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Chemokines, Osteopontin, ICAM-1 Gene Expression in Cultured Rat Mesangial Cells

To investigate whether MCP-1, CINC, RANTES, osteopontin and ICAM-1 mRNA could be induced in cultured rat mesangial cells by interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and lipopolysaccharide (LPS), and whether MCP-1 and CINC gene expression could be modulated by dexamethasone, Northern blot assays were performed. IL-1β induced MCP-1, CINC, RANTES and ICAM-1 gene expression in a time dependent manner. IL-1β-induced MCP-1, CINC and ICAM-1 mRNA amount were maximal at 3 hours exposure around 14.5, 15.7, 2.2 folds increase and IL-1β-induced RANTES mRNA at 24 hours around 2.0 folds. TNF-α and LPS also induced MCP-1 and ICAM-1 gene expression. TNF-α also induced RANTES gene expression but LPS did not. On the other hand, IL-1β, TNF-α and LPS had little effect on osteopontin gene expression but fetal calf serum could increase osteopontin mRNA. Dexamethasone suppressed the IL-1β-induced MCP-1 and CINC mRNA. These results suggest that, through these gene expressions, mesangial cells are able to communicate directly or indirectly with macrophages or neutrophils, which may lead to glomerulosclerosis.

Key Words : *Glomerular mesangium; MCP-1; RANTES; CINC; Osteopontin; ICAM-1*

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INTRODUCTION

The role of macrophages in the pathogenesis of glomerulonephritis is becoming apparent (1). Proinflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), which are secreted mainly by infiltrating macrophages, are likely to modulate the biologic activities of intrinsic renal cells, especially mesangial cells, which can also recruit and activate macrophages by secreting macrophage specific chemotactic factors. Mesangial cells are also capable of generating a number of cytokines including IL-1β, growth factors and inflammatory mediators which may be involved in local inflammation and tissue damage. Thus the interaction between macrophages and mesangial cells may be important in the initiation and progression of renal injury in glomerulonephritis (2).

Recently a superfamily of chemokines which can recruit and activate leukocytes at sites of inflammation, has been identified. They are basic heparin-binding polypeptides with a molecular weight ranging from 8 to 10 kDa and divided into two subfamily based on the disposition of the first two of the four conserved cysteine residues. The C-X-C class chemokines consisting of molecules such as interleukin-8, GRO/CINC, ENA-78, NAP-2 and MIP-2 are chemoattractant primarily for neutrophils whereas the C-C class chemokines such as MCP-1, RANTES, MIP-1, and I-309 appear to attract mainly monocytes. In addition to their chemotactic activities, most of the chemokines also activate several functions of leukocytes, such as degranulation and oxidant burst, modulation of leukocyte adhesiveness, vasoconstriction, vascular permeability and angiogenesis (3).

Osteopontin which was originally isolated as a matrix molecule in bone, is now known to be produced by a variety of cell types including intrinsic renal cells, mainly by tubular epithelium. Osteopontin is also known as a possible chemotactic factor for monocyte and as an important mediator in tubulointerstitial injury in glomerulonephritis (4). But little is known about the gene expression of osteopontin in mesangial cells.

For the accumulation of macrophages in tissues, expression of adhesion molecules on the tissue is also necessary (2). Among the adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) appears to play a major role in glomerulonephritis. Increased glomerular expres-

sion of ICAM-1 is a common finding in the various forms of glomerulonephritis and generally the intensity of ICAM-1 correlates with disease activity (5).

Besides proinflammatory cytokines such as IL-1β and TNF-α, lipopolysaccharide (LPS), a potent proinflammatory component of bacterial cell wall, can promote glomerular inflammation by stimulating mesangial cell production of several cytokines and growth factors.

In this study, we investigated whether MCP-1 (monocyte chemoattractant protein), RANTES (regulated upon activation, normal T cell expressed and secreted), CINC (cytokine induced neutrophil chemoattractant), osteopontin and ICAM-1 gene expression could be induced in cultured rat mesangial cells by IL-1β, LPS and TNF-α, and whether MCP-1 and CINC gene expression could be modulated by dexamethasone.

