

Induction of Monocyte Chemoattractant Protein-1 by Albumin Is Mediated by Nuclear Factor κ B in Proximal Tubule Cells

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Abstract. The transcription and translation of monocyte chemoattractant protein-1 (MCP-1), a CC chemokine, are increased in proximal tubule epithelial cells (PTC) stimulated with pathophysiologically relevant concentrations of albumin. The purpose of this study was to investigate whether nuclear factor κ B (NF κ B)/Rel proteins play a role in albumin-induced MCP-1 transcription. Confluent monolayers of rat PTC in primary culture were stimulated with delipidated bovine serum albumin. NF κ B, the NF κ B inhibitory protein (I κ B), and MCP-1 transcription were assessed using electrophoretic mobility shift assays, Western immunoblotting, semiquantitative reverse transcription-PCR, and ribonuclease protection assays. Activation of NF κ B by delipidated bovine serum albumin (15 mg/ml) was detectable within 2 h, maximal after 8 h, and maintained for at least 16 h of continuous exposure. Supershift analysis showed that the activated proteins were composed of

p50/p50, p50/p65, and p50/c-Rel dimers. Cytoplasmic I κ B α levels were decreased 30 min after stimulation and returned to unstimulated levels by 4 to 8 h. I κ B β levels were decreased at 2 h and there was no recovery until 8 h. Inhibition of NF κ B with pharmacologic agents (*N*-tosyl-phenylalanine chloromethyl ketone and dexamethasone) and an antisense oligonucleotide to the rat p65 subunit of NF κ B significantly reduced MCP-1 transcription. The 3.6-kb 5' flanking region of the rat MCP-1 gene was cloned and sequenced, and two putative κ B binding sites were identified within the enhancer region. Therefore, albumin increased NF κ B and reduced I κ B levels in PTC, and MCP-1 expression was dependent on NF κ B activation. It is concluded that the activation of NF κ B/Rel proteins modulates chemokine production in PTC in response to albumin and is likely to have an important role in the mediation of tubulointerstitial injury in proteinuric renal disease.

The accumulation of extracellular matrix proteins in the cortical interstitium is a consistent feature of chronic glomerular diseases (CGD) and a strong determinant of renal prognosis (1,2). Injury to proximal tubule epithelial cells (PTC) and interstitial inflammation have been proposed as prerequisites for the development of matrix deposition and interstitial fibrosis (3). Excessive glomerular filtration of urinary proteins and their effects on PTC may in part explain the pathogenesis of tubular injury and interstitial inflammation (4), and the severity of proteinuria is correlated with that of tubulointerstitial injury in both human and experimental proteinuric renal disease (1,3,4). Evidence supporting a central role for proteinuria and the PTC in causing interstitial inflammation has been derived from *in vitro* studies demonstrating that urinary proteins, at concentrations similar to those of proteinuric urine, cause PTC to produce chemotactic cytokines and matrix proteins and to alter their expression of surface adhesion molecules (5–13). Furthermore, certain components of proteinuric urine, such as holotransferrin, are known to induce damage in PTC (14).

PTC secrete the CC chemokine monocyte chemoattractant

protein-1 (MCP-1) across the basolateral membrane in response to albumin and transferrin (9). MCP-1 is an important chemoattractant for macrophages and T lymphocytes, which are the predominant inflammatory cells found in the interstitium in CGD (15,16). Increasing evidence suggests that MCP-1 may have an important role in the regulation of interstitial inflammation and possibly other processes related to matrix deposition (17). The signal transduction pathways of MCP-1 gene activation are tissue- and stimulus-specific, and the intracellular mechanisms by which albumin upregulates MCP-1 in PTC have not been characterized (18).

The promoter region of the human MCP-1 gene contains putative binding sites for a number of transcription activators, including the ubiquitous nuclear factor κ B (NF κ B)/Rel family (19). NF κ B proteins normally exist in the cytosol as dimers bound to inhibitory proteins (I κ B); after exposure to different stimuli, I κ B undergoes proteolysis, allowing NF κ B to enter the nucleus and activate genes encoding chemokines and other proteins (20). For example, in mesangial cells stimulated with interleukin-1 (IL-1), MCP-1 expression is correlated with the activation of NF κ B (21,22). Furthermore, lipopolysaccharide (LPS) and interferon- γ have been shown to activate NF κ B in a murine PTC line (23). Recently, Zoja and coworkers (13) reported that albumin activated NF κ B in the LLC-PK₁ cell line and that this was correlated with the expression of RANTES, a cytokine that also belongs to the chemokine superfamily.

There are multiple potential signal transduction pathways by which MCP-1 might be upregulated, and those involved in the

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upregulation of MCP-1 by albumin have not been previously investigated. Therefore, the aim of this study was to investigate the potential role of NF κ B in albumin-induced MCP-1 transcription in rat PTC in primary culture, by (1) examining the effect of albumin on NF κ B activation and I κ B kinetics; (2) examining the effect of NF κ B inhibition on MCP-1 gene expression; and (3) cloning and sequencing the 5' flanking region of the rat MCP-1 gene to identify potential κ B binding sites.

Materials and Methods

Isolation and Culture of Rat PTC

PTC were isolated and cultured from normal male Wistar rats using isopycnic centrifugation. Cells were grown in Dulbecco's modified Eagle's medium supplemented with epidermal growth factor (10 ng/ml) and insulin (5 μ g/ml), in a 5% CO₂/95% O₂ environment (9,14). The PTC origin of these cells was verified in previous experiments (9). Experiments were commenced after cells had reached confluence, which was usually 5 to 6 d after the isolation procedure.

Reagents

Dulbecco's modified Eagle's medium, endotoxin-free delipidated bovine serum albumin (dBSA) (fraction V), dexamethasone, *N*-tosyl-phenylalanine chloromethyl ketone (TPCK), LPS (*Escherichia coli*), and all other cell culture reagents were purchased from Sigma-Aldrich (Sydney, Australia). In previous studies, it was determined that both dBSA and the lipidated form of bovine serum albumin (BSA) stimulate MCP-1 transcription in rat PTC (9). Additional experiments confirmed that this fraction of dBSA dissolved in medium is almost completely free of endotoxin (<0.1 ng/ml), as assessed using the *Limulus* amoebocyte assay (Sigma-Aldrich). Studies in our laboratory showed that concentrations of LPS at or below this level do not induce MCP-1 gene transcription in PTC.

Analysis of MCP-1 Transcription

Total RNA was isolated using RNazol B (Tel-TEST, Inc., Friendwood, TX) as described previously (11). The transcription of MCP-1 mRNA in PTC was assessed using both semiquantitative reverse transcription (RT)-PCR and RNase protection assays. The design of primers and conditions for RT-PCR have been described previously (9). Ten percent of the PCR product was loaded onto a 1.2% agarose gel, which was stained with ethidium bromide (0.5 μ g/ml) and photographed (Polaroid 665 film) over ultraviolet light. The bands on the negative film were densitometrically scanned (personal densitometer; Molecular Dynamics, Sunnyvale, CA), and the signal intensity for MCP-1 was expressed relative to that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase.

