Angiotensin II regulation of vascular endothelial growth factor and receptors Flt-1 and KDR/Flk-1 in cyclosporine nephrotoxicity

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Background. Vascular endothelial growth factor (VEGF) is involved in angiogenesis, wound healing and inflammation. VEGF exerts its effect via the tyrosine kinase receptors Flt-1 and KDR/Flk-1. We have previously shown that VEGF is up-regulated in a chronic cyclosporine (CsA) nephrotoxicity model. Our current study examined the role of angiotensin II (Ang II) blockade with enalapril (E) or losartan (L) on VEGF in this model.

Methods. Pair-fed salt-depleted rats were administered vehicle, CsA, CsA + nilvadipine, CsA + hydralazine/hydrochlorthiazide (HCTZ), CsA + E or CsA + L, and were sacrificed at 7 or 28 days. Physiologic and histologic changes were studied in addition to the mRNA expression of VEGF and its receptors Flt-1 and KDR/Flk-1 by Northern blot, and the protein expression of VEGF by Western blot.

Results. While all groups achieved similar blood pressures and creatinine clearances, the amelioration in nephrotoxicity was observed only with Ang II blockade. VEGF mRNA and protein expressions increased with CsA and became significantly reduced with Ang II blockade. Flt-1 expression was similar in all groups; it decreased early and remained low. On the other hand, KDR/Flk-1 mRNA expression was higher at seven days in all groups, except in the +E and +L groups where it was significantly lower, and then became further down-regulated at 28 days.

Conclusions. The increased VEGF expression in chronic CsA nephrotoxicity seems to be related to up-regulation of Ang II. In addition, VEGF probably exerted its effect via the KDR/Flk-1 receptor. The actions of VEGF in this model remain speculative, but may be related to its effect on macrophage infiltration or matrix deposition.

Vascular endothelial growth factor (VEGF) is an endothelial cell mitogen that promotes angiogenesis, in-

creases vascular permeability, and is chemotactic for monocytes [1]. VEGF also plays a role in wound healing, inflammation and tissue remodeling. Hypoxia and hypoglycemia are major stimulators of VEGF expression [2]. Also, VEGF production is stimulated by a number of cytokines and growth factors including transforming growth factor-β (TGF-β), fibroblast growth factor, platelet-derived growth factor (PDGF), keratinocyte growth factor, angiotensin II (Ang II), insulin-like growth factor-1 (IGF-1) and reactive oxygen species (ROS) [3]. VEGF exerts much of its action by binding selectively and with high affinity to two tyrosine kinase receptors designated as Flt-1 (*fms*-like tyrosine kinase, also known as VEGFR-1) and Flk-1 (fetal liver kinase-1, also known as KDR or VEGFR-2). Flt-1 binds VEGF with high affinity but is poorly expressed, while KDR/Flk-1 binds VEGF with somewhat lower affinity but is more readily detected [4]. VEGF receptors undergo dimerization and autophosphorylation after ligand binding, leading to activation of intracellular signal molecules such as mitogen-activated protein (MAP) kinase and phospholipase C. Until recently, most studies described VEGF receptors expression as being specific to endothelial cells. However, there are now several reports of non-endothelial cells expressing VEGF receptors [5].

The clinical use of cyclosporine (CsA) is limited by chronic nephrotoxicity, which is characterized by striped tubulointerstitial fibrosis and afferent arteriolar hyalinosis [6]. While acute nephrotoxicity results from intrarenal vasoconstriction, the mechanism leading to chronic CsA nephrotoxicity remains unclear [7]. To study chronic CsA nephrotoxicity, a reproducible animal model was developed with physiologic and histologic features that resemble the human renal lesion described in patients on long-term CsA therapy. Our previous studies have shown that TGF-β1 is involved in the fibrosis of this model [8]. Apoptosis also plays a central role [9]. More recently, we demonstrated an up-regulated expression

of VEGF mRNA and protein along with an increased expression of Flt-1 and KDR/Flk-1 in this model [10].

In the kidney, VEGF is constitutively expressed primarily on glomerular visceral epithelial and tubular cells, but its function is not fully delineated [3–5, 11]. Flt-1 and KDR/Flk-1 are present not only on renal endothelial cells, but also on human mesangial and rat renal tubular epithelial cells [3–5, 12]. VEGF plays a role in endothelial development, in enhancing endothelial cell permeability and in post-injury glomerular regeneration. Recently, an increased VEGF expression was shown in renal biopsies from recipients with chronic rejection [13]. Of note is that VEGF is increased in conditions associated with macrophage infiltration, and that both chronic renal allograft rejection and chronic CsA nephrotoxicity show a heavy monocytic infiltration.

Angiotensin II plays an important role in pathological extracellular matrix (ECM) accumulation in models of progressive glomerulosclerosis and interstitial fibrosis, independent of its effect on glomerular pressure [14]. On the other hand, angiotensin-converting enzyme (ACE) inhibitors and angiotensin II subtype 1 (AT_1) receptor antagonists can reverse or attenuate fibrosis in a number of animal and human kidney diseases [15]. In kidney transplantation, several lines of evidence suggest a role for the renin-angiotensin system (RAS) in the development and progression of chronic allograft injury [16]. We have previously shown that Ang II blockade resulted in the amelioration of CsA-induced fibrosis and in the decrease in the expression of TGF-β1 and matrix proteins [17]. These data, together with the recent findings that Ang II stimulates VEGF production [18], prompted us to investigate whether Ang II regulates the production of VEGF and the expression of receptors Flt-1 and KDR/Flk-1 in a model of chronic CsA nephrotoxicity.

METHODS

Experimental design

Thirty-two male Sprague-Dawley rats (Charles River, Wilmington, MA, USA) weighing 225 to 250 g were used. They were housed in individual cages in a temperature and light controlled environment, received a low salt diet (0.05% sodium, Teklad Premier, WI, USA) and were allowed free-access to water. After one week on low salt diet, they were assigned to the following experimental groups ($N = 5$ to 6/group) and were sacrificed at 7 or 28 days.

(1) Vehicle (VH): Rats received subcutaneous (SC) injection of olive oil 1 mL/kg/day.
(2) CsA: Rats received a daily SC injection of CsA 15 mg/kg.
(3) CsA + N: Rats received both a daily SC injection of CsA 15 mg/kg and of nilvadipine 0.5 mg/kg.

(4) CsA + HH: Rats received a daily SC injection of CsA 15 mg/kg plus hydralazine 10 mg/kg/day and hydrochlorthiazide (HCTZ) 10 mg/kg/day (both by matrix-driven delivery pellet).
(5) CsA + E: Rats received a daily SC injection of CsA 15 mg/kg, and enalapril 10 mg/kg by gavage.
(6) CsA + L: Rats received a daily SC injection of CsA 15 mg/kg, and losartan 10 mg/kg by gavage.

This dosage and route of administration for enalapril and losartan were selected because they were respectively previously shown to significantly block ACE activity and AT_1 receptors [17].

