Overexpression of Vascular Endothelial Growth Factor and the Development of Post-Transplantation Cancer

Aninda Basu, I,2,3 Alan G. Contreras, I,2,3 Dipak Datta, I,2,3 Evelyn Flynn, I,2,3 Liling Zeng, 4
Herbert T. Cohen, 1 David M. Briscoe, I,2,3 and Soumitro Pal I,2,3

1Division of Nephrology, Children's Hospital Boston; 2Transplantation Research Center, Children's Hospital Boston and Brigham and Women's Hospital; 3Department of Pediatrics, Harvard Medical School; and 4Renal Section, Department of Medicine, Boston Medical Center and Boston University School of Medicine, Boston, Massachusetts

Abstract
Cancer is an increasing and major problem after solid organ transplantation. In part, the increased cancer risk is associated with the use of immunosuppressive agents, especially calcineurin inhibitors. We propose that the effect of calcineurin inhibitors on the expression of vascular endothelial growth factor (VEGF) leads to an angiogenic milieu that favors tumor growth. Here, we used 786-O human renal cancer cells to investigate the effect of cyclosporine (CsA) on VEGF expression. Using a full-length VEGF promoter-luciferase construct, we found that CsA markedly induced VEGF transcriptional activation through the protein kinase C (PKC) signaling pathway, specifically involving PKCζ and PKCδ isoforms. Moreover, CsA promoted the association of PKCC and PKCζ with the transcription factor Sp1 as observed by immunoprecipitation assays. Using promoter deletion constructs, we found that CsA-mediated VEGF transcription was primarily Sp1 dependent. Furthermore, CsA-induced and PKC-Sp1–mediated VEGF transcriptional activation was partially inhibited by von Hippel-Lindau protein. CsA also promoted the progression of human renal tumors in vivo, wherein VEGF is overexpressed. Finally, to evaluate the in vivo significance of CsA-induced VEGF overexpression in terms of post-transplantation tumor development, we injected CT26 murine carcinoma cells (known to form angiogenic tumors) into mice with fully MHC mismatched cardiac transplants. We observed that therapeutic doses of CsA increased tumor size and VEGF mRNA expression and also enhanced tumor angiogenesis. However, coadministration of a blocking anti-VEGF antibody inhibited this CsA-mediated tumor growth. Collectively, these findings define PKC-mediated VEGF transcriptional activation as a key component in the progression of CsA-induced post-transplantation cancer. [Cancer Res 2008;68(14):5689–98]

Introduction
Cancer is a common and major problem after organ transplantation (1–3). Malignant tumors develop in 15% to 20% of graft recipients within 10 years and contribute to morbidity and mortality in these patients (4). There are three major ways by which malignant tumors may develop in transplant recipients: de novo occurrence, recurrent malignancy, or transmission of malignancy from the donor (4). Some forms of cancer (e.g., cancers of kidney and skin and lymphoma) increase markedly after kidney transplantation as compared to the general population or comparable patients on dialysis (1, 2). De novo nonlymphoid cancers are a major cause of late death in liver transplant recipients (5). Thus, the prevention of cancer should be a major goal of future therapy after organ transplantation.

Immunosuppressive agents used in transplant recipients may play a critical role in the tumorigenic process. These agents are thought to compromise immune surveillance mechanism(s) for tumor cells (2, 6, 7) and/or interfere with normal DNA repair mechanisms (1). In particular, use of calcineurin inhibitors, including cyclosporine (CsA) and FK506, has significantly increased the incidence of post-transplantation cancer (7, 8). Hojo and colleagues (7) showed that CsA promotes cancer progression by direct cellular effect(s) through transforming growth factor-β (TGF-β) production that is independent of its effect on the immune system of the host. Similarly, FK506 has been shown to promote the proliferation of tumor cells through TGF-β (9). Koehl and colleagues (10) reported that CsA augments the growth of tumor cells in vivo in doses that are sufficient to inhibit allograft rejection, and although they suggested a role of TGF-β in this process, they could not rule out the possible involvement of other angiogenic factors. Guba and colleagues (11) suggested that CsA may induce the expression of an angiogenic cytokine, like vascular endothelial growth factor (VEGF), but did not show its function in the development of post-transplantation cancer.

