Renal Cancer Resistance to Antiangiogenic Therapy Is Delayed by Restoration of Angiostatic Signaling

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Abstract

Treatment of metastatic renal cell cancer (RCC) with antiangiogenic agents that block vascular endothelial growth factor (VEGF) receptor 2 signaling produces tumor regression in a substantial fraction of patients; however, resistance typically develops within 6 to 12 months. The purpose of this study was to identify molecular pathways involved in resistance. Treatment of mice bearing either 786-0 or A498 human RCC xenografts with sorafenib or sunitinib produced tumor growth stabilization followed by regrowth despite continued drug administration analogous to the clinical experience. Tumors and plasma were harvested at day 3 of therapy and at the time of resistance to assess pathways that may be involved in resistance. Serial perfusion imaging, and plasma and tumor collections were obtained in mice treated with either placebo or sunitinib alone or in combination with intratumoral injections of the angiostatic chemokine CXCL9. Sunitinib administration led to an early downmodulation of IFN-γ levels as well as reduction of IFN-γ receptor and downstream angiostatic chemokines CXCL9 to 11 within the tumor. Intratumoral injection of CXCL9, although producing minimal effects by itself, when combined with sunitinib resulted in delayed resistance in vivo accompanied by a prolonged reduction of microvascular density and tumor perfusion as measured by perfusion imaging relative to sunitinib alone. These results provide evidence that resistance to VEGF receptor therapy is due at least in part to resumption of angiogenesis in association with reduction of IFN-γ-related angiostatic chemokines, and that this resistance can be delayed by concomitant administration of CXCL9.

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Introduction

The pathogenesis of renal cell carcinoma (RCC) frequently involves the inactivation of the von Hippel Lindau (VHL) tumor suppressor gene that encodes an E3 ubiquitin ligase complex that targets hypoxia-inducible factor for proteasome-mediated degradation. In the setting of VHL inactivation, a large repertoire of hypoxia inducible genes including vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) are overexpressed (1). This increase in proangiogenic factors, even in the absence of hypoxia, likely accounts for the nearly unique sensitivity of RCC to treatment with small molecule tyrosine kinase inhibitors (TKI) of the VEGF receptor (VEGFR) such as sunitinib and sorafenib. Treatment of patients with RCC with these agents frequently leads to tumor regression, but resistance to treatment typically develops within one year, substantially limiting their benefit (2–6).

This acquired “evasive” resistance to VEGF pathway inhibition has been observed in multiple preclinical models and tumor types (7). In these settings proposed resistance mechanisms include an increase in alternative proangiogenic factors such as interleukin-8 (IL-8) and basic fibroblast growth factor as seen in the setting of anti-VEGFR2 antibody therapy (8, 9). The empty basement membrane sheaths and pericyte changes seen by Mancuso et al. could also provide the scaffold for tumor angiogenesis in the resistant setting (10). Other suggested mechanisms include selection of cells that can better tolerate hypoxia (11) and, in the setting of intrinsic resistance to VEGF inhibition, the recruitment of CD11b and Gr1-positive bone marrow–derived proangiogenic cells (12). Elucidation of the mechanisms underlying the acquired resistance to VEGFR blockade in RCC may contribute to the development of novel therapeutic approaches that could enhance the efficacy of VEGFR inhibitors in this patient population.

IFN-γ signaling leads to the production of three angiostatic chemokines, CXCL9 to 11 (mig, IP-10, and ITAC;...
These chemokines are highly expressed in RCC relative to normal kidney and have been associated with favorable prognosis in patients with RCC (14, 15). Moreover, CXCR3, the receptor for CXCL9 to 11, is much more highly expressed in RCC than in normal kidney (16), and its expression is associated with improved disease-free survival following nephrectomy (17). Functional studies of CXCL9 and 10 also show that both chemokines exhibit antitumor activity in mouse models of lung cancer and RCC, respectively (18, 19). Prior studies from our group have shown that resistance to VEGFR blockade is accompanied by restoration of angiogenesis. We also noted a loss of IFNγ-regulated chemokines at day 3 of treatment. Consequently, because of the known angiostatic function of these chemokines, we hypothesized that this loss might contribute to the acquired resistance to VEGFR blockade. We investigated this possibility by testing the potential value of restoration and maintenance of angiostatic chemokines in delaying the acquired resistance to VEGFR blockade in murine human tumor xenograft models.

