Expression of angiopoietins in renal epithelial and clear cell carcinoma cells: regulation by hypoxia and participation in angiogenesis

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Yamakawa, Midori, Louis X. Liu, Adam J. Belanger, Taro Date, Takayuki Kuriyama, Mark A. Goldberg, Seng H. Cheng, Richard J. Gregory, and Canwen Jiang. Expression of angiopoietins in renal epithelial and clear cell carcinoma cells: regulation by hypoxia and participation in angiogenesis. Am J Physiol Renal Physiol 287: F649–F657, 2004. First published June 15, 2004; 10.1152/ajprenal.00028.2004.—The hereditary von Hippel-Lindau (VHL) syndrome predisposes sufferers to highly vascularized tumors such as renal clear cell carcinoma (RCC) and central nervous system hemangioblastoma. In RCC4 and RCC786–0 VHL+ cells with VHL mutations, the protein of hypoxia-inducible factor-1α (HIF-1α) is constitutively stabilized and the mRNA levels of HIF target genes, including vascular endothelial growth factor (VEGF), are elevated. However, the expression of angiopoietins in these cells and their involvement in angiogenesis are not well known. In this study, we compared the mRNA levels of angiopoietins in human kidney proximal tubule epithelial (RPTE) and RCC4 and RCC786–0 VHL− cells. In RPTE cells, angiopoietin-4 (Ang-4) expression was selectively induced by hypoxia or by expression of a hybrid form of HIF-1α. Under normoxic conditions, the mRNA levels of Ang-4 were higher in RCC4 than RPTE cells. Angiopoietin-1 expression was detectable in RCC4 and RCC786–0 VHL− cells but not RPTE cells. In RCC786–0 VHL− cells, which were stably transfected with a wild-type copy of VHL, the mRNA levels of VEGF and Ang-4 were suppressed and the hypoxic response was restored. We also demonstrated that stimulation of endothelial cell migration by conditioned medium harvested from RCC4 cells was inhibited by a soluble Tie-2 receptor. These results suggest that the angiopoietin/Tie-2 system may participate in the angiogenic response to hypoxia in renal tissues and in tumor angiogenesis in renal carcinoma.

hypoxia-inducible factor-α ; von Hippel-Lindau; vascular endothelial growth factor; Tie-2 receptor

Hypoxia is a fundamental stimulus for angiogenesis through activation of VEGF transcription and stabilization of its mRNA (9, 29). Hypoxia-induced VEGF transcription is mediated by hypoxia-inducible factor-1 (HIF-1), a heterodimeric basic helix-loop-helix-PAS transcription factor (31, 35). HIF-1 is composed of two subunits, HIF-1α and HIF-1β (aryl hydrocarbon nuclear translocator). While the β-subunit protein is constitutively present, the transcription and stability of the α-subunit are precisely controlled by intracellular oxygen concentration. The mRNA levels of HIF target genes are negatively regulated by the von Hippel-Lindau syndrome tumor-suppressor protein (VHL) (16, 21, 26). VHL targets the HIF-1α subunit for proteolysis after the hydroxylation of specific prolyl residues within the HIF-1α oxygen-dependent degradation domain. Hypoxia reduces the enzymatic activity of the prolyl hydroxylases and thereby increases HIF-1α protein levels (8, 30). In the VHL mutant renal carcinoma cell lines (e.g., RCC4), the mRNA levels of VEGF and other HIF target genes are increased under normoxic conditions, due to elevated levels of HIF-1α (16, 21, 26).

