Calcineurin Inhibitors Modulate CXCR3 Splice Variant Expression and Mediate Renal Cancer Progression

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ABSTRACT
Calcineurin inhibitors (CNI) are used to prevent inflammatory diseases and allograft rejection. However, little is known about the mechanism(s) underlying their ability to promote the development and recurrence of cancer. Recent studies suggested that the chemokine receptor CXCR3 may play important roles in tumorigenesis. CXCR3 has two splice variants with opposite functions: CXCR3-A promotes cell proliferation, and CXCR3-B inhibits cell growth. Here, we explored the effects of CNI on the expression and function of CXCR3 splice variants. Compared with normal renal tissues and renal epithelial cells, human renal cancer tissues and renal cancer cell lines demonstrated higher expression of CXCR3-A and markedly lower expression of CXCR3-B. In human renal cancer cells (786-0 and Caki-1) and renal epithelial cells, CNI markedly downregulated the expression of CXCR3-B, whereas expression of CXCR3-A was unchanged. This CNI-mediated downregulation of CXCR3-B resulted in increased proliferation and migration of renal cancer cells; CNI-mediated cell proliferation involved signaling through G proteins, perhaps via CXCR3-A. Finally, it was observed that CNI treatment increased the growth of human renal tumors in vivo, and the expression of CXCR3-B was significantly decreased in these tumors. In summary, these observations suggest that CNI may mediate the progression of human renal cancer by downregulating CXCR3-B and by promoting proliferative signals, likely through CXCR3-A. Targeting CXCR3 splice variants or the signaling pathways downstream of CXCR3 receptors may provide a therapeutic strategy for the prevention of CNI-mediated renal cancer progression.


Calcineurin is a serine/threonine phosphatase that is activated by cellular calcium.1,2 In T cells, it has been demonstrated that calcineurin activity is necessary for the expression of several cytokines through the dephosphorylation of a family of transcription factors known as nuclear factor of activated T cells (NFAT).3–5 A major function of calcineurin inhibitors (CNI) is to prevent the activation of NFAT and thereby to suppress T cell activation.6 This immunosuppressive activity of CNI established their use in the treatment of a variety of inflammatory diseases.7,8 In the field of organ transplantation, the therapeutic introduction of these inhibitors revolutionized the treatment and prevention of acute allograft rejection.7,9

Nevertheless, recent studies clearly identified a major association between the use of CNI and the development and recurrence of different types of cancer in both transplant and nontransplant patients.10–14 The immunosuppressive effect of CNI may result in impaired immune surveillance for
neoplastic cells and may increase susceptibility to oncogenic viral infections. CNI may also directly cause DNA damage to cells and interfere with normal DNA repair mechanisms; however, independent of these effects, it has been found that commonly used CNI cyclosporine (CsA) and FK506 may promote tumor growth through the induced expression of different genes, including TGF-β, IL-10, and angiogenic cytokines (e.g., vascular endothelial growth factor). Thus, understanding the mechanism of CNI-mediated tumor development may allow a safer and effective use of these inhibitors in patients.

Chemokines are small, cytokine-like, secreted proteins (8 to 11 kD) that have chemoattractant properties and are well established to function in the recruitment of leukocytes into inflamed tissues. The biologic effects of chemokines are mediated through specific G-protein–coupled receptors, primarily expressed on leukocytes, but also on nonlymphoid cells, including endothelial and epithelial cells. The interaction between chemokines and their receptors has been found to be important in various acute and chronic inflammatory processes; however, over the past few years, it has been demonstrated that chemokines and their receptors may also be functional in tumor development.

The chemokine CXCL10 (also known as IP-10) is a T cell chemoattractant and is classically thought to have tumor inhibitory properties via its effects to elicit T cell–dependent mechanisms of tumor destruction as well as its effects to inhibit tumor angiogenesis. Nevertheless, paradoxically, some recent reports have indicated that CXCL10 can also promote tumor growth. This led to a controversy whether this chemokine may function as an anti- or protumorigenic agent. We recently reported that this controversy in the function of CXCL10 is in part related to alternative splicing of its receptor (CXCR3). CXCR3 exists as two novel variants called CXCR3-A and CXCR3-B, each of which mediates different intracellular signals, and has select functions: CXCR3-A promotes chemotaxis and cell proliferation, whereas CXCR3-B signals for growth inhibition. Therefore, any changes in the relative balance in the expression of CXCR3 splice variants may play a critical role in regulating different cellular functions in response to CXCR3-A/B-binding chemokines.

