Transforming Growth Factor β1 Is a Target for the von Hippel-Lindau Tumor Suppressor and a Critical Growth Factor for Clear Cell Renal Carcinoma

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ABSTRACT

The von Hippel-Lindau (VHL) tumor suppressor gene is mutated in patients with VHL disease and in the majority of patients with sporadic clear cell renal carcinoma (RCC). Overexpression of transforming growth factor (TGF)-β has been observed in patients with several cancers, including RCCs, with serum and urine levels correlating inversely with prognosis. We have demonstrated that the VHL tumor suppressor gene product represses TGF-β mRNA and protein levels (~3–4-fold) in 786-O RCC cells by decreasing the TGF-β mRNA half-life. Exogenously added TGF-β1 did not suppress the growth of 786-O cells in vitro, nor did the addition of neutralizing antibody (Ab) against TGF-β have any effect. Indeed, 786-O cells were found to express no TGF-β type II receptor protein, thus allowing them to escape from the negative growth control of TGF-β. In contrast to the in vitro data, neutralizing Ab to TGF-β inhibited tumorigenesis and, in some cases, repressed established 786-O tumors in athymic mice. Immunohistochemistry for von Willebrand's factor revealed a 3–4-fold lower tumor microvessel count in the mice treated with TGF-β Ab compared to controls, suggesting that the Ab was inhibiting angiogenesis. Our findings indicate that TGF-β1 is a novel target for the VHL tumor suppressor and that antagonizing its paracrine action may provide novel avenues for treatment of RCCs as well as other tumors that secrete TGF-β1.

INTRODUCTION

TGF-β1 is a polypeptide growth factor that belongs to a large family of structurally related growth factors referred to as the TGF-β superfamily. It is a multifunctional cytokine that plays a major role in morphogenesis, development, and tissue repair and in the pathogenesis of fibrotic diseases (1, 2). Whereas TGF-β1 was originally described as an inducer of anchorage-independent growth in fibroblasts, it is known to be a potent inhibitor of epithelial cell growth, although it can stimulate the growth of certain tumor cells (3, 4). The role of TGF-β in human malignancies is complex (2), and both paracrine and autocrine actions need to be assessed. TGF-β1 is elevated in several cancers including RCCs (5–7). Some tumor cells are sensitive to the growth-suppressive effects of TGF-β, whereas others are not (6, 8–11). In a recent study, 20 of 20 primary RCCs and 30 of 30 RCC cell lines expressed TGF-β1, and the majority of the cell lines were resistant to the growth-suppressive effect of exogenous TGF-β1 (12). Moreover, serum and urine levels of TGF-β1 and tissue expression of TGF-β1 mRNA in several cancers correlate inversely with prognosis, suggesting an important paracrine role for TGF-β1 in promoting tumor progression and possibly metastasis in vivo (13–17). RCC is the most common cancer of the kidney, occurring in over 27,000 individuals in the United States each year, and it is responsible for over 11,000 deaths annually (18). The treatment of RCC remains frustrating to the oncologist, and locally unresectable, metastatic disease has a dismal prognosis. There is a tremendous need to understand the basic biology of RCC and develop better therapeutic options. Most sporadic and hereditary RCCs (VHL -disease associated) show mutation and/or loss of both copies of the VHL gene (18). pVHL is lost in early atypical cysts, suggesting that pVHL might play a gatekeeper role in RCC development, analogous to the APC gene product in colon cancer (19, 20). VHL -disease-associated tumors are typically hypervascular and target genes identified to date include VEGF, TGF-α, and platelet-derived growth factor β, all of which have proangiogenic effects (21–24). TGF-β1 is another gene that is significantly involved in angiogenesis (25). Although TGF-β1 has been found to be elevated in RCCs (5–7, 12, 14), there has been no link to date with the VHL tumor suppressor, and no functional role has been ascribed to TGF-β1 for RCC growth in vivo.