Materials and Methods

**Reagents**

CXCL9 antibody was obtained from R&D. IFNγ receptor 1 (IFNγR1), CXCL10, CXCL11, and GAPDH antibody for Western Blot were obtained from Abcam. The anti-vinculin antibody was from Sigma. Horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-mouse, goat anti-rabbit) were obtained from Cell Signaling Technology. For immunohistochemistry, CD34 was from Abcam, rabbit anti-goat from Dako, and IFNγR from Santa Cruz.

**Plasma protein analysis**

The Multiplex bead kit for cytokine measurement was purchased from BioRad Laboratories, Inc. Standard curves for each mediator were generated, ranging from 2 to 32,000 pg/mL. Plasma samples were incubated with 50 μL of antibody-coupled microsphere sets for 1 hour at room temperature. Freshly diluted secondary detection antibody (25 μL; 1 μg/mL) were added and incubated at room temperature for 1 hour. Streptavidin-phycoerythrin (50 μL; x1) was added, followed by an incubation for 10 minutes at room temperature. After each step incubation of samples with microsphere sets, secondary detection antibody, and streptavidin-phycoerythrin, a filtering step and three washing steps using a vacuum manifold were done. Each well was analyzed on a Bioplex Protein Array System (BioRad Laboratories, Inc.) according to the manufacturer’s instructions. All protein concentrations are given in picograms (pg) per milliliter.

**Protein extraction and Western blot analysis**

Tumor tissues were homogenized in lysis solution (Cell Signaling Technology) supplemented with sodium fluoride (10 μmol/L; Fisher) and phenylmethylsulfonyl fluoride (100 μg/mL; Sigma-Aldrich). After sonication for 10 seconds, cell debris was removed by centrifugation at 12,000 g for 20 minutes at 4°C. Protein concentration was determined by bicinchoninic acid protein assay reagent (Pierce). Lysates were fractionated in either 8 or 12% SDS-polyacrylamide gels, and the separated proteins were transferred to nitrocellulose. The blots were probed for the proteins of interest with specific antibodies followed by a second antibody–HRP conjugate and then incubated with SuperSignal chemiluminescence substrate (Pierce).

**Cell culture**

A498 and 786-O, two VHL-deficient human RCC cell lines (20), were obtained from the American Type Culture Collection and cultured for <1 month, and aliquots were frozen. Fresh frozen aliquots were used for each experiment. A498 was grown in Eagle’s MEM. 786-O cells were cultured in RPMI 1640 medium (Cellgro). All media were supplemented with 2 mmol/L L-glutamine, 10% FCS, and 1% streptomycin (50 μg/mL), and cells were cultured at 37°C with 5% CO2. Growth and morphology of both lines was observed and noted to be consistent with prior descriptions of the lines; no further genetic characterization was done.

**Tumor xenograft induction**

For s.c. xenograft tumor models, female athymic nude/beige mice (Charles River Laboratories) were used. The mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions. All experiments were approved by the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center.

RCC cell lines were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with medium containing 10% fetal bovine serum, and the cells were washed once in serum-free medium and resuspended in PBS. Only suspensions consisting of single cells with >90% viability were used for the injections.

