

# Protein Overload Induces Fractalkine Upregulation in Proximal Tubular Cells through Nuclear Factor $\kappa$ B– and p38 Mitogen-Activated Protein Kinase–Dependent Pathways

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**Abstract.** Investigated was the effect of high albumin concentrations on proximal tubular cell expression of fractalkine. Human proximal tubular cells (HK-2) were incubated with human serum albumin (HSA), which induced a dose-dependent increase in fractalkine mRNA associated with increased levels of both membrane-bound and soluble forms of the protein. To evaluate the role of nuclear factor  $\kappa$ B (NF- $\kappa$ B) activation in HSA-induced fractalkine mRNA, HK-2 cells were infected with a recombinant adenovirus encoding the natural inhibitor of NF- $\kappa$ B, I $\kappa$ B $\alpha$ ; a 43% reduction of fractalkine mRNA levels resulted. Similarly, when cells were infected with the recombinant adenovirus expressing dominant negative mutant of the I $\kappa$ B kinase 2, a 55% inhibition of

fractalkine mRNA was achieved. p38 mitogen-activated protein kinase was activated by HSA and was involved in NF- $\kappa$ B–dependent transcription of fractalkine. In kidneys of mice with bovine serum albumin overload proteinuria, fractalkine mRNA levels were 2.3-fold greater than those of controls. Fractalkine expression was also induced in tubular epithelial cells in this model. Anti-CXCR1 antibody treatment limited interstitial accumulation of mononuclear cells. Protein overload is a promoter of fractalkine gene induction mediated by NF- $\kappa$ B and p38 activation in proximal tubular cells. Fractalkine might contribute to direct mononuclear cells into peritubular interstitium and enhance their adhesion property, which in turn would favor inflammation and disease progression.

Chronic kidney diseases are increasing at an alarming rate worldwide and are emerging as a public health problem, such that the provision of renal replacement therapy absorbs a considerable amount of health care budgets. Progressive nephropathies are characterized by both a highly enhanced glomerular permeability to proteins, in turn leading to proteinuria, and concomitant tubulointerstitial damage (1). Among cellular mechanisms that may underlie interstitial inflammation, it has been suggested that abnormally filtered proteins may have intrinsic renal toxicity and may serve as an early fosterer in tubular cells by activating the synthesis of vasoactive and proinflammatory substances (2). Thus, proximal tubular cells in culture were induced by exposure to high plasma protein concentrations to upregulate and release endothelin 1 (3) and chemokines such as monocyte chemoattractant protein 1 (MCP-1) (4,5), RANTES (regulated upon activation, normal T cell expressed and secreted) (6), and IL-8 (7) into the basolat-

eral medium, a polarized type of secretion that *in vivo* would promote monocyte and lymphocyte recruitment into the renal interstitium. Like many other proinflammatory genes, MCP-1 and RANTES genes are controlled by the transcription factor nuclear factor  $\kappa$ B (NF- $\kappa$ B) (8). In resting cells, NF- $\kappa$ B exists in an inactive form in the cytoplasm bound to the inhibitory protein I $\kappa$ B $\alpha$ . Upon cell activation by stimuli, I $\kappa$ B is phosphorylated by the I $\kappa$ B kinase (IKK) complex, ubiquitinated, and degraded, allowing NF- $\kappa$ B translocation for binding to DNA motifs in gene promoters (9,10). In proximal tubular cells, albumin and IgG activated NF- $\kappa$ B in a dose-dependent manner, followed by the upregulation of MCP-1 and RANTES, which was suppressed by NF- $\kappa$ B inhibitors (5,6,11). Evidence in chronic nephropathy induced by renal mass reduction and in passive Heymann nephritis in rats revealed that excess uptake of plasma proteins in proximal tubular cells precedes because the early stage is subsequently associated with an inflammatory reaction (12). Proteinuria over time is also associated with a remarkable increase in NF- $\kappa$ B activity in proximal tubular cells, paralleled by upregulation of renal MCP-1 mRNA, heralding the local accumulation of monocytes, macrophages, and T cells (13).

Fractalkine (CX3CL1) displays differential properties compared with other chemokines described so far. It contains a chemokine domain with three amino acids between the first two cysteine residues (CX3C), followed by an extended mu-

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cinlike stalk, a transmembrane domain, and an intracellular domain (14,15). Fractalkine can exist in two forms: it can be membrane anchored, or it can shed soluble glycoprotein upon proteolytic cleavage from the membrane (14). Thus, in addition to functioning as chemoattractant, fractalkine appears to behave as a unique inducer of firm adhesion for cells expressing its receptor CX3CR1, including monocytes, T lymphocytes, and natural killer cells (16,17). Unlike most chemokines, fractalkine is not synthesized by leukocytes (14).

The emerging role of fractalkine in renal inflammation is credited by experimental and clinical data. Fractalkine mRNA was upregulated in the glomerular endothelium of rats with anti-GBM glomerulonephritis, whereas leukocytes expressed high levels of CX3CR1 and anti-CX3CR1 antibodies blocked leukocyte infiltration and attenuated injury (18). Fractalkine was overexpressed in interstitial areas in patients with glomerulonephritis in correlation with the degree of lymphocyte infiltration (19). Tubular epithelial cells can also be induced to express fractalkine, as revealed by the strong signal of fractalkine mRNA in proximal tubuli in kidneys of patients with allograft rejection (20). Moreover, *in vitro*, upon TNF- $\alpha$  stimulation, proximal tubular cells expressed fractalkine that supported adhesion of CX3CR1-positive monocytes and natural killer cells, suggesting a role for fractalkine in leukocyte adhesion at tubular sites (21).

Here we approached the hypothesis that fractalkine could be upregulated in proximal tubular cells as a consequence of albumin load, contributing to interstitial inflammation. Specifically, we assessed *in vitro* whether albumin may stimulate fractalkine expression in human proximal tubular cells; and we assessed whether such induction was dependent on NF- $\kappa$ B. We then investigated whether signaling by p38 mitogen-activated protein kinase (MAPK), one of the major transduction pathways in inflammation (22,23), was required for NF- $\kappa$ B dependent fractalkine expression. To evaluate the relevance in disease, we determined the renal expression of fractalkine mRNA and protein, and the effect of functional blockade of the CX3CR1 receptor on mononuclear cell trafficking in a mouse model of protein overload proteinuria.

## Materials and Methods

### Cell Culture and Incubation

HK-2 cells are a permanent, well characterized human proximal tubular cell line (24) and were obtained from the American Type Culture Collection (Rockville, MD). They were grown in DMEM/F-12 plus 5% FCS supplemented with L-glutamine (2 mmol/L), penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), 3,3',5-triiodo-L-thyronine (5 pg/ml), hydrocortisone (5 ng/ml), prostaglandin E1 (5 pg/ml), epidermal growth factor (10 ng/ml), insulin (5  $\mu$ g/ml), transferrin (2.5  $\mu$ g/ml), and sodium selenite (3.3 ng/ml). For experiments, cells were preincubated with serum-free DMEM/F-12 for 24 h.

To investigate the effect of protein overload on fractalkine mRNA expression, confluent HK-2 were exposed for 6, 15, 24, and 48 h to medium alone (control) or 10 mg/ml of human serum albumin (HSA, A5843; Sigma Chemical, St. Louis, MO; endotoxin content <0.1 ng/mg by Limulus Amebocyte test). For dose-response experiments, the cells were incubated with HSA (1, 5, and 10 mg/ml) for 15 h. At

the end of the incubation, total RNA was obtained for Northern blot analysis.

