

# Human Platelets Stimulate Mesangial Cells to Produce Monocyte Chemoattractant Protein-1 via the CD40/CD40 Ligand Pathway and May Amplify Glomerular Injury

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**Abstract.** Platelets are thought to play an important role in the initiation and the progression of a variety of glomerulonephritides. This study examined whether platelets induce production of monocyte chemoattractant protein-1 (MCP-1), a chemokine involved in leukocyte recruitment and glomerular injury, by cultured human mesangial cells (MC). To this end, platelets isolated from normal human donors were cocultured with MC at various ratios. MCP-1 synthesis was evaluated by quantitative real-time PCR and enzyme-linked immunosorbent assay. Platelets at 1:100 ratio (MC to platelets) induced an approximately 20-fold increase in mesangial MCP-1 mRNA and protein expression through an obligatory cell-to-cell contact–de-

pendent mechanism. Importantly, blockade of the CD40/CD40 ligand (CD40L) pathway with neutralizing antibodies decreased MCP-1 production by approximately 60%. It was confirmed that CD40 was functionally expressed on MC. Gel-shift assays and inhibitors of phosphorylation were used to demonstrate that activation of p38 mitogen-activated protein kinase, protein tyrosine kinases, and nuclear factor- $\kappa$ B activation were essential for MCP-1 production. These data indicate that platelet/MC contact stimulates the production of MCP-1 and may contribute to glomerular inflammatory responses by recruiting leukocytes from the peripheral blood.

Platelets are thought to play an important role in the initiation and the progression of glomerular injury in a variety of glomerulonephritides (GN) (1). Platelets are among the first cells to infiltrate the glomeruli, as shown in a variety of animal models, including Habu snake venom–induced proliferative GN (2), experimental diabetic nephropathy (3), or immune complex nephritis (4,5). Improvement of renal function and reduced glomerular cell proliferation was observed in immune complex nephritis (4) and a model of diabetic nephropathy (3) after treatment with anti-platelet antibodies. Depletion of platelets decreased the release of inflammatory mediators in acute nephrotoxic serum nephritis in rats (6). In humans, circumstantial data, such as decreased platelet survival and increased renal platelet sequestration, suggest a role of platelets in the pathogenesis of diffuse proliferative lupus GN (7). *In vitro* and *in vivo* studies have shown that growth factors found in abun-

dance in platelets, such as platelet-derived growth factor (PDGF) and transforming growth factor- $\beta$  (TGF- $\beta$ ), induce mesangial proliferation and matrix accumulation respectively that are observed in the course of glomerular injury (8). Anti-platelet agents improve long-term renal function in membranoproliferative GN (9).

In addition to soluble factors, platelets may also mediate glomerular injury by direct cell contact with glomerular mesangial cells (MC). Structural characteristics in glomeruli, such as defects of glomerular basement membrane between MC and blood lumen and porous glomerular endothelial cells, render direct contact between platelets and MC possible. In addition, injured glomerular endothelial cells in GN may further facilitate direct contact between platelets and MC. Coculture experiments have shown that contact with platelets induces chemokine production by vascular endothelial cells or myeloid leukocytes (10–12). Similar interactions between platelets and MC could therefore feasibly promote glomerular inflammation.

A recent study (13) has identified that platelets express CD40 ligand (CD40L) on their surface. CD40L, a transmembrane protein structurally related to tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), was originally identified on activated T cells. Interaction of CD40L on T cells with CD40 on B cells is of paramount importance for the development and function of the humoral immune system (14). CD40 is also found on monocytes, macrophages, and endothelial cells, suggesting that

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CD40L has a broader immunologic function *in vivo* (15). Upregulation of CD40 on MC has been observed in renal biopsies of patients with inflammatory glomerulonephritides such as lupus nephritis and IgA nephropathy (16). CD40/CD40L interactions between infiltrating mononuclear cells and resident renal cells are thought to play an important role in the pathogenesis in immune-mediated glomerulonephritis (17). Interestingly, CD40L-dependent platelet/endothelial cell interactions may induce the latter to secrete chemokines and express adhesion molecules (13). Similar platelet/MC interactions have not previously been explored.

Monocyte chemoattractant protein-1 (MCP-1) plays a crucial role in the pathogenesis of immune-mediated glomerulonephritides. Cultured renal parenchymal cells, including MC and renal tubular epithelial cells, produce MCP-1 in response to proinflammatory cytokines (18–20). Inhibition of MCP-1 by neutralizing antibodies attenuates macrophage influx in various experimental nephritides, decreases histologic glomerular damage, and reduces proteinuria (21–23). MCP-1-deficient lupus-prone mice demonstrate improved survival and a dramatic reduction in macrophage recruitment, renal pathology, and proteinuria. Notably renal Ig/C3 deposits are not diminished in MCP-1-deficient lupus-prone mice, suggesting an uncoupling of the inflammatory responses from deposition and activation of immune reactants (24).

We used coculture experiments and inhibitors of phosphorylation in this study to address whether platelets induce MCP-1 production by cultured MC and to identify the mechanisms involved in this process. We now report that platelets induce MCP-1 production in part through the CD40/CD40L pathway and identify key molecules involved in intracellular signaling pathways in MC.