VEGF is the most potent angiogenic factor described to date, playing important roles in tumor development (12, 13). It is expressed by tumor cells, endothelial cells, and a variety of cell types (13). VEGF is expressed in significant amounts in transplant recipients. Induced VEGF expression may mediate inflammatory cell trafficking into allografts (14, 15) and may promote both acute and chronic allograft rejection (16, 17). It is possible that high levels of VEGF expression in transplant recipients may provide an environment in which microtumors can grow more efficiently due to enhanced VEGF-induced angiogenesis. The effect of CsA on VEGF expression may therefore be a risk factor for the development of post-transplantation cancer.

The calcineurin complex consists of three subunits, the catalytic A, the regulatory B, and calmodulin (18). Cellular CalC binds to both calmodulin and the B subunit, displacing the inhibitory COOH-terminal peptide from the active site of the catalytic A subunit (19). This process activates the catalytic subunit for its function as serine/threonine phosphatase, resulting in the activation of the nuclear factor of activated T cells (NFAT) family of transcription factors (20). Although NFAT is functional in many cell
types, it has been best studied in T-cell activation responses, where it induces different cytokines, like interleukin-2 (21). It has also been suggested that the NFAT pathway may either induce or repress the expression of several angiogenic factors, including VEGF (20, 22–24). Thus, the calcineurin inhibitor CsA binds to cyclophilin, a cytoplasmic protein, and the resultant complex binds to the regulatory B subunit of calcineurin and prevents the activation of NFAT (25). This process may alter the regulatory switch for VEGF-induced angiogenesis, resulting in increased VEGF expression and tumor angiogenesis. However, the molecular mechanism by which CsA may mediate VEGF overexpression is completely unknown.

In the present study, we show that CsA can directly promote the transcriptional activation of VEGF in human renal cancer cells, involving the protein kinase C (PKC) signaling pathway, particularly the atypical and novel PKC isoforms. We also observed that CsA augments tumor growth after cardiac transplantation in vivo and that this effect is in part dependent on increased VEGF expression.

**Materials and Methods**

**Reagents.** CsA (Novartis) and FK506 (Astellas) were purchased from Children’s Hospital Boston pharmacy. The mitogen-activated protein kinase (MAPK) kinase (MEKK) inhibitor PD98059, the phosphatidylinositol 3-kinase (PK3) inhibitor LY294002, the generalized PKC inhibitor calphostin C, and the classic PKC inhibitor Go6976 were obtained from Calbiochem. The small interfering RNA (siRNA) for hypoxia-inducible factor-2α (HIF-2α) and its control were purchased from Qiagen.

**Development of blocking antibody to murine VEGF.** The development of our blocking anti-murine VEGF antibody (by D.M.B.) was performed using reagents and techniques similar to those previously described (14). Briefly, noble rats were immunized with the NH2-terminal amino acid sequence of murine VEGF (CAPITTEGFQKSHAEVYQTSY) coupled with keyhole limpet hemocyanin using the maleimido-benzyl-α-hydroxysuccinimid ester cross-linker. Hybridoma fusion products were generated according to standard protocols and were screened based on their anti-VEGF (peptide) absorbance reading in ELISA. Secondary screens showed two IgG-producing clones, 2G1L-2A05 and 1F07-1C02, which were subcloned and purified. In Western blots, anti-VEGF 2G1L-2A05 bound murine VEGF with high affinity, producing a band of appropriate size similar to that using a commercially available polyclonal rabbit anti-human/mouse antibody (Santa Cruz Biotechnology). We also found that anti-VEGF monoclonal antibody 2G1L-2A05 blocked murine VEGF-induced proliferation of endothelial cells in vitro using an assay previously described (14) and that 2G1L-2A05 neutralized VEGF function in vivo in our standardized VEGF-induced angiogenesis assay (14).

**Cell culture.** The human renal cancer cell lines (786-O and Caki-1) and the murine colon adenocarcinoma cell line (CT26) were obtained from American Type Culture Collection. The cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (Hyclone Laboratories). 786-O clonal cell lines stably transfected with either pFLAG-CMV-2 (Neo cells containing empty vector with neo cassette) or pFLAG-CMV-2/VHL [von Hippel-Lindau (VHL) cells containing wild-type (wt)-VHL] were generated according to standard protocols and were cultured in complete epithelial medium (REGM BulletKit).