On day 7 or 28, systolic blood pressure was measured by tail plethysmography (Natsume Seisakusho Co. Ltd., Tokyo, Japan) and 24-hour urine samples were collected in metabolic cages (Nalge Company, Rochester, NY, USA). The following day, the rats were anesthetized with intraperitoneal ketamine, the abdomen was opened through a midline incision and the aorta was cannulated retrogradely below the renal arteries with an 18-gauge needle. With the aorta occluded by ligation above the renal arteries and the renal veins opened by a small incision for outflow, the kidneys were perfused with 20 mL of cold heparinized saline. The left kidney was removed and processed for light microscopy and protein analysis. After removing the right kidney, the cortex was dissected from the medulla, and the cortex was processed for RNA analysis. The animals were then euthanized by deep anesthesia with ketamine followed by exsanguination.

Drugs

Cyclosporine A (Novartis Research Institute, East Hanover, NJ, USA) was diluted in olive oil to a final concentration of 15 mg/mL. VH consisted of an identical volume of olive oil. Losartan (Du Pont Merck Pharmaceutical, Wilmington, DE, USA) was dissolved in sterile water and orally given to animals at 10 mg/kg/day. Enalapril (Merck, Rahway, NJ, USA) was diluted in sterile water and orally given to animals at 10 mg/kg/day. Nilvadipine (Fujisawa, Osaka, Japan) was diluted in ethanol/olive oil and subcutaneously given to animals at 0.5 mg/kg/day. Matrix-driven delivery pellet of hydralazine and hydrochlorthiazide (Innovative Research of America, Toledo, OH, USA) were subcutaneously implanted in animals and released at 10 mg/kg/day, respectively.

Functional studies

Blood was collected from the jugular vein in plastic syringes transferred to metal-free tubes and chilled on ice. Plasma was harvested immediately by centrifugation at 4°C and stored at −70°C until determined. Urinary and plasma creatinine was measured by a Cobas Autoanalyzer (Roche Diagnostics, Div. Hoffman-La Roche

Inc., Nutley, NJ, USA). Creatinine clearance (C_{Cr}) was calculated using a standard formula. CsA blood level was measured by a monoclonal radioimmunoassay for CsA (Incstar Co., Stillwater, MN, USA).

Histology

Tissue was fixed in 10% buffered formalin and embedded in paraffin. Sections 2 to 4 μ thick were stained with periodic acid-Schiff (PAS) reagent and trichrome stain and examined by light microscopy. The histologic findings were subdivided into two categories: interstitial fibrosis and arteriolopathy. The findings of interstitial fibrosis consisted of matrix-rich expansion of the interstitium with distortion and collapse of the tubules and thickening of the tubular basement membranes. Features of interstitial inflammation included mononuclear infiltrates, edema and vacuolization of interstitial cells. Arteriolopathy consisted of hyalinization and destruction of afferent arterioles and terminal portions of interlobular arteries. A minimum of 20 fields at $\times$100 magnification were assessed and graded in each biopsy by an observer masked to treatment groups using a color image analyzer (Nikon E400, Nikon Inc., Tokyo, Japan; Pixera Professional digital camera, Los Gatos, CA, USA; Macintosh Powerbook G3, NIH Image v. 1.5). Interstitial fibrosis was estimated by counting the percentage of injured areas per field of cortex and scored semiquantitatively using the following: 0 = normal interstitium; 0.5 = <5% of areas injured; 1 = 5 to 15%; 1.5 = 16 to 25%; 2 = 26 to 35%; 2.5 = 36 to 45%; and 3 = >45%. Arteriolar hyalinosis was semiquantitatively assessed by counting the percentage of juxtaglomerular afferent arterioles available for examination with a minimum of 100 glomeruli per biopsy assessed: 0 = no arterioles injured; 0.5 = <15%; 1 = 15 to 30%; 1.5 = 31 to 45%; 2 = 46 to 60%; 2.5 = 61 to 75%; and 3 = >75%.

Northern blot analysis

Renal tissue was finely minced with a razor blade on ice then homogenized in TRIzol reagent (Gibco Brl, Grand Island, NY, USA). RNA extraction was performed per the manufacturer's protocol. After resuspension in Tris-EDTA buffer, RNA concentrations were determined using spectrophotometric readings at Absorbance$_{260}$. RNA 30 μg were electrophoresed in each lane in 0.9% agarose gels containing 2.2 mol/L formaldehyde and 0.2 mol/L Mops (pH 7.0) and transferred to a nylon membrane (ICN Biomedicals, Costa Mesa, CA, USA) overnight by capillary blotting. Nucleic acids were cross-linked by ultraviolet irradiation (Stratagene, La Jolla, CA, USA). The membranes were prehybridized for two hours at 42°C with 50% formamide, 10% Denhardt's solution, 0.1% sodium dodecyl sulfide (SDS), 5 $\times$ standard saline citrate (SSC), and 200 μg/mL denatured salmon sperm DNA. They were hybridized at 42°C for 18 hours with cDNA probes labeled with ^{32}P-dCTP by random oligonucleotide priming (Boehringer Mannheim, Mannheim, Germany). Blots were washed in 2 $\times$ SSC, 0.1% SDS at room temperature for 15 minutes, and in 0.1 $\times$ SSC, 0.1% SDS at 50°C for 15 minutes. Films were exposed at -70°C for different time periods to ensure linearity of densitometric values and exposure time. Autoradiographs were scanned on an imaging densitometer (GS-700; Bio-Rad Laboratories, Hercules, CA, USA). The density of bands for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used to control for differences in the total amount of RNA loaded onto each gel line. Equal loading was further ensured by the ethidium bromide gel inspection. For quantitative purposes, values were divided by the density of bands for GAPDH in the same lane. The VEGF cDNA probe used was a 384 bp *Not* I-*Eco*RI murine cDNA fragment of clone pT7T3D-Pac (American Type Culture Collection, Rockville, MD, USA). The rat GAPDH cDNA probe (plasmid pBluescript KS II) was a gift from J.M. Blanchard [19]. Flt-1 and KDR/Flk-1 cDNA probes were derived from rat reverse transcription-polymerase chain reaction (RT-PCR) products. For Flt-1, both sense and antisense primers were located in the extracellular region: sense 5'-AGCCCACCTCTC TATCCGCTGG, antisense 5'-GGCGCTTCCGAATC TCTA. For KDR/Flk-1, the primers were sense 5'-AGC TTGGCTCACAGGCAACATCGG (located in the sixth IgG-like domain), antisense 5'-TGGCCCCGCTTAAC GGTCCGT (located between the transmembrane region and the intracellular domain). For RT-PCR, 5 μg total RNA were combined with 2 μL random primers (Gibco BRL) and 2 μL of Moloney murine leukemia virus (MMLV) reverse transcriptase per the manufacturer's instructions. RT product 1 to 2 μL was amplified using the Perkin-Elmer thermal cycler (Norwalk, CT, USA). Cycles used for PCR for both Flt-1 and KDR/Flk-1 were 94°C for five minutes (once), 94°C for one minute, 58°C for one minute, 72°C for one minute (30 cycles), and 72°C for five minutes (once). PCR products were electrophoresed in 1% agarose gel and photographed under ultraviolet light. The cDNA probes obtained were sequenced and the correct sequences were confirmed. The PCR products were cloned using the Original TA Cloning kit (Invitrogen, Carlsbad, CA, USA) per the manufacturer's instructions.