We describe here that TGF-β1 is a novel target gene for pVHL and that pVHL-regulates the TGF-β1 gene at the posttranscriptional level. Furthermore, we provide evidence that antagonizing the effects of TGF-β1 suppresses RCC in vivo through an antiangiogenic mechanism.

MATERIALS AND METHODS

Plasmids and Cell Culture

We stably transfected RCC cells (786-O cells from the American Type Culture Collection) lacking the wt VHL gene (26) with an expression vector containing full-length VHL cDNA epitope-tagged in the NH2 terminus with FLAG sequence (pCMV2-FlagVHL) that has been described previously (22, 23). Pooled clones from these cell lines were used. 786-O clonal cell lines stably transfected with either pRC (786-O Neo), pRC-HAVHL (786-O HA VHL), or pRC-HAVHL (1–115), which lacks aa 116–213 (786-O HA ΔVHL), were gifts from W. Kaelin and have been described previously (26). 786-O FLAG VHL, 786-O Neo, 786-O HA VHL, and 786-O HA ΔVHL were grown in DMEM with 10% FBS and supplemented with G418. The wt 786-O and CCL-64 (mink lung epithelial cells) were grown in DMEM with 10% FBS. Transfected 786-O cells were a TGF-β1 (human lung carcinoma) and a 600-bp PCR-amplified fragment from human actin cDNA.

RNA Extraction and Northern Blot Analysis

These experiments were done as described previously (23). The probes used were a TGF-β1 2.1-kb EcoRI fragment (phTGF-β2 from the American Type Culture Collection) and a 600-bp PCR-amplified fragment from human actin cDNA.

ELISA Analysis of TGF-β1 Secretion

ELISA of cell culture supernatants obtained from confluent cultures was performed using the manufacturer’s recommended procedures (Quantakine kit; R&D Systems). All samples and standards were run in triplicate. Color intensity was measured at 450 nm using a spectrophotometric plate reader. Activation of latent TGF-β1 to immunoreactive TGF-β1 was performed according

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3 The abbreviations used are: TGF, transforming growth factor; RCC, clear cell renal carcinoma; VHL, von Hippel-Lindau; Ab, antibody; pVHL, VHL gene product; FBS, fetal bovine serum; wt, wild-type; aa, amino acid(s); VEGF, vascular endothelial growth factor; NK, natural killer; IL, interleukin; bFGF, basic fibroblast growth factor; GADPH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin.
to the manufacturer’s recommendations, and all samples were measured before (active TGF-β1) and after activation (latent TGF-β1).

**Western Blot Analysis**

Cells were grown to 70–80% confluence and lysed in lysis buffer as described previously (22, 23). Equal amounts of cell lysates (50 μg), as determined by the Bradford assay, were loaded. Abs included TGF-β type II receptor-specific Ab (Santa Cruz Biotechnology, Santa Cruz, CA), actin Ab (Santa Cruz Biotechnology), and VHL Ab (a gift from W. Kaelin).

**Nuclear Run-on in Vitro Transcription Assay**

Nuclei isolation and in vitro transcription were performed as described previously (22, 23). Probes were either gel-purified inserts from plasmids or generated by PCR. The TGF-β1 probe used (∼2 kb) contained the entire coding region of TGF-β1, including 779 bp of the 5' untranslated sequence and 216 bp of the 3' untranslated sequence. The amount of sample hybridized to the TGF-β1 probe was normalized by dividing the TGF-β1 signal by that of GAPDH within each experiment.

**Assessment of Growth Properties after Exposure to TGF-β1**

DNA synthesis was studied in triplicate by [3H]thymidine incorporation into DNA. Cells (2 × 10⁵) were plated in 24-well plates in DMEM with 0.5% FBS. At 24 h after plating, the medium was replaced with or without TGF-β1 (1 or 10 ng/ml; R&D Systems) and anti-TGF-β1, -β2, -β3 monoclonal Ab (10 μg/ml; Genzyme) for 48 h. In vitro and in vivo neutralization of this TGF-β1 Ab has been published previously (27–29). During the last 4 h, 2 μCi of [3H]thymidine (DuPont New England Nuclear) were added. Cells were then washed twice in PBS and lysed in 5% NaOH. [3H]-Thymidine incorporation of the cells was assessed in a liquid scintillation counter (Beckman).