**Plasmids.** A 2.6 kb VEGF promoter-luciferase construct in pGL2 basic vector (Promega), containing the full-length VEGF promoter sequence (−2,361 to +298 bp relative to the transcription start site), and the two deletion constructs (0.35 and 0.07 kb) of the 2.6 kb VEGF promoter were used in transient transfection assays (26, 27). The kinase inactive PKCζ plasmid (PKCζ KW) was a generous gift from Alex Toker (Beth Israel Deaconess Medical Center, Boston, MA; ref. 27). The kinase inactive PKCζ plasmid (PKCζ KR) was a generous gift from Rakesh Dutta (Dana-Farber Cancer Institute, Boston, MA; ref. 27).

**Transfection assays.** Cells were plated at 2 × 10⁶ per well in six-well plates, and were transfected with expression plasmids using the Effectene transfection reagent (Qiagen; ref. 28). The total amount of transfected plasmid DNA was normalized using a control empty expression vector. Transfection efficiency was determined by cotransfection of the β-galactosidase gene and measurement of β-galactosidase activity. For luciferase assays, cells were harvested 24 h after transfection and luciferase activity was measured using a standard assay kit (Promega) in a luminometer. The relative luciferase activity units were calculated as (light emission from experimental sample – light emission of lysis buffer alone) / micrograms of cellular protein in the sample (29).

**Immunoprecipitation assays.** Immunoprecipitations were performed with 0.5 mg of total protein at antibody excess using anti-Sp1 (Santa Cruz Biotechnology). Immunocomplexes were captured with protein A–Sepharose beads (Amersham Pharmacia Biotech), and bead-bound proteins were subjected to Western blot analysis using either anti-PKCζ or anti-PKCζ (Santa Cruz Biotechnology).

**Western blot analysis.** Protein samples were run on SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (NE Life Sciences Product, Inc.; ref. 28). The membranes were incubated with anti-VEGF/anti–β-actin, anti–phosphorylated PKCζ/α, anti–total PKCζ/α, anti–HIF-2α, or anti-VHL (Santa Cruz Biotechnology) and subsequently incubated with peroxidase-linked secondary antibody. The reactive bands were detected by chemiluminescence (Pierce).

**PKC assay.** PKC activity was assayed in the presence of phosphatidylserine by measuring the incorporation of 32P into histone after standard methodology (27).

**Electrophoretic mobility shift assay.** Nuclear extracts were prepared using a nuclear extract kit (Active Motif). Electrophoretic mobility shift assay (EMSA) was performed using a lightshift chemiluminescent EMSA kit (Pierce) using the biotin-labeled Sp1 oligonucleotide duplex (Integrated DNA Technologies). The unlabeled Sp1 consensus oligonucleotide (for competition) and Sp1 antibody (for supershift) were purchased from Santa Cruz Biotechnology.

**Quantification of VEGF.** The concentrations of human and mouse VEGF in tissue culture supernatants were determined using Quantikine VEGF immunoassay kits (R&D Systems).

**In vivo tumor development.** Human renal cancer cells (786-O) were injected s.c. into immunodeficient (nu/nu) mice. To evaluate the growth of tumors in allograft recipients, murine tumor cells (CT26) were injected s.c. into BALB/c mice 1 wk before heart transplantation. Tumor volume was measured using a digital caliper at regular intervals. The volume was estimated by following a standard method (10), using the formula \( V = \frac{1}{2} \times a \times b^2 \), wherein \( a \) is the short axis and \( b \) is the long tumor axis.

**Heart transplantation.** BALB/c mice were used as recipients of fully MHC mismatched C57BL/6 donor hearts. Vascularized intra-abdominal heterotopic heart transplantation was performed as described (14). Donor hearts were monitored daily (by measuring palpation) for the development of rejection.

**RNA isolation and real-time PCR.** Total RNA was prepared using the RNeasy isolation kit (Qiagen), and cDNA was synthesized using cloned AMV first-strand synthesis kit (Invitrogen). To analyze VEGF expression, real-time PCR was performed using the Assays-on-Demand Gene Expression product (TaqMan, Mammalian Gene Collection probes) according to the manufacturer’s instructions (Applied Biosystems). As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified.

Gene-specific primers were obtained from Applied Biosystems. \( C_t \) value (the cycle number at which emitted fluorescence exceeded an automatically determined threshold) for gene of interest (VEGF) was corrected by the \( C_t \) value for GAPDH and expressed as \( \Delta C_t \). Data were reported as fold change in mRNA amount, which was calculated as follows: (fold change) = \( 2^{\Delta C} \) (wherein \( X = \Delta C \), for control group – \( \Delta C \), for experimental group).
Immunohistochemistry. To stain the tumor vessels, tissue sections were incubated first with anti-mouse CD31 (BD PharMingen) and then with a species-specific horseradish peroxidase–conjugated secondary antibody using standard protocol (28). Specimens were developed in 3-aminoethylcarbazole and were counterstained in Gill's hematoxylin. Vessel densities were quantified by grid-counting method at ×400 magnification.

