SU5416 Selectively Impairs Angiogenesis to Induce Prostate Cancer-specific Apoptosis

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Abstract

We have previously demonstrated the differential expression in tumor-associated blood vessels of two vascular endothelial growth factor receptors (VEGFRs), VEGFR1 and VEGFR2, during initiation and progression of prostate cancer in the genetically engineered transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse model. In our “progression switch” model, expression of VEGFR1 is associated with early and more differentiated disease, whereas expression of VEGFR2 is associated with advanced and more poorly differentiated disease. To test the hypothesis that stage-specific inhibition of vascular endothelial growth factor signaling could be used as therapy for autologous prostate cancer, we initiated a preclinical trial with SU5416, a potent antiangiogenic small molecule inhibitor of VEGFR associated tyrosine kinase activity. In our early intervention trial, administration of SU5416 to TRAMP mice did not appear to influence angiogenesis or tumor progression between 10 and 16 weeks of age, a time corresponding to high levels of VEGFR1 expression. In our late intervention trial, however, we observed a significant decrease in tumor-associated mean vessel density, increased apoptotic index, and pronounced regions of cell death when SU5416 was administered to TRAMP mice between 16 and 22 weeks of age, a time corresponding to high levels of VEGFR2 expression. These results clearly demonstrate that therapy directed specifically against the VEGFR signaling axis can dramatically impair angiogenesis and induce apoptosis of autologous spontaneous and progressive prostate cancer.

Introduction

Angiogenesis is a requisite event for the growth of solid tumors and metastasis (1, 2), and previous studies on prostate cancers have demonstrated a correlation between microvessel density, pathological stage, and Gleason score (3, 4). In addition, expression of the proangiogenic ligand VEGF has been demonstrated in cells representing normal, benign, and malignant prostate phenotypes (5, 6). Rapid development and evaluation of therapies designed to inhibit the angiogenic axis would certainly facilitate translational research. Unfortunately, it has been difficult to study the dynamic process of angiogenesis during the natural history of most clinical cancers, including prostate cancer. Therefore, we have chosen the autologous TRAMP model for our preclinical studies.

The TRAMP model was developed by placing expression of the SV40 early genes (Tag) under the temporally and spatially restricted prostate-specific control of the minimal rat probasin promoter (7). Prostate cancer in the TRAMP mice closely mimics the natural history of clinical disease, and the mice reproducibly develop spontaneous, progressive, metastatic, and hormone-refractory disease. As early as 6–12 weeks of age, the prostate glands from TRAMP mice can display mild to severe hyperplasia of the prostate epithelium, resembling PIN (7). By 30 weeks of age, 100% of TRAMP mice will display primary and metastatic prostate cancer. Using this model, we previously demonstrated an angiogenic “initiation switch” corresponding to the recruitment of vasculature into emerging PIN lesions. It was interesting to note that this early switch was concurrent not only with the expression of VEGFR1 but also with the expression of hypoxia-inducible factor-α in tumor tissue (8). Furthermore, we demonstrated evidence for a late angiogenic “progression switch” defined by decreased expression of VEGFR1 and increased expression of VEGFR2 and VEGF ligand concomitant with the transition of differentiated adenocarcinomas to higher-grade, poorly differentiated cancers. The observations in TRAMP mice were predictive and consistent with data obtained from representative clinical samples (8).

Because the VEGF axis is clearly important for vasculogenesis and angiogenesis (9–12), and we have been able to study the process of angiogenesis at the molecular level in a transgenic model, we undertook a study of the ability of a
small molecule inhibitor of VEGFR tyrosine kinase activity to inhibit angiogenesis and the progression of spontaneous autochthonous prostate cancer in a preclinical setting. We chose SU5416 because this compound had been shown to inhibit ligand-dependent phosphorylation of the VEGFR tyrosine kinase domain in NIH 3T3 cells, human umbilical vascular endothelial cell migration, and the growth of a number of tumor lines in xenograft and transplantation settings (13). Furthermore, SU5416 had been shown to inhibit angiogenesis, metastasis, and tumor proliferation as well as increase tumor and endothelial cell apoptosis in a mouse model of colon cancer metastasis (14).