The RNase protection assays were performed using a lysate ribonuclease protection assay kit (Ambion, Austin, TX). The single-stranded sense RNA probe for MCP-1 was prepared by amplification of the PCR product and incorporation of the T7 phage promoter sequence. The 18S RNA probe was purchased (Ambion), and both probes were radiolabeled with ³²P using a commercial kit (for *in vitro* transcription with MAXIScript; Ambion). Cells were suspended in lysis solution (1 \times 10⁷ cells/ml), and 45 μ l of lysis solution was hybridized overnight with 50,000 cpm of either MCP-1 or 18S RNA ³²P-labeled antisense RNA probes, at 37°C. Ten microliters of RNase cocktail were added to the sample and incubated for 30 min at 37°C, to remove single strands of RNA. Excess protein was removed by the addition of 20 μ l of 10% sodium sarcosyl and 10 μ l of proteinase K

and incubation for 30 min at 37°C. The RNA was precipitated with 500 μ l of isopropanol. MCP-1 RNA/RNA duplexes were separated by electrophoresis on 5% polyacrylamide gels. The gels were dried and then exposed to x-ray film for 1 to 2 h at -80°C. The density of the signals was determined by scanning as described, and the results for MCP-1 were expressed relative to those for 18S RNA.

Extraction of Nuclear Proteins and Electrophoretic Mobility Shift Assay

Nuclear proteins were extracted from PTC using methods described by Dingnam *et al.* (24), with minor modifications. Approximately 1 \times 10⁶ cells were scraped from culture plates and transferred to microcentrifuge tubes. Cells were lysed with buffer A (10 mM Hepes, pH 7.9, 1.5 mM KCl, 10 mM MgCl₂, 0.5 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], and 0.01 mM β -mercaptoethanol [BME]) and homogenized with 10 even strokes of a hand-held glass homogenizer (Thomas Scientific, Swedesboro, NJ). Release of nuclei was monitored during this step by visual inspection using light microscopy (magnification, \times 400). The homogenate was centrifuged for 6 min at 6000 rpm at 4°C and was washed with low-salt buffer C (20 mM Hepes, 25% glycerol, 1.5 mM MgCl₂, 0.2 mM ethylenediaminetetraacetate [EDTA], 0.5 mM DTT, 1 mM PMSF, 0.01 mM KCl, and 0.5 mM BME). Fifty microliters of high-salt buffer C (low-salt buffer C plus 1.2 M NaCl) were added, and the mixture was incubated on ice for 30 min. Fifty microliters of buffer D (20 mM Hepes, 19% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF, and 0.5 mM BME) were then added, and the mixture was centrifuged for 10 min at 13,000 rpm at 4°C. The supernatant (containing nuclear protein) was collected, and its protein concentration was determined using the Bradford method (BioRad Laboratories, Richmond, CA), with BSA as the external standard. The concentrations of the nuclear proteins were generally >2 μ g/ μ l. If the concentration was <1 μ g/ μ l, the nuclear extract was concentrated on a filter (G-10; Millipore, Sydney, Australia) at 2800 \times g for 40 min at 4°C. The nuclear proteins were diluted to a standard concentration (3 μ g/ μ l) and stored in aliquots at -70°C.

Double-stranded oligonucleotides (Promega, Sydney, Australia) containing human NF κ B consensus binding sites (5'-AGTTGAGGG-GACTTCCAGG-3') were radiolabeled using T4 kinase (Promega) and [³²P]ATP (Amersham Life Science, Sydney, Australia) and were purified by centrifugation over G-50 Sephadex spin columns. These columns remove any free [³²P]ATP. Five micrograms of nuclear protein were incubated with 50,000 cpm of ³²P-labeled NF κ B probe for 20 min at room temperature, in binding buffer (10 mM Hepes, pH 7.9, 1 mM EDTA, pH 8.0, 50 mM KCl, 12% glycerol, 1 mM DTT, and 0.015 mg/ml poly(dI-dC)-poly(dI-dC)). The samples were loaded onto a 7% polyacrylamide gel with 1 \times TAE buffer (40 mM Tris-acetate, 1 mM EDTA), and electrophoresis was performed at 100 V for 1 h. The gel was dried under vacuum and exposed to x-ray film for 2 to 4 h. The specificity of the reaction was assessed by competition reactions in which a 100-fold molar excess of unlabeled NF κ B probe was added to the binding reaction 10 min before the addition of radiolabeled probe.

NF κ B/Rel proteins exist as dimers (20); therefore, in additional experiments the composition of activated NF κ B was analyzed using supershift assays. In these studies, 1 μ l of antibodies reactive with the rat p50, p65, or cRel protein (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with the reaction mixture for 30 min before the addition of radiolabeled NF κ B probes. Electrophoresis was performed as described, and the autoradiographs were analyzed for reductions in signal intensities and the presence of supershifted bands.

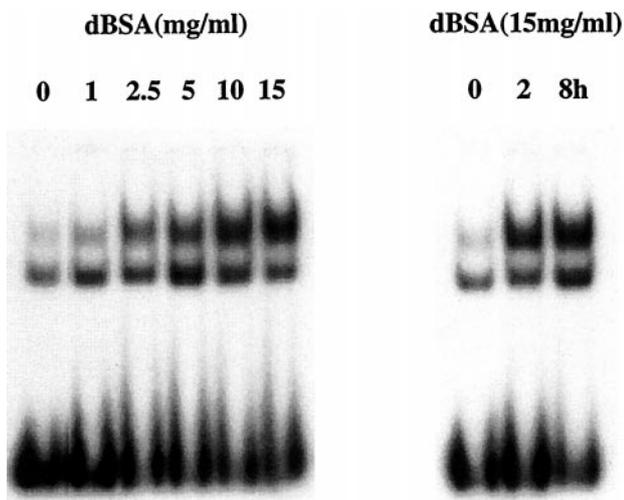


Figure 1. Activation of nuclear factor κ B (NF κ B), determined by electrophoretic mobility shift assay (EMSA), in proximal tubule cells (PTC) in response to delipidated bovine serum albumin (dBSA). EMSA results show the dose–response pattern for NF κ B binding to 32 P-labeled κ B DNA in nuclear extracts from PTC stimulated with dBSA (0 to 15 mg/ml) for 8 h and the time course for NF κ B binding activity after exposure to dBSA (15 mg/ml) for 0, 2, or 8 h. The bands observed in the EMSA were demonstrated as being NF κ B-specific by the addition of excess unlabeled NF κ B probe as competitor (Figure 2). The results are representative of experiments that were repeated twice.

