistical analysis was conducted on a Kaplan-Meier survival curve, using the log-rank test (13).

Results

Functional Status of TGF-β Signaling in Transfected BM. Transfection efficiency into primary BM cells using the above approach was consistently greater than 90% as assayed by GFP expression (12). Results of the functional analysis of these transfected BM cells have been reported earlier (12). Briefly, when the TβRIIDN-BM control group was monitored for a total of 2 months, the lung tissue of untreated control mice indicated a significant tumor burden evident in metastases throughout the tissue. However, the TβRIIDN-BM-treated group had fewer metastatic lesions in the lungs of nonsurviving mice and virtually no discernable lesions in the lungs of mice surviving throughout the duration of the experiment. These results strongly suggest that mice transplanted with BM with targeted blockade of TGF-β signaling generate potent antitumor immunity in C57Bl/6 mice challenged with highly metastatic, nonimmunogenic tumor cells.

To determine the efficacy of the TβRIIDN-BM treatment on metastatic tumor formation in a model of prostate cancer, we subsequently challenged TβRIIDN-BM-treated male C57Bl/6 mice with i.v. administration of 5 × 10^5 TRAMP-C2 cells and monitored the mice similarly as described above. At 3 weeks postchallenge, macroscopic tumor formation was difficult to detect in either the treated or untreated controls, indicating that the TRAMP-C2 tumor cells were not as aggressive in their formation of metastatic lung foci as were the B16-F10 tumor cells. However, on further examination of histological specimens of mice sacrificed at 21 days post tumor challenge, microscopic metastatic lesions were already visible in the GFP group but not in the TβRIIDN group (data not shown). A second group of mice was tumor challenged and monitored for a period of 8 weeks, by which point the survival of the wild-type and GFP control mice was 0% (0 of 5, each group by week 7; Fig. 2A), whereas the survival of the TβRIIDN-BM treated cohort was 100% (5 of 5). By week 9, one animal in the TβRIIDN-BM group died, leaving the overall survival rate of 80% (4 of 5) for this group. Results of statistical analysis, using the log-rank test, indicated P < 0.05 between the TβRIIDN-BM and the other two control groups. Postmortem analysis of the untreated or vector-control-treated animals indicated a significant tumor burden evident in the lungs of each mouse (Fig. 2B), whereas the lungs of
TβRIIDN mice remained metastases free. From these data, we conclude that targeting immune TGF-β signaling with BM-directed retroviral therapy is an effective means of preventing metastatic prostate tumor growth in mice.

**TβRIIDN Mice Generate Specific Antitumor CTLs in Vivo.** To determine whether the antitumor response generated by transplant of TβRIIDN-BM is tumor-specific, we collected splenocytes from TβRIIDN-BM- and GFP-BM-tumor-challenged mice at 3 weeks post-tumor challenge and assayed the ability of CTLs to lyse B16-F10 cells *in vitro* using a standard 51Cr release assay. Results from the CTL assay indicated a significant increase in tumor-specific lysis of melanoma cells in splenocytes from TβRIIDN-BM-transplanted mice compared with GFP control-treated counterparts (Fig. 3A), suggesting that the antitumor phenotype in TGF-β signaling pathway-deficient mice is at least partially caused by CTL activity and not simply a result of broader, nonspecific immune stimulation of treated mice. Likewise, a 51Cr release assay performed on labeled TRAMP-C2 cells by splenocytes recovered from TβRIIDN-BM- and GFP-BM-transplanted mice (Fig. 3B) indicate that tumor-specific cytolysis is generated by the retroviral blockade of TGF-β signaling.

**Discussion**

Results of the present study demonstrate that disruption of the TGF-β signaling pathway in BM cells using a gene therapy approach confers an antitumor phenotype on treated mice. Targeting of TGF-β-mediated immunosuppression has been used previously to show that the blockade of normal TGF-β signaling pathways confers an antitumor effect in a variety of tumor models, either via modulation of tumor TGF-β production in a tumor vaccine approach or via the systemic down-regulation of available TGF-β cytokine in the serum, and has been used in a variety of tumor therapies to combat both primary and secondary tumor growth. *Ex vivo* transfer of an antisense TGF-β construct into isolated tumor cells followed by reimplantation into the brain of rats with established gliomas has been shown to result in complete eradication of the tumors *in vivo* (14), and a similar approach has been used successfully to confer immunogenicity to a prostate tumor model in the Dunning rat (15). Systemic administration of anti-TGF-β antibody and IL-2 shows a significant decrease in number and size of metastatic B16 tumor lesions (16), suggesting that TGF-β immunosuppression can be at least partially overcome by a general TGF-β signal blockade. This latter approach, including similar approaches such as soluble TGF-β type II receptor therapy (17), although providing a rationale for a TGF-β-targeted approach in cancer therapy, may be ultimately limited in its ability to mediate antitumor effects at sites in which the delivery of a soluble therapeutic agent may be insufficient to block TGF-β present at high concentrations in tumor microenvironments.