Western blot analysis

Frozen (-70°C) kidney tissue sections embedded in Tissue-Tek O.C.T. (Miles Inc., Diagnostics Div., Elkhart, IN, USA) were processed for protein extraction as previously described. Equal amounts of protein were resolved on 15% SDS-PAGE gel (Bio-Rad) and then electroblotted to nitrocellulose membranes (Bio-Rad). After blocking the membranes for one hour with TBS-T, they

were incubated with goat polyclonal VEGF antibody (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibody was anti-goat (1:2000 dilution; Santa Cruz Biotechnology) conjugated with alkaline phosphatase. The detection of signal employed a colorimetric subtraction kit (Zymed, San Francisco, CA, USA).

Immunohistochemical staining

Four-micron sections of formalin-fixed, paraffin-embedded tissue were cleared with xylene and hydrated using decreasing concentrations of ethanol. For VEGF immunostaining, the primary antibody used was a rabbit polyclonal antibody to human VEGF (Santa Cruz Biotechnology). To identify macrophages, a mouse monoclonal IgG1 antibody to rat macrophages (ED1; Serotec, Oxford, UK) was used. Staining for VEGF and ED1 was performed in three representative animals from the VH-, CsA-, and CsA + L-treated rats sacrificed at 28 days. Control experiments included omission of either the primary or secondary antibody.

All sections for VEGF staining were subjected to antigen retrieval using immersion in a 0.1 mol/L citrate buffer (pH 6.0), followed by heating in an electrical pressure cooker for three minutes (total time 30 min). All steps were carried out at room temperature. The primary VEGF antibody was applied overnight at a dilution of 1:100. This was followed sequentially by incubation for 30 minutes with a secondary anti-rabbit IgG antibody (Sigma, St. Louis, MO, USA) at a dilution of 1:300 and streptavidin peroxidase-conjugated (Dako, Carpenteria, CA, USA), with color development using diaminobenzidine (DAB; Dako) for five minutes.

Immunostaining for macrophages was carried out using a slide autostainer (Ventana, Tucson, AZ, USA). All steps were performed at 37°C. Slides were pretreated by Protease 2 enzyme digestion for 12 minutes. The primary antibody (ED1) was applied at a dilution of 1:800 for 32 minutes. This was followed by incubation for 8 minutes with a secondary anti-mouse IgG antibody (Vector Labs, Burlingame, CA, USA) at a dilution of 1:300. Detection steps were then performed using the basic DAB kit from Ventana.

Statistical analysis

Results are presented as mean ± standard error. Comparisons between groups were done by analysis of variance (ANOVA; the Kruskal-Wallis test followed by Tukey test). The level of statistical significance was chosen as $P < 0.05$.

RESULTS

Physiologic studies

Values for serum creatinine, creatinine clearance, systolic blood pressure, and CsA whole blood trough levels

Table 1. Physiologic changes in the experimental groups

	Blood pressure *mm Hg*	CsA blood level *ng/mL*	Serum creatinine *mg/dL*	Creatinine clearance *mL/min/100 g*
7 days				
VH	152 ± 2	0 ± 0	0.59 ± 0.04	0.36 ± 0.01
CsA	149 ± 8	2752 ± 136	0.63 ± 0.02	0.30 ± 0.01
CsA + N	122 ± 2[a]	2693 ± 204	0.65 ± 0.07	0.27 ± 0.05
CsA + HH	106 ± 6[a]	2425 ± 282	0.72 ± 0.06	0.32 ± 0.03
CsA + E	108 ± 6[a]	2819 ± 351	1.11 ± 0.20[a]	0.19 ± 0.04[a]
CsA + L	98 ± 4[a]	3391 ± 326	1.45 ± 0.16[a]	0.15 ± 0.02[a]
28 days				
VH	143 ± 2	0 ± 0	0.57 ± 0.02[a]	0.39 ± 0.02[a]
CsA	144 ± 4	5164 ± 270	1.02 ± 0.06	0.20 ± 0.01
CsA + N	125 ± 5[a]	5256 ± 309	1.06 ± 0.07	0.22 ± 0.02
CsA + HH	98 ± 6[a]	4295 ± 293	1.14 ± 0.06	0.18 ± 0.01
CsA + E	90 ± 4[a]	4488 ± 658	1.85 ± 0.16[a]	0.11 ± 0.01[a]
CsA + L	93 ± 4[a]	4374 ± 688	2.37 ± 0.41[a]	0.09 ± 0.01[a]

Data are mean ± SEM of 5-6 animals. Abbreviations are: VH, placebo; CsA, cyclosporine; N, nilvadipine; HH, hydralazine-hydrochlorthiazide; E, enalapril; L, losartan.

[a] $P < 0.05$ vs. CsA only

are summarized in Table 1. There were no significant differences in body weight or in the rate of weight gain suggesting that food intake was comparable in all groups (data not shown). CsA blood levels increased progressively and, at the end of each treatment period, similar levels were achieved. CsA-induced hypertension was not observed in this model. Despite that, addition of losartan or enalapril caused an early sustained and significant decrease in blood pressure ($P < 0.05$ at 7 and 28 days vs. CsA-only group). In order to control for the effect of blood pressure on interstitial fibrosis, rats were randomized to receive CsA + N or CsA + HH for 7 or 28 days with blood pressure reductions similar to the CsA + E and CsA + L groups being achieved. All CsA-treated animals had a significant increase in their serum creatinine by day 28 ($P < 0.05$ vs. VH group) and experienced a decline in their glomerular filtration rates as estimated by creatinine clearance (C_{Cr}). This was present by day 7 but became more significant at 28 days ($P < 0.05$ vs. VH group). Animals treated with CsA + N or CsA + HH had a significantly higher GFR compared to those treated with CsA + E or CsA + L ($P < 0.05$). There were no significant differences in C_{Cr} between the CsA only, CsA + N and CsA + HH groups.

Histologic changes

The histologic changes observed are summarized in Table 2. VH-treated rats demonstrated a normal kidney histology (Fig. 1A, B). By contrast, the kidneys of CsA-treated rats developed a striped pattern of tubulointerstitial fibrosis and tubular atrophy (Fig. 1C) in addition to afferent arteriolar hyalinosis (Fig. 1D). When the extent of the histological changes was graded using a 0 to 3+ semiquantitative score, most of the differences were observed at 28 days (Table 2). The concurrent administra-

Table 2. Histologic semiquantitative scoring

	Tubulointerstitial fibrosis		Arteriolopathy	
	7 days	28 days	7 days	28 days
VH	0 ± 0	0 ± 0[a]	0.1 ± 0.1	0.2 ± 0.1[a]
CsA	0 ± 0	1.5 ± 0.3	0.1 ± 0.1	1.8 ± 0.2
CsA + N	0 ± 0	1.3 ± 0.3	0.1 ± 0.1	1.7 ± 0.2
CsA + HH	0.1 ± 0.1	2.1 ± 0.2	0.1 ± 0.1	2.1 ± 0.1
CsA + E	0 ± 0	0.8 ± 0.2[a]	0.1 ± 0.1	0.3 ± 0.1[a]
CsA + L	0 ± 0	0.7 ± 0.1[a]	0.1 ± 0.1	0.3 ± 0.1[a]

Data are mean ± SEM of 5–6 animals. Abbreviations are VH, placebo; CsA, cyclosporine; N, nilvadipine; HH, hydralazine-hydrochlorthiazide; E, enalapril; L, losartan.