Western Blot Analysis

NF κ B activity is primarily regulated by the turnover of I κ B proteins in the cytosol. Therefore, Western immunoblotting was performed to analyze the kinetics of I κ B proteins. Cells were lysed with buffer (0.1 M Tris, pH 6.8, 0.1% Nonidet P-40, 10% BME, 4 mM EDTA, and 1 mM orthovanadate). Protein contents of the samples were determined as described above. Samples containing 10 μ g of protein were loaded into each lane of a gel and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. The proteins were transferred to a nitrocellulose filter for electrophoresis performed for 1 h at 100 V. The membrane was incubated sequentially with 5% BSA (to block nonspecific binding sites) and then primary polyclonal antibodies (rabbit anti-I κ B α and anti-I κ B β , 1:1000; Santa Cruz Biotechnology), for 1 h each, at room temperature. Blots were washed with Tris buffer and then incubated with peroxidase-conjugated sheep anti-rabbit IgG (1:1000; Amersham) for 1 h at room temperature. Reactivity was detected with ECL detection reagent (Amersham). The molecular weights of the immunoreactive bands were estimated by comparison with known molecular standards (Amersham).

Cloning of the Rat p65 Gene and Treatment with Antisense and Sense Oligonucleotides

Antisense oligonucleotide against the p65 subunit has been used to specifically inhibit NF κ B in *in vitro* studies (21). The specific sequence of the rat p65 gene has not been reported; therefore, to design oligonucleotides, the gene was cloned and sequenced using the rapid amplification of cDNA ends (RACE) method. 5'-RACE was performed on mRNA isolated from rat PTC, using a commercial kit according to the instructions provided by the manufacturer (Marathon cDNA amplification kit; Clontech, Palo Alto, CA). Briefly, a full-length cDNA library from PTC was generated from 1 μ g of mRNA by

cDNA synthesis and adaptor ligation. RACE reactions were performed on a full-length cDNA library using adaptor primers and specific primers that were designed from highly conserved regions identified by comparison with the mouse and human sequences. The 5'-RACE PCR products were subjected to electrophoresis, and single bands were excised and purified using Wizard PCR Preps DNA purification resin (Promega). Fragments from the RACE reaction were cloned into a T/A-type cloning vector. The sequence was determined using an autosequencer (Applied Biosystems; BioRad) and was analyzed by comparison with known sequences for the human and mouse p65 genes using the BLAST program (National Center for Biotechnology Information).

Antisense and sense oligonucleotides to the rat p65 subunit were designed according to the cloned 5' end fragment of the rat p65 gene.

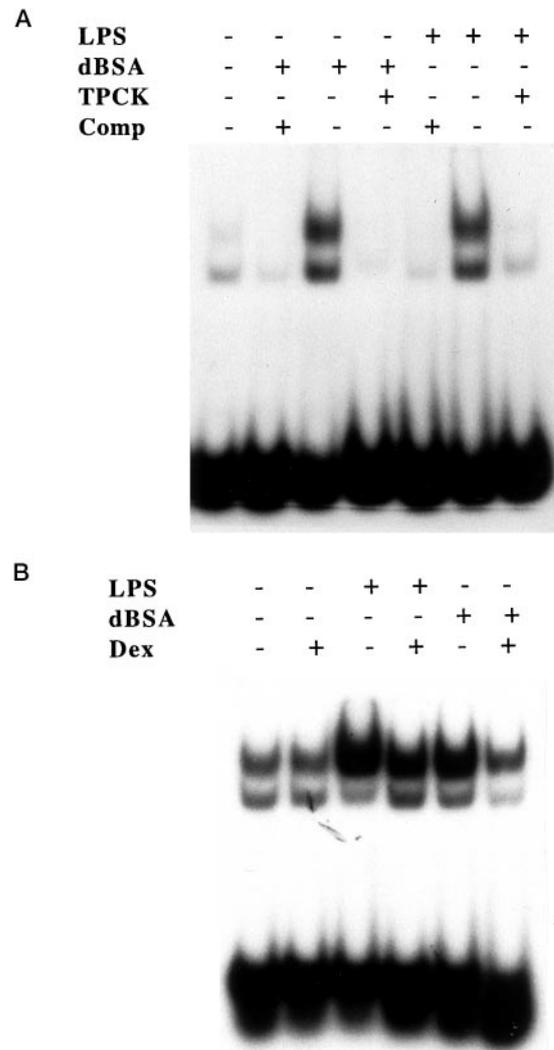


Figure 2. EMSA showing the effects of *N*-tosyl-phenylalanine chloromethyl ketone (TPCK) (100 mM) and dexamethasone (100 mM) on lipopolysaccharide (LPS) (5 mg/ml)- and dBSA (15 mg/ml)-induced NF κ B activity. (A) TPCK completely inhibited NF κ B binding activity after stimulation with LPS or dBSA. Excess unlabeled NF κ B oligonucleotide added as competitor (Comp) blocked NF κ B-specific binding. (B) Dexamethasone (Dex) inhibited NF κ B binding activity after stimulation with dBSA but only partially inhibited that after stimulation with LPS. The results are representative of experiments that were repeated three times.

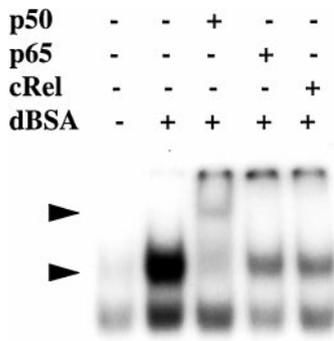


Figure 3. Supershift assay showing the interaction of anti-p50, anti-p65, and anti-cRel antibodies with NFκB protein stimulated by dBSA. The anti-p50 antibody nearly completely abolished the NFκB bands. The anti-p65 and anti-cRel antibodies only partially reduced the NFκB bands. Lower arrowhead, band containing p50, p65, and cRel dimers; upper arrowhead, supershifted band.

Phosphorothioate analogs of the antisense oligodeoxynucleotide correspond to the 5' end of p65 mRNA and include one codon upstream from the translation initiation site. Antisense (5'-GGGAAACA-GATCGTCCATGGC-3') and sense (5'-GCCATGGACGATCTGTT-TCCC-3') oligonucleotides were synthesized on a DNA synthesizer (Beckman, Sydney, Australia). Preliminary experiments were performed with a range of concentrations (0.1 to 50 μM) to assess the optimal concentration of oligonucleotide to inhibit NFκB. Antisense and sense oligonucleotides were added to cells 5 h before the cells were treated with dBSA (15 mg/ml). Cells were then incubated with dBSA and oligonucleotides for an additional 8 h, after which RNA analysis and electrophoretic mobility shift assay (EMSA) were performed as described.

