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OVEREXPRESSION OF VASCULAR ENDOTHELIAL GROWTH FACTOR AND THE DEVELOPMENT OF POST-TRANSPLANTATION CANCER

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Abstract

Cancer is an increasing and major problem following 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 786-0 human renal cancer cells to investigate the effect of Cyclosporine (CsA) on VEGF expression. Utilizing a full-length *VEGF* promoter-luciferase construct, we found that CsA markedly induced *VEGF* transcriptional activation through the PKC signaling pathway, specifically involving PKC ζ and PKC δ isoforms. Moreover, CsA promoted the association of PKC ζ and PKC δ 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 pVHL. CsA also promoted the progression of human renal tumors *in vivo*, where 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, *VEGF* mRNA expression, and also enhanced tumor angiogenesis. However, co-administration 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.

Keywords

Transplantation; cancer; VEGF; angiogenesis

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INTRODUCTION

Cancer is a common and major problem following organ transplantation (1-3). Malignant tumors develop in 15-20% 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 non-lymphoid 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 following 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 et al (7) demonstrated 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 host's immune system. Similarly, FK506 has been shown to promote the proliferation of tumor cells through TGF- β (9). Koehl et al (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 et al (11) suggested that CsA may induce the expression of an angiogenic cytokine, like vascular endothelial growth factor (VEGF), but did not demonstrate 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 micro tumors 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 Ca^{2+} binds to both calmodulin and the B subunit, displacing the inhibitory C-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). While NFAT is functional in many cell types, it has been best studied in T cell activation responses, where it induces different cytokines, like IL-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 pathway may exert both positive and negative regulatory signals on different angiogenic molecules. 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 demonstrate 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 observe that CsA augments tumor growth following cardiac transplantation *in vivo*, and that this effect is in part dependent on increased VEGF expression.

MATERIALS AND METHODS

Reagents

Cyclosporine (CsA) (Novartis, East Hanover, NJ) and FK506 (Astellas, Deerfield, IL) were purchased from Children's Hospital Boston pharmacy. The MEKK inhibitor PD98059, the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002, the generalized PKC inhibitor Calphostin C and the classical PKC inhibitor Go6976 were obtained from Calbiochem (La Jolla, CA). The small interfering RNA (siRNA) for HIF-2 α and its control were purchased from Qiagen (Valencia, CA).

Development Of Blocking Antibody To Murine VEGF

The development of our blocking anti-murine VEGF antibody (by DMB) was performed using reagents and techniques similar to those previously described (14). Briefly, noble rats were immunized with the N-terminal amino acid sequence of murine VEGF (CAPTTEGEQKSHEVIKFMVDVYQRSY) coupled with keyhole limpet hemocyanin (KLH) using the maleimidobenzyl-N-hydroxysuccinimide ester (MBS) crosslinker. Hybridoma fusion products were generated according to standard protocols, and were screened based on their anti-VEGF (peptide) optical density reading in ELISA. Secondary screens demonstrated two IgG producing clones, 2G11-2A05 and 1F07-1C02, which were subcloned and purified. In Western blots, anti-VEGF 2G11-2A05 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, Santa Cruz, CA). We also found that anti-VEGF monoclonal antibody 2G11-A05 blocked murine VEGF-induced proliferation of endothelial cells *in vitro* using an assay previously described (14), and that 2G11-A05 neutralized VEGF function *in vivo* in our standardized VEGF-induced angiogenesis assay (14).

Cell Culture

The human renal cancer cell lines (786-0 and Caki-1) and the murine colon adenocarcinoma cell line (CT26) were obtained from American Type Culture Collection, Manassas, VA. The cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT). 786-0 clonal cell lines stably transfected with either pFLAG-CMV2 (Neo cells, containing empty vector with *neo* cassette), or pFLAG-CMV2-VHL (VHL cells, containing wt-VHL) were grown in complete medium supplemented with G418 (0.5 mg/ml) (26). Human renal proximal tubular epithelial cells (TEC) were purchased from Clonetics (Walkersville, MD) and were cultured in complete epithelial medium (REGM BulletKit, MD).