Statistical analysis. Statistical evaluation for data analysis was determined by Student's t test. Differences with \( P < 0.05 \) were considered statistically significant.

Results

CsA promotes the transcriptional activation of VEGF through the PKC pathway. We first evaluated whether CsA can induce VEGF expression in cancer cells. As renal cancer is common among transplant recipients (1–3), we made use of an established human renal cancer cell line (786-0) for our in vitro studies. The 786-0 cells were treated with either increasing concentrations of CsA or vehicle, and the expression of VEGF was examined by Western blot analysis. As shown in Fig. 1A, CsA significantly induced VEGF protein expression in these cells. We then evaluated whether CsA can regulate VEGF promoter activity in 786-0 and also in normal renal TECs. The cells were transiently transfected with a 2.6-kb full-length VEGF promoter-luciferase construct and treated with either increasing concentrations of CsA or vehicle. The effect of CsA on VEGF promoter activity was assessed by luciferase assay. As shown in Fig. 1B, CsA significantly induced VEGF transcriptional activation in a dose-dependent manner compared with vehicle-treated controls in 786-0 cells and in TEC. Thus, CsA may regulate VEGF transcription in both renal cancer and renal epithelial cells.

![Figure 1](https://www.aacrjournals.org/doi/figure/10.1158/0008-5472.CAN-08-0211)

**Figure 1.** CsA promotes VEGF transcriptional activation. **A,** 786-0 cells were treated with different concentrations (1.0 and 5.0 μg/mL) of CsA or with the vehicle alone (control) for 24 h. Whole-cell lysates were prepared, and Western blot analysis was performed using anti-VEGF to quantitate VEGF protein expression (top). The expression of \( β \)-actin in these cells was analyzed by Western blot analysis using anti–\( β \)-actin (bottom). The bar graph below the Western blot illustrates the relative expression of VEGF by densitometry, wherein the signals were standardized to the expression of the internal control \( β \)-actin. Representative of three independent experiments. **Columns,** average of relative intensity of VEGF expression from three different blots; **bars,** SD. **B,** 786-0 and TEC cells were transfected with the full-length (2.6 kb) VEGF promoter-luciferase construct (1.0 μg), cultured for 12 h, and then treated overnight (12 h) either with different concentrations (1.0 and 5.0 μg/mL) of CsA or with the vehicle alone (control). C, 786-0 cells were pretreated with the pharmacologic kinase inhibitors PD98059 (10 μmol/L), LY294002 (10 μmol/L), or calphostin C (250 nmol/L). Control cells were pretreated with the vehicle alone. D, 786-0 cells were pretreated either with different concentrations (50–250 nmol/L) of calphostin C or with the vehicle alone. 

\( C \) and \( D \), pretreated cells were transfected with the 2.6-kb VEGF promoter-luciferase construct (1.0 μg), cultured for 12 h, and then treated overnight (12 h) either with CsA (5.0 μg/mL) or with the vehicle alone (control), in the absence or presence of the kinase inhibitors. 

B to D, the cells were harvested after 12 h of CsA treatment, and fold increase in luciferase activity was calculated as the relative luciferase counts from the CsA-treated or pharmacologic inhibitor-treated group of cells compared with that of cells treated with the vehicle alone. The data reflect three independent experiments. **Columns,** average of triplicate readings of two different samples; **bars,** SD. * \( P < 0.05 \) compared with vehicle-treated control (A–D); **, \( P < 0.05 \) compared with CsA-treated but pharmacologic inhibitor–untreated group (C and D).
We next determined the role of intermediary signaling molecules in CsA-mediated VEGF transcriptional activation. It has been shown that CsA may regulate kinases, such as c-Jun NH2 kinase, MAPK, and PKC (30–32). To evaluate the roles of these kinases, we transfected 786-0 cells with the 2.6-kb VEGF promoter-luciferase construct and treated them with CsA in the absence or presence of pharmacologic inhibitor; vehicle-treated cells served as controls. As shown in Fig. 1C, CsA significantly induced VEGF transcription compared with vehicle-treated controls; although PD98059 (a MEKK inhibitor) and LY294002 (a PI3K inhibitor) had no significant effect, calphostin C (a generalized PKC inhibitor) significantly inhibited CsA-mediated VEGF transcription. Furthermore, we observed that the effect of calphostin C on CsA-mediated VEGF transcription was dose dependent (Fig. 1D). These results suggest that PKC is an important intermediary signaling molecule in CsA-mediated VEGF transcriptional activation.