To establish RCC tumor xenografts, 786-O or A498 tumor cells were injected s.c. (1 × 107 cells) into the flanks of 6- to 8-week-old mice that were of 20 g average body weight. Tumors developed in >80% of the mice and were usually visible within a few days of implantation. Sorafenib (80 mg/kg; Bayer) or sunitinib (additive-free, 53.6 mg/kg; Pfizer) was administered 6 of 7 days per week by gavage beginning when the tumors had grown to a diameter of 12 mm as per Sabir et al. (21) and Schor-Bardach et al. (22). Tumors were measured daily while on treatment, and the day of resistance was recorded. Resistance was defined as an increase in tumor diameter by 2 mm from its pretreatment size of 12 mm. This difference represents the smallest increase in size that could be reproducibly measured by calipers and is roughly analogous to the clinical criteria for resistance (20% growth by Response Evaluation Criteria for Solid Tumors).
used in patients. Furthermore, this difference was previ-
ously shown to be associated with restored angiogenesis
in this model (22). Tumor long and short axes were mea-
sured, and long axis and tumor volume were followed to
determine growth curves. Treatment was continued until
tumors grew to 20 mm, at which point the mice were sac-
rificed. Tumor tissue was obtained pretreatment, during
response, and at time of resistance for various analyses
described below.

**CXCL9 administration**

CXCL9 (1 ug in 200 uL; R&D Systems) was injected
into the central portion of tumor xenografts three times
weekly as described previously (18). Control mice re-
ceived injections of PBS, the vehicle in which the CXCL9
was dissolved. Injections began when tumors reached
12 mm and were given either alone or concomitant with
sunitinib as described above. Tumors were measured
daily, and time to resistance (growth by 2 mm) and
20 mm were ascertained. Tumor tissue was obtained
during response and at time of resistance for the vari-
ous tumor tissue analyses described below.

**Immunohistochemistry**

For CD34 analysis, 4-um-thick sections were prepared
from formalin-fixed, paraffin-embedded tumor speci-
mens. Sections were deparaffinized, rehydrated, and
heated with a pressure cooker to 125°C for 30 seconds
in citrate buffer for antigen retrieval. After cooling to
room temperature, sections were incubated in 3% hy-
drogen peroxide for 5 minutes to quench endogenous
peroxidase, and then for 20 minutes in Dako serum-free
HRP-labeled polymer anti-mouse for 30 minutes, fol-
lowed by rabbit anti-goat secondary antibody for
1 hour, was applied at a 1:100 dilution to sections for 1 hour.
Detection
was done by incubating with Dako EnVision + System
HRP-labeled polymer anti-
rabbit for 30 minutes, followed by 3,3′-diaminobenzidine
chromogen. Slides were scanned using the Scan-
scope XT (Aperio Technologies Inc.) and analyzed
using a modified microvessel analysis algorithm (Aperio
Technologies Inc.).

For IFNγR staining, frozen sections were used. Sec-
tions were fixed in -20°C acetone for 5 minutes and then
air-dried. Sections were incubated in 3% hydrogen perox-
ide for 5 minutes to quench endogenous peroxidase. The
anti-IFNγR antibody (Santa Cruz Biotechnology) was
applied at a 1:50 dilution to sections for 1 hour. Detection
was done by incubating with Dako EnVision + System
HRP-labeled polymer anti-mouse for 30 minutes, fol-
lowed by 3,3′-diaminobenzidine chromogen.

**Tumor perfusion imaging**

Tumor perfusion imaging [arterial spin labeled (ASL)
magnetic resonance imaging (MRI)] was done as previ-
ously described (22). ASL sequence raw data were saved
and transferred to the analysis workstation for image re-
construction by using custom software written within the
Interactive Data Language (Research Systems). The ASL
difference image, between average label and control
images, was then converted to quantitative tumor perfu-
sion as previously described (23).

To determine tumor perfusion, a region of interest was
drawn freehand around the peripheral margin of the tu-
mor by using an electronic cursor on the reference image
that was then copied to the perfusion image. The mean
blood flow for the tumor tissue within the region of inter-
est was derived, and image window and level were fixed.
A 16-color table was applied in 10 mL/100 g/minute
increments ranging from 0 to 160 mL/100 g/minute, with
flow values represented as varying shades of black, blue,
green, yellow, red, and purple, in order of increasing
perfusion.

