Anti-Inflammatory Effect of Hepatocyte Growth Factor in Chronic Kidney Disease: Targeting the Inflamed Vascular Endothelium

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Recent studies show that hepatocyte growth factor (HGF) has potent anti-inflammatory effects in multiple animal models of disease in various organ systems, including the kidney, suggesting that HGF may suppress a common proinflammatory process. The aim of this study was to examine the molecular mechanism of HGF's anti-inflammatory actions in a model of chronic kidney disease. Beginning 2 wk after subtotal nephrectomy, rats received a continuous infusion of recombinant HGF, neutralization of endogenous HGF by daily injection of an anti-HGF antibody, or preimmune IgG for an additional 2 wk. The effects on inflammation and injury were examined. HGF administration ameliorated whereas neutralizing endogenous HGF worsened renal inflammation in remnant kidneys. This was accompanied by parallel alterations in endothelial activation and inflammation, marked respectively by *de novo* E-selectin expression in renal vascular endothelium and leukocyte adhesion to endothelium. *In vitro*, HGF abrogated monocyte adhesion to TNF-α–activated endothelial monolayers and suppressed endothelial expression of E-selectin, which depended on NF-κB signaling. In addition, HGF suppressed NF-κB reporter gene activity that was induced by TNF-α and counteracted TNF-α–elicited NF-κB interaction with κB elements at the E-selectin gene level. Dissection of the NF-κB signaling cascade revealed that suppression of NF-κB depended on HGF's inhibitory action on NF-κB and IκB phosphorylation and IκB degradation. *In vivo*, continuous infusion of exogenous HGF markedly diminished sequestration of circulating fluorescence-labeled macrophages in the remnant kidney, mimicking the action of an E-selectin blocking antibody. These findings suggest that HGF has potent and direct anti-inflammatory effects on the basis of suppression of NF-κB activation and downstream endothelial inflammation.

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Most forms of chronic kidney disease (CKD) progress inexorably to ESRD, which carries considerable morbidity and mortality. Although the initiators of CKD vary, it generally is accepted that secondary processes that are common to all renal diseases ensue, establishing a vicious cycle of nephron destruction, progressive glomerulosclerosis, and tubulointerstitial fibrosis (1–3). A growing body of evidence suggests that renal inflammation is one of the key secondary processes that drive progression (1–5) and that vascular endothelial activation is an early and indispensable event in the recruitment of inflammatory cells to the renal parenchyma (6–8). In CKD, specific pathogenic stimuli such as anti–endothelial cell antibodies, dyslipidemia, and advanced glycosylation end products combine with overproduction of multiple soluble mediators (*e.g.*, TNF-α, IL-1β) to activate renal vascular endothelial cells, causing them to transit to a proinflammatory phenotype (9). Activated endothelial cells express multiple adhesion molecules, facilitating the migration of leukocytes to the inflamed area (10–13). This process involves several steps: Rolling, sticking, diapedesis, and chemotaxis. Rolling is the earliest step and is required for leukocyte margination and arrest on the activated endothelium (12,14). Rolling is mediated by the selectin family of adhesion molecules, including endothelial (E-selectin), platelet (P-selectin), and leukocyte (L-selectin). Both P- and L-selectins are constitutively expressed, whereas *de novo* expression of E-selectin occurs only in response to proinflammatory stimulation and is crucial for leukocyte trafficking (15).

Hepatocyte growth factor (HGF) is a multifunctional, pleiotropic growth factor that ameliorates chronic renal injury in a variety of models, including remnant kidney (16–19), unilateral ureteral obstruction (20,21), and diabetic nephropathy (22). Various mechanisms have been proposed to explain this beneficial effect, including antiapoptosis (20), enhanced matrix degradation (16,17), and blocking of epithelial-to-mesenchymal transition (21). Recently, others (19,23) and we (18,24) have provided compelling evidence to suggest that HGF also has direct anti-inflammatory effects, and this may play a role in its renoprotective action. HGF also ameliorates acute and chronic inflammation in a variety of nonrenal disease models, including inflammatory bowel disease (25–30) and airway inflammation (31). This suggests that HGF regulates key inflammatory events that are common to many diseases and organ systems.

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Nevertheless, the exact mechanisms by which HGF suppresses inflammation are largely unknown. To explore this process further, we examined the effects of HGF on chronic renal inflammation in the rat remnant kidney model. We found that HGF blocks endothelial inflammation by suppressing expression of E-selectin.

Materials and Methods

Cell Culture Studies

Human umbilical vein endothelial cells (HUVEC) were purchased from VEC Technologies (Rensselaer, NY) and maintained in MCDB-131 complete medium. HUVEC (two to eight passages) were seeded on gelatin-coated (1.5%) cultures at approximately 80% confluence. After growth for 24 h in complete medium, cells underwent serum starvation for 6 h in Medium 199. Human recombinant HGF (Genentech, South San Francisco, CA) and human recombinant TNF-α (R&D Systems, Minneapolis, MN) were added to the culture with fresh serum-free medium at a final concentration of 100 and 0.1 ng/ml, respectively, or as otherwise indicated.

Human monocytes (THP-1) and rat alveolar macrophages (RAM) were purchased from American Type Culture Collection (ATCC; Manassas, VA) and were cultured, respectively, in RPMI supplemented with 10% FBS and Ham's F12K that contained 15% FBS. For fluorescence-viable labeling, THP-1 and RAM cells (1×10^7) were incubated in medium that contained 5 μg/ml Calcein-AM (Invitrogen, Carlsbad, CA) at 37°C for 30 min. Excess dye was removed by washing three times with PBS.

Animal Studies

Study 1 was designed to determine how manipulation of HGF activity affects endothelial inflammation in an experimental chronic renal disease. Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) that initially weighed 180 to 210 g were housed in an approved animal care facility and fed a standard diet. Five-sixths renal ablation was performed as described previously (13–15). Two weeks after surgery, rats were randomly assigned to one of three groups ($n = 7$; Figure 1A): (*1*) HGF-treated group: Rats received recombinant human HGF as a continuous intravenous infusion *via* an implanted osmotic minipump into the jugular vein at a dose of 100 μg/kg per d for 14 d; (*2*) anti-HGF antibody (α-HGF)–treated group: Rats received daily injections of a high-titer, rabbit polyclonal anti–HGF-neutralizing antibody intravenously through the tail vein for 14 d; or (*3*) preimmune IgG-treated group: Rats received a continuous intravenous infusion of saline *via* minipump as well as daily tail-vein injections of rabbit preimmune IgG. Sham-operated rats with no renal ablation served as normal controls ($n = 7$). Two weeks after treatment, rats were killed.

Study 2 was performed to test how HGF or E-selectin blockade affects macrophage adhesion to the endothelium *in vivo* in the remnant kidney. Figure 1B provides a summary of the treatments that were given to each of the five groups ($n = 5$). For examination of macrophage adhesion *in vivo*, rats were anesthetized, a tracheostomy was performed, and catheters were inserted into the jugular vein and carotid artery. All rats received an infusion of plasma as described previously (13–15) to maintain euvolemic conditions. Rats also received a bolus injection of a rabbit anti–E-selectin–blocking antibody (Santa Cruz Biotechnology, Santa Cruz, CA), preimmune IgG, or saline into the left carotid artery. Four hours later, fluorescence-labeled RAM cells (1×10^4) suspended in 1 ml of normal saline were infused into the carotid artery as a bolus injection. Rats were killed 30 min after macrophage administration.