**Role of atypical and novel PKC isoforms in CsA-mediated VEGF transcription.** Members of the PKC family can be subdivided into three major classes: classic, atypical, and novel PKC isoforms (33). In the next series of experiments, we sought to determine which PKC isoforms may be important for CsA-mediated VEGF transcription. First, we used Go6976, a selective inhibitor of the classic PKC family (such as PKCα and PKCβ ref. 34). The 786-0 cells were transfected with the 2.6-kb VEGF promoter-luciferase construct and treated with CsA in the absence or presence of Go6976. Vehicle-treated cells served as control. We found that Go6976 failed to inhibit CsA-mediated VEGF transcriptional activation and rather, to our surprise, somewhat increased

![Figure 2](image-url)
VEGF transcription (Fig. 2A). Thus, the classic PKC isoforms are not intermediaries in CsA-mediated VEGF transcriptional activation. In fact, they may facilitate a negative feedback loop for VEGF expression.

Next, we wished to evaluate the roles of PKCζ and PKCd, members of the atypical and novel PKC families, respectively (33), in CsA-mediated VEGF transcription. The 786-0 cells were cotransfected with the 2.6-kb VEGF promoter-luciferase construct and either a specific dominant-negative mutant of PKCζ/PKCδ or the empty expression vector. After transfection, the cells were treated with either CsA or vehicle. As shown in the Fig. 2B, the dominant-negative mutants of both PKCζ and PKCd dose-dependently inhibited CsA-mediated VEGF transcriptional activation compared with the empty vector–transfected and vehicle-treated controls. To next assess whether CsA increases the phosphorylation of PKCζ or PKCd, 786-0 cells were treated with either different concentrations of CsA or vehicle. Western blot analysis was performed using a phospho-specific antibody to either PKCζ or PKCd. We found that CsA increased the phosphorylation of both PKCζ and PKCd compared with vehicle-treated controls (Fig. 2C). However, there was no change in the amount of total PKCζ or PKCd in these cells after CsA treatment. In separate experiments, we also found that CsA increased the kinase activity of both PKCζ (Fig. 2D) and PKCd (data not shown) compared with vehicle-treated controls. Together, these findings suggest that PKCζ and PKCd may act as critical intermediaries in CsA-induced VEGF transcription.

CsA-mediated VEGF transcription involves Sp1 but not HIF-α. The transcription factor Sp1 is an established regulator of VEGF expression (26, 27). Moreover, we have previously observed that PKC can form complex with Sp1 to facilitate VEGF transcriptional activation (27). Because CsA increases PKCζ and PKCd phosphorylation, we next evaluated whether CsA could promote the association between these two PKC isoforms and Sp1.
By immunoprecipitation, we observed that CsA treatment indeed promoted the association of both PKCζ and PKCδ with Sp1 in 786-0 cells (Fig. 3A). A similar level of association of both phosphorylated PKCζ and phosphorylated PKCδ with Sp1 was also found after CsA treatment (data not shown). By EMSA, we found that CsA treatment of 786-0 cells promoted the binding of Sp1 to a specific DNA probe containing an Sp1-binding site, and the induced binding of Sp1 to the probe was confirmed by both supershift and competition assays (Fig. 3B). Thus, CsA-induced and PKC-Sp1–mediated pathways may play an important role in VEGF transcription.

