Pressure-induced expression of monocyte chemoattractant protein-1 through activation of MAP kinase

TAKESHI SUDA, AKIHiko OSAJIMA, MASAHITO TAMURA, HIROAKI KATO, MASAKO IWAMOTO, TAKAYUKI OTA, KAORI KANEGAE, HIROSHI TANAKA, HIROFUMI ANAI, NARUTOSHI KABASHIMA, MASAIRO OKAZAKI, and YASUHIDE NAkASHIMA

The Second Department of Internal Medicine, University of Occupational and Environmental Health, School of Medicine, Kitakyushu, Japan

Pressure-induced expression of monocyte chemoattractant protein-1 through activation of MAP kinase.

Background. In glomerular hypertension, mesangial cells (MC) are subjected to at least two physical forces: a high pressure and mechanical stretch. In 5/6 nephrectomized rat, a model of progressive glomerular sclerosis associated with glomerular hypertension, monocyte chemoattractant protein-1 (MCP-1) is expressed in glomeruli, suggesting the possible role of MCP-1 in the pathogenesis of glomerular sclerosis; however, whether pressure directly affects MCP-1 expression remains undetermined. Here we examined the effects of pressure on MCP-1 expression in cultured rat MC and the signal transduction pathways that lead to MCP-1 expression.

Methods. Pressure was applied to MC by instilling compressed helium gas into sealed plates. MCP-1 mRNA and protein levels in MC were detected by reverse transcription-polymerase chain reaction (RT-PCR) or Northern blotting and ELISA or Western blotting, respectively. Mitogen-activated protein (MAP) kinase activity was measured with the catalytic activity of p42/p44 MAP kinase and anti-phospho p42/p44 MAP kinase antibody. A transient transfection assay that specifically modulates MAP kinase (MEK) activity was carried out.

Results. MCs subjected to external pressure expressed MCP-1 mRNA rapidly and transiently with the peak level noted at 10 minutes and 80 mm Hg pressure. MCP-1 protein levels in cell lysates and culture medium also significantly increased after pressure loading. Pressure rapidly increased the phosphorylation level and activity of p42/p44 MAP kinase. Treatment of MC with a MAP kinase inhibitor, PD98059, suppressed levels of both pressure-induced MAP kinase activities and MCP-1 mRNA expression. The constitutively activated type of MEKI induced MCP-1 expression (13.7-fold) even in non-pressurized MC.

Conclusions. Our results indicate that pressure per se can induce MCP-1 via activation of MAP kinase pathway, suggesting that glomerular hypertension might be involved in the progression of renal diseases through the expression of MCP-1 in MC.

Glomerular capillary hypertension is an important causative factor of glomerular sclerosis [1, 2]. Since the mesangial area occupies a central location and is surrounded by glomerular capillaries, increased glomerular pressure results in at least two major effects: a high pressure and stretch on mesangial cells (MC) [3, 4]. Using the pressure apparatus and helium gas [5], as modified by our laboratory [6], we have previously reported that increased pressure induces overexpression of platelet-derived growth factor-B (PDGF-B) mRNA in MC and that pressure-induced MC proliferation is inhibited by neutralizing antibodies against PDGF [6]. These results suggest that PDGF-B plays an important role in the pressure-induced MC proliferation. Similar results have been reported following exposure of MC to stretching forces (abstract; Kaname et al, J Am Soc Nephrol 8:402A, 1997), suggesting the involvement of PDGF in the pathogenesis of glomerular sclerosis induced by physical forces, as seen in glomerular hypertension [7].

In the 5/6 nephrectomized rat, a model of progressive glomerular sclerosis associated with glomerular hypertension, infiltration of monocytes/macrophages into the glomeruli has been reported to occur following MC proliferation and PDGF expression in glomeruli, leading to progression of glomerular sclerosis [7–9]. These findings suggest that the influx of monocytes/macrophages in glomeruli may participate in the development of glomerular sclerotic changes induced by elevated glomerular pressure [7, 9]. Schlondorff et al have reported that various chemokines exhibit strong chemoattractant activities for monocytes/macrophages and postulated their involvement in inflammatory cell recruitment into injured tissues [9]. Among the chemokines, monocyte chemo-
attrac tant protein-1 (MCP-1), a member of the C-C group, is a potent chemokine for monocytes/macrophages and plays an active role in renal injury [9]. MCP-1 is expressed in a variety of renal cell types, including MC in human and experimental glomerulonephritis [9–13]. Using immunohistochemistry, Schiller and Moran showed increased glomerular MCP-1 expression in 5/6 nephrectomized rats prior to the infiltration of monocytes/macrophages into glomeruli [14]. They also demonstrated that a low protein diet reduced both the MCP-1 expression and infiltration of monocytes/macrophages, suggesting that elevated glomerular pressure directly affects MCP-1 expression on glomerular cells, especially in MC. To our knowledge, however, no studies have previously examined the effects of pressure per se on MCP-1 expression in MC.