Fractalkine protein expression was evaluated by Western blot analysis both in cell lysates from HK-2 cells treated with HSA (10 mg/ml) for 24 h and in the supernatant of cells exposed to HSA for 15, 24, 36, and 48 h. Fractalkine expression in HK-2 cells was also studied by fluorescence confocal microscopy at 24 h.

To study intracellular signaling pathways that regulate fractalkine gene transcription in HK-2 cells loaded with HSA, we assessed the potential role of NF- $\kappa$ B first by determining NF- $\kappa$ B activity in nuclear extracts from HK-2 cells treated with HSA (10 mg/ml) for 30 min, in the presence or absence of the NF- $\kappa$ B inhibitor pyrrolidine dithiocarbamate (PDTC, 25  $\mu$ mol/L, Sigma) (5) added 1 h before HSA; and second, by evaluating the effect of NF- $\kappa$ B inhibitor on fractalkine mRNA expression. Cells were treated with PDTC (25  $\mu$ mol/L) 1 h before and during incubation with HSA (10 mg/ml, 15 h); in this experimental condition, PDTC had no toxic effect, as evaluated by viable cell counts by Trypan blue dye exclusion (control,  $1.9 \times 10^6$  cells; PDTC,  $2.1 \times 10^6$  cells; HSA,  $2 \times 10^6$  cells; HSA + PDTC,  $2 \times 10^6$  cells). Third, we transfected HK-2 cells with a recombinant adenovirus coding for I $\kappa$ B $\alpha$ , the natural inhibitor of NF- $\kappa$ B (25), or a dominant negative mutant of the I $\kappa$ B kinase 2 (IKK2), a kinase that acts as an upstream activator of NF- $\kappa$ B (26). To elucidate the role of p38 MAPK in HSA-induced NF- $\kappa$ B activation and subsequent fractalkine expression, first, phosphorylation of p38 MAPK was assessed by Western blot in HK-2 cells treated with HSA (10 mg/ml) for 15 min, 30 min, 3 h, and 6 h. Second, the effect of the p38 inhibitor SB202190 (20  $\mu$ mol/L, Calbiochem-Novabiochemical, La Jolla, CA) (27) was evaluated in HK-2 cells transfected with NF- $\kappa$ B luciferase reporter gene and loaded with HSA (15 h). Third, the effect of blockade of p38 function on fractalkine gene and protein expression was studied in cells exposed to HSA in the presence of the p38 inhibitor. SB202190 is effective and specific as chemical inhibitor of the catalytic activity of p38 MAPK by competitive binding to the ATP pocket in p38 (28–30).

### Northern Blot Analysis

Total RNA was isolated from HK-2 cells or from whole murine kidney tissue by the guanidinium isothiocyanate/cesium chloride procedure. Total RNA (10  $\mu$ g for cells and 20  $\mu$ g for murine kidney) was then fractionated on 1.2% or 1.6% agarose gel and blotted onto synthetic membranes (Zeta-probe; Bio-Rad, Richmond, CA). Human and rat fractalkine cDNA probes were cloned by PCR by the following oligonucleotides: sense primer 5'-CCTGGATGCAGCCTCAGT, antisense primer 5'-GGACAATCCAAGGGAGAGGTG for the human probe (0.7 kb); sense primer 5'-CCTCGTGTCTCCTG-GCTTTG, antisense primer 5'-GATCCTGGCAGCTTCTGAATG for the rat probe (0.8 kb). The resulting PCR products were labeled with  $\alpha$ - $^{32}$ P dCTP by random-primed method. Hybridization was performed overnight at 65°C in 0.25 mol/L Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2, 7% SDS. Filters were washed twice for 30 min with 20 mmol/L Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2, 5% SDS and two times for 10 min with 20 mmol/L Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2, 1% SDS at 65°C. Membranes were subsequently probed with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, taken as internal standard of equal loading of the samples on the membrane. Fractalkine mRNA optical density was normalized to that of the constitutively released GAPDH gene expression.

### Western Blot Analysis

HK-2 cells were lysed in lysis buffer (Tris-HCl 0.02 mol/L, pH 7.5, NaCl 0.15 mol/L, EDTA 5 mmol/L, NP40 0.5%) with protease

inhibitor cocktail tablets (Boehringer Mannheim, Mannheim, Germany). Protein concentration was determined by the Bradford assay with the Bio-Rad protein assay reagent. Proteins were separated on 7.5% to 10% (fractalkine) or 12% (p38) polyacrylamide gels by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked overnight at 4°C with PBS containing 5% BSA, 1 to 3% rabbit serum, and 0.05% Tween20. The blots were incubated for 1 h in PBS containing 1% BSA and 0.05% Tween20 with the following primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA): goat polyclonal antibody against a peptide mapping to the chemokine domain of fractalkine (1:200, C-18), mouse monoclonal IgM antibody p-p38 (1:400), and mouse monoclonal IgG antibody p38 (1:200). After incubation with secondary antibodies (horseradish peroxidase-conjugated rabbit anti-mouse IgG or rabbit anti-goat IgG) for 1 h in PBS 1× and 0.05% Tween20, protein bands were detected by Super-Signal chemiluminescent substrate (Pierce, Milan, Italy). As positive control, 40 ng of purified recombinant human fractalkine (R&D Systems, Minneapolis, MN, obtained in a mouse myeloma cell line) were loaded onto gel; whole-cell lysate of NIH/3T3 heat-shocked cells (Santa Cruz Biotechnology) was used as standard control of p-p38 signal. For detection of cleaved soluble fractalkine, supernatants of HSA treated HK-2 cells were concentrated with Centricon-10 (Millipore, Bedford, MA).

#### *Immunofluorescence Staining for the Detection of Fractalkine in HK-2 Cells*

HK-2 cells grown to 80% confluence on glass coverslips were fixed in 3% paraformaldehyde plus 2% sucrose in PBS pH 7.4 for 20 min at 37°C. The cells were washed and rinsed with PBS, and nonspecific binding sites were saturated in blocking solution (2% FCS, 2% BSA in PBS) for 1 h. The cells were incubated with the primary anti-fractalkine antibody for 2 h at room temperature, washed, and then incubated with FITC-conjugated rabbit anti-goat IgG antibody for 1 h at room temperature. Negative control experiments with secondary antibody FITC-conjugated rabbit anti-goat IgG alone were performed. Coverslips were washed, mounted in 1% *N*-propyl-gallate in 50% glycerol, 0.1 mol/L Tris-HCl, pH 8, and examined under confocal inverted laser microscopy (Insight Plus, Meridian Instruments, Okemos, MI). Representative fields were digitized with millions of colors and printed.

#### *Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay*

Nuclear extracts were prepared from HK-2 cells with the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce/Celbio, Pero, Italy) according to the manufacturer's instructions. To minimize proteolysis, all buffers contained protease inhibitor cocktail (Boehringer Mannheim). The protein concentration was determined by the Bradford assay with the Bio-Rad protein assay reagent.

Electrophoretic mobility shift assays (EMSAs) were performed as described previously (5,6) with the kB DNA sequence of the Ig gene (5'-CCGGTCAGAGGGGACTTTCGAGACT; the core kB sequence is underlined). Nuclear extracts (2 µg) were incubated with 50 kcpm of <sup>32</sup>P-labeled NF-κB oligonucleotide in a binding reaction mixture (10 mmol/L Tris-HCl pH 7.5, 80 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 5% glycerol, 1.5 µg of poly(dI-dC)) for 30 min on ice. In competition studies, a 100-fold molar excess of unlabeled oligonucleotide was added to the binding reaction mixture as indicated, before the addition of the labeled kB probe.