We next determined if Sp1 facilitates CsA-mediated VEGF transcription. Besides Sp1, HIF-α is another well-established transcription factor for VEGF expression (26, 35). We used two 5′ deletion constructs of the 2.6-kb full-length VEGF promoter-luciferase plasmid (Supplementary Fig. S1; ref. 26): a 0.35-kb construct that has a deleted HIF-α-binding site but intact Sp1-binding sites and a 0.07-kb construct that has deleted binding sites for both HIF-α and Sp1. The 2.6-kb construct has binding sites for both HIF-α and Sp1. We transfected 786-0 cells with each of the VEGF promoter-luciferase constructs and evaluated the effect of CsA on VEGF transcriptional activation by measuring luciferase activity. Vehicle-treated cells served as controls. We observed that there was a marked decrease in basal luciferase activity with the 0.35-kb construct compared with the 2.6-kb construct; however, the relative fold induction of each construct after CsA treatment was similar (~3-fold; Fig. 3C). These results suggest that HIF-α plays a major role in basal activation of the VEGF promoter in 786-0 cells, but it is unlikely to be involved in CsA-mediated VEGF transcription. In contrast, there was a marked reduction in basal luciferase activity with the 0.07-kb construct compared with the 0.35-kb construct and there was no change in the activation of the 0.07-kb promoter after CsA treatment (Fig. 3C). These results support a major role for Sp1 in both basal and CsA-mediated VEGF transcription.

Although we found that CsA may promote VEGF transcription in a HIF-α–independent manner, we next confirmed our findings using siRNA. It is established that in 786-0 cells, HIF-1α is undetectable but HIF-2α is active (36). Thus, we first knocked down HIF-2α in 786-0 cells using gene-specific siRNA and then

![Figure 4](image-url)

**Figure 4.** pVHL inhibits CsA-induced VEGF transcriptional activation. 786-0 and Caki-1 cells (A) and Neo and wt-VHL cells (B) were transfected with the 2.6-kb VEGF promoter-luciferase construct and cultured for 12 h. The cells were then treated overnight (12 h) either with CsA (5.0 μg/mL) or with the vehicle alone. After CsA treatment, the cells were harvested and fold increase in luciferase activity was calculated as the relative luciferase counts from the CsA-treated group of cells compared with cells treated with the vehicle alone. The data reflect three independent experiments. Columns, average of triplicate readings of two different samples; bars, SD. *, P < 0.05 compared with respective vehicle-treated controls; **, P < 0.05 compared with either CsA-treated 786-0 or CsA-treated Neo cells. B, bottom, expression of pVHL in wt-VHL cells was confirmed by Western blot analysis. C, Neo and wt-VHL cells were treated either with CsA (5.0 μg/mL) or the vehicle alone for 2 h. The cells were lysed and the extracts were immunoprecipitated with anti-Sp1. Immunoprecipitates (IP) were captured by protein A–Sepharose beads and were separated by SDS-PAGE. Western blot (Blot) was performed with anti-PKCζ or anti-Sp1. Bottom, total lysate input of PKCζ. Representative of three independent experiments with similar findings.
studied the effect of CsA on the induction of full-length 2.6-kb *VEGF* promoter in these cells as described above. As shown in Fig. 3D, CsA promoted *VEGF* transcriptional activation in HIF-2α knockdown cells to a similar level as observed in control siRNA-transfected cells, although there was a decrease in basal promoter activity. The knockdown of HIF-2α was confirmed by Western blot analysis (Fig. 3D, right). Together, these results suggest that CsA can induce *VEGF* transcription in a HIF-α-independent manner and that PKCζ-mediated and PKCδ-mediated binding of Sp1 to the *VEGF* promoter may be one of the important regulatory factors in this induction process.

**Role of *VHL* tumor suppressor gene in CsA-mediated *VEGF* transcription.** *VHL* has a critical role in the pathogenesis of renal cell carcinoma (37). It is known that 786-0 cells lack *VHL* (37, 38), whereas the Caki-1 renal cancer cell line retains the gene (38). Thus, we first tested whether CsA could promote the activation of the 2.6-kb *VEGF* promoter in Caki-1 cells, as observed in 786-0 cells. We found that, although there was induction in CsA-mediated *VEGF* promoter activity in *VHL*-containing Caki-1 cells, the effect was much lower compared with 786-0 cells lacking *VHL* (Fig. 4A).

To dissect the specific role of *VHL*, we made use of 786-0 cells stably transfected with either empty vector (Neo) or wt-*VHL* (26) and studied the effect of CsA on *VEGF* promoter activity in these cells. As shown in Fig. 4B, CsA promoted ~3-fold increase in the 2.6-kb *VEGF* promoter activity in Neo cells; however, the activation was blunted in wt-*VHL* cells. In addition, we observed that in contrast to Neo cells, CsA could not increase the association between PKCζ and Sp1 in wt-*VHL* cells (Fig. 4C).