Mitogen-activated protein (MAP) kinase is an important mediator of growth-mediated signal transduction in a variety of cells, including MC [15–19]. Several signaling pathways such as p42/p44 MAP kinase are involved in the regulation of MCP-1 expression [20–25]. We and others have shown that pressure is a potent activator of p42/p44 MAP kinase as well as protein kinase C (PKC) and tyrosine kinase in MC [6, 26], suggesting the possible role of p42/p44 MAP kinase in the pressure-induced MCP-1 expression. In the present study, we examined whether externally applied pressure alone induces MCP-1 expression in cultured rat MC. We also studied whether p42/p44 MAP kinase is involved in these pressure-induced events.

METHODS

Materials

Platelet-derived growth factor-B was obtained from Sigma (St. Louis, MO, USA). PD98059, calphostin C, and wortmannin were from Calbiochem (San Diego, CA, USA). The p42/p44 MAP kinase enzyme assay system and [γ-32P] ATP (37 MBeq/mmol) were from Amersham Life Science (Arlington Heights, IL, USA). Monoclonal anti-phospho-p44/p42 MAP kinase and polyclonal anti-p42/p44 MAP kinase antibodies were purchased from New England Biolabs (Beverly, MA, USA). Monoclonal anti-hemagglutinin (HA) antibody (12CA5) was obtained from Boehringer Mannheim (Indianapolis, IN, USA). Polyclonal anti-rat MCP-1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Mesangial cell cultures

Rat glomerular MC were isolated from glomeruli harvested from four-week-old Wistar rats by the differential sieving method and were previously described [27, 28]. MC were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 200 μg/mL penicillin, 100 μg/mL streptomycin at 37°C in 5% CO2 incubator. To set the cells in a quiescent stage, MC were maintained in DMEM containing 0.5% fetal calf serum for 48 hours. Cells were used between 5 and 13 passages.

Pressure loading apparatus

We used the pressure loading apparatus described previously by our laboratory (Miwa Co., Tokyo, Japan) [6]. The apparatus consisted of a resealable steel chamber with inlet and outlet ports. The inlet port was connected through a tube to a reservoir of compressed helium, while the exit port was connected through a tube to a sphygmomanometer and air-release valve. Compressed helium gas was pumped into the chamber to raise internal chamber pressure. During the delivery of helium gas into the apparatus, no prepacked room air was released so that the partial pressures of the gases originally present in the chamber, such as O2, N2, and CO2, were kept constant [29], consistent with the Boyle-Charle law, as described previously [5]. The plates used for our experiments were placed on a warm plate (37°C) inside the chamber. The partial pressure of O2, temperature, and pH of the incubation medium in the plates remained constant throughout the experiments.

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) and Northern blotting

Total RNA was extracted by using an RNeasy kit (Qiagen, Hilden, Germany). The first-strand cDNA was synthesized from 1 μg of total RNA in 50 mmol/L Tris-HCl buffer (pH 8.3) containing 200 ng random hexamers, 3 mmol/L MgCl2, 400 U murine Moloney leukemia virus reverse-transcriptase, 500 μmol/L dNTP, 15 mmol/L dithiothreitol (DTT), and 75 mmol/L KCl in a final volume of 7.5 μL (1 hour at 37°C) as described previously [28]. Each sample was assayed for MCP-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA in separate tubes using specific primers for PCR; MCP-1; sense: 5'-TATGCAGGTCTCTGTACGC-3', antisense; 5'-AAATGTGTGAACCAGGATTCACA-3', GAPDH; sense: 5'-TCCCTCAAGATTGTCAGCAA-3', antisense; 5'-AGATCCAAACGGATACTATT-3'. PCR was performed by incubating 1 μg of the sample cDNA with 68 mmol/L KCl, 45 mmol/L Tris-HCl (pH 8.3), 15 mmol/L DTT, 9 mmol/L MgCl2, 80 μg/mL bovine serum albumin (BSA), 2.5 U Taq DNA polymerase, 1.8 mmol/L dNTP, and 40 pmol of each primer in a final volume of 25 μL. PCR was carried out under the following conditions; 1 minutes at 94°C, 45 seconds at 60°C, and 45 seconds at 72°C. For MCP-1 and GAPDH, 30 and 25 cycles were used, respectively. Primer sets for MCP-1 and GAPDH generated 595 and 308 bp products, respectively. The samples were then subjected to agarose gel electrophoresis and stained with ethidium bromide to visualize DNA...
bands, followed by scanning densitometry (FB1200S; Canon, Tokyo).