In all animal experiments, kidneys were excised for analysis. One portion was fixed in 10% phosphate-buffered formalin for morphology and immunohistochemistry analyses. Another part was frozen immediately for cryostat sectioning. The remainder was snap-frozen in liquid nitrogen and stored at −80°C for RNA and protein extraction. For quantification of the fluorescence RAM cells that were sequestrated in the kidney, kidney homogenates with equal amount of protein (100 μg) were subjected to fluorometric analysis in a fluorescence plate reader as described below.

Morphologic Studies

Formalin-fixed kidneys were embedded in paraffin and prepared in 3-μm-thick sections. Immunohistochemical staining for ED-1 was performed as described previously (18). The antibody for ED-1 was purchased from Serotec (Oxford, UK). Indirect immunofluorescent staining of E-selectin was carried out on methanol/acetone-fixed (1:1) frozen cryostat sections using rabbit polyclonal anti–E-selectin antibody (Santa Cruz Biotechnology). Frozen sections were double stained for aminopeptidase P, a marker for vascular endothelial cells, using a specific murine mAb, JG-12 (Bender MedSystems GmbH, Vienna, Austria), which has been shown to bind specifically to endothelial cells of blood vessels in rat kidney (32). The Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 594 goat anti-mouse antibody (Invitrogen) were as used as

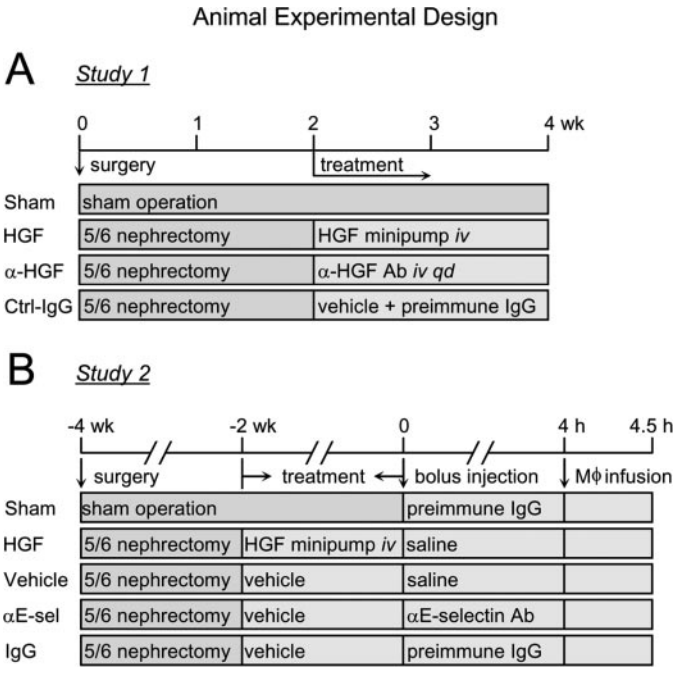

Figure 1. Schematic diagrams illustrate experimental design for animal studies. (A) In Study 1, 2 wk after renal ablation or sham operation (Sham), rats were treated for another 2 wk with minipump continuous intravenous (iv) infusion of hepatocyte growth factor (HGF), daily (qd) tail-vein injection of anti-HGF antibody (α-HGF), or minipump vehicle infusion plus daily tail-vein injection of preimmune IgG (Ctrl-IgG). (B) In Study 2, 2 wk after renal ablation or sham operation (Sham), rats were treated for another 2 wk with minipump continuous intravenous infusion of HGF or vehicle before a bolus injection of saline, anti–E-selectin antibody (αE-sel), or preimmune IgG through the carotid artery. Four hours later, all rats were infused with Calcein-AM–labeled rat macrophages and killed after 30 min.

secondary antibody. As a negative control, the primary antibody was replaced by nonimmune serum from the same species; no staining occurred. Finally, all sections were counterstained with 4′,6-diamidino-2-phenylindole or Evans blue and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). For visualization of fluorescence monocytes that were sequestrated in the tissue, cryostat sections were fixed with methanol/acetone and then subjected to counterstaining. Morphologic results were interpreted with a Nikon Microphot-FX fluorescence microscope equipped with a Spot II digital camera.

Static Monocytic Adhesion Assay

Adhesion studies were performed with the human monocytic cell line THP-1 under static conditions (33). Fluorescence-labeled THP-1 cells were resuspended in Medium 199 (1×10^6 cells/ml). For static adhesion assays, HUVEC monolayers with equal cell numbers in 12-well plates were treated with HGF and/or TNF-α for the stated intervals. HUVEC cultures were washed twice with PBS before addition of 1 ml (1×10^6) of labeled THP-1 cells per well. The plates were incubated for 30 min at 37°C. After incubation, the monolayers were washed gently three times with PBS and photomicrographs were taken using a phase-contrast microscope. Adherent monocytes were lysed with RIPA buffer (1% Nonidet P-40, 0.1% SDS, 100 μg/ml PMSF, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 2 μg/ml aprotin, 2 μg/ml leupeptin, and 5 mM EDTA in PBS), and fluorescence intensity was measured by a Spectramax GEMINI EM fluorescence plate reader (Molecular Devices, Sunnyvale, CA) at an excitation wavelength of 485 nm and emission at 530 nm. HUVEC monolayers that were adhered with nonlabeled THP-1 cells served as negative controls.

Transient Transfection and Reporter Gene Assay

The reporter construct pGL-3κB-Luc, a firefly luciferase reporter gene driven by three copies of an NF-κB consensus sequence, was provided by Dr. Fu (Division of Gastroenterology, Brown Medical School). HUVEC were transfected by electroporation using the Amaxa HUVEC Nucleofector kit (Amaxa GmbH, Koeln, Germany). A fixed amount (50 ng) of internal control reporter *Renilla reniformis* luciferase driven under thymidine kinase (TK) promoter (pRL-TK; Promega, Madison, WI) was co-transfected for normalizing the transfection efficiency. After transfection with equal amounts of reporter constructs, HUVEC were seeded and treated as indicated. Total cell lysates were collected, and luciferase activity was determined using the Dual-Glo luciferase assay kit (Promega). The relative NF-κB transactivation activity was assessed as the fold change of firefly luciferase activity after normalization for *Renilla reniformis* luciferase activity.

DNA Affinity Precipitation Assay

The interaction between NF-κB and DNA was examined by DNA affinity precipitation assay as described elsewhere (34) with minor modifications. After various treatments, HUVEC were collected and washed with PBS. The nuclear extracts were prepared with the NE-PER nuclear extraction kit (Pierce, Rockford, IL). A 50-μg sample of the nuclear lysate was mixed with 2× Laemmli's sample buffer, boiled, and used for Western blotting as an input fraction. The residual lysate was used for the DNA precipitation assay. The 5′-biotinylated, double-stranded oligonucleotides (0.2 nM) that contained two putative NF-κB *cis* elements at the nucleotide position −129 to −80 bp of human E-selectin promoter (5′-CGTGGATATTCCCGGGAAAGTTTTTGGATG-CCATTGGGGATTTCCTCTTT-3′; Genbank accession no. M64485) were mixed with 200 μg of nuclear extracts. DNA affiliated protein then was pulled down by streptavidin-agarose and washed with ice-cold PBS that contained 0.5% Triton X-100 and 5 mM EDTA. Proteins were eluted from the beads by the addition of 2× Laemmli's sample buffer, followed by boiling for 10 min and immunoblot analysis with specific anti–NF-κB p65 antibody (Santa Cruz Biotechnology).