In the present study, we demonstrated the therapeutic efficacy of targeting progenitors of leukocyte populations in the BM with retroviral particles that specifically blocked TGF-β signaling by expressing a dominant negative TGF-β type II receptor with a truncated cytoplasmic domain. The lack of formation of metastatic lesions in TβRIIDN-BM-treated mice after i.v. administration with highly metastatic B16-F10 cells emphasizes the importance of the TGF-β signaling pathway to tumorigenicity *in vivo*, even in the case of tumor cells with aggressive growth properties and little natural immunogenicity. Likewise, the lack of metastatic lesion formation in TβRIIDN-

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**Fig. 2.** TβRIIDN-BM-treated mice showing antitumor capacity against TRAMP-C2 mouse prostate cancer tumor challenge. 5 × 10⁵ TRAMP-C2 prostate adenocarcinoma cells were injected via the tail vein into TβRIIDN-BM-treated mice, and the mice were monitored for morbidity and mortality. A, survival of wild-type (untreated), GFP, and TβRIIDN-transplanted mice post-tumor challenge (n = 5/group), expressed as the Kaplan-Meier curve. (P < 0.05 by the log-rank test for the TβRIIDN group versus the control or GFP group; Ref. 13). B, lung tissue from TβRIIDN-BM- and GFP-BM-treated mice at 6 weeks post-tumor challenge indicating metastatic tumor foci (arrows).

**Fig. 3.** Generation of tumor-specific killing in TβRIIDN-BM-transplanted mice. Splenocytes from tumor-challenged mice were collected and stimulated for 5 days with irradiated B16-F10 mouse melanoma cells (A) or with TRAMP-C2 mouse prostate carcinoma cells (B) before being cocultured with 51Cr-labeled targets at the indicated E:T ratios. Samples were analyzed in duplicate (A) or triplicate (B) wells.
BM-treated animals after a challenge with TRAMP-C2 cells, a murine model of prostate cancer, supports the idea that this antitumor approach is viable in a range of cancers of different tissue origins. The potency of TGF-β as an immunoregulatory cytokine that is critical for the maintenance of immune homeostasis also necessitates the careful application of perturbations in the TGF-β signaling processes for cancer immunotherapy. The potential for the generation of widespread autoimmunity and inflammation, which is generated in the processes for cancer immunotherapy. The potential for the generation of autoimmunity and inflammation, which is generated in the processes for cancer immunotherapy.

The potential for the generation of a lack of cytotoxic effector activity, particularly with regard to the careful application of perturbations in the TGF-β pathways in immune cells (12), makes it essential that the approach described here be maximized for its utility as an antitumor therapy but modified so as to minimize potential autoimmune side effects against host tissue. Mice that are deficient in TGF-β1 cytokine display a massive auto-inflammatory phenotype and quickly succumb to systemic damage in a variety of tissues (18, 19), whereas other transgenic models, restricted to TGF-β-signal abrogation in the immune compartment or single lineages including T (20) and B cells (21), similarly result in dysregulation of immune function. The retroviral approach to therapeutic gene delivery can be enhanced by vectors that offer a regulatory mechanism to control expression of the transgene and/or survival of transgene-positive cells, whether through the use of on/off systems responsive to pharmacological agents (e.g., tetracycline) or through the use of suicide gene elements present in the integrated viral genome.

We submit that the results presented here represent a viable approach to the problem of tumor escape from immune surveillance using readily available retroviral gene transfer technology, and we suggest that this approach could potentially be coupled with other immunostimulatory protocols that generate tumor-specific lymphocyte responses but that, to date, have had only mixed results because of a lack of cytotoxic effector activity, particularly with regard to distant metastatic tumor foci, as a result of TGF-β-mediated immunosuppression. The hematopoietic stem-cell gene therapy approach, already established as a viable means for the delivery of therapeutic genes to cells of the immune system, provides a legitimate and characterized target for TGF-β signaling-directed therapy for a potentially wide variety of cancers.

References


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