Plasmids

A 2.6-kb *VEGF* promoter-luciferase construct in pGL2 basic vector (Promega, Madison, WI), containing the full-length *VEGF* promoter sequence (base pairs -2361 to +298 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 PKC ζ plasmid (PKC ζ KW) was a generous gift from Alex Toker (Beth Israel Deaconess Medical Center, Boston, MA) (27). The kinase inactive PKC δ plasmid (PKC δ KR) was a generous gift from Rakesh Dutta (Dana Farber Cancer Institute, Boston, MA) (27).

Transfection Assays

Cells were plated at 2×10^5 cells/well in 6-well plates, and were transfected with expression plasmids using the Effectene transfection reagent (Qiagen) (28). The total amount of transfected plasmid DNA was normalized using a control empty expression vector. Transfection efficiency was determined by co-transfection of the β -galactosidase gene and by measurement of β -galactosidase activity. For luciferase assays, cells were harvested 24 hours 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, Piscataway, NJ), and bead-bound proteins were subjected to Western blot analysis using either anti- PKC ζ or anti- PKC δ (Santa Cruz Biotechnology).

Western Blot Analysis

Protein samples were run on SDS-polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane (NEN Life Sciences Product Inc, Boston, MA) (28). The membranes were incubated with anti-VEGF/anti- β -actin, anti-phospho PKC ζ/δ , anti-total PKC ζ/δ , anti-HIF-2 α or anti-VHL (Santa Cruz Biotechnology), and subsequently incubated with peroxidase-linked secondary antibody. The reactive bands were detected by chemiluminescence (Pierce, Rockford, IL).

PKC Assay

PKC activity was assayed in the presence of phosphatidylserine by measuring the incorporation of ^{32}P into histone following standard methodology (27).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared using a nuclear extract kit (Active Motif, Carlsbad, CA). EMSA was performed using a lightshift chemiluminescent EMSA kit (Pierce), utilizing the biotin-labeled Sp1 oligonucleotide duplex (Integrated DNA Technologies, Coralville, IA). 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, Minneapolis, MN).

In Vivo Tumor Development

Human renal cancer cells (786-0) were injected subcutaneously in immunodeficient (nu/nu) mice. To evaluate the growth of tumors in allograft recipients, murine tumor cells (CT26) were injected subcutaneously into BALB/c mice one week 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 $V = \pi / 6 \times a^2 \times b$, where a was the short and b the long tumor axis. Mice were sacrificed at designated times following injection or if complications occurred, which included signs of inactivity, cachexia, or decreased responsiveness.

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, Carlsbad, CA). 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, Foster City, CA). As an internal control, *GAPDH* mRNA was amplified. Gene-specific primers were obtained from Applied Biosystems. Ct value (the cycle number at which emitted fluorescence exceeded an automatically determined threshold) for gene of interest (*VEGF*) was corrected by the Ct value for *GAPDH* and expressed as ΔCt . Data were reported as fold change in mRNA amount, which was calculated as follows: (fold change) = 2^X (where $X = \Delta Ct$ for control group - ΔCt for experimental group).

Immunohistochemistry

To stain the tumor vessels, tissue sections were incubated first with anti-mouse-CD31 (BD Pharmingen, San Diego, CA), and then with a species-specific horseradish peroxidase-conjugated secondary antibody using standard protocol (28). Specimens were developed in 3-amino ethylcarbazole and were counterstained in Gill's hematoxylin. Vessel densities were quantified by grid-counting method at 400X 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 Protein Kinase C (PKC) Pathway

We first evaluated whether Cyclosporine (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 the Figure-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 tubular epithelial cells (TEC). 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 Figure-1B, CsA significantly induced *VEGF* transcriptional activation in a dose-dependent manner as compared to vehicle-treated controls in 786-0 cells and in TEC. Thus, CsA may regulate *VEGF* transcription in both renal cancer and renal epithelial cells.

