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See commentary by Ebos and Pili, p. 3719

Rapid Angiogenesis Onset after Discontinuation of Sunitinib Treatment of Renal Cell Carcinoma Patients

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Abstract

Purpose: To investigate the angiogenic changes in primary tumor tissue of renal cell carcinoma (RCC) patients treated with VEGF-targeted therapy.

Experimental Design: Phase II trials of VEGF pathway–targeted therapy given before cytoreductive surgery were carried out with metastatic RCC patients with the primary tumor *in situ* to investigate the necessity of nephrectomy. Primary tumor tissues were obtained and assessed for angiogenesis parameters. Results were compared with similar analyses on untreated tumors.

Results: Sunitinib or bevacizumab pretreatment resulted in a significant reduction of microvessel density in the primary tumor. Also, an increase in vascular pericyte coverage was found in sunitinib-pretreated tumors, consistent with efficient angiogenesis inhibition. Expression of several key regulators of angiogenesis was found to be suppressed in pretreated tissues, among which VEGFR-1 and VEGFR-2, angiopoietin-1 and angiopoietin-2 and platelet-derived growth factor-B. In addition, apoptosis in tumor and endothelial cells was induced. Interestingly, in sunitinib-pretreated tissues a dramatic increase of the number of proliferating endothelial cells was observed, which was not the case in bevacizumab-pretreated tumors. A positive correlation with the interval between halting the therapy and surgery was found, suggesting a compensatory angiogenic response caused by the discontinuation of sunitinib treatment.

Conclusion: This study describes, for the first time, the angiostatic response in human primary renal cancers at the tissue level upon treatment with VEGF-targeted therapy. Discontinuation of treatment with tyrosine kinase inhibitors leads to accelerated endothelial cell proliferation. The results of this study contribute important data to the ongoing discussion on the discontinuation of treatment with kinase inhibitors. *Clin Cancer Res; 18(14); 1–11. ©2012 AACR.*

Introduction

New blood vessel formation is a critical aspect of tumor progression (1, 2). Angiogenesis inhibitors have now established their role in the clinical practice of cancer therapy. Most applications of antiangiogenic compounds are in combination with other treatment strategies (3–7). Metastatic renal cell carcinoma (mRCC) is treated with the antiangiogenic tyrosine kinase inhibitor sunitinib as a monotherapy strategy. This cancer type is therefore an attractive disease to study the effect of exposure of tumor tissue to treatment with angiogenesis inhibitors. However, as surgery is often the first treatment pillar, the effect of angiogenesis inhibitors on the primary tumor tissue and blood vessels has never been extensively studied in patients.

In mRCC sunitinib treatment leads to progression-free survival (PFS) of 11.5 months and an overall survival of 26 months (8), which may extend up to 4 years with adequate sequential therapy (9). Increased effectivity has renewed the controversy surrounding cytoreductive nephrectomy for patients with primary mRCC. Downsizing of the primary tumor occurs frequently, and additional cytoreductive nephrectomy may not change clinical outcome. However, 91% of patients included in the pivotal phase III trial of sunitinib versus IFN had a nephrectomy (10). It is therefore unknown whether the reported outcome applies to mRCC patients with the primary tumor *in situ*. Retrospective data suggest that cytoreductive nephrectomy as an adjunct to targeted therapy is beneficial in selected patients with good performance and intermediate Memorial Sloan Kettering Cancer Centre (MSKCC) risk (11). Presurgical targeted

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Translational Relevance

Current cancer treatment with tyrosine kinase inhibitors often involves treatment discontinuations due to potentially significant side effects. It is now shown that this results in a compensatory boost of angiogenesis. These data support the clinical observation of rapid regrowth during the 2-week break from sunitinib therapy in some patients. As these results are not observed in patients pretreated with bevacizumab, possibly due to the long half-life of this antibody-based drug, clear differences in endothelial growth kinetics exist as a function of agent choice. The therapeutic and mechanistic relevance of this regrowth are not clear, as both agents show a salutary effect in clinical practice. The current results raise the possibility that tumor growth during therapy, as well as delayed regrowth after therapy, are important parameters for improving patient outcome. Linking these tissue-based findings to clinically relevant readouts will be important.

therapy may help to further identify those who suffer from early progression and may not benefit from surgery (12). In addition, it provides primary tumor tissue for translational research into prognostic and predictive biomarkers. Two large-phase III trials (CARMENA, EORTC SURTIME) now investigate the role and sequence of cytoreductive nephrectomy in patients receiving sunitinib (13). Previously, we conducted a phase II trial to investigate downsizing of the primary tumor following 2 courses of sunitinib. Secondary endpoints focused on safety of presurgical sunitinib and on evaluating this strategy to identify patients who progress rapidly and may not benefit from cytoreductive nephrectomy. The clinical outcome has recently been reported, including data from a U.K. trial in a meta-analysis (14, 15).

Part of the study was to investigate angiogenesis parameters in primary tumor tissues of RCC patients preoperatively treated with sunitinib. We compared the data retrospectively with a similar study in which pretreatment was carried out with bevacizumab (16). We found that preoperative treatment with either sunitinib or bevacizumab resulted in measurable inhibition of angiogenesis, as assessed by suppressed microvessel density, increased apoptosis and pericyte coverage of microvessels, and repression of several molecular angiogenic key regulators. Interestingly, although the number of proliferating endothelial cells, a more acute feature of ongoing angiogenesis, was suppressed by bevacizumab, we found an increased number of growing endothelial cells after sunitinib, depending on the length of the time interval between halting the therapy and nephrectomy. This study describes, for the first time, the angiogenic changes in human primary renal cancers at a tissue level and contributes important data to the ongoing discussion on the discontinuation of treatment with tyrosine kinase inhibitors.