[a]$P < 0.05$ vs. CsA only

tion of losartan or enalapril with CsA dramatically and equally improved the tubulointerstitial fibrosis ($P < 0.05$) and the afferent arteriolopathy ($P < 0.01$) when compared to the CsA-only treated group (Fig. 1 E, F). On the other hand, CsA-induced tubulointerstitial fibrosis was not affected by the concurrent use of nilvadipine and worsened with the hydralazine-HCTZ combination (Table 2). Of note is that the beneficial effect of Ang II blockade was more significant in preventing CsA-induced arteriolopathy than in ameliorating the tubulointerstitial fibrosis.

There was also a difference in the number of ED1-positive macrophages at 28 days (Fig. 2). In the VH-treated rats, there were very few macrophages in the interstitium. However, CsA-treated kidneys displayed large numbers of macrophages infiltrating the tubulointerstitium. On the other hand, those animals treated with CsA and losartan showed only rare macrophages. The number of macrophages identified in the glomeruli was not different between the three groups.

VEGF expression

The mRNA expression of VEGF was clearly up-regulated in the CsA-treated rats as compared to the VH-treated rats (Figs. 3 and 8). This effect was seen early at 7 days ($P < 0.05$), and it progressively increased and continued to be significant in the CsA-treated kidneys at 28 days ($P < 0.05$). Ang II blockade with losartan or enalapril equally decreased VEGF mRNA expression ($P < 0.05$) by approximately 40 to 50% at 7 days and by 22 to 24% at 28 days compared to the CsA only group. To assess whether the changes in the mRNA expression were translated into changes in the synthesis of VEGF peptide, Western blots were performed on tissue homogenates derived from the whole cortex. The protein expression of VEGF was similar to its mRNA (Figs. 4 and 8) in that it increased at day 7 ($P < 0.05$) and more so at day 28 ($P < 0.05$) in CsA-treated rat kidneys when compared to VH-treated rats. Similarly, Ang II blockade with losartan or enalapril equally and significantly decreased VEGF peptide production ($P <$

0.05 vs. CsA only group) by approximately 35 to 40% at 7 days and by 27 to 28% at 28 days. Of note is that the effect of Ang II blockade was observed more significantly early, at 7 days, when compared to 28 days. While nilvadipine decreased the mRNA and protein expressions of VEGF at both 7 and 28 days, the difference did not achieve statistical significance. On the other hand, treatment with hydralazine/HCTZ resulted in no change in VEGF mRNA or protein expression.

The immunohistochemical staining for VEGF at 28 days is shown in Figure 5 and was mostly present in the corticomedullary junction and in the outer medullary areas. CsA-treated rats exhibited a strong, diffuse and granular cytoplasmic staining for VEGF. This was mostly seen in the tubulointerstitium with strongly positive (more than 90%) staining in both proximal and distal tubular epithelial cells. There was also positive staining of occasional glomerular podocytes. This was in sharp contrast with VH-treated rats that showed only focal, faint and non-specific cytoplasmic staining for VEGF in tubular epithelial cells. On the other hand, treatment of CsA rats with losartan reduced greatly the intensity of VEGF staining in tubular epithelial cells. In addition, VEGF-positive cells colocalized in serial sections with the ED1 marker for macrophages.

Expression of Flt-1 and KDR/Flk-1 receptors

The mRNA expressions of the Flt-1 and KDR/Flk-1 receptors were similar in the VH-treated groups and were lower than the levels observed in CsA only-treated kidneys (Figs. 6–8). CsA treatment increased the mRNA expression of Flt-1 and KDR/Flk-1 early at seven days ($P < 0.01$ vs. VH group); CsA increased KDR/Flk-1 expression to a greater extent than Flt-1. Flt-1 receptor became significantly down-regulated early ($P < 0.05$ vs. VH) in all the treated groups and its expression seemed to be independent of the antihypertensive agent used (Figs. 6 and 8). Its expression remained low and unchanged at 28 days and no specific effect was observed with Ang II blockade. On the other hand, the behavior of the KDR/Flk-1 mRNA receptor was somewhat different (Figs. 7 and 8). It declined with time and was higher at 7 days compared to 28 days possibly due to VEGF down-regulating its own receptor. There was a specific effect observed with Ang II blockade where both enalapril and losartan significantly decreased KDR/Flk-1 mRNA expression at both time points studied ($P < 0.05$ vs. VH group). However, a similar effect was not observed in the CsA + N and CsA + HH groups where there was a trend toward a lower KDR/Flk-1 expression, but the difference did not achieve statistical significance.