## Materials and Methods

### Antibodies and Cytokines

The following Abs, recombinant cytokines, and proteins were used in this experiment: mouse monoclonal anti-human CD154 (CD40L) (Ansell, Bayport, MN); mouse monoclonal anti-human CD62P (P-selectin) (R&D systems, Minneapolis, MN); mouse monoclonal anti- $\beta$ 1-integrin (clone M13, kindly provided by Dr. Kenneth Yamada, NIDR, NIH, Bethesda, MD); mouse monoclonal anti-human CD40 (Biosource, Camarillo, CA); biotin-conjugated goat anti-mouse immunoglobulins; R-PE-conjugated goat anti-mouse immunoglobulins (Dako, Carpinteria, CA); recombinant human TNF- $\alpha$  (rTNF- $\alpha$ ); rIFN- $\gamma$  (both from R&D systems); and trimeric human CD40L/leucine-zipper fusion protein (rCD40L) (a gift from Immunex, Seattle, WA).

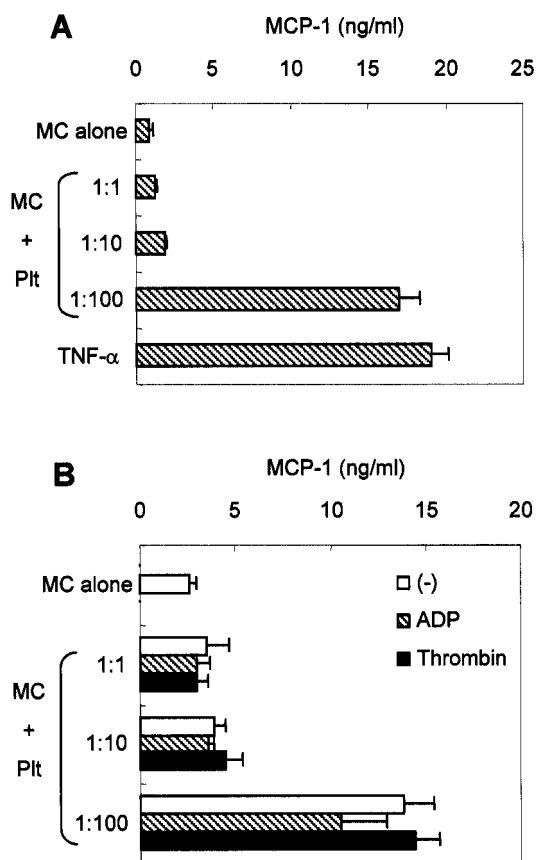
### Preparation of Platelets

Platelets were carefully isolated as described elsewhere with slight modification (25). In brief, platelet-rich plasma (PRP) was isolated from freshly drawn citrated whole blood after centrifugation ( $200 \times g$  for 20 min). PRP was recentrifuged ( $500 \times g$  for 20 min) in the presence of acid-citrate-dextrose (ACD; 15% vol/vol). The supernatant was discarded, and Tris-EDTA saline (10 mM Tris, 1 mM EDTA, and 150 mM NaCl) was used to resuspend the platelet pellet. Platelet suspension was centrifuged ( $500 \times g$  for 20 min), the supernatant was discarded, and the platelet pellet was resuspended in phosphate-

buffered saline (PBS). For the experiment that used the pre-stimulated platelets (Figure 1B and 3), platelets were activated with 0.2 U/ml of human thrombin (Sigma, St. Louis, MO) or 1  $\mu$ M of ADP (Sigma) for 10 min, washed twice with PBS, and then resuspended in PBS. In other experiments (Figure 3B), platelets were fixed for 10 min in 1% paraformaldehyde (Sigma) in PBS at 4°C with gentle agitation; this preserves membrane integrity but prevents secretory activity. To test the isolation-induced activation of platelets, P-selectin expression was examined by FACS analysis and found to be less than 5% (data not shown). In contrast, after the stimulation with thrombin or ADP, more than 90% of cells were positive for P-selectin. Contamination of leukocytes was assessed microscopically and found to be negligible.

### Human Mesangial Cells (MC)

Human MC were established and characterized as reported previously (26). Cells were cultured in Waymouth medium (Life Technologies, Grand Island, NY) supplemented with 17% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin,



**Figure 1.** (A) Platelets induce monocyte chemoattractant protein-1 (MCP-1) upregulation by mesangial cells (MC) at the ratio of 1:100. (B) Prestimulation of platelets with either thrombin or ADP is not required. In panel A, platelets were isolated from normal human donors and cocultured with MC at various ratios for 24 h. Alternatively, MC were stimulated with 10 ng/ml of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) for 24 h (A). In panel B, platelets were pre-stimulated with 0.2 U/ml of thrombin or 1  $\mu$ M ADP, washed, and then cocultured with MC. MCP-1 production in supernatants was determined by enzyme-linked immunosorbent assay (ELISA). Data are means  $\pm$  SE from three separate experiments.

cin, 2 mM L-glutamine, 2 mM sodium pyruvate, 1% (vol/vol) nonessential amino acids, and 26  $\mu\text{g/ml}$  of bovine insulin (all Life Technologies). Three independent cell lines were employed during passages 5 through 12.

### Platelet/MC Coculture

Platelets were prepared to 1, 10, or  $100 \times 10^5$  /ml in Waymouth medium containing 1% FBS. MC were harvested at 80% confluence and cultured in 24-well plates at  $6 \times 10^4$  cells/0.6 ml per well in 1% FBS/Waymouth medium in duplicate for 16 h. Subsequently prepared 0.6 ml of platelets was added to  $6 \times 10^4$  of MC, resulting in 1:1, 1:10, or 1:100 ratio of coculture (MC:platelets). In the experiments examining the effect of cell contact, identical parallel cultures were established in which platelets were separated from MC by a 0.4- $\mu\text{m}$  pore size semipermeable membrane (Biocoat, Becton Dickinson Labware, Bedford, MA) while sharing the same medium. In the experiments, which examined the effect of rTNF- $\alpha$ , rIFN- $\gamma$ , and rCD40L, MC were thawed in a 24-well plate at the same concentration with coculture experiment for 16 h for the adherence. Then, MC were further incubated at final volume of 1.2 ml/well in the presence or absence of various cytokines for 24 h. After 24-h incubation at 37°C, MCP-1 synthesis in supernatants and MC CD40 expression were determined by enzyme-linked immunosorbent assay (ELISA, R&D systems) and FACS analysis, respectively.