Material and Methods

Patient characteristics and tissue used for evaluation

Primary tumors from patients with clear-cell mRCC treated with presurgical sunitinib ($n = 21$) from a retrospective study and 2 phase II clinical studies were used for evaluation and compared with tissue from a phase II trial with presurgical bevacizumab ($n = 29$). Clear-cell RCC tissues from nontreated patients were used as controls ($n = 70$). The 3 trials were carried out independently and contained protocols for investigating angiogenesis in primary tumor tissue. Subsequently, a retrospective collective analysis of available tumor samples was carried out. Patient characteristics of the trials are presented in Table 1. One of the phase II trials was designed to investigate the effects of preoperative sunitinib treatment in patients with primary metastatic clear-cell RCC. The tissues of 12 primary tumors from this trial were evaluated. The main objective of the study (registered under EudraCT 2006-006491-38, https://eudract.ema.europa.eu/) was to investigate the response rate of the primary tumor following pretreatment with sunitinib. Details of the trial and patient characteristics have been published previously (14, 15). Briefly, patients with histologically confirmed mRCC of clear-cell subtype with a resectable asymptomatic primary *in situ* were included and received sunitinib at 50 mg/d for 2 cycles of each 4 weeks on treatment and 2 weeks off treatment. At completion of the second cycle, patients underwent cytoreductive nephrectomy as per protocol 1 day after discontinuation of sunitinib. Three patients stopped earlier because of adverse events. Another 9 primary tumors were provided from a second phase II study of presurgical sunitinib in patients with primary clear-cell mRCC. These patients were restaged after one cycle of systemic therapy, began a second cycle of systemic therapy with sunitinib, and discontinued therapy 1 day before nephrectomy (clinicaltrials.gov identifier: NCT00715442).

Previous data from a retrospective study (17) suggested that a 1-day interval was safe. In this study, 17 patients were evaluated, who had received sunitinib at various lengths (3 to 11 courses) with discontinuation 2 to 21 days before surgery. From 3 of these patients who underwent surgery, we used tissues to evaluate whether there was an association with blood vessel changes and the time interval of presurgical discontinuation of sunitinib. To these were added the 3 patients from the phase II trial that interrupted sunitinib earlier than 1 day before surgery (total number of patients who discontinued therapy between 4–21 days before surgery $n = 6$).

To compare the findings in tumor tissue following presurgical sunitinib, 29 primary tumors were provided from a phase II trial of presurgical bevacizumab (clinicaltrials.gov identifier: NCT00113217). The trial was similar in design and included patients with primary metastatic clear-cell RCC. Patients received 4 doses of bevacizumab administered intravenously every 14 days and discontinued bevacizumab 28 days before surgery. Characteristics and details of the trial have been published (16).

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Table 1. Patient characteristics from 3 phase II trials of presurgical VEGF-targeted therapy from which the primary tissues were used for analysis in this study

Presurgical drug	Sunitinib (EudraCT 2006-006491-38)[a]	Sunitinib (NCT00715442)[a]	Bevacizumab (NCT00113217)[a]
Number of patients	12	9	29
Age	56 (range: 39–75)	61 (range: 49–76)	61 (range: 41–74)
Gender			
Male	10	6	22
Female	2	3	7
MSKCC risk score			
Intermediate	11	6	25
Poor	1	3	4
Number of metastatic sites			
1	2	5	2
2	5	3	17
3	5	1	6
4			4
Metastatic sites			
Lung	10	9	25
Bone	5	1	7
Lymph node	4	4	17
Liver	3		2
Other	5		19
Median reduction primary tumor (percent longest diameter)	11% (range: +2.2%–36%)	5% (range 0%–10%)	1% (range +11%–33%)
PFS (median and range)	8.5 mo (range: 4–48 mo)	14 mo (range: 3–40 mo)	5.5 mo (range: 1–24 mo)
Overall survival (median and range)	20.5 mo (range: 5–48 mo)	35 mo (range: 6–40 mo)	18.6 (range 3–40 mo)

[a]Clinical trial identifier (clinicaltrials.gov).

Immunohistochemistry

To investigate microvessel density and the quantity of proliferating endothelial cells, CD31/34 and Ki-67 double staining (18) were carried out (sunitinib pretreatment, $n = 21$, and bevacizumab pretreatment, $n = 29$). RCC clear-cell tissues without pretreatment ($n = 70$) were used as controls. For the CD31/34 and Ki-67 double staining, paraffin sections (6-μm thickness) were deparaffinized in xylene and rehydrated in alcohol series. To block endogenous peroxidase activity, sections were treated with 3% H_2O_2 in methanol for 20 minutes, after which antigen retrieval was carried out by heating the sections in a Tris-EDTA buffer (10 mmol/L Tris-1 mmol/L EDTA, pH 8) for 15 minutes in a microwave. Subsequently, the slides were incubated for 30 minutes in 0.5% bovine serum albumin in PBS, blocking nonspecific antigen binding. Sections were incubated for 1 hour with a rabbit-polyclonal Ki-67 (Neomarker, dilution 1:50), followed by a polyclonal biotin-labeled swine anti-rabbit IgG (Dako; 1/200) for 30 minutes and avidin–biotin complex horseradish peroxidase (HRP; Dako; 1/500) for 30 minutes. Diaminobenzidine (Sigma) with 0.03% $NiCl_2$ was used as a black chromogen to be able to distinguish the black stained proliferating nuclei from the brown melanin. After the second primary antibody incubation of a mixture of CD31 (Dako; 1/50) and CD34 (Monosan; 1/200) of 1 hour, followed by a biotin-labeled goat anti-mouse IgG (Dako; 1/200) for 30 minutes and another 30 minutes incubation with an avidin–biotin complex AP (Dako; 1/200), the slides were developed with alkaline phosphatase substrate kit III (Vector Laboratories). The slides were treated with insulmount (Klinipath) to prevent alkaline phosphatase bleaching and after 12 hours, mounted with enthalan (Merck).