Cloning of the 5'-Flanking Region of the Rat MCP-1 Gene and Detection of NFκB Binding Sites

The human and mouse, but not the rat, MCP-1 genes are known to contain putative binding sites for NFκB proteins. Therefore, the 5'-flanking region was isolated with the genome Walker kit (Clontech). Five genomic Walker libraries were constructed from genomic DNA isolated from rat PTC. The rat genomic DNA was separately digested with five different restriction enzymes (*EcoRV*, *ScaI*, *DraI*, *PvuII*, and *SspI*) and ligated to the adaptor. After construction, the libraries were amplified by using Tth polymerase (Clontech) with an adaptor primer and a gene-specific primer (designed according to the cDNA sequence of MCP-1). The primary PCR mixture was diluted and used as a template for nested PCR, using a nested adaptor primer and a nested gene-specific primer. This produced a single major PCR product from three of five libraries. Three PCR fragments were cloned and analyzed, using an autosequencer (Applied Biosystems), from the known sequence at the 5' end of the MCP-1 cDNA to the unknown adjacent genomic DNA. For detection of the transcription start site, cDNA was prepared from PTC mRNA by RT, after adaptor ligation. A 5'-RACE reaction was performed using a specific primer (designed according to the MCP-1 cDNA sequence) and an adaptor primer. The fragments from the RACE reaction were cloned into a T/A-type cloning vector and sequenced. The transcription start site was defined as the first oligonucleotide of the 5' end MCP-1 cDNA.

The binding sites for NFκB were surveyed in the sequence of the 5'-flanking region using MatInspector computer program (25). To confirm the specificity of these sites, an EMSA was performed as

described above, using nonmutant and mutant oligonucleotides designed according to the elucidated sequences.

Statistical Analyses

Data are presented as mean ± SD of three to five separate experiments in which values were determined in triplicate. ANOVA and Fisher least significant difference method were used for comparisons among multiple means, and the unpaired *t* test was used for comparisons between two means. A *P* value of <0.05 was considered significant.

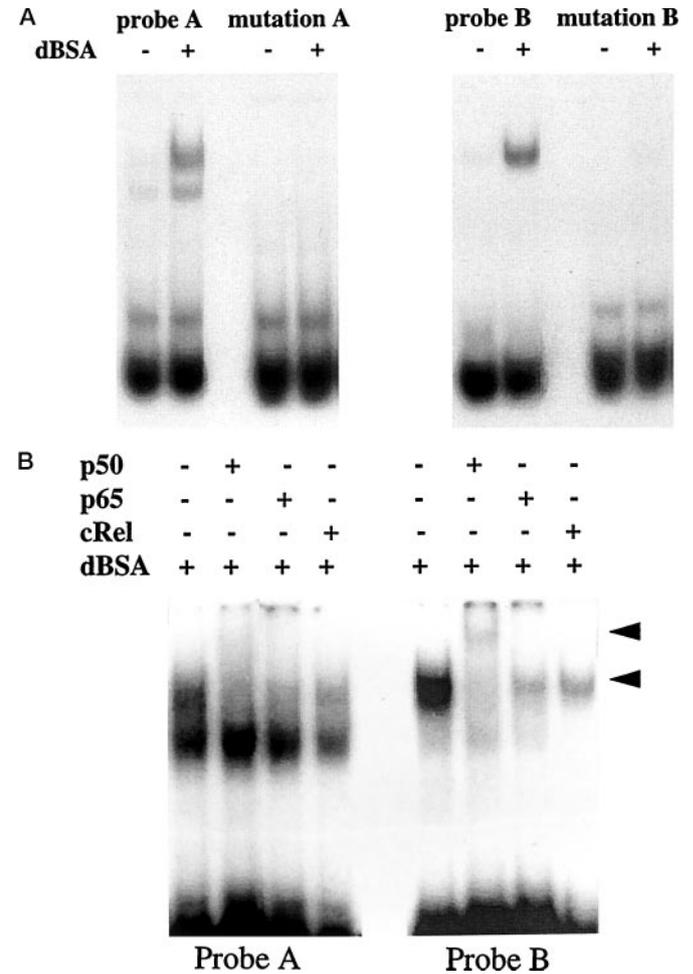


Figure 4. NFκB binding to the rat monocyte chemoattractant protein-1 (MCP-1) 5'-flanking region. Two κB consensus sequences (sites A and B) were located in the region that is identical to the enhancer region of the human MCP-1 gene. Oligonucleotide probes (probe A, GAATGGGAATTTCCACAC; probe B, GTCTGG-GAACTTCCAATG) containing these two MCP-1 κB consensus sequences and their mutations (mutation A, GAATGccATTTCACAC; mutation B, GTCTGGGAACtCggAATG) were incubated with nuclear extracts from stimulated (15 mg/ml dBSA) or unstimulated PTC. (A) dBSA increased the NFκB binding activity with probe A and probe B but not mutation A or mutation B. (B) Supershift analysis using specific probes that bind to the κB consensus sites of rat MCP-1 showed that the activated NFκB bands were composed of p50, p65, and cRel. Lower arrowhead, band containing p50/p65, p50/cRel, and p50/p50 dimers; upper arrowhead, supershifted band.

Results

Activation of NF κ B by dBSA

In unstimulated PTC, mild binding activity to NF κ B was detected in nuclear extracts using EMSA (Figure 1). In dose-response studies, as little as 2.5 mg/ml dBSA activated NF κ B and maximal activation occurred at concentrations of 15 mg/ml. In time-course studies, dBSA-induced NF κ B activity was seen by 2 h and was maximal after 8 h of continuous exposure. The NF κ B signal intensity was sustained for up to 16 h of continuous exposure to dBSA (data not shown). The addition of excess unlabeled NF κ B oligonucleotide resulted in complete absence of the band in autoradiographs, thus confirming the specificity of the reaction (Figure 2). In contrast, the signal intensity was not affected by incubation with an irrelevant oligonucleotide (activator protein-2 consensus oligonucleotide). The time-course and dose-response patterns for NF κ B activation corresponded to those for MCP-1 gene transcription that we described previously (11).

Supershift analysis was used to elucidate the composition of the activated NF κ B proteins. Incubation of extracts with the anti-p50 antibody nearly abolished the signal intensity in the autoradiographs and caused formation of a more slowly migrating, supershifted band. Incubation with the anti-p65 or anti-c-Rel antibodies, however, only partially reduced the signal intensity. This suggested that dBSA-activated NF κ B contained predominantly p50 dimers (p50/p65, p50/c-Rel, and p50/p50) and to a lesser extent p65 and c-Rel dimers (Figure 3). Supershift analysis using specific probes that bound to κ B consensus sites of rat MCP-1 also demonstrated that the composition of the activated NF κ B included p65, p50, and c-Rel (Figure 4B).

Reduction of Cytosolic I κ B Levels

To define the kinetics of regulatory proteins involved in NF κ B nuclear translocation, parallel studies were performed. Exposure to dBSA led to a rapid but transient decrease in I κ B α , which returned to control levels within 2 h (Figure 5A). I κ B β levels also decreased after 2 h of stimulation with dBSA; however, unlike I κ B α levels, no recovery was seen by 8 h (Figure 5B).

