Flt3-Ligand Induces Transient Tumor Regression in an Ectopic Treatment Model of Major Histocompatibility Complex-negative Prostate Cancer

Richard P. Ciavarrà, Kenneth D. Somers, Roy R. Brown, William F. Glass, Patricia J. deAngelis Consolvo, George L. Wright, and Paul F. Schellhammer

Abstract

We assessed the in vivo efficacy of Flt3-ligand (Flt3-L) treatment in C57Bl/6 mice bearing a well-established MHC class I-negative prostate carcinoma TRAMP-C1. Flt3-L immunotherapy was initiated approximately 30 days after tumor inoculation, a time when ≥80% of the mice had palpable TRAMP-C1 tumors. Treatment with Flt3-L at 10 μg/day for 21 consecutive days suppressed TRAMP-C1 tumor growth and induced tumor stabilization (P = 0.0337). Enhanced tumor regression was demonstrated at a higher dose of 30 μg/day (P < 0.0001). Tumors excised from mice treated with Flt3-L were smaller than carrier-treated controls and contained a more pronounced mixed inflammatory cell infiltrate primarily composed of mφ. In regenerator mice, tumors reappeared at the site of injection when Flt3-L therapy was terminated. When the experiment was repeated with MHC class I-positive TRAMP-C1 cells, tumor stabilization and/or regression was again observed after treatment (P < 0.0001); however, once again, tumors reappeared after the termination of therapy despite an extended treatment schedule (35 days). MHC class I-negative variants were present in tumors isolated from carrier- and Flt3-L-treated mice, and this phenotype could be reversed by IFN-γ treatment in vitro. Thus, Flt3-L treatment of mice with preexisting transplantable prostate tumors results in tumor regression that is dose-dependent and accompanied by a pronounced mixed-cell inflammatory tumor infiltrate. However, disease relapse was invariably observed after the termination of therapy, which suggests that Flt3-L treatment of advanced MHC− prostate cancers will require adjuvant modalities to achieve a durable response.

Introduction

Prostate cancer is the most commonly diagnosed cancer in men. Approximately 179,000 new cases and over 37,000 deaths were estimated in the United States in 1999 (1). These statistics underscore the need to identify new targets and develop innovative approaches for treatment. Although prostate cancer responds to androgen deprivation initially, successful therapy is generally lacking when androgen-independence inevitably occurs. Immune-based strategies represent a promising approach for the treatment of both localized and metastatic prostate cancer. Tumor-associated peptide antigens have been identified that act as targets for CTLs. A number of peptide vaccines for generation of active immune responses to human cancers are being tested in animal models or are in Phase I clinical trials. A Phase II trial using autologous DCs pulsed with HLA-A2-restricted prostate-specific membrane antigen peptides reported several partial responses in patients with advanced prostate cancer (2). However, DC-based immunotherapeutic approaches are predicated on the expression of HLA class I molecules that present tumor antigens to effector T cells. A major limitation to successful immunotherapy is down-regulation of MHC class I molecules by prostate tumor cells and the resultant escape from T-cell recognition (3). The demonstration of complete loss of HLA class I expression in 34% of primary prostate cancers and 80% of lymph node metastases, in contrast to normal MHC class I expression in benign prostatic hyperplasia (4), raises potential obstacles to the efficacy of MHC class I-based immunotherapy and provides a rationale for focusing on strategies that circumvent the need for class I MHC-mediated recognition of prostate cancer cells by CTL.

Flt3-L is a recently described member of a small family of growth factors that stimulate the proliferation of hematopoietic stem cells (reviewed in Ref. 5). In vivo administration of Flt3-L dramatically increased: (a) the number of hematopoietic progenitors in a variety of organs (for example, bone marrow, peripheral blood, and spleen), which resulted in enhanced myelopoiesis and B lymphopoiesis; and (b) the number of functional DCs that accumulated in the spleen and lymphoid tissue (6, 7). Expansion of the resident DC population was not restricted to lymphoid tissue; enhanced numbers were also detected in organs such as lung, liver, and bone marrow. Because DC are considered professional antigen-presenting cells, Flt3-L treatment may represent a novel strategy to manipulate the immune system. This notion has been supported by recent studies that indicate that Flt3-L can augment tumor resistance in several mouse tumor models (8, 9). Analysis of the cellular mechanisms involved in tumor regression has suggested that Flt3-L treatment augmented specific antitumor immune responses. Thus, studies with murine tumor models suggest that even when the tumor is weakly immunogenic and grows progressively in its syngeneic host, prudent manipulation of the immune system can prevent the growth of an implanted tumor. It should be noted, however, that in these studies, immunotherapeutic intervention was not attempted in animals with preexisting disease, a more realistic clinical scenario.