Angiopoietins are ligands for the endothelium-specific receptor tyrosine kinase Tie-2 (7). Angiopoietin-1 (Ang-1) plays an important role in the assembly of newly formed vasculature and in the maintenance of vascular integrity (6, 32). The role of angiopoietin-2 (Ang-2) in angiogenesis is highly dependent on the presence of other angiogenic factors, particularly VEGF (13, 24). In the absence of VEGF, Ang-2 antagonizes the activation of Tie-2 by Ang-1 and causes endothelial cell apoptosis and vascular regression. In the presence of VEGF, Ang-2 destabilizes the preexisting vasculature and consequently makes it more responsive to angiogenic stimuli (14). When coadministered with VEGF, both Ang-1 and Ang-2 are capable of augmenting angiogenesis (2). Recently, human angiopoietin-4 (Ang-4) and mouse angiopoietin-3 (Ang-3) were cloned (33). Like Ang-1, Ang-4 binds to the Tie-2 receptor and functions as an agonist. Ang-3, the mouse counterpart of human Ang-4, antagonizes Tie-2 activation by Ang-1. However, Ang-4 protects cultured endothelial cells against apoptosis induced by serum starvation and inhibits thrombin-induced increase in permeability. Ang-4 also stimulates endothelial cell migration and tube formation in vitro, indicating that Ang-4 is capable of functioning as Ang-1 (37). Expression of Ang-2 and Tie-2 is upregulated by hypoxia in endothelial (25, 27, 36) and other cells (19, 30, 38). In cultured human endothelial cells, hypoxia induces Ang-4 expression via a HIF-mediated mechanism (37). In vascular smooth muscle cells, Ang-1 expression is also increased after hypoxia and reoxygenation (28). In rats exposed to hypoxia, Ang-1 expression is decreased in the lung, liver, cerebellum, and heart, but mRNA levels of Ang-2 and Ang-3 in the lung, kidney, and diaphragm are increased (1). Ang-2 expression is upregulated in metastases, which plays a critical role in the initial regression of these co-opted vessels and subsequent robust angiogenesis (13, 14). These observations suggest that angiopoietins participate in the cellular response to hypoxia and play a role in tumor angiogenesis.

The hereditary VHL cancer syndrome predisposes sufferers to highly angiogenic tumors such as renal clear cell carcinoma.
(RCC) (4, 18). Independently of cellular hypoxia, mutations of the VHL gene result in elevated mRNA levels of angiogenic genes including VEGF (11, 16, 21, 26). However, expression of angiopoietins in VHL mutant RCC cells and their potential role in angiogenesis are not known. In this study, we examined the mRNA levels of angiopoietins in human kidney proximal tubule epithelial (RPTE) and RCC4 cells maintained under various conditions. We then assessed the involvement of the angiopoietin/Tie-2 system in the enhancement of endothelial tube formation induced by conditioned medium harvested from RCC4 and RCC786–0 VHL− cells. We demonstrated that in RPTE cells, Ang-4 expression was selectively induced by hypoxia or expression of a hybrid form of HIF-1α. The mRNA levels of Ang-1 and Ang-4 were elevated in RCC4 and RCC786–0 VHL− cells under normoxic conditions, compared with RPTE cells. Our results suggest that the angiopoietin/Tie-2 system may participate in the angiogenic response to hypoxia in renal tissues and in tumor angiogenesis in renal carcinoma.

MATERIALS AND METHODS

Construction of adenoviral vectors. Ad2/HIF-1α/VP16, which encodes a hybrid form of HIF-1α, was constructed using an E1-deleted Ad2/E4ORF6 backbone (wild-type E2 and E3, and deletion of E4 except for ORF6), as described previously (17, 22). Ad2 nucleotide sequences between 337 and 4021 were replaced with the cytomegalovirus enhancer-promoter, the HIF-1α/VP16 hybrid, and the SV40 sequences between 357 and 4021 were replaced with the cytomega-

except for ORF6), as described previously (17, 22). Ad2 nucleotide sequences between 337 and 4021 were replaced with the cytomegalovirus enhancer-promoter, the HIF-1α/VP16 hybrid, and the SV40 polyadenylation signal. The HIF-1α/VP16 hybrid, which is composed of the DNA-binding and dimerization domains from HIF-1α and the transactivation domain of HSV VP16, was constructed by truncating HIF-1α at amino acid 390 and then joining the VP16 fragment (amino acid 413 to 490) downstream (34). Ad2/CMVVEV was constructed in a similar manner to that for Ad2/HIF-1α/VP16 except that Ad2/CMVEV lacked a transgene.