**RCC Tumor Model**

The 6–8-week-old NIH-3-nude beige xid mice (Harlan Sprague Dawley) and nude mice (Harlan Sprague Dawley) were given s.c. injections in the right flank of 3 million 786-O cells in a 100-μl volume. Tumors appeared approximately 2 weeks after implantation. Tumor size was measured using calipers, and tumor volume was calculated using the following formula: volume = width² × length/2. Tumor volume ranged from 150–200 mm³. The animals were randomized into two groups of four mice each with comparable tumor size. Mice were then injected i.p. with 100 μg of monoclonal anti-TGF-β1, -β2, -β3 Ab (the same Ab used in vitro and described above) or control nonspecific IgG (Sigma) on alternate days. When treatment was terminated, animals were sacrificed, and tumors from each mouse were removed and divided into two pieces: one piece was fixed in 10% formalin and paraffin-embedded; and the other was cryopreserved in OCT. The paraffin-embedded specimens were stained with H&E and Masson’s trichrome stain and evaluated by light microscopy.

**Immunohistochemistry**

Factor VIII Staining/Microvessel Count. Six-μm-thick sections from cryopreserved tumor specimens were stained for endothelial cells using Factor VIII Ab (DAKO), using a standard immunoperoxidase technique as described previously (30). The microvessel count was done according to the method of Delahunt et al. (31). After the area of highest neovascularization was identified under low power (×10), individual microvessels were counted on four adjacent high-power fields (×40), and the mean microvessel count was determined. Any area showing positive staining for Factor VIII was considered to be a countable vessel, regardless of whether or not a distinct lumen was visible. In the tumors in which the microvasculature formed a dense network, each distinct branch was interpreted as a single vessel.

**RESULTS**

**VHL but not Mutant VHL Suppresses Endogenous TGF-β1 mRNA and Protein Levels in Stably Transfected RCC Cell Lines.**

Several candidate genes known to be up-regulated in RCCs (VEGF, TGF-α, IL-6, epidermal growth factor receptor, and TGF-β1) were analyzed by Northern blot in the 786-O RCC cell lines. The 786-O RCC cells, derived from a patient with sporadic metastatic RCC, lack wt pVHL (26). Strikingly, TGF-β1 mRNA levels were repressed ~4-fold in 786-O RCC cell lines stably transfected with wt VHL (786-O HAVHL) compared with levels in the same cell lines transfected with an empty vector (786-O Neo) or transfected with a mutant VHL lacking aa 116–213 (786-O HAVHL; Fig. 1A). To address the possibility of clonal variation, we confirmed the findings in wt 786-O cells and in 786-O FLAG VHL cells generated in our laboratory (Fig. 1A). Levels of protein expression from the stably transfected constructs were assessed by Western blot (Fig. 1B). ELISA confirmed that VHL-transfected cells secreted ~3–4-fold less latent and active TGF-β1 protein in the culture supernatant (Fig. 1C). These data indicate that both TGF-β1 mRNA and protein are repressed by pVHL, indicating that TGF-β1 mRNA is a target for pVHL.

**TGF-β1 Is Regulated by pVHL at the Posttranscriptional Level.**

TGF-β1 is regulated by a number of different factors including phorbol esters, estrogens, retinoic acid, steroids, and itself (32). The regulation of TGF-β1 expression occurs at both the transcriptional and posttranscriptional levels. Nuclear run-on in vitro experiments showed that the reintroduction of wt VHL in 786-O cells did not alter the transcription of TGF-β1 (Fig. 2A). Similarly, mutant VHL did not alter the transcription of the TGF-β1 gene (Fig. 2A). Experiments performed in the presence of α-amanitin showed an inhibition of TGF-β1 transcription, indicating the specificity of our experimental conditions (Fig. 2A). A shorter TGF-β1 probe (~700 bp in the 5' untranslated region) gave similar results (data not shown). Our nuclear run-on data suggest that the regulation of TGF-β1 by VHL is predominantly posttranscriptional, e.g., at the level of RNA splicing, nuclear export, or mRNA stability.

