Soluble Tie2 and Flt1 Extracellular Domains in Serum of Patients with Renal Cancer and Response to Antiangiogenic Therapy

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ABSTRACT

Antiangiogenesis drugs can be difficult to evaluate because they produce disease stabilization rather than tumor regression. Markers of endothelial mass in tumors may be of value to monitor therapy and evaluate such drugs. Soluble domains of the endothelial receptor tyrosine kinases, sTie2 (angiopoietin receptor) and sFlt1 (vascular endothelial growth factor receptor-1) were analyzed by sandwich ELISA in serum samples from 43 patients with advanced renal cancer before and 1 month after antiangiogenic therapy with razoxane. Pretreatment sFlt1 levels were 0.77 ng/ml ± 0.48 (SD) and sTie2 74.3 ng/ml ± 15 (SD). Pretreatment sFlt1 levels above the median were associated with a lesser chance of stable disease ($P = 0.04$) and poorer survival ($P = 0.01$). Fall of sTie2 on treatment was associated with stable disease ($P = 0.05$) and improved survival ($P = 0.04$). The soluble receptors measured weeks before response were assessed and correlated with response and survival, showing they may be useful to monitor and develop antiangiogenic therapy.

INTRODUCTION

Angiogenesis is a key step in tumor growth, important for enlargement of the primary tumor and for the spread and growth of metastases. Renal cancer is one of the most vascular tumors and commonly presents with metastatic disease. High levels of VEGF[2] have been reported in primary renal tumors and serum of patients with renal cancer (1), probably related to the mechanism of transformation involving the Von-Hippel Lindau syndrome gene (2). Mutations in the VHL gene occur in the majority of hypernephromas or clear cell tumors, and this has been shown to result in up-regulation of VEGF production under normoxic conditions such that levels are similar to those normally induced by hypoxia (3).

Although cytokines such as interleukin-2 and IFN-α have antitumor activity in renal cancer and can prolong survival (4), the majority of patients with metastases die within 1 year. Thus, new treatment approaches are needed, and renal cancer represents a prime target for antiangiogenesis therapy.

One of the problems with antiangiogenesis therapy is assessing response, because inhibition of new vessel formation may not cause tumor regression but may stabilize tumor growth (5). Such responses are not readily recognized by standard response criteria; therefore, additional markers of antiangiogenic effects are desirable.

Tumor vascular endothelium shows up-regulation of receptors for angiogenic factors, including the VEGF receptor 2 (Kdr) and angiopoietin receptor, Tie2 (6). Two other endothelial receptors for angiogenic factors, the VEGF receptor 1 (Flt1; Ref. 7) and the orphan receptor Tie 1, are also up-regulated by hypoxia (8). These receptors, in common with many other tyrosine kinases, have external domains that can be cleaved or shed and released into the circulation as described for sTie1. A soluble variant of Tie2, presumably resulting from proteolytic activity, has also been demonstrated.[3] A soluble form of VEGF-receptor 1 (sFlt1), on the other hand, is known to be produced by alternate splicing. Thus, assays to detect soluble forms of these receptors may be markers of tumor vascular endothelial cell mass, and drugs that reduce or inhibit the endothelial growth may result in stabilization or reduction of such markers.

We have developed recently (9) assays for the soluble domains of Flt1 (sFlt1) and Tie2 (sTie2) and analyzed their concentrations in serum samples from patients with advanced renal cancer in a prospective study of the antiangiogenic drug, razoxane. Razoxane was initially synthesized as an EDTA analogue and found to inhibit metastasis and normalize blood vessels. It is p.o. active, has been used to treat a range of solid tumors, and is also a radiosensitizer (9, 10–14). We have related basal levels of soluble receptors and change on therapy to response to razoxane and survival. The results show that such markers may be useful as a new approach to evaluating antiangiogenesis therapy.

We recently conducted a Phase II trial of razoxane in advanced renal cancer and found 29% of patients had stable disease for 4 months or longer. To relate potential new markers of angiogenesis to use of angiogenesis inhibitors clinically, we have studied soluble sFlt1 and sTie2 (15).

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[2] The abbreviations used are: VEGF, vascular endothelial growth factor; CI, confidence interval; LDH, lactate dehydrogenase; VHL, Von-Hippel Lindau.

[3] D. Marmé, P. Reusch, and B. Barleon, unpublished results.