#### *Overexpression of Adenovirally Encoded IκBα and dnIKK2 in HK-2 Cells*

Subconfluent HK-2 cells were incubated with recombinant adenovirus coding for IκBα or dnIKK2 (the gift of R. de Martin, Department of Vascular Biology and Thrombosis Research, University of Vienna) (25,26) or with a control adenovirus (Ad null control) at a multiplicity of infection of 200 in DMEM/F-12 without serum for 3 h at 37°C. The adenovirus was washed off, and cells were maintained in fresh medium without serum for 24 h. Then cells were exposed to 10 mg/ml HSA for additional 15 h and processed for fractalkine mRNA expression (Northern blot analysis). Transfection did not affect cell viability.

The inhibitory effects of both constructs on NF-κB activity was verified by EMSA experiments performed in nuclear extracts from HK-2 cells transfected for 3 h with recombinant adenovirus coding for IκBα or dnIKK2, followed by 2 h in fresh medium and then stimulated for 30 min with TNF-α (100 U/ml), a potent inducer of NF-κB activation. Densitometric analysis of NF-κB complexes indicated that the constructs inhibited NF-κB DNA binding activity by 68% and 71%, respectively.

#### *Transient Transfection and Reporter Luciferase Gene Assay*

Subconfluent HK-2 cells were transfected with 1 µg NF-κB luciferase reporter gene (Stratagene; M-Medical, Florence, Italy) by the Superfect transfection reagent following manufacturer's protocol (Quiagen, Milan, Italy). Three hours after transfection, the reporter gene was washed off and cells were maintained in fresh medium without serum for 24 h. Then cells were exposed to 10 mg/ml HSA for additional 15 h. The p38 inhibitor SB202190 (27) was added 1 h before and during stimulation with HSA. Cells were subsequently lysed in 1× reporter lysis buffer for 15 min at room temperature. The lysates were cleared by centrifugation. The luciferase activity was measured according to standard protocols (Stratagene; M-Medical), with a Lumat LB9507 luminometer (EG&G Berthold, Bad Wildbad, Germany). Induced luciferase activities were normalized on the basis of protein contents and expressed as fold stimulation compared with unstimulated controls.

#### *Murine Model of Protein-Overload Proteinuria*

BSA-overload proteinuria was induced in C57BL/6 × 129 mice (Charles River Italia, Calco, Italy) weighing 20 to 22 g at the beginning of the study. Animal care and treatment were conducted in accordance with the institutional guidelines that are in compliance with national (Decreto Legislativo n.116, Gazzetta Ufficiale suppl 40, February 18, 1992, Circolare n.8, Gazzetta Ufficiale 14 July 1994) and international laws and policies (EEC Council Directive 86/609, OJL358-1, December 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). Animals were housed in a constant temperature room with a 12-h dark/12-h light cycle and were fed a standard diet. Mice were uninephrectomized under anesthesia 5 d before BSA injections were initiated (31). Low-endotoxin BSA (Sigma A-9430) was dissolved in saline and given 5 days a week intraperitoneally at the dosage of 10 mg/g body weight for 4 wk. Control mice received the same volume of saline. Renal fractalkine mRNA and protein expression were evaluated in 3 mice with overload proteinuria and 3 controls at 4 wk. Additional mice with overload proteinuria were used to study whether treatment with anti-fractalkine receptor antibody could reduce the accumulation of monocytes or macrophages into the renal interstitium. Beginning on day 1 of BSA injection, uninephrectomized mice received daily intraperito-

neally injections of 100  $\mu$ l (2  $\mu$ g) of polyclonal rabbit anti-rat CX3CR1 antibody (Torrey Pines Biolabs, San Diego, CA) (32) ( $n = 4$ ), saline ( $n = 4$ ), or control rabbit serum ( $n = 3$ ) for up to 4 wk.

Twenty-four-hour urine samples were collected in metabolic cages and proteinuria was determined by modified Coomassie blue G dye-binding assay for proteins with bovine serum albumin as standard (33). Renal function was assessed as BUN and creatinine in serum samples with an enzymatic ultraviolet rate device (Sincron CX-5; Beckman, Fullerton, CA).

### Renal Histology

The removed kidneys were fixed overnight in Dubosq-Brazil, dehydrated in alcohol, and embedded in paraffin. Kidney samples were sectioned at 3- $\mu$ m intervals, and the sections were stained with Masson trichrome, hematoxylin and eosin, and periodic acid–Schiff reagent. Tubular (atrophy, casts, and dilation) and interstitial changes (fibrosis and inflammation) were graded from 0 to 4+ (0, no change; 1+, changes affecting less than 25% of the sample; 2+, changes affecting 25% to 50% of the sample; 3+, changes affecting 50% to 75% of the sample; and 4+, changes affecting 75% to 100% of the sample). At least 100 glomeruli were examined for each animal, and the extent of glomerular damage was expressed as the percentage of glomeruli presenting sclerotic lesions. All renal biopsy samples were analyzed by the same pathologist, who was unaware of the nature of the experimental groups.

### Immunohistochemical Analysis

Renal tissue was fixed overnight in Dubosq Brazil, dehydrated in alcohol, and embedded in paraffin. Paraffin was removed for 3- $\mu$ m sections, and the samples were taken through graded alcohols to PBS. Fractalkine staining was detected by alkaline phosphatase–Vector Red technique. Sections were incubated in bovine trypsin at 37°C for 30 min, washed in PBS, blocked with 1% PBS-BSA, and incubated overnight at 4°C with primary antibody (goat anti-mouse fractalkine C-20, 20  $\mu$ g/ml; Santa Cruz). After washes in PBS, they were incubated with biotinylated horse anti-goat IgG (10  $\mu$ g/ml; Vector Laboratories, Burlingame, CA) for 30 min at room temperature. Then the sections were incubated with streptavidin-alkaline phosphatase conjugate (1:500; Roche Diagnostic, Monza, Italy) for 30 min at room temperature. After washes in PBS, Vector Red substrate (Vector Laboratories; SK 5100) was applied for 20 min at room temperature.

Rat monoclonal antibody against a cytoplasmic antigen present in mouse monocytes and macrophages (F4/80, 5  $\mu$ g/ml; Serotec, Oxford, UK) was used for the detection of infiltrating cells by immunoperoxidase technique. Sections were incubated for 30 min with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol to quench endogenous peroxidase. Then the tissue was permeabilized in 0.1% Triton X-100 in PBS 0.01 mol/L, pH 7.2, for 30 min and then incubated with normal goat serum (Vector Laboratories) for 30 min. Primary antibody was incubated overnight at 4°C, followed by the secondary antibody (biotinylated goat anti-rat IgG; Vector Laboratories) and avidin-biotin peroxidase complex solution, and finally development with DAB. The sections were counterstained with Harris hematoxylin. Negative controls were obtained by omitting the primary antibody. F40/80-labeled cells were counted in at least 10 randomly selected high-power microscopic fields ( $\times$ 400) per each animal.

### Statistical Analyses

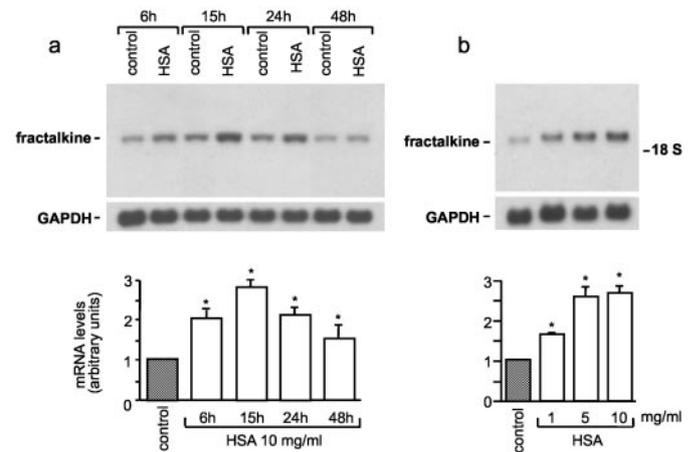
The results are expressed as mean  $\pm$  SEM. Statistical analysis was performed by ANOVA, followed by the nonparametric Kruskal-

Wallis test for multiple comparisons. Statistical significance level was defined as  $P < 0.05$ .