We next studied TGF-β1 mRNA stability in 786-O Neo, 786-O HAVHL, and 786-O HA ΔVHL cells by measuring the decay of TGF-β1 mRNA in the presence of the transcriptional inhibitor actinomycin D. The TGF-β1 mRNA half-life was 11.48 h in VHL-transfected cells compared to 39.36 h in mutant VHL-transfected cells and 25.92 h in empty vector-transfected cells (Fig. 2B). Proof that actinomycin D was active during the early time points of the experiments came from probing the blots with a c-myc probe: the signal fell dramatically and was undetectable at 2 h (data not shown). These results suggest that pVHL destabilizes TGF-β1 mRNA.

**786-O RCC Cells Are Unresponsive to Exogenous TGF-β1 in Vitro and Lack Functional TGF-β Type II Receptor.**

TGF-β1 binds with high affinity to the type II receptor. This binding is followed by recruitment of the type I receptor and subsequent intracellular signal transduction (2). We checked whether the RCC cell lines were growth responsive to exogenous TGF-β1. We found that the growth of 786-O cells was unaffected by exogenous TGF-β1 at 1
were grown to 70–80% confluence in 100-mm dishes (Fig. 1A) after 10 days. Interestingly, one of the four tumors in the treated group had a striking difference in tumor size when compared to controls (Fig. 2). We then examined the expression of the TGF-β type II receptor protein in 786-O cells. Western blot analysis revealed no TGF-β type II receptor protein in 786-O Neo and 786-O HAVHL cells. Mink lung epithelial cells expressing functional TGF-β type I and II receptors were used as a positive control (Fig. 3B). These data suggest that the loss of type II receptor expression in 786-O cells is responsible for their unresponsiveness to TGF-β in vivo.

Neutralizing Ab Against TGF-β Inhibits Angiogenesis and RCC Tumor Growth in Athymic Mice. To assess the biological significance of elevated TGF-β1 levels in RCCs, we used a xenograft athymic mouse model, because there is no syngeneic model for VHL-associated RCCs. Because TGF-β affects T-cell and NK cell activity, we injected 786-O RCC cells s.c. into the thigh region of nude beige xid (T-, B-, and NK cell-deficient) mice and allowed the tumors to grow to 150–200 mm³. A total of 100 μg of the TGF-β monoclonal Ab (used in Fig. 3A) or a control Ab were injected i.p. on alternate days for five doses. Animals treated with TGF-β Ab showed a striking difference in tumor size when compared to controls (Fig. 4A) after 10 days. Interestingly, one of the four tumors in the treated group regressed to a small nodule of <50 mm³ (data not shown). At the end of the experiment, the mice were sacrificed, and the tumors were harvested and pathologically examined. There was no difference between treated and control groups on H&E stain (no infiltrating cells noted) and on Masson’s trichrome stain. Areas of patchy necrosis in the rapidly growing control tumors were noted (data not shown). Importantly, Factor VIII staining showed a 3–4-fold decrease in the number of microvessels in the treated group (Fig. 4B and C). Furthermore, the proliferative index of the treated group was 3-fold lower than in the controls, with no change in the apoptotic index (Fig. 4C). These data suggest that the primary mechanism of tumor suppression through the use of TGF-β neutralizing Ab is the inhibition of angiogenesis.

To assess whether tumor cells would develop resistance to TGF-β Ab treatment and whether larger tumors (250–300 mm³) could be treated with the same dose, we conducted a separate experiment. As before, therapy with TGF-β Ab (two animals) or control Ab (two animals) was stopped after 10 days (Fig. 4D). Both tumors regressed in the treated group over this time period but regrew rapidly upon cessation of therapy. Therapy was reinstituted when the mean tumor size was 280 mm³, and tumor growth was again suppressed with a second course of TGF-β Ab. Collectively, these data suggest that antagonizing TGF-β in vivo significantly inhibits RCC tumors in athymic mice, with regressions seen in 4 of 10 mice treated. Moreover, these tumors do not develop resistance to this therapy, consistent with the microvessel count data suggesting inhibition of angiogenesis as the therapeutic mechanism involved.