We next determined the role of intermediary signaling molecules in CsA-mediated *VEGF* transcriptional activation. It has been demonstrated that CsA may regulate kinases, such as JNK, 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

absence/presence of pharmacological inhibitor; vehicle-treated cells served as controls. As shown in the Figure-1C, CsA significantly induced *VEGF* transcription as compared to 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 (Figure-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: classical, atypical and novel PKC isoforms (33). In 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 classical PKC family (such as PKC α and PKC β) (34). The 786-0 cells were transfected with the 2.6-kb *VEGF* promoter-luciferase construct, and treated with CsA in absence/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 *VEGF* transcription (Figure-2A). Thus, the classical PKC isoforms are not intermediaries in CsA-mediated *VEGF* transcriptional activation. In fact, they may facilitate a negative feed-back loop for *VEGF* expression.

Next, we wished to evaluate the roles of PKC ζ and PKC δ , members of the atypical and novel PKC families respectively (33), in CsA-mediated *VEGF* transcription. The 786-0 cells were co-transfected with the 2.6-kb *VEGF* promoter-luciferase construct and either a specific dominant-negative mutant of PKC ζ /PKC δ or the empty expression vector. Following transfection, the cells were treated with either CsA or vehicle. As shown in the Figure-2B, the dominant-negative mutants of both PKC ζ and PKC δ dose-dependently inhibited CsA-mediated *VEGF* transcriptional activation, as compared to the empty vector-transfected and vehicle-treated controls. To next assess whether CsA increases the phosphorylation of PKC ζ or PKC δ , 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 PKC ζ or PKC δ . We found that CsA increased the phosphorylation of both PKC ζ and PKC δ as compared to vehicle-treated controls (Figure-2C). However, there was no change in the amount of total PKC ζ or PKC δ in these cells following CsA treatment. In separate experiments, we also found that CsA increased the kinase activity of both PKC ζ (Figure-2D) and PKC δ (data not shown) as compared to vehicle-treated controls. Together, these findings suggest that PKC ζ and PKC δ 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). Since CsA increases PKC ζ and PKC δ 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 PKC ζ and PKC δ with Sp1 in 786-0 cells (Figure-3A). A similar level of association of both phospho-PKC ζ and phospho-PKC δ with Sp1 was also found following 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 (Figure-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, hypoxia inducible factor- α (HIF- α) is another well-established transcription factor for *VEGF* expression (26,35). We utilized two 5' deletion constructs of the 2.6-kb full-length *VEGF* promoter-luciferase plasmid (Supplementary Figure-1) (26); a 0.35-kb construct which 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 following CsA treatment was similar (~3-fold) (Figure-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 following CsA treatment (Figure-3C). These results support a major role for Sp1 in both basal as well as 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-1 α is undetectable, but HIF-2 α is active (36). Thus, we first knocked down HIF-2 α in 786-0 cells using gene-specific siRNA, and then 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 Figure-3D, CsA promoted *VEGF* transcriptional activation in HIF-2 α knock-down cells to a similar level as observed in control siRNA-transfected cells, although there was a decrease in basal promoter activity. The knock down of HIF-2 α was confirmed by Western blot analysis (Figure-3D, right panel). Together, these results suggest that CsA can induce *VEGF* transcription in a HIF- α -independent manner; and that PKC ζ - and PKC δ -mediated binding of Sp1 to the *VEGF* promoter may be one of the important regulatory factors in this induction process.