**Results**

**Modulation of IFNγ signaling with VEGFR inhibitor therapy**

786-O-derived tumors were implanted into mice as
described in Materials and Methods. Treatment with su-
unitinib or sorafenib was initiated at a tumor size of
12 mm in long axis. Tumors exhibited a period of growth
stabilization followed by growth resumption despite con-
tinued therapy as previously described (22). Plasma was
collected from untreated mice (n = 12), on day 3 (n = 16),
and at resistance (n = 13). Twenty-seven cytokines were
screened using human-specific panels and 23 cytokines
were screened using murine-specific panels. Changes in
several cytokines were noted in both the tumor and the
stroma at the time of resistance (Supplementary Table S1),
including a significant decrease in the production of IFNγ
(P = 0.018 for day 3 versus resistant, and 0.0120 for un-
treated versus resistant; Fig. 1A). Human cytokines are
tumor derived in this system; these findings suggest that
tumor-derived IFNγ may be downmodulated with resis-
tance. Other significant changes were observed in several
immune cytokines, including decreased human granulo-
cyte macrophage colony-stimulating factor (GM-CSF)
and granulocyte colony-stimulating factor, and, as previ-
ously reported, increased human IL-8 at resistance (24).
Significant changes in murine IL-3, IL-4, IL-13, tumor
carcinosis factor α, and GM-CSF were also noted (Supple-
mentary Fig. S1).

To further interrogate this cytokine pathway, expression
of IFNγR was measured in the tumors from mice in the
absence of therapy or with treatment with either su-
unitinib or sorafenib. There was abundant expression
of IFNγR in untreated tumors and a loss of expression
of IFNγR both at day 3 of therapy and at the time of re-
stance in mice receiving either sorafenib or sunitinib
(Fig. 1B). These data suggest that a loss of IFNγ signaling
can accompany resistance to therapy. Furthermore, im-
munohistochemical analysis showed dramatic down-
regulation of IFNγR expression in tumors derived from
mice that were treated with 3 days of sunitinib as com-
pared with untreated tumors (Fig. 1C).
To further define the changes in the IFNγ pathway that accompany resistance to sorafenib and sunitinib, the expression of the angiostatic chemokines regulated by IFNγ was analyzed. Western analysis of the tumors at day 3 of therapy and at the time of resistance showed a loss of all three chemokines (CXCL9–11) relative to untreated tumors (Fig. 2). This is consistent with the hypothesis that VEGFR TKI treatment leads to a decrease in IFNγ signaling.

### Chemokine administration delays the development of resistance to sunitinib

The treatment-induced loss of angiostatic chemokines led to the hypothesis that resistance could be delayed with chemokine replacement. To study this, CXCL9 was injected into the 786-O tumors either alone or concomitant with sunitinib gavage. CXCL9 was selected because of the prior data that this chemokine exhibits activity in murine RCC models (18). Figure 3 shows the growth of tumors treated with vehicle gavage, sunitinib plus intratumoral PBS, sunitinib plus intratumoral CXCL9, or intratumoral CXCL9 alone. Although CXCL9 alone and treatment with sunitinib alone slowed tumor growth, this effect was further enhanced by the combination of sunitinib and injections of CXCL9 (Fig. 3). Tumors treated with sunitinib plus PBS increased by 2 mm in 11.2 ± 1.3 days and this was extended to 18.9 ± 3.2 days with the addition of CXCL9 (P = 0.001). Treatment with CXCL9 alone exhibited a similar effect as sunitinib alone (Fig. 3). Thus, the early administration of CXCL9 with sunitinib delays resistance to sunitinib.
CXCL9 treatment prolongs antiangiogenic effect of sunitinib

To assess the mechanism by which CXCL9 prolongs the effect of sunitinib, 786-O–derived tumors were analyzed at the time of sacrifice. Tumors were harvested from both the sunitinib-plus-PBS and the sunitinib-plus-CXCL9 groups at the average time when the tumors in the mice treated with sunitinib plus PBS reached 20 mm in size (day 43 ± 2.4 posttreatment). Figure 4 shows representative immunohistochemical sections of CD34 assessment of microvessel density (MVD) of mice treated with vehicle, CXCL9, sunitinib plus PBS, and sunitinib plus CXCL9 (Fig. 4A–D, respectively). Although CXCL9 itself had no significant effect on MVD, the average MVD of tumors treated with sunitinib plus CXCL9 was 40% lower than tumors treated with sunitinib plus PBS (P = 0.014).