**DISCUSSION**

We report several novel findings: (a) we have identified TGF-β1 as a target for tumor suppressor pVHL in RCC cells; (b) we have shown that this regulation is at the level of mRNA stability; and (c) we have addressed the biological significance of elevated TGF-β1 in RCCs both in vitro and in vivo. We have shown that 786-O RCC cells lack functional TGF-β type II receptors, are growth resistant to the action of TGF-β1, and are unaffected by a neutralizing Ab to TGF-β in vivo. In contrast, Ab to TGF-β in vivo inhibited angiogenesis, leading to 786-O RCC tumor growth arrest and regression.

The human VHL gene encodes a 213-aa protein and is expressed in all tissues. VEGF, platelet-derived growth factor, and glucose transporter-1 were the first target genes identified for pVHL (21, 23, 24). We have recently found that TGF-α is another target gene for pVHL (22). Here we report that both TGF-β1 mRNA and protein levels are suppressed by wt VHL in two 786-O cell transfectants. These data provide the first connection between a tumor suppressor product and TGF-β1. Given the biological actions of TGF-β1, its up-regulation in cells with mutant VHL could promote tumor progression in multiple ways: by promoting angiogenesis; by enhancing stromal proliferation; by increasing collagenase and gelatinase B activity; and by suppressing the immune response to the tumor (detailed below). Indeed, the concomitant dysregulation in RCCs of TGF-α, VEGF, and TGF-β1, which are all VHL targets, may be particularly efficacious in this context.

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**Fig. 1.** wt pVHL inhibits TGF-β1 expression. A, total RNA (20 μg) from wt 786-O cells and 786-O cells transfected with expression vectors containing empty vector (Neo), HA-tagged VHL (HA VHL), HA-tagged mutant VHL lacking an 116–213 (HA ΔVHL), and FLAG-tagged VHL (Flag VHL) was size separated, blotted, and probed with 32P-labeled TGF-β1 cDNA. VHL transfection significantly repressed the TGF-β1 message (~2.5 kb). Fold inhibition was calculated by densitometry using actin (bottom panel) as a normalization control. B, expression of HA-tagged and FLAG-tagged pVHL and pΔVHL was confirmed by Western analysis using a monoclonal VHL Ab. Each lane contains 20 μg of whole cell lysate from the stably transfected 786-O cells indicated in A. C, 786-O Neo, 786-O HAVHL, and 786-O ΔVHL cells (as described in A) were grown to 70–80% confluence in 100-mm dishes (~1 × 10⁶ cells) and cultured in 10 ml of DMEM without FBS for 48 h. The supernatant was collected and cleared by centrifugation. Levels of TGF-β1 in the medium were assayed by ELISA using the Quantakine kit (R&D Systems). Results show a 3–4-fold down-regulation of latent and active TGF-β1 protein in the supernatant of cells transfected with VHL. Results are expressed as the mean ± SE of three independent experiments and were statistically significant (P < 0.01 using ANOVA).
Fig. 2. pVHL decreases TGF-β1 mRNA stability in stably transfected RCC cell lines. A, pVHL does not modify the transcription rate of the TGF-β1 gene. The autoradiogram shows one representative experiment. Nuclei from 786-O Neo, 786-O HAVHL, and 786-O HA ΔVHL cells were isolated, and nuclear run-on assays were performed in the presence of [32P]UTP for 30 min. The nascent 32P-labeled transcripts were hybridized to slots of filter-bound TGF-β1, actin, and GAPDH cDNA fragments. A control experiment with the addition of α-amanitin in the in vitro transcription assay confirmed that the detected transcripts were produced by RNA polymerase II. The filters were scanned, and radioactivity was measured on a Molecular Dynamics PhosphorImager. The transcription rate of TGF-β1 was normalized to the transcription rate of GAPDH within each experiment. The relative TGF-β1 transcription rate was 94.35 in the VHL-expressing cells and 103.88 in the ΔVHL-expressing cells, compared to an arbitrary value of 100 in the Neo-transfected cells (values were not statistically different). B, pVHL decreases the half-life of the endogenous TGF-β1 message in stably transfected cells. Subconfluent 786-O cells were incubated with actinomycin D (5 μg/ml) for various time points from 0–24 h. Total RNA was isolated and analyzed by Northern blot hybridization with 32P-labeled TGF-β1. The TGF-β1 message was quantitated by densitometry, normalized to 28S RNA, and plotted in a graph against time. Linear regression analysis indicated that the half-life of TGF-β1 was 25.92 h in 786-O Neo cells, 39.36 h in 786-O ΔVHL cells, and 11.48 h in 786-O VHL cells. Results are the mean of four independent experiments, and the regression coefficients (r) are shown.