### Inhibition of Chemokine Synthesis by Antibodies or Chemical Inhibitors

In the blocking experiment with neutralizing Abs, coculture of MC and unstimulated platelets was carried out at the ratio of 1:100 (MC:platelets) in the presence or absence of 10  $\mu\text{g/ml}$  Abs against P-selectin,  $\beta$ 1-integrin, CD40L, or IgG<sub>1</sub> of irrelevant specificity (Sigma). All antibodies were added to MC simultaneously with platelets. In the experiments that used pyrrolidine derivative of dithiocarbamate (PDTC, Sigma), SB203580, PD98059 (both from Calbiochem-Novabiochem, La Jolla, CA), and genistein (Sigma), MC were preincubated with those inhibitors for 2 h, and then washed four times with medium to remove added inhibitors completely. Coculture of MC and unstimulated platelets were subsequently performed at 1:100 ratio for 24 h. MCP-1 synthesis was determined by ELISA in the supernatants after 24 h.

### Quantitative Real-Time PCR

MCP-1 mRNA expression in coculture was determined by real-time PCR, following the manufacturer's protocol. Briefly, total RNA was isolated from coculture of MC and unstimulated platelets (1:100 ratio) in 6-well plates after a variety time of incubation using RNeasy Mini kit (Qiagen, Valencia, CA). cDNA was synthesized from 2  $\mu\text{g}$  of total RNA by Superscript preamplification system for first strand cDNA system (Life Technologies), using oligo-dT primers. Subsequently, real-time PCR was performed in the ABI Prism 7700 Sequence Detection System (Perkin Elmer Applied Systems, Foster City, CA) using the SYBR Green I PCR kit (Perkin Elmer Applied Systems). Each reaction contained 25  $\mu\text{l}$  of the 2 $\times$  SYBR green Master Mix, 300 nM primers (MCP-1, forward: 5'-GAT CTC AGT GCA GAG GCT CG-3', reverse: 5'-TGC TTG TCC AGG TGG TCC AT-3';  $\beta$ -actin, forward: 5'-GAA CTT TGG GGG ATG CTC GC-3', reverse: 5'-CGG GAA ATC GTG CGT GAC AT-3') (27), 5  $\mu\text{l}$  of a 1:10 dilution of the cDNA prepared above, and water to 50  $\mu\text{l}$ . The reactions were incubated at 94°C for 10 min to activate the Amplitaq Gold polymerase (Perkin Elmer Applied Systems) followed by 40 cycles of 30 s at 94°C, 30 s at 60°C, and 60 s at 72°C. The ABI Prism

7700 Sequence Detection System software determined relative mRNA expression of MCP-1 and  $\beta$ -actin in each samples, based on the standard curve described below. MCP-1 mRNA expression in each sample were finally determined after correction with  $\beta$ -actin expression. Standard curve was generated as follows (28). In brief, cDNA was prepared from MC stimulated with 100 ng/ml of LPS (Sigma) for 8 h. Using this cDNA as template, PCR products for MCP-1 and  $\beta$ -actin were prepared with same primers. Each reaction contained 5  $\mu\text{l}$  of the 10 $\times$  PCR buffer; 500 nM forward and reverse primers; 0.5  $\mu\text{l}$  of Taq Gold polymerase; 1  $\mu\text{l}$  of the cDNA; and water to 50  $\mu\text{l}$ . The reactions were incubated at 94°C for 10 min to activate the Amplitaq Gold polymerase followed by 40 cycles of 30 s at 94°C, 30 s at 60°C, and 60 s at 72°C. After confirming specific single band on the agarose gels, serial dilutions (tenfold) of these PCR products ( $10^{-5}$ - $10^{-9}$ ) were prepared and then amplified simultaneously with samples from coculture using SYBR green. A standard curve was determined by the ABI Prism 7700 Sequence Detection System software. The expression of MCP-1 and  $\beta$ -actin in each sample was quantitated in separate wells with the respective primers. No PCR products were detected in the real-time PCR procedure without reverse transcription, indicating that the contamination of genomic DNA was negligible. Gels of the PCR products after quantification of MCP-1 or  $\beta$ -actin by real-time PCR showed a single band (152 and 711 bp, respectively) with the expected size (data not shown).

### Flow Cytometry

CD40 expression on MC was determined by FACS analysis. Briefly, MC were harvested with trypsin/EDTA and stained for CD40 expression by incubating the cells on ice with anti-human CD40 antibody or an isotype-matched control, followed by R-PE-conjugated goat anti-mouse immunoglobulins. CD40 expression was analyzed using CELLQUEST (Becton Dickinson).