To visualize apoptosis in tissue sections, staining was carried out with anti-PARP p85 Fragment polyclonal antibody directed against the 85-kDa caspase-cleaved fragment (p85) of human PARP (Promega). Sections were subjected to antigen retrieval using citrate for 10 minutes at 95°C. To block nonspecific binding sites, sections were incubated with normal goat serum (5% in PBS) for 10 minutes followed by incubation o/n with anti-PARP p85 antibody. After incubation in Powervision-goat anti-rabbit HRP (Klinipath), peroxidase activity was shown by incubation in 3,3-diaminobenzidine tetrachloride (Sigma). Finally, sections were counterstained with hematoxylin, dehydrated and mounted in Pertex (Sigma-Aldrich). Between incubation steps, the sections were extensively rinsed in PBS. Within each test, isotype-matched control antibodies were included and found to be negative. All sections were examined by 2 investigators in a double-blind manner.

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Immunohistochemical analyses

Microvessel density was determined by 2 to 4 independent observers in 10 randomly selected fields (200×), and presented as the amount of blood vessels/mm^2. A second parameter for angiogenesis was evaluated through detection of active neovascularization. To that end, a similar approach was followed to enumerate the amount of proliferating endothelial cells.

For quantification of pericyte coverage, the number of vessels per high-power field lacking association of smooth muscle actin (SMA)-positive cells was quantified. PARP expression was quantified as negative (no staining cells in the tissue, given value: 0), <2% of positive cells (given value: 1), or >2% of positive cells (given value: 2). Scores between the groups were compared and used for statistical analyses.

Immunofluorescence multicolor staining

Triple staining was carried out as described previously with minor modifications (19, 20). In brief, acetone-fixed cryosections were preincubated for 30 minutes in 5% (v/v) normal goat serum (Sanquin, Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) and incubated for 30 minutes in a mixture of mouse–anti-CD31 (1:100 final dilution, Dako A/S), mouse-anti CD34 (1:50, Monoson) and rabbit anti-Ki67 (1:200; Dako); this was followed by subsequent incubation steps in biotin-labeled goat-anti mouse (1:400; Dako), normal mouse serum (1:100; Sanquin), fluorescein isothiocyanate (FITC)-conjugated mouse anti-CD3 (1:20; Dako) and a mixture of Alexa 568–labeled streptavidin (1:80; Molecular Probes Europe BV) and Alexa 633–labeled goat anti-rabbit antibody (1:800; Molecular Probes) before mounting in Vectashield (Vector Laboratories). Between incubation steps, the sections were rinsed extensively in PBS. Within each test, isotype-matched control antibodies were included and found to be negative.

Confocal laser-scanning microscopy analysis

FITC, Alexa 568, and Alexa 633 signals were collected separately on a LEICA TCS SP confocal system (Leica Microsystems) equipped with an argon–krypton–helium/neon laser. Images were taken using a ×40 numerical aperture × 1.4 objective. Color photomicrographs were taken from electronic overlays.

RNA isolation and cDNA synthesis

Total RNA was isolated from ten 20-μm thick sections using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Samples, a selection of tissues of which frozen tissue was available, included sunitinib pretreated (different time intervals of discontinuation, $n = 12$) and clear-cell RCC without pretreatment as control ($n = 5$). For RNA isolation, the acidic phenol extraction method was used according to laboratory protocol. Briefly, frozen tissue was lysed with TRIzol (Invitrogen), and RNA was extracted with NaAC 4.5 pH (3 mol/L), acidic phenol 4.5 pH (AM9720; Ambion), and chloroform (0.1:1:0.2). RNA concentrations were measured using the NanoDrop-2000 Spectrophotometer (Thermo Scientific). One μg total RNA was incubated for 5 minutes at 70°C, and cDNA synthesis was carried out for 1.5 hours at 42°C with 400 U of M-MLV reverse transcriptase RNase H (Promega) in 20 μL of 1× first strand buffer (Promega) and 1 mmol/L dNTPs in the presence of 10 U RNase inhibitor rRNasin (Promega) and 0.5 μg random primers (Promega). The reverse transcriptase activity was inactivated by incubation at 95°C for 5 minutes, and following addition of 1xTE up to a final volume of 50 μL, the cDNAs were stored at −20°C.

Real-time quantitative RT-PCR

The primers used for real-time quantitative PCR (qRT-PCR) were targeted against reference genes *β-actin*, *cyclophilin A*, *β-2 microglobulin*, and the following angiogenesis-related genes: *VEGFR-1* and *VEGFR-2*, *(VEGF)-A*, placental growth factor (PLGF), angiopoietin (ANG)-1 and ANG-2, EGF, and platelet-derived growth factor (PDGF)-B. Also primers for VE-cadherin and CD31 were used where indicated. All primers were designed to meet several requirements concerning GC-content, annealing temperature, and amplicon length and synthesized by Sigma-Genosys. Real-time qRT-PCR was carried out on a CFX96 (Bio-Rad) using the iQ SYBR Green PCR mix (Bio-Rad). The PCR reaction was carried out in a 25 μL volume containing 1.5 μL cDNA, 1× iQ SYBR Green PCR mix (Bio-Rad), and 400 nmol/L of each primer. The PCR profile was as follows: 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 30 seconds at 60°C. The C_t values and specific melting point of the amplicons were analyzed using CFX Manager (Bio-Rad). The parameter C_t (threshold cycle) was defined as the cycle number at which the fluorescent signal passed a fixed value (threshold) above baseline. Expression relative to the reference genes was calculated using the $2^{-dC}{}_t$ method (21).

Statistical analysis

Statistical calculations were carried out with SPSS software. We used the Student t test for statistical analyses of Figs. 1–4. Regression analyses were used for the correlation analyses in Fig. 5. Statistical significance was set in all analyses at $P < 0.05$.

Results

Clinical studies

The outcome of patients from the retrospective study following pretreatment with sunitinib and the 2 phase II trials of presurgical sunitinib and bevacizumab have been published previously (14, 16, 17). Briefly, in the retrospective analysis of 17 patients, 4 had a partial response, 12 had stable disease, and 1 had progressive disease. No further outcome data were reported (17).