regard, because all have proangiogenic actions, and TGF-α, in addition, can act to directly stimulate tumor growth through an autocrine loop. The mechanism of action of pVHL is poorly understood. It has been shown that pVHL interacts in vitro with the regulatory subunits elongin B and elongin C of the transcription elongation complex (elongin SIII); however, none of the target genes identified thus far, including TGF-β1, appears to be regulated at the level of transcriptional elongation. Moreover, VHL interacts with Hs-Cul-2; its yeast homologue Cdc 53 is part of a ubiquitin protein ligase complex that targets cell cycle proteins for degradation by the ubiquitin proteolytic pathway (33, 34). Collectively, these data point to a role for complexes containing pVHL, elongin B, elongin C, and Cul-2 to target certain proteins, perhaps RNA-binding proteins, involved in the degradation of target mRNAs (33). Interestingly, VEGF, TGF-α, and TGF-β1 are all regulated largely at the level of the mRNA half-life, and it will be interesting to investigate whether the pVHL-elongin-Cul-2 complex is involved in controlling their degradation through some common mechanism, especially because the mutant COOH terminus VHL truncation that lacks the elongin C binding site (ΔVHL) did not repress either of these targets (this study and Refs. 21–24).

Lack of a functional type II receptor and associated rearrangements of the gene or aberrations in transcription or in posttranscriptional steps have been described in several human tumors, including gastric, colon, breast, and T-cell malignancies (8, 35–39), but not in RCC. In this report, we have shown that 786-O cells do not express the type II receptor protein and are functionally resistant to the TGF-β signal transduction. Studies are in progress to characterize the nature of the genetic defect leading to the absence of the type II receptor protein in RCC.

TGF-β1 is up-regulated in several cancers. To date, studies correlating prognosis with TGF-β1 levels in different cancers have not been consistent, although inverse associations have been found in hepatic, renal, cervical, and prostate cancer (13–17, 40). Some cancer cells also secrete activated TGF-β1 (41); however, RCCs predominantly secrete latent TGF-β1 (7, 12, 17). Moreover, when compared to primary cancers, cell lines derived from metastatic cancers can show greater TGF-β1 production and resistance to its growth-inhibitory effect but increased collagenase activity (42).