Role Of von Hippel-Lindau (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), while 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* (Figure-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 Figure-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 PKC ζ and Sp1 in wt-VHL cells (Figure-4C). These results suggest that the VHL protein (pVHL) may inhibit CsA-mediated *VEGF* transcriptional activation likely through the prevention of increased association between PKC ζ 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 following CsA treatment (Figure-5A). Tumor cells were injected subcutaneously in immunodeficient (nu/nu) mice. The mice (n=5 in each group) were then treated either with CsA (10 mg/kg/day) or with vehicle as control for 25 days. We observed that from day 5 following tumor injection, CsA treatment enhanced tumor growth as compared to vehicle-treated controls (Figure-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 as compared to vehicle-treated controls (Figure-5C). CsA treatment also markedly increased tumor vessel density as observed by CD31 staining (Figure-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 syngenic tumor cell line (CT26), which can form VEGF-dependent angiogenic tumors. We found that these cells express HIF-1, as observed by others (39), while 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 Figure-2). CT26 cells were then subcutaneously 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 pre-existing tumor cells are present in patients undergoing transplantation. Following cardiac transplantation, the mice (n=5 in each group) were treated either with CsA (10 mg/kg/day) or with vehicle as control. Treatment was continued for 14 days (i.e., up to 22 days following 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 Figure-6A, tumor size was significantly higher in the CsA-treated group as compared to the vehicle-treated control group on days 15 and 22 following 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 (Figure-6B). We also found that tumor vessel density was markedly increased in the CsA-treated group, as observed by CD31 staining (Figure-6C).

We next evaluated whether neutralization of VEGF can attenuate CsA-induced post-transplantation tumor growth. We used the same *in vivo* model of post-transplantation cancer as described above. Following CT26 tumor cell injection and cardiac transplantation, the mice (n=5 in each group) were treated with CsA (10 mg/kg/day) in absence or presence of a murine blocking anti-VEGF antibody; vehicle- 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 as compared to control (data not shown). As shown in the Figure-6D, CsA promoted tumor growth as compared to 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 pro-angiogenic and anti-angiogenic effects. CsA may inhibit VEGF-induced angiogenesis either alone or in presence of some angiogenesis inhibitors (22,41). In contrast, Shihab et al (42) demonstrated that during CsA-induced nephrotoxicity, VEGF and its receptors are overexpressed. Gottsch et al (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 it may induce the growth of micro tumors under immune suppressed conditions. Another possibility is that CsA-induced VEGF expression may be an indirect effect through TGF- β . 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 demonstrated in this study.

Apart from inhibiting NFAT, the calcineurin inhibitors may also regulate other signaling molecules involved in VEGF expression. Pan et al (46) recently showed that CsA inhibits carabin, a novel endogenous inhibitor of calcineurin. Carabin may also inhibit the Ras signaling pathway, suggesting CsA can activate Ras (a known inducer of VEGF) through the inhibition of carabin. Cho et al (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 (LAT). In this study, we have shown for the first time that CsA activates PKC ζ and PKC δ isoforms in human renal cancer cells. Our observations show that blockade of the PKC ζ and PKC δ pathways inhibits CsA-induced *VEGF* transcriptional activation. We have also found that CsA can promote the association of PKC ζ and PKC δ with the transcription factor Sp1, and can induce Sp1 DNA-binding activity. We have previously reported that PKC ζ can phosphorylate Sp1 (27), and thus we suggest that CsA may activate Sp1 through its association with PKC ζ .

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-2 α is stabilized in these cells (36). Our findings suggest that HIF-2 α is not involved in CsA-mediated *VEGF* transcription. However, others have demonstrated that CsA may regulate gene expression through either degradation of HIF-1 α or prevention of HIF-1 α protein accumulation (48,49). In this study, we have found that CsA-induced *VEGF* transcription is mediated primarily through the PKC ζ -Sp1 and PKC δ -Sp1 pathways. We have also observed that CsA-mediated *VEGF* transcription is partially inhibited by wt-pVHL, which prevents the association between PKC ζ 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 appears 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 PKC δ .

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 demonstrate 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure-1. CsA promotes VEGF transcriptional activation

(A) The 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 hours. Whole cell lysates were prepared, and Western blot analysis was performed using anti-VEGF to quantitate VEGF protein expression (upper panel). The expression of β -actin in these cells was analyzed by Western blot analysis using anti- β -actin (lower panel). The bar graph below the Western blot illustrates the relative expression VEGF by densitometry, where 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*, $\pm$ SD.