Cell culture. Human RPTE cells were obtained from Clonetics (San Diego, CA) and were cultured in REGM [basal medium (REBM) plus hydrocortisone, epidermal growth factor, epinephrine, insulin, triiodothyronine, transferrin, gentamycin-amphotericin, and amino acids] provided by Clonetics. Human umbilical vein endothelial cells (HUVECs) were also purchased from Clonetics and cultured and supplemented in EGM2 [basal medium (EBM) supplemented with 2% fetal bovine serum (FBS) and SingleQuots (epidermal growth factor, hydrocortisone, VEGF, basic fibroblast growth factor, insulin growth factor-1, ascorbic acid, heparin, gentamycin-amphotericin B)] provided by Clonetics. RCC4, RCC786–0 VHL−, and RCC786–0 VHL− cells were renal carcinoma cell lines and maintained in Dulbecco’s modified Eagle’s medium supplemented with arginine, asparagines, l-glutamine, and 10% (vol/vol) FBS (10, 15, 26).

Hypoxia and viral infection. Cells were cultured under normoxic conditions (2% O2, 5% CO2, 37°C) or placed in a hypoxia chamber (% O2, 5% CO2, 37°C) for 24 h. Separate groups of cells under normoxic conditions were infected with Ad2/HIF-1α/VP16 or Ad2/CMVEV at a multiplicity of infection (MOI) of 100 for 6 h and then maintained under normoxic conditions for an additional 42 h. The cells under various conditions were harvested with RNAzol B (Tel-test), and total RNA was prepared.

TaqMan analysis. TaqMan 5′-nucleate fluorescent quantitative PCR was performed as described previously using an ABI Prism 7700 Sequence Detector (Foster City, CA) (3, 23). Briefly, primers and probes (Table 1) were designed according to ABI-PerkinElmer guidelines and synthesized by Operon (Alameda, CA). Standard curves for each gene of interest were performed in duplicate. The mRNA levels of the gene of interest were normalized to 18S rRNA and expressed in either arbitrary units or as fold-increases over controls.

Generation of conditioned medium and endothelial tube formation assay. RTPE and RCC4 cells were maintained under normal culture conditions. After they reached confluence, the cells were maintained in fresh EBM for an additional 24 h. Separate groups of RTPE cells were infected with adenoviral vectors for 4 h, washed three times with PBS, and finally maintained in fresh EBM for an additional 20 h. The conditioned medium was then collected and filtered using a 0.2-μm filter.

The angiogenic response to various treatments was assessed using an in vitro capillary/tubelike structure formation assay (37). HUVECs were seeded onto 48-well cell culture plates coated with Matrigel (BD Bioscience) at 104 cells/well and maintained in each conditioned medium supplemented with 2% FBS in the presence or absence of a soluble Tie-2 receptor (Tie2Fc; Sigma) at 20 μg/ml or (Z)-3-[2,4-Dimethyl-3-(ethoxycarbonyl)pyrrol-5-yl]methylenylindol-2-one (VEGFR kinase inhibitor I; Calbiochem, San Diego, CA) at 1 μM. The capillary/tubelike structures were visualized by light microscopy 8 h later and analyzed using NIH ImageJ software.

Statistical analysis. Data are expressed as means ± SE; n is the number of samples examined. Data were analyzed by ANOVA, followed by a modified Student’s t-test. An unpaired Student’s t-test was used in experiments involving two groups only. A probability value of <0.05 was considered statistically significant.

RESULTS

Selective upregulation of Ang-4 by hypoxia in normal human kidney epithelial cells. To examine whether hypoxia regulates the expression of angiogenic genes in human RPTE cells, we measured the mRNA levels of VEGF, Ang-1, Ang-2, and Ang-4. Under normoxic conditions, low levels of VEGF and Ang2 and Ang-4 mRNA were detected by TaqMan analysis (Fig. 1). Subjecting the RPTE cells to hypoxia for 24 h significantly increased the mRNA levels of VEGF and Ang-4. In contrast, Ang-2 mRNA levels remained unchanged in response to hypoxia. Ang-1 mRNA was not detectable by TaqMan analysis in these cells maintained under normoxic conditions or subjected to hypoxia (data not shown). These results suggest that in human RPTE cells Ang-4 is selectively upregulated by hypoxia.