MATERIAL AND METHOD

Mesangial cell culture

Glomeruli were isolated from 4 to 6 week old Sprague-Dawley rats by the differential sieving method, as previously described (6). After exposure to 0.1% collagenase (Gibco, Gaithersburg, USA), the glomeruli were placed in a tissue culture flask and cultured in RPMI 1640 culture media supplemented with 11.5 mM HEPES, 26.2 mM sodium bicarbonate, penicillin (100 U/ml), streptomycin (100 μg/ml), fungizone (250 ng/ml) and 20% fetal calf serum at 37℃ in 5% CO_2. The media were changed every 3 days and cells were subcultured by exposing to 0.05% trypsin and 0.02% EDTA, when they became confluent. Characterization of mesangial cells and culture purity were assessed by phase contrast microscopy and by staining, which revealed characteristic actin-positive cytoskeletal components that were negative for cytokeratin and factor VIII antigens.

After mesangial cells were cultured for 48 hours in serum free RPMI media, each stimulant such as IL-1β (1 ng/ml), TNF-α (5 ng/ml), and LPS (1 μg/ml) was added and incubated. Just in case that gene expression was not induced by IL-1β, TNF-α, and LPS, mesangial cells were also stimulated by fetal calf serum (FCS, 20%) which acted as a potent nonspecific stimulator, as a second experiment.

Total RNA was extracted before and 3, 6, 24, 48 hours after stimulation. Recombinant human IL-1β and TNF-α were obtained from R&D system. Lipopolysaccharide and dexamethasone were obtained from Sigma Chemical Company.

RNA isolation and Northern blot hybridization

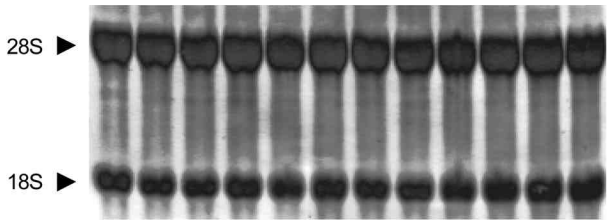

Fig. 1. Methylene blue staining of ribosomal RNA, demonstrating integrity and equivalent loading of RNA.

Total cellular RNA was isolated by using the Tri reagent kit® (Molecular Research Center, INC, Cincinnati, OH, USA). Amount of RNA was quantitated by absorbance at 260 nm and purity of RNA was assessed by absorbance ratio at 260/280 nm.

RNA (10 μg/lane) was electrophoresed through 1% agarose, 2.2 M formaldehyde denaturing gel with Mopp buffer, followed by capillary transfer to nylon membranes. After transfer, RNA integrity was assessed by methylene blue staining (Fig. 1). After baking for 2 hours at 80℃, the filters were prehybridized at 65℃ in 0.5 M $NaHPO_4$ buffer, pH 7.0, containing 1mM EDTA, 7% sodium dodecyl sulfate, and 1% bovine serum albumin. Then the filters were hybridized with ^{32}P labelled cDNA probes and 100 μg/ml salmon sperm DNA at 65℃ overnight. cDNA probes were labelled with ^{32}P by the random primer method (Megaprime™ DNA labelling system, Amersham International plc, England). The filters were exposed to film and autoradiographed at -80℃ after being washed. Intensities of the bands on autoradiogram were quantitated by the scanning laser densitometry (GS-670 imaging densitomter, Bio-Rad). The filters were also reprobed with GAPDH cDNA to correct for variation in RNA loading and transfer efficiency. mRNA levels were calculated as a density ratio.

All data were expressed as mean±SE.

Generation of cDNA probes

cDNA probes were made by reverse transcription-polymerase chain reaction (RT-PCR) method. RT was performed under the following conditions: As a template, total RNA extracted from IL-1β-stimulated rat mesangial cells were used for first-strand cDNA synthesis with 100 U Moloney murine leukemia virus H- reverse transcriptase (Superscript®, BRL, Gaithersburg, MD, USA), 0.5 μg oligo $(dT)_{15}$, 20U RNAsin, 10 mM dithiothreitol, 0.5 mM dNTP and 1% bovine serum albumin in the buffer provided by the manufacturer in a total volume of 20 μl at 42℃ for 1 hour. After RT, PCR was performed in PCR buffer containing 0.25 mM dNTP, 12.5 pmol of each primer and 1.25 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA) in a total volume of 50 μl

with initial denaturation at 94℃ for 3.5 minutes, followed by 30 cycles with the following sequential steps: denaturation at 94℃ for 1.5 minutes, annealing at 54℃ for 1.5 minutes and extension at 72℃ for 1.5 minutes. Samples were incubated at 72℃ for an additional 8 minutes before completion. Each PCR primer set was synthesized (from Korea Biotech, Inc, Korea) based on the published cDNA sequences for rat MCP-1 (7), RANTES (direct submission to GeneBank), CINC (8), osteopontin (9), ICAM-1 (10) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (11).