To further assess the angiogenic capability of the treated tumors, serial tumor perfusion imaging by ASL MRI was done. Figure 5A shows a time course of tumor (786-O) perfusion in a mouse treated with vehicle, CXCL9, sunitinib plus PBS, or sunitinib plus CXCL9. Whereas sunitinib plus PBS and sunitinib plus CXCL9 produced initial loss of perfusion compared with control followed by recovery of perfusion at the time of resistance, treatment with sunitinib plus CXCL9 suppressed perfusion longer and to a greater extent. Resumption of perfusion was seen on week 3 (days 22–26) and was significant by week 6 (days 42–46; P = 0.03) in sunitinib-plus-PBS-treated mice, whereas mice treated with sunitinib plus CXCL9 exhibited a greater reduction in tumor perfusion that was maintained at both week 3 (days 22–26) and week 6 (days 42–46) of therapy (P = 0.03 and 0.04, respectively). In contrast to tumor perfusion in mice treated with sunitinib plus PBS, tumor perfusion at time of resistance to sunitinib plus CXCL9 never attained the pretreatment perfusion levels. These observations are consistent with the CD34 immunohistochemical data. Tumor perfusion exhibited little change over time in mice treated either with PBS alone or CXCL9 alone (Fig. 5A and B).

CXCL9 restoration delays resistance in a second xenograft model

To confirm these findings and extend them to another tumor model, mice bearing xenograft tumors derived from A498 cells were treated with sunitinib plus PBS or sunitinib plus CXCL9. As noted in the 786-O–derived tumors, CXCL9 when initiated with sunitinib extended the duration of relative tumor stability as measured by time to increase by 2 mm. Tumors treated with the combination of CXCL9 and sunitinib showed a period of stability of 39.8 ± 6.1 days compared with 22.2 ± 4.1 days for sunitinib plus PBS (P = 0.0008; Fig. 6A). CXCL9 itself also slowed tumor growth but not to as great an extent as the sunitinib-containing treatments. Serial ASL MRI was done on mice treated with vehicle, CXCL9 alone, sunitinib plus PBS, and sunitinib plus CXCL9. Both administration of sunitinib plus PBS (P ≤ 0.001) and CXCL9 alone (P = 0.001) significantly reduced perfusion relative to vehicle
Representative images show that the combination of sunitinib and CXCL9 resulted in a greater reduction in tumor blood flow and lower tumor perfusion at all time points relative to sunitinib plus PBS (Fig. 6B and Supplementary figure, \( P = 0.001 \)). As in 786-O tumors, ASL MRI–measured tumor perfusion in sunitinib-plus-CXCL9-treated mice never returned to the pretreatment perfusion level. Levels of IFN-\( \gamma \)R and CXCL9 were downmodulated in the A498 tumors with exposure to sunitinib (Fig. 6C). In contrast to 786-O tumors, however, the majority of A498 tumors exhibited increases in these molecules at the time of resistance to sunitinib. This early, but not sustained, loss of angiostatic chemokines is consistent with the need for early supplementation of sunitinib with CXCL9.

