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

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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 nKp. In regressor 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 have recently been developed. 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 cytoketerin, 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% NuSerum (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 carcinoma, B6 male mice were injected s.c. or i.d. with 5 × 10^6 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 Immunix Corporation (Seattle, WA). Tumor volumes were obtained two times a week by the measurement of bisecting tumor diameters. When the experiments were terminated, tumor volumes were determined directly on the excised tumors, and the data were analyzed by factorial repeated measures ANOVA. The biological activity of the Flt3-L was confirmed by flow cytometric 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 (K^b, D^b) and class II (I-A^b) 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, which 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 Ser-4, a rat antimouse mAb (University of Oxford, Oxford, United Kingdom; Ref. 13). Sections were then incubated with a biotin-conjugated antirat IgG (Vector Laboratories, 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.

**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 (12). Inhibition of a fibrosarcoma was reported to be dramatically augmented in mice treated daily with 30 µg (12).

Tumor volumes were obtained twice a week by measuring bisecting diameters. Data in this and subsequent experiments are expressed as the mean ± SD in mice treated with either carrier or Flt3-L. There was a significant difference in tumor growth between carrier- and Flt3-L-treated groups by ANOVA analysis (P < 0.0037). B, mice were inoculated i.d. in the ventral chest wall with 5 × 10^6 TRAMP-C1 cells. A similar treatment schedule was followed as described above with the exception that mice were treated with either 0.1% NMS (C) or a high dose (30 µg/injection) of Flt3-L (○). Control mice received 21 daily injections of 0.3 ml of carrier i.p.; experimental animals were given 21 daily injections i.p. of Flt3-L at 10 µg/injection. Tumor volumes were obtained twice a week by measuring bisecting diameters. Data in this and subsequent experiments are expressed as the mean ± SD in mice treated with either 0.1% NMS (C) or a high dose (30 µg/injection) of Flt3-L (○). Once TRAMP-C1 tumors became palpable, mice were necropsied at the end of therapy for histopathology and final measurements of the excised tumors. The differences in tumor growth between these two groups were highly significant by ANOVA analysis (P = 0.0001).

Marked tumor growth inhibition was achieved although TRAMP-C1 tumors expressed little class I (H-2K^b, H-2D^b) and no class II (I-A^b), 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 regressive 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, reinitiation 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) effector cells (data not shown). However, a pronounced infiltrate of monocytes/macrophages (Ser-4) 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. 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 observation that needs to be explored further.

**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

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**Fig. 2.** Histopathology of TRAMP-C1 carcinoma in mice treated with either carrier or Flt3-L. A–C, the histopathology of TRAMP-C1 prostate carcinoma implanted i.d. over the chest wall of mice treated with vehicle. The tumors implanted in control animals were composed of spindle cells that infiltrated diffusely throughout skeletal muscle without visible inflammatory changes. At high magnification (C), occasional tumor cells could be observed invading myocytes (arrow). A, $\times 27$; B, $\times 66$; C, $\times 133$. D–F, the histopathological appearance of TRAMP-C1 carcinoma implanted i.d. over the chest wall in mice treated with Flt3-L. Tumors in these animals were smaller (D) and often associated with scar tissue containing calcified debris (arrow). At the interface between the tumors and adjacent tissues such as skeletal muscle (E, F), there was occasionally a prominent round-cell infiltrate. The infiltrate had a band-like distribution around invaded skeletal muscle bundles. Whether these are tumor cells or inflammatory cells is not known. D, $\times 27$; E, $\times 66$; F, $\times 133$. G–I, Immunohistochemical staining of TRAMP-C1 tumors after 21 days of therapy. TRAMP-C1 tumors excised from either carrier- (A and B) or Flt3-L-treated mice (C) were stained with either normal rat IgG (A) or a rat antimouse mAb (B and C). For this study, mAbs were detected with Ser-4 mAb, but similar staining patterns were observed with other mAb-specific mAbs (data not shown). Control rat IgG caused no staining (G), but Ser-4 mAb stained abundant cells within both the vehicle- and Flt3-L-treated tumors. Tumors from carrier-treated mice contained occasional nonstaining foci (H, arrow). $\times 66$. 2083
either carrier (\textit{A}) or recombinant murine IFN-\textalpha (\textit{B}) with tumor peptides or tumor-derived RNA, tumor progression most likely will ensue because class I anti-
gens are immunologically silent and avoid destruction by antitumor T cells.

The absence of infiltrating lymphoid elements (CD4\textsuperscript{+}, CD8\textsuperscript{+}, B220\textsuperscript{+}) 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 ter-
mminated. 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\textsuperscript{+}) and B cells (B220\textsuperscript{+}) and a predominant population that strongly stained with a cocktail of anti-
mouse m\textPhi mAb (data not shown). Consistent with immunohisto-
chemical studies, TRAMP-C1 tumors from carrier-treated mice also contained a prominent population that stained with anti-m\textPhi 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 respon-
sible 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-2\textsuperscript{d}) CTLs failed to kill TRAMP-C1 target cells in a standard 4-h isotope release assay.\textsuperscript{4} This resistance seems to be unrelated to the quantity of alloantigen expressed on the surface of TRAMP-C1 cells because up-regulation of class I antigens by IFN-\gamma did not render TRAMP-C1 target cells susceptible to alloreactive CTL.\textsuperscript{2} However, activated peritoneal exudate m\textPhi and alloreactive CTL can induce apoptotic cell death in TRAMP-C1 targets after an overnight incubation.\textsuperscript{4} Studies to assess whether m\textPhi 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\textsuperscript{\textast} 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

4. Unpublished observations.
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