(B) The 786-0 and TEC cells were transfected with the full-length (2.6-kb) VEGF promoter-luciferase construct (1.0 µg), cultured for 12 hours, and then treated overnight (12 hours) either with different concentrations (1.0 and 5.0 µg/ml) of CsA or with the vehicle alone (control).

(C) The 786-0 cells were pretreated with the pharmacological kinase inhibitors PD98059 (10 µM), LY294002 (10 µM), or Calphostin C (250 nM). Control cells were pretreated with the vehicle alone. (D) The 786-0 cells were pretreated either with different concentrations (50-250 nM) of Calphostin C or with the vehicle alone. In (C) and (D), the pretreated cells were transfected with the 2.6-kb VEGF promoter-luciferase construct (1.0 µg), cultured for 12 hours, and then treated overnight (12 hours) either with CsA (5.0 µg/ml) or with the vehicle alone (control), in absence/presence of the kinase inhibitors. In (B), (C), and (D), the cells were harvested after 12 hours of CsA treatment, and fold increase in luciferase activity was calculated as the relative luciferase counts from the CsA-treated or pharmacological 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*, $\pm$ SD. In (A-D), *, $p < 0.05$ compared with vehicle-treated control; and in (C) and (D), **, $p < 0.05$ compared with CsA-treated but pharmacological inhibitor-untreated group.

Figure-2. CsA promotes VEGF transcriptional activation through PKC ζ or PKC δ

(A) The 786-0 cells were pretreated either with different concentrations (250-1000 nM) of Go6976 or with the vehicle alone. The pretreated cells were transfected with the 2.6-kb VEGF promoter-luciferase construct (1.0 μ g), cultured for 12 hours, and then treated overnight (12 hours) either with CsA (5.0 μ g/ml) or with the vehicle alone (control), in absence/presence of the kinase inhibitors. Following 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 that of cells treated with the vehicle alone. The data reflect three independent experiments. *Columns*, average of triplicate readings of two different samples; *bars*, $\pm$ SD. *, $p < 0.05$ compared with vehicle-treated control. (B) The 786-0 cells were co-transfected with the 2.6 kb VEGF promoter-luciferase construct, and a dominant-negative (DN) mutant of either PKC ζ or PKC δ . Control cells were co-transfected with the VEGF promoter-luciferase construct and the empty expression vector. Following transfection, the cells were cultured for 12 hours, and then treated overnight (12 hours) either with CsA (5.0 μ g/ml) or with the vehicle alone (control). Following 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 that of cells transfected with the empty vector, and treated with the vehicle alone. The data reflect three independent experiments. *Columns*, average of triplicate readings of two different samples; *bars*, $\pm$ SD. *, $p < 0.01$ compared with vehicle-treated control, and **, $p < 0.01$ compared with empty vector-transfected and CsA-treated group. (C) and (D) The 786-0 cells were treated either with different concentrations (1.0 and 5.0 μ g/ml) of CsA or with the vehicle alone for 2 hours. In (C), the cells were lysed, and Western blot analysis using anti-phospho-PKC ζ or anti-phospho-PKC δ was performed to quantitate the expression of phospho-PKC ζ and phospho-PKC δ respectively (first and third panels). The expression of total cellular PKC ζ or PKC δ was analyzed by Western blot analysis using anti-PKC ζ or anti-PKC δ (second and fourth panels). Representative of three independent experiments with similar findings. In Supplementary Figure-3, a bar graph illustrates the relative expression phospho-PKC ζ and phospho-PKC δ by densitometry, where the signals were standardized to the expression of total PKC ζ and total PKC δ (from three different experiments). Although not shown, a similar level of phosphorylation of both PKC ζ and PKC δ was observed even at later time points of CsA treatment (up to 12 hours). In (D), the cells were lysed, and PKC ζ was immunoprecipitated from cell lysate using anti-PKC ζ . Using immunoprecipitated PKC ζ , a kinase assay was performed as described in the methods. The data for the PKC ζ kinase activity are expressed as fold induction with respect to the activity

in vehicle-treated control cells. The data reflect three independent experiments, resulting from duplicate readings of two different samples. *Columns*, average value of PKC ζ kinase activity; *bars*, +/- SD. *, $p < 0.05$ compared with vehicle-treated control.