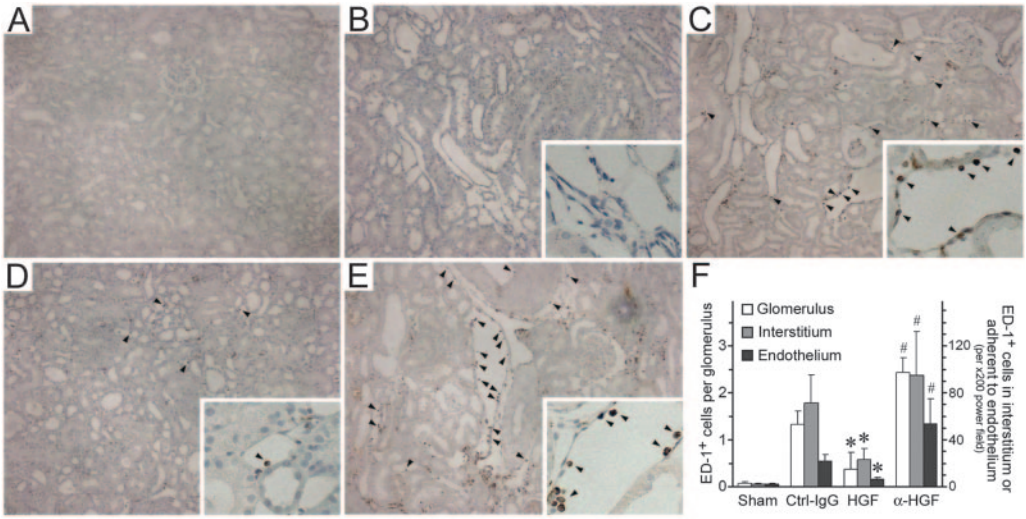

Figure 2. HGF supplement suppresses whereas HGF blockade enhances renal inflammation, accompanied by parallel alternations in endothelial inflammation, marked by adhesion of immune competent cells to renal vascular endothelium. (A through E) Representative micrographs of ED-1 immunohistochemistry staining depicting macrophages adherent to renal vascular endothelium (arrowheads) in kidney sections from sham-operated rats (B) and remnant kidney rats treated with preimmune IgG (Ctrl-IgG; A and C), HGF (D), or an anti-HGF antibody (α-HGF; E). Sections processed with nonimmune serum from the same species as the primary antibody were examined as a negative control (A) for immunohistochemistry. (E) Counting of ED-1–positive cells in glomeruli (□), tubulointerstitium (▨), and those adherent to vascular endothelium (■) in kidney sections from each group. *$P < 0.05$, #$P < 0.05$ *versus* other groups with remnant kidney disease ($n = 7$). Magnifications: ×100 in A through E; × 400 for inserts.

Chromatin Immunoprecipitation Assay

The *in situ* interaction between NF-κB and E-selectin promoter in HUVEC was examined by chromatin immunoprecipitation assay (35) by using a commercially available kit (Upstate Biotechnology, Charlottesville, VA) according to the manufacturer's instructions. Briefly, HUVEC were fixed and cross-linked with 4% formaldehyde. After collection of cell pellets and sonication, aliquots of the samples were set aside as input fraction, and the rest were subjected to immunoprecipitation using an anti–NF-κB p65 antibody or preimmune IgG and Protein A agarose that were used to pull down the immune complexes. After elution, precipitated chromatin as well as the input fraction was heated at 65°C to reverse cross-links, and the DNA was extracted with Qiagen PCR purification kit (Qiagen, Valencia, CA). DNA sequences that spanned the κB responsive elements in the promoter region of E-selectin gene were amplified by PCR using specific primers (forward 5′-GGCATGGACAAAGGTGAAGT-3′; reverse 5′-CAGCCAAGAACAGCTGAACA-3′; Genbank accession no. M64485) for a number of cycles in the exponential phase as estimated in pilot experiments. DNA samples that were extracted from input fractions were amplified in parallel for normalization. PCR products that were resolved in approximately 1.5 to 2% agarose gels were photographed under ultraviolet light.

Semiquantitative Reverse Transcription–PCR

Total RNA was prepared and reverse transcription–PCR was performed as before (18) using specific primers for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and E-selectin (forward 5′-TTATGATGAGGCCAGTGCTTATTG-3′; reverse, 5′-CTTTTTGCCTATTGTTGGGTTCAC-3′; Genbank accession no. NM_000450) for a number of cycles in the exponential phase as estimated in pilot experiments. PCR products that were resolved in approximately 1.5 to 2% agarose gels were photographed under ultraviolet light.

Western Immunoblot Analysis

Rat kidneys were homogenized and HUVEC monolayers were lysed in RIPA buffer supplemented with protease inhibitors. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Sigma, St. Louis, MO). Samples with equal amounts of total protein (40 to 80 μg/ml) were fractionated by 7.5 to 10% SDS–polyacrylamide gels under reducing condition and analyzed by Western immunoblot as described previously (16). The antibodies against E-selection, NF-κB p65, IκBα, and actin were purchased from Santa Cruz Biotechnology, and those for phosphorylated NF-κB p65 and phosphorylated IκBα were purchased from Cell Signaling Technology (Beverly, MA).

Statistical Analyses

Immunoblot bands were scanned and the integrated pixel density was determined using a densitometer and the National Institutes of Health image analysis program. All data are expressed as mean ± SD. Statistical analysis of the data from multiple groups was performed by ANOVA followed by Student-Newman-Kuels tests. Data from two groups were compared by *t* test. $P < 0.05$ was considered significant.

Results

HGF Attenuates Endothelial Inflammation in Rat Remnant Kidney

Endothelial cell activation and leukocyte-to-vascular endothelial adhesion are the initial steps in the genesis of an interstitial inflammatory infiltrate (6,7). As shown in Figure 2, immunohistochemistry staining for ED-1, a rat macrophage marker, demonstrated that adhesion of inflammatory cells to vascular endothelium was evident in rat remnant kidney 4 wk after renal ablation (Figure 2C). Adhesion of ED-1–positive cells was markedly reduced by HGF treatment (Figure 2D) and

