Abstract. Acute peritonitis, in which peritoneal mesothelial cells are directly exposed to bacterial components, is a major cause of peritoneal dysfunction in continuous ambulatory peritoneal dialysis patients. We have previously shown that Toll-like receptors (TLR) are expressed in kidney cells, and LPS induces TLR4-dependent chemokine production in tubular epithelial cells. The present work was designed to investigate the involvement of TLR, especially TLR4, in the lipid A–mediated chemokine production by murine peritoneal mesothelial cells (MPMC). A primary cell culture of MPMC from C3H/HeN mice (wild-type mouse; LPS sensitive) and from C3H/HeJ mice (containing a point mutation of TLR4; LPS hyposensitive) was established. The expression profile of the TLR family and their accessory molecules, CD14 and MD-2, which are requisite for the LPS signaling pathway, was examined by RT-PCR, Northern blot test, and immunohistochemical staining. Synthetic lipid A–mediated chemokine production by MPMC was studied. The involvement of MAP kinase family (ERK, JNK, and p38 mitogen-activated protein kinase) and nuclear factor (NF)-κB in these processes was also studied. MPMC constitutively express TLR4, CD14, and MD-2. A prominent induction of monocyte chemotactic protein–1 (MCP-1) and macrophage inflammatory protein (MIP)-2 by MPMC was detected after lipid A stimulation and was strictly dependent on TLR4. Furthermore, TLR4-dependent chemokine production followed by leukocyte influx into the peritoneal cavity was also confirmed in vivo after stimulation with LPS. mRNA expression of MCP-1 was abolished by NF-κB inhibition, but were not affected by the inhibition of ERK, JNK, or p38. As compared with MCP-1, MIP-2 mRNA expression was inhibited by a high dose of curcumin but not by NF-κB decoy oligodeoxynucleotide and individual inhibitions of MAP kinase, suggesting that the additional signaling pathway with NF-κB might be involved in mRNA expression of MIP-2. These show that TLR4 is directly involved in the production of MCP-1 and MIP-2 by MPMC in a NF-κB–dependent manner, but the process does not require any MAP kinase activation. The results provide a candidate molecular target in prevention of it.

Peritonitis in peritoneal dialysis (PD) patients still remains a problem and major cause of transfer to hemodialysis, even though the rate has dramatically improved (1). The importance of bacterial peritonitis as a leading causal agent of peritoneal fibrosis (PF) is well established (2). In such situations, infiltration of inflammatory cells such as neutrophils and macrophages is observed in peritoneal fluids and is accompanied by the elevation of several chemokines such as monocyte chemoattractant protein–1 (MCP-1) and IL-8 (3,4). In contrast, regulated upon activation normal T cell expressed and secreted (RANTES) and TNF-α could not be detected in peritoneal dialysate effluents from PD patients with peritonitis (5,6). Several cytokines such as IL-1β, TNF-α, and INF-γ activate peritoneal mesothelial cells and induce certain chemokine production from these cells in vitro (7). Chemokine expression by cytokine-activated mesothelial cells and leukocyte infiltration in the peritoneal cavity and peritoneal tissues are important for the induction and development of peritonitis and PF.

Toll-like receptors (TLR) play important roles in the initial recognition of bacterial components in the host defense system and ten members of TLR have been reported so far (8,9). Among them, TLR2 recognizes bacterial components such as lipoprotein, peptidoglycan, and lipoteichoic acid. In contrast, TLR4 principally recognizes LPS and mediates the LPS signal transduction in collaboration with other molecules such as CD14, MD-2, myeloid differentiation factor 88 (MyD88), and so on (9). Besides LPS, TLR4 also recognizes other exogenous and endogenous ligands, including respiratory syncytial virus, heat-shock proteins, fibronectin, fibrinogen, and hyaluronic acid (10–16). It is thought that by binding these ligands, TLR4 is supposed to be directly involved in a variety of tissue injuries such as atherosclerosis, myocardial dysfunction, and ozone-induced lung injuries (17–19). Recently, Cunningham et al.
shown that LPS-induced acute renal failure is also mediated by TLR4.