In the current study, we randomly assigned [C57BL/6 TRAMP × FVB]F1 mice to one of three trials. In the early intervention trial, the mice received SU5416 for 6 weeks beginning at 10 weeks of age. At this time, the tumor-associated vasculature expresses predominantly VEGFR1, and the mice display predominately PIN lesions and well-differentiated adenocarcinoma. At the end of the trial, the tumors in the mice receiving SU5416 were not found to be significantly different than the tumors in the mice that received vehicle alone. In the late intervention trial, mice received SU5416 for 6 weeks beginning at 16 weeks of age. At this time, significant levels of VEGF have been found in the serum and tumor samples, the tumor-associated vasculature expresses predominantly VEGFR2, and the mice displayed mostly advanced disease. In contrast to the early intervention study, the consequence of SU5416 therapy in the late intervention setting was a significant reduction in tumor-associated IMVD. Furthermore, in those mice receiving SU5416, there was a trend toward lower-grade tumors with a significant increase in tumor apoptotic index. To determine the consequence of SU5416 therapy on advanced prostate cancer, we also performed a regression trial where mice received SU5416 or vehicle alone, beginning at the time they first developed a palpable abdominal mass. However, the mice receiving SU5416 therapy in the regression trial did not demonstrate an increase in survival time or a reduction in metastatic burden or tumor grade when compared with mice that received vehicle alone. Taken together, our data clearly demonstrate that inhibition of VEGFR-associated signaling by the small molecule inhibitor SU5416 could specifically and dramatically influence the natural history of prostate cancer, but only when administered within a specific window of opportunity corresponding to the expression of VEGFR2 in the tumors of the progressive autochthonous TRAMP model.

Materials and Methods

Transgenic Mice. TRAMP mice heterozygous for the PB-Tag transgene were maintained in a C57BL/6 background (Harlan Sprague Dawley, Inc., Indianapolis IN; Ref. 7) and crossed with nontransgenic FVB mice to obtain both transgenic and nontransgenic [C57BL/6 × FVB] F1 males. Isolation of tail DNA and PCR screening were performed as described previously (7). Approximately half of each prostate specimen was used for histological analysis and subsequent pathological grading according to a previously described scheme (15), as recently modified (16). Remaining tissues were stored at −80°C and used for protein analysis. Mice were monitored daily for signs of distress or morbidity. All experiments were conducted using the highest standards for humane care in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Treatment with SU5416. TRAMP mice were randomized into six cohorts as shown in Table 1. Where appropriate, animals received 50 mg/kg SU5416 in DMSO i.p. twice a week. Control littermates received vehicle alone. Mice in cohort A received vehicle from 10 weeks to 16 weeks of age; mice in cohort B received SU5416 from 10 weeks to 16 weeks of age; mice in cohort C received vehicle from 16 weeks to 22 weeks of age; and mice in cohort D received SU5416 from 16 weeks to 22 weeks of age. Mice in cohorts E and F were treated after palpation of tumor by two investigators, one of whom was blind to cohort. The mice in cohorts D or E received SU5416 or DMSO, respectively, twice a week until they were sacrificed. All mice were sacrificed 3 days after the last dose.

Immunohistochemistry. Immunohistochemistry was performed as described previously (8). Where appropriate,
slides were incubated with a rat monoclonal antibody specific for CD31/PECAM-1 (PharMingen, San Diego, CA) at a 1:50 dilution overnight at 4°C or a rabbit polyclonal antibody specific for Ki67 (Novocastra Laboratories) at a 1:100 dilution for 1 h at room temperature. All slides were subsequently washed several times in PBS and incubated with a 1:100 dilution of biotin-conjugated goat antirabbit IgG (PharMingen) or a 1:2000 dilution of biotin-conjugated goat antirabbit IgG (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. TUNEL staining was performed according to the manufacturer’s protocol for fixed tissue (Roche).

IMVD was determined as described previously (8). Briefly, the number of intraductual vessels was determined by counting three high-power (×200) fields of the highest vascular density. To determine proliferation or apoptosis index, the number of Ki67- or TUNEL-stained cells, respectively, was counted in representative ×200 fields. Statistical analysis was performed by nonparametric ANOVA multiple comparisons using Fisher’s least significant difference.