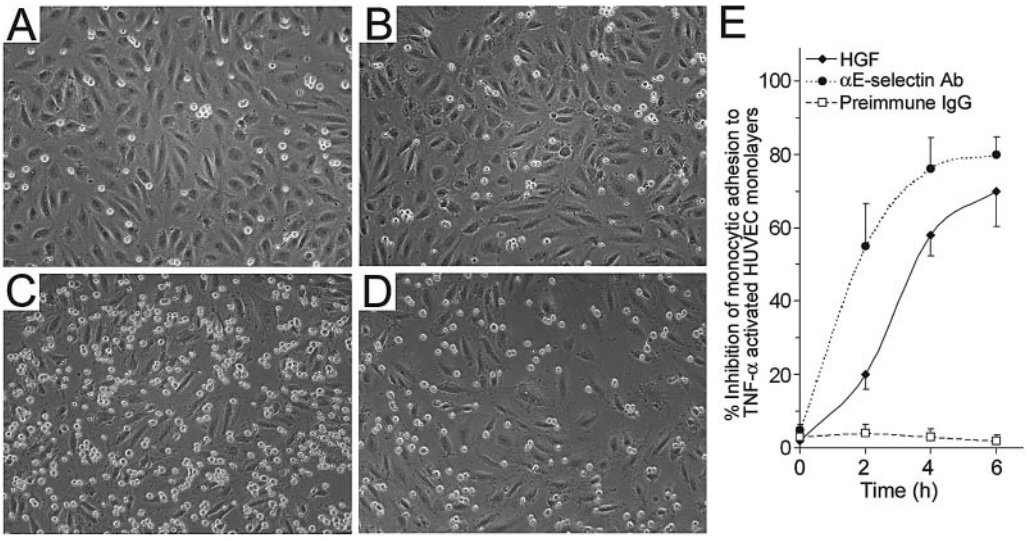

Figure 3. HGF abrogates monocytic adhesion to endothelial cell monolayers that is activated by TNF-α, mimicking the action of E-selectin–blocking antibody. Human umbilical vein endothelial cells (HUVEC) were pretreated with vehicle (A and C) or 100 ng/ml HGF (B and D) for 30 min before addition of 0.1 ng/ml TNF-α (C and D) or vehicle (A and B). After various intervals (4 h for A and B, and 2, 4, and 6 h for E), HUVEC monolayers were washed and Calcein-AM–labeled THP-1 cells were applied. Before TNF-α stimulation, a rabbit anti–E-selectin antibody (2 μg/ml) or preimmune IgG (2 μg/ml) was given to demonstrate the role of E-selectin in monocyte adhesion. (A and B) Representative microphotographs demonstrate THP-1 adhesion to HUVEC monolayers after different treatments. (E) Aliquots of cell lysates were subjected to fluorometric analysis to quantify the inhibitory effect of various maneuvers on THP-1 adhesion ($n = 3$).

significantly increased after HGF neutralization by a specific antibody (Figure 2E). Absolute counting of ED-1–positive macrophages in the renal sections revealed that the extent of macrophage-to-endothelial adhesion correlated with the magnitude of interstitial inflammation and was modulated by HGF (Figure 2F). This is consistent with the important role of endothelial activation in the renal inflammatory response (6).

Adhesion of Monocytes to TNF-α–Activated Endothelial Cells Is Attenuated by HGF

The direct effects of HGF on leukocyte adhesion to activated endothelial cells were investigated. HUVEC monolayers were pretreated with HGF or vehicle and then stimulated by the proinflammatory cytokine, TNF-α, for different intervals. Calcein-AM-labeled human monocytes (THP-1 cells) were applied for static adhesion assay. Microphotographs showed that very few cells were adherent to vehicle-treated (Figure 3A) or HGF-treated (Figure 3B) endothelial monolayers. TNF-α increased THP-1 cell adherence (Figure 3C), and this effect was significantly blunted by HGF (Figure 3D). Fluorometric analyses of cell lysates were consistent with the microscopic observations and revealed time-dependent suppression of monocytic adhesion to activated HUVEC by HGF (Figure 3E).

After endothelial activation, E-selectin is induced and captures circulating leukocytes (10). To determine whether E-selectin was responsible for the static adhesion that we observed in endothelial cell cultures *in vitro*, we used a rabbit polyclonal anti–E-selectin antibody to block the adhesion molecule. Shown in Figure 3E, preimmune IgG from the same species as the anti–E-selectin antibody had little effect on THP-1 adhesion to TNF-α–activated HUVEC at all time points. In contrast, the E-selectin blocking antibody significantly reduced monocytic adhesion in a pattern that was similar but more potent than HGF. This suggests that the effect of HGF on monocyte-to-endothelial adhesion is consistent with E-selectin blocking.

De Novo *Expression of E-Selectin in Cultured Endothelial Cells Is Suppressed by HGF*

The direct effect of HGF on endothelial expression of E-selectin in HUVEC was examined. E-selectin expression was minimal under basal conditions but was induced beginning 1 to 2 h after exposure to TNF-α. HGF suppressed TNF-α–induced expression of E-selectin in a dosage- and time-dependent manner; the maximal inhibitory effect was observed at 500 ng/ml and at 4 h, respectively (Figure 4, A and B). Reverse transcription–PCR demonstrated that HGF also reduced E-selectin mRNA levels, suggesting that HGF suppresses E-selectin primarily at the transcriptional level (Figure 4C). Of note, TNF-α–induced E-selectin expression was markedly suppressed by HGF regardless of whether HGF was added before, simultaneously with, or after TNF-α stimulation (Figure 4C).

HGF Supplement Decreases Vascular Endothelial E-Selectin Expression in Rat Remnant Kidney

For examination of whether HGF also regulates endothelial E-selectin expression in the diseased kidney *in vivo*, remnant kidneys from rats in which HGF action was unaltered, augmented, or blocked were stained fluorescently for E-selectin. Shown in Figure 5A, there was minimal expression in sham-operated kidney; however, prominent focal E-selectin expression was evident in remnant kidneys primarily in the interstitial area (Figure 5, B and E) and, to a lesser extent, in glomerular tufts (Figure 5F). E-selectin staining co-localized with the vascular endothelial marker JG-12 (Figure 5, E and F), suggesting that E-selectin was expressed predominantly by renal vascular endothelium. Continuous HGF infusion for 2 wk markedly reduced E-selectin staining (Figure 5C) and decreased its absolute abundance as estimated by immunoblot (Figure 5G) and densitometry (Figure 5H). Conversely, neutralization of endogenous HGF by a specific antibody significantly enhanced E-selectin staining (Figure 5D) and substantially increased its expression level (Figure 5, G and H).

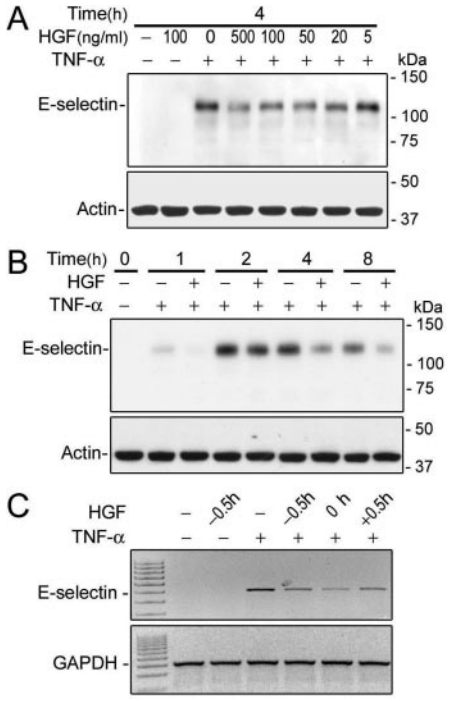

Figure 4. HGF suppresses TNF-α–induced expression of E-selectin in cultured endothelial cells. (A and B) HUVEC were pretreated with HGF (100 ng/ml or otherwise indicated) or vehicle for 30 min before stimulation by TNF-α (0.1 ng/ml). Cell lysates were harvested at different time points after TNF-α and analyzed for E-selectin by Western immunoblot. Cytoplasmic β-actin was used as a standard molecule for normalization. (C) HGF (100 ng/ml) treatment was initiated at different time points relative to TNF-α (0.1 ng/ml). E-selectin message was examined by reverse transcription–PCR 4 h later.