The initial event in the pathogenesis of peritoneal inflammation or fibrosis in continuous ambulatory peritoneal dialysis (CAPD) patients is the direct exposure of peritoneal mesothelial cells to PD solutions. Many factors can directly activate peritoneal mesothelial cells during CAPD. They include bacterial components such as LPS, the PD solutions themselves (glucose degrading products, AGE, and pH), and several other factors released from peritoneal tissues (22,23). These stimuli induce the expression of several growth factors and chemokines, which is followed by the induction of neoangiogenesis and peritoneal sclerosis (24,25).

In a previous report, we showed that LPS significantly induced the major C-C chemokines MCP-1 and RANTES in murine tubular epithelial cells (MTEC) in a TLR4-dependent manner and clarified the signal pathway that produces these chemokines (26). These observations prompted us to study the role of peritoneal mesothelial cells in producing chemokines or cytokines and inducing peritoneal inflammation and fibrosis in the same manner as tubular epithelial cells (27). However, the precise mechanisms of the TLR4-dependent chemokine production in peritoneal mesothelial cells have not been explored so far.

In this study, we focused on the role of TLR4 in the chemokine production after stimulation with lipid A, an active part of LPS, and on the signal transduction of these chemokines in vitro. We also examined the biologic role of TLR4 in murine peritonitis in vivo in C3H/HeJ and C3H/HeN mice.

Materials and Methods

Mice

C3H/HeJ and C3H/HeN mice were purchased from Japan SLC (Shizuoka, Japan). The C3H/HeJ strain is hyposensitive to LPS because of a point mutation in the TLR4 gene. Mice of the C3H/HeJ strain were found to have a defective response to bacterial endotoxin. Although C3H/HeJ mice have a expression of TLR4, they lack the role of peritoneal mesothelial cells in producing chemokines or cytokines and inducing peritoneal inflammation and fibrosis in the same manner as tubular epithelial cells (27). However, the precise mechanisms of the TLR4-dependent chemokine production in peritoneal mesothelial cells have not been explored so far.

Regent and Antibodies (Ab)

A synthetic lipid A analog (ONO-4007) was a gift from ONO Pharmaceuticals (Osaka, Japan). The lipid moiety, lipid A, is an active part of LPS (29). Anti-cytokeratin Ab was obtained from Enzo Diagnostics (Shizuoka, Japan). The C3H/HeJ strain is hyposensitive to LPS because of a point mutation in the TLR4 gene. Mice of the C3H/HeJ strain were found to have a defective response to bacterial endotoxin. Although C3H/HeJ mice have a expression of TLR4, they lack the role of peritoneal mesothelial cells in producing chemokines or cytokines and inducing peritoneal inflammation and fibrosis in the same manner as tubular epithelial cells (27). However, the precise mechanisms of the TLR4-dependent chemokine production in peritoneal mesothelial cells have not been explored so far.

In this study, we focused on the role of TLR4 in the chemokine production after stimulation with lipid A, an active part of LPS, and on the signal transduction of these chemokines in vitro. We also examined the biologic role of TLR4 in murine peritonitis in vivo in C3H/HeJ and C3H/HeN mice.

RT-PCR

Total RNA was prepared with TRiZol reagent (Life Technologies BRL, Rockville, MD). One microgram of total RNA was reverse-transcribed and PCR was performed with a One Step RNA PCR kit (TaKaRa Biomedicals, Osaka, Japan). The primers were: mouse TLR1 sense, CGAAGCTTGTGTGACATCA; mouse TLR1 antisense, GGGAAACTGATGGTGCGTC; mouse TLR2 sense, CAGCTTAAAGGGCGGTTCAGAG; mouse TLR2 antisense, TGGAGAGACGCCAGCTCTTGCTCA; mouse TLR3 sense, ATGTTTCAGTGCATCGGATT; mouse TLR3 antisense, AAACATTCCCTCCTCGAAACAC; mouse TLR4 sense, AGTGGGCTCAAGGAACACAGAGAC; mouse TLR4 antisense, CTTTACCGCTCTTCTACCC; mouse TLR5 sense, GAATTCCTTAAGGCGGTGTTA; mouse TLR5 antisense, GGAAGATAAAGCGGTGCTG; mouse TLR6 sense, AGTGTGCCCAAGTTTGCGAACA; mouse TLR6 antisense, AGGAAACACCCAGTATAGC; mouse TLR9 sense, CCAGAGCTCTTCGTGAGAACC; mouse TLR9 antisense, GATTAGAAAGTGCGGCGTTGT.