The sequences of each primer were as follows:

MCP-1:	forward primer 5' GATCTCTGCAGCTGCATCC 3'
	backward primer 5' CTTCTCTGGGTTGGCACAC 3'
RANTES:	forward primer 5' GATCTCTGCAGCTGCATCC 3'
	backward primer 5' CTTCTCTGGGTTGGCACAC 3'
CINC:	forward primer 5' CAGAGCACCATGGTCTCAGC 3'
	backward primer 5' ACGACCATCGATGAAACGC 3'
Osteopontin:	forward primer 5' CCTCCTGTCTCCCGGTGAA 3'
	backward primer 5' CTCGGCACTATCGATCGCA 3'
ICAM-1:	forward primer 5' TTCGTTCCCAGAGCGAGTG 3'
	backward primer 5' CACCGTGGGCTTCACACTT 3'
GAPDH:	forward primer 5' TCACCATCTTCCAGGAGCG 3'
	backward primer 5' CTGCTTCACCACCTTCTTGA 3'

PCR products were electrophoresed on an ethidium bromide-stained 1% agarose gel. Gene specific bands were visualized with UV. The sequences of the RT-PCR-generated 424 nucleotide(nt) MCP-1, 232-nt RANTES, 492-nt CINC, 710-nt osteopontin, 680-nt ICAM-1, 572-nt GAPDH matched completely with that of the corresponding rat cDNA sequences (data not shown). Each PCR products in agarose gel were extracted using Jetsorb gel extraction kit (Genomed Inc, USA). Purified PCR products were used as probes for Northern hybridization.

RESULTS

Chemokine gene expressions such as MCP-1, CINC and RANTES in mesangial cells

IL-1β induced MCP-1, CINC and RANTES gene expression in a time dependent manner (Fig. 2, 3). IL-1β-induced MCP-1 and CINC mRNA amount were maximal at 3 hour exposure around 14.5±0.4, 15.7±0.7 folds increase respectively (Fig. 2) and IL-1β-induced RANTES mRNA at 24 hours around 2.00±0.04 folds relative to unstimulated control (Fig. 3). Their expression remained elevated for 48 hours. TNF-α and LPS also induced MCP-1 gene expression (Fig. 4). TNF-α increased RANTES mRNA, maximally at 24 hours around 2.00±0.29 folds but LPS had little effect on RANTES gene expression (Fig. 3).

Osteopontin and ICAM-1 gene expression in mesangial cells

IL-1β, TNF-α, and LPS increased ICAM-1 gene expression in a time dependent manner (Fig. 5). IL-1β in-

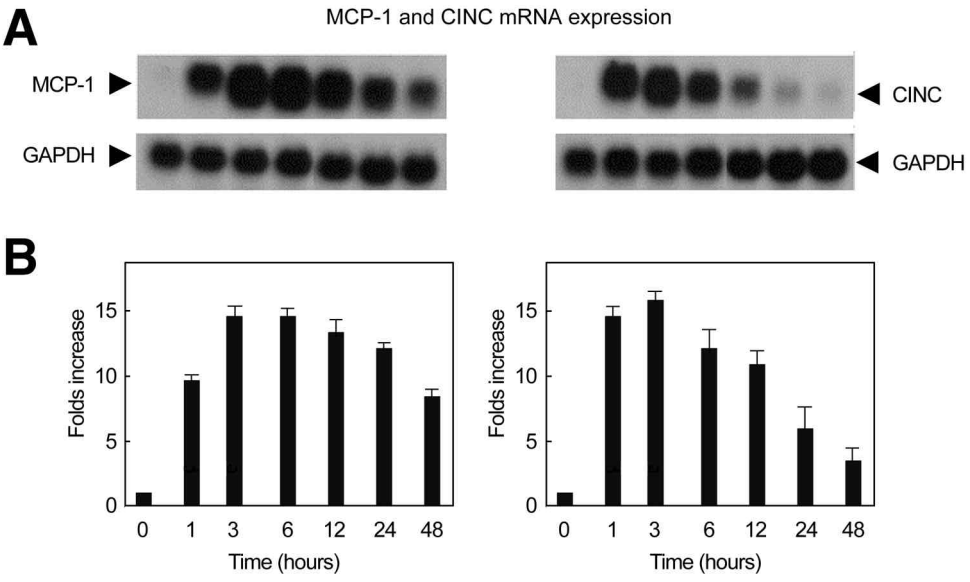

Fig. 2. Time dependent induction of MCP-1 and CINC gene expression by IL-1β.
A) A representative of autoradiography.
B) Densitometric quantitation normalized by GAPDH mRNA
Data were expressed as mean ± SE, (n=3).