HGF Affects NF-κB–Mediated E-Selectin Expression in Endothelial Cells

NF-κB activation and subsequent transactivation of downstream proinflammatory genes is a fundamental mechanism that initiates and promotes inflammation in chronic renal disease (1–4,36). Two specific inhibitors for NF-κB, n-tosyl-l-phenylalanine chloromethylketone and pyrrolidine dithiocar-

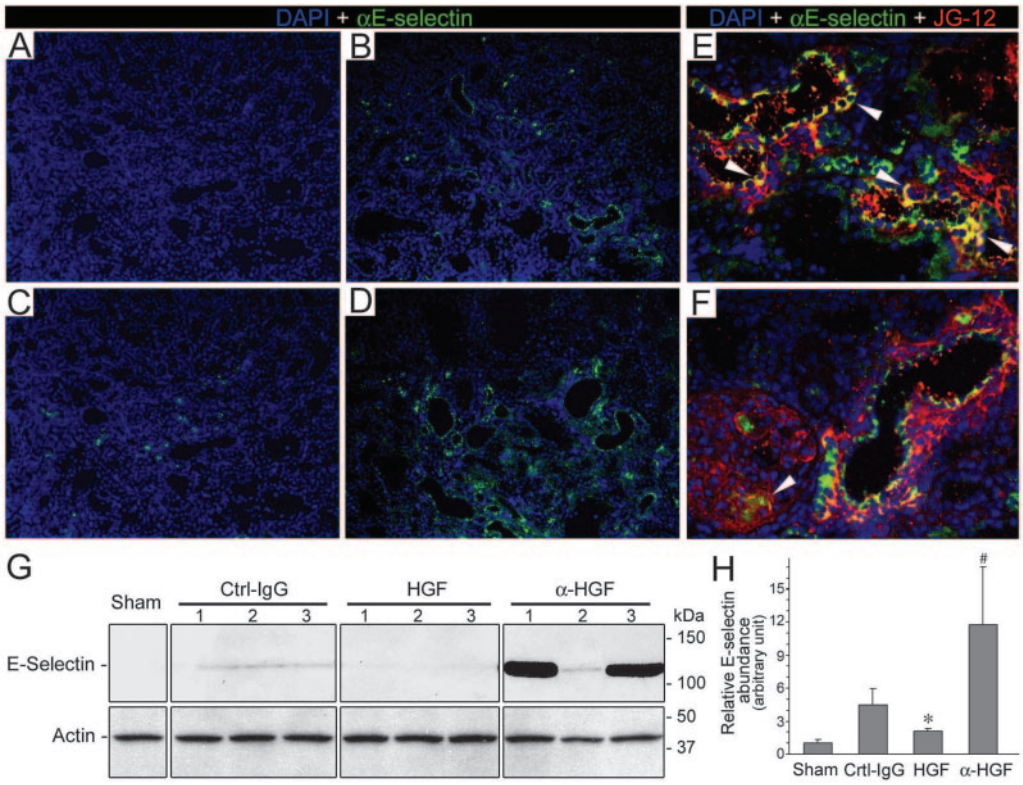

Figure 5. HGF infusion inhibits and HGF neutralization induces renal vascular endothelial expression of E-selectin. (A through E) Representative micrographs showing immunofluorescent staining for E-selectin (arrowheads) in kidney sections from sham-operated rats (A) and remnant kidney rats treated with preimmune IgG (Ctrl-IgG; B, E, and F), HGF (C), or anti-HGF antibody (α-HGF; D). (E and F) Co-localization of E-selectin (green) with the rat vascular endothelial maker JG-12 (red) in the interstitium (E) and glomerulus (F). Tissue sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). (G) Representative immunoblots for E-selectin in kidney homogenates. (H) Arbitrary units of E-selectin abundance in immunoblot analysis expressed as densitometric ratios of E-selectin/actin as folds above that found in the normal control group. $^{*}P < 0.05$, $^{\#}P < 0.05$ *versus* other disease groups (n = 7). Magnifications: ×100 in A through D; ×400 in E and F.

bamate, reduced *de novo* expression of E-selectin in endothelial cells (Figure 6), consistent with the hypothesis that NF-κB regulates E-selectin expression. To explore further whether HGF suppression of E-selectin is mediated by NF-κB inhibition, we examined the effect of HGF on NF-κB–mediated transcriptional activity in HUVEC. HUVEC were transiently transfected with a reporter gene construct driven by three copies of an NF-κB consensus sequence. Shown in Figure 7A, 3κB promoter activity essentially was unchanged by HGF treatment but increased significantly after TNF-α stimulation. HGF co-treatment significantly blunted TNF-α's effect. The transcriptional activity of NF-κB is fundamentally affected by its interaction with κB *cis* elements. To determine whether HGF regulates NF-κB by altering DNA binding, we performed DNA affinity precipitation assay on cell lysates by using an oligonucleotide that contained two tandem copies of a κB *cis* element in the human E-selectin promoter region to pull down NF-κB with binding activity. Immunoblot analysis demonstrated that HGF alone had only a minor effect on constitutive binding of NF-κB p65 to the exogenous oligo (Figure 7B). TNF-α promoted total input as well as oligo-affiliated NF-κB p65, whereas HGF reduced NF-κB p65 binding that was elicited by TNF-α without altering total NF-κB abundance. Chromatin immunoprecipitation assay was used to examine further the inhibitory effect of HGF on NF-κB p65-DNA interaction *in situ* at the target gene (E-selectin) level. Untreated or HGF-treated HUVEC showed no NF-κB to E-selectin gene binding. In contrast, TNF-α treatment induced recruitment of NF-κB to the E-selectin promoter, and this effect was abrogated by HGF (Figure 7C). Collectively,

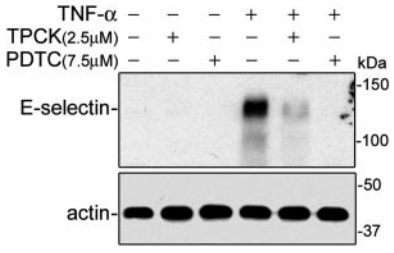

Figure 6. De novo expression of endothelial E-selectin is regulated by NF-κB. HUVEC were pretreated with n-tosyl-l-phenylalanine chloromethylketone (TPCK) or pyrrolidine dithiocarbamate (PDTC), two selective NF-κB inhibitors, for 30 min before TNF-α activation. Cell lysates were harvested 4 h later for immunoblot analysis of E-selectin or actin.

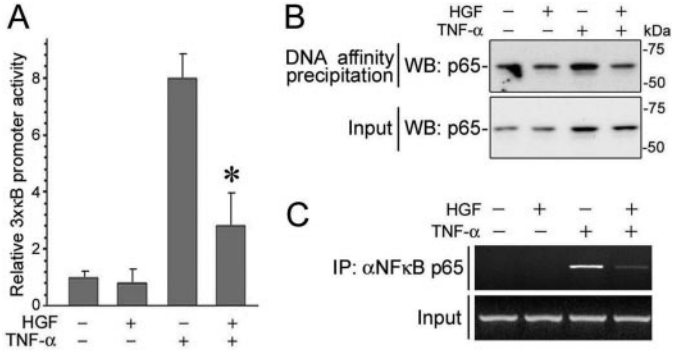

Figure 7. HGF suppresses the transcription activity of NF-κB and alters NF-κB–E-selectin gene interactions. (A) HUVEC were transiently transfected with a reporter gene driven by three copies of an NF-κB consensus sequence. After 24 h, cells were treated with HGF (100 ng/ml) and/or TNF-α (0.1 ng/ml) for another 24 h before cell lysates were prepared for luciferase assay. Relative luciferase activity (fold induction) is presented. $^*P < 0.05$ *versus* TNF-α stimulation alone ($n = 3$). (B) HUVEC were treated with HGF and/or TNF-α as described in A. After 4 h, cell lysates were subjected to DNA affinity precipitation assay using synthesized oligonucleotides detailed in Materials and Methods. Precipitated or input fractions were analyzed by immunoblot for NF-κB p65. (C) After 4 h of treatment as described in B, HUVEC were fixed and subjected to chromatin immunoprecipitation assay. DNA was extracted from precipitated or input fractions and underwent PCR amplification in the exponential phase using primers that were specific for E-selectin gene.