Immunohistological Evaluation of Mouse Peritoneum

To detect the expression of TLR4 in the peritoneum, LPS (500 µg/body) was injected into the peritoneal cavity of the C3H/HeN mice. Two-micrometer-thick frozen sections of mouse peritoneum were fixed in cold acetone and used for the immunohistologic evaluation. The TLR4 expression in the peritoneum was detected by an indirect immunohistotechnique as described previously (31).
Northern Blot Analysis
cDNA was synthesized from 2 μg of total RNA derived from RAW 264.7 cells by RT-PCR. The synthesized PCR products were used as the specific probes. The primers were: mouse MCP-1 sense, GTGAGCTTAGCTTCTCTCTCCACCAG; mouse MCP-1 antisense, CACGGATCCTTTACGGGACAACTTCACATTCAAA; mouse macrophage inflammatory protein (MIP)-2 sense, GTGAAGCTTAGCG; mouse MCP-1 antisense, CACG-GATCTTTCCAGGACCGAGT. cDNA fragments containing the full coding regions of mouse MD-2 (32) were also synthesized by RT-PCR and used as the specific probes. Northern Standard blot was performed as described previously (26).

ELISA
To quantify the level of MCP-1 and MIP-2 protein under the different experimental conditions, the total MCP-1 and MIP-2 protein was measured in the culture supernatant with a commercial sandwich ELISA kit for MCP-1 and MIP-2 (Techne, Minneapolis, MN) according to the manufacturer’s instructions.

Study of Leukocyte Recruitment and Chemokine Concentration of Peritoneal Fluid in Murine Peritonitis
C3H/HeN and C3H/HeJ mice were intraperitoneally administered LPS (100 μg/body) in 2.0 ml sterile PBS. Mice were killed 15 h later after LPS administration and peritoneal exudate cells were harvested. Smear specimens were stained with Giemsa solution, and leukocyte numbers were assessed by differential cell counts. MCP-1 and MIP-2 concentrations in the peritoneal cavity lavaged fluids were quantified by the same ELISA method.

Western Blot Analysis
For the detection of MAPK phosphorylation and MAPK in the MPMC, SDS-PAGE and immunoblotting were performed as described previously (33).

Immune Complex Kinase Assay
Immune complex kinase assay was performed as described previously (26). In brief, cell lysates were incubated with 0.4 μg polyclonal anti-JNK1 Ab, polyclonal anti-p38 MAPK Ab, or polyclonal anti-ERK1/2 Ab for 2 h at 4°C and then with protein A-Sepharose beads (Amersham Pharmacia Biotech) for an additional 1 h. The kinase reaction was initiated by the addition of 40 μl of kinase buffer with 20 mM ATP, 5 μCi[γ-32P]ATP, and 0.5 μg of myelin basic protein (Sigma) for ERK, GST—c-Jun for JNK, or GST-ATF2 for p38 MAPK. The reaction was terminated, and samples were boiled and resolved by SDS-PAGE. The fixed gel was exposed to an IP reader in a BAS system.

Electrophoretic Mobility Shift Assay (EMSA)
MPMC were pretreated with various concentrations of curcumin for 1 h followed by 30-min stimulation with 1 μg/ml lipid A. Subsequently, nuclear extracts were prepared from cells as described previously (34). An oligonucleotide containing the nuclear factor (NF)-κB sense sequence (5'-AGT TGA GGG GAC TTT CCC AGG C-3') was used as the probe for EMSA. Approximately 1 × 10⁴ cpm of 32P-labeled oligonucleotide, 10 μg of nuclear extract, and 1 μg of poly(dI/dC) were added to the binding buffer (10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 4% glycerol) and incubated for 30 min at 4°C. The reaction mixture was run in a 5% nondenaturing polyacrylamide gel at 4°C with Tris-borate-EDTA buffer (90 mM Tris-borate, 2 mM EDTA).