In the phase II study of presurgical sunitinib only 1 primary tumor responded partially by RECIST (4.5%). Median reduction of longest diameter was −9.5% (range 2.2 to −36%). A greater than 10% reduction of diameter was significantly associated with a high probability to survive 2 years ($P = 0.01$). At metastatic sites, 7 patients developed a

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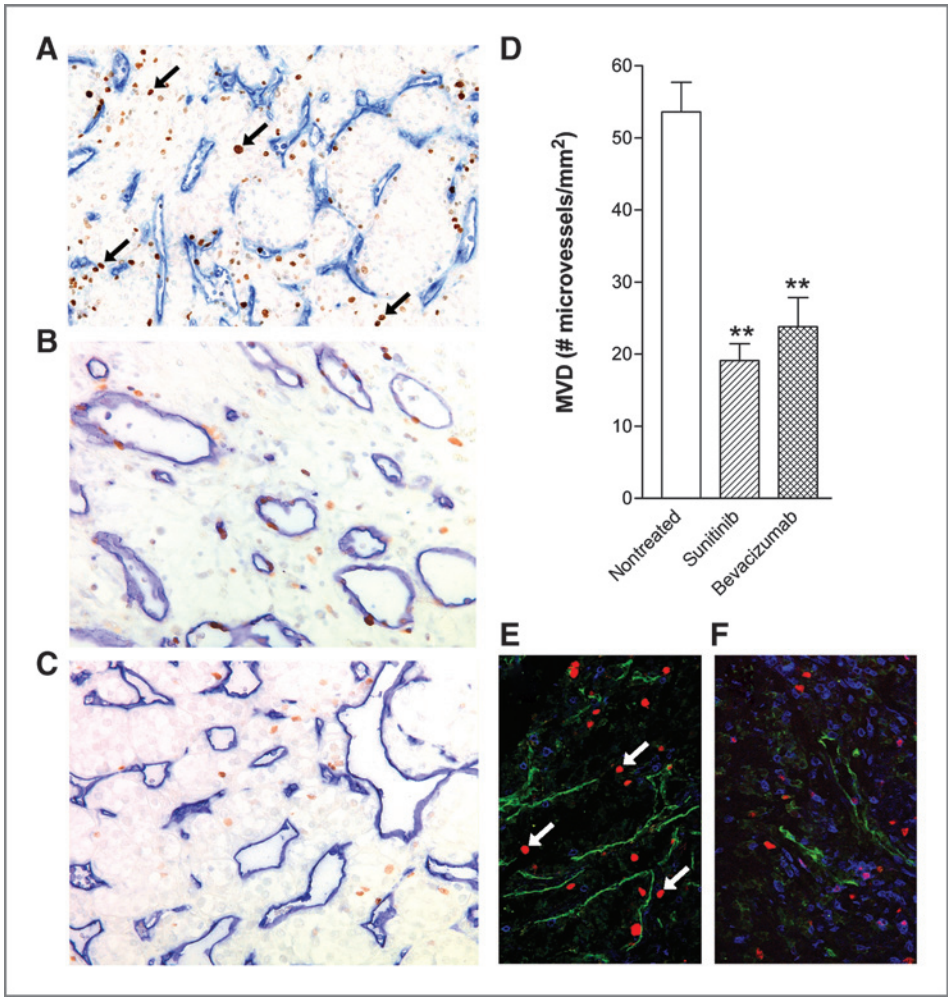

Figure 1. Suppressed microvessel density in the primary tumor after 2 cycles of sunitinib treatment. Primary tumor tissue sections of patients with RCC ($n = 21$) were stained with a mixture of CD31/CD34 antibodies to visualize blood vessels (blue) and with anti–Ki-67 (brown, arrows) to monitor proliferation status of both the endothelial and tumor cell compartments. Representative tissues of untreated ($n = 70$, A), sunitinib treated ($n = 24$, B), and bevacizumab treated ($n = 29$, C) tissues are shown (n values are the number of patient tissues with interpretable stainings). D shows the quantification (means ± SEM) of all 3 patient groups (**, $P = 0.001$). E (untreated) and F (sunitinib treated) represent immunofluorescence images, CD31/34 showing in green, Ki-67 in red, and CD3 in blue. Arrows indicate Ki-67-positive nuclei of proliferating endothelial cells.

partial response (31.8%), 7 stable disease (31.8%), and 8 progressive disease (36.4%). By MSKCC all patients had intermediate risk. Median PFS was 7 months (range 0–41 months). Median follow-up was 23 months (range 2–41 months). Median overall survival has not been reached (14).

In the presurgical phase II bevacizumab trial, 52 patients were enrolled on study and 50 were included in the analysis. By MSKCC criteria, 82% of patients had intermediate-risk features and 18% had poor-risk features. Forty-two patients underwent nephrectomy. Median PFS was 11.0 months (95% CI, 5.5–15.6 months). Median overall survival was 25.4 months (95% CI, 11.4 months to not estimable; ref. 16).

Sunitinib treatment inhibits primary tumor microvessel density

Primary tumor tissue sections of patients with RCC were stained with a mixture of CD31 and CD34 antibodies to visualize blood vessels and with anti–Ki-67 to monitor proliferation status of both the endothelial and tumor cell compartments. Untreated RCC tissues were observed to have a high microvessel density and large numbers of proliferating tumor cells (Fig. 1A and E). Preoperative sunitinib treatment resulted in a markedly suppressed number of blood vessels in the primary tumor (Fig. 1B, D, and F, $P < 0.001$ for both sunitinib and bevacizumab) as well as an inhibition of the number of proliferating tumor cells (Fig. 1B and F). A histologic observation indicated that the fewer blood vessels in the treated tumors were larger and more often seemed to have a lumen, as compared with vessels in untreated tumors (Fig. 1B). The less compressed aspect of the treated vessels is expected to be a sign of normalized interstitial pressure in the tumor tissue. A similar study on RCC tumor tissues of patients treated with preoperative bevacizumab showed similar results (Fig. 1C and D).