Standard Northern blotting was performed as described [30]. cDNA probe for GAPDH was purchased from Clontech (Palo Alto, CA, USA). Rat MCP-1 cDNA was constructed using the PCR as described previously in this article. The PCR product was subcloned into pCR-TOPO (Invitrogen, Groningen, The Netherlands), and the sequence was confirmed. RNA samples were subjected to 1% formaldehyde-agarose gels, transferred onto nylon membranes, and covalently cross-linked to the membrane with ultraviolet light. The membranes were hybridized with a cDNA probe for MCP-1 labeled with [32P]dCTP by using Rediprime II (Amersham Pharmacia Biotech, Buckinghamshire, UK) and then rehybridized with a probe for GAPDH. The membranes were subsequently exposed to Hyperfilm-MP (Amersham Pharmacia Biotech) with an intensifying screen at −70°C.

ELISA for MCP-1

Monocyte chemoattractant protein-1 concentrations in the culture supernatants of MC were measured by using an enzyme-linked immunosorbent assay (ELISA) kit (Cosmo Bio, Tokyo, Japan) following the method recommended by the manufacturer. Briefly, cells were exposed to 80 mm Hg pressure for 10 minutes and incubated for up to 24 hours. Culture supernatants (50 μL) were added into flat-bottomed 96-well ELISA plates coated with goat anti-rat MCP-1 serum together with biotinylated anti-MCP-1 solution and incubated for 30 minutes at room temperature. Stabilized chromogen (100 μL) was added to each well and incubated for additional 30 minutes. After adding stop solution (100 μL), the absorbance at 450 nm was analyzed by using a plate reader (model 450; BioRad, Hercules, CA, USA). The minimum sensitivity of ELISA for MCP-1 was 8.0 pg/mL.

Western blotting

Western blotting was performed as described previously [31]. Briefly, MC grown to subconfluence were serum starved for 48 hours. After exposure of cells to pressure, they were lysed with RIPA lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.5, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, and 2 mmol/L ethylenediaminetetraacetic acid (EDTA)] containing 2 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 50 μg/mL soybean trypsin inhibitor, 20 mmol/L iodoacetamide, 50 mmol/L sodium fluoride, and 1 mmol/L sodium orthovanadate. The lysates were clarified by centrifugation at 14,000 × g for 15 minutes at 4°C. Protein concentrations were determined spectrophotometrically using protein concentration reagent (BioRad), and samples were adjusted to equal protein concentrations and volumes. Samples were resolved onto 12 to 15% polyacrylamide gels and electro-}

phoretically transferred to nitrocellulose membrane (Novex, San Diego, CA, USA) for 90 minutes at 150 mA. The filters were incubated with blocking buffer (5% non-fat dry milk; alternatively, 5% BSA for anti-p44/p42 MAP kinase antibody), in T-TBS (50 mmol/L Tris, 150 mmol/L NaCl, and 0.5% Tween 20, pH 7.4) for one hour and then incubated overnight in a cold room with either MCP-1 (1:2000), anti-phospho-p44/p42 MAP kinase (1:1000), anti-p44/p42 MAP kinase (1:2000), or 12CA5 (1:400) antibodies. Blots were visualized by the enhanced chemiluminescence (ECL) reaction (Amersham Life Science).

p42/p44 MAP kinase assay

The activity of p42/p44 MAP kinase was assayed using a synthetic peptide (KRELVEPTPAGEAPNQLLR; Amersham Life Science) as a specific MAP kinase substrate, based on the assay protocol recommended by the manufacturer. After exposure of serum-starved MC grown on a six-well plate to 80 mm Hg pressure, MC were lysed with ice-cold lysis buffer (10 mmol/L Tris-HCl, pH 7.4, 20 mmol/L NaCl, 2 mmol/L EGTA, 2 mmol/L DTT, 1 mmol/L orthovanadate, 1 mmol/L phenylmethylsulfonylfluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin) and centrifuged at 14,000 × g for 15 minutes at 4°C. The supernatants (~1 μg protein) were incubated with 1.2 mmol/L [γ-32P]ATP (1 μCi) and 2 mmol/L substrate peptide in 75 mmol/L HEPES buffer, pH 7.4, containing 1.2 mmol/L MgCl2 for 30 minutes at 30°C. The resultant solution was applied to a phosphocellulose membrane (Amersham), followed by extensive washing in 1% acetic acid and then in H2O. The radioactivity trapped on the membrane was measured in a liquid scintillation Coulter counter (LS7000; Beckman, Fullerton, CA, USA).