Synthesis of Decoy Oligodeoxynucleotide (ODN) and Transfection
Sequences of the phosphorothioate ODN utilized were as follows: NF-κB decoy ODN (consensus sequences are underlined): 5'-CCCTGAGGGATTTCCCTCC-3', 3'-GGAACTCCCTAAAGGGAGG-5'. Scrambled decoy ODN: 5'-TTGCGTACCTGACTTACCC-3', 3'-AACGGCATGGAGCTAATCGG-5'. NF-κB decoy ODN have been shown to bind the NF-κB transcriptional factor (35). The single-stranded ODN were annealed for 2 h while the temperature descended from 80 to 25°C and the ODN concentration was quantified by spectrophotometry. MPMC were received the latest passage 24 h before transfection. NF-κB decoy ODN were transfected into MPMC by Lipofectamine (Invitrogen, Rockville, MD) according to the manufacturer’s instructions.

Statistical Analyses
All values are given as mean ± SD. When a significant difference was detected, the statistical analysis was further performed by Fisher’s F test or Scheffé’s F test between two groups. A P value of <0.05 was taken to indicate a significant difference.

Results
Gene and Protein Expression of TLR in MPMC
The primary culture cells used were positive for vimentin and cytokeratin staining (data not shown), indicating that they were peritoneal mesothelial cells (30). Figure 1A shows the TLR family gene expression in MPMC as detected by RT-PCR. TLR1, 2, 3, 4, 5, and 6 mRNA were expressed constitutively in MPMC and at the same levels as in the mouse macrophage cell line (RAW264.7) without stimulation. In contrast, TLR9 mRNA expression was undetectable in MPMC. Figure 1B shows the results of the immunohistochemical staining of TLR4 protein of MPMC (a) and in the peritoneal cavity of MPMC (b) after LPS stimulation (c) without LPS stimulation (d). The punctate staining of TLR4 was detected on the cell surface of the MPMC without stimulation. The linear staining of TLR4 in the peritoneal mesothelial layer was detected in the parietal peritoneum in the C3H/HeN mice. Figure 1C shows the staining of TLR4 protein in the peritoneum after LPS stimulation (b) or without LPS stimulation (c) in vivo. The punctate staining of TLR4 was detected on the cell surface of the MPMC without stimulation. The linear staining of TLR4 in the peritoneal mesothelial layer was detected in the parietal peritoneum in the C3H/HeN mice. Figure 1C shows that TLR4 mRNA was constitutively expressed in MPMC and lipid A stimulation did not change the mRNA expression of TLR4.

Synthetic Lipid A Directly Induces Chemokine Expression in MPMC
Northern blot analysis was performed to examine the profile of the chemokine production induced by lipid A in MPMC. MCP-1, MIP-2, MIP-1α, MIP-1β, and RANTES are representative inflammatory chemokines that play important roles in the induction and progression of peritonitis by recruiting inflammatory cells into the peritoneum (36). MPMC were stimulated with lipid A for 1 or 8 h and the total RNA was
RAW264.7, a control mouse macrophage cell line, was used as a positive control. MPMC and RAW 264.7 cells were cultured under basal conditions. Total mRNA was extracted and RT-PCR was performed as described in Materials and Methods. Identification of TLR4 in MPMC and mouse peritoneum. (a) Immunofluorescence staining of TLR4 in MPMC from C3H/HeN mice. Original magnification, ×200. (b) Immunofluorescence staining of TLR4 in parietal peritoneum from C3H/HeN mice 24 h after LPS stimulation (500 μg/body). Original magnification, ×200. (c) Immunofluorescence staining of TLR4 in parietal peritoneum from C3H/HeN mice without LPS stimulation. (C) Gene expression of TLR4 in MPMC after lipid A stimulation. MPMC were stimulated with 1 μg/ml of lipid A. Total mRNA was extracted and analyzed by Northern blot hybridization. The gene expression of GAPDH is shown as a quantitative control.