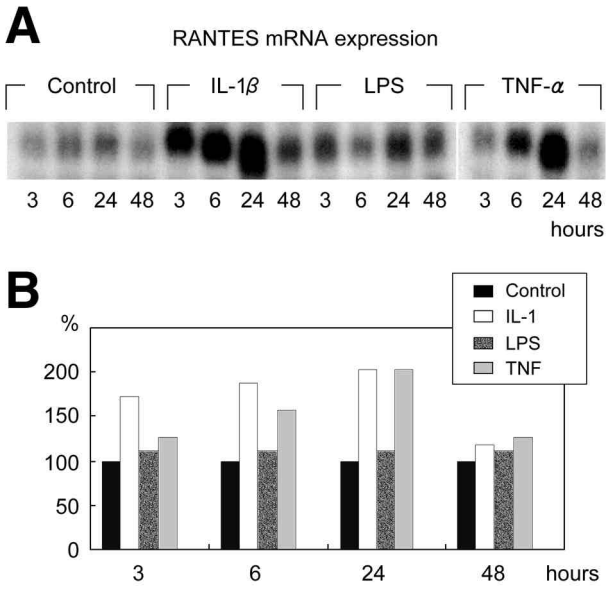

Fig. 3. Time dependent induction of RANTES mRNA expression in rat mesangial cells.
A) A representative of autoradiography
B) Densitometric quantitation of RANTES mRNA normalized by GAPDH mRNA (n=3).

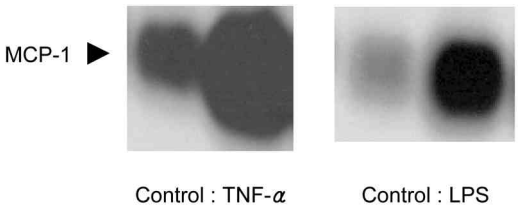

Fig. 4. Induction of MCP-1 gene expression by TNF-α (5 ng/ml) or LPS (1 μg/ml).

duced-ICAM-1 mRNA amount was maximal at 3 hour exposure around 2.2±0.1 folds increase and remained elevated for a 48 hour period. On the other hand, IL-1β, TNF-α, LPS had little effect on osteopontin gene expression but FCS could increase osteopontin mRNA around 2 folds over 48 hour period (Fig. 6).

Effect of dexamethasone on the IL-1β-induced MCP-1 and CINC gene expression

Dexamethasone suppressed the IL-1β-induced MCP-1 and CINC mRNA by about 73.7±2.2%, 40.0±5.1% respectively at a concentration of 10^{-6} M (Fig. 7) and inhibited IL-1β-induced MCP-1gene expression in a dose dependent manner (10^{-6}-10^{-10} M) (Fig. 8).

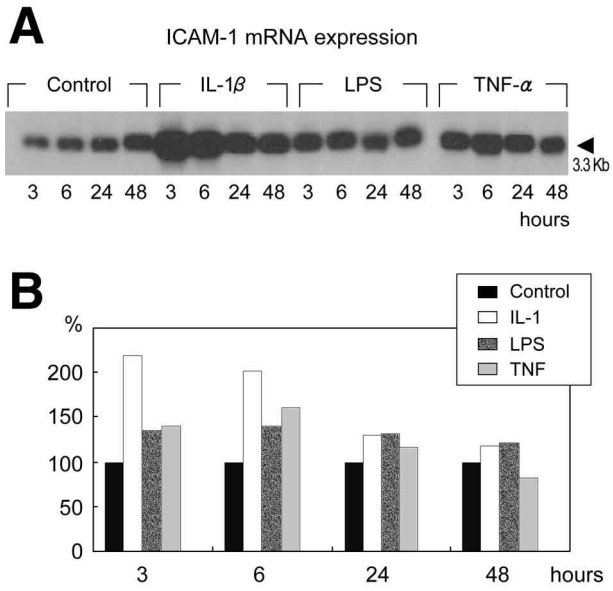

Fig. 5. Time dependent induction of ICAM-1 mRNA expression in rat mesangial cells.
A) A representative of autoradiography
B) Densitometric quantitation of ICAM-1 mRNA normalized by GAPDH mRNA: Data were expressed as a percentage control with the mean control value being 100% (n=3).