Plasmids and transfections

Three plasmids containing pMCL•HA-tagged MEK1 (wild-type and constitutively activated type) were kindly provided by Dr. N.G. Ahn (Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO, USA) [32]. Puromycin-resistant plasmid pHA262pur was obtained from Dr. Heinte Riele (Division of Molecular Carcinogenesis, The Netherlands Cancer Institute), [33]. Either wild-type or constitutively activated HA-MEK1 (8 μg) was transfected into MC using the cationic liposome (lipofectamine; GIBCO BRL, Gaithersburg, MD, USA)-mediated transfection method together with pHA262pur (1 μg) [28, 34]. To increase the expression of transfected genes, 5 mmol/L sodium butyrate was added to the culture medium. Cells expressing HA-MEK1 were selected as described previously [34]. Briefly, at 24 hours after transfection, cells were maintained for two days in culture media containing 1 μg/mL puromycin, and cultured for an additional 24 hours in the regular culture
First, the dynamics of the pressure effects on the expression of MCP-1 mRNA were examined in cultured MC. We have previously shown that the maximal increase in DNA synthesis and cell proliferation occurred at a pressure level of 80 mm Hg [6]. Application of a similar pressure to MC caused a rapid increase in the expression of MCP-1 mRNA at 5 minutes with a peak noted at 10 minutes but decreased thereafter to the basal level at 30 minutes by RT-PCR (Fig. 2A, B). Similar increased mRNA expression of MCP-1 was also observed by using Northern blot analysis (Fig. 2C). In contrast, MCP-1 mRNA levels in control cells (not subjected to pressure) did not change throughout the experiment (data not shown). Since it has been reported that MC produce MCP-1 in response to various cytokines such as PDGF-B [25], we used PDGF-B as a positive control. Treatment of MC with PDGF-B (25 ng/mL) for 10 minutes significantly increased MCP-1 expression up to 4.6 ± 1.7-fold, which was twofold higher than that of 80 mm Hg pressure stimulation (Fig. 3).

Using Western blotting and ELISA, we examined MCP-1 protein in cell lysates and culture supernatants. We therefore amplified 1 μg of each RNA by 21 to 27 cycles in the following experiments. Similarly, PCR for GAPDH was performed using 21 to 27 cycles or by incubating serially lower amounts of RNA (data not shown). Accordingly, 1 μg of RNA and 25 cycles were selected for amplification of GAPDH.

First, the dynamics of the pressure effects on the expression of MCP-1 mRNA were examined in cultured MC. We have previously shown that the maximal increase in DNA synthesis and cell proliferation occurred at a pressure level of 80 mm Hg [6]. Application of a similar pressure to MC caused a rapid increase in the expression of MCP-1 mRNA at 5 minutes with a peak noted at 10 minutes but decreased thereafter to the basal level at 30 minutes by RT-PCR (Fig. 2A, B). Similar increased mRNA expression of MCP-1 was also observed by using Northern blot analysis (Fig. 2C). In contrast, MCP-1 mRNA levels in control cells (not subjected to pressure) did not change throughout the experiment (data not shown). Since it has been reported that MC produce MCP-1 in response to various cytokines such as PDGF-B [25], we used PDGF-B as a positive control. Treatment of MC with PDGF-B (25 ng/mL) for 10 minutes significantly increased MCP-1 expression up to 4.6 ± 1.7-fold, which was twofold higher than that of 80 mm Hg pressure stimulation (Fig. 3).