In this study, we show that CNI can downregulate the expression of the growth-inhibitory CXCR3-B receptor in human renal cancer cells, without altering growth-promoting CXCR3-A. In the absence of CXCR3-B, renal cancer cells undergo increased proliferation and migration. These results suggest a mechanism underlying the association between CNI use in transplant patients and the progression of human renal cancer.

RESULTS

Expression Profiles of CXCR3-A and CXCR3-B in Human Renal Tumor Tissues and in Renal Cancer Cell Lines

We first analyzed the expression profiles of CXCR3-A and CXCR3-B in human renal cell carcinoma (RCC) tissues by real-time PCR using gene-specific primer–probe sets. We examined a total of 12 RCC tissues (six low-stage and six high-stage tumors), compared with normal renal tissues. We observed that the expression of the growth-promoting CXCR3-A was markedly higher (approximately 5- to 170-fold increase) in all tumors as compared with normal renal tissues (Figure 1A). In contrast, the expression of the growth-inhibitory CXCR3-B in renal tumor tissues (both low- and high-stage) was consistently at lower levels of expression (approximately 25 to 90% decrease) than those observed in normal renal tissues (Figure 1B).

We next examined the expression profiles of the CXCR3 splice variants in two well-established human renal cancer cell lines (786-O and Caki-1) compared with normal renal tubular epithelial cells (REC). We observed that CXCR3-A was mark-
edly higher and CXCR3-B was lower in both cancer cell lines as compared with normal REC (Figure 2, A and B).

We also examined the expression of known CXCR3-binding chemokines (CXCL9, CXCL10, CXCL11, and CXCL4) in 786-0 and Caki-1 cell lines compared with normal REC. It is known that CXCL9, CXCL10, and CXCL11 interact with both CXCR3-A and CXCR3-B splice variants; however, CXCL4 binds selectively to the growth-inhibitory CXCR3-B. Whereas CXCL9 was very low in expression (data not shown), we found that CXCL10 and CXCL11 were significantly higher in both 786-0 and Caki-1 cells compared with REC (Figure 2C). In contrast, the expression of CXCL4 was markedly lower in 786-0 cells (Figure 2C). Together, these observations demonstrate that there are completely different expression patterns of CXCR3 splice variants and CXCR3-binding ligands in human renal cancer cells, compared with normal REC.

CNI Downregulate the Expression of CXCR3-B in Human Normal REC and Renal Cancer Cells

We next wished to evaluate the effect of CNI on the expression of CXCR3-A and CXCR3-B in normal REC and in renal cancer cells (786-0 and Caki-1). The cells were treated with either CsA (0.1 and 1.0 μg/ml) or FK506 (0.01 and 0.10 μg/ml) or vehicle alone, and gene expression was measured by real-time PCR. As shown in Figure 3, A and B, both CsA and FK506 significantly downregulated the growth-inhibitory CXCR3-B (P < 0.05) in all cell lines as compared with vehicle-treated controls; however, there was no significant change in the expression of growth-promoting CXCR3-A in these cells after CNI treatment (Figure 3C). These observations suggest that CNI may differentially regulate CXCR3 splice variants and may have an impact on renal cancer progression.