Table 1. Primers and probes for TaqMan 5′-nucleate fluorescent quantitative PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
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<tbody>
<tr>
<td>Ang-1</td>
<td>CCAAGCTAGAAATGTTATTTGAGG</td>
<td>AAGCAGGCTGACGGTTTACCC</td>
<td>CCCATTCAGGCCAACCTTGGC</td>
</tr>
<tr>
<td>Angel-2</td>
<td>CAGAGGGAGGCTGCCAGAG</td>
<td>GCAGGAGCTGACGGTTTACCC</td>
<td>CCCATTCAGGCCAACCTTGGC</td>
</tr>
<tr>
<td>Ang-4</td>
<td>CAGAGGGAGGCTGCCAGAG</td>
<td>TCGGAGCTGACGGTTTACCC</td>
<td>CCCATTCAGGCCAACCTTGGC</td>
</tr>
<tr>
<td>VEGF</td>
<td>CTTTCTTCTGTCTGTTAACCC</td>
<td>TCGGAGCTGACGGTTTACCC</td>
<td>CCCATTCAGGCCAACCTTGGC</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>GGCGCTGACGGAAATAATC</td>
<td>TTTCGAGGCTGACGGTTTACCC</td>
<td>CCCATTCAGGCCAACCTTGGC</td>
</tr>
</tbody>
</table>

Ang-1, Ang-2, and Ang-4: angiopoietin-1, -2, and -4, respectively. The primers and probes for the measurement of the mRNA levels of human Ang-1, Ang-2, Ang-4, VEGF, and 18S rRNA were designed according to ABI-PerkinElmer (Foster City, CA) guidelines and synthesized by Operon (Alameda, CA).
We next assessed whether hypoxic activation of VEGF and Ang-4 in human RPTE cells could be mimicked by adenovirus-mediated expression of a constitutively stable form of HIF-1α/H9251/VP16. Human RPTE cells were infected with Ad2/HIF-1α/H9251/VP16 (100 MOI), an adenoviral vector encoding a constitutively active form of HIF-1α, or Ad2/CMVEV, an empty vector (17). Expression of the hybrid HIF-1α significantly increased the mRNA levels of VEGF and Ang-4 (Fig. 2), whereas Ang-2 expression was not altered. Ang-1 mRNA remained undetectable by TaqMan analysis. The same dose of Ad2/CMVEV had no effect, suggesting that the increase in VEGF and Ang-4 expression after infection with Ad2/HIF-1α/VP16 was not due to viral infection per se (Fig. 1). These results suggest that hypoxic upregulation of Ang-4 expression is mimicked by adenovirus-mediated expression of HIF-1α/VP16, similarly to that observed for VEGF in human RPTE cells.

Elevation of Ang-1 and Ang-4 mRNA levels in RCC4 cells under normoxic conditions. The expression of several HIF-1 target genes including VEGF is increased in VHL-deficient renal carcinoma cells under normoxic conditions (11, 16, 21, 26). RPTE cells are believed to give rise to renal carcinoma. In this study, we compared the mRNA levels of VEGF and angiopoietins between RCC4 and RPTE cells. As expected, the basal level of VEGF mRNA under normoxic conditions was significantly greater in RCC4 cells than in human RPTE cells (Fig. 3). The mRNA levels of Ang-4 were also higher (20-fold) in RCC4 than in RPTE cells. Under normoxic conditions, Ang-2 mRNA levels were not significantly different between the two cell types.

The mRNA levels of both VEGF and Ang-4 in RCC4 cells were not increased in response to hypoxia (Fig. 4), reflecting the fact that the HIF-1α proteins were already constitutively stabilized under normoxic conditions in these cells. However, infection with Ad2/HIF-1α/VP16 further increased VEGF and Ang-4 mRNA levels, presumably due to the presence of additional exogenous hybrid HIF-1α. Infection with Ad2/CMVEV had no effect. Neither hypoxia nor adenovirus-mediated expression of HIF-1α/VP16 altered the mRNA levels of Ang-2 in RCC4 cells. Hence, the mRNA levels of Ang-4 and VEGF in RPTE cells were increased in response to hypoxia or adenovirus-mediated expression of a constitutively stable form of HIF-1α.