## Results

### Albumin Upregulates Fractalkine mRNA in Human Proximal Tubular Cells

We studied the ability of albumin to modulate the expression of fractalkine mRNA in human proximal tubular HK-2 cells. Cells were incubated for 6, 15, 24, and 48 h with serum-free medium containing 10 mg/ml HSA (5). As shown in Figure 1a, a single 3.3-kb fractalkine mRNA transcript of low intensity was detected in unstimulated HK-2 cells used as control. Fractalkine mRNA expression in unstimulated cells slightly increased from 6 to 48 h. Albumin promoted an increase in message levels at 6 h, which peaked at 15 h, remained elevated at 24 h, and declined after 48 h (2.0-, 2.8-, 2.1-, and 1.5-fold increase over control of corresponding time). The effect of increasing concentrations of HSA (1, 5, and 10 mg/ml, 15 h) on fractalkine mRNA is depicted in Figure 1b. A 1.7-fold increase in transcript levels was observed with 1 mg/ml HSA and a further increase with 5 and 10 mg/ml (2.6- and 2.7-fold, respectively).



**Figure 1.** Effect of albumin on fractalkine mRNA expression in HK-2 cells. (a) Time course. (top) Northern blot experiments were performed with total RNA isolated from HK-2 cells exposed to medium alone (control) or human serum albumin (HSA) (10 mg/ml) for 6, 15, 24, and 48 h. Data shown are representative of five experiments. (bottom) Densitometric analysis of autoradiographic signals for fractalkine. The optical density of autoradiographic signals was quantified and calculated as the ratio of fractalkine to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Results (mean  $\pm$  SEM) are expressed as fold increase over control (considered as 1) of corresponding time. \* $P < 0.01$  versus control. (b) Dose response. (top) Total RNA isolated after a 15-h period of culture with medium alone (control) or HSA (1, 5, and 10 mg/ml). Data shown are representative of five experiments. (bottom) Fractalkine mRNA expression was analyzed after normalization for GAPDH expression. Results (mean  $\pm$  SEM) are expressed as fold increase over control (considered as 1) in densitometric arbitrary units. \* $P < 0.01$  versus control.

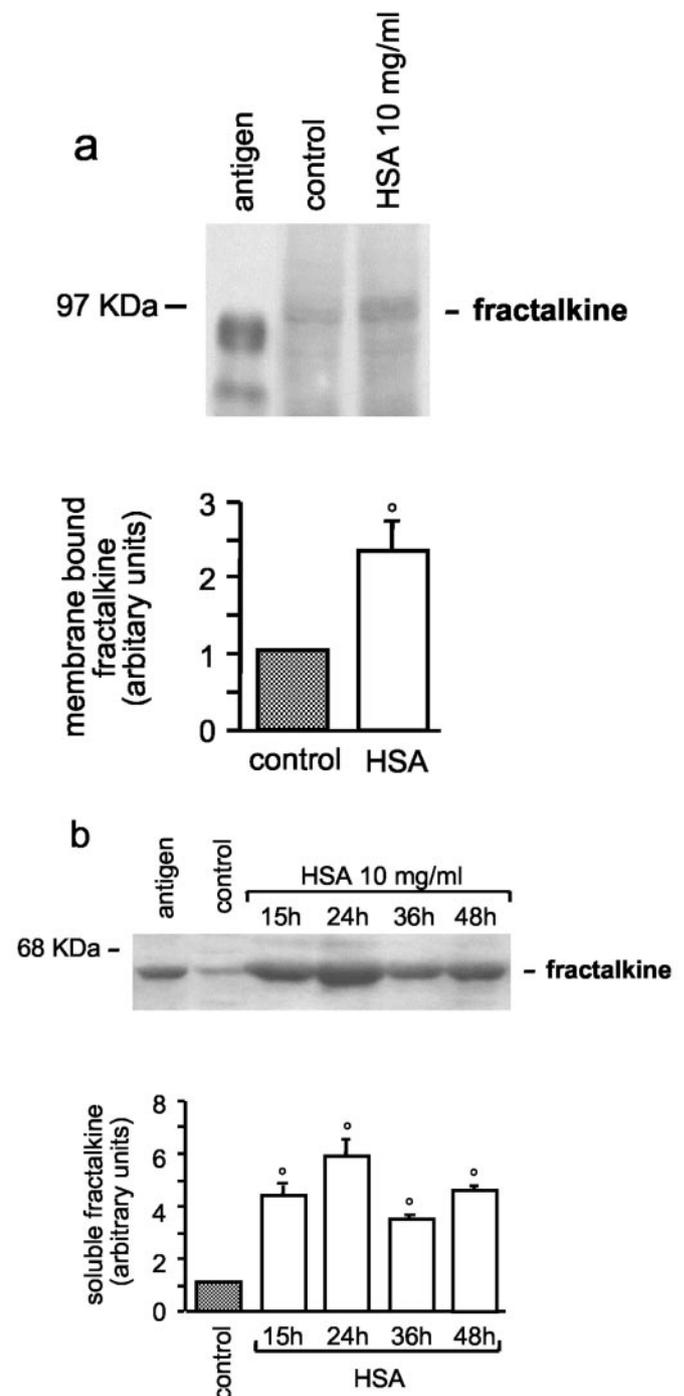
### Albumin Increases Both Membrane-Bound and Soluble Forms of Fractalkine Protein

To determine whether the induction of fractalkine mRNA resulted in increased synthesis of fractalkine protein, Western blot tests were conducted with antibody specifically directed against the internal domain of fractalkine. Membrane-bound form of fractalkine was detected in HK-2 cell lysates running with a molecular size of approximately 95 kD (Figure 2a). The expression of fractalkine was increased 2.4-fold within 24-h incubation with 10 mg/ml HSA. In addition to cell-bound fractalkine, HK-2 cells also produced the cleaved form of fractalkine (Figure 2b). Actually, cells were found to constitutively express a low level of an approximately 65-kD soluble fractalkine protein. After stimulation with HSA, soluble fractalkine was strongly detected at 15 h, reaching a peak at 24 h. This increase was still evident at 48 h. By immunofluorescence experiments, we confirmed overexpression of surface-associated fractalkine with a granular pattern distribution in proximal tubular cells loaded with albumin (Figure 3).

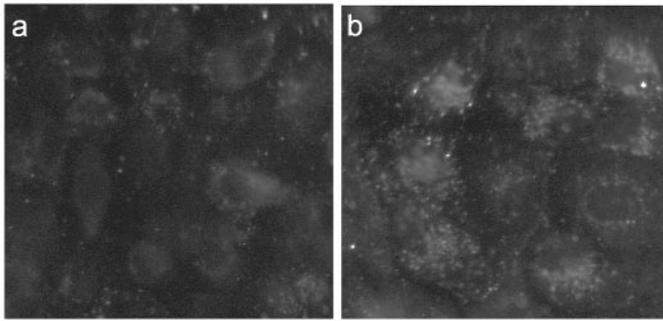
### Upregulation of Fractalkine mRNA in Response to Albumin Is Dependent on NF- $\kappa$ B Activation

Nuclear extracts from HK-2 cells were assayed for NF- $\kappa$ B DNA binding activity with a radiolabeled specific oligonucleotide probe. Consistent with our previous studies (5,6), unstimulated cells displayed low basal levels of NF- $\kappa$ B binding activity, whereas 30-min incubation with HSA lead to a substantial rise in NF- $\kappa$ B activity (Figure 4). The specificity of binding reaction was confirmed in competition experiments by the ability of excess unlabeled (cold) NF- $\kappa$ B oligonucleotide to inhibit binding.