Table 1 Characteristics of the patient population

	Treatment response	
No. of patients (% in row)	No change	Progressive disease
Total no. of patients	11	30
Sex		
Female	1	10
Male	10	20
Age		
<60	7	15
≥60	4	15
Performance status:		
0	9	17
1 or 2	2	13
Pretreatment		
Surgery		
Yes	1	13
No	10	17
Chemotherapy		
Yes	1	2
No	10	28
Radiotherapy		
Yes	3	4
No	8	26
LDH		
≤250	10	23
>250	1	7
No. of metastatic sites		
1	5	12
≥2	6	18
Time from diagnosis		
<1 year	6	5
≥1 year	5	25
High pretreatment sTie2	4	16
Low pretreatment sTie2	7	14
High pretreatment sFlt1	2	17
Low pretreatment sFlt1	9	13
Risk factors		
1	9	9
2	1	16
3	1	5

MATERIALS AND METHODS

Patients. Forty-three patients with advanced metastatic renal cancer were entered into a Phase II trial of oral razoxane. The protocol was approved by the local ethics research committee, and written informed consent was obtained from all of the patients. Razoxane was given 125 mg twice daily for 5 days each week in monthly cycles. Baseline staging investigations were repeated at 8 weekly intervals. Patients were categorized into risk groups depending on whether they had one, two, or three of the following poor prognostic factors: a performance status of 0 *versus* 1 or 2; time from diagnosis of more than 1 year or equal to and less than 1 year; and number of sites of metastasis was one *versus* more than one. Because it was anticipated that antiangiogenic therapy may produce stable disease as a main end point, we made a prospective definition of stable disease as 4 months without progression. The patient pretreatment characteristics categorized by response are shown in Table 1. Two patients were not evaluated for response; one withdrew for personal reasons, and one withdrew because of side effects. They are included in the survival analysis.

Controls. Forty-four patients (controls) had preoperative blood samples taken before surgery for benign disease, and 10 blood donors had repeated sampling of eight samples over 5 days (80 samples in total) to assess day-to-day variability of the assays.

Assays. Soluble Flt1 (sFlt1) was measured in a sandwich ELISA as described previously (16) with minor modifications. Mouse monoclonal antibody 11G2 was used as capture antibody at a concentration of 2 μg/ml. Recombinant sFlt1, comprising the NH_2-terminal five immunoglobulin-like loops of the extracellular domain, was used for calibration. Detection of bound receptor was performed with a polyclonal rabbit antiserum at a dilution of 1:1500. Incubation with a biotinylated goat antirabbit IgG polyclonal antibody followed by streptavidin-alkaline phosphatase resulted in color development.

A soluble form of Tie2[4] was measured in a sandwich ELISA using mouse monoclonal antibody αTEK16 as capture and the biotinylated monoclonal antibodies αTEK2 and αTEK9 as detection antibodies. The recombinant extracellular domain of Tie2, fused to human Fc (sTie2-Fc), was used as calibrator.

RESULTS

Blood Donor and Benign Disease Values Compared with Renal Cancer Patients before Razoxane. The mean sTie2 in blood donors was 55.2 ng/ml (± 7.2; SD), which was significantly lower than that in the pretreatment renal patients (74.3 ng/ml ± 15; $P > 0.0001$; unpaired *t* test). The mean sTie2 in controls was 56 ng/ml (± 6.4; SD), which was significantly lower than that in the renal cancer patients ($P > 0.0001$). The mean sFlt1 value in blood donors was 0.16 ng/ml (± 0.08; SD), which was significantly lower than that in the pretreatment renal patients (0.77 ng/ml ± 0.98; $P > 0.0001$; unpaired *t* test). The mean sFlt1 in controls was 0.65 ng/ml (± 0.41; SD), which was significantly lower than that in the renal patients ($P < 0.0001$). Thus, the proportional elevation of sTie2 was greater than that of sFlt1 in renal cancer patients.

Correlation of Pretreatment and on Treatment sTie2 and sFlt1 Levels. Forty-three patients had pretreatment samples, and 33 patients had paired on treatment samples. Pretreatment sTie2 values were 74.3 ng/ml (mean, SD ± 15) and after 1 month of treatment were 78.5 ng/ml (mean, SD ± 17.9). Pretreatment sFlt1 values were 0.77 ng/ml (mean, SD ± 0.98) and after 1 month of treatment 0.74 ng/ml (mean, SD ± 0.8).

There were significant correlations of the two receptors with each other, both pretreatment and on treatment. Also, for each individual receptor there was a stronger correlation of the pretreatment with the on-therapy values than between the receptors (Table 2).