Inhibition of NF κ B

The protease inhibitor TPCK attenuated MCP-1 gene transcription and NF κ B activation after exposure to LPS or dBSA. The anti-inflammatory agent dexamethasone also reduced both NF κ B activity and MCP-1 gene transcription after exposure to dBSA (Figures 2 and 6). However, pretreatment with dexamethasone only partially reduced NF κ B and MCP-1 levels in cells stimulated with LPS.

The 5' end cDNA for the rat NF κ B p65 subunit was amplified and sequenced to 254 bp, containing the translation start codon. The sequence of this fragment is 92% identical to the mouse p65 gene and 89% identical to the human p65 gene (Figure 7). The sequence was accepted by GenBank (accession no. AF079314). Antisense oligonucleotide to p65 at a concentration of 0.5 μ M attenuated MCP-1 expression in PTC exposed to dBSA. In contrast, sense oligonucleotide (0.5 to 2

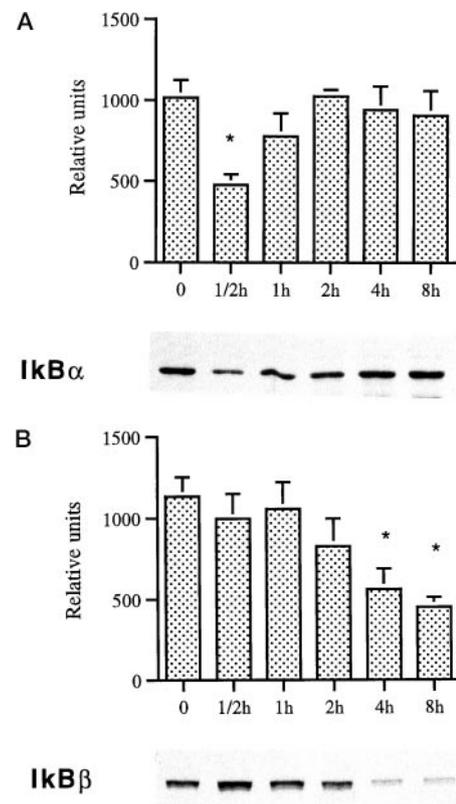


Figure 5. Western blots of cytoplasmic extracts from PTC exposed to dBSA (15 mg/ml). Inhibitory protein α (I κ B α) levels decreased rapidly, with resynthesis at 1 h (A), whereas I κ B β levels decreased later (after 2 h) and did not recover (B). The Western blots are representative of experiments that were repeated three times. * $P < 0.05$. Values are mean \pm SD.

μ M) had no effect on MCP-1 expression. MCP-1 mRNA expression was measured by semiquantitative RT-PCR and was further confirmed by RNase protection assays (Figure 8).

MCP-1 Gene

The 5'-flanking region of the rat MCP-1 gene was isolated from three of five genomic libraries (digested with *Dra*I, *Pvu*II, and *Ssp*I) and sequenced extensively up to 3657 bp. This sequence was accepted by GenBank (accession no. AF079313). The 5'-flanking region of the rat MCP-1 sequence is 65% homologous to the human MCP-1 sequence (Figure 9A). The rat MCP-1 gene has 74% homology with the human MCP-1 gene in the enhancer region and 73% homology with the human MCP-1 gene in the promoter region. Two κ B consensus sites (sites A and B) were located in the sequence that corresponds to the human MCP-1 enhancer region. The sequences of these two κ B consensus sites are identical to those of the human MCP-1 gene (Figure 9B). κ B consensus sequence A is identical to the MHC class II-associated variant-chain κ B sequence (GGGAATTTCC). Unlike the human MCP-1 gene, there is no κ B consensus site in the promoter region of the rat MCP-1 gene (Figure 9C).

An EMSA was performed to test whether NF κ B binds to the κ B consensus sequences in response to dBSA. Oligonucleotide

probes (probe A, GAATGGGAATTTCCC; probe B, GTCTGGGAAGTTCCAATGC) containing these two MCP-1 κB consensus sequences and their mutations (mutation A, GGAATGGccATTTCCACCAC; mutation B, GTCTGGGAAGTcggAATGC) were used to examine the specificity of NFκB binding to the rat MCP-1 gene. ³²P-labeled probe A and its mutation A, as well as probe B and its mutation B, were incubated with nuclear extracts from PTC stimulated or not with dBSA (15 mg/ml). dBSA increased the specific binding activity of NFκB to probe A and probe B but not mutation A or mutation B (Figure 4).

Discussion

Increasing evidence suggests that proteinuria may be an independent mediator of disease progression in CGD, rather than simply a marker of the severity of glomerular injury (4).

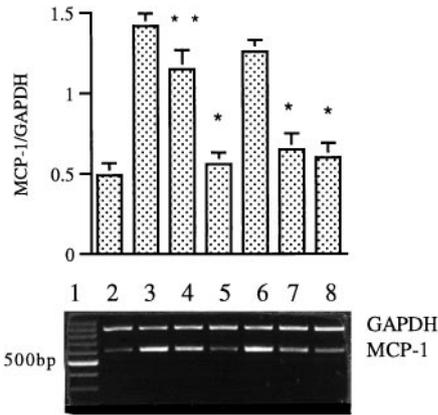


Figure 6. Effects of TPCK (100 mM) and dexamethasone (100 mM) on MCP-1/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) ratios in cells stimulated for 8 h with either LPS (5 mg/ml) or dBSA (15 mg/ml). TPCK and dexamethasone significantly reduced MCP-1 expression after stimulation with either LPS or dBSA. Lane 1, molecular markers; lane 2, unstimulated cells; lane 3, LPS; lane 4, LPS and dexamethasone; lane 5, LPS and TPCK; lane 6, dBSA; lane 7, dBSA and dexamethasone; lane 8, dBSA and TPCK. Results are from three separate experiments, each performed in triplicate. Values are mean ± SD. *P < 0.05 versus stimulated control; **P < 0.05 versus stimulated and unstimulated controls.

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Rat p65 GGTCTCAGCTGCGACCCCGGCCCGCCCGGGACCCCTGGCCATGGACGA
Human p65 GGGCCCAGCTGCGACCCCGGCCCGCCCGGGACCCCGGCCATGGACGA
Rat p65 TCTGTCTTCCCTCATCTTTCCCTCAGAGCCAGCCAGGCTTCTGGGCCAT
Human p65 ACTGTTCCTCCCTCATCTTCCCGGCAGAGCCAGCCAGGCTTCTGGCCCT
Rat p65 ATGTGGAGATCATTGAGCAGCCCAAGCAGCGGGGCATGCGTTCCGTTAC
Human p65 ATGTGGAGATCATTGAGCAGCCCAAGCAGCGGGGCATGCGTTCCGTTAC
Rat p65 AAGTGCAGGGCCGCTCTCAGGCAGTATTCTCTGGGAGAGAAGCACAGA
Human p65 AAGTGCAGGGCCGCTCTCAGGCAGTATTCTCTGGGAGAGAAGCACAGA
Rat p65 TACCACTAAGACGCACCCAA
Human p65 TACCACCAAGACCCACCCAA
    
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Figure 7. Alignment of the rat p65 cDNA fragment and human p65 cDNA. The rat sequence has 89% homology with the human p65 sequence. The start codon is in bold type and the oligonucleotide sequence for the antisense study is underlined.