Pericyte coverage of microvessels and apoptosis after angiostatic pretreatment of RCC

Recently formed angiogenic tumor endothelium is often characterized by a low level of pericyte coverage (22, 23). Treatment of tumors with angiogenesis inhibitors in animal models is reported to normalize this phenotype resulting in more pericyte-supported vessels (22). We investigated whether this phenomenon is also observable in human

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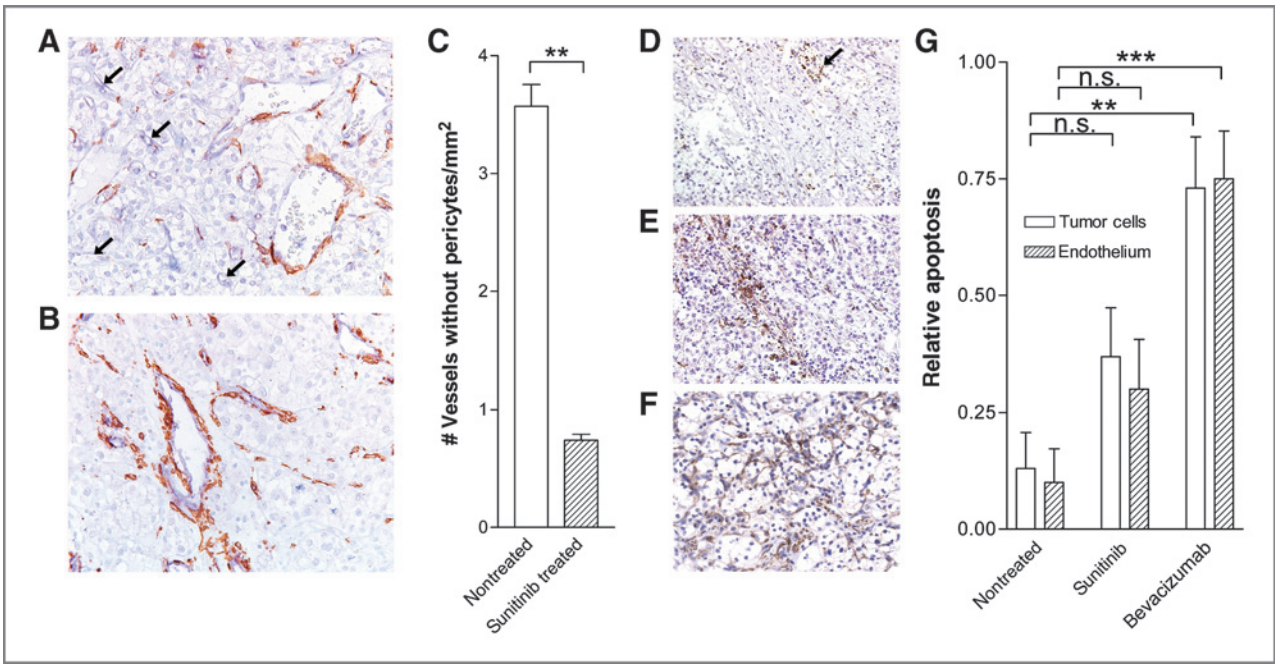

Figure 2. Pericyte coverage and apoptosis is enhanced after sunitinib treatment. A and B, CD31/34 (blue) and SMA (brown) stained RCC tissues. Arrows in A indicate small blood vessels that are not associated with pericytes. C, quantification (means ± SEM) of the number of vessels lacking pericyte coverage (identified by arrows, ** represents statistical significance, $P < 0.0001$). D–F, PARP staining (arrow in D) of nontreated, sunitinib-treated, and bevacizumab-treated tumors, respectively. G, quantification of the PARP staining in tumor and endothelial cell compartments (mean ± SEM, **, $P < 0.002$; ***, $P < 0.001$).

primary tumors treated with sunitinib and bevacizumab, by double staining for CD31/34 and SMA. In the tumors of nontreated control RCC patients, many microvessels were found lacking support of pericytes (Fig. 2A). Interestingly, in the sunitinib-treated tumors, this number was markedly lower, showing a 4.8-fold decrease and suggesting a significant antiangiogenic and vascular maturization effect (Fig. 2A–C).

Staining for PARP was used to monitor for apoptosis induction by the treatment. A small percentage of PARP staining (1%–2%) cells was visible in the nontreated control tumors (Fig. 2D). Sunitinib treatment led to an enhanced number (approximately 3-fold increase) of $PARP^+$ apoptotic cells, both in tumor cells as well as in endothelial cells, although for neither compartment this reached significance (Fig. 2E and G). This parameter increased by more than 7-fold in the tumors of patients treated with bevacizumab and did reach significance for both the tumor ($P < 0.01$) and endothelial cell compartments ($P < 0.001$, Fig. 2F and G).

In addition, we analyzed a potential correlation with pre- and posttreatment volume of tumor necrosis for some of the sunitinib-pretreated tumors. We were not able to find an association (data not shown).

Sunitinib treatment suppresses several key angiogenesis regulators in the primary tumor

To get more insight into the molecular regulation of the effect of sunitinib, real-time quantitative RT-PCR was carried out on RNA of frozen tumor tissue of sunitinib-treated RCC patients, and results were compared with nontreated cryotissue. It was found that gene expression of VEGFR-1 and VEGFR-2, ANG-1 and ANg-2, and PDGF-B was significantly suppressed in the sunitinib-treated tumors. In contrast, VEGF itself and 2 other cytokines PLGF and EGF remained unchanged. Expectedly, the message for the endothelial specific markers VE-cadherin and CD31 were suppressed, independently confirming the inhibition of vessel density after sunitinib treatment (Fig. 3).

Increased number of proliferating endothelial cells after sunitinib, but not after bevacizumab treatment

Because ongoing angiogenesis can also be quantified by the number of proliferating endothelial cells, we quantified the amount of $CD31/34^+/Ki\text{-}67^+$ cells. Considering all the suppressed parameters in pretreated tumors, as described above, we expected a decrease in the number of proliferating endothelial cells after the sunitinib treatment. Interestingly, however, a markedly increased number of proliferating endothelial cells was observed by standard immunohistochemistry, as well as fluorescence microscopy (Fig. 4A and C, respectively) in the tumors of patients that received sunitinib pretreatment. A larger than 4-fold significant ($P < 0.003$) increase in Ki-67–positive endothelial nuclei was noted (Fig. 4D) in these patients as compared with nontreated control patients. This increase of proliferating endothelial cells was not observed in bevacizumab-pretreated patients (Fig. 4B and D).