Relation between Pretreatment sTie2 and sFlt1 Levels and Response to Razoxane Treatment. In this study, two patients (one of those with paired samples) were not evaluable for response. In 41 patients, there were 11 with stable disease for 4 months or longer and 30 with progressive disease. The on-treatment samples were analyzed at 4 weeks, before final as-

[4] P. Reusch, B. Barleon, K. Weindel, G. Martiny-Baron, A. Gödde, G. Siemeister, and D. Marmé. Identification of a soluble form of the angiopoietin receptor 7LE-2 released from endothelial cells and present in human blood, submitted for publication.

Table 2 Correlation of pre- and post-treatment markers with each other

Correlation coefficient (*P*)	Pre-sTie2	Pre-sFlt1	Post-sTie2
Pre-sFlt1	0.36 (0.02)		
Post-sTie2	0.70 (0.00)	0.34 (0.05)	
Post-sFlt1	0.47 (0.01)	0.62 (0.001)	0.64 (0.001)

sessment of response at 16 weeks (the full details of the trial have been submitted elsewhere).

Elevated pretreatment sFlt1 was associated with a lesser chance of stable disease, using the median value as a cut point (9 of 11 patients with stable disease had levels below the median; 13 of 30 patients with progressive disease had levels below the median; $P = 0.04$; χ^2 test). Using continuous data, the odds ratio for progression was 0.17 (95% CI, 0.03–0.92; *P*, 0.04) low *versus* high in univariate analysis. Pretreatment sTie2 results were not related to response.

Multivariate analysis of sFlt1 for relation to response showed it was the second most significant factor after time from diagnosis (odds ratio, 0.17 for low sFlt1; $P = 0.08$).

Change of sTie2 and sFlt1 on Treatment and Response to Therapy. The 32 evaluable patients with paired samples were analyzed for the relationship of increase or decrease of sFlt1 or sTie2 and response. Overall, there was no significant difference in the pre- *versus* post-levels. However, for those with progressive disease, the sTie2 levels rose significantly (mean, 77.2 ± 14.2 SD to 83.6 ± 17.5 SD ng/ml; $P = 0.02$), and in those with stable disease, there was a nonsignificant fall (mean, 72.8 ± 19.2 to 66.9 ± 14.6 SD). The mean value of sTie2 was increased by 6.3 ± 12.9 ng/ml in patients with progressive disease and decreased by 5.8 ± 11.8 ng/ml in patients with stable disease. The change in sTie2 before and after the treatment was significantly different between patients with progressive disease and stable disease, $P = 0.03$. The pretreatment and posttreatment levels of sFlt1 did not change with response.

The percentage change compared with the baseline was also calculated. Changes in sTie2 were significant in univariate analyses, with a hazard ratio of 6.4 for an increase *versus* decrease ($P = 0.05$). However, the CI was wide because the numbers in the response group are small. In multivariate analyses of sTie2, including risk group 1 *versus* groups 2 and 3, sTie2 remained significant ($P = 0.035$) in contrast to the risk group category ($P = 0.112$). sFlt1 changes were not significantly associated with response.

The Relationship of Pretreatment sFlt1 and sTie2 and Change in Markers to Survival. In an univariate Cox model, the pretreatment value of sFlt1 in all of the 43 patients was significant in predicting the patient's survival. The hazard ratio was 2.24 (95% CI, 1.17–4.3; $P = 0.015$; Fig. 1*A*). The separation of survival groups is similar to that occurring with LDH (Fig. 1*B*) or performance status (Fig. 1*C*). Pretreatment sTie2 was not significant.

In multivariate analyses, nephrectomy ($P = 0.01$), LDH ($P = 0.03$), and performance status ($P = 0.01$) were significant, but sFlt1 was not, although the hazard ratio was similar to univariate analysis (1.55; Table 3).

In multivariate analyses including nephrectomy, LDH, and performance status, change in sTie2 was a significant prognostic factor for survival ($P = 0.04$; hazard ratio, 0.44 for fall in sTie2) with *P*s of 0.1, 0.06, and 0.05 for the other factors, respectively (Table 3).

DISCUSSION

This is the first study to analyze pretreatment soluble receptors for Flt1 and Tie2 and changes in them and to relate them to response to antiangiogenic therapy. The results show that each receptor has a different relationship to response and survival. Pretreatment sFlt1 levels were related to response and survival, but percentage change on therapy was not. In contrast, pretreatment sTie2 levels were not related to response or survival, but percentage change was related to both. This suggests that each receptor is measuring a different aspect of tumor vascular biology. There was a significant association of the concentration of the receptors with each other. There are many possible explanations for this, one being that they reflect a common origin for a component of each one from vessels. However, detailed evaluation of normal and tumor distribution will be necessary to further understand the relationship.