Downregulation of CXCR3-B Is Associated with Increased Signals for Renal Cancer Cell Proliferation

We next questioned whether downregulation of growth-inhibitory CXCR3-B expression would result in a reciprocal increase in renal cancer cell proliferation. We transfected Caki-1 and 786-0 cells with CXCR3-B–specific small interfering RNA (siRNA), which selectively inhibited CXCR3-B mRNA expression; however, the efficiency of knockdown was greater in Caki-1 cells (approximately 80% knockdown; Supplemental Figure 1) versus 786-0 cells (approximately 50% knockdown, data not shown). Thus, for our siRNA transfection experiments, we used Caki-1 cells. As shown in Figure 4A, transfection of Caki-1 cells with CXCR3-B siRNA (25 and 50 nM) resulted in a marked increase in cell proliferation, as compared with control siRNA-transfected cells. Next, we transfected Caki-1 cells with a CXCR3-B overexpression plasmid, which resulted in an approximately 250-fold increase in the gene expression in these cells (Supplemental Figure 2). We found that the overexpression of CXCR3-B resulted in a decrease in cancer cell proliferation (Figure 4B). Similar results were obtained in 786-0 cells (data not shown). Together, these
results suggest that CXCR3-B mediates a growth-inhibitory signal in renal cancer cells, and the inhibition of CXCR3-B expression may withdraw this inhibitory signal to promote cell proliferation.

**Figure 3.** CNI downregulate the expression of CXCR3-B in human renal cancer cells. (A through C) REC, 786-0, and Caki-1 cells were treated with CsA (0.1 and 1.0 μg/ml), FK506 (0.01 and 0.10 μg/ml), or vehicle alone and incubated for 18 h. Total RNA was isolated from these cells and reverse-transcribed. Fold changes in mRNA expression of CXCR3-B in response to CsA (A) or FK506 (B) was measured by real-time PCR. Fold changes in mRNA expression of CXCR3-A in response to CsA was also measured by real-time PCR (C). Data reflect three independent experiments. Columns are average of triplicate readings of two different samples; bars are ±SD. In A and B, *P < 0.05 versus vehicle-treated cells.

**Figure 4.** Inhibition of CXCR3-B expression is associated with increased proliferation of human renal cancer cells. (A) Caki-1 cells were transfected with either the control or the CXCR3-B siRNA (25 and 50 nM). (B) Caki-1 cells were transfected with either CXCR3-B overexpression plasmid (1.0 and 1.5 μg/ml) or the empty expression vector. All of the cells in A and B were incubated for 72 h and subjected to cell proliferation assay by measuring [3H]thymidine incorporation within the cells as described in the Concise Methods section. Data reflect three independent experiments. Columns are average of triplicate readings (cpm) of the samples; bars are ±SD. In A and B, *P < 0.05 versus either control siRNA- or empty vector-transfected cells.

**CNI-Mediated Downregulation of CXCR3-B in Renal Cancer Cells Promotes Cell Proliferation and Migration**

We next tested whether inhibition of CXCR3-B expression by CNI can induce renal cancer cell proliferation and migration. Normal REC or 786-0 cells were treated with the increasing concentrations (0.1 to 1.0 μg/ml) of either CsA or FK506 or vehicle alone, and a cell proliferation assay was performed. As shown in Figure 5, A and B, CsA treatment markedly induced cell proliferation in both REC and 786-0 cells, compared with vehicle-treated controls. Similar results were obtained using FK506 (data not shown). To examine further the role of CXCR3-B–mediated signaling in renal cancer cell prolifera-
tion, we made use of the ligand CXCL4, known to interact specifically with CXCR3-B.37,38 We observed that treatment of REC and 786-0 cells with CXCL4 markedly suppressed the proliferation of both cell types; however, CXCL4 did not inhibit CsA-mediated cell proliferation (Figure 5, A and B). These results suggest that CNI may promote cancer cell proliferation by downregulating CXCR3-B expression (as observed in Figure 3, A and B), and in absence of CXCR3-B, CXCL4 cannot inhibit CsA-mediated renal cancer cell proliferation.

Next, we evaluated whether CNI could promote the migration of renal cancer cells by altering the expression of CXCR3 splice variants. We treated 786-0 cells with CXCL4 markedly suppressed the proliferation of both cell types; however, CXCL4 did not inhibit CsA-mediated cell proliferation (Figure 5, A and B). These results suggest that CNI may promote cancer cell proliferation by downregulating CXCR3-B expression (as observed in Figure 3, A and B), and in absence of CXCR3-B, CXCL4 cannot inhibit CsA-mediated renal cancer cell proliferation.