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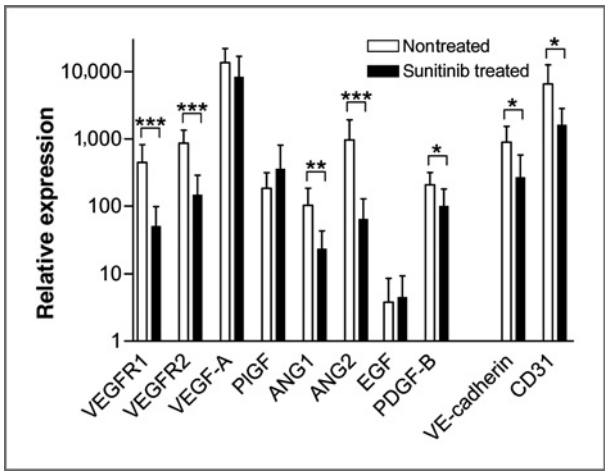

Figure 3. Regulation of gene expression in the primary tumor tissue after treatment with sunitinib. Real-time quantitative PCR was carried out for 10 angiogenesis-related genes, that is, VEGF receptor-1 and VEGF receptor-2, VEGF-A, PLGF, ANG-1 and ANG-2, EGF, PDGF-B, VE-cadherin, and CD31. Measurements were carried out in RNA preparations of cryotumor tissue of sunitinib-treated ($n = 12$) and untreated ($n = 5$) patients. Relative expression is shown (mean ± SEM) compensated for the expression of 2 household genes (β-actin, cyclophilin A). Statistical significance is indicated by *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Relationship to discontinuation of therapy

An explanation for the enhanced number of growing endothelial cells after sunitinib treatment is that all patients stopped therapy before surgery, and that halting the treatment with sunitinib may induce and unleash a compensatory boost of endothelial cell proliferation. To investigate this possibility, correlation between the number of proliferating endothelial cells and the number of days from halting therapy to the moment of cytoreductive surgery was investigated. Indeed, a strong and significant positive correlation ($P < 0.001$) was found (Fig. 5A). This correlation was not observed for microvessel density, a parameter that would take more time to adapt (Fig. 5B). Clinical observations of rapid regrowth of tumors after discontinuation of sunitinib treatment were reported. We believe that the boost of endothelial growth may explain this observation.

To understand the molecular regulation of this response, the real-time qPCR data, as described above, were also correlated to the interval to surgery. Interestingly, for VEGFR-2, ANG-2 (both plotted in Fig. 5C), PLGF, and PDGF-B, a significant positive association between the number of proliferating endothelial cells and the number of days from halting therapy to the moment of cytoreductive surgery was observed (Table 2), further supporting the concept of a rapid onset of angiogenesis after discontinuation of sunitinib therapy.

Discussion

Historically, the treatment of mRCC was unsatisfying, with a lack of meaningful response to most chemotherapeutic agents and radiotherapy. With the advent of agents targeting VEGF signaling, a substantial improvement in response rate and PFS was observed. As an example, sunitinib showed an impressive response rate of approximately 40% (10). Sunitinib is a tyrosine kinase inhibitor that blocks the signaling pathways of a series of growth factor receptors, among which are VEGFR-1 and VEGFR-2, PDGFR-b, c-KIT, and FLT-3. Half-life of this agent is 40 to 60 hours. The "off-target" effects of sunitinib conspire to produce considerable side effects in patients receiving these agents (24). These side effects make it necessary to administer therapy with an interrupted cycle of 4 weeks on treatment and 2 weeks off treatment. There is clinical evidence that metastases occasionally regrow considerably during the 2-week treatment break (25, 26). Elegant preclinical work has shown that treatment of the RIP-Tag2 mouse tumors or implanted Lewis lung carcinomas with sunitinib resulted in depletion of the tumor endothelial cells, but that rapid regrowth occurred when treatment was discontinued (27). These findings parallel what may be occurring in the human tumors assessed in the current study. Bevacizumab, which is an anti–VEGF-A antibody approved for the treatment of advanced RCC, has a half-life of 21 days. This significant difference in pharmacokinetics can have a significant effect on tumor endothelial growth kinetics.

We found that angiogenesis is clearly inhibited in the primary tumor in response to sunitinib. Several lines of evidence support this observation: (i) A lower tumor microvessel density was present in sunitinib-treated patients, as

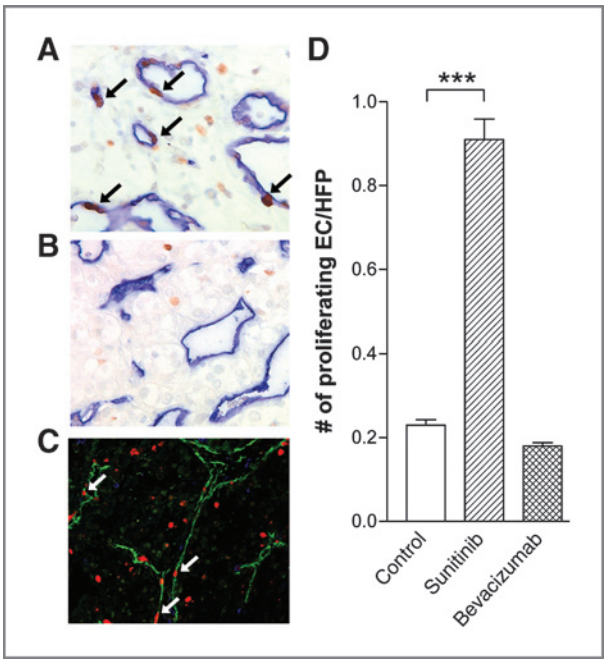

Figure 4. Enhanced numbers of proliferating endothelial cells in tissues of sunitinib-treated patients. CD31/34 and Ki-67 staining by standard light microscopy (blue and brown, respectively, arrows identify proliferating nuclei of endothelial cells) and immunofluorescence microscopy (green and red, respectively, arrows identify proliferating endothelial cells) in tumor tissues of sunitinib-treated patients. Number of nuclei of double stained cells is quantified in C (mean ± SEM, ***, $P = 0.0031$).