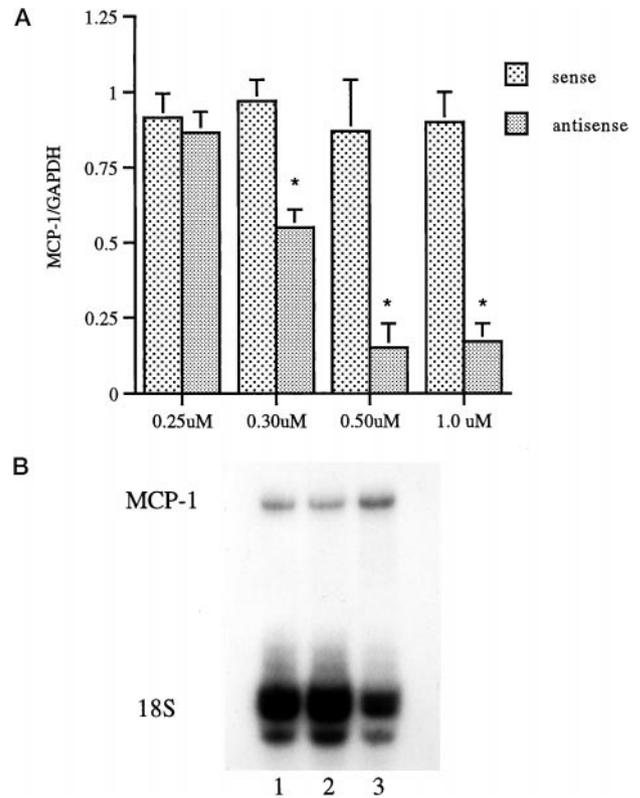


Figure 8. Inhibition of MCP-1 by p65 antisense oligonucleotides. (A) Expression of MCP-1 mRNA, determined by reverse transcription-PCR, in PTC exposed to dBSA and varying concentrations of p65 antisense oligonucleotides (0.25 to 1 μM) for 8 h. Results are from four separate experiments, each performed in triplicate. Values are mean ± SD. *P < 0.05 versus control (p65 sense oligonucleotide and dBSA). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (B) Expression of MCP-1 mRNA determined by RNase protection assay. PTC were treated with dBSA (15 mg/ml) and 0.5 μM p65 antisense or sense oligonucleotide for 8 h. Lane 1, untreated cells; lane 2, p65 antisense oligonucleotide and dBSA; lane 3, p65 sense oligonucleotide and dBSA as control. Results are representative of two separate experiments. Values are mean ± SD. *P < 0.05 versus control.

Previously, we demonstrated that albumin at pathophysiologically relevant concentrations induced transcription and translation of MCP-1, as well as its secretion across the basolateral membrane of PTC, without causing cellular toxicity (9). However, signal transduction pathways involved in this effect of albumin on MCP-1 had not yet been characterized. The results of the study presented here demonstrate that albumin-induced upregulation of MCP-1 involves the nuclear translocation of NFκB. Several pieces of evidence support this contention, as follows: (1) albumin activated NFκB in a time- and dose-dependent manner, which was correlated with MCP-1 gene transcription; (2) inhibition of NFκB with either TPCK or dexamethasone reduced MCP-1 expression; (3) specific inhibition of NFκB using antisense oligonucleotides against the rat p65 subunit completely attenuated MCP-1 expression in cells stimulated with dBSA; and (4) the 5'-flanking region of the rat MCP-1 gene contained two putative binding sites for κB proteins.



Figure 9. (A) Sequence of the 5'-flanking region of the rat MCP-1 gene. The 3657-bp nucleotide sequence of the 5'-flanking region sequence of the rat MCP-1 gene is 65% homologous with the human MCP-1 sequence. The consensus sequences for κB binding (sites A and B) are underlined. The ATG initiation codon is in bold type. (B) Alignment of the enhancer regions of the rat and human MCP-1 genes. Nucleic acid identity between the rat and human genes (vertical lines) is 74%. Two NFκB binding sites exist in the enhancer regions of both rat and human MCP-1 genes and show 100% homology. (C) Alignment of the promoter regions of the rat and human MCP-1 genes. The rat MCP-1 gene has 73% homology with the human gene in the promoter region. NFκB consensus sequences exist in the human MCP-1 promoter region but not in the rat gene.

Our results are in agreement with previous reports documenting that NF κ B can be activated by albumin in PTC (13). This study is unique in that these results have been confirmed in tubular cells in primary culture, and it has been shown that albumin-induced NF κ B activation is also involved in the regulation of MCP-1. Furthermore, this study has provided additional information regarding the effect of albumin on NF κ B. First, continuous exposure to albumin caused sustained activation of NF κ B for as long as 16 h, whereas only earlier time points (15 to 30 min) were previously reported (13,23). Although our study should also be regarded as short term, it is conceivable that chronic activation of NF κ B in PTC by urinary proteins could take place *in vivo*. In support of this possibility, we recently showed that rats with proteinuric interstitial inflammation in adriamycin-induced nephrosis have increased levels of renal cortical activated NF κ B 4 wk after disease induction (26). Analysis of activated NF κ B proteins using gel retardation assays showed them to be composed of p50, p65, and cRel dimers. This is consistent with the findings of Zoja *et al.* (13), who demonstrated that activated NF κ B was composed of a triad of p65/p65 homodimers and p50/cRel and p50/p65 heterodimers in LLC-PK₁ cells stimulated with either albumin or tumor necrosis factor- α (TNF- α). However, Amoah-Apraku *et al.* (23) found that LPS activated primarily p65 and to a lesser extent p50 proteins but not cRel in murine PTC. These observations may be of particular relevance because the composition of translocated NF κ B seems to be important in the selectivity of gene activation with different stimuli. For example, NF κ B activation in murine PTC was required but not sufficient for inducible nitric oxide synthase induction, possibly because of the absence of cRel in these cells. cRel was identified as being a critical factor for inducible nitric oxide synthase transcription in a macrophage cell line as well (23). p65, cRel, and their dimers are thought to be important for MCP-1 gene activation, whereas p50 has no effect on MCP-1 gene activation (27,28) and p50 homodimers seem to have a negative regulatory effect on IL-2 gene activation (29).