**Western Blot Analysis.** Blots were prepared, and immunoblot analysis performed as described previously (8). Briefly, total cell lysates were prepared by tissue homogenization in radioimmunoprecipitation assay buffer. Approximately 40 μg of protein from each tumor sample were denatured in loading buffer by boiling for 10 min and loaded onto a 7.5% or 12% SDS-polyacrylamide gel. When appropriate, blots were incubated overnight at 4°C in 3% nonfat dry milk in Tris-buffered saline Tween (8) with a rabbit polyclonal antibody specific for VEGF (Ab-1; 1:400 dilution; NeoMarkers, Fremont, CA), a rabbit polyclonal antibody specific for VEGFR1/Flt-1 (C-17; 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), a rabbit polyclonal antibody specific for VEGFR2/Flk-1 (N-931; 1:1000 dilution; Santa Cruz Biotechnology), or a monoclonal antibody specific for β-actin (AC-74; 1:5000 dilution; Sigma). After several washes, filters were incubated with either horseradish peroxidase-conjugated antirabbit or antimouse IgG antibody (Amersham, Piscataway, NJ) diluted 1:5000 for 1 h at room temperature in 3% nonfat dry milk in Tris-buffered saline Tween (8). After several washes, the filters were developed with enhanced chemiluminescence detection system (Pierce, Rockford, IL) according to the manufacturer’s recommended protocol and exposed to X-ray film (XAR-1; Eastman Kodak, Rochester, NY).

**Results**

During our studies of the molecular mechanisms involved in angiogenesis during the initiation of progression of prostate cancer, we have determined that the angiogenic switch could be resolved to distinct early “initiation” and late “progression” events (8). Furthermore, we established that expression of individual components of the angiogenic switch was a function of tumor progression in both TRAMP and clinical cancer, and we proposed that the VEGF axis was a logical target for intervention and regression therapy and that functional abrogation of VEGFR signaling would be an effective therapy for prostate cancer. To this end, we examined the consequence of antiangiogenic therapy with the VEGFR inhibitor SU5416 in TRAMP mice.

**SU5416 Therapy.** The compound SU5416 is a small molecule inhibitor of the VEGFR tyrosine kinase receptors. We performed three trials in cohorts of TRAMP mice that received either 50 mg/kg SU5416 i.p. or vehicle control. In the early and late intervention trials, mice were dosed twice a week for 6 weeks. The early intervention trial initiated treatment at 10 weeks of age. The late intervention trial was initiated when mice were 16 weeks of age. In the regression trial, mice were treated only when they developed palpable abdominal masses, and these mice were sacrificed when they displayed obvious signs of distress. In all trials, mice were sacrificed 3 days after the last treatment, at which time serum, prostate, pelvic lymph nodes, and lung tissues were collected. The pathology for each of the prostatic lobes (dorsal, lateral, and ventral) was determined independently, and the percentage of total pathology was calculated from all of the samples within each cohort for each of the three trials. Similar to previous reports (13), a small decrease in body weight was noted during the first week of treatment. However, we observed no significant differences in animal gross weight between the treated and control groups after 3 weeks of treatment.

**Early Intervention Trial.** Mice in the early intervention trial received SU5416 beginning at 10 weeks of age, and all mice were sacrificed at 16 weeks of age. After necropsy, we determined prostate wet weights, the incidence of metastasis, tumor IMVD, the degree of cellular proliferation, apoptosis, and tumor pathology. As shown in Table 1, we were unable to detect significant differences in prostate wet weights between samples procured from treated or control mice. Remarkably, well-differentiated prostate cancer was the predominant lesion in both the control (87%) and treated (86%) mice. There were no statistical differences in the incidence of high-grade PIN or poorly differentiated disease. Similarly, we were unable to determine statistically significant differences between the incidence of metastatic disease detected in pelvic lymph nodes in the treated and control animals or in the IMVD, proliferation, or apoptotic indices.

**Late Intervention Trial.** Mice in the late intervention trial received SU5416 beginning at 16 weeks of age, and all mice were sacrificed at 22 weeks of age. We determined prostate wet weights, the incidence of metastasis, tumor IMVD, the degree of cellular proliferation, apoptosis, and tumor pathology as described above. Although we were unable to detect significant differences in prostate wet weights between samples procured from treated or control mice, the number of samples with PIN lesions was greater in treated mice than in control mice, whereas well-differentiated cancer in the treated cohort (36%) was lower than that in the control cohort (53%). Based on these observations, it appears that SU5416 administered during the progressive phase of prostate cancer in TRAMP significantly slowed the progression of PIN to well-differentiated cancer but not the progression of well-differentiated cancer to poorly differentiated or metastatic cancer. Notably, although the incidence of advanced and metastatic disease was not significantly different between the treated and control cohorts, there was a significant increase in the apoptotic index in tumors from the SU5416-
treated animals compared with the controls ($P < 0.001$; Table 1).