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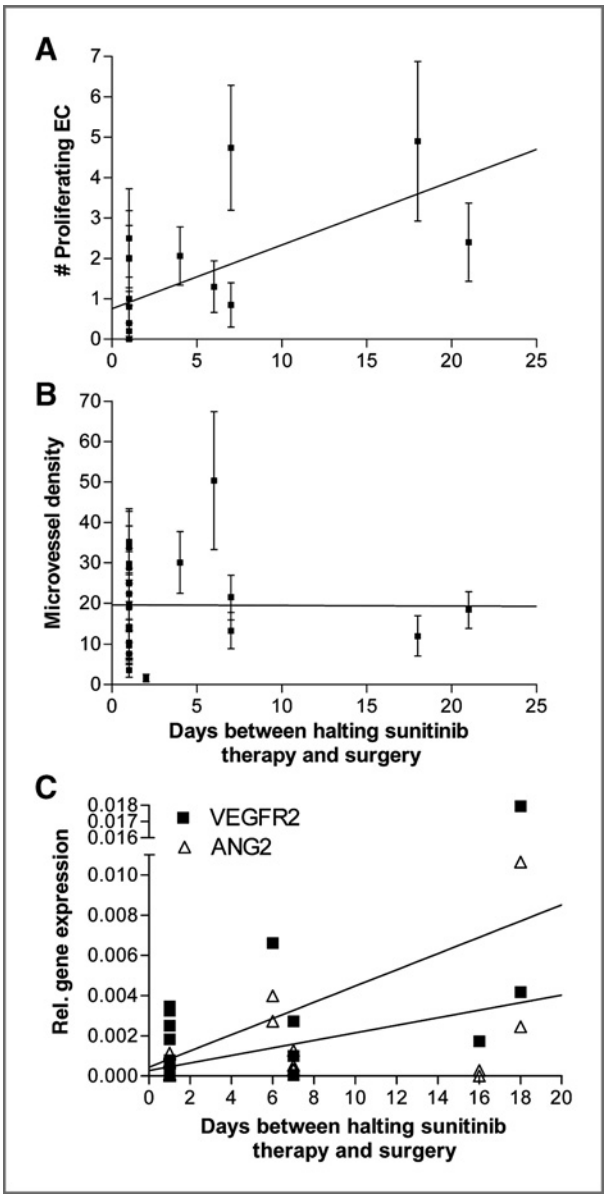

Figure 5. Onset of angiogenesis after discontinuation of sunitinib treatment. Positive correlation ($P < 0.001$) between the number of proliferating endothelial cells with the time interval between treatment stop and cytoreductive surgery (A) and lack of this correlation with microvessel density (B). C represents the linear regression analyses of the gene expression with the time period between halting the sunitinib therapy and surgery. The correlation plots in C shows the most significantly correlating genes *VEGFR2* and *ANG2*. Correlation and *P* values for these and the other tested genes are shown in Table 2.

compared with nontreated controls. This further supports the observations by Mancuso and colleagues (27) that the plasticity of vessels can be very high. Turnover of tumor cells is also high, illustrated by the number of proliferating tumor cells. The high activity in the tumor tissue underlies the rapid responses in vessel density. (ii) The number of highly angiogenic blood vessels, visible by their lack of pericyte coverage, was significantly reduced in sunitinib-treated

Table 2. Correlations between qPCR-based gene expression assessment and the number of days from halting sunitinib therapy

	R (correlation)[a]	Slope[a]	*P*
VEGFR1	0.302	0.0001	0.184
VEGFR2	0.580	0.0004	0.006*
VEGF-A	−0.263	−0.0125	0.25
PLGF	0.521	0.0012	0.015*
ANG1	0.093	0.0000	0.689
ANG2	0.593	0.0002	0.005*
EGF	−0.014	0.0000	0.952
PDGFB	0.502	0.0002	0.02*

[a]Correlation assessments conducted for VEGF receptor-1 and VEGF receptor-2, VEGF-A, placental growth factor, ANG-1 and ANG-2, EGF, platelet-derived growth factor, respectively. *Statistical significance.

tumors. This is a somewhat paradoxical finding, as PDGF inhibition would be expected to decrease pericyte coverage, but this effect may be counterbalanced by the normalizing effect of VEGF blockade in RCC. In addition, our study enumerated the number of vessels without pericytes, representing the population of newly formed vessels, rather than the total number of pericytes. We conclude that vessel neoformation is inhibited. As we did not enumerate the total number of pericytes in the tissue, we cannot draw any conclusion on the direct effect of sunitinib on pericytes. Treatment of primary tumors with combination VEGF and PDGF receptor therapy in an animal model of breast cancer led to decreased pericyte coverage and increased number of metastases (28). Whether the association between pericyte coverage and clinical outcome holds true for RCC needs to be further studied. (iii) Sunitinib induced an increased number of PARP-positive cells, not only in the tumor cell compartment but also in the endothelial cell fraction, suggesting death of newly formed blood vessels. (iv) The expression of several key angiogenesis regulators is found to be high in nontreated tumor tissues and markedly repressed as a result of sunitinib treatment.