To examine whether lipid A directly increases the chemokine mRNA, the MPMC were pretreated with cycloheximide, a protein synthesis inhibitor, before lipid A stimulation. As shown in Figure 2B, cycloheximide failed to inhibit the LPS-induced MCP-1 and MIP-2 mRNA expressions in the MPMC. These results suggested that lipid A directly stimulated the MPMC to express MCP-1 and MIP-2.

Because accessory molecules such as CD14 and MD-2 are essential for the LPS signal pathway, the expression of these molecules in MPMC was examined. A significant amount of CD14 protein was detected in the membrane fraction of MPMC by Western blot analysis (Figure 2C). In addition, MD-2 mRNA was also expressed in MPMC as determined by the Northern blot analysis (Figure 2C).
**TLR4 Is Required for Lipid A–Mediated Chemokine Expression by MPMC**

We next examined whether the expression of MCP-1 and MIP-2 was dependent on TLR4 in vitro. MPMC were isolated from C3H/HeN mice and from C3H/HeJ mice, which have a TLR4 mutation and are hyporesponsive to LPS (28). Northern blot analysis showed that lipid A induced the expression of MCP-1 and MIP-2 mRNA in a dose-dependent manner in MPMC from the cells of C3H/HeN mice. In contrast, these increases in the chemokine expression were completely abrogated in MPMC from C3H/HeJ mice (Figure 3). In the time-course assays, MCP-1 mRNA expression reached the peak level at 2 or 4 h and then gradually decreased (Figure 4). In contrast, the induction of MIP-2 was much earlier, around 1 h after stimulation in MPMC from C3H/HeN mice. As in the dose-dependency experiments, lipid A also did not induce the expression of these chemokines in the cells of C3H/HeJ mice in any of the time courses (Figure 4).

The effects of several cytokines on the expression of MCP-1 and MIP-2 in MPMC were also studied. As shown in Figure 5, lipid A, TNF-α, and IL-1β significantly induced the expression of MCP-1 and MIP-2 by the MPMC. In contrast, INF-γ had no effects on chemokine production in MPMC. The chemokine production by lipid A stimulation was completely abolished only in MPMC from C3H/HeJ mice.

**Leukocyte Recruitment and Chemokine Production in Response to LPS through TLR4 In Vivo**

We examined whether leukocyte recruitment into the peritoneal cavity was actually dependent on TLR4 in response to LPS in vivo. The number of leukocytes in the peritoneal cavity from C3H/HeN mice was dramatically increased as compared with that of C3H/HeJ mice, especially for the number of macrophages (Figure 6A). Chemokine productions induced by LPS administration were also studied. As shown in Figure 6B, the productions of MCP-1 and MIP-2 were significantly increased in the peritoneal cavity lavaged fluids from C3H/HeN mice.

**MAPK Are Not Involved in the MCP-1 and MIP-2 mRNA Induction in MPMC**

LPS activates MAPK signaling pathways including p38 kinase, JNK, and ERK in various cell types (37–39). To confirm that lipid A activates MAPK signaling pathways in MPMC, Western blot analysis was performed with phosphospecific against p38 kinase, JNK, and ERK. As shown in Figure 7, Lipid A induced the rapid activation of the MAPK pathway that peaked 5 to 15 min after the stimulation in the MPMC of C3H/HeN mice. No phosphorylated bands were detected in the cells from C3H/HeJ mice, suggesting that the activation was dependent on TLR4.

In the next experiments, the cells from C3H/HeN mice were pretreated with specific inhibitors of the MAPK signaling pathways to clarify whether the MAPK pathway was involved in the MCP-1 and MIP-2 production after lipid A stimulation. Before lipid A stimulation, the MPMC were pretreated with specific inhibitors of p38 (SB208530), JNK (SP600125), or...
ERK (PD98059). Although each inhibitor effectively blocked the activity of the target signaling molecule in a dose-dependent manner, they did not affect significantly mRNA expression of MCP-1 or MIP-2 after lipid A stimulation in the triplicate experiment (Figure 8).