To establish whether upregulation of fractalkine mRNA in response to HSA was dependent on NF- $\kappa$ B activation, we studied the expression of the chemokine in proximal tubular cells exposed to HSA in the presence of the NF- $\kappa$ B inhibitor PDTC (25  $\mu$ M). By EMSA experiments, PDTC inhibited NF- $\kappa$ B binding activity (Figure 4). As reported in Figure 5, PDTC reduced by 40% the increase of fractalkine transcript levels in response to HSA stimulation. In addition, we used a genetic approach to specifically inhibit NF- $\kappa$ B activation by acting on two regulatory proteins. Before exposure to albumin, HK-2 cells were infected with a recombinant adenovirus encoding I $\kappa$ B $\alpha$  (25), an inhibitory protein that sequesters NF- $\kappa$ B into cytoplasm avoiding its translocation to the nucleus. Overexpression of I $\kappa$ B $\alpha$  resulted in 43% reduction of HSA-induced fractalkine mRNA transcript levels (Figure 6a). Similarly, when tubular cells were infected with the dominant negative mutant of the I $\kappa$ B kinase 2 (IKK2), which fails in promoting the dissociation of I $\kappa$ B $\alpha$  from NF- $\kappa$ B (26), a 55% inhibition of fractalkine mRNA was obtained (Figure 6b). No difference was observed in unstimulated cells infected with the control adenovirus (Ad null control) in respect to control cells transfected with Ad.I $\kappa$ B $\alpha$  or Ad.dnIKK2 (Figure 6). Taken together, these data imply that activation of NF- $\kappa$ B is at least in part responsible for the fractalkine gene upregulation in our setting.



**Figure 2.** Western blot analyses of membrane-bound and soluble fractalkine protein expression in HK-2 cells. (a) Membrane-bound fractalkine in cell lysates from HK-2 cells exposed to 10 mg/ml human serum albumin (HSA) for 24 h. Corresponding densitometric analysis of the autoradiograph is shown. Representative Western blot analysis of  $n = 3$  experiments.  $^{\circ}P < 0.05$  versus control. (b) Time course of soluble fractalkine expression in the supernatant of HK-2 cells exposed to 10 mg/ml HSA. Densitometric analysis of the autoradiographic signals of fractalkine is shown. Results (mean  $\pm$  SEM) are expressed as fold increase over control (considered as 1) in densitometric arbitrary units.  $^{\circ}P < 0.05$  versus control. Data are representative of three experiments.



**Figure 3.** Fractalkine expression in HK-2 cells treated with human serum albumin (HSA). Confocal immunofluorescence micrographs of HK-2 cells treated with control medium (a) or HSA (10 mg/ml, 24 h) (b) and stained for fractalkine. Data are representative of three experiments.

#### Activation of p38 MAPK by Albumin

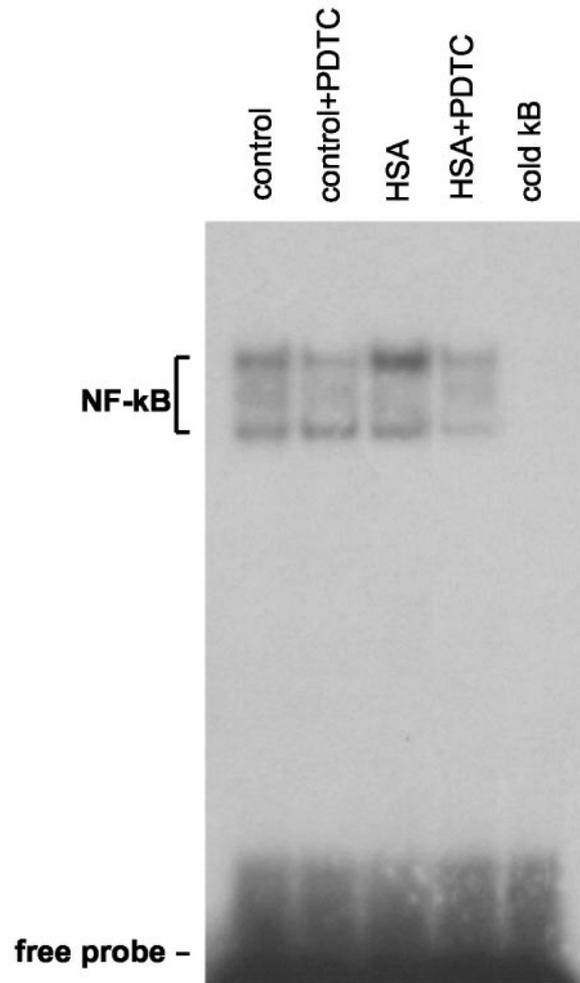
On the basis of the evidence that in other cellular systems, cytokine-induced p38 MAPK phosphorylation regulated the transcriptional activity of NF- $\kappa$ B (27,34), we investigated whether albumin overload induced activation/phosphorylation of p38 MAPK, which in turn could mediate NF- $\kappa$ B–dependent fractalkine upregulation. By Western blot test, albumin-loaded HK-2 cells exhibited a marked p38 phosphorylation within 15 min (2.4-fold over control) that further increased up to 4- and 5.5-fold over control at 3 and 6 h, respectively (Figure 7a). Next, to address whether albumin could modulate NF- $\kappa$ B activity by inducing p38 MAPK activation, we evaluated the effect of the p38 inhibitor SB202190 in albumin-treated HK-2 cells transfected with a vector encoding the luciferase reporter gene driven by a promoter containing consensus sequence for NF- $\kappa$ B. Results showed that albumin increased luciferase activity by threefold in respect to control and that SB202190 significantly reduced the luciferase activity (Figure 7b).

The effect of the p38 inhibitor was then assessed on both fractalkine mRNA and protein. As shown in Figure 7c, treatment of HK-2 cells with SB202190 resulted in a partial, although significant, inhibition (27%) of fractalkine mRNA transcript levels induced by albumin. A similar inhibitory effect (31%) was observed for fractalkine protein expression in this setting (Figure 7d).

#### Renal Fractalkine mRNA and Protein Expression in Mice with Overload Proteinuria

Mice receiving repeated injections of BSA developed proteinuria averaging  $29 \pm 5$  mg/d ( $P < 0.05$  versus control:  $8 \pm 1$  mg/d) at 4 wk. Light microscopy changes at 4 wk consisted of tubular hyaline casts and protein droplets in proximal tubular epithelial cells (mean score for tubular damage, 1), mild and focal hypercellularity in the interstitium, and focal segmental hyalinosis and sclerosis affecting on average 7% of glomeruli. Later on, after 11 wk of BSA injections, tubulointerstitial damage and glomerulosclerosis further worsened in this model (35).