The activation and regulation of NF κ B dimers are tightly controlled by a family of protein monomers termed I κ B. Stimuli that activate NF κ B result in proteolysis of I κ B and thus a reduction in the concentration of these proteins in the cytosol. I κ B α is the most well-studied member of this family and has the highest affinity for cRel- and p65-containing NF κ B dimers (19). The recently identified I κ B β has the same affinity for NF κ B proteins as does I κ B α . However, reduction of I κ B β concentrations has been demonstrated after stimulation with IL-1 or LPS but not TNF- α or phorbol-12-myristate-13-acetate (30). In the study presented here, albumin led to a rapid but transient reduction in I κ B α levels and a more persistent decrease in I κ B β levels, a pattern resembling that of cells stimulated with either IL-1 or LPS. The reappearance of I κ B α is probably attributable to NF κ B activation of the I κ B α gene. This has been proposed to be an internal counter-regulatory mechanism to prevent excessive activation of NF κ B (20). This phenomenon has been reported in other cell types and may result in I κ B α levels equal to or exceeding those found in unstimulated cells. In our study, the fact that NF κ B levels were

not reduced by the time I κ B α levels recovered may be explained by synthesis of new NF κ B protein. The mechanisms underlying sustained activation of NF κ B by dBSA are not clear. These results indicate that the rapid degradation of I κ B α could account for activation of NF κ B in the early stages. The persistent degradation of I κ B β could contribute to more prolonged activation of NF κ B in the later stages. Sustained activation of NF κ B in response to dBSA might be explained by continued production of NF κ B proteins escaping the inducible control exerted by limited amounts of I κ B (29).

MCP-1 transcription is known to directly involve NF κ B activation in synovial, endothelial, smooth muscle, and mesangial cells (21,28,31). Furthermore, the expression of MCP-1 has been correlated with activation of NF κ B in *in vivo* models of immune complex nephritis and inflammation (32,33). However, it is known that MCP-1 gene expression is cell- and stimulus-specific; therefore, it was not possible to assume that NF κ B would be involved in MCP-1 induction by albumin in PTC. In this study, gene transcription was reduced by inhibition of NF κ B activation by two different methods. Inhibition with dexamethasone and TPCK attenuated MCP-1 gene expression, as assessed by both RT-PCR and RNase protection assays. Similar results were obtained with antisense oligonucleotides against the rat p65 subunit, which were used to specifically block NF κ B synthesis. This provided strong evidence that MCP-1 transcription, as induced by albumin, does depend on the activation of NF κ B in PTC. To strengthen this proposition, we analyzed the 5'-flanking region of the rat MCP-1 gene. Although human and mouse MCP-1 promoter regions are known to contain putative κ B binding sites, the rat gene had not been defined. Therefore, in our study, it was necessary to clone and sequence the 5'-flanking region of the rat MCP-1 gene. The fragment showed 65% homology with the human gene, and two putative κ B binding sites were identified in the region identical to the human enhancer region. To prove the functional significance of these NF κ B binding sites and to conclusively demonstrate the direct role of NF κ B, transfection studies using MCP-1 reporter constructs will be required.

The specific molecular pathways by which albumin activated NF κ B and subsequently MCP-1 are not known. Albumin uptake in PTC is receptor-mediated, involving the transmembrane molecule megalin (34). Lysosomal autolysis after excessive intake of reabsorbed proteins may cause sublethal cell damage, and cellular injury is known to activate NF κ B and MCP-1. In fact, tubular injury frequently accompanies interstitial inflammation in CGD. However, in recent studies, we found that holotransferrin but not albumin caused cell membrane damage (14), but both proteins increased MCP-1 transcription in PTC when tested individually (9). These data suggest the possibility that individual urinary proteins have unique mechanisms of signal transduction. At least in LLC-PK₁ cells, albumin- and IgG-induced NF κ B activation is inhibited by the antioxidant pyrrolidone dithiocarbamate, suggesting that reactive oxygen species generation or a pyrrolidone dithiocarbamate-sensitive transcription site is involved (13). Whether protein kinase C, cAMP, and protein

tyrosine kinase pathways, which are known to be critical intracellular components of MCP-1 signal transduction in mesangial cells (22), are involved in PTC exposed to urinary proteins requires further investigation.

Substantial evidence has established that NF κ B proteins have a role in controlling a wide variety of genes regulating inflammation, immunologic responses, and cell growth and differentiation. Although the prospect was not investigated in this study, it is possible that albumin and other urinary proteins may also affect genes, other than MCP-1 or RANTES, that are known to be regulated by NF κ B. For example, albumin and proteinuric urine from nephrotic rats induces PTC hypertrophy (35), and it is possible that these effects involve *c-myc*, a gene induced by NF κ B (19). Furthermore, albumin induces the transcription of IL-1 β , TNF- α , IL-10, and IL-6, genes that are known to be regulated by NF κ B (26,36). However, it should be noted that albumin and oxidized lipoproteins cause the production of fibronectin in PTC (7,11). In addition to being a matrix protein, fibronectin is a potent chemoattractant and is not known to be regulated by NF κ B (37). Therefore, it is possible that transcription factors in addition to NF κ B are involved in albumin signaling pathways.

In summary, these studies provide additional evidence linking proteinuria and tubulointerstitial injury in CGD. Specifically, the data support the hypothesis that MCP-1 induction by albumin in PTC is dependent on the activation of NF κ B. Furthermore, the identification of the rat p65 gene and the 5'-flanking region of the rat MCP-1 gene could facilitate the exploration of the effects of NF κ B and other transcription-regulating factors on MCP-1 gene expression, as well as the design of interventions to modulate the production of MCP-1 in interstitial inflammation.