Tie2 is known to be up-regulated in tumor vasculature (17, 18) and has two ligands, angiopoietin 1 and 2, involved in stabilization or remodeling of vessels. In brain tumors, Tie2 was specifically up-regulated and expressed in endothelium, as was the ligand angiopoietin 2 in a subset of vessels, in contrast to angiopoietin 1, which was in tumor cells (18). Angiopoietin 1 produced sprouting angiogenesis *in vitro* (19), and both ligands are involved in different steps of angiogenesis (20). VEGF is an important factor cooperating with angiopoietin 2 to maintain new vessel development (21, 22) and was necessary to demonstrate the effects of both Tie2 ligands in corneal assays (23). Tie2 is also important in embryonic growth for angiogenesis (24). The use of soluble Tie2 external domain delivered systemically blocked tumor vessel growth in a rat cutaneous tumor model (25) and also growth of primary tumors and metastases (26). However, the effects of the endogenous external domain are unknown. It is clear that the majority must originate in normal tissues, because the concentrations in normal controls were quite similar to those in the patients.[5] This is consistent with the finding that the receptor Tie2 is expressed constitutively on endothelial cells, even on the resting vasculature, and can be shed from the cells by a proteolytic process, as already described for the related receptor Tie1. Thus, the pretreatment levels of sTie2 most likely reflect the presence of the receptor throughout the body. The changes of sTie2 during antiangiogenic therapy would then reflect the activation status of the tumor vessels.

The change in serum levels at 1 month correlated with the conventional staging examination at 4 months, suggesting that the changes were an early reflection of differences in blood vessel activation and, hence, in tumor growth. Production of more normal vessels, as shown in animal models for razoxane (9), would be expected to down-regulate Tie2.

[5] D. Marmé, P. Reusch, and B. Barleon, unpublished data.