Multiple biological actions of TGF-β1 in the etiology of cancer can be invoked. The proangiogenic effects of TGF-β1 in potentiating tumor progression are likely to be important. Targeted disruption of either the TGF-β1 gene or its type II receptor results in defective placental vasculogenesis (43, 44). TGF-β1 regulates other angiogenic molecules, such as VEGF, TGF-α, and VEGF receptor flk-1 (1, 45), and has very recently been shown to inhibit the generation of the antiangiogenic protein an-
Fig. 4. Antitumor effect of neutralizing Ab against TGF-β in RCC tumors in nude mice. A, nude beige mice were injected s.c. with 786-O tumor cells. When the tumor volume was approximately 150 mm$^3$, therapy was started with neutralizing Ab against TGF-β (treated animals, $n = 4$) or control IgG (controls, $n = 4$) on alternate days (days 1–9), as indicated by arrows. The tumor size was measured on alternate days (days 1–11). Each time point represents the mean ± SE of four mice in each group. The difference (*) in tumor size on day 11 between the treated animals and controls was statistically significant ($P < 0.01$ using ANOVA). B, micrograph of s.c. tumors immunostained with Factor VIII under low power (×10). The control tumors show numerous microvessels (indicated by an arrow) forming a dense, lacy network (top panel), whereas the treated tumors show isolated microvessels (bottom panel). C, histological sections of tumors from TGF-β Ab-treated and control Ab-treated specimens were stained for Factor VIII (microvessel count) and Ki-67 (proliferative index) and assessed for apoptosis (apoptotic index). The differences (*) between treated and control tumors were statistically significant ($P < 0.05$) for the microvessel count and proliferation index, but not for the apoptotic index. D, cyclical therapy with TGF-β Ab. Nude mice (nu/nu) were injected s.c. with 786-O tumor cells. When the tumor volume was approximately 100 mm$^3$, therapy was started with TGF-β Ab ($n = 2$) or control Ab ($n = 2$) on alternate days (days 1–9), as shown by arrows. Therapy was then stopped and re-started from days 19–27, as shown by arrows. Tumors were measured on alternate days. Each time point represents the average results from two mice. The tumor size of the two control mice were 90 mm$^3$ (day 1), 467 mm$^3$ (day 13), and 1030 mm$^3$ (day 29; point not shown in the graph because it would be off the scale).
TGF-β1 is a Target for vHL

giostatin via modulation of the plasminogen/plasmin system (46). Although TGF-β1 inhibits endothelial cell proliferation in vitro, its overall effect in vivo is proangiogenic (25). An explanation of these seemingly discrepant results is that TGF-β1 plays an important role in the resolution phase of angiogenesis by directly inhibiting endothelial cell growth and migration and reducing extracellular proteolysis. Stable transfection of TGF-β1 confers a growth advantage on Chinese hamster ovary cells in vivo but not in vitro, accompanied by an increase in capillary density; local administration of neutralizing Ab to TGF-β1 reduced both capillary density and tumor growth (47). However, a large quantity of Ab (5 mg) was used to neutralize the overexpressed TGF-β1 produced by cancer cells, in contrast to the 100 μg of Ab used to antagonize endogenous TGF-β1 in our experiments. TGF-β1-mediated angiogenesis is contextual, i.e., in the presence of positive regulators such as VEGF and bFGF, an additive or synergistic angiogenic response is noted. Also, the effect of TGF-β1 on endothelial cell function is concentration dependent, i.e., VEGF- and bFGF-induced capillary invasion in an in vitro three-dimensional model was dependent on the concentration of TGF-β1 and was highest at 200–500 pg TGF-β1/ml (25). These facts may explain why the neutralizing Ab against TGF-β1 did not inhibit angiogenesis in a breast cancer model (48), whereas in the 786-O RCC model, VHL mutation leads to the up-regulation of multiple factors (VEGF, TGF-α, and TGF-β1) perhaps potentiating each other’s angiogenic effects.

Despite rapid progress in characterizing cell surface receptors for TGF-β, little is known about the function of TGF-β receptors in endothelial cells, and virtually nothing is known about the role of TGF-β ligand-receptor interactions in the regulation of angiogenesis in vivo. Endothelial cells respond equally to TGF-β1 and TGF-β3 and respond poorly, if at all, to TGF-β2, possibly due to the low affinity of endoglin (type III receptor) for TGF-β2 in endothelial cells. Although the Ab we used is type specific (against TGF-β1, TGF-β2, and TGF-β3), the effect we have seen is probably mediated by the neutralization of TGF-β1 because 786-O RCC cells do not express TGF-β3 and express very low levels of TGF-β2 mRNA (data not shown).