**NF-κB Activation Regulates Lipid A–Induced MCP-1 or MIP-2 mRNA Upregulation**

To further analyze the molecular mechanisms of the lipid A–induced chemokine productions in MPMC, we used a high dose of curcumin as an inhibitor of NF-κB. It has been reported that a small dose of curcumin (5 to 10 μM) inhibits JNK activation, but NF-κB translocation to the nucleus is inhibited at higher doses (50 μM or higher) (40). Lipid A–induced NF-κB activation was completely inhibited at a dose of 50 μM curcumin in MPMC as determined by EMSA (Figure 9A). At the same time, the expression of lipid A–induced MCP-1 and MIP-2 mRNA was also abolished at the same concentration of curcumin (Figure 9A). A low dose of curcumin (10 μM) did not effectively inhibit the chemokine expression, which was consistent with the result using a specific inhibitor of the JNK

**Figure 4.** Time course of lipid A–induced gene expression of chemokines (MCP-1 and macrophage inflammatory protein [MIP]-2) in murine peritoneal mesothelial cells (MPMC) from C3H/HeN and C3H/HeJ mice. MPMC were stimulated with 1 μg/ml of lipid A for the indicated time. Total mRNA was extracted and analyzed by Northern blot hybridization. MCP-1 mRNA (A) and MIP-2 mRNA (B) were measured. The intensities of the MCP-1 and MIP-2 signals were factored for the GAPDH signal, and the results expressed as bar charts. The mean intensities of the control groups were designated as an arbitrary value of 1. Data are mean ± SD. n = 3. *P < 0.05.

**Figure 5.** Effect of cytokine stimulation on MCP-1 and macrophage inflammatory protein (MIP)-2 protein expression by murine peritoneal mesothelial cells (MPMC). The cells from C3H/HeN and C3H/HeJ mice were cultured for 12 h with 1 μg/ml lipid A, 10 ng/ml TNF-α, 10 ng/ml INF-γ, or 10 ng/ml IL-1β. The culture supernatants were collected and MCP-1 (top) and MIP-2 (bottom) protein concentrations were measured by ELISA. Data are mean ± SD. n = 3. *P < 0.05.
pathway (SP600125) (Figure 8). A total of 25 μM of curcumin inhibited gene expression of MCP-1 was much higher than that of MIP-2. Experiments of transfection with NF-κB decoy ODN into MPMC were performed for more specific inhibition of NF-κB activation. NF-κB decoy ODN have been reported to block the binding of NF-κB transcriptional factor resulting in the inhibition of gene transcription (35). As shown in Figure 9B, transfection of NF-κB decoy ODN (10 μM) significantly inhibited protein expression of MCP-1 after lipid A stimulation. Control scrambled decoy ODN did not alter MCP-1 expression. In contrast, NF-κB decoy ODN had no inhibitory effects on MIP-2 expression.

**Discussion**

Dysfunction of the peritoneal membrane and PF are major complications of CAPD. There are several factors directly involved in the process of peritoneal dysfunction or PF. First, local production of VEGF induces neoangiogenesis and functional changes of the vessels in the peritoneal. A specific VEGF inhibitor successfully blocked PF in a rat model of PF (41). Second, the proliferation and transdifferentiation of peritoneal fibroblasts might be directly involved in PF by the induction of TGF-β and collagen gene expression (27,42,43). Third, chemokines locally produced by peritoneal macrophages, fibroblasts, and mesothelial cells mediate peritoneal inflammation (4,44,45). Of interests, peritoneal mesothelial cells have been reported to produce all of the substances listed above including several chemokines. We therefore focused on the role of peritoneal mesothelial cells as proinflammatory cells that mediate peritoneal inflammation.