DISCUSSION

We have previously shown that the expression of VEGF and its receptors Flt-1 and KDR/Flk-1 was up-
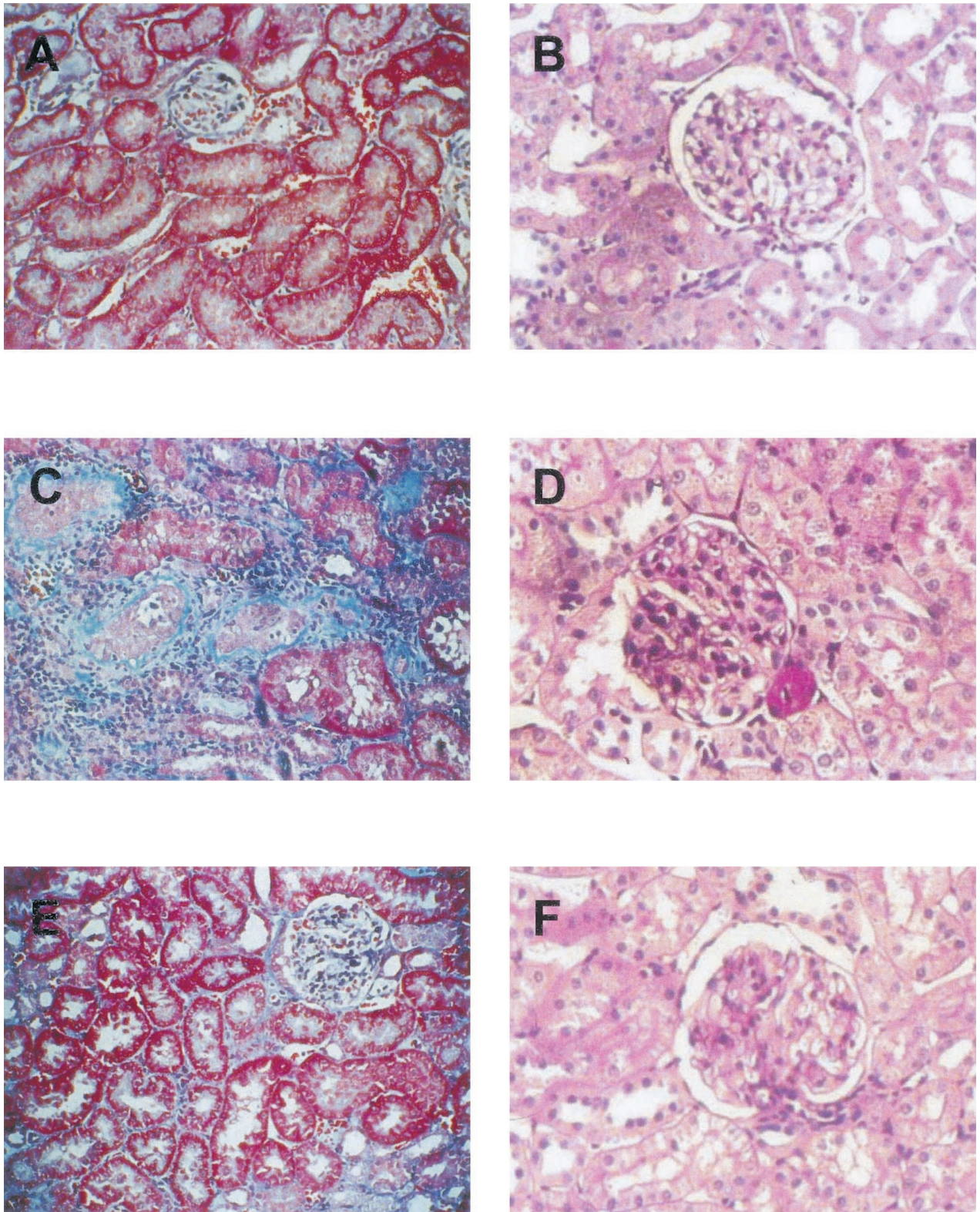
Fig. 1. Histologic changes. Photomicrographs showing the renal cortex of a salt-depleted rat given vehicle (VH; *A, B*), cyclosporine A (CsA) 15 mg/kg/day (*C, D*), or a combination of CsA 15 mg/kg/day and losartan 10 mg/kg/day (*E, F*). VH-treated rats display normal kidney histology (A, B). In rats treated with CsA, extensive interstitial fibrosis and tubular atrophy (C) and afferent arteriolopathy (D) are seen. When both CsA and losartan are given in combination, these changes are markedly reduced (E, F). Trichrome (A, C, E), PAS (B, D, F). Original magnification ×200.

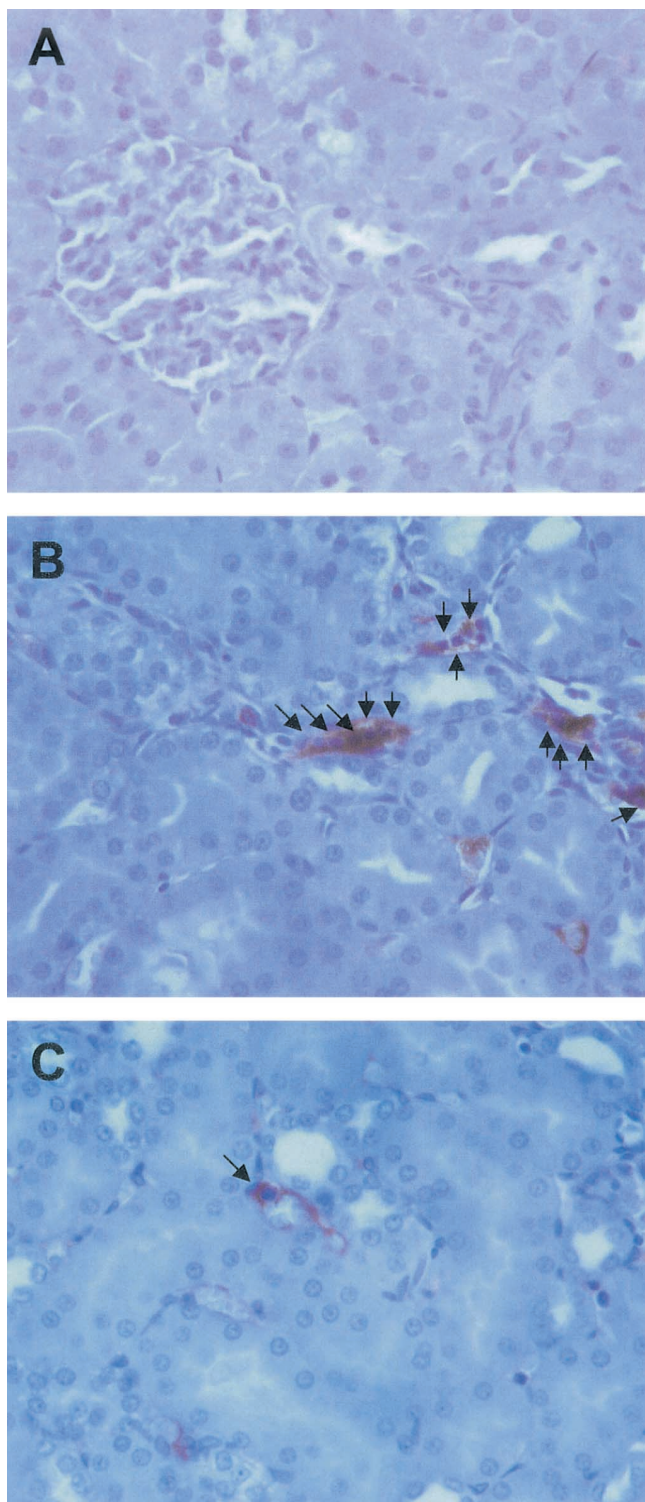**Fig. 2. Infiltration by macrophages in chronic CsA nephrotoxicity.** Kidney sections from VH-treated (*A*), CsA-treated (*B*) and CsA + L-treated (*C*) rats for 28 days were stained with ED1, an antibody to macrophages. While VH-treated rats had very few macrophages (A), CsA-treated kidneys displayed large numbers of macrophages infiltrating the tubulointerstitium (B). On the other hand, those animals treated with CsA and losartan showed only rare macrophages (C). Original magnification ×400.
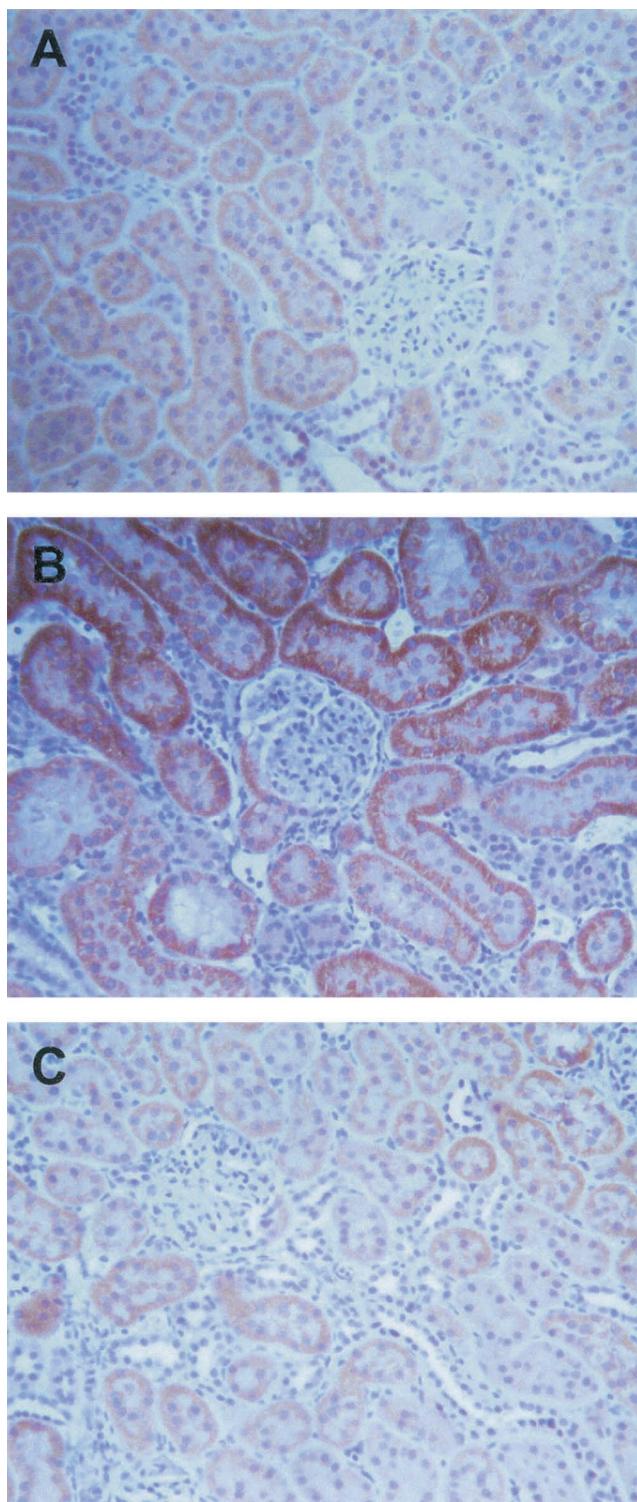