Renal fractalkine gene expression as evaluated by Northern blot analysis is shown in Figure 8. In the kidney of control mice, a single 3.0-kb fractalkine mRNA transcript of low intensity was

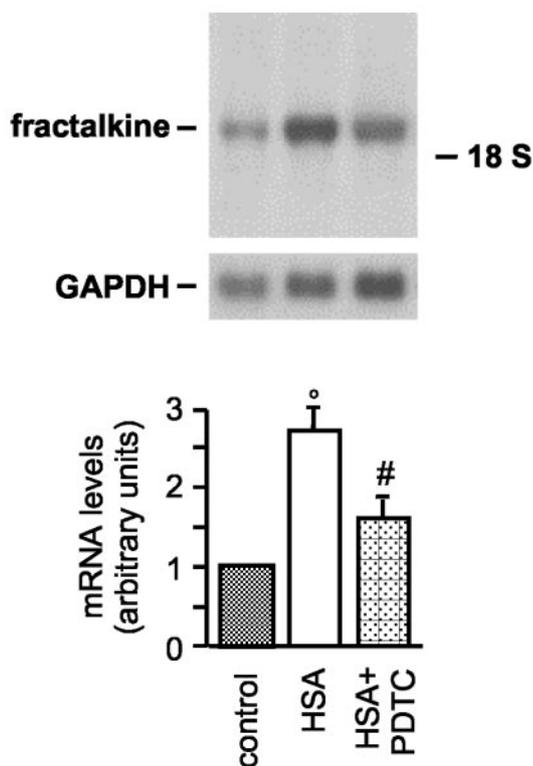


**Figure 4.** NF- $\kappa$ B activity in nuclear extracts from HK-2 cells treated with human serum albumin (HSA). HK-2 cells were exposed for 30 min to control medium or HSA (10 mg/ml) in the presence or absence of pyrrolidine dithiocarbamate (PDTC, 25  $\mu$ mol/L) added 1 h before HSA. To demonstrate the specificity of binding of the NF- $\kappa$ B oligonucleotide, a 100-fold molar excess unlabeled (cold) nucleotide was used to compete with the labeled NF- $\kappa$ B probe for binding to nuclear proteins. Results are representative of three experiments.

detected. In mice with protein-overload proteinuria, message levels were 2.3-fold greater than those of controls. No specific immunostaining for fractalkine was observed in the normal kidney. In contrast, kidneys of mice given 4 wk of BSA injection revealed fractalkine staining, which was confined to tubular epithelial cells in a focal distribution (Figure 9, a through c). The staining within the cells was predominantly diffuse; however, a focal increase in the intensity could be detected in the basal cell region in proximal tubuli. Brush borders did not show any significant concentration of fractalkine staining (Figure 9, a and b).

#### Effect of Anti-CX3CR1 Antibody on the Interstitial Accumulation of Inflammatory Cells in Mice with Overload Proteinuria

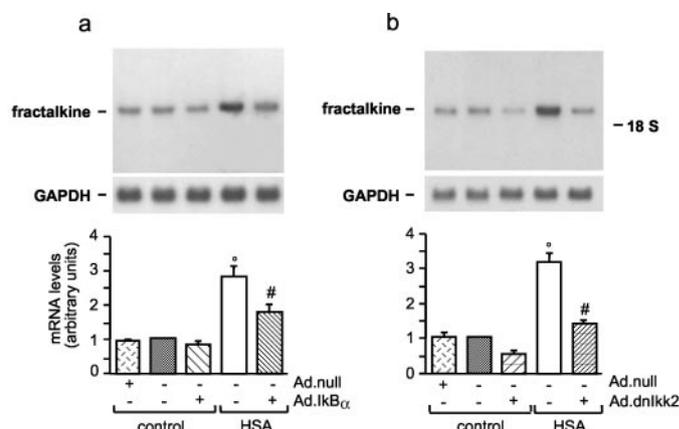
To determine the functional significance of fractalkine upregulation in inducing accumulation of mononuclear cells in



**Figure 5.** Effect of pyrrolidine dithiocarbamate (PDTC) on fractalkine mRNA expression induced by albumin overload. (top) HK-2 cells were treated with PDTC (25  $\mu$ mol/L) 1 h before and during 15 h incubation with human serum albumin (HSA) (10 mg/ml). (bottom) The optical density of autoradiographic signals was quantified and calculated as the ratio of fractalkine to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Results (mean  $\pm$  SEM) are expressed as fold increase over control (considered as 1) in densitometric arbitrary units. <sup>o</sup> $P < 0.05$  versus control; <sup>#</sup> $P < 0.05$  versus HSA. Data are representative of three experiments.

protein overload proteinuria, mice were treated daily up to 4 wk with an antibody against the fractalkine receptor CX3CR1, beginning on day 1 of BSA injection. Kidneys were analyzed for F4/80 positive monocytes and macrophages by immunohistochemical technique. A marked accumulation of F4/80-positive cells was present in the renal interstitium of untreated proteinuric mice ( $37 \pm 3$  cells per high-power field,  $P < 0.05$  versus control). The distribution of F4/80-positive monocytes and macrophages, albeit irregular, was confined to peritubular areas (Figure 9d). Kidneys of normal mice did not exhibit signs of cellular infiltration (Figure 9e). Anti-CX3CR1 antibody treatment reduced, although not to a statistically significant degree (22%), the interstitial accumulation of mononuclear cells ( $29 \pm 5$  cells per high-power field), in the absence of significant effects on proteinuria ( $25 \pm 5$  versus untreated:  $33 \pm 7$  mg/d) and tubular and glomerular changes by histology (data not shown). Treatment with rabbit nonimmune serum did not modify the number of F4/80-positive monocytes and macrophages in the renal interstitium.

Mice with protein overload proteinuria at 4 wk had a mild impairment of renal function, as indicated by increased BUN



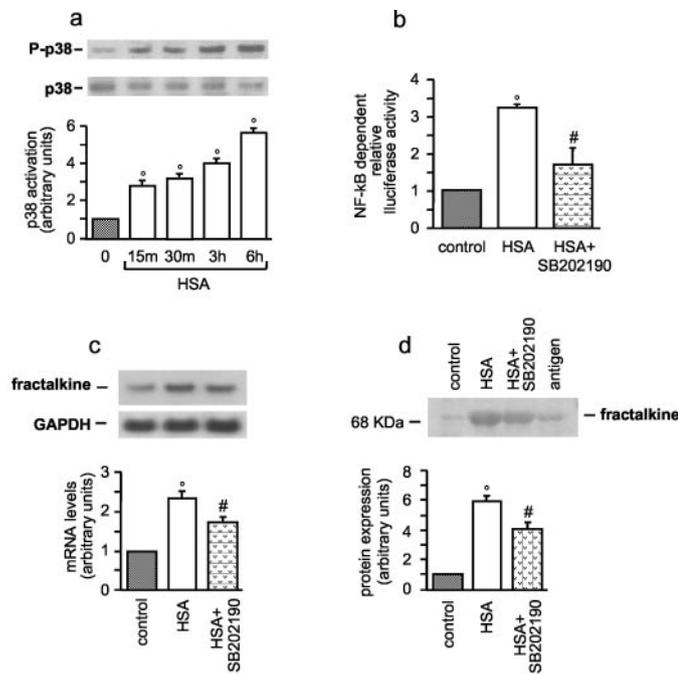
**Figure 6.** Fractalkine mRNA upregulation induced by albumin is inhibited by adenovirus-mediated gene transfer of I $\kappa$ B $\alpha$  or dominant negative mutant of IKK2. (top) HK-2 cells were left untreated or infected with rAd. I $\kappa$ B $\alpha$  (a) or rAd.dnIKK2 (b) for 3 h; cells were then exposed to medium alone or to human serum albumin (HSA) (10 mg/ml, 15 h). Representative Northern blot analysis of three experiments. (bottom) Corresponding densitometry of the autoradiographs reported above. The optical density of autoradiographic signals was quantified and calculated as the ratio of fractalkine to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The mRNA levels of HK-2 cells infected with adenoviruses were determined by assuming the optical density of control as unit. <sup>o</sup> $P < 0.05$  versus control; <sup>#</sup> $P < 0.05$  versus HSA.

and serum creatinine levels in respect to controls (BUN,  $41 \pm 2$  versus  $31 \pm 2$  mg/dl,  $P < 0.05$ ; creatinine,  $0.44 \pm 0.02$  versus  $0.34 \pm 0.02$  mg/dl). Renal function was ameliorated after treatment with the anti-CX3CR1 antibody because BUN and serum creatinine values decreased ( $33 \pm 3$  mg/dl;  $0.39 \pm 0.07$  mg/dl, respectively) as compared with untreated mice with overload proteinuria.