Using Western blotting and ELISA, we examined MCP-1 protein in cell lysates and culture supernatants produced by MC after application of the pressure stimulus (80 mm Hg) for 10 minutes. MCP-1 protein levels in MC increased significantly at one hour after pressure loading, and the levels remained unchanged up to 12 hours (Fig. 4A). In addition, the mean concentration of MCP-1 protein in the culture supernatants after 12 hours with ELISA was significantly higher (163.3 ± 27.8%) than in the control (without pressure; P < 0.05, N = 3; Fig. 4B). In contrast, basal MCP-1 production by MC remained unchanged throughout the experiments. We also examined the effect of different pressure levels on MCP-1 mRNA expression. Expression of MCP-1 mRNA significantly increased when the chamber pressure was increased for 10 minutes. However, only pressures more than 60 mm Hg influenced the expression of MCP-1 mRNA, with a maximal induction noted at 80 mm Hg (Fig. 5). Interestingly, MCP-1 mRNA level at 100 mm Hg was less than that at 80 mm Hg, suggesting that the physical pressure for maximal induction of MCP-1 is 80 mm Hg. Based on the results shown in Figures 2 and 5, a pressure stimulus of 80 mm Hg applied for 10 minutes was selected in the remaining experiments described later in this article.

**RESULTS**

**Pressure-induced MCP-1 mRNA expression and MCP-1 protein secretion by MC**

To confirm the accuracy of the mRNA quantity amplified by RT-PCR, PCR for MCP-1 was performed by differential PCR cycles (Fig. 1A) or by incubating serially lower amounts of mesangial RNA (Fig. 1B), which revealed a dose- and cycle-dependent increase in the PCR product. We therefore amplified 1 μg of each RNA by 30 cycles in the following experiments. Similarly, PCR for GAPDH was performed using 21 to 27 cycles or by incubating serially lower amounts of RNA (data not shown). Accordingly, 1 μg of RNA and 25 cycles were selected for amplification of GAPDH.

First, the dynamics of the pressure effects on the expression of MCP-1 mRNA were examined in cultured MC. We have previously shown that the maximal increase in DNA synthesis and cell proliferation occurred at a pressure level of 80 mm Hg [6]. Application of a similar pressure to MC caused a rapid increase in the expression of MCP-1 mRNA at 5 minutes with a peak noted at 10 minutes but decreased thereafter to the basal level at 30 minutes by RT-PCR (Fig. 2A, B). Similar increased mRNA expression of MCP-1 was also observed by using Northern blot analysis (Fig. 2C). In contrast, MCP-1 mRNA levels in control cells (not subjected to pressure) did not change throughout the experiment (data not shown). Since it has been reported that MC produce MCP-1 in response to various cytokines such as PDGF-B [25], we used PDGF-B as a positive control. Treatment of MC with PDGF-B (25 ng/mL) for 10 minutes significantly increased MCP-1 expression up to 4.6 ± 1.7-fold, which was twofold higher than that of 80 mm Hg pressure stimulation (Fig. 3).

Using Western blotting and ELISA, we examined MCP-1 protein in cell lysates and culture supernatants produced by MC after application of the pressure stimulus (80 mm Hg) for 10 minutes. MCP-1 protein levels in MC increased significantly at one hour after pressure loading, and the levels remained unchanged up to 12 hours (Fig. 4A). In addition, the mean concentration of MCP-1 protein in the culture supernatants after 12 hours with ELISA was significantly higher (163.3 ± 27.8%) than in the control (without pressure; P < 0.05, N = 3; Fig. 4B). In contrast, basal MCP-1 production by MC remained unchanged throughout the experiments. We also examined the effect of different pressure levels on MCP-1 mRNA expression. Expression of MCP-1 mRNA significantly increased when the chamber pressure was increased for 10 minutes. However, only pressures more than 60 mm Hg influenced the expression of MCP-1 mRNA, with a maximal induction noted at 80 mm Hg (Fig. 5). Interestingly, MCP-1 mRNA level at 100 mm Hg was less than that at 80 mm Hg, suggesting that the physical pressure for maximal induction of MCP-1 is 80 mm Hg. Based on the results shown in Figures 2 and 5, a pressure stimulus of 80 mm Hg applied for 10 minutes was selected in the remaining experiments described later in this article.

**Role of MAP kinase and PKC on pressure-dependent overexpression of MCP-1 mRNA**

The next series of experiments investigated the intracellular signaling pathways involved in pressure-induced MCP-1 expression. Because external pressure application is known to activate MAP kinase and/or PKC, leading to MC proliferation [6, 35], MC were treated with inhibitors of p42/p44 MAP kinase kinase (MEK; 25 μmol/L PD98059), PKC (1 μmol/L calphostin C), or a
increase in MCP-1 mRNA expression. Both inhibitors had no effect on the basal levels of MCP-1 mRNA (data not shown). In contrast, wortmannin (500 nmol/L) failed to inhibit pressure-induced increase in MCP-1 mRNA expression. These results suggest that MAP kinase and PKC pathways are involved in mediating the pressure-induced increase in MCP-1 mRNA expression (Fig. 6).