TGF-β1 may also enhance carcinogenesis by suppressing the immune response (1, 2, 48). In our nude beige mice RCC model, there are no T cells, B cells, and NK cells; hence, we believe that tumor suppression is predominantly a result of the antiangiogenic effect, as suggested by the microvessel count data. Experiments in syngeneic RCC models might elucidate an additional therapeutic benefit of helper and cytolytic T-cell activity and NK cell activity upon neutralization of TGF-β1 action. In this context, antagonizing TGF-β1 action may be particularly appropriate for the treatment of RCCs in man, because these tumors secrete numerous immunomodulatory cytokines in addition to TGF-β1, including IL-10 and IL-6 (14). These cytokines diminish T-cell responses to the tumor, and neutralizing part of their activity may therefore show unusual benefit in renal cancer therapy.

Several additional features of our tumor data are worth highlighting. First, anti-TGF-β1 therapy regressed established tumors. In 4 of 10 treated animals, tumors diminished to a size of ∼50 mm³, reminiscent of the results seen after therapy with endostatin, an antiangiogenic COOH terminus ~20-kDa fragment of collagen XVIII (49, 50). As with endostatin, the tumors regress quickly upon cessation of therapy and could again be treated successfully. These data demonstrate the lack of drug resistance but highlight the need for either chronic therapy or multimodality therapy (see below) to effect a cure. Second, on a molar basis, anti-TGF Ab was ~60× more effective than endostatin, based on published data in non-RCC (51) as well as our studies with endostatin in RCC using the same 786-O xenograft model (52). This number could be further increased with a higher-affinity Ab, or one with improved pharmacokinetics. Third, because endostatin appears to inhibit the initiation phase of angiogenesis (cell proliferation and migration; Ref. 52), whereas TGF-β1 is important in the resolution phase, it is conceivable that endostatin in combination with methods to neutralize TGF-β activity may be additive or synergistic. Similarly, TGF-β1 neutralization in combination with chemotherapy, immunotherapy, or radiation makes eminent sense. Also, it is noteworthy that the acquisition of TGF-β unresponsiveness correlates well with tumor progression (12, 42, 53), so that anti-TGF-β therapy will be the most useful in advanced disease, when other modalities are less likely to be effective. Fourth, there is the possibility that TGF-β1 induced by the process of surgery itself (54) or by radiation (55) may stimulate metastatic growth, especially if the metastatic lesions are resistant to the growth-inhibitory activity of TGF-β2 (42). Along these lines, O’Reilly (56) has shown that a combination of TGF-β1 and bFGF administered systemically can cause the regrowth of small tumor rests after endostatin therapy. These data point to the potential importance of monitoring TGF-β1 levels in a cancer patient and assessing tumor profiling of TGF-β and its receptors and suggests the possible use of anti-TGF-β therapy either at the time of primary tumor removal or at surgery in a patient with cancer in remission or before and peri radiation therapy. Counterweighing this would be the potential deleterious effects of such therapy on wound repair. Finally, other approaches to negating the effect of TGF-β1 may be more efficacious or practical than the use of neutralizing Ab. Decorin, a proteoglycan known to bind TGF-β (57), latency associated peptide, soluble TGF-β receptors, antisense oligonucleotides, and TGF-β peptide antagonists (58) may serve this purpose.

In conclusion, we have shown that TGF-β1 is a target for the pVHL and that repression of the TGF-β1 message occurs predominantly at a posttranscriptional level. Identification of potential RNA-binding proteins and destabilizing elements in the TGF-β1 mRNA will help elucidate the function of pVHL. Whereas VHL mutations occur early in RCC development, an important secondary genetic event in the TGF-β1 signaling pathway leads to the abrogation of TGF-β type II receptor expression and resistance to the antiproliferative effects of TGF-β1. Moreover, we have shown that the biological significance of elevated TGF-β1 in RCCs is to stimulate angiogenesis and that neutralizing TGF-β activity can regress established RCCs without the development of drug resistance. Blocking the paracrine effects of TGF-β1 may provide novel treatment strategies for RCCs and other cancers that secrete TGF-β1.

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