Fig. 5. Immunohistochemical staining for VEGF: Kidney sections from VH-treated (*A*), CsA-treated (*B*) and CsA+L-treated (*C*) rats for 28 days were stained with an antibody to VEGF. (B) CsA-treated rats exhibited a strong and diffuse tubular epithelial staining. (A) In contrast, the staining for VH-treated rats was faint and non-specific. (C) Treatment with losartan of CsA rats greatly reduces the intensity of VEGF staining. Original magnification ×400.
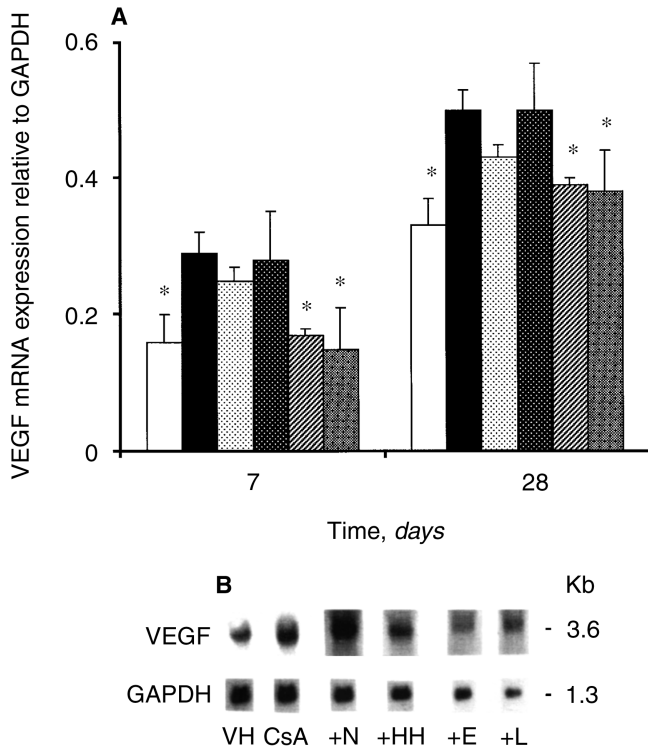
Fig. 3. Northern blot expression of vascular endothelial growth factor (VEGF) mRNA. Total RNA was isolated from whole cortex at 7 and 28 days and hybridized with a cDNA probe to VEGF. (*A*) Results of densitometric analysis after correcting for GAPDH mRNA. Symbols are: (□) placebo (VH); (■) cyclosporine (CsA); (▦) CsA + nilvadipine (N); (▦) CsA + hydralazine-hydrochlorthiazide (HH); (▧) CsA + enalapril (E); (■) CsA + losartan (L). *P < 0.05 vs. CsA only group. (*B*) A representative Northern blot. *N* = 5 to 6 per group.
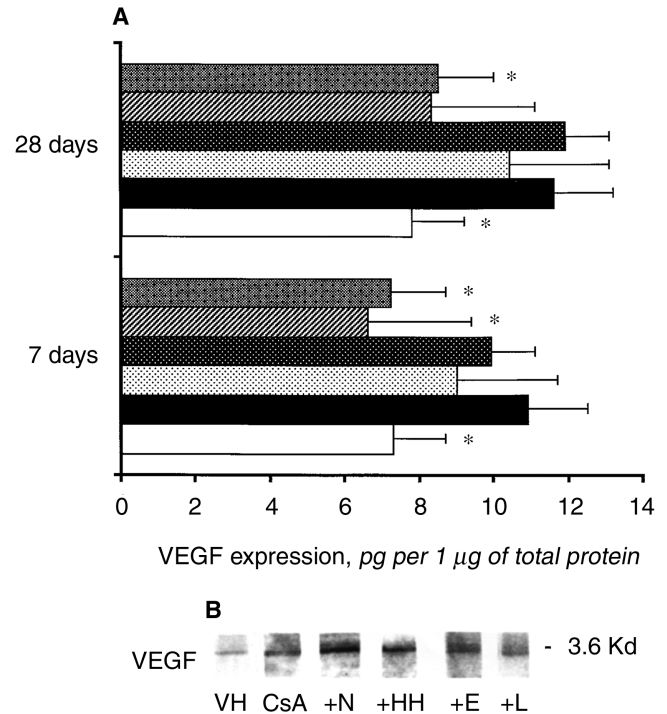

Fig. 4. Vascular endothelial growth factor (VEGF) protein expression by Western blot. Protein was isolated from whole cortex at 7 and 28 days and hybridized with a goat polyclonal anti-mouse VEGF antibody. Results of the densitometric analysis of equal amounts of protein are shown (*A*). *P < 0.05 vs. CsA only group. Symbols are: (■) CsA + L; (▧) CsA + E; (▦) CsA + HH; (▦) CsA + N; (■) CsA; (□) VH. Abbreviations are in Fig. 3. (*B*) Representative Western blot. *N* = 5 to 6 per group.