Pressure-induced p42/p44 MAP kinase activation

The next step focused on the effect of pressure on p42/p44 MAP kinase activation and the resultant increase in the expression of MCP-1. For this purpose, we examined the role of p42/p44 MAP kinase in pressure-induced increase in MCP-1 mRNA expression. The catalytic activity of p42/44 MAP kinase was assayed by using a synthetic peptide as a specific substrate for MAP kinase. Pressure at 80 mm Hg resulted in a rapid increase in p42/p44 MAP kinase activity at one minute (3.2 ± 0.2-fold). However, the increase was transient and decreased to the basal level within five minutes. In contrast, the level of p42/p44 MAP kinase activity in nonpressurized
MC remained unchanged for up to 10 minutes (Fig. 7A). To further confirm the activation of p42/p44 MAP kinase by pressure, we examined the phosphorylation levels of p42/p44 MAP kinase using anti-phospho p42/p44 MAP kinase antibody. Similar to the results obtained in the kinase assay, p42/p44 MAP kinase phosphorylation levels also significantly increased by pressure loading compared with the levels of nonpressurized MC. Treatment of MC with PD98059 (25 μmol/L) abrogated the pressure-induced p42/p44 MAP kinase activation (Fig. 7B, C), suggesting MEK-dependent activation of p42/p44 MAP kinase.

Effects of wild-type and constitutively activated MEK1 on MCP-1 mRNA expression

To investigate the role of p42/p44 MAP kinase activation in pressure-induced MCP-1 mRNA expression, we used a transient transfection assay that specifically modulates MEK activity, which is immediately upstream of p42/p44 MAP kinase. First, the effects of transfected MEK1 constructs on downstream p42/p44 MAP kinase activities were assayed by Western blotting for phospho-p42/p44 MAP kinase (Fig. 8A). Constitutively activated MEK1 markedly increased the phosphorylation of p42/p44 MAP kinase without pressure. Wild-type MEK1 also activated MAP kinase; however, the activation level was less than that of constitutively activated MEK1. We also confirmed similar expression levels of transfected MEK1 constructs by Western blotting for HA (Fig. 8B). Constitutively activated MEK1 was of a smaller molecular weight due to the truncation, as described previously [32]. Interestingly, constitutively activated MEK1 increased MCP-1 mRNA expression up to 13.7 ± 7.5-fold even without pressure loading (Fig. 9). These results indicate that activation of the MEK/MAP kinase pathway plays an important role in MCP-1 expression.
Fig. 6. Effects of inhibitors of MEK, PKC, and phosphatidylinositol 3-kinase on pressure-induced MCP-1 mRNA expression in cultured MC. MCs were treated without or with either PD 98059 (25 μmol/L), calphostin C (1 μmol/L), or wortmannin (500 nmol/L) for 30 minutes before and during 80 mm Hg pressure-loading for 10 minutes. The expressions of MCP-1 and GAPDH mRNAs were analyzed by RT-PCR (A). Relative MCP-1 mRNA expression levels are expressed relative to control levels (with pressure) after normalization with GAPDH. Data represent the mean ± SD of three independent experiments (B). †P < 0.001 vs. control (pressure +).

Fig. 7. Effects of pressure on ERK/MAP kinase activation in cultured MC. (A) Effects of pressure on MAP kinase activity. After exposure of quiescent MC to 80 mm Hg pressure for indicated time intervals, the catalytic activity of p42/44 MAP kinase was analyzed using a specific MAP kinase substrate as described in the Methods section. Symbols are: (●) pressure apparent; (○) control. Values represent mean ± SD (N = 4). *P < 0.05; **P < 0.01; †P < 0.001 vs. the respective control group. (B and C) Effects of pressure on p42/p44 MAP kinase phosphorylation. MC were treated without or with PD 98059 (25 μmol/L) for 30 minutes before and during application of 80 mm Hg pressure for one minute. Cell extracts (40 μg protein) were subjected to Western blotting with either anti-phospho p44/42 MAP kinase antibody or anti-MAP kinase antibody (B). Relative p42/p44 MAP kinase phosphorylation levels were determined from Western blotting by densitometric analysis and expressed relative to control levels (without pressure) (C). Values represent mean ± SD (N = 3). †P < 0.001 vs. pressure +.