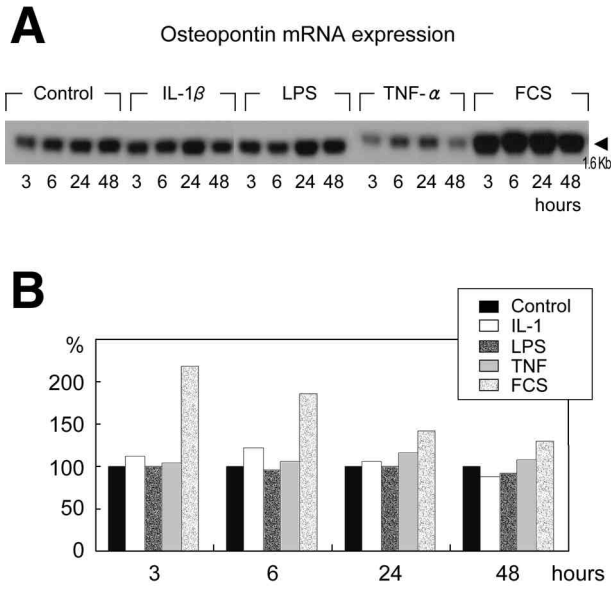

Fig. 6. Time dependent induction of osteopontin mRNA expression in rat mesangial cells.
A) A representative of autoradiography
B) Densitometric quantitation of osteopontin mRNA normalized by GAPDH mRNA (n=3).

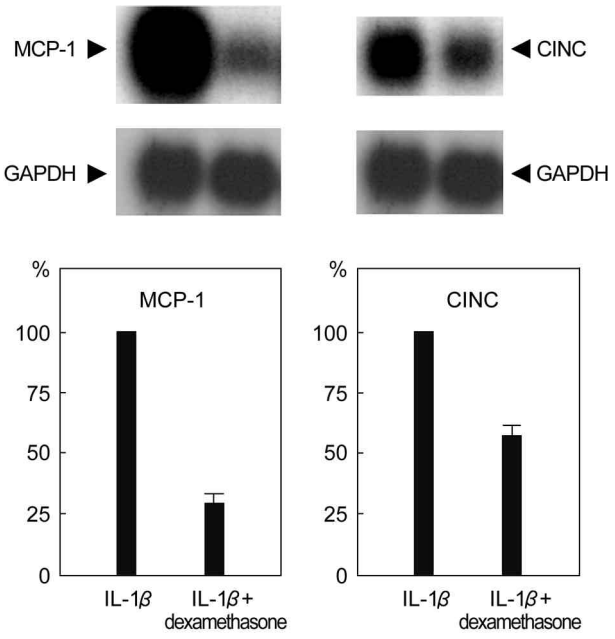

Fig. 7. Suppression of IL-1β induced-MCP-1 and CINC gene expression by dexamethasone. Mesangial cells were stimulated by 1 ng/ml IL-1β for 3 hours with or without 10^{-6} M dexamethasone. Data were expressed as mean ± SE (n=3).

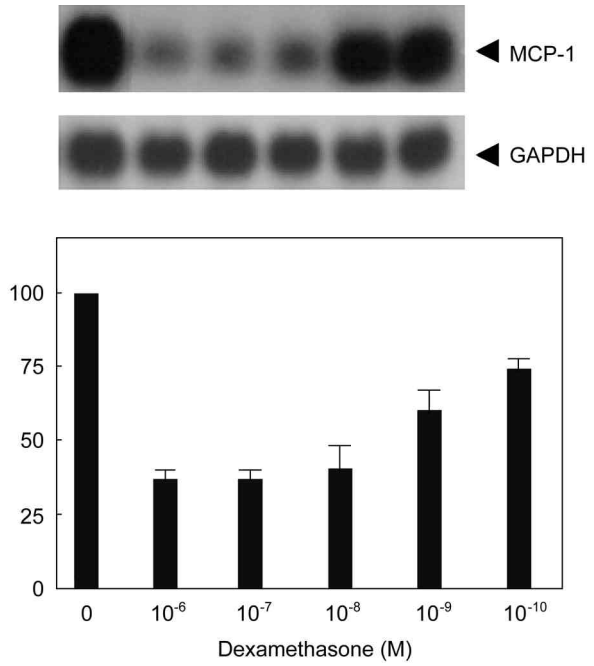

Fig. 8. Dose dependent suppression of IL-1β induced-MCP-1 gene expression by dexamethasone. Data were expressed as mean ± SE (n=2).