Fig. 1. Effect of hypoxia on the expression of VEGF and angiopoietins in human kidney proximal tubule epithelial cells. Cells were maintained under normoxic conditions (open bars) or subjected to hypoxia (filled bars). mRNA levels of VEGF, angiopoietin (Ang)-2, and Ang-4 were measured by TaqMan analysis and normalized to 18S RNA. Ang-1 was not expressed under these conditions. Values means ± SE expressed as arbitrary units; n = 3. *P < 0.05 and **P < 0.01 compared with normoxia.

Fig. 2. Effect of adenovirus-mediated expression of hypoxia-inducible factor-1α (HIF-1α)/VP16 on the mRNA levels of VEGF angiopoietins in human kidney proximal tubule epithelial cells. mRNA levels of VEGF (A), Ang-2 (B), and Ang-4 (C) were measured by TaqMan analysis in cells maintained under normoxia (N) or infected with Ad2/EV (EV) or Ad2/HIF-1α/VP16 (VP16) at a multiplicity of infection of 100. Ang-1 was not expressed under these conditions. mRNA levels were normalized to 18S RNA and expressed as arbitrary units. Values are means ± SE; n = 3. *P < 0.05 compared with normoxia.
of HIF-1α. In RCC4 cells, Ang-4 and VEGF expression was constitutively increased, compared with their RPTE counterparts, whereas hypoxia did not result in a further increase in the mRNA levels of these two genes in these cells. These results suggest that hypoxic upregulation of Ang-4 expression is likely mediated by HIF-1.

Interestingly, Ang-1 was detected in RCC4 but not RPTE cells (Fig. 3). Neither hypoxia nor adenovirus-mediated expression of HIF-1α/VP16 further increased Ang-1 mRNA levels in RCC4 cells. Ang-1 expression in RPTE cells was also not detectable under normoxic or hypoxic conditions and was not induced by adenovirus-mediated expression of HIF-1α/VP16. These results suggest that expression of Ang-1 in RCC4 cells may not be associated with elevated HIF-1 levels.

Suppression of Ang-4 mRNA levels in RCC786–0 VHL− cells by stable transfection with a wild-type copy of VHL. Previous studies demonstrated that expression of wild-type VHL in RCC786–0 VHL− cells suppresses the mRNA levels of HIF-1 target genes, including VEGF under normoxic conditions, and restores the hypoxic upregulation (15, 18, 26). In this study, similar to that observed with RCC4 cells, the basal levels of VEGF and Ang-4 mRNA under normoxic conditions were significantly elevated but did not increase further in response to hypoxia in RCC786–0 VHL− cells (Fig. 5A). Stable transfection with a wild-type copy of VHL significantly reduced the mRNA levels of both VEGF and Ang-4 under normoxic conditions and restored the hypoxic response. These results provide additional evidence that upregulation of Ang-4 expression in RCC cells is likely mediated by elevated HIF levels due to VHL deficiencies. Expression of both Ang-1 and Ang-2 was also detected. However, there was no significant difference between RCC786–0 VHL− and RCC786–0 VHL+ cells.

Contribution of angiopoietins to angiogenesis induced by secreted growth factors from RPTE cells expressing HIF-1α/VP16. To examine the contribution of the angiopoietin/Tie-2 system to the angiogenic response of RPTE cells to elevated levels of HIF-1α, we assessed the effect of a soluble Tie-2 receptor on endothelial cell tube formation. RPTE cells were maintained in EBM for 24 h or infected with either Ad2/CMVEV or Ad2/HIF-1α/VP16 for 4 h and maintained in fresh
EBM for an additional 20 h. The conditioned media were then harvested. HUVEC cultures on Matrigel were maintained in the various conditioned medium supplemented with 2% FBS under normoxic conditions for 8 h. HUVECs cultured in the conditioned medium harvested from control RPTE donor cells developed tubelike structures (Fig. 6). However, the tubelike structures were more extensive in cells cultured with conditioned medium harvested from RPTE cells infected with Ad2/HIF-1α/H9251/VP16, whereas Ad2/CMVEV had no effect. These results suggest that expression of HIF-1α/H9251/VP16 in RPTE cells enhances endothelial cell tube formation, probably through secreted growth factors. Furthermore, addition of either a soluble Tie-2 receptor (Tie-2Fc) or VEGFR2 kinase inhibitor I significantly reduced the increase in tubelike structures induced by conditioned medium from RPTE cells infected with Ad2/HIF-1α/VP16. These results suggest that the angiopoietin/Tie-2 system is involved in angiogenesis by HIF-1α-induced release of angiogenic factors from RPTE cells.