Discussion

Treatment of patients with the VEGFR TKIs sorafenib and sunitinib can lead to periods of tumor stability, but resistance to therapy is inevitable. We used a mouse model to define mechanisms by which resistance develops and found that components of the IFN-\( \gamma \) signaling pathway are lost with sunitinib or sorafenib therapy. Our data also show that CXCL9 treatment delays resistance to sunitinib in 786-O- and A498-derived tumors.
Figure 5. A, serial tumor perfusion in a representative tumor treated with vehicle as control, CXCL9 alone, sunitinib + PBS, or sunitinib + CXCL9 as measured by ASL MRI. Red lines, regions of tumor. The tumor size was measured with long and short axes (in mm) and the mean blood flow (in mL/100 g/minute) are shown below each image. Color scale, range of perfusion values from 0 to 160 mL/100 g/minute. B, average perfusion with SE (n ≥ 3 mice in all arms). The mice treated with sunitinib + PBS exhibited decreased perfusion that began to resume by week 3 (*, P = 0.04 for comparison of vehicle versus sunitinib treated mice; £, P = 0.03 for comparison of day 3 versus day 45 of therapy with sunitinib + PBS). In contrast, mice treated with sunitinib + CXCL9 exhibited a greater reduction in tumor perfusion than with sunitinib + PBS that was maintained at week 3 (days 22–26) and week 6 (days 42–46; ¶, P = 0.03 and $, P = 0.04, respectively).
Figure 6. A, growth curves of average volume of A498-derived xenograft tumors treated with sunitinib + PBS, sunitinib + CXCL9, CXCL9, or vehicle, with SE (n = 5 in all arms). As compared with untreated controls or sunitinib-treated mice, the growth of tumors treated with sunitinib + CXCL9 exhibit a longer time to grow by 2 mm shown in the accompanying table and a prolonged time of overall tumor growth (days of tumor growth from 12 mm to 14 mm: sunitinib + PBS versus sunitinib + CXCL9; §, P = 0.0008). CXCL9 also slowed tumor growth but to a lesser extent than sunitinib + CXCL9 (*, P < 0.0001 for CXCL9 versus vehicle; #, P < 0.0001 for CXCL9 versus sunitinib + CXCL9). B, representative set of perfusion images from a set of mice treated with vehicle as control, CXCL9 alone, sunitinib + PBS, or sunitinib + CXCL9 (representative of 3 mice per arm). Red lines, regions of tumor. The tumor size was measured with long and short axes (in mm) and the mean blood flow (in mL/100 g/minute) are shown below each image. Color scale, range of perfusion values from 0 to 160 mL/100 g/minute. C, Western analysis for IFNγR and CXCL9 in A498 tumors in representative untreated tumors (Un), day 3 of sunitinib (D3), and at the time of resistance (R). Comparisons were done by Student’s t-test.
CXCL9 Delays Resistance to VEGFR Inhibition

and that one mechanism by which this occurs is by prolongation of the antiangiogenic effects of sunitinib. These data suggest that angiostatic pathways are suppressed as a result of VEGFR TKI therapy and set the stage for the subsequent development of resistance to therapy.

In the setting of VEGFR inhibition, RCC tumors undergo extensive necrosis (22). In the setting of this necrosis and accompanying hypoxia/nutrient deprivation, tumors may undergo compensatory changes, including the induction of salvage angiogenesis pathways. We propose that the environmental stress resultant from a rapid and dramatic reduction in tumor vasculature and VEGFR signaling is particularly conducive to the development of such molecular changes. We hypothesize that one of these molecular changes is the loss of IFNγR and that this loss is linked to the downmodulation of angiostatic chemokines. Thus, this revascularization of VEGF-deprived tumors is likely physiologically distinct from de novo angiogenesis.

Within just a few days of therapy, RCC xenografts seemed particularly vulnerable to agents that could prevent salvage angiogenesis. In fact, we show an early loss (by day 3 of treatment) of IFNγR and CXCL9, which led to our hypothesis that treatment with CXCL9 must be administered early. Thus, we administered sunitinib and CXCL9 concurrently in an effort to overcome possible changes resulting from this early loss of angiostasis. Although other recently published studies have shown that antiangiogenic therapy can lead to increased production of angiogenic molecules and increased invasiveness and metastatic potential (25, 26), we show that antiangiogenic therapy also leads to downmodulation of angiostatic signaling.

CXCR3, the receptor for CXCL9, is expressed on both tumor cells and endothelium. CXCR3 signaling in other tumor types has proinvasive properties (27). By contrast, RCC xenografts in our models did not show accelerated growth compared with untreated tumors when injected with single-agent CXCL9. This is consistent with the finding that CXCR3 expression confers a favorable prognosis in patients with localized RCC (17). Future studies could explore CXCR3 expression in the tumors, including the spliced subtype expression as well as the potential role for direct effects of CXCL9 on tumor cells. Additionally, CXCL9 has been shown to function in leukocyte activation via its interaction with CXCR3 on Th1 cells. Although our experiments used immunocompromised mice, the possibility remains that CXCL9 also recruits immune cells to the tumor, but in preliminary experiments we did not see evidence of increased lymphocyte recruitment to the tumors (data not shown). Exclusion of an immune role for CXCL9 would likely require studies in more severely immunodeficient mice such as RAG KO mice. Finke et al. noted an increase in type I IFNγ producing cells after treatment with sunitinib (28). Further studies will be required to understand the relationship between this finding and the loss of IFNγR signaling that we have seen.