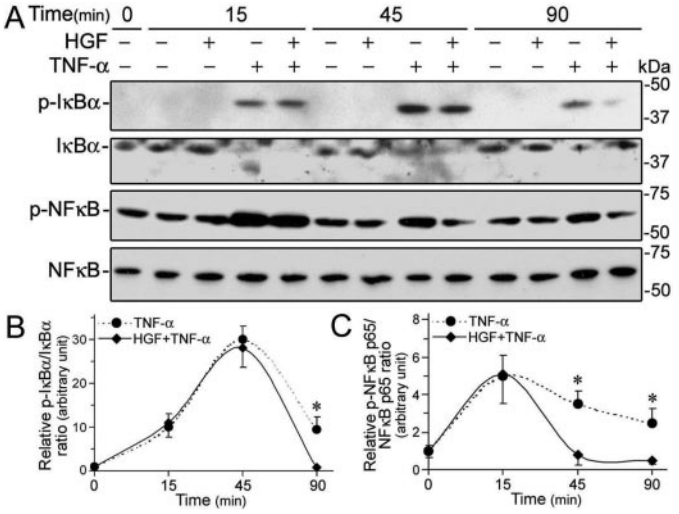

Figure 8. HGF intercepts NF-κB signal transduction in endothelial cells. (A) HUVEC were treated with HGF (100 ng/ml) and/or TNF-α (0.1 ng/ml) for various intervals. Cell lysates were subjected to immunoblot analysis for phosphorylated or total IκBα or NF-κB p65. (B and C) Arbitrary units of relative IκB or NF-κB activity expressed as the ratio of phosphorylated/total IκBα or NF-κB p65 as fold of nontreated cells at the same time points. $^*P < 0.05$ *versus* HGF treatment at the same time point ($n = 3$).

these findings suggest that HGF suppresses NF-κB-mediated TNF-α–induced E-selectin expression.

HGF Intercepts NF-κB Signal Transduction in Endothelial Cells

To further unravel the mechanism by which HGF suppresses NF-κB–meditated E-selectin expression, we examined NF-κB signal transduction (Figure 8A). As documented previously (37), TNF-α induced a rapid activation of the NF-κB pathway in HUVEC, as evidenced by phosphorylation of IκBα and NF-κB p65 as well as degradation of IκB. HGF had little effect at early time points; however, beginning at 45 min, HGF inhibited NF-κB p65 phosphorylation and, to a lesser extent, IκBα phosphorylation so that the restoration of IκB clearly was accelerated at 90 min. To quantify more accurately NF-κB signaling activity, we calculated densitometric ratios of phosphorylated to total IκBα (Figure 8B) and NF-κB (Figure 8C) and confirmed that HGF intercepts TNF-α–induced IκB and NF-κB activation at a late phase.

HGF Suppresses E-Selectin–Mediated Macrophage Sequestration in Rat Remnant Kidney

For examination of the functional consequences of HGF suppression of E-selectin *in vivo*, fluorescence-labeled RAM cells were infused into nephrectomized rats after 2 wk of HGF or vehicle administration. Shown in Figure 9A, a significant number of fluorescent RAM cells were observed in the tubulointerstitial area in vehicle-treated rats; most were adherent to the microvasculature endothelium with some migrating into the renal parenchyma (Figure 9C). In contrast, very few fluorescence cells were noted in the HGF-treated group (Figure 9B). Blocking E-selectin with a specific antibody (Figure 9E) also reduced RAM sequestration as compared with the group that was given preimmune IgG (Figure 9D). Fluorometric analysis of kidney homogenates corroborated the morphologic findings (Figure 9F). These data suggest that suppression of E-selectin contributes to HGF's inhibitory effect on inflammatory sequestration in CKD.

Discussion

Several recent studies suggest that HGF suppresses both acute and chronic inflammation in a variety of organ systems and disease models (18,19,23–31); however, the mechanism is still unclear. Our study demonstrates that in addition to suppressing overall inflammation, HGF modulates endothelial activation and inflammation in the remnant kidney, suppressing E-selectin expression and leukocyte adhesion. Moreover, this results from HGF's inhibitory effect on activation of NF-κB.

Renal inflammation is a complex but well-orchestrated response to injury that results in the migration of immune cells from the circulation to renal parenchyma (38). A large number of cellular and molecular components are involved in the genesis of an inflammatory infiltrate; however, attachment of leukocytes to endothelium, mediated by the selectin family of adhesion molecules, is the initial event in leukocyte extravasation (6–9). Upon endothelial activation, expression of E-selectin

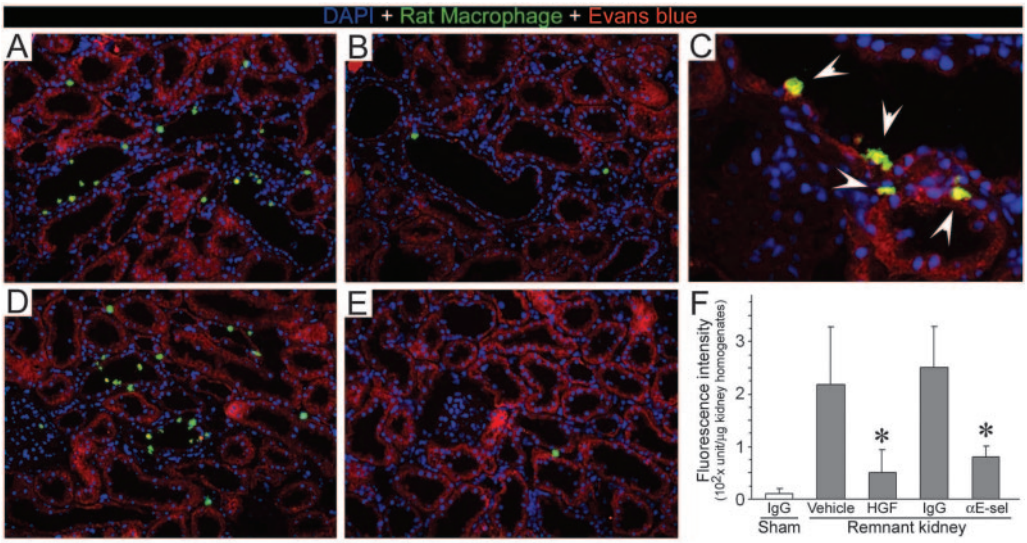

Figure 9. HGF suppresses E-selectin–mediated inflammatory cell sequestration in rat remnant kidney. (A through E) Fluorescence micrographs show overall sequestration (A, B, D, and E) and detailed adhesion and transmigration (C; arrowheads) of infused macrophages in rat remnant kidney. Two weeks after renal ablation, rats received vehicle (A and C through E) or HGF *via* a continuous minipump infusion (B) for another 2 wk. Then rats were infused with Calcein-AM–labeled rat macrophages. After 30 min, remnant kidneys were harvested. For estimation of the functional role of E-selectin, a specific anti–E-selectin blocking antibody (αE-sel; E) or preimmune IgG (D) was injected 4 h before macrophage infusion. Tissues were counterstained with DAPI and Evans blue. Sham-operated rats (Sham) were treated with vehicle and injected with preimmune IgG before macrophage infusion. (E) Aliquots of kidney homogenates from differently treated groups with an equal amount of total protein were subjected to fluorometry to quantify sequestered rat alveolar macrophages cells. $^*P < 0.05$ *versus* vehicle- or IgG-treated rats with remnant kidney disease ($n = 5$). Magnifications: ×100 in A, B, D, and E; ×400 in C.

is induced and is thought to be crucial in establishing rolling and stable arrest of leukocytes on the activated vascular endothelium early in the inflammatory response (10–15,39). In support of this, although both are expressed on the endothelium, E-selectin but not P-selectin is required for development of adjuvant-induced arthritis in the rat (40). In the kidney, an anti–E-selectin blocking antibody or E-selectin gene disruption protects mice from ischemia-reperfusion–induced acute renal failure (41). E-selectin also is considered to play a crucial role in the pathogenesis of chronic inflammation (42). For example, transgenic mice that produce a soluble E-selectin that competitively inhibits the binding of inflammatory cells to E-selectins on the endothelium are resistant to bleomycin-induced chronic pulmonary inflammation and lung fibrosis (43). Blocking E-selectin also decreases adventitial inflammation and intimal hyperplasia in the rat carotid artery after balloon injury (44). These findings suggest that maneuvers that suppress E-selectin are beneficial in both acute and chronic inflammatory diseases.