These results suggest that the *VHL* protein (pVHL) may inhibit CsA-mediated *VEGF* transcriptional activation likely through the prevention of increased association between PKCζ and Sp1. However, we cannot rule out the presence of a pVHL-independent pathway that may also be involved in CsA-mediated *VEGF* transcription.
CsA promotes renal tumor growth in vivo. We next examined whether CsA can promote renal tumor growth in vivo. We used 786-0 human renal cancer cells in which VEGF secretion was found to be increased after CsA treatment (Fig. 5A). Tumor cells were injected s.c. in immunodeficient (nu/nu) mice. The mice (n = 5 in each group) were then treated either with CsA (10 mg/kg/d) or with vehicle as control for 25 days. We observed that from day 5 after tumor injection, CsA treatment enhanced tumor growth compared with vehicle-treated controls (Fig. 5B). Tumors were harvested on day 25 and evaluated for VEGF expression. By real-time PCR, we observed that CsA treatment significantly induced VEGF expression in the tumor compared with vehicle-treated controls (Fig. 5C). CsA treatment also markedly increased tumor vessel density as observed by CD31 staining (Fig. 5D). Thus, CsA may promote tumor growth in vivo and the overexpression of VEGF by the tumor cells as observed in our in vitro studies may be critical in mediating enhanced tumor angiogenesis. However, we cannot rule out a direct effect of CsA on endothelial cell proliferation.

CsA promotes post-transplantation tumor growth in VEGF-dependent manner. To evaluate the in vivo significance of CsA-induced VEGF overexpression in terms of post-transplantation tumor development, we modified a previously reported mouse model of post-transplantation cancer (10). We made use of a syngeneic tumor cell line (CT26) that can form VEGF-dependent angiogenic tumors. We found that these cells express HIF-1, as observed by others (39), although there was no expression of pVHL (data not shown). We first confirmed that treatment of CT26 cells with increasing doses of CsA markedly induced VEGF expression/secretion in these cells (Supplementary Fig. S2). CT26 cells were then s.c. injected into BALB/c mice, and fully MHC mismatched cardiac transplants (C57BL/6) were performed in these mice 7 days later. This model may mimic a clinical situation in which few preexisting tumor cells are present in patients undergoing transplantation. After cardiac transplantation, the mice (n = 5 in each group) were treated either with CsA (10 mg/kg/d) or with vehicle as control. Treatment was continued for 14 days (i.e., up to 22 days after tumor injection). We found that vehicle-treated mice rejected allografts within 10 days, whereas there was a significant prolongation of allograft survival in the CsA-treated group (data not shown). Tumor volume was monitored on alternate days. As shown in the Fig. 6A, tumor size was significantly higher in the CsA-treated group compared with the vehicle-treated control group on days 15 and 22 after tumor injection.

Tumors were harvested on day 22 and were initially evaluated for VEGF expression. We observed a significant increase in VEGF mRNA expression within tumors in the CsA-treated animals compared with vehicle-treated mice (Fig. 6B). We also found that tumor vessel density was markedly increased in the CsA-treated group, as observed by CD31 staining (Fig. 6C).

We next evaluated whether neutralization of VEGF can attenuate CsA-induced post-transplantation tumor growth. We used the same in vivo model of posttransplantation cancer as described above. After CT26 tumor cell injection and cardiac transplantation, the mice (n = 5 in each group) were treated with CsA (10 mg/kg/d) in the absence or presence of a murine blocking anti-VEGF antibody; vehicle-treated and IgG isotype–treated mice served as controls. Treatments were continued until day 30 of tumor injection. We found that treatment with anti-VEGF alone resulted in some inhibition of tumor volume compared with control (data not shown). As shown in the Fig. 6D, CsA promoted tumor growth compared with vehicle-treated controls and blockade of VEGF substantially inhibited CsA-induced tumor development. However, the anti-VEGF treatment did not inhibit the tumor volume to the level of control, suggesting the possible roles of additional factors in CsA-mediated tumorigenic process, as proposed by others (7). Nevertheless, these findings suggest that CsA can promote the development of post-transplantation cancer through its effect on VEGF expression and VEGF-induced angiogenesis.

Discussion

Although calcineurin inhibitors are excellent immunosuppressive agents to inhibit allograft rejection, they may promote the growth of different tumors (7, 9–11, 40). In this study, we define a mechanism in human cancer cells by which CsA can promote tumor growth through VEGF overexpression and angiogenesis, having direct relevance for the development of post-transplantation cancer. Although not shown, we have found that FK506, another calcineurin inhibitor (25), also induces VEGF overexpression in these cells.