regulated in chronic CsA nephrotoxicity [10]. These changes were seen only in rats on low salt diet in which nephrotoxicity developed and not in rats on normal salt diet that remained disease free. This led to the suggestion that CsA nephrotoxicity, and not merely CsA administration, is responsible for the enhanced VEGF expression. Since a low salt diet activates the RAS, we examined the effect of Ang II blockade on VEGF expression. Our prior study showed that Ang II blockade ameliorated chronic CsA nephrotoxicity [17]. Our current study shows a similar beneficial effect, namely a reduction in interstitial fibrosis and arteriolopathy. The protective effect of RAS blockade on the kidney was dissociated from its renal hemodynamic effects, since there was worsening of the GFR in the losartan and enalapril-treated rats, a finding similar to previous observations in the same model [17, 20]. Part of the beneficial effects of Ang II blockade also could have been related to its antihypertensive effect. However, more interestingly, VEGF mRNA expression and peptide production were specifically and significantly reduced by Ang II blockade. This effect was seen early, persisted at 28 days, and was independent of blood pressure since a comparable decrease in blood pressure with the use of nilvadipine or with hydralazine/HCTZ failed to achieve similar results. In addition, the effects of enalapril and losartan on kidney histology and VEGF expression were comparable.

Previous reports have demonstrated that Ang II is a potent stimulus for VEGF in vascular smooth muscle, cardiac and retinal endothelial, and mesangial cells [21–26]. This action is Ang II concentration-dependent and is rapid in onset [26]. Ang II exerts its multiple actions via specific receptors. To date, most of the recognized biological actions of Ang II are transduced via the AT_1 receptor [27]. In this model, the RAS is clearly activated and the effects exerted by CsA on VEGF were abolished by losartan, suggesting the involvement of the AT_1 receptor. This is similar to other studies that showed that AT_1 receptor antagonists, but not AT_2 antagonists, are capable of inhibiting completely the effect of Ang II on VEGF [28]. Our results do not necessarily contradict recent findings that VEGF infusion improved CsA-associated hypertension in rats on a high-salt diet since, unlike our model, the RAS in this setting was blunted [29]. Also, we did not investigate the mechanism through which Ang II affected VEGF expression. It could be due to an inhibition in mRNA transcription and/or a decrease
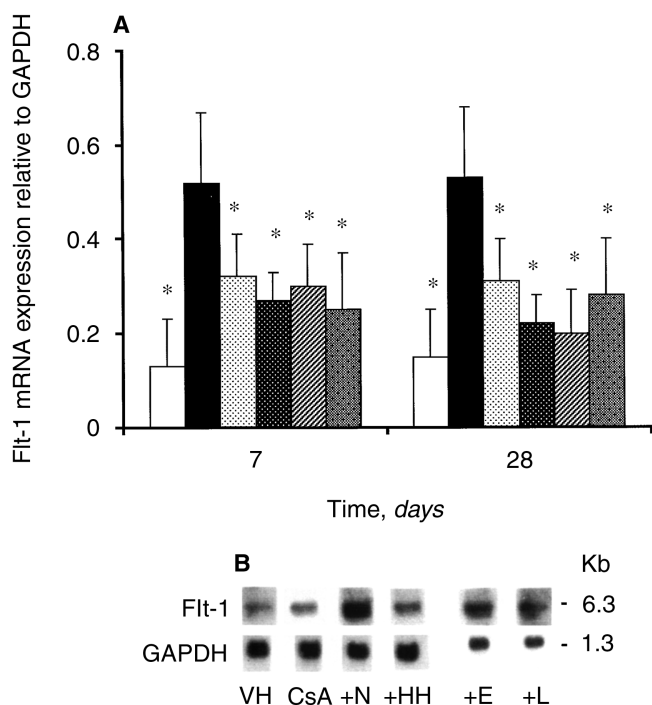
Fig. 6. Northern blot expression of Flt-1 mRNA. Total RNA was isolated from whole cortex at 7 and 28 days and hybridized with a cDNA probe to Flt-1. (*A*) Results of densitometric analysis after correcting for GAPDH mRNA. Symbols are: (□) VH; (■) CsA; (▦) CsA + N; (▥) CsA + HH; (▨) CsA + E; (▩) CsA + L. Abbreviations are in Figure 3 legend. (*B*) Representative Northern blot. *N* = 5-6 per group. **P* < 0.05 vs. CsA only group.
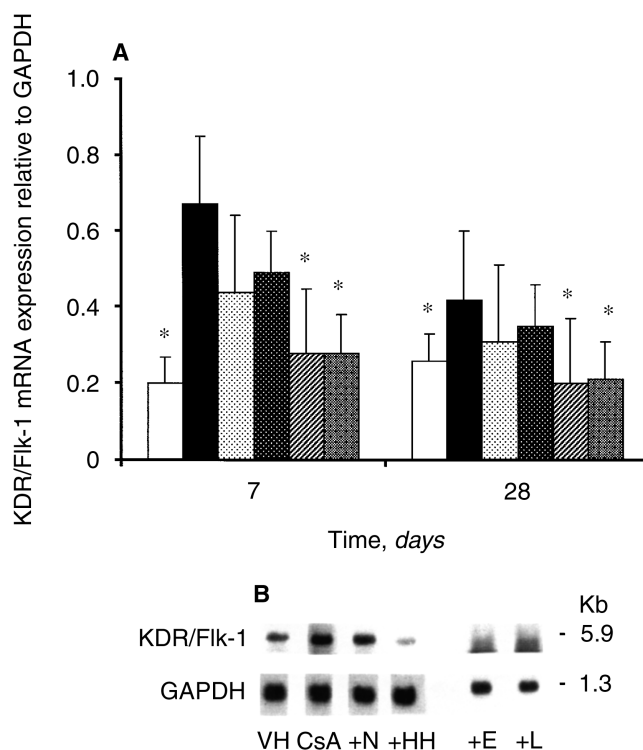

Fig. 7. Northern blot expression of KDR/Flk-1 mRNA. Total RNA was isolated from the whole cortex at 7 and 28 days and was hybridized with a cDNA probe to KDR/Flk-1. (*A*) Results of densitometric analysis after correcting for GAPDH mRNA. Symbols are: (□) VH; (■) CsA; (▦) CsA + N; (▥) CsA + HH; (▨) CsA + E; (▩) CsA + L. Abbreviations are in Figure 3 legend. (*B*) Representative Northern blot. *N* = 5 to 6 per group. **P* < 0.05 vs. CsA only group.

in mRNA stability. In hypoxia, VEGF was increased in human epithelial cells through increasing mRNA transcription and stability [30]. On the other hand, Ang II was able to activate VEGF transcription in bovine retinal pericytes without changing the mRNA half-life [25]. It is interesting to note that the VEGF gene contains a number of motifs in the 3′ untranslated region involved in regulating mRNA stability [31].