In this study, HGF inhibited TNF-α–induced expression of E-selectin in cultured endothelial cells in both a rescue and a preventive manner. *In vivo* in rat remnant kidneys, HGF infusion suppressed whereas HGF neutralization enhanced E-selectin expression in the renal vascular endothelium, and this was associated with a marked reduction in interstitial inflammation and injury. Functionally, an E-selectin blocking antibody mimicked the effects of HGF, reducing monocytic adhesion to endothelial monolayers that had been activated by TNF-α, as well as macrophage sequestration in the remnant kidney. These data suggest that the anti-inflammatory and renoprotective effects of HGF depend at least in part on suppression of endothelial E-selectin.

Our study also examined the mechanism by which HGF regulates E-selectin expression in endothelial cells. E-selectin is an NF-κB–dependent gene that is induced in response to a variety of proinflammatory cytokines (37). Sequence analysis reveals the presence of multiple putative κB elements in the promoter region of the E-selectin gene, and interaction between NF-κB and κB elements in the E-selectin promoter is required for E-selectin expression (45). Previously, we reported that HGF suppressed NF-κB activation in tubular epithelial cells *in vivo* in the remnant kidney (18). Reasoning that HGF also might suppress E-selectin *via* the NF-κB pathway, we examined the effects of HGF on multiple aspects of the regulation of NF-κB–mediated E-selectin expression in endothelial cells. We found that HGF modulated TNF-α–induced activation of this pathway at multiple points. First, HGF counteracted NF-κB–dependent transcriptional activity that was induced by TNF-α. Second, binding of NF-κB to an exogenous oligonucleotide that bore the consensus κB elements was overridden by HGF. Third, HGF regulated NF-κB binding to κB elements *in situ* on the E-selectin promoter level. In-depth dissection of the NF-κB signaling pathway showed that HGF suppressed NF-κB activity primarily by intercepting phosphorylation of IκB and NF-κB and inhibiting IκB degradation.

The finding that inhibition of NF-κB signal transduction by HGF peaks only after some time of exposure suggests that it

might be a secondary event. This finding is partly consistent with Min *et al.* (46), who reported that HGF had barely discernible effects on IκBα phosphorylation in HUVEC after TNF-α or IL-1β stimulation for 10 min, but they failed to make observations at later time points. Similarly, in our study of renal tubular epithelial cells (18), HGF suppression of NF-κB activity as assessed by nuclear translocation also occurred at a late phase. The identity of the transducer that mediates this HGF suppression of NF-κB is unknown at present and merits further study.

Conclusion

We have found that exogenous HGF ameliorates whereas blocking endogenous HGF worsens progressive chronic renal inflammation in rat remnant kidney disease. This is accompanied by parallel alternations in endothelial activation, marked by *de novo* E-selectin expression in renal vascular endothelium and leukocyte adhesion to the inflamed endothelium. The inhibitory effect of HGF on endothelial E-selectin expression is mediated by NF-κB inhibition. Our findings suggest that HGF's renoprotective effect is attributable, at least in part, to potent and direct anti-inflammatory activity suppressing endothelial inflammation *via* NF-κB.

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References

1. Remuzzi G, Bertani T: Pathophysiology of progressive nephropathies. *N Engl J Med* 339: 1448–1456, 1998
2. Eddy AA: Molecular basis of renal fibrosis. *Pediatr Nephrol* 15: 290–301, 2000
3. Eddy AA: Progression in chronic kidney disease. *Adv Chronic Kidney Dis* 12: 353–365, 2005
4. Eddy AA: Interstitial macrophages as mediators of renal fibrosis. *Exp Nephrol* 3: 76–79, 1995
5. Sean Eardley K, Cockwell P: Macrophages and progressive tubulointerstitial disease. *Kidney Int* 68: 437–455, 2005
6. Briscoe DM, Cotran RS: Role of leukocyte-endothelial cell adhesion molecules in renal inflammation: In vitro and in vivo studies. *Kidney Int Suppl* 42: S27–S34, 1993
7. Brady HR: Leukocyte adhesion molecules: Potential targets for therapeutic intervention in kidney diseases. *Curr Opin Nephrol Hypertens* 2: 171–182, 1993
8. Takano T, Brady HR: The endothelium in glomerular inflammation. *Curr Opin Nephrol Hypertens* 4: 277–286, 1995
9. Stenvinkel P: Endothelial dysfunction and inflammation: Is there a link? *Nephrol Dial Transplant* 16: 1968–1971, 2001
10. Luscinskas FW, Gimbrone MA Jr: Endothelial-dependent mechanisms in chronic inflammatory leukocyte recruitment. *Annu Rev Med* 47: 413–421, 1996
11. Carlos TM, Harlan JM: Leukocyte-endothelial adhesion molecules. *Blood* 84: 2068–2101, 1994
12. Bevilacqua MP, Nelson RM, Mannori G, Cecconi O: Endothelial-leukocyte adhesion molecules in human disease. *Annu Rev Med* 45: 361–378, 1994
13. Kevil CG: Endothelial cell activation in inflammation: Lessons from mutant mouse models. *Pathophysiology* 9: 63–74, 2003
14. Nathan C: Points of control in inflammation. *Nature* 420: 846–852, 2002
15. Ley K: The role of selectins in inflammation and disease. *Trends Mol Med* 9: 263–268, 2003
16. Gong R, Rifai A, Tolbert EM, Centracchio JN, Dworkin LD: Hepatocyte growth factor modulates matrix metalloproteinases and plasminogen activator/plasmin proteolytic pathways in progressive renal interstitial fibrosis. *J Am Soc Nephrol* 14: 3047–3060, 2003
17. Dworkin LD, Gong R, Tolbert E, Centracchio J, Yano N, Zanabli A, Esparza A, Rifai A: Hepatocyte growth factor ameliorates progression of interstitial fibrosis in rats with established renal injury. *Kidney Int* 65: 409–419, 2004
18. Gong R, Rifai A, Tolbert EM, Centracchio JN, Bisawas P, Dworkin LD: Hepatocyte growth factor ameliorates renal interstitial inflammation in rat remnant kidney by modulating tubular expression of MCP-1 and RANTES. *J Am Soc Nephrol* 15: 2868–2881, 2004
19. Tanaka T, Ichimaru N, Takahara S, Yazawa K, Hatori M, Suzuki K, Isaka Y, Moriyama T, Imai E, Azuma H, Nakamura T, Okuyama A, Yamanaka H: In vivo gene transfer of hepatocyte growth factor to skeletal muscle prevents changes in rat kidneys after 5/6 nephrectomy. *Am J Transplant* 2: 828–836, 2002
20. Mizuno S, Matsumoto K, Nakamura T: Hepatocyte growth factor suppresses interstitial fibrosis in a mouse model of obstructive nephropathy. *Kidney Int* 59: 1304–1314, 2001
21. Yang J, Liu Y: Blockage of tubular epithelial to myofibroblast transition by hepatocyte growth factor prevents renal interstitial fibrosis. *J Am Soc Nephrol* 13: 96–107, 2002
22. Mizuno S, Nakamura T: Suppressions of chronic glomerular injuries and TGF-beta1 production by HGF in attenuation of murine diabetic nephropathy. *Am J Physiol Renal Physiol* 286: F134–F143, 2004
23. Molnar C, Garcia-Trevijano ER, Ludwiczek O, Talabot D, Kaser A, Mato JM, Fritsche G, Weiss G, Gabay C, Avila MA, Tilg H: Anti-inflammatory effects of hepatocyte growth factor: Induction of interleukin-1 receptor antagonist. *Eur Cytokine Netw* 15: 303–311, 2004
24. Gong R, Rifai A, Dworkin LD: Activation of PI3K-Akt-GSK3beta pathway mediates hepatocyte growth factor inhibition of RANTES expression in renal tubular epithelial cells. *Biochem Biophys Res Commun* 330: 27–33, 2005
25. Arthur LG, Kuenzler KA, Schwartz MZ: Hepatocyte growth factor ameliorates inflammatory bowel disease in a rat model. *J Gastrointest Surg* 7: 1062–1068, 2003
26. Arthur LG, Schwartz MZ, Kuenzler KA, Birbe R: Hepatocyte growth factor treatment ameliorates diarrhea and bowel inflammation in a rat model of inflammatory bowel disease. *J Pediatr Surg* 39: 139–143, 2004
27. Oh K, Iimuro Y, Takeuchi M, Kaneda Y, Iwasaki T, Terada N, Matsumoto T, Nakanishi K, Fujimoto J: Ameliorating effect of hepatocyte growth factor on inflammatory bowel disease in a murine model. *Am J Physiol Gastrointest Liver Physiol* 288: G729–G735, 2005
28. Ohda Y, Hori K, Tomita T, Hida N, Kosaka T, Fukuda Y, Miwa H, Matsumoto T: Effects of hepatocyte growth factor on rat inflammatory bowel disease models. *Dig Dis Sci* 50: 914–921, 2005