### Electrophoretic Mobility Shift Assay (EMSA)

Nuclear proteins were isolated by the methods described previously with slight modification (29). Briefly, cocultures of MC and unstimulated platelets were performed at the ratio of 1:100 in 75-cm<sup>2</sup> flask for 1 h. Alternatively, MC were incubated for 1 h with 10 ng/ml of TNF- $\alpha$  or 100 ng/ml of LPS (Sigma) instead of platelets. After the treatment, cells were washed with ice-cold PBS, harvested by scraping, then spun-down by centrifugation. Pellets were resuspended in 1 ml of hypotonic buffer (10 mM Hepes, pH 7.9, containing 10 mM NaCl, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF). After 15-min incubation on ice, 60  $\mu\text{l}$  of 10% NP-40 was added. Cells were then vortexed vigorously for 15 s and then centrifuged for 1 min at 12,000 rpm at 4°C. After removing supernatants, the pellets were resuspended in 500  $\mu\text{l}$  of extraction buffer (20 mM Hepes, pH 7.9, containing 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF). After incubation for 15 min on ice, samples were vortexed vigorously for 15 s and then centrifuged at 12,000 rpm for 10 min. The supernatants containing nuclear protein were used for EMSA after determining protein concentration with the Bradford method (BioRad Protein Assay kit; BioRad Laboratories, Hercules, CA). EMSA of nuclear factor- $\kappa$ B (NF- $\kappa$ B) was carried out by use of Gel Shift Assay Systems (Promega, Madison, WI), following manufacturer's protocol. Briefly, double-stranded oligonucleotide containing the NF- $\kappa$ B binding element (5'-AGT TGA GGG GAC TTT CCC AGG C-3') was end-labeled using [ $\gamma$ -32P]ATP (NEN Life Science Products, Boston, MA) and T4 polynucleotide kinase and then purified through G-25 spin columns (BioRad). Nuclear extracts (2  $\mu\text{g}$  of protein) were incubated with radiolabeled probes for 20 min



at room temperature in 10  $\mu$ l of binding buffer (10 mM Tris-HCL, pH 7.5, containing 50 mM NaCl, 1 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 0.5 M DTT, 0.05 mg/ml poly (dl-dC)(dl-dC), 4% glycerol). Specific controls included unlabeled NF- $\kappa$ B or Sp-1 (5'-ATT CGA TCG GGG CGG GGC GAG C-3') consensus oligonucleotides at 100-fold excess. The samples were separated on a nondenaturing 4% polyacrylamide gel in Tris-Borate buffer, dried, and then analyzed by autoradiography.

### Statistical Analysis

All experiments were repeated at least three times. Results are presented as the mean  $\pm$  SEM from three separate experiments where indicated. Statistical significance, where indicated, was determined by *t* test. A value of *P* < 0.05 was considered to represent a statistically significant difference between two groups.

## Results

### Platelets Induce Upregulation of MCP-1 Production by MC

We first examined whether platelets induce MCP-1 production by MC. Platelets purified from normal human subjects were cocultured for 24 h with MC at various ratios. Platelets, at 1:100 ratio (MC:platelets), enhanced MCP-1 production by 18-fold above the basal level released by MC alone (Figure 1A). This was comparable to levels induced by recombinant TNF- $\alpha$ . We did not observe further increases in MCP-1 production by higher ratios up to 1:500 (data not shown).

We next examined the effect of prestimulation of platelets with ADP or thrombin. Platelets were isolated and stimulated with thrombin or ADP for 10 min. Activation of platelets was verified by the induction of P-selectin expression (data not shown). After washing, platelets were cocultured with MC at various ratios as before. Pretreatment of platelets with thrombin or ADP did not further enhance the expression of MCP-1 (Figure 1B), indicating that specific prior activation of platelets was not required. Stimulated platelets alone did not synthesize MCP-1 (data not shown).

We next determined whether the upregulation of MCP-1 production occurred at the transcriptional level. Platelets (without prior stimulation) were cocultured with MC at the ratio of 1:100. At various time points, cells were harvested and mRNA was extracted and quantified by real-time PCR. Unstimulated MC expressed low levels of basal MCP-1 mRNA. Platelets enhanced MCP-1 mRNA expression 22-fold at 6 h, which was sustained up to 12 h (Figure 2). Taken together, these data demonstrate that platelets induce MCP-1 upregulation by MC at both protein and mRNA levels. The magnitude of this response could not be further enhanced by specific prior *in vitro* platelet stimulation. This may have reflected activation of critical pathways during platelet isolation. This could have varied between donors, we therefore elected to stimulate platelets (judged by P-selectin expression) in subsequent mechanistic experiments to ensure maximal stimulation had been achieved in each experiment.

### Direct Cell-to-Cell Contact between Platelets and MC Is Essential for MCP-1 Production

Stimulation of MC by platelets could be mediated either by direct cell-to-cell contact or through soluble factors. We there-