**Regression Trial.** To determine the consequence of SU5416 therapy on established advanced disease, we initiated a regression trial where animals received treatment after detection of a palpable abdominal mass. In this study, TRAMP mice received either 50 mg/kg SU5416 or vehicle twice a week until they were sacrificed due to signs of distress. As shown in Table 1, there were no significant differences in prostate wet weights or tumor stage between the samples procured from treated and control mice. Furthermore, the incidence of metastasis was 100% in both the control and treated groups.

**SU5416 Therapy Increased Apoptosis and Decreased IMVD in Poorly Differentiated Tumors.** We examined a subset of poorly differentiated tumors from SU5416-treated animals in the late intervention study by histology and found them to exhibit large areas of apoptosis with morphologically visible apoptotic blood vessels and pockets of viable tumor cells (Fig. 1, A and D). We used the TUNEL method to visualize the areas of apoptosis in the samples procured from treated and control mice (Fig. 1, B and E). We determined with a 4',6-diamidino-2-phenylindole nuclear stain that live tumor cells still surrounded the few remaining viable blood vessels (Fig. 1, C and F). The extent of apoptosis found in the tumors of control animals was representative of the most poorly differentiated TRAMP tumors. These observations clearly show that SU5416 reduced the incidence of viable blood vessels and increased apoptosis in the more advanced tumors.

To quantitate the phenotype observed in the late regression trial, we determined the IMVD in the tumor samples procured from the treated and untreated mice. As shown in Fig. 1G, we observed the IMVD in poorly differentiated tumors from treated animals (average = 9.67/field) to be significantly lower than IMVD in poorly differentiated tumors from control animals (average = 12.74/field; $P < 0.05$). In fact, tumor-specific apoptosis was significantly increased when we compared poorly differentiated tumors of SU5416-treated animals (18.69%) with tumors from animals receiving vehicle alone (5.48%; $P < 0.0005$; Fig. 1H). Consistent with the hypothesis that inhibition of the VEGF axis would influence tumor growth by inhibiting vasculogenesis and inducing apoptosis, we observed no significant difference in tumor cell proliferation between poorly differentiated tumors of treated or control mice (Fig. 1I).

**Consequence of SU5416 on Expression of VEGF Axis.** Because SU5416 is an inhibitor of VEGF signaling, we considered the possibility that this compound could influence the expression of the VEGF axis. To this end, immunoblot analysis was performed on samples representative of well and poorly differentiated tumors procured from animals in the late intervention trial. As shown in Fig. 2, expression of VEGFR1 was readily detectable by immunoblotting in sam-

![Image](https://example.com/image1.png)
TRAMP mouse model. The spontaneous autochthonous genetically engineered VEGFR-associated tyrosine kinase activity, SU5416, using prostate cancer. To this end, we initiated a preclinical trial VEGF signaling could be used as therapy for autochthonous was to test the hypothesis that stage-specific inhibition of VEGFR2 were differentially expressed during prostate cancer. We have demonstrated previously (8) that VEGFR1 and appear to influence the expression of VEGF, VEGFR1, or based on these observations, treatment with SU5416 did not appear to influence the expression of VEGF, VEGFR1, or VEGFR2.

Consequences of SU5416 Therapy in TRAMP Survival. To determine the consequences of SU5416 treatment on survival, TRAMP mice were treated with SU5416 or vehicle control upon palpation of tumor, and this treatment was continued until the mice were sacrificed due to signs of distress. The time between palpation and sacrifice was plotted against the survival frequency (Fig. 3). Unfortunately, in this study we were unable to demonstrate a significant survival advantage in mice that received SU5416 once a palpable tumor had already developed.

Discussion
We have demonstrated previously (8) that VEGFR1 and VEGFR2 were differentially expressed during prostate cancer progression in TRAMP. Hence, the purpose of this study was to test the hypothesis that stage-specific inhibition of VEGF signaling could be used as therapy for autochthonous prostate cancer. To this end, we initiated a preclinical trial with a potent antiangiogenic small molecule inhibitor of VEGFR-associated tyrosine kinase activity, SU5416, using the spontaneous autochthonous genetically engineered TRAMP mouse model.