29. Numata M, Ido A, Moriuchi A, Kim I, Tahara Y, Yamamoto S, Hasuike S, Nagata K, Miyata Y, Uto H, Tsubouchi H: Hepatocyte growth factor facilitates the repair of large colonic ulcers in 2,4,6-trinitrobenzene sulfonic acid-induced colitis in rats. *Inflamm Bowel Dis* 11: 551–558, 2005
30. Hanawa T, Suzuki K, Kawauchi Y, Takamura M, Yoneyama H, Han GD, Kawachi H, Shimizu F, Asakura H, Miyazaki JI, Maruyama H, Aoyagi Y: Attenuation of mouse acute colitis by naked hepatocyte growth factor gene transfer into the liver. *J Gene Med* 8: 623–635, 2006
31. Ito W, Kanehiro A, Matsumoto K, Hirano A, Ono K, Maruyama H, Kataoka M, Nakamura T, Gelfand EW, Tanimoto M: Hepatocyte growth factor attenuates airway hyperresponsiveness, inflammation, and remodeling. *Am J Respir Cell Mol Biol* 32: 268–280, 2005
32. Matsui K, Nagy-Bojarsky K, Laakkonen P, Krieger S, Mechtler K, Uchida S, Geleff S, Kang DH, Johnson RJ, Kerjaschki D: Lymphatic microvessels in the rat remnant kidney model of renal fibrosis: Aminopeptidase p and podoplanin are discriminatory markers for endothelial cells of blood and lymphatic vessels. *J Am Soc Nephrol* 14: 1981–1989, 2003
33. Martens CL, Cwirla SE, Lee RY, Whitehorn E, Chen EY, Bakker A, Martin EL, Wagstrom C, Gopalan P, Smith CW, Tate E, Koller KJ, Schatz PJ, Dower WJ, Barrett RW: Peptides which bind to E-selectin and block neutrophil adhesion. *J Biol Chem* 270: 21129–21136, 1995
34. Saeki K, Yuo A, Suzuki E, Yazaki Y, Takaku F: Aberrant expression of cAMP-response-element-binding protein ('CREB') induces apoptosis. *Biochem J* 343: 249–255, 1999
35. Pan F, Ye Z, Cheng L, Liu JO: Myocyte enhancer factor 2 mediates calcium-dependent transcription of the interleukin-2 gene in T lymphocytes: A calcium signaling module that is distinct from but collaborates with the nuclear factor of activated T cells (NFAT). *J Biol Chem* 279: 14477–14480, 2004
36. Guijarro C, Egido J: Transcription factor-kappaB (NF-kappaB) and renal disease. *Kidney Int* 59: 415–424, 2001
37. Boyle EM Jr, Kovacich JC, Canty TG Jr, Morgan EN, Chi E, Verrier ED, Pohlman TH: Inhibition of nuclear factor-kappaB nuclear localization reduces human E-selectin expression and the systemic inflammatory response. *Circulation* 98: II282–II288, 1998
38. Baud L, Fouqueray B, Bellocq A: Switching off renal inflammation by anti-inflammatory mediators: The facts, the promise and the hope. *Kidney Int* 53: 1118–1126, 1998
39. Bevilacqua MP, Nelson RM. Selectins. *J Clin Invest* 91: 379–387, 1993
40. Issekutz AC, Mu JY, Liu G, Melrose J, Berg EL: E-selectin, but not P-selectin, is required for development of adjuvant-induced arthritis in the rat. *Arthritis Rheum* 44: 1428–1437, 2001
41. Singbartl K, Ley K: Protection from ischemia-reperfusion induced severe acute renal failure by blocking E-selectin. *Crit Care Med* 28: 2507–2514, 2000
42. Welply JK, Keene JL, Schmuke JJ, Howard SC: Selectins as potential targets of therapeutic intervention in inflammatory diseases. *Biochim Biophys Acta* 1197: 215–226, 1994
43. Azuma A, Takahashi S, Nose M, Araki K, Araki M, Takahashi T, Hirose M, Kawashima H, Miyasaka M, Kudoh S: Role of E-selectin in bleomycin induced lung fibrosis in mice. *Thorax* 55: 147–152, 2000
44. Gotoh R, Suzuki J, Kosuge H, Kakuta T, Sakamoto S, Yoshida M, Isobe M: E-selectin blockade decreases adventitial inflammation and attenuates intimal hyperplasia in rat carotid arteries after balloon injury. *Arterioscler Thromb Vasc Biol* 24: 2063–2068, 2004
45. Schindler U, Baichwal VR: Three NF-kappaB binding sites in the human E-selectin gene required for maximal tumor necrosis factor alpha-induced expression. *Mol Cell Biol* 14: 5820–5831, 1994
46. Min JK, Lee YM, Kim JH, Kim YM, Kim SW, Lee SY, Gho YS, Oh GT, Kwon YG: Hepatocyte growth factor suppresses vascular endothelial growth factor-induced expression of endothelial ICAM-1 and VCAM-1 by inhibiting the nuclear factor-kappaB pathway. *Circ Res* 96: 300–307, 2005