Monocyte Chemoattractant Protein-1 Expression Is Enhanced by Granulocyte-Macrophage Colony-stimulating Factor via Jak2-Stat5 Signaling and Inhibited by Atorvastatin in Human Monocytic U937 Cells*

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The proinflammatory cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) is expressed in inflammatory and atherosclerotic lesions. GM-CSF is known to enhance monocytic expression of monocyte chemoattractant protein-1 (MCP-1). However, the molecular mechanism(s) by which GM-CSF up-regulates the MCP-1 expression remains to be clarified. Thus, in this study, we examined our hypothesis that GM-CSF up-regulates the MCP-1 expression via Jak2-Stat5 signaling pathway. In human monocytic cell line U937, GM-CSF increased MCP-1 expression in protein and mRNA levels. Furthermore, analysis of the GM-CSF promoter element revealed that the STAT5 (signal transducer and activator of transcription-5) transcription factor binding site, located between −152 and −144 upstream of the transcription start site, as well as Janus kinase-2-mediated Stat5 activation were necessary for the GM-CSF-induced transcriptional up-regulation of the MCP-1 gene. This GM-CSF-induced MCP-1 expression, measured as both protein and mRNA levels, was down-regulated by atorvastatin, a 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor. However, this decrease in MCP-1 expression was not at the transcriptional level of MCP-1 gene but rather at the level of the stability of MCP-1 mRNA. These results indicate that GM-CSF regulates MCP-1 expression via Janus kinase-2-Stat5 pathway and by a novel regulatory mechanism of statins to reduce inflammatory reactions by down-regulating the expression of monocytic MCP-1, which promotes atherosogenesis.

Inflammatory events are involved in the pathogenesis of atherosclerosis (1, 2), and T-lymphocytes and monocytes/macrophages are abundant in the atherosclerotic lesion (3). Monocytes/macrophages are present in all stages of atherosclerosis, playing a central role in atherogenesis; their multiple functions include migration and production of growth factors, cytokines, and matrix-degrading enzymes as well as uptake of modified lipoproteins (4). Specifically, the adhesion and migration of circulating monocytes to endothelial cells and the subendothelial microenvironment are important for the initiation of atherogenesis (5).

An association between matrix turnover and migration of many types of cells may be involved in atherogenesis, and the production of matrix-degrading enzymes, such as matrix metalloproteinase (MMP), from monocytes is one of the key events to enhance the migratory action of the monocytes (6). Granulocyte-macrophage colony-stimulating factor (GM-CSF) has been reported to enhance MMP-1, MMP-9, and MMP-12 expression as well as stimulate the activation of RhoA and integrin clustering in monocytes (7–9). Thus, GM-CSF is thought to play pivotal roles in various monocyte functions, including migration in atherogenesis (7, 9). On the other hand, studies of blockage of the monocyte chemoattractant protein-1 (MCP-1) signaling pathway by MCP-1 knock-out and MCP-1 receptor knock-out have provided direct evidence of a critical role for MCP-1 and its receptor in monocyte migration during atherogenesis (10–12). The atherogenic effect of MCP-1 is mainly explained by its potent chemoattractive effect on monocytes. Since our previous study demonstrated that monocytic MCP-1 expression is up-regulated by GM-CSF, we speculated that there was a synergistic effect of MCP-1 and GM-CSF on monocytic migration (9). However, the molecular mechanism(s) by which GM-CSF enhances MCP-1 expression in monocytes is not clearly understood.

There is growing evidence that some beneficial effects of 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors (statins) are independent of their effects on lowering lipid levels. Statins have pleiotropic effects, such as improvement of endothelial dysfunction, increased nitric oxide bioavailability, anti-inflammatory activity, and antioxidant properties (13, 14). In particu-
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lar, anti-inflammatory effects would be beneficial for prevention of atherosclerosis. In fact, atorvastatin therapy reduces blood levels of proinflammatory cytokines, such as tumor necrosis factor-α, interleukin (IL)-1, IL-6, and MCP-1 in hypercholesterolemic patients and in those with acute coronary syndrome (15, 16