To measure tumor vasculature, we analyzed tumor expression of CD34 by immunohistochemistry and carried out tumor perfusion imaging using ASL MRI. We show here that CD34 staining correlates with ASL MRI perfusion, giving us confidence to use this imaging modality to assess response and resistance to antiangiogenic therapy. One noteworthy advantage of ASL MRI over CD34 is that it enables serial imaging of tumors as opposed to comparing distinct tumors removed from different animals at different time points. Although plasma biomarker analyses have shown that there are changes in plasma cytokines that may predict for antitumor activity (29), there are advantages to having a visual representation of the angiogenic status of a tumor in an individual patient. This could provide early clues to the timing and means by which escape from therapy occurs. Our data show that a hallmark of sunitinib treatment is dramatically reduced tumor perfusion followed by a restoration of tumor perfusion accompanied by tumor regrowth. Prior work from our group has shown similar findings with sorafenib treatment (22). We did note, however, that the restored tumor perfusion at the time of resistance never reaches the pretreatment levels. This finding suggests that angiogenesis-independent factors may also contribute to resistance to VEGFR blockade. For example, metabolic changes in a tumor may occur that limit its oxygen requirements relative to the treatment-naïve setting (30).

The biological significance of the downmodulation of CXCL9 and IFNγR noted shortly after beginning treatment with a VEGFR antagonist is unclear. The fact that these angiostatic molecules are expressed by tumor cells in untreated mice and even overexpressed in some tumors that have developed resistance to sunitinib indicates that their presence is not an absolute deterrent to tumor growth and vascularization. This may be especially true in the resistant setting, in which new angiogenic pathways may dominate over the angiostatic forces. CXCL9 initiated concurrently with the sunitinib, however, prolonged the duration of sunitinib-induced growth arrest and delayed the revascularization and resumption of tumor perfusion that otherwise rapidly ensues in mice treated with sunitinib alone. This observation indicates that the early phases of tissue remodeling induced by VEGFR blockade are affected by the presence of CXCL9 and that the disappearance of this chemokine from the tissue facilitates the re-establishment of the tumor microcirculation and the development of resistance to VEGFR antagonists. Thus, the early loss of CXCL9 and IFNγR expression seen in the A498 tumors is likely the dominant predictor of the utility of chemokine supplementation and the observation that these molecules are re-expressed at resistance may indicate that other proangiogenic factors can be sufficient to mediate tumor growth even in the presence of enhanced expression of angiostatic factors such as CXCL9.

Although intratumoral CXCL9 injection validates the concept that sunitinib resistance can be delayed with CXCR3 ligands, this method of delivery is not amenable to clinical practice. Future studies that would enable translation to the clinical setting could involve the application...
of a CXCR3 agonist. Additionally, the potential use of systemic CXCL9 could be explored as could treatment of patients with IFNγ- or IFNγ-inducing agents such as IL-12. Although we find that tumor cells lose IFNγR and may not be able to respond to IFNγ treatment by producing CXCL9-11, it is possible that other nontumor cell types maintain the ability to upregulate the angiostatic chemokines in response to IFNγ despite antiangiogenic therapy. We are currently exploring this issue.

The inability to sustain the initial tumor stabilization or regression induced by VEGF pathway blockers is arguably the most vexing problem now encountered by oncologists who care for patients with RCC. Our studies suggest that relative loss of CXCL9 is one of the molecular mechanisms that curtail the initial effectiveness of VEGFR blockers. Future studies with augmentation of angiostatic pathways might lead to the elucidation of therapeutic approaches that extend the effectiveness of sunitinib, sorafenib, or other VEGF pathway blockers in RCC and possibly other tumors.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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