Figure-3. CsA promotes VEGF transcriptional activation in an Sp1-dependent but a HIF-independent manner

(A) The 786-0 cells were treated either with different concentrations (1.0 and 5.0 μ g/ml) of CsA or the vehicle alone for 2 hours. The cells were lysed and the extracts were immunoprecipitated with anti-Sp1. Immunoprecipitates (IP) were captured by protein A-sepharose beads, and were boiled in SDS buffer and separated by SDS-PAGE. Western blot (Blot) was performed with anti-PKC ζ , anti-PKC δ or anti-Sp1. The bottom panels represent the total lysate inputs of PKC ζ and PKC δ . Representative of three independent experiments with similar findings. (B) The 786-0 cells were treated either with different concentrations (1.0 and 5.0 μ g/ml) of CsA or the vehicle alone for 2 hours. Nuclear extracts were prepared from these cells. EMSA was performed with the nuclear extracts of 786-0 cells using a chemiluminescent kit as described in methods. The probe used was a biotin-labeled oligonucleotide duplex containing an Sp1 binding site. The Sp1 antibody (1.0 μ g) or the control IgG, or the unlabeled Sp1 consensus oligonucleotide (10-fold molar excess) was added to the binding reaction mixture of the indicated samples to either supershift or compete away the CsA (5.0 μ g/ml)-induced specific protein-DNA complex formation (right panel). Representative of three independent experiments with similar findings. (C) The 786-0 cells were transfected with the 2.6-kb, 0.35-kb, or 0.07-kb VEGF promoter-luciferase construct, and cultured for 12 hours. (D) The 786-0 cells were transfected either with HIF-2 α siRNA (25 nM) or control siRNA for 48 hours. The cells were then transfected with the 2.6-kb VEGF promoter-luciferase construct, and cultured for 12 hours. In (C) and (D), the cells were then treated overnight (12 hours) either with CsA (5.0 μ g/ml) or with the vehicle alone. Following CsA treatment, the cells were harvested and the luciferase activity of each group of cells was calculated as described in methods. The data reflect three independent experiments. Columns, average of triplicate readings of two different samples; bars, $\pm$ SD. *, $p < 0.01$ compared with vehicle-treated controls of the respective promoter constructs. In (D), the knock down of HIF-2 α was confirmed by Western blot analysis following 48 hours of siRNA transfection (upper right corner).

Figure-4. pVHL inhibits CsA-induced *VEGF* transcriptional activation

(A) The 786-0 and Caki-1 cells, and (B) the Neo and wt-VHL cells were transfected with the 2.6-kb *VEGF* promoter-luciferase construct, and cultured for 12 hours. The cells were then treated overnight (12 hours) either with CsA (5.0 μ g/ml) or with the vehicle alone. Following 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*, $\pm$ SD. *, $p < 0.05$ compared with respective vehicle-treated controls, and **, $p < 0.05$ compared with either CsA-treated 786-0 or CsA-treated Neo cells. In the lower panel of (B), the expression of pVHL in wt-VHL cells was confirmed by Western blot analysis. (C) The Neo and wt-VHL cells were treated either with CsA (5.0 μ g/ml) or the vehicle alone for 2 hours. 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-PKC ζ or anti-Sp1. The bottom panel represents the total lysate input of PKC ζ . Representative of three independent experiments with similar findings.

Figure-5. CsA promotes the growth of human renal tumors *in vivo* with induced expression of VEGF

(A) The 786-0 cells were serum starved, and then treated with different concentrations (1.0 and 5.0 µg/ml) of CsA or with the vehicle alone (control) for 24 hours. CsA-induced VEGF release in cell culture medium was measured by ELISA as described in methods. The data reflect three independent experiments. *Columns*, average value of VEGF release from triplicate readings of two different samples; *bars*, +/- SD. *, $p < 0.01$ compared with vehicle-treated controls.