Transgenic animal models of prostate cancer that reproduce the heterogeneity of human prostatic disease have recently been developed. In the TRAMP model developed by Greenberg et al. (10), the rat probasin promoter drives the expression of SV40 Tag that is restricted to the epithelial cells of the prostate gland of C57Bl/6 mice. TRAMP mice and prostate tumor-derived cell lines (TRAMP-C1) provide a useful model in which to evaluate innovative strategies designed to augment the host immune system and to foster a better understanding of the role of host immunity in tumor growth and metastasis (11). We have, therefore, used TRAMP-C1 cells to develop an ectopic treatment model to assess the efficacy of Flt3-L immunotherapy in the treatment of an established, highly invasive, MHC− prostate tumor.

Materials and Methods

Cell Line and Cell Culture. TRAMP-C1 cells were established from a prostate tumor from a TRAMP mouse and were kindly provided by Norman Greenberg (Baylor College of Medicine, Houston, TX). TRAMP-C1 are...
tumorigenic in syngeneic C57Bl/6 (B6) recipients, express cytokeratin, E-cadherin, and androgen receptor but do not express SV40 Tag (11). TRAMP-C1 cells were cultured in DMEM supplemented with 5% fetal bovine serum, 5% NuSera (Collaborative Biomedical), 5 μg/ml insulin, and 0.01 nm dihydrotestosterone and were used at passages 8 and 16.

**Animal Treatment Model.** To determine whether Flt3-L therapy can induce regression of a preexisting prostate cancer, B6 male mice were injected s.c. or i.d. with 5 × 10⁶ syngeneic TRAMP-C1 cells, and Flt3-L immunotherapy was initiated when the majority (=80%) of mice had developed palpable tumors at the inoculation site (approximately 30–35 days after tumor inoculation). Treatment consisted of 21 consecutive i.p. injections of either carrier (0.1% normal mouse serum in PBS) or Flt3-L, a kind gift from Immunex Corporation (Seattle, WA). Tumor volumes were obtained two times a week by the measurement of bisecting tumor diameters during the treatment period. When the experiment was terminated, tumor volumes were determined directly on the excised tumors, and the data were analyzed by factorial repeated measures ANOVA to determine the efficacy of the therapy against prostate cancer. A subset of mice was also treated with either carrier or Flt3-L but did not receive TRAMP-C1 cells. After 10 days of treatment, the number of splenic DCs (CD11c⁺ I-Aãβ⁺) was determined by two color flow cytometry procedures to verify the biological activity of the Flt3-L.

**Flow Cytometry.** To monitor the expression of class I and II antigens on TRAMP-C1 cells after growth in vivo, TRAMP-C1 tumors were minced with scalpels, dissociated with a digestion cocktail (12) with continuous stirring for 30 min at 37°C (12), and then washed extensively before flow cytometric analysis. MHC antigens were detected using biotin-conjugated mAbs to MHC class I (Kb, Db) and class II (I-Aß) antigens and FITC-conjugated streptavidin. All of the antibodies were obtained from PharMingen (San Diego, CA) and were used according to the manufacturer’s instructions. Up-regulation of class I molecules was achieved by incubating TRAMP-C1 cells with 10 units/ml IFN-γ (PeproTech, Rocky Hill, NJ) for 48 h prior to analysis by flow cytometry.

**Histological and Immunohistochemical Analyses.** For histological analysis, TRAMP-C1 tumors, were fixed in 10% neutral buffered formalin, embedded in Paraplast, and then stained with H&E. To characterize lymphocyte-infiltrating TRAMP-C1 tumors, frozen tumor sections (8 um) were fixed with acetone (2 min), air-dried, and incubated with either normal rat IgG (Rockland, Gilbertsville, PA) or 4-β, a rat antimonos IgM, kindly provided by Dr. Paul Crocker (University of Oxford, Oxford, United Kingdom; Ref. 13). Sections were then incubated with a biotin-conjugated antirat IgG (Vector Laboratories, CA), and bound complexes were detected with a Vectastain Elite ABC (peroxidase) kit using 3,3′-diaminobenzidine as a substrate (Vector Laboratories) following the manufacturer’s instructions. Images were captured with a SV Micro digital camera and were assembled with Adobe Photoshop and PowerPoint software.