DISCUSSION

The present study demonstrates, to our knowledge for the first time, that externally applied pressure per se can induce MCP-1 mRNA expression in MC, and that MCP-1 production is pressure dependent and accompanied by a rapid and transient activation of p42/p44 MAP kinase. Pressure-induced MCP-1 expression was suppressed by pretreatment of cells with a MEK inhibitor. We also demonstrated that the constitutive-active form of MEK1 effectively increased MCP-1 expression without pressure loading. These results strongly indicate that...
pressure induces MCP-1 expression in MC through activation of p42/p44 MAP kinase.

Monocyte chemoattractant protein-1 mRNA expression was observed within 5 minutes of application of external pressure, reaching a peak level at 10 minutes, but returned to the control levels by 30 minutes. These results indicate that MCP-1 expression induced by externally applied pressure was rapid and of a transient nature. Our results are different from previous reports showing that the induction of MCP-1 mRNA by other stimuli such as inflammatory cytokines and glucose occurs after a few hours and lasts for more than several hours [9, 22, 24, 36]. The unique feature of pressure-induced MCP-1 expression might be due to differences in applied stimuli, employed signaling cascades, and cell types used. Furthermore, using Western blotting and ELISA, we also confirmed that MCP-1 protein production within MC followed the secretion of its protein into the culture medium after pressure load (Fig. 4). The difference in the time course of MCP-1 mRNA and protein levels might be due to differences in degradation rates; MCP-1 mRNA degrades rapidly, whereas its protein is relatively stable. Our results suggest that elevated pressure, similar to inflammatory cytokines [9, 24, 36], can directly induce mesangial MCP-1 expression. Also, induction of MCP-1 mRNA expression was dependent on a critical level of externally applied pressure. Our results that MCP-1 expression levels at high pressure (100 mm Hg) were less than those at 80 mm Hg indicate that 80 mm Hg is the most effective physical pressure level for MCP-1 induction. These findings are consistent with those of our previous study demonstrating maximal increases in DNA synthesis and MC proliferation in MCs exposed to external pressure of 80 mm Hg [6]. In pathological conditions in vivo, MC are persistently subjected to high pressure [2]. Although it remains to be clarified whether the rapid induction of MCP-1 observed in our study plays a pathogenic role in vivo, our previous and these findings that application of pressure to MC resulted in a pressure level-dependent (~80 mm Hg) induction of MCP-1 expression as well as MC growth through the induction of PDGF [6] strongly suggest that glomerular
hypertension is likely to play important roles in the progression of glomerular diseases.

Several signaling pathways such as p42/p44 MAP kinase, PKC, activator protein-1 (AP-1), and nuclear factor-B (NF-B) are involved in the regulation of MCP-1 expression [22–25, 37–44]. The present study design used inhibitors of MEK, PD98059, and that of PKC, calphostin C, to examine the likely intracellular mechanism that mediates the pressure-induced MCP-1 expression. Our results showed that PD98059 and calphostin C significantly inhibited pressure-induced MCP-1 mRNA expression, whereas PI 3-kinase inhibitor, wortmannin, had no inhibitory effects. These results suggest that p42/p44 MAP kinase and PKC pathways—but not that of PI 3-kinase—are likely to be involved in the pressure-induced MCP-1 expression in MCs. The involvement of p42/p44 MAP kinase in this process was further supported by our findings that stimulation by PDGF-B, which is known to induce a rapid activation of MAP kinase (within 5 min) [16], significantly increased the levels of MCP-1 mRNA (~2-fold compared with the level seen during application of external pressure), as described previously [9, 24, 36]. Our results also showed that externally applied pressure rapidly increased the catalytic activity of p42/p44 MAP kinase with the peak level noted at one minute (3-fold). Furthermore, using Western blotting, pressure rapidly increased p42/p44 MAP kinase phosphorylation levels, and those levels were significantly inhibited by MEK inhibitor PD98059, findings that are similar to those reported by Kawata et al [26]. Our results are consistent with the previous findings reported by Chien, Li and Shyy [37], which showed that PD98059, a MAP kinase inhibitor, abrogated angiotensin II-induced MCP-1 gene expression in endothelial cells. However, recent studies also have shown the importance of p38 MAP kinase in cytokine-stimulated MCP-1 expression in several cell types, including human MC [45]. Although the reason for the discrepancy in MAP kinases signaling is not clear at present, it may reflect species differences or differences in the applied stimulus.