## Discussion

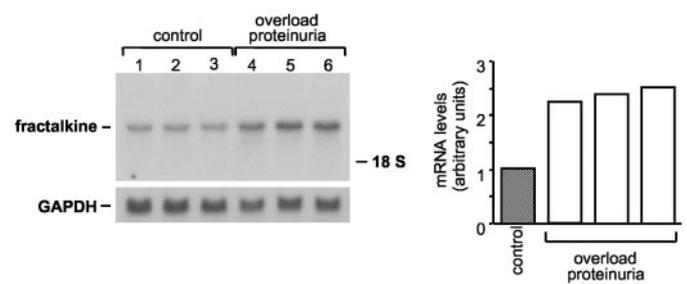
The study presented here explored fractalkine upregulation by proximal tubular cells as a potential trigger for the recruitment of mononuclear cells into the renal interstitium. Our results indicate that fractalkine gene expression in tubular cells is actually enhanced in the proteinuric setting. Evidence of induction of both soluble and membrane-bound forms in cultured tubular cells would suggest that here, fractalkine acts both as a chemoattractant and as an adhesion molecule inducing leukocyte adhesion and retention. A recent study documented that cytokine-activated tubular cells overexpressed membrane-bound fractalkine, which is responsible for the adhesion of inflammatory mononuclear cells (21).

We proceeded to characterize the intracellular response to ultrafiltered proteins as the stimulatory factor and intracellular pathways of fractalkine upregulation, still largely undefined so far. There is one study showing that fractalkine transcription was dependent on the activation of NF- $\kappa$ B in endothelial cells stimulated by cytokines and endotoxin (36). Our group previously documented activation of NF- $\kappa$ B and induction of NF- $\kappa$ B-dependent inflammatory chemokines in proximal tubular



**Figure 7.** (a) Activation of p38 mitogen-activated protein kinase (MAPK) by albumin in HK-2 cells. HK-2 cells were incubated with 10 mg/ml of human serum albumin (HSA) for 15 min, 30 min, 3 h, and 6 h. Cell lysates were analyzed by immunoblotting using antibody against phosphorylated form of p38 MAPK (upper panel). The blots were stripped and re probed with an antibody, anti-nonphosphorylated p38 (bottom), to confirm equal loading of the proteins on the gel. Relative p38 activity was quantified by densitometric evaluation of autographs and is depicted as mean  $\pm$  SEM of three independent experiments. \* $P < 0.05$  versus control. (b) Effect of pharmacologic inhibition of p38 MAPK on HSA-induced NF- $\kappa$ B-dependent promoter activity. HK-2 cells were transfected for 3 h with NF- $\kappa$ B luciferase reporter gene. Then cells were maintained in fresh medium without serum for 24 h before exposure to 10 mg/ml HSA for additional 15 h. The p38 inhibitor SB202190 (20  $\mu$ mol/L) was added 1 h before and during stimulation with HSA. Relative luciferase activity is expressed as fold stimulation by assuming control as unit. Data are mean  $\pm$  SEM (three experiments). \* $P < 0.05$  versus control; # $P < 0.05$  versus HSA. (c) Effect of p38 MAPK inhibitor on HSA-induced fractalkine mRNA expression. HK-2 cells were treated with the p38 inhibitor SB202190 1 h before and during 15 h incubation with HSA (10 mg/ml). Representative Northern blot analysis of three experiments. The optical density of autoradiographic signals was quantified and calculated as the ratio of fractalkine to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Results (mean  $\pm$  SEM) are expressed as fold increase over control (considered as 1) in densitometric arbitrary units. (d) Effect of p38 MAPK inhibitor on HSA-induced fractalkine production. Western blot analysis of fractalkine protein in supernatant from HK-2 cells exposed to 10 mg/ml HSA for 24 h in presence or absence of SB202190. Corresponding densitometric analysis of the autoradiograph is shown. Representative Western blot analysis of three experiments. \* $P < 0.05$  versus control; # $P < 0.05$  versus HSA.

cells exposed to protein overload (5,6). Reactive oxygen species were identified as second messengers, in that the treatment of albumin-laden tubular cells with antioxidants, by reducing

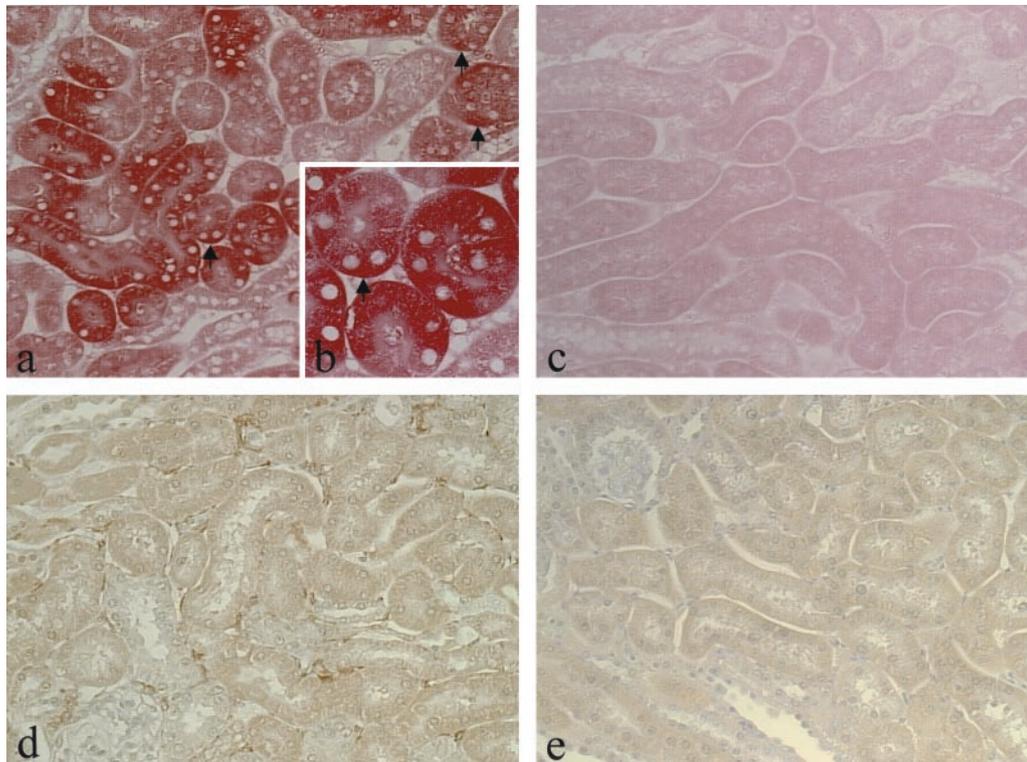


**Figure 8.** Renal fractalkine mRNA expression is upregulated in mice with protein overload proteinuria. (left) Northern blot experiments were performed by total RNA isolated from kidneys of mice with protein overload proteinuria induced by 4-wk injections of BSA (10 mg/g body weight) or control mice. (right) Densitometric analysis of autoradiographic signal for fractalkine. The optical density of the autoradiographic signal was quantified and calculated as the ratio of fractalkine to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Results are expressed as fold increase over control (considered as 1).

hydrogen peroxide generation, inhibited NF- $\kappa$ B activation and abolished MCP-1 mRNA upregulation (5).