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These results again support our previous observation and suggest that CsA may promote renal cancer cell migration by inhibiting CXCR3-B; and in absence of CXCR3-B, CXCL4 cannot inhibit CsA-mediated cell migration.

**CNI-Mediated Renal Cancer Cell Proliferation Involves Gι Proteins**

CXCR3-A–mediated signaling involves Gι proteins.28,37,40 Here, we first examined whether inhibition of CXCR3-B expression in renal cancer cells could promote cell proliferation via Gι protein–mediated responses. We transfected Caki-1 cells with either a control or CXCR3-B–specific siRNA and performed a cell proliferation assay in the absence or presence of the Gι protein inhibitor pertussis toxin (PTX). As shown in Figure 6A, the knockdown of CXCR3-B in these cells increased cell proliferation as compared with control siRNA-transfected cells. In contrast, CXCL4 markedly inhibited cell migration, compared with the vehicle-treated controls; however, CXCL4 did not inhibit migration of cells treated with CsA (Figure 5C).
CXCR3-B siRNA-transfected cells was significantly inhibited (Figure 6A). This suggests a role for Gi proteins in this proliferation process, likely involving CXCR3-A.

Furthermore, when we treated the control siRNA-transfected Caki-1 cells (expressing both CXCR3-A and CXCR3-B) with CXCL10, there was no significant change in cell proliferation, compared with untreated controls (Figure 6A); however, when we treated the cells with PTX to inhibit CXCR3-A-mediated responses, we found that CXCL10 significantly inhibited cancer cell proliferation (Figure 6A). We suggest that this decreased cell proliferation is probably mediated via unopposed growth-inhibitory CXCR3-B, which does not involve Gi proteins.28,37,40

Next, we analyzed the role of Gi proteins in CNI-mediated renal cancer cell proliferation. We treated the 786-0 cells with CsA in the absence or the presence of PTX, and we performed a cell proliferation assay. We observed that CsA treatment promoted cell proliferation as compared with vehicle-treated controls, and CsA-mediated cell proliferation was significantly inhibited by PTX (Figure 6B). Thus, CNI may induce a Gi protein–dependent signaling mechanism, which may promote cell proliferation in the absence of CXCR3-B, and likely involves CXCR3-A.

CNI Promotes the Growth of Human Renal Tumors In Vivo and Downregulates the Expression of CXCR3-B in Tumor Tissues

We next examined whether CsA can promote human renal tumor growth in vivo, and we evaluated the expression profiles of CXCR3-A and CXCR3-B in these tumor tissues. Human renal cancer cells were injected subcutaneously into immunodeficient (nu/nu) mice, and the mice were treated either with CsA (10 mg/kg per d) or with vehicle alone for 30 d. We observed that CsA treatment markedly enhanced tumor volume compared with vehicle-treated controls (Figure 7A). Tumors were harvested on day 30, and we evaluated them for the expression of CXCR3-A and CXCR3-B. As shown in Figure 7B, CsA treatment significantly decreased the expression of CXCR3-B in the tumor compared with vehicle-treated controls. In contrast, there was no significant change in CXCR3-A expression in the tumor after CsA treatment. Together, these in vivo observations support our in vitro findings and suggest that in the presence of CNI, there may be an increased protumorigenic signal through unopposed CXCR3-A, allowing an accelerated progression of existing tumors.