**Results**

Fig. 1A demonstrates that TRAMP-C1 tumors were not detected until approximately 1 month after transplantation. However, once palpable, the tumors grew rapidly in control mice that were given carrier alone. In contrast, the growth rate of TRAMP-C1 was suppressed in mice that were given Flt3-L immunotherapy. Although higher doses (≥10 μg) of Flt3-L do not increase DC numbers in lymphoid organs, regression or growth inhibition of a fibrosarcoma was reported to be dramatically augmented in mice treated daily with 30 μg Flt3-L (9). To assess the efficacy of this dose, we followed the schedule described above with the exception that the tumor was transplanted i.d. This site was chosen to facilitate tumor detection so that immunotherapy could be initiated earlier in the disease. However, tumor inoculation in the i.d. site did not facilitate earlier detection because palpable tumors were still not detected until approximately one month after transplantation and, once detected, grew rapidly in mice treated with carrier (Fig. 1B). In contrast, TRAMP-C1 tumors in mice treated with Flt3-L grew initially but then manifested a dramatic growth arrest and subsequent shrinkage (Fig. 1B). In some animals, tumors were no longer palpable at the inoculation site. Histological analysis (Fig. 2) of TRAMP-C1 tumors excised from carrier-treated mice revealed highly undifferentiated sheets of cells that regularly invaded surrounding tissue such as fat and muscle (Fig. 2, A–C). In some animals, the tumor had spread extensively into the leg, eroded bone (femur), and invaded marrow. Two animals died before immunotherapy was completed with evidence of urinary obstruction and hydronephrosis.

Marked tumor growth inhibition was achieved although TRAMP-C1 tumors expressed little class I (H-2Kb, H-2Db) and no class II (I-Aãβ), as assessed by flow cytometry at the time of inoculation (data not shown). Although it was difficult to palpate tumors in the Flt3-L–treated animals, intact tumor cells could still be detected by histopathology at the inoculation site (Fig. 2D). Most of the animals in these studies were necropsied after immunotherapy so that histological and immunohistochemical studies could be performed on the spleen and tumor. Thus, for most of these animals, we do not know whether regressor mice were cured or whether tumor progression would have ensued after termination of Flt3-L treatment. However, three mice in each group in the 30 μg/dose experiment were maintained for long-term study. All of the three Flt3-L–treated mice had disease recurrence about 2 weeks after therapy was terminated, which suggests that a sufficient memory response did not develop in these mice. Furthermore, reinstitution of Flt3-L immunotherapy failed to induce tumor regression (data not shown).

To gain insight into what cell types may have contributed to tumor regression, animals were necropsied immediately after 21 days of continuous Flt3-L therapy and tumors were excised for histopathological and immunohistochemical investigation. Histological analysis revealed a peripheral, mixed inflammatory cell infiltrate with evidence of scar tissue (not shown) at this location in tumors excised from mice treated with Flt3-L (Fig. 2, D–F). Immunohistochemical evaluation of tumors obtained from mice after 21 daily injections of Flt3-L failed to reveal the presence of significant numbers of infiltrating T and B cells as well as DCs (data not shown). Lymphoid cells expressing perforin were also not detected in TRAMP-C1 tumors, which indicated that the tumor was not significantly infiltrated with cytotoxic (natural killer cells, CTLs) effectors cells (data not shown). However, a pronounced infiltrate of monocytes/macrophages (Mac-1⁺, F4/80⁺, CD11b⁺) was present in TRAMP-C1 tumors that were obtained from mice treated with either carrier or Flt3-L (Fig. 2, G–I). These data suggest that TRAMP-C1 cells elicit a robust innate (inflammatory) response that may become tumoricidal/cytostatic in the presence of Flt3-L.

It could be argued that the absence of class I on TRAMP-C1 cells precluded the development of tumor-specific immunity and immuno-
logical memory. This may explain why tumor progression ensued after termination of Flt3-L therapy. We, therefore, repeated the experiment with an early passage (passage 8) of TRAMP-C1 cells that expressed class I antigens at the time of inoculation. We also extended the treatment schedule to 35 days to determine whether more prolonged treatment with Flt3-L would prevent tumor recurrence once therapy was terminated. As shown in Fig. 3, Flt3-L treatment again resulted in a dramatic growth inhibition (P = 0.0001) of established TRAMP-C1 tumors. However, despite the presence of class I antigens on the initial transplanted tumor cells (Fig. 4A), termination of Flt3-L therapy was associated with the recurrence of TRAMP-C1 tumors in tumor regressor mice. Flow cytometric analysis of TRAMP-C1 cells recovered from mice that were given either carrier or Flt3-L indicated that most of the cells now did not express either H-2Kb or H-2Db class I antigens (Fig. 4A). However, the loss of class I expression was reversible; class I− TRAMP-C1 cells rapidly up-regulated class I in the presence of IFN-γ (10 units/ml for 24 h) in vitro (Fig. 4B).