(B) 1.0×10^6 human renal tumor cells (786-0) were injected subcutaneously in nude mice, and they ($n=5$ in each group) were treated either with CsA (10 mg/kg/day) or with vehicle as a control. Treatment was continued for 25 days, and tumor volume was monitored at regular intervals using a digital caliper. Tumors were harvested 25 days after tumor injection. The data reflect three independent experiments. *Points*, average value of tumor volume; *bars*, +/- SD. The CsA-treated group had average tumor volumes that were significantly different than the vehicle-treated group ($p < 0.05$).

(C) Total RNA was isolated from harvested tumor tissues (day 25) and reverse transcribed. Fold change in VEGF mRNA expression was measured by real time PCR using gene-specific primers for human VEGF. The data reflect three independent experiments, resulting from duplicate readings of two different samples. *Columns*, average value of VEGF mRNA expression; *bars*, +/- SD. *, $p < 0.05$ compared with vehicle-treated controls.

(D) Representative photomicrographs showing the immunohistochemical expression of CD31 in harvested tumor tissues (day 25) (magnification: 400X). The right panel represents the mean vessel density calculated by standard grid counting of CD31-positive vessels at 400X magnification. The data reflect three independent experiments, in which three to four non-overlapping fields of each specimen were analyzed in a blinded manner. *Columns*, average value of vessel counts; *bars*, +/- SD. *, $p < 0.05$ compared with vehicle-treated controls.

Figure-6. CsA promotes the development of VEGF-induced post-transplantation cancer (A) 0.5×10^6 syngenic tumor cells (CT26) were injected subcutaneously in BALB/c mice, and fully MHC mismatched cardiac transplants were performed in these animals 7 days later. Following cardiac transplantation, the mice ($n=5$ in each group) were treated either with CsA (10 mg/kg/day) or with vehicle as a control. Treatment was continued for 14 days (i.e. up to 22 days following tumor injection), and tumor volume was monitored using digital caliper on alternate days. Tumors were harvested after 22 days of tumor injection. The data reflect three independent experiments. *Columns*, average value of tumor volume; *bars*, $\pm$ SD. *, $p<0.01$ compared with vehicle-treated control. (B) Total RNA was isolated from harvested tumor tissues (day 22), and reverse transcribed. Fold change in *VEGF* mRNA expression was measured by real time PCR using gene-specific primers for murine *VEGF*. The data reflect three independent experiments, resulting from duplicate readings of two different samples. *Columns*, average value of *VEGF* mRNA expression; *bars*, $\pm$ SD. *, $p<0.01$ compared with vehicle-treated control. (C) Representative photomicrographs showing the immunohistochemical expression of CD31 in harvested tumor tissues (day 22) (magnification: 400X). The right panel represents the mean vessel density calculated by standard grid counting of CD31-positive vessels at 400X magnification. The data reflect three independent experiments, in which three to four non-overlapping fields of each specimen were analyzed in a blinded manner. *Columns*, average value of vessel counts; *bars*, $\pm$ SD. *, $p<0.05$ compared with vehicle-treated control. (D) 0.5×10^6 syngenic tumor cells (CT26) were injected subcutaneously in BALB/c mice, and fully MHC mismatched cardiac transplants were performed in these animals 7 days later. Following cardiac transplantation, the mice ($n=5$ in each group) were treated either with i) CsA (10 mg/kg/day) + control IgG, ii) CsA (10 mg/kg/day) + anti-VEGF and iii) vehicle. Treatments were continued until the mice were sacrificed (up to 30 days following tumor injection). Tumor volume was monitored on alternate days. *Points*, average value of tumor volume; *bars*, $\pm$ SD. On days 22-30, the CsA + control IgG-treated group had average tumor volumes that were significantly higher than the vehicle-treated group ($p<0.05$), and the CsA + anti-VEGF-treated group had average tumor volumes that were significantly lower than the CsA + control IgG-treated group ($p<0.05$).