Potential involvement of angiopoietins in angiogenesis of highly vascularized clear renal carcinoma. We next assessed whether conditioned medium harvested from RCC4 cells was more proangiogenic than that from RPTE cells. RPTE or RCC4 cells were maintained under normoxic conditions in EBM for 24 h, and the conditioned media were harvested. HUVECs were seeded on Matrigel and cultured in the conditioned medium supplemented with 2% FBS under normoxic conditions for 8 h. Tubelike structures were more extensive in cells cultured with conditioned medium harvested from RCC4 cells than RPTE cells (Fig. 7). These results suggest that secreted growth factors from RCC4 cells stimulate endothelial cell tube formation. Furthermore, addition of either Tie-2Fc or VEGFR2 kinase inhibitor I significantly reduced the increase in tubelike structures induced by conditioned medium from RCC4 cells, suggesting that both VEGF and angiopoietin/Tie-2 contribute to angiogenesis stimulated by secreted growth factors from RCC4 cells.

**DISCUSSION**

Angiopoietins play an important role in the assembly and maintenance of a functional vasculature. The expression of Ang-1 and Ang-2 is regulated by hypoxia (25, 27, 28, 30, 36, 38). However, hypoxic regulation of the expression of specific angiopoietins appears to be tissue and species dependent (1, 25, 36). These observations raise the possibility that angiopoietins are involved in the angiogenic response to hypoxia, a process that requires the orchestration of a variety of endothelial growth factors and receptors. Upregulation of Ang-2 and downregulation of Ang-1 by hypoxia may prepare endothelial cells for the initial phase of angiogenesis. An increase in Ang-1 expression may also be required for maturation and maintenance of newly formed vessels (1, 25, 27, 28). Recently, we reported that Ang-4 was selectively upregulated by hypoxia in human endothelial cells, which is mediated by HIF-1α (37). In this study, we demonstrated that expression of Ang-4 in human renal epithelial cells was also upregulated by hypoxia. In contrast, the mRNA levels of Ang-2 remained unchanged. Ang-1 expression was not detectable in RPTE cells under normoxic or hypoxic conditions. Adenovirus-mediated expression of HIF-1α/VP16 also increased Ang-4 mRNA levels, supporting a role for HIF-1 in hypoxia-induced expression of Ang-4 in epithelial cells. It has been proposed that in rats Ang-2 functions as an Ang-1 antagonist in an organ-specific manner and that Ang-3 may substitute for Ang-2 in certain organs as the primary Ang-1 antagonist (1). In human
endothelial cells, Ang-4 is capable of functioning as Ang-1 (37). Our results suggest that in human kidney epithelial cells, Ang-4 may participate in the angiogenic response to hypoxia by substituting for Ang-1 as a Tie-2 agonist and acting on adjacent endothelial and other cells.