In the early intervention trial, administration of SU5416 to TRAMP mice for 6 weeks beginning at 10 weeks of age was not observed to significantly slow prostate cancer growth, inhibit tumor progression, or cause tumor regression. At first, these results appear inconsistent because SU5416 is known to inhibit VEGFR1 activity, and early prostate cancers readily express detectable levels of VEGFR1. However, early tumors do not produce appreciable levels of VEGF (8), and this observation raises the distinct possibility that the VEGFR1 expressed in these early cancers, likely through a hypoxia-induced hypoxia-inducible factor-α mechanism (17), is not actually activated, and therefore SU5416 therapy would not be expected to be significantly effective. Unfortunately, we are not currently able to determine the phosphorylation status of VEGFR1 in such PIN lesions. Nevertheless, these observations indicate that early angiogenesis in TRAMP is not strictly dependent on activation of VEGFR1 and that SU5416 was unable to influence the growth of prostate cancer in this early intervention trial.

In the late intervention study, in contrast to the early intervention study, therapy with SU5416 administered for 6 weeks resulted in a significant increase in apoptotic blood vessels with a concomitant reduction in vessel density in poorly differentiated tumors. Importantly, these advanced, poorly differentiated tumors express high levels of both VEGF and VEGFR2 (see Fig. 2). This data clearly support the conclusion that SU5416 is very effective at blocking VEGFR2 signaling, resulting in significant endothelial cell death in vivo. Moreover, these results are in general agreement with independent studies in the RIP1-Tag2 transgenic mouse model of pancreatic islet carcinogenesis demonstrating a decrease in angiogenesis and increase in apoptosis after treatment with antiangiogenic agents including SU5416 (18, 19). It is interesting to note, however, that VEGF may play different roles in the RIP1-Tag2 and TRAMP models. In the RIP1-Tag2 model, expression of matrix metalloproteinase 9 in the developing tumors released VEGF from the extracellular matrix to initiate angiogenesis, and administration of SU5416 was able to prevent VEGF-dependent tumor development (19). In contrast, VEGF does not appear to be required for the initiation of angiogenesis in the TRAMP model, even though VEGF signaling is critically important for vessel survival in more poorly differentiated tumors (8). These differences in early VEGF dependence likely reflect some tissue-specific angiogenic events, especially because SV40 T/t is the predisposing lesion in both models. Crossing TRAMP with a conditional VEGF knockout should help to resolve the requirement of VEGF during early angiogenesis. These studies are currently under way.

If VEGF is not the “initiation switch” driving angiogenesis in TRAMP, then what is? One likely candidate is FGF-2, a potent proangiogenic factor. In fact, we have recently dem-
onstrated that FGF-2 (as well as FGF-7) is expressed by TRAMP tumors during progression (20, 21) and that FGF-R1 is expressed in tumor vasculature during progression of prostate cancer in TRAMP (20). These data raise the possibility that a combination of therapies directed against the FGF-Rs (FGF-R1 and probably FGF-R2) as well as VEGF could prove additive, if not synergistic.

Despite our observations that SU5416 significantly increased apoptosis in blood vessels and reduced blood vessel density, tumors were observed to grow after SU5416 therapy. One possible explanation for this continued growth is that the blood vessels in these late-stage cancers were actually mosaic, lined by both tumor and endothelial cells, and therefore only those expressing VEGF receptors represented valid targets for SU5416. In fact, it has been shown that nonendothelial cells could comprise 15% of the lining of vessels in a colon carcinoma xenograft model (22). This possibility is further supported by our observations that only a small fraction of blood vessels in late-stage cancers actually expressed PECAM-1, and these vessels demonstrated significantly lower IMVD in those animals treated with SU5416 (Fig. 1G). In fact, our observation that a small number of the non-PECAM-1-expressing vessels actually contained RBCs strongly supports a conclusion that these mosaic vessels are functional.

In this study we have demonstrated a drug-dependent response in poorly differentiated tumors and a significant increase in tumor-associated apoptosis as a consequence of SU5416 therapy. These data support the hypothesis that inhibition of the VEGF axis, specifically VEGF activation of VEGF2, could slow tumor progression and cause vascular apoptosis in progressive autochthonous disease. However, our data also demonstrate that SU5416 therapy alone was insufficient to completely inhibit the progression of prostate cancer or prolong survival in the TRAMP model.

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References

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