Bacterial peritonitis is one of the major causes of peritoneal dysfunction and morbidity in CAPD patients. LPS derived from gram-negative organisms is a potent mediator of peritoneal inflammation and could be a trigger of the orchestrated production of chemokines. However, peritoneal dysfunction by gram-negative bacterial peritonitis is not so common, but aseptic peritonitis is often reported in CAPD patients (46). Furthermore, the damaged peritoneal tissues and massive fibrin depositions are also thought to be additional causal factors for peritoneal dysfunction or PF (10). From these previous findings, we have postulated that peritoneal mesothelial cells are endowed with specialized receptors that recognize both bacterial components and nonbacterial ligands. The most relevant candidates seemed to be TLR, especially TLR4. Because TLR4 recognizes many nonbacterial ligands, TLR4 might be a key molecule that activates peritoneal mesothelial cells continuously in aseptic conditions as well as in a bacterial peritonitis.
However, the precise mechanisms of the TLR4-dependent chemokine production in peritoneal mesothelial cells have not been explored so far. In the study presented here, we used lipid A as a selective ligand for TLR4 to explore the TLR4 dependent signaling pathway in MPMC in vitro.

Because CAPD patients use PD solutions with a high concentration of glucose, their peritoneal mesothelial cells are continuously exposed to oxidative stresses, resulting in fibrin and necrotic cells that also the possible candidates for TLR4 ligands. Furthermore, contaminating bacterial LPS derived from the systemic circulation (21) or from the gastrointestinal tract might continuously stimulate the mesothelial cells without any apparent bacterial infection. It is thus interesting to speculate that MPMC is directly activated by endogenous or exogenous TLR4 ligands and contribute to the development of irreversible peritoneal injuries during the long course of CAPD treatment.

In the study presented here, we found that MPMC constitutively expressed mRNA for TLR1, 2, 3, 4, 5, and 6, but not for TLR9. Especially, TLR4 was expressed in MPMC without any cell stimulation. In addition, MPMC expressed both CD14 and MD-2 that are indispensable for LPS signal transduction through TLR4 indicating that MPMC constitutively express all of the molecules required for LPS signal transduction.

From the studies that used mesothelial cells from TLR4-mutant mice, our data indicated that the lipid A–induced expression of MCP-1 and MIP-2 in MPMC was completely dependent on TLR4. On the other hand, MIP-1α, MIP-1β, and RANTES were not induced by lipid A stimulation in MPMC. By use of specific MAPK inhibitors or a high dose of curcumin it was revealed that, in MPMC, the LPS-mediated MCP-1 and MIP-2 mRNA expression was not dependent on the three MAPK signaling pathways (JNK, p38, and ERK), but was dependent on NF-κB activation. Recently, we have shown that LPS or lipid A stimulates the expression of C-C chemokines via TLR4 in cultured tubular epithelial cells and we also clarified its signal transduction pathways (26).

Figure 8. Inhibitory effects of selective MAPK inhibitors on the induction of MCP-1 and macrophage inflammatory protein (MIP)-2 mRNA in murine peritoneal mesothelial cells (MPMC) from C3H/HeN mice after lipid A stimulation. (A) MPMC were pretreated with the indicated concentrations of SB203580 (p38 specific inhibitor) for 1 h. (Upper three panels) Total mRNA was extracted after 1-h stimulation with 1 μg/ml lipid A and analyzed for MCP-1 and MIP-2 by Northern blot hybridization. GAPDH is shown as a control. (Lower two panels) After 15 min of stimulation with lipid A, p38 activation was measured by in vitro kinase assay with GST-ATF2 as the substrate. MPMC were pretreated as in the upper three panels. Total p38 protein levels are shown as controls. (B) MPMC were analyzed with SP600125 (JNK-specific inhibitor), and JNK activation was measured with GST-c-jun as a substrate. Western blot result with anti-JNK antibodies (Ab) is shown as a control. (C) MPMC were analyzed with PD98059 (ERK-specific inhibitor) and inhibition of ERK phosphorylation was measured by Western blot analysis with anti–phospho-ERK Ab. ERK is shown as a control. The intensities of the MCP-1 and MIP-2 signals were factored for the GAPDH signal, and the results expressed as bar charts. The mean intensities of the control groups were designated as an arbitrary value of 1. Data are mean ± SD. n = 3. *P < 0.05.
increase was dependent on NF-κB activation but not on the three MAPK signaling pathways. In contrast, JNK and p38 MAPK activation pathways were involved in the LPS-mediated RANTES mRNA expression in MTEC, but not in MPMC.