**DISCUSSION**

In this study, we show that CNI can promote the progression of human renal cancer by downregulating the expression of the growth-inhibitory receptor CXCR3-B in cancer cells, which leads to increased cell proliferation and migration. It is now established that immunosuppressed individuals, including patients with an organ transplant, are at increased risk for either de novo cancer development or recurrence/progression of cancer.12,13,15 Furthermore, cancers that develop in transplant recipients are often more aggressive than those in the general population.41 CNI, both CsA and FK506, are thought to play a critical role in the development/recurrence of cancer in immunosuppressed individuals. Apart from mediating immune escape of tumor cells, CNI may promote cancer through direct cellular effects involving different cytokines10,19–23 or by a re-
The chemokine receptor CXCR3 and its ligands are important players in the development and progression of human tumors by facilitating migration and proliferation of malignant cells. In this study, we showed that CXCR3 and its ligands are overexpressed in human renal cancer, one of the major cancers after solid organ transplantation. The two splice variants of CXCR3 mediate distinctly different signals and have completely opposite functions in tumor cells as well as in a variety of normal cell types, such as lymphocytes, endothelial cells, and airway epithelial cells; CXCR3-A promotes chemotaxis and cell proliferation, whereas CXCR3-B mediates growth inhibition. Thus, it is likely that signaling through CXCR3-B in renal cancer cells may inhibit cell growth. We have observed that in the presence of CXCL4, the specific ligand for CXCR3-B, renal cancer cells undergo reduced proliferation; however, when the cancer cells are treated with CNI (CsA or FK506), the expression of CXCR3-B (but not CXCR3-A) is downregulated, which results in increased cell proliferation and migration. CNI-mediated downregulation of CXCR3-B may involve a factor(s) regulating mRNA splicing or mRNA stability or possibly could involve other novel signaling mechanisms that need to be investigated.

In a recent report, Petrai et al. showed that the growth-inhibitory signal through CXCR3-B is mediated by the p38 mitogen-activated protein kinase pathway. Although CXCR3-B is expressed at low levels in tumor tissues as compared with normal tissues, CXCR3-B-mediated negative signals may have a predominant effect over the growth-promoting positive signals through CXCR3-A and may thus prevent tumor progression. We suggest that with further reduction in the levels of CXCR3-B, these negative signals for growth inhibition are withdrawn, which may facilitate an accelerated progression of existing tumors through the interaction of CXCR3-A and its ligands.

It is established that CXCR3-A and CXCR3-B may involve differential G protein coupling. Our findings indicate that treatment with CNI inhibits CXCR3-B and induces G protein-mediated signals promoting renal cancer cell proliferation, likely through CXCR3-A. In addition, we suggest that in the absence of CXCR3-B, the CXCR3-binding ligands (CXCL9, CXCL10, and CXCL11) that are found to be overexpressed in renal cancer cell lines and renal cancer tissues (data not shown) can interact in an autocrine manner with the growth-promoting CXCR3-A with higher affinity. This may promote a positive signal for the recurrence and progression of renal cancer in CNI-treated immunosuppressed patients. We and others have shown that G protein–dependent and CXCR3-mediated signals can activate the phosphatidylinositol-3-kinase/Akt and extracellular signal–regulated kinase 1/2 signaling pathways to promote cell proliferation; however, an alternative explanation is that there are other novel receptors that may also interact with CXCR3-binding ligands and may be involved in CNI-mediated renal cancer cell proliferation.

In summary, although the problem of cancer in immunosuppressed patients, including transplant recipients, has been documented for some time, new approaches to deal with neoplasms in these patients are difficult to implement. Treatment of metastatic renal cancer in transplant recipients is largely ineffective because of immunosuppressive therapy. Thus, a treatment regimen that monitors the risk for recurrence/progression of renal cancer in transplant patients would be of high value. Our studies clearly suggest that changes in the expression profile of CXCR3 splice variants in normal renal epithelial cells could be used to monitor the risk for recurrence/progression of renal cancer in transplant recipients.
and renal cancer cells after CNI treatment may play a role in the progression of renal cancer likely through CXCR3-A. Furthermore, in previous studies, we observed that CXCR3-A–mediated cancer cell proliferation might involve the Akt signaling pathway.28 We suggest that a potent inhibitor of Akt-mediated signaling events, such as rapamycin, may block the growth-promoting responses through CXCR3-A. Thus, monitoring the relative expression and/or targeting CXCR3 splice variants or its signals or ligands might serve to prevent the progression of CNI-mediated renal cancer, particularly in transplant recipients.