To confirm further the involvement of 42/p44 MAP kinase in the pressure-induced MCP-1 expression, either wild-type or constitutively active MEK1 were transfected into MC. Interestingly, the constitutively activated form of MEK1 effectively induced MCP-1 expression even without externally applied pressure, suggesting that activation of p42/p44 MAP kinase itself can induce MCP-1 gene expression. These results strongly indicate the involvement of the p42/p44 MAP kinase signal pathway in pressure-induced MCP-1 expression. This study also found that pressure-induced MCP-1 expression was inhibited by genistein, a tyrosine kinase inhibitor (data not shown), which may support our earlier results indicating that pressure promoted a rapid increase in tyrosine kinase [6]. Thus, our results suggest that pressure-induced MCP-1 expression is mediated, at least partly, by a rapid activation of p42/p44 MAP kinase pathway through tyrosine kinase.

Using the selective PKC inhibitor calphostin C, pressure-induced MCP-1 mRNA expression was abolished by calphostin C as well as PD98059, suggesting the involvement of PKC-dependent pathway in pressure-induced MCP-1 expression. However, pretreatment of MC with calphostin C failed to inhibit pressure-induced increase in p42/p44 MAP kinase phosphorylation levels (data not shown). These results suggest that PKC is not located upstream of p42/44 MAP kinase and that the PKC pathway modifies pressure-induced MCP-1 expression via a pathway independent of p42/p44 MAP kinase or below the level of MAP kinase in MCs, as suggested previously [46]. Thus, PKC as well as p42/p44 MAP kinase may play a major role in the pressure-induced MCP-1 expression. Our results are supported by those of earlier studies, which showed enhanced MCP-1 expression by other mechanical forces such as strain and shear stress through activation of PKC in human and bovine endothelial cells [43, 47].

Previous studies have shown that the genes encoding MCP-1 as well as PDGF in bovine aortic endothelial cells contain the shear-stress responsive element (SSRE) that responds to shear stress [47]. Furthermore, various ion channels such as a stretch-activated ion channels and/or a mechanosensitive ion channel also are thought to be involved in the transduction signals from mechanical forces to cell membrane in some tissues including MC [48, 49]. However, to our knowledge, there are no studies that have previously demonstrated the presence of gene elements related to pressure such as SSRE or pressure-related ion channels in MCs. At present, it is not clear how the cell senses increased external pressure. Previously we have shown that external pressure increased PDGF-B mRNA levels at three hours after application, with a peak level occurring at six hours [6]. In this respect, we cannot exclude the possible involvement of cytokines such as PDGF in pressure-induced events. However, based on our previous [6] and present results of apparent time lag between the expression of PDGF-B and that of MCP-1, it is unlikely that PDGF-B induces the early (10 min) enhanced expression of MCP-1 demonstrated in the present study. Further studies are required to identify the mechanisms involved in the perception of externally applied pressure stimulus.

In conclusion, the present study demonstrates that pressure stimuli per se produced a rapid and transient induction of MCP-1 expression in MC, through the activation of p42/p44 MAP kinase pathway. Our results point to the importance of lowering blood pressure in glomerular hypertension to modulate mesangial MCP-1 expression.
ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Education, Science and Culture of Japan (A.O., No. 11617066, M.T., No. 13770616), the Renal Anemia Foundation, Japan (A.O., M.T.), Kaibara Morikazu Medical Science Promotion Foundation (M.T.) and the Fukushima Cancer Association, Japan (M.T.). Part of this manuscript was presented in abstract form at 32nd and 33rd annual meeting of the American Society of Nephrology. The authors thank Ms. Akiko Sugimoto for technical assistance.

APPENDIX

Abbreviations used in this article are: ELISA, enzyme-linked immunosorbent assay; MC, mesangial cells; MCP-1, monocyte chemotactic protein-1; MEF, mitogen-activated protein kinase kinase; p42/p44 MAP kinase, p42/p44 mitogen-activated protein kinase; PDGF, platelet-derived growth factor; RT-PCR, reverse transcription-polymerase chain reaction.

Reprint requests to Akiko Osajima, M.D., Ph.D., The Second Department of Internal Medicine, University of Occupational and Environmental Health, School of Medicine, 1-1 Iseigaoka, Yahatanishi, Kitakyushu, 807-8555, Japan. E-mail: mdoasa@cncl.uoeh-u.ac.jp

REFERENCES


44. SHYY YJ, LI YS, KOLATTUKUDY PE: Activation of MCP-1 gene expression is mediated through multiple signaling pathways. Biochem Biophys Res Commun 192:693–699, 1993