Leukocyte infiltration in the inflamed tissues is characterized by an early influx of neutrophils followed by the replacement with more sustained population of monocytes and/or macrophages. This switch in the phenotype of leukocytes from neutrophils to macrophages is important for the development of inflammation and tissue fibrosis (31). It has been shown that IL-6 (47) and INF-γ (7), are the major cytokines that control this critical step by regulating the profile of chemokine production from C-X-C chemokine (IL-8 and MIP-2) to C-C chemokine (MCP-1). In particular, sIL-6R in combination with IL-6 suppresses C-X-C chemokine (responsible for neutrophil recruitment) production but promotes the secretion of C-C chemokine (responsible for macrophage recruitment) (47).

We observed the increased expression of MIP-2 and MCP-1 mRNA in MPMC by lipid A stimulation in a TLR4-dependent manner. There was a time lag in the expression of these chemokines from MPMC. The peak levels of MIP-2 and MCP-1 mRNA expression in MPMC were 1 h and 4 h after lipid A stimulation, respectively. We did not observe the direct connections between TLR4 and IL-6 or INF-γ in MPMC. However, these observations strongly suggest that TLR4 signaling by lipid A per se also contribute the switch in the pattern of leukocyte infiltration by the delayed expression of MCP-1 in an acute peritoneal inflammation.

For the inhibition of NF-κB, a high dose of curcumin and a more specific inhibitor, NF-κB decoy ODN, were used. Curcumin (diferuloylmethane) has shown anti-inflammatory properties to regulate transcription factors such as AP-1, NF-κB, and many signaling pathway such as protein kinase C and EGF-receptor tyrosine kinase (48). Inhibition of NF-κB by a high dose of curcumin and NF-κB decoy ODN completely abolished MCP-1 mRNA production, showing that MCP-1 production in response to lipid A is dependent on NF-κB activation in MPMC. In contrast, MIP-2 mRNA expression was inhibited by a high dose of curcumin but not by NF-κB decoy ODN or individual MAPK (ERK, JNK and p38) inhibitors, suggesting that the synergistic activation of NF-κB and AP-1 might be important for lipid A–induced MIP-2 mRNA expression in MPMC. Similar observation has recently reported in macrophages. Kim et al. (49) clearly demonstrated that LPS-induced MIP-2 expression in murine macrophages was dependent on the activation of NF-κB and c-Jun.

Recently we succeeded to block the tubulointerstitial inflammation and fibrosis in rat model of protein overload through interference of the binding of MCP-1 and its receptor by in vivo transfection with MCP-1 mutant gene (7ND) (50). Because MCP-1 and MIP-2 are closely linked with organ fibrosis as well as inflammation, these chemokines and the related molecules in their signaling pathway are good targets for more specific and selective therapies of PF.

In summary, we have demonstrated that a functional TLR4 and its accessory molecules, CD14 and MD-2, were expressed in MPMC. A notable induction of MCP-1 and MIP-2 by
MPMC was detected after lipid A stimulation and was strictly dependent on TLR4. LPS actually induced the productions of these chemokines followed by leukocyte recruitment into the peritoneal cavity in C3H/HeN mice in vivo. Lipid A-stimulated MCP-1 production in MPMC was completely dependent on NF-κB activation but not on MAPK signal pathways. The activation of TLR4 in peritoneal mesothelial cells might result in a massive influx of leukocytes by releasing MIP-2 and MCP-1 in the peritoneal cavity, leading to the development of peritoneal dysfunction or PF. The signal pathway through TLR4 may be one of the promising therapeutic targets for treating peritoneal dysfunction.

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