DISCUSSION

We found that, in mesangial cells, MCP-1, CINC and ICAM-1 gene expressions were induced by IL-1β, TNF-α, and LPS in a time dependent manner. RANTES gene expression was also induced by IL-1β and TNF-α. On the other hand, IL-1β, TNF-α and LPS had little effect on osteopontin gene expression, which was clearly different to that for MCP-1, CINC, RANTES and ICAM-1. But FCS which served as a nonspecific stimulator, could induce osteopontin mRNA in mesangial cells. Thus the capability of these chemotactic factors and ICAM-1 production by mesangial cells could contribute to the recruitment of monocytes and neutrophils into glomeruli. Through these cross-talks between mesangial cells and macrophages, cell proliferation, extracellular matrix expansion and even glomerulosclerosis might be developed in glomerulonephritis.

Our results were consistent with those of previous reports for MCP-1 (12, 13), RANTES (14), ICAM-1 (15). But the gene expression of CINC or osteopontin in mesangial cells has not been widely known. CINC, the rat homologues of human GROα, is known to act as a functional chemoattractant for neutrophils (16). In general, neutrophil accumulation at inflammatory sites is thought to be mediated in part by locally produced chemoattractants such as C5a fragment of serum complement and leukotriene B4. The findings that CINC gene expression could be induced by IL-1β, TNF-α, and LPS in mesangial cells, implied that infiltration of neutrophil could occur without complement activation, which suggested a possible role of CINC as an important mediator in the pathogenesis of non-immunologic glomerular injury.

Osteopontin which was originally isolated as a matrix molecule in bone, is now known to be produced by a variety of cell types including renal tubular epithelium, macrophages, T cell lymphocytes and smooth muscle cells. Osteopontin is a highly acidic glycosylated phosphoprotein with RGD sequences. The biological roles of osteopontin seem to be involved in facilitating cell attachment and spreading by interacting with vitronectin receptor and also acting as chemoattractant for macrophages as well as smooth muscle cells. In kidney, osteopontin is produced mainly by cortical tubular epithelium and also known as an important mediator in tubulointerstitial injury in glomerulonephritis. A number of cytokines, such as TGF-α, EGF, IL-1β, TNF-α and PDGF have been shown to stimulate osteopontin synthesis in various cells of non-renal origin (4). We confirmed that, in rat mesangial cells, IL-1β, TNF-α and LPS had little effect on osteopontin gene expression but fetal calf serum could increase osteopontin mRNA. The fact that osteopontin mRNA could be induced also in mesangial cells, implied a possible role of glomerular osteopontin in the pathogenesis of glomerular inflammation and injury.

It has been known that some chemokine gene expressions show differential time response to different stimuli, which may be involved in the temporal association of renal injury (17). Our results showed that there were no time differences in maximal gene expression of MCP-1, CINC, ICAM-1, no matter what the stimuli were (IL-1β or TNF-α or LPS). Maximal induction time was around 3 hours after stimulation. But maximal induction time of RANTES gene expression by IL-1β and TNF-α was observed at 24 hours exposure.

Dexamethasone, used as anti-inflammatory and immunomodulatory agents, exerts its effects by modulating the expression of many cytokine genes such as IL-1, IL-2, TNF-α, interferon-β, interferon-γ and chemokines. We confirmed that dexamethasone suppressed the IL-1β induced-MCP-1 gene expression in a dose dependent manner and also suppressed the IL-1β induced-CINC mRNA. The mechanisms of dexamethasone-mediated suppression of CINC gene expression have been suggested as involving blocks of cytokine-induced nuclear translocation and DNA binding to NF-kB (18).

In conclusion, our results demonstrated that, through MCP-1, CINC, RANTES, osteopontin and ICAM-1, mesangial cells would be able to communicate directly or indirectly with macrophages or neutrophils, which might lead to cell proliferation, extracellular matrix expansion and glomerulosclerosis.

Acknowledgements

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