There is convincing evidence in vitro and in vivo that Ang II increases TGF-β [32] and that Ang II blockade decreases TGF-β [14]. Along those lines, we have shown that Ang II blockade decreases TGF-β1 in chronic CsA nephrotoxicity [17]. Since TGF-β induces VEGF in several cell types [1, 3, 26], it is conceivable that Ang II-induced VEGF could be mediated via Ang II-induced release of TGF-β. However, two lines of evidence negate this possibility: (*1*) Ang II actions to increase VEGF occur rapidly and earlier than the time course for TGF-β induction by Ang II [18]; (*2*) the released TGF-β1 is in the latent form by bioassays [22]. These observations do not necessarily exclude the possibility that more prolonged Ang II stimulation, by allowing time for TGF-β production, could increase VEGF directly via the AT_1 receptor and indirectly via TGF-β.

We also investigated the effect of Ang II blockade on

VEGF receptors expression. KDR/Flk-1 decreased in a manner similar to VEGF, an effect not seen in the other groups. On the other hand, the behavior of Flt-1 was independent of Ang II. Based on these findings, we conclude that VEGF probably exerted its biological effect in this model via KDR/Flk-1. It also is possible that Ang II may directly regulate KDR/Flk-1 gene expression. Recently, in a report investigating Ang II activity in retinal endothelial cells, Ang II potentiated VEGF-induced angiogenic activity through KDR/Flk-1 but had no effect on Flt-1 [21]. These two receptors display differences in function; for example, KDR/Flk-1-expressing cells show changes in morphology, chemotaxis, and mitogenicity upon VEGF stimulation, whereas Flt-1-expressing cells lack such response [1, 32]. Also, gene knockout experiments suggest differences in vascular development [1]. It is thought that Flt-1 is not a signal receptor, but rather a "decoy" receptor that negatively regulates VEGF by sequestering it and rendering it less available to KDR/Flk-1 [33]. However, subsequent studies indicated that Flt-1 interacts with some signal transducing proteins [32, 34]. Nevertheless, most studies support the hypothesis that interaction with KDR/Flk-1 is a critical
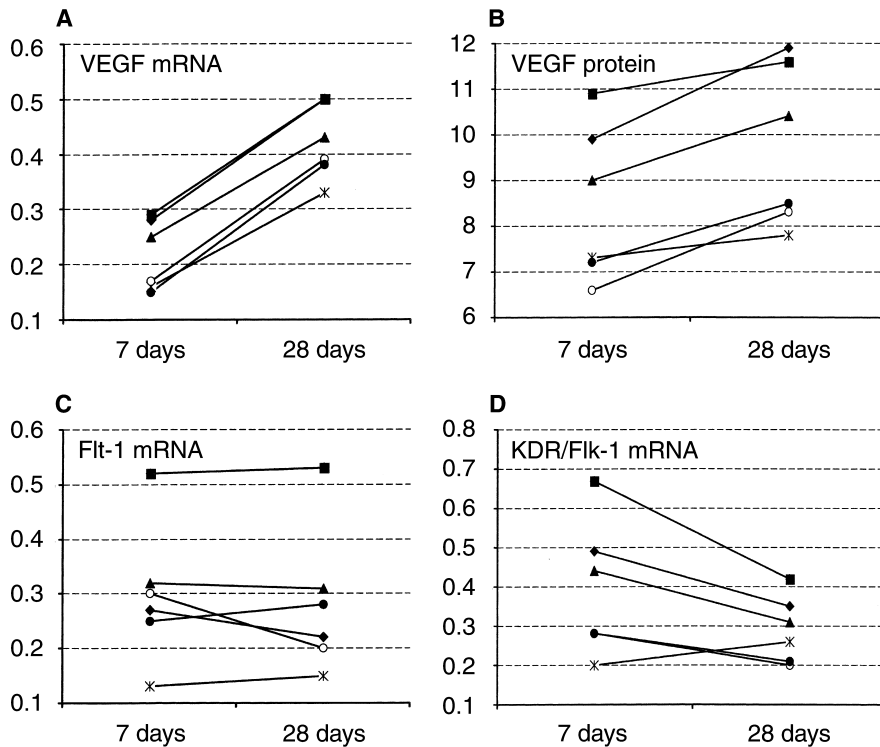
Fig. 8. Time course for the expression of VEGF and its receptors Flt-1 and KDR/Flk-1. Symbols are: (×) placebo; (■) CsA; (▲) CsA + N; (◆) CsA + HH; (○) CsA + E; (●) CsA + L. Abbreviations are in Figure 3 legend. $N = 5$ to 6 per group.

requirement to induce the full spectrum of VEGF biological responses. In support of this, VEGF mutants that bind selectively to KDR/Flk-1 are fully active endothelial cell mitogens and anti-idiotypic antibodies that activate KDR/Flk-1 promote tumor angiogenesis [35, 36]. In addition, it used to be believed that VEGF receptors were restricted to the endothelium and that VEGF exerts its activity in an autocrine fashion. However, recent findings confirmed the presence of VEGF receptors on nonendothelial cells and suggested that VEGF acts also in a paracrine fashion [37].

What effect VEGF actually has on chronic CsA nephrotoxicity remains unclear because the renal actions of VEGF remain speculative. A study in patients with chronic renal disease noted an up-regulated VEGF expression in the interstitium, but the signals were reduced in globally sclerosed glomeruli [38]. These results are consistent with a temporal sequence in which VEGF expression precedes renal fibrosis and is then down-regulated or disrupted after fibrosis. Further studies are needed to determine whether VEGF is in fact a signaling molecule involved in enhancing glomerular sclerosis or interstitial fibrosis. More recently, VEGF was shown to activate MAP kinase and to enhance collagen synthesis in human mesangial cells [39]. Of interest, VEGF also was reported to induce interstitial collagenase in endothelial cells but not in fibroblasts [40]. Thus, both matrix synthesis and degradation may be regulated by VEGF through contrasting actions on renal cells. Moreover, we know that VEGF is chemotactic for macrophages and is increased in conditions associated with macrophage infiltration. In human chronic rejection, VEGF-positive cells in the interstitium colocalized with macrophages [13]. It is also important to note that our CsA model displays a mononuclear infiltrate that precedes interstitial fibrosis and that is improved by Ang II blockade [17]. In this experiment, the number of mononuclear cells increased with CsA administration and later declined with losartan treatment. Moreover, VEGF expression colocalized with that of ED-1 positive cells in serial sections. This suggests that either VEGF is produced by macrophages or that it is contributing to their recruitment into the interstitium. Moreover, Ang II is known to play a role in the progression of glomerular and tubulointerstitial fibrosis [41]. Thus, it is possible that Ang II blockade, by decreasing VEGF, is capable of decreasing monocytic attraction, thereby aborting a self-perpetuating loop of myofibroblast proliferation and interstitial fibrosis.

The results reported herein raise some intriguing questions. Since VEGF is a potent endothelial cell mitogen, it is possible that Ang II-induced VEGF production is a compensatory mechanism directed at replacing the damaged endothelium caused by CsA. In addition, it is possible that VEGF plays a role, either independently or through Ang II, in CsA-induced fibrosis. This may be related either directly through its effect on matrix remodeling or indirectly through its chemotactic effect

on monocytes. More studies are needed to define the interactions between Ang II, TGF-β and VEGF, and to delineate their individual roles in this model. In addition, studies are needed to determine the clinical consequences of decreased VEGF with Ang II blockade.

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