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References

- Ridson RA, Sloper JC, De Wardener HE: Relationship between renal function and histological changes found in renal biopsy specimens from patients with persistent glomerular nephritis. *Lancet* 1: 363–366, 1968
- Nath KA: Tubulointerstitial changes as a major determinant in the progression of renal damage. *Am J Kidney Dis* 20: 1–17, 1992
- Eddy AA: Experimental insights into the tubulointerstitial disease accompanying primary glomerular lesions. *J Am Soc Nephrol* 5: 1273–1287, 1994
- Remuzzi G, Ruggenenti P, Benigni A: Understanding the nature of renal disease progression. *Kidney Int* 51: 2–15, 1997
- Ong ACM, Jowett TP, Morrhead JF, Owen JS: Human high density lipoproteins stimulate endothelin-1 release by cultured human renal proximal tubular cells. *Kidney Int* 46: 1315–1321, 1994
- Zoja C, Morigi M, Figliuzzi M, Bruzzi I, Oldroyd S, Benigni A, Ronco P, Remuzzi G: Proximal tubular synthesis and secretion of endothelin-1 on challenge with albumin and other urinary proteins. *Am J Kidney Dis* 26: 934–941, 1995
- Burton CJ, Combe C, Walls J, Harris KPG: Fibronectin production by human tubular cells: The effect of apical protein. *Kidney Int* 50: 760–767, 1996
- Kees-Folts D, Sadow JL, Schreiner GF: Tubular catabolism of albumin is associated with the release of an inflammatory lipid. *Kidney Int* 45: 1697–1709, 1994
- Wang Y, Chen J, Chen L, Tay YC, Rangan GK, Harris DCH: Induction of monocyte chemoattractant protein-1 in proximal tubular cells by urinary protein. *J Am Soc Nephrol* 10: 1537–1545, 1997
- Thakkar H, Burrin JM, Newman DJ: RBP and α 1M cause a more potent and differential cytokine expression than albumin in human proximal tubular cells [Abstract]. *J Am Soc Nephrol* 8: 487A, 1997
- Roh DD, Kamanna VS, Kirschenbaum MA: Atherogenic lipoproteins enhance murine cortical epithelial cell fibronectin protein synthesis and gene expression. *Am J Nephrol* 18: 83–88, 1998
- Arrizabalaga P, Sole M, Quinto IL, Ascaso C: Intercellular adhesion molecule-1 mediated interactions and leucocyte infiltration in IgA nephropathy. *Nephrol Dial Transplant* 12: 2258–2262, 1997
- Zoja C, Donadelli R, Colleoni S, Figliuzzi M, Bonazzola S, Morigi M, Remuzzi G: Protein overload stimulates RANTES production by proximal tubular cells depending on NF- κ B activation. *Kidney Int* 53: 1608–1615, 1998
- Chen L, Boadle R, Harris DCH: Toxicity of holotransferrin but not albumin in proximal tubule cells in primary culture. *J Am Soc Nephrol* 9: 77–84, 1998
- Boucher A, Droz D, Adafer E, Noel LH: Characterization of mononuclear subsets in renal cellular interstitial infiltrates. *Kidney Int* 29: 1043–1049, 1986
- Danoff TM: Chemokines in interstitial injury. *Kidney Int* 53: 1807–1808, 1998
- Lloyd CM, Minto AW, Dorf ME, Proudfoot A, Wells TN, Salant DJ, Gutierrez-Ramos JC: RANTES and MCP-1 play an important role in the inflammatory phase of crescentic nephritis but only MCP-1 is involved in crescent formation and interstitial fibrosis. *J Exp Med* 185: 1371–1380, 1997
- Ueda A, Okuda K, Ohno S, Shirai A, Igarashi T, Matsunaga K, Fukushima J, Kawamoto S, Ishigatsubo Y, Okubo T: NF- κ B and Sp1 regulate transcription of monocyte chemoattractant protein-1 gene. *J Immunol* 153: 2052–2055, 1994
- Baueerle PA, Henkel T: Function and activation of NF- κ B in the immune system. *Annu Rev Immunol* 12: 141–179, 1994
- Thanos D, Maniatis T: NF κ B: A lesson in family values. *Cell* 80: 529–532, 1995
- Rovin BH, Dickerson JA, Tan LC, Herbert CA: Activation of nuclear factor- κ B correlates with MCP-1 expression by human mesangial cells. *Kidney Int* 48: 1263–1271, 1995
- Rovin BH, Dickerson JA, Tan LC, Fassler J: Modulation of IL-1 induced chemokine expression in human mesangial cells through alterations in redox status. *Cytokine* 9: 178–186, 1997
- Amoah-Apraku B, Chandler LJ, Harrison JK, Tang SS, Ingelfinger JR, Guzman NJ: NF- κ B and transcriptional control of renal epithelial-inducible nitric oxide synthase. *Kidney Int* 48: 674–682, 1995
- Dingnam JD, Lebovitz RM, Roeder RG: Accurate transcription

- initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* 11: 1475–1489, 1983
25. Quandt K, Frech K, Karas H, Wingender E, Werner T: MatInd and MatInspector: New fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res* 23: 4878–4884, 1995
 26. Rangan GK, Wang Y, Tay YC, Harris DCH: N-Acetylcysteine does not attenuate established tubulointerstitial inflammation [Abstract]. In: *Proceedings of the 34th Annual Scientific Meeting*, Auckland, New Zealand, Australian and New Zealand Society of Nephrology, 1998, p A61
 27. Ueda A, Ishigatsubo Y, Okubo T, Yoshimura T: Transcriptional regulation of human monocyte chemoattractant protein-1 gene: Cooperation of two NF- κ B sites and NF- κ B/subunit specificity. *J Biol Chem* 272: 31092–31099, 1997
 28. Martin T, Cardarelli PM, Parry GC, Felts KA, Cobb RR: Cytokine induction of monocyte chemoattractant protein-1 gene expression in human endothelial cells depends on the cooperative action of NF- κ B and AP-1. *Eur J Immunol* 27: 1091–1097, 1997
 29. Grimm S, Baeuerle P: The inducible transcription factor NF- κ B: Structure-function relationship of its protein subunits. *Biochem J* 290: 297–308, 1993
 30. Thompson JE, Philips RJ, Erdjument-Bromage H, Tempst P, Ghosh G: I κ B regulates the persistent response in biphasic activation of NF κ B. *Cell* 80: 573–582, 1995
 31. Landry DB, Couper LL, Bryant SR, Lindner V: Activation of the NF- κ B and I κ B system in smooth muscle cells after rat arterial injury: Induction of vascular cell adhesion molecule-1 and monocyte chemoattractant protein-1. *Am J Pathol* 151: 1085–1095, 1997
 32. Morrissey JJ, Klahr S: Enalapril decreases nuclear factor κ B activation in the kidney with ureteral obstruction. *Kidney Int* 52: 926–933, 1997
 33. Ruiz-Ortega M, Bustos C, Hernandez-Presa MA, Lorenzo O, Plaza JJ, Egido J: Angiotensin II participates in mononuclear cell recruitment in experimental immune complex nephritis through nuclear factor- κ B activation and monocyte chemoattractant protein-1 synthesis. *J Immunol* 161: 430–439, 1998
 34. Cui S, Verroust PJ, Moestrup SK, Christensen EI: Megalin/gp330 mediates uptake of albumin in renal proximal tubule. *Am J Physiol* 271: F900–F907, 1996
 35. Burton CJ, Bevington A, Harris KPG, Walls J: Growth of proximal tubular cells in the presence of albumin and proteinuric urine. *Exp Nephrol* 2: 345–350, 1994
 36. Tay YC, Wang Y, Rangan GK, Harris DCH: Proinflammatory cytokine expression in rat proximal tubules in primary culture [Abstract]. In: *Proceedings of the 34th Annual Scientific Meeting*, Auckland, New Zealand, Australian and New Zealand Society of Nephrology, 1998, p A55
 37. Dean DC: Expression of the fibronectin gene. *Am J Respir Cell Mol Biol* 8: 463–470, 1989