The most important finding of this study is the enhanced number of proliferating endothelial cells in the tumors of sunitinib-treated patients, which was not observed in bevacizumab-treated patients. This parameter has been described as a better parameter of ongoing angiogenesis than microvessel density (29, 30). One explanation is that an apparent induction of angiogenesis is illustrated by the sunitinib-induced synchronization of endothelial cells in a certain stage of cell cycle, for example, G_2–M, in which cells are positive for Ki-67. Such a situation has been suggested for treatment of endothelial cells with the angiogenesis inhibitor platelet factor-4 (31). This would then mean that the cells are not growing and undergoing perfect angiostasis. Staining of cell cultures for DNA with propidium iodide, before and after sunitinib exposure, however, did not reveal

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any synchronization of cell cycle (data not shown). The only other explanation is therefore the presence of a compensatory boost of angiogenesis after discontinuation of the treatment, a conclusion that is supported by the finding of the molecular upregulation of angiogenesis mediators (Figs. 3 and 5). The observed rapid onset of neovascularization after discontinuation of presurgical treatment of patients in the tumor tissue may partly play a role in the clinical observation of rapid progression in 36% of patients pretreated with sunitinib within a period of a median of 29 days following nephrectomy in primary metastatic disease (15, 32). This relatively high frequency within a short period of time with most patients (73%) stabilizing again under reinitiation of treatment suggests that discontinuation rather than resistance to sunitinib is the cause. However, in preclinical models and clinical practice, intermittent higher dose therapy with short-term discontinuation has been beneficial. In an animal xenograft model, higher dose intermittent sorafenib was superior to continuous sorafenib in controlling growth of 786-0 cells implanted in the flank of nu/nu mice (33). A randomized study comparing sunitinib 50 mg on a 4 week on, 2 week off schedule versus 37.5 mg continuous dosing showed a trend toward improved outcome for the intermittent dosage group (34). Therefore, the relative benefit of higher dose therapy with breaks versus continuous lower dose treatment may be in favor of intermittent therapy, except for the subset of patients with highly aggressive disease who experience a significant rebound effect. Another explanation may be that the commonly practiced discontinuation of 2 weeks is too short a period in the majority of patients to observe clinical progression. Case reports have been published of rapid disease progression days after discontinuation of sunitinib (25, 26), but this does not reflect clinical reality in the majority of patients. We observed in the period of presurgical interruption, which was shorter than 2 weeks with the exception of 2 cases, that though endothelial cell proliferation increases in primary tumor tissue, microvessel density did not. This suggests that a longer time off drug would be needed before an increase in microvessel density would ensue and subsequently translate into clinical progression. As most intermittent treatment protocols reinitiate therapy after 2 weeks, it is likely that endothelial cell proliferation is abrogated before onset of new blood vessels. Currently, presurgical treatment protocols require a period off treatment in the recovery period following cytoreductive surgery. Together with presurgical discontinuation, this may add up to 6 weeks treatment interruption and may be longer in those with surgical complications. These patients may therefore be at risk of disease progression. The ongoing EORTC SURTIME trial compares immediate cytoreductive nephrectomy followed by an uninterrupted treatment with sunitinib 4 weeks on and 2 weeks off, until progression, to delayed cytoreductive nephrectomy after 3 cycles of sunitinib (4 weeks on, 2 weeks off) with a period off treatment of 4 weeks before continuation of treatment. The results of this trial will provide further data on the potential significance of prolonged treatment interruption in mRCC. Some insight into the effect of treatment discontinuation can also be elucidated from patients who have achieved CR after treatment with antiangiogenic therapy, with or without metastasectomy (35, 36), though it has to be acknowledged that these are highly selected patients with a favorable course of the disease. In each study, a number of individuals showed disease recurrence, underscoring the fact that these agents are not cytocidal against tumor epithelial cells and may eventually need to be restarted in the majority of patients. In another series of patients who had stable disease or better with antiangiogenic therapy, treatment discontinuation because of toxicity resulted in 63% of the patients progressing after a median of 10 months (37). Of these, 32% developed new metastatic sites.

This study has a number of limitations. The number of patients in the pretreated groups is very small and may not allow evaluation of statistically meaningful correlations between the expression of certain angiogenesis genes and outcome parameters or the time off treatment before surgery. Although in individual patients with a very short survival and rapid disease progression, upregulation of genes was comparable with the pattern observed in the untreated control group, these findings have to be interpreted with caution. The observed upregulation may reflect a primary or very early resistance to the antiangiogenic effect of sunitinib or rapid proliferation after withdrawal of sunitinib, but other confounding factors cannot be ruled out. Tumor heterogeneity may play an important role in the variation of expression with different patterns in the same primary tumor at the time of sampling. Ideally, pretreatment tissue would be compared with posttreatment tissue in the same patients. Unfortunately, in most cases, acquiring a sufficiently large pretreatment sample for robust analysis is quite difficult. In addition, a very recent publication described significant intratumor heterogeneity in RCC and suggests that pretreatment tumor samples may not represent the areas from which metastases originated (38). Consequently, they may not be representative when assessing pre- and posttreatment tumor changes, which may have implications for the interpretation of results from genomic profiling. In this study, we therefore carried out interindividual comparisons using full tumor sections of treated and untreated patients. Although intraindividual comparisons using pretreatment biopsy materials would have been possible, it was felt that having larger tumor areas to compare would be more accurate than comparing the small cores of Tru cut biopsies, which may indeed not be representative.

Acquiring tissue for analysis is obviously a challenge, and better techniques are needed. Ideally, noninvasive measures of early success with antiangiogenic therapy should be developed. These could include imaging modalities and measure of circulating factors and cells. Dynamic contrast ultrasound can be used to assess response to antiangiogenic therapy by evaluating area under the curve of blood flow (39). Imaging with 111In bevacizumab is also being developed and may be a useful tool for measuring tumor response (40). Multiplex bead-based analysis platforms

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may allow noninvasive measurement of the changes in tumor microenvironment induced by therapy (41).

Although these speculations may be preliminary, this work shows that different angiogenesis expression patterns can be detected between pretreated and untreated tumors and between different antiangiogenic agents. It is clear that larger numbers of patients are needed to rule out intertumoral variations and define association with clinical benefit.

Disclosure of Potential Conflicts of Interest

A. Bex has received honoraria from Speakers Bureau of Pfizer and is a consultant and advisory board member of Pfizer. E. Jonasch has received commercial research grant from Pfizer and is a consultant and advisory board member of Genentech.

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Study supervision: A.W. Griffioen, A. Bex

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