Here, NF- $\kappa$ B regulation on fractalkine mRNA expression in response to albumin was demonstrated by two approaches. First, we found that the antioxidant and NF- $\kappa$ B inhibitor PDTC significantly decreased fractalkine mRNA induction after albumin challenge. Then, because NF- $\kappa$ B activity is regulated by the I $\kappa$ B, which sequesters NF- $\kappa$ B in the cytoplasm, avoiding its translocation to the nucleus (9,10), we evaluated whether overexpression of I $\kappa$ B $\alpha$  could reduce fractalkine gene expression in albumin-loaded proximal tubular cells. This was the case in HK-2 cells infected with a recombinant adenovirus encoding I $\kappa$ B $\alpha$ . In addition, we used a recombinant adenovirus that allows highly efficient gene transfer to express a kinase-negative mutant of IKK2 that acts as a dominant negative molecule (26). IKK2 is a specific kinase that, by activation-induced phosphorylation of I $\kappa$ B $\alpha$ , allows dissociation of I $\kappa$ B $\alpha$ /NF- $\kappa$ B complex (37). Actually, adenovirus-mediated expression of dnIKK2 impaired the nuclear translocation of p65 NF- $\kappa$ B and DNA binding of the transcription factor in stimulated endothelial cells, resulting in inhibition of proinflammatory gene expression (26). Accordingly, our finding that dnIKK2 inhibited by 55% albumin-induced fractalkine upregulation indicates that fractalkine overexpression by proximal tubular cells on proteinuric challenge is, at least in part, dependent on NF- $\kappa$ B activation.

Other non-NF- $\kappa$ B pathways are likely to be involved in such transcriptional regulatory pathways. Within the 15-kb genomic sequence of the fractalkine gene obtained from GenBank besides NF- $\kappa$ B, binding sequences for other factors, including AP-1, p53, p300, and SP-1, can be predicted in the promoter region. In this regard, AP-1 has been recently reported to mediate TNF- $\alpha$ -stimulated fractalkine expression in rat mesangial cells, as indicated by reduced fractalkine levels upon treatment with AP-1 inhibitor (38). In addition, experiments with human cancer cells have documented that fractalkine is a direct transcriptional target of p53 (39).



**Figure 9.** Changes in fractalkine protein expression and F4/80 positive mononuclear cells in kidney taken from mice with protein overload proteinuria. Immunohistochemical staining of fractalkine in kidney in a mouse with protein overload proteinuria at week 4 shows localization to proximal tubular cells (a, b), in contrast to absence of staining in control mouse (c). More intense staining can be noted in the basal region of the cells (arrows). Macrophage accumulation is seen in peritubular areas in kidney after protein overload (d). (e), control kidney. Original magnification, a, c, d, and e,  $\times 400$ ; b,  $\times 1000$ .

To further elucidate mechanisms underlying NF- $\kappa$ B-dependent fractalkine expression in proximal tubular cells, we focused on p38 MAPK. Studies over the last few years have demonstrated that different MAPK cascade pathways contribute to the transmission of extracellular signals that can finally result in phosphorylation of transcription factors and alterations in gene expression (23). p38 is a member of the MAPK family primarily activated by cytokines and cellular stress (22) that was recognized to regulate NF- $\kappa$ B-dependent transcription of proinflammatory genes (27,34). It was shown in different cell types that p38 MAPK was critical for cytokine-induced expression of chemokines (40,41), and that the p38 MAPK-dependent pathway interfered directly with the transcriptional activity of NF- $\kappa$ B factors in the transcriptional complex, resulting in chemokine production (27). p38 MAPK phosphorylates the basal transcription factor TATA binding protein (TBP), a step required for TBP binding to the TATA box and to the p65 subunit of NF- $\kappa$ B. Such newly formed complex actually induces NF- $\kappa$ B transcriptional activity (42). Recently, induction of MCP-1 expression in endothelial cells was reported to require activation of NF- $\kappa$ B signaling, nuclear accumulation of p65 and subsequent recruitment of transcriptional coactivators such as CBP/p300 (27). p38 MAPK regulated the coactivator function and facilitated the proper assembly and activity of the complex. In the study presented here, we first documented rapid p38 activation in proximal tubular cells

exposed to albumin. Treating the cells with the p38 inhibitor SB202190 inhibited transcription of NF- $\kappa$ B promoter/luciferase reporter gene construct, consistent with a role of p38 as regulator in this pathway. Finally, data that the p38 inhibitor reduced both fractalkine mRNA and protein overexpression reflect NF- $\kappa$ B-dependent expression of the fractalkine gene via p38 MAPK cascade.

In line with the finding that excess albumin upregulates fractalkine expression in cultured proximal tubular cells is the evidence of fractalkine gene induction in the kidney of mice with protein-overload proteinuria. Fractalkine staining was also detected in tubular epithelial cells in a focal distribution, in contrast to control mouse kidneys. That fractalkine of tubular cell origin may have contributed to mononuclear cell accumulation into the interstitium is further suggested here by the evidence that the treatment of mice with overload proteinuria with an antibody against the fractalkine receptor CX3CR1 limited the number of monocytes and macrophages infiltrating the renal interstitium. Previous studies in rats with anti-glomerular basement membrane glomerulonephritis reported that blocking CX3CR1 resulted in the reduction of glomerular leukocytes and crescents (18). The reduction in inflammatory cells was not statistically significant but remarkable (22%) in the protein overload model, considering that in other models blocking or inhibiting CC or CXC chemokines decreased

monocyte infiltration by 28% to 47% while at the same time ameliorating proteinuria (43,44).

The most likely explanation for limited benefit of anti-fractalkine receptor antibody treatment is the persistent stimulation by locally produced MCP-1, RANTES, and complement or other ultrafiltered inflammatory components (45). Lack of complete CX3CR1 inhibition might concur to limiting the efficacy of treatment, as also suggested in the cardiac allograft (32). Notably, the recent demonstration that the majority of leukocytes infiltrating the kidney in human renal diseases expressed CX3CR1 (46) is in support of an important role for fractalkine-CX3CR1 interactions in clinical settings. Finally, the finding that the reduction of interstitial accumulation of mononuclear cells in albumin-loaded mice treated with anti-CX3CR1 antibody was associated with amelioration of renal function is consistent with the evidence that the number of T cells and macrophages infiltrating the renal interstitium correlates with the declining renal function (47), to the extent that treatments that decreased interstitial mononuclear cell infiltration and activation protected from renal function impairment (48,49). Thus, experimental evidence is available showing that in the rat model of anti-glomerular basement membrane disease administration of IL-1 receptor antagonist prevented renal function impairment while inhibiting interstitial mononuclear cell infiltration (48). In addition, in rats with puromycin aminonucleoside nephrosis, prednisolone treatment of established disease markedly suppressed interstitial mononuclear cell infiltration in association with improved creatinine clearance (49).

In summary, we have shown that in human proximal tubular cells in culture, 1) albumin dose-dependently induced fractalkine mRNA; 2) upregulation of fractalkine mRNA was associated with enhanced levels of both membrane-bound and soluble form of the protein; 3) albumin-induced fractalkine expression was, at least in part, dependent on NF- $\kappa$ B activation; and 4) p38 MAPK was activated by albumin and was involved in NF- $\kappa$ B-dependent fractalkine transcription. Moreover, we have documented that fractalkine mRNA and protein expression increased in the kidney of mice with protein overload proteinuria, and that anti-CX3CR1 antibody treatment reduced, at least to a limited degree, the accumulation of monocytes and macrophages into the renal interstitium.

These findings indicate protein overload as a promoter of fractalkine gene induction partly mediated by NF- $\kappa$ B and p38 activation in proximal tubular cells. Fractalkine might contribute to direct mononuclear cells into peritubular interstitium and enhance their adhesion property, in turn favoring interstitial inflammation and disease progression.

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