Some previous studies have suggested that CsA may have both proangiogenic and antiangiogenic effects. CsA may inhibit VEGF-induced angiogenesis either alone or in the presence of some angiogenesis inhibitors (22, 41). In contrast, Shihab and colleagues (42) showed that, during CsA-induced nephrotoxicity, VEGF and its receptors are overexpressed. Gottsch and colleagues (43) observed that CsA can promote angiogenesis in corneal ulcers. However, CsA may mediate completely opposite effects on the same signaling pathway in two different cell types (44).

We suggest some possible mechanisms for the effect of CsA and other calcineurin inhibitors on VEGF overexpression. One possibility is that while CsA treatment blocks the calcineurin/NFAT signaling pathway, it may also suppress negative regulators of VEGF expression and angiogenesis as proposed by others (20, 23). Thus, in the absence of any negative regulator, VEGF may be overexpressed in transplant patients and induce the growth of micromalignants under immunosuppressed conditions. Another possibility is that CsA-induced VEGF expression may be an indirect effect through TGF-β/Sp1 signaling. CsA is a potent inducer of TGF-β (7), and it has been reported that TGF-β can stimulate VEGF transcription (45). However, CsA can also directly promote VEGF transcriptional activation as shown in this study.

Apart from inhibiting NFAT, the calcineurin inhibitors may also regulate other signaling molecules involved in VEGF expression. Pan and colleagues (46) recently showed that CsA inhibits carabin, a novel endogenous inhibitor of calcineurin. Carabin may also inhibit the Ras signaling pathway, suggesting that CsA can activate Ras (a known inducer of VEGF) through the inhibition of carabin. Cho and colleagues (47) reported that, while CsA or FK506 suppress calcineurin, they may also unleash the PKC signaling pathways to promote the expression of the linker for activation of T cells. In this study, we have shown for the first time that CsA activates PKCζ and PKCδ isoforms in human renal cancer cells. Our observations show that blockade of the PKCζ and PKCδ pathways inhibits CsA-induced VEGF transcriptional activation. We have also found that CsA can promote the association of PKCζ and PKCδ with the transcription factor Sp1 and can induce Sp1 DNA-binding activity. We have previously reported that PKCζ can phosphorylate Sp1 (27) and, thus, we suggest that CsA may activate Sp1 through its association with PKCζ.
It is established that HIF-α and pVHL play major roles in the regulation of VEGF in renal cancer. The 786-0 cells lack VHL (37, 38), and in the absence of pVHL, HIF-2α is stabilized in these cells (36). Our findings suggest that HIF-2α is not involved in CsA-mediated VEGF transcription. However, others have shown that CsA may regulate gene expression through either degradation of HIF-1α or prevention of HIF-1α protein accumulation (48, 49). In this study, we have found that CsA-induced VEGF transcription is mediated primarily through the PKCζ-Sp1 and PKCδ-Sp1 pathways. We have also observed that CsA-mediated VEGF transcription is partially inhibited by wt-pVHL, which prevents the association between PKCζ and Sp1. It has been shown that pVHL can also directly bind to Sp1 and may prevent Sp1-mediated gene transcription (26). We and others have previously reported that pVHL can bind to atypical PKC isoforms and either prevent their membrane translocation (34) or promote their degradation (50), supporting our present findings. The effect of CsA on VEGF expression was smaller in pVHL-intact cells compared with pVHL-deficient cells. Thus, loss of pVHL, as occurs early in the course of renal cell carcinoma development, may sensitize a cell to CsA-induced growth-promoting effects. However, although pVHL seems to be a critical regulator of CsA-mediated VEGF expression, we cannot rule out a pVHL-independent pathway that may involve other PKC isoforms, like PKCδ.

In summary, the mechanism(s) underlying the development of post-transplantation cancer should be thoroughly evaluated, such that select agents can be used to target cancer development. Our in vitro and in vivo studies in this report clearly show the role of overexpressed VEGF in the development of CsA-induced post-transplantation cancer. Thus, targeting the pathways that promote VEGF overexpression in response to calcineurin inhibitors might
serve as novel therapeutics for the prevention and treatment of post-transplantation cancer.

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No potential conflicts of interest were disclosed.

References

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Aninda Basu, Alan G. Contreras, Dipak Datta, et al.


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