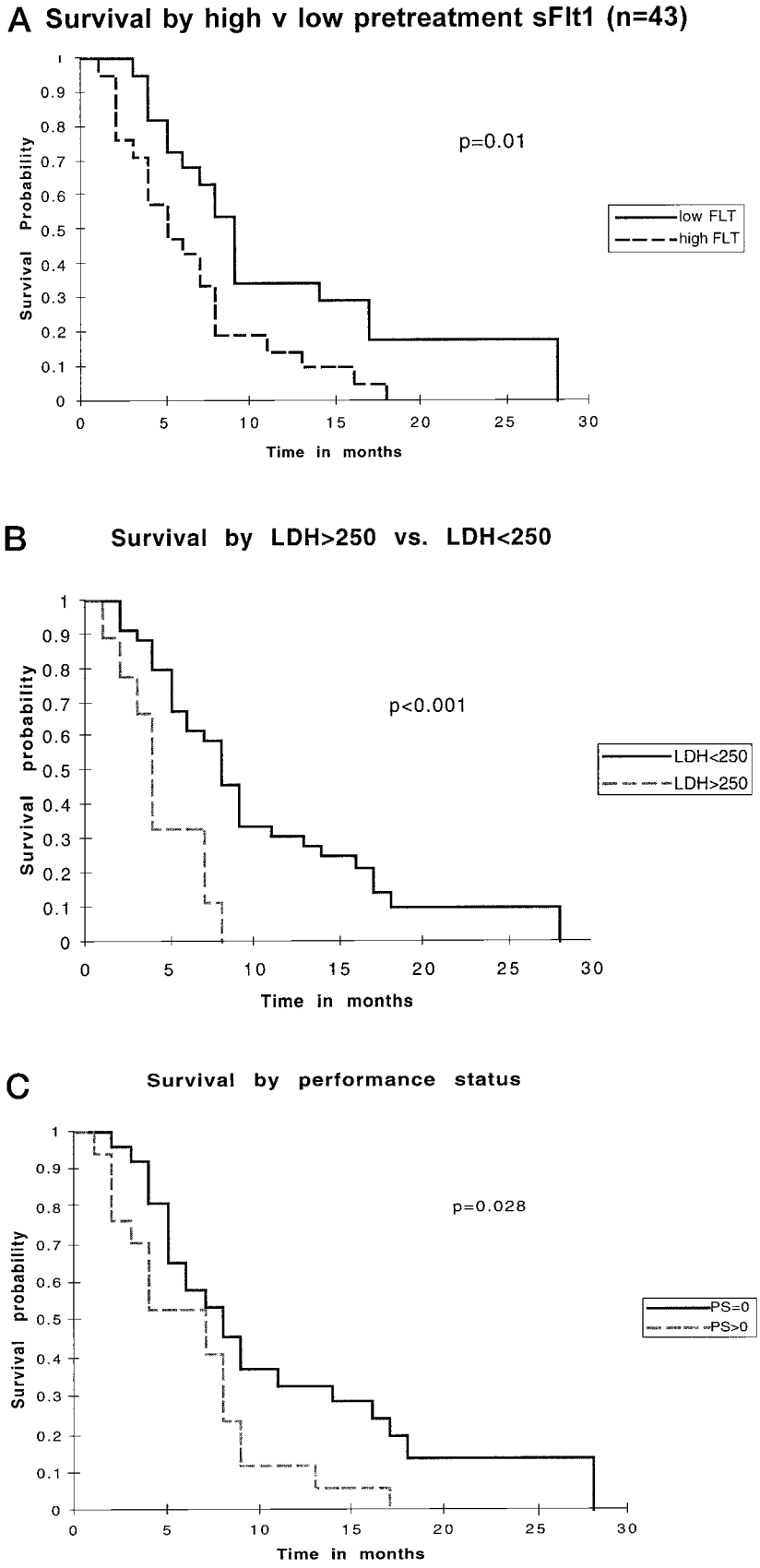

Fig. 1 Survival curves for patients stratified by sFlt1 (*A*), LDH (*B*), or performance status (*C*).

Table 3 Multivariate analysis of soluble angiogenic receptors and survival

	Comparison	Hazard ratio	*P*	95% CI
All of the patients ($n = 43$)				
Pre-sFlt1	High *vs.* low	1.55	0.25	0.73–3.29
Pre-sTie2	High *vs.* low	0.71	0.38	0.33–1.53
Radiotherapy	Yes *vs.* no	0.44	0.12	0.16–1.22
Nephrectomy	Yes *vs.* no	0.34	0.01	0.15–0.79
LDH	>250 *vs.* ≤250	2.80	0.03	1.11–7.04
Performance status	1 *vs.* 0	2.57	0.01	1.23–0.37
33 patients with serial assays				
Change sTie2	Decrease *vs.* increase	0.41	0.04	0.17–0.98
Nephrectomy	Yes *vs.* no	0.39	0.11	0.12–1.23
LDH	>250 *vs.* ≤250	3.09	0.06	0.94–10.2
Performance status	≤1 *vs.* 0	2.29	0.05	1.00–5.24

In contrast, Flt1 is expressed on vessels but also on macrophages, which are a significant component of most tumors, including renal cancers. Flt1 is expressed in the stroma of renal cancer (27). Also, several tumor types have been reported to express the Flt1 receptor on the epithelial component, *e.g.*, ovarian and bladder cancer. Thus, soluble domains may reflect both components and renal tumor mass rather than being markers for vessels alone. In other tumor types, macrophages have been associated with poor prognosis (28), and they produce many proangiogenic factors. Flt1 may be present in the tumor, the vessels, or the stroma. Because VEGF has been shown to up-regulate Flt1, this might also explain high pretreatment values. Thus, pretreatment levels may be associated with poor prognosis and an additional nonvascular component. The latter would not respond to antiangiogenic therapy and would preclude use of the marker to assess response. Nevertheless, this group of patients with high pretreatment sFlt1 could be candidates for anti-VEGF therapy (29).

As with Tie2, soluble Flt1 has been used to inhibit tumor angiogenesis in experimental models (30), and the function of the soluble receptor is unknown. However, either soluble receptor can decrease angiogenesis, showing the close interaction of the pathways *in vivo.*

The overall changes in the markers were small, and this reflects the common finding on antiangiogenesis treatment of producing stable disease. Nevertheless, the markers that were measured weeks before the response was assessed correlated with response and survival in different ways.

Flt1 may be a useful marker to analyze drugs targeted to macrophages such as linomide that inhibit their migration into tumors. These studies need to be extended to different tumor types, because the vascular bed may differ in each organ, and the host microvascular environment can determine the morphology and function of the tumor vasculature, including expression of Flt1 in the vessels (31). Other conditions associated with angiogenesis and cell migration, *e.g.*, pregnancy, can result in release of a soluble extracellular domain from Flt1, as recently reported by Banks *et al.* (32), although it is not known if this is the same as the domain we have detected.

Our results suggest that these markers may be of value in assessing antiangiogenic treatments targeting different components of the vasculature or angiogenic process and could be incorporated into future assessments of antiangiogenic therapy.

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