**CONCISE METHODS**

**Reagents**
The CNI CsA (Novartis, East Hanover, NJ) and FK506 (tacrolimus; Astellas, Deerfield, IL) were purchased from the Children’s Hospital Boston pharmacy. The G, inhibitor PTX was purchased from Calbiochem (La Jolla, CA). The siRNA for CXCR3-B and its respective control were purchased from Invitrogen (Carlsbad, CA). Recombinant CXCL10 and CXCL4 (PF-4) were purchased from R&D Systems (Minneapolis, MN).

**Tissue Samples**
Twelve tissue samples of RCC were obtained from surgical specimens of patients who underwent surgery at the University Hospital (Würzburg, Germany). The protocol to obtain tissue samples was approved by the review board of the hospital. Tumor tissues were graded (stages I through IV) according to Robson staging system. Normal renal tissues were obtained from normal parts of the surgical specimens, and the normalcy of these tissues was confirmed by histology.

**Cell Culture**
The human renal cancer cell lines (786-0 and Caki-1) were obtained from American Type Culture Collection (Manassas, VA). The cells were grown in RPMI-1640 supplemented with 10% FBS (HyClone Laboratories, Logan, UT). Human renal proximal tubular epithelial cells were purchased from Clonetics (Walkersville, MD) and were cultured in complete epithelial medium (REGM BulletKit, Walkersville, MD), as supplied, according to recommended instructions.

**Plasmid**
CXCR3-B overexpression plasmid (pcDNA3-CXCR3-B) was a gift from Paola Romagnani (University of Florence, Florence, Italy).46

**Transfection Assays**
The cells were transfected with the expression plasmids using the Effectene transfection reagent (Qiagen, Valencia, CA). 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 under the control of cytomegalovirus immediate early promoter and by measurement of β-galactosidase activity. The cells were transfected with the siRNA using Lipofectamine-2000 (Invitrogen).

**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 mRNA expression, we performed real-time PCR 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, 18S mRNA was amplified and analyzed under identical conditions. Gene-specific primer–probe sets for human CXCR3-A/CXCR3-B/CXCR3-binding ligands/18S were obtained from Applied Biosystems.37 Ct value (the cycle number at which emitted fluorescence exceeded an automatically determined threshold) for gene of interest was corrected by the Ct value for 18S and expressed as ΔCt. Data were measured as fold changes of mRNA amount, which was calculated as follows: (fold changes) = 2^X (where X = ΔCt for control group − ΔCt for experimental group).

**Cell Proliferation Assay**
Cells (5 × 10^3) were seeded and grown in 96-well plates. [3H]Thymidine (0.5 μCi/well) was added for the final 15 h before cell harvesting. [3H]Thymidine incorporation was measured using a microplate scintillation and luminescence counter (Perkin Elmer/Wallac, Boston, MA).

**In Vitro Wound-Healing Cell Migration Assay**
Cell migration assays were performed as described previously.50 Cultured cells were allowed to form a confluent monolayer in a six-well plate in the presence of 1% serum. The wound was made by scraping a conventional pipette tip across the monolayer. The cells were either vehicle-treated or treated with CsA/CXCL4 and allowed to heal for 36 h. Pictures of representative wells were taken under the microscope at 0 and 36 h after wounding. The relative distance traveled by the leading edge from 0 to 36 h was assessed using Photoshop software.

**In Vivo Tumor Development**
Human renal cancer cells (786-0) were injected subcutaneously in immunodeficient (nu/nu) mice. Either CsA (10 mg/kg per d) or the vehicle was then administered intraperitoneally to these mice. Tumor volume was measured using a digital caliper at regular intervals. The volume was estimated by following a standard method,21 using the formula: V = \( \pi / 6 \times a^2 \times b \), where \( a \) is the short and \( b \) is the long tumor axis. Mice were killed at designated times after injection or when complications occurred, which included signs of inactivity, cachexia, or decreased responsiveness. All animal works were approved by the animal care and use committee at Children’s Hospital Boston and in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

**Statistical Analysis**
Statistical evaluation for data analysis was determined either by t test for two groups of data or by one-way ANOVA for three or more groups. Differences with \( P < 0.05 \) were considered statistically significant.
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DISCLOSURES

None.

REFERENCES


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