**Discussion**

We have documented a dramatic dose-dependent growth inhibition of an established prostate tumor line in syngeneic male mice at two ectopic sites (s.c. and i.d.) in response to Flt3-L therapy. This was achieved despite the fact the tumor was well established, devoid of MHC class I antigens, and highly malignant, rapidly invading surrounding muscle and bone. Moreover, Flt3-L induced tumor regression in animals bearing MHC class I− and class I− tumors, which suggested that this therapy also induced a potent inflammatory response that, heretofore, has not been appreciated. This is an important
either carrier (E) or experiment has been repeated two times with similar results. Recombinant murine IFN-γ therapy were highly significant by ANOVA analysis (differences in tumor growth between these two groups immediately after termination of the arrow), and tumor cell growth was monitored in the remaining animals. The differences in tumor growth between these two groups immediately after termination of the therapy were highly significant by ANOVA analysis (P = 0.0001).

The absence of infiltrating lymphoid elements (CD4+; CD8+; B220+) as well as cytotoxic effector cells in either carrier- or Flt3-L-treated mice suggests that TRAMP-C1 tumors elicited a weak T-cell-mediated antitumor immune response, a phenomenon that may be related to the paucity of MHC class I antigens on TRAMP-C1. The failure to develop sufficient T-cell memory may explain why long-term cures were not achieved when Flt3-L immunotherapy was terminated. Alternatively, Flt3-L may have induced a lymphocytic infiltrate before our immunohistochemical analysis. This latter hypothesis is currently being tested by phenotyping tumor infiltrating lymphocytes at earlier time points during Flt3-L immunotherapy. Preliminary histological analysis of digested TRAMP-C1 tumors suggest that tumors that were isolated from mice treated with Flt3-L but not with vehicle contained a marked mononuclear infiltrate 7 and 14 days after the initiation of therapy. Flow cytometric analysis indicated that this population contained T (primarily CD8+) and B cells (B220+) and a predominant population that strongly stained with a cocktail of anti-murine mAb (data not shown). Consistent with immunohistochemical studies, TRAMP-C1 tumors from carrier-treated mice also contained a prominent population that stained with anti-mAb. On the basis of these initial studies, we propose that Flt3-L induces both an inflammatory as well as a tumor-specific immune response.

The effector mechanism(s) elicited by Flt3-L therapy, which is responsible for tumor regression/stabilization, remains to be identified. If TRAMP-C1 cells display sufficient tumor antigen to induce a tumor-specific immune response, CTL recognition of tumor antigens may be compromised at the effector-cell stage by the progressive loss of MHC as the tumor progresses to a more neoplastic phenotype. Furthermore, TRAMP-C1 tumor cells seem to be relatively resistant to the cytolytic activity of CTL and natural killer cells. For example, alloreactive (anti-H-2b) CTls failed to kill TRAMP-C1 target cells in a standard 4-h isotope release assay. However, activated peritoneal exudate mAb and alloreactive CTL can induce apoptotic cell death in TRAMP-C1 targets after an overnight incubation. Studies to assess whether mAb- and CTL-infiltrating TRAMP-C1 tumors possess similar activities are currently being investigated.

In summary, we have documented that Flt3-L immunotherapy can induce transient tumor regression of an advanced, MHC prostate cancer implanted at two ectopic sites. Unfortunately, this therapy alone did not induce long-term immunity because disease recurrence ensued after the termination of treatment. Thus, combination therapy may be required to drive clonal expansion/differentiation of tumor-specific T cells and the development of a memory response. Combination immunotherapy is currently being evaluated in this prostate tumor model.

References


Unpublished observations.
Flt3-Ligand Induces Transient Tumor Regression in an Ectopic Treatment Model of Major Histocompatibility Complex-negative Prostate Cancer

Richard P. Ciavarra, Kenneth D. Somers, Roy R. Brown, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/60/8/2081

Cited articles
This article cites 13 articles, 7 of which you can access for free at:
http://cancerres.aacrjournals.org/content/60/8/2081.full.html#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/60/8/2081.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.