Some tumors originate as small avascular structures, which must then induce the development of new vessels to grow beyond a few millimeters in size (12). Other malignancies and metastases rapidly co-opt existing host vessels to form an initially well-vascularized tumor mass, followed by Ang-2-induced regression of these co-opted vessels. The remaining avascular tumor is then rescued by robust angiogenesis, due to upregulation of both VEGF and Ang-2 (13, 14). These studies highlight the importance of angiopoietins in tumor angiogenesis. The hereditary VHL syndrome predisposes sufferers to highly vascularized tumors such as clear cell carcinoma (4, 18). In addition to being an adaptive response to a hypoxic microenvironment, genetic alterations of the VHL gene result in the
loss of VHL-mediated proteolysis of HIF-1α. RCC4 and RCC786–0 VHL− cells, which were derived from a VHL mutant renal carcinoma, express high levels of HIF-1α and/or HIF-2α protein under normoxic conditions (15, 18, 26). The mRNA levels of HIF-1 target genes, including VEGF, are elevated under normoxic conditions (11, 16, 21, 26). In this study, we found that the mRNA levels of Ang-1 and Ang-4 in RCC4 and RCC786–0 VHL− cells were also elevated under normoxic conditions compared with normal kidney epithelial cells. These studies are the first to demonstrate the elevated expression of Ang-4 under normoxic conditions in tumor cells. The hypoxia-induced upregulation of Ang-4 mRNA levels seen in normal kidney epithelial cells did not occur in RCC4 and RCC786–0 VHL− cells. In RCC786–0 VHL+ cells, which were stably transfected with a wild-type copy of VHL, the mRNA levels of VEGF and Ang-4 were suppressed and the hypoxic response was restored. These results in RCC4 and RCC786–0 VHL− cells suggest that, as with VEGF, Ang-4 expression is regulated by VHL through HIF-1. The current study did not examine whether HIF activates Ang-4 transcrip-
tion by binding to the Ang-4 promoter/enhancer directly. The precise molecular mechanism by which HIF-1 upregulates Ang-4 mRNA levels remains to be determined. The upregulation of Ang-4 expression under normoxic conditions is attributable to the high levels of HIF-1α proteins due to the mutant VHL in RCC4 and RCC786–0 VHL− cells. However, upregulation of Ang-1 mRNA levels in RCC4 and RCC786–0 VHL− cells cannot be explained by elevated levels of HIF-1α proteins, because neither hypoxia nor overexpression of HIF-1α/VP16 affected Ang-1 expression in RPTE cells. One may speculate that other alterations rather than elevated levels of HIF-1α proteins per se influenced Ang-1 expression in RCC4 and RCC786–0 VHL− cells. It should be noted that Currie et al. (5) reported increased expression of Ang-1 and Ang-2 in human renal carcinoma tissues/cells compared with their normal kidney counterparts but did not detect Ang-4 expression in these cells. The discrepancy between our results and their observations remains to be determined.

The tubulilike structures formed by HUVECs seeded on Matrigel were more extensive with conditioned medium harvested from RCC4 cells than RPTE cells, suggesting that RCC4 cells stimulate endothelial cell tube formation, probably through paracrine signaling by secreted growth factors. This increase in tubulilike structures was significantly reduced by Tie-2Fc, indicating the contribution of the angiopoietin/Tie-2 system. Furthermore, the enhancement of endothelial tube formation by RCC4 cells was mimicked by adenosvirus-mediated expression of HIF-1α/VP16 in RPTE cells. It should be noted that our studies did not examine the contribution of individual angiopoietins to the enhancement of endothelial tube formation by conditioned medium harvested from RCC4 cells or RPTE cells expressing HIF-1α/VP16. We previously reported that Ang-1 and Ang-4 increased tubular tube formation of human coronary and pulmonary artery endothelial cells at comparable concentrations, whereas Ang-2 failed to stimulate angiogenesis in the particular in vitro setting (37). However, the bioactivity of Ang-4 in HUVECs has not been examined. The relative contribution of individual angiopoietins, especially the importance of Ang-4 in the angiogenic response to conditioned medium harvested from RCC4 cells or RPTE cells expressing HIF-1α/VP16, remains to be defined. Nonetheless, our results support the notion that angiopoietins are involved in angiogenesis of highly vascularized clear renal carcinoma.

In summary, Ang-4 was selectively upregulated by hypoxia or overexpression of HIF-1α/VP16 in RPTE cells. Ang-4 mRNA levels were also elevated in VHL protein-deficient RCC4 and RCC786–0 VHL− cells, suggesting regulation of Ang-4 by VHL through HIF-1. We also demonstrated that the enhancement of endothelial cell tube formation induced by conditioned medium from RCC4 cells under normal culture conditions or RPTE cells expressing HIF-1α/VP16 was significantly inhibited by Tie-2Fc, suggesting the participation of the angiopoietin/Tie-2 system in angiogenesis of highly vascularized clear renal carcinoma.

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