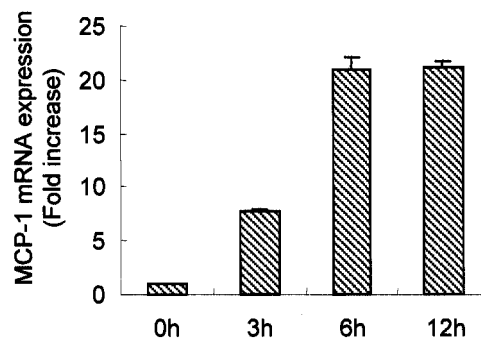


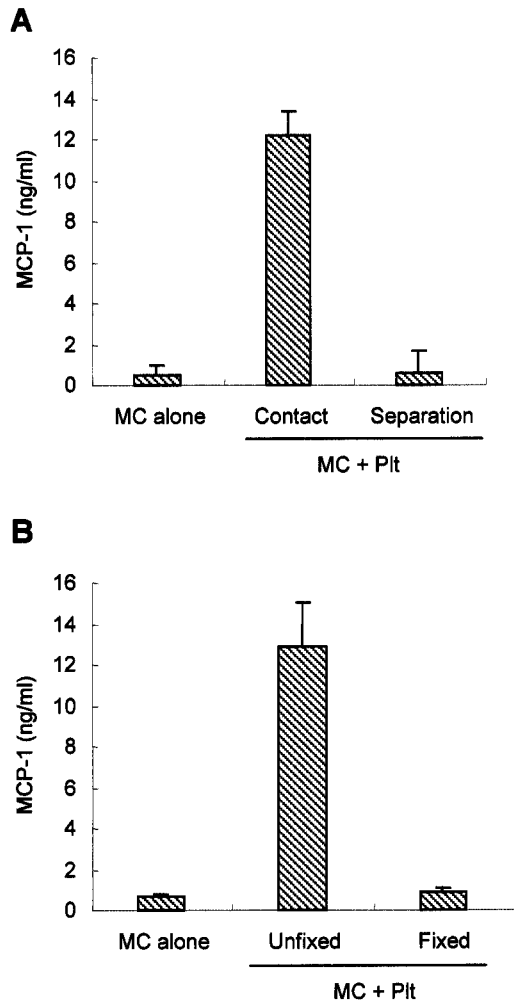
Figure 2. Platelets induce upregulation of MCP-1 mRNA by MC. MC were cocultured with platelets at the ratio of 1:100 for various times, and then MCP-1 and  $\beta$ -actin mRNA expressions were analyzed by real-time PCR. Fold increase in MCP-1 expression at each time point, relative to 0 h, is shown after correction with  $\beta$ -actin expression. Data are means  $\pm$  SE from three separate experiments.

fore determined whether platelets could induce MC MCP-1 release when separated by a semipermeable membrane that prevents direct cell-to-cell contact. Platelets were incubated with thrombin for 10 min and then cocultured with MC for 24 h at a 1:100 ratio in contact with or separated from MC. As shown in Figure 3A, separation of platelets from MC abrogated MCP-1 upregulation by MC, suggesting a role for cell-surface molecules. ADP-stimulated platelets also failed to induce MCP-1 when separated from MC (data not shown). These data implicate direct cell-to-cell contact in platelet-mediated MCP-1 upregulation in MC.

To address this further, platelets activated with thrombin were fixed with PFA to preserve membrane integrity but prevent secretion of soluble factors. Fixed, activated platelets were cocultured with MC at the ratio of 1:100 for 24 h. Figure 3B demonstrates that fixed activated platelets did not induce MCP-1 upregulation. Fixed activated platelets failed to secrete MCP-1 production, even at the ratio of 1:500 (data not shown). This suggests that cell-surface molecules alone are not sufficient and that soluble factors from activated platelets are also required for MCP-1 synthesis by MC.

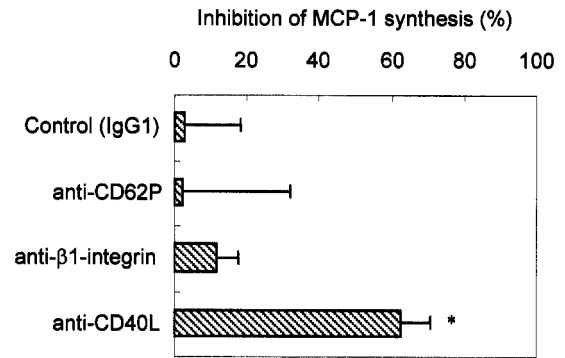
### CD40/CD40L Pathway is Involved in Platelet-Mediated MCP-1 Production by MC

We next sought to identify molecules involved in platelet/MC interactions. P-selectin plays a crucial role in leukocyte or endothelial cell activation by platelet binding (11,12), and  $\beta$ 1-integrins are implicated in platelet interactions with extracellular matrix (30). Activated platelets also express surface CD40L through which platelets can induce proinflammatory responses by endothelial cells (13). We therefore examined the effect of neutralizing P-selectin,  $\beta$ 1-integrins, and CD40L in coculture experiments. Platelets were cocultured with MC at the ratio of 1:100 in the presence or absence of antibodies for 24 h. Inhibition of CD40/CD40L reduced MCP-1 induction by approximately 60%, whereas blocking of P-selectin or  $\beta$ 1-integrins did not significantly affect the results (Figure 4). Although immunohistochemical analysis has shown that CD40

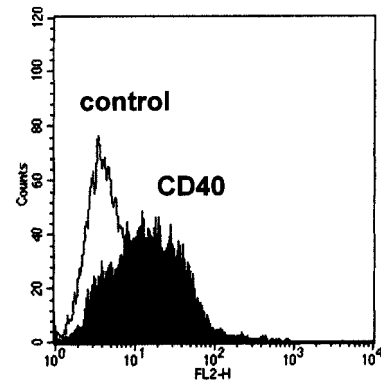


**Figure 3.** (A) Direct cell-to-cell contact between platelets and MC is essential for MCP-1 upregulation. (B) Fixed activated platelets do not upregulate MCP-1 production by MC. In panel A, MC were cocultured in contact with or separated by a porous membrane insert from pre-stimulated platelets at 1:100 ratio for 24 h. In panel B, platelets were stimulated with thrombin and fixed with 1% PFA to preserve membrane integrity but prevent secretion of soluble factors. MC were subsequently cocultured with fixed or unfixed activated platelets at 1:100 ratio for 24 h. MCP-1 production in supernatants was determined by ELISA. Data are means ± SE from three separate experiments.

is expressed on MC in renal biopsy from patients with a variety of glomerulonephritides (16), its expression on cultured MC has not been identified. Therefore we examined CD40 expression on MC by FACS analysis, and we found that MC constitutively expressed CD40 (Figure 5). In addition, inflammatory cytokines, such as TNF-α and interferon-γ (IFN-γ), or coculturing with platelets at the ratio of 1:100 increased CD40 expression (Table 1). To further examine the role of CD40 for MC activation, we stimulated MC with recombinant CD40L (rCD40L). MC were stimulated with 3 μg/ml of rCD40L for 24 h in the presence or absence of 500 U/ml of IFN-γ, which is known to function synergistically with CD40L in the monocyte/macrophage activation (15). In preliminary studies, the



**Figure 4.** Inhibition of MCP-1 synthesis in MC/platelet coculture by neutralizing Abs. MC were cocultured with platelets at 1:100 ratio for 24 h in the presence or absence of neutralizing Abs for 24 h. Percent inhibition was calculated as follows: 100 – 100 × (MCP-1 synthesis with neutralizing Ab/MCP-1 synthesis without neutralizing Ab). Data are means ± SE from three separate experiments. \* Statistically significant difference as compared with control (*P* < 0.01).



**Figure 5.** MC constitutively express CD40. MC were stained with anti-human CD40 antibody or an isotype-matched control, followed by R-PE-conjugated goat anti-mouse immunoglobulins. CD40 expression was determined by FACS analysis. Representative data of three distinct experiments are shown. The left-hand histogram represents the staining with control Abs.

optimal concentrations of rCD40L and IFN-γ were determined. Although rCD40L alone did not upregulate MCP-1, rCD40L in combination with IFN-γ enhanced MCP-1 synthesis by twofold above basal levels in response to IFN-γ alone (Figure 6). These data suggest that CD40 is expressed on MC and is involved in their activation by platelets to release MCP-1.

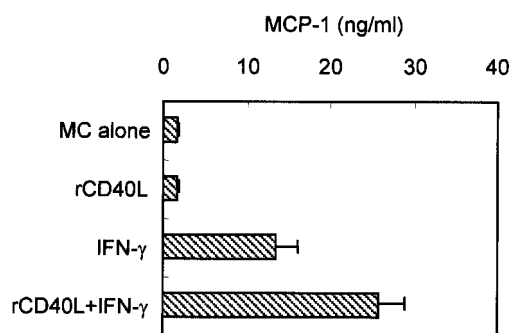
*Platelets Induce Mesangial MCP-1 Upregulation through NF-κB Activation*

MCP-1 expression is regulated both in a stimulus-specific and a tissue-specific manner (31). NF-κB plays an essential role for MCP-1 upregulation (32). We performed gel-shift assays to examine the molecular mechanisms by which platelets activate MC. Platelets significantly enhanced NF-κB activation above basal levels detected in resting MC (Figure 7A). Binding was inhibited by adding cold probe against NF-κB,

**Table 1.** Effect of cytokines or coculture with platelets on MC CD40 expression<sup>a</sup>

Stimuli	CD40 (MFI)
Medium	21.0
rTNF- $\alpha$ (10 ng/ml)	51.1
rIFN- $\gamma$ (500 U/ml)	43.3
Platelets (MC:platelets = 1:100)	119.7

<sup>a</sup> Shown is the MFI of CD40 expression on mesangial cells (MC) after culturing for 24 h in the presence or absence of rTNF- $\alpha$ , rIFN- $\gamma$ , or a 100 excess of platelets. Similar results were obtained from three different experiments.

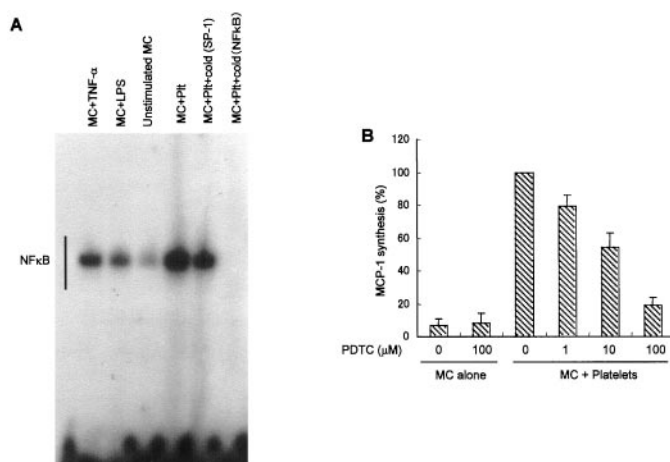


**Figure 6.** rCD40L enhances MCP-1 production by MC in the presence of interferon- $\gamma$  (IFN- $\gamma$ ). MC were cultured to adherence at  $6 \times 10^4$  cells/0.6 ml per well in 24-well plates in duplicate for 16 h. Cells were subsequently stimulated with rCD40L (3  $\mu$ g/ml), IFN- $\gamma$  (500 U/ml), or both. After 24 h, MCP-1 synthesis was determined by ELISA. Data are mean  $\pm$  SE from three separate experiments.

but not against SP-1, indicating specificity. To further investigate the involvement of NF- $\kappa$ B activation, we used PDTC, a specific inhibitor of NF- $\kappa$ B. PDTC reduced MCP-1 upregulation in a dose-dependent manner (Figure 7B). Collectively these data demonstrate that platelet contact induce NF- $\kappa$ B activation in MC, which in turn is essential for MCP-1 synthesis.

### Platelets Induce MCP-1 Production through p38 MAPK and PTK

Previous studies have shown that p38 MAPK activation is required in cytokine-induced MCP-1 expression (33,34). Similarly TNF- $\alpha$  or interleukin-1 (IL-1) induces MCP-1 production through protein tyrosine kinases (PTK), but not protein kinase C (PKC) or cAMP-protein kinase A (PKA) (35,36). To examine whether platelets induce MCP-1 synthesis by MC through activation of p38 MAPK and/or PTK, we used SB203580 or genistein, which are inhibitors of p38MAPK or PTK, respectively. As a control we used PD98059, an inhibitor of ERK1/2. MC were preincubated with these inhibitors for 2 h and then washed extensively with medium. In preliminary studies, the concentrations of inhibitors to achieve the maximal effects were established. Treatment with those inhibitors did not alter the basal expression of MCP-1 by MC. Platelets were

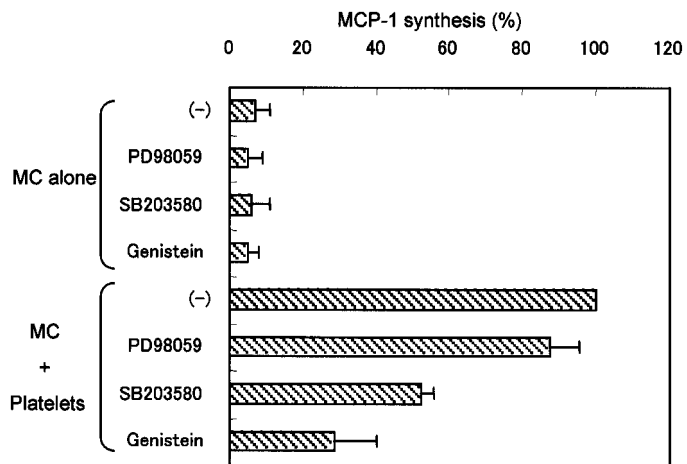


**Figure 7.** (A) Platelet contact induces nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation by MC. (B) PDTC, the specific inhibitor of NF- $\kappa$ B, reduced MCP-1 production in coculture in a dose-dependent manner. In panel A) MC were cocultured with platelets at 1:100 ratio for 1 h. Alternatively, MC were incubated in the presence or absence of LPS (100 ng/ml) or TNF- $\alpha$  (10 ng/ml) for 1 h. Nuclear extracts were subjected to electrophoretic mobility shift assay (EMSA) to examine NF- $\kappa$ B activation. Specificity was checked with an unlabeled probe (cold) against NF- $\kappa$ B or Sp-1. In panel B, MC pretreated with various concentrations of PDTC were cocultured with platelets at the ratio of 1:100 for 24 h. MCP-1 production was determined by ELISA. MCP-1 production in coculture without PDTC is shown as 100%. Data are means  $\pm$  SE from three separate experiments.

added to inhibitor-treated MC at a final ratio of 1:100. SB203580 and genistein reduced MCP-1 production by 40 and 70%, respectively (Figure 8), whereas PD98059 had no effect. These data suggest that platelets induce MCP-1 synthesis by MC through activation of p38 MAPK and PTK.

### Discussion

MCP-1 is known to play a predominant role in monocyte/macrophage recruitment into glomeruli in a variety of glomerulonephritides. Macrophages infiltrating glomeruli in turn stimulate resident glomerular cells to induce (1) mesangial proliferation and matrix expansion through macrophage-derived growth factors, such as PDGF and TGF- $\beta$ , and (2) upregulation of adhesion molecule and chemokine secretion through inflammatory cytokines, such as TNF- $\alpha$  and IL-1 $\beta$ , to facilitate further infiltration of leukocytes. Herein, we report that platelets induce MCP-1 production by MC in part through interactions involving CD40/CD40L and that activation of NF- $\kappa$ B, p38 MAPK, and PTK are involved in this process. Whereas an 100-fold excess of platelets is required to induce this phenomenon, these findings demonstrate a novel mechanism that could be important *in vivo*. Under pathologic conditions whereby the composition of the extracellular matrix may be altered and inflammatory molecules and other cells, such as macrophages, T cells, or blood-derived polymorphonuclear cells, are present, a smaller number of platelets might be enough to stimulate MC. Our findings indicate that platelets are not only regulators of intraglomerular coagulation but also



**Figure 8.** p38 MAPK and protein tyrosine kinases (PTK) are involved in mesangial MCP-1 upregulation induced by platelet binding. MC were pretreated with 50  $\mu$ g/ml PD98059, SB203580, or genistein for 1 h. In preliminary studies, the concentrations of inhibitors to achieve the maximal effects were determined. Medium was changed four times to remove added inhibitors completely. Subsequently, coculture of MC and platelets were performed at the ratio of 1:100. ELISA determined MCP-1 synthesis after 24 h. MCP-1 production in coculture without inhibitors is shown as 100%. Data are means  $\pm$  SE from three separate experiments.

key modulators of glomerular inflammatory responses. Platelet-mediated MCP-1 production by MC may be an important mechanism whereby platelets contribute to the amplification and progression of glomerular injury.

Previous studies exploring the pathogenesis of vascular inflammation and atherosclerosis have documented activation of vascular endothelial cells or myeloid leukocytes by platelet binding (10–12). In these studies, contact with activated platelets was shown to induce MCP-1 production by endothelial cells or leukocytes. Coculture of platelets and MC resulted in upregulation of osteopontin, cyclooxygenase 2 (COX2), and MCP-1 mRNA expression by MC (37). In the present study, we have considerably extended such observations and have defined potential pathways whereby such effects are mediated. We show that MC express CD40 *in vitro* and that such expression is functionally involved in cell-to-cell interactions. Critically however, we demonstrate that both cell-to-cell contact and soluble factors were implicated. Thus, pre-stimulated platelets did not induce MCP-1 by MC when separated from MC, indicating that direct contact between platelets and MC is essential. The most plausible explanation for this is that signals mediated by cell contact are indispensable for MCP-1 production. The effects of direct cell-to-cell contact may be bidirectional, resulting in stimulation of platelets to release soluble mediators or upregulate adhesion molecule expression (such as CD40L and P-selectin) and thus further amplify the platelet-induced stimulation of MC. Commensurate with this, rCD40L in combination with IFN- $\gamma$  enhanced MCP-1 production by MC, whereas rCD40L alone was ineffective (Figure 6). These data indicate that functional CD40 is expressed on cultured MC

and that signals mediated by CD40 enhance MCP-1 production.

As platelet expression of CD40L increases upon activation of platelets with ADP or thrombin, we expected, but did not find, higher levels of MC MCP-1 production induced by activated platelets. This may have reflected isolation effects that varied from donor to donor. In addition, MC produce extracellular matrix (including collagen, an activator of platelets) in their surrounding microenvironment, and this by itself may be enough to induce maximum CD40L expression on platelets. Alternatively, in the presence of other stimuli, baseline levels of CD40L similar to those found on unstimulated platelets may be sufficient for optimal activation of MC.

Several studies have shown that CD40/CD40L interaction plays important roles in the pathogenesis of immune-mediated glomerulonephritides. In animal models of lupus nephritis, anti-CD40L antibodies ameliorate nephritis even when administered after disease onset (38). Our data suggest that the protective effect of anti-CD40L in this disease may partly reflect inhibition of MCP-1 production after platelet/MC interactions. CD40L-positive leukocytes are rarely observed in glomeruli as compared with the interstitium in proliferative lupus nephritis (16), further strengthening the idea that platelet-bound CD40L might be the predominant ligand source for CD40 on MC. A functional soluble form of CD40L has recently been reported (39,40), suggesting that platelets may stimulate MC through either soluble or cell-surface CD40L. In contrast to data obtained in other cell types (11,12), blockade of P-selectin on platelets did not decrease MCP-1 production in coculture. Although we used doses of the anti-P-selectin mAb that can be expected to almost completely block surface-expressed P-selectin, mesangial production of MCP-1 was not significantly decreased. Considering the redundancy between P-selectin and other similar adhesion molecules, these data do not exclude a modest contribution by P-selectin.

MCP-1 expression is regulated in both a stimulus-specific and a tissue-specific manner (31). Although several lines of evidence suggest that NF- $\kappa$ B activation is essential, recent studies have shown that MCP-1 induction by PDGF-BB is dependent on proximal Sp-1 but not on NF- $\kappa$ B activation (32). We have demonstrated that platelet binding induces high levels of NF- $\kappa$ B activation that in turn is required for MCP-1 production. Platelets were surprisingly more potent in NF- $\kappa$ B activation than TNF- $\alpha$  or LPS. This also might have resulted from strong and rather unique effects of the direct cell-to-cell contact, together with soluble inflammatory mediators. Accordingly in our previous study, which examined the interaction between monocytes and MC, both cell-to-cell contact and soluble factors were required for the maximum activation of the latter (18). Other studies have shown that platelet contact induces chemokine or adhesion molecule expression by endothelial cells or leukocytes through NF- $\kappa$ B activation (10,12). NF- $\kappa$ B plays a critical role in inflammatory response through induction of a variety of proinflammatory genes. Thus platelet binding to MC may lead to the amplification of inflammatory responses through the expression not only of MCP-1 but also of other inflammatory molecule expression dependent upon



NF- $\kappa$ B. Ligation of CD40 by CD40L activates NF- $\kappa$ B through TNF receptor-associated factors (TRAF) (41). It is thus possible that CD40L upregulates MCP-1 through NF- $\kappa$ B activation; alternatively, activated platelets contain IL-1–like activity which is also a strong inducer of NF- $\kappa$ B activation (42).

Previous studies have demonstrated that TNF- $\alpha$  induces MCP-1 production by HUVEC through p38 MAPK, but not through other MAPK, such as Jun N-kinase (JNK) or ERK1/2 (33). We have identified that p38 MAPK is involved in platelet-mediated MCP-1 production by MC as treatment of MC with a specific inhibitor of p38 MAPK decreased MCP-1 by 50% (Figure 7). Although it is still unclear how activation of p38 MAPK leads to induction of MCP-1 expression, a recent study suggested that p38 MAPK is involved in the activation of NF- $\kappa$ B in the cytoplasm as well as in modulating its transactivating potential in the nucleus (43). The finding that an inhibitor of PTK reduced MCP-1 production by 70% demonstrates that PTK also play a role in platelet-mediated MCP-1 induction by MC. Ligation of CD40 is reported to activate p38 MAPK and PTK, such as lyn, in other types of cells (44,45). Therefore, we have also examined if rCD40L alone can directly induce their activation by Western blot using specific antibodies against phospho-p38 MAP kinase (Cell Signaling Technology, Beverly, MA) or phosphotyrosine (clone 4G10; Upstate Biotechnology, Lake Placid, NY). rCD40L alone did not induce any detectable upregulation of p-p38 MAPK and phosphotyrosine (data not shown). This does not completely exclude the possibility that CD40L activates at low levels p38 MAPK and PTK, considering the sensitivity of Western blotting and the absence of co-stimulation from activated platelets.

In summary, we have shown that platelet contact induces mesangial expression of MCP-1, in part through the activation of CD40/CD40L, NF- $\kappa$ B, p38 MAPK, and PTK. As MCP-1 plays a key role in the infiltration of macrophages into inflamed glomeruli and glomerular injury, we suggest that platelet/MC adhesion may represent an important mechanism for the amplification and the perpetuation of glomerular inflammatory responses in a variety of glomerulonephritides. Inhibition of the interaction between platelets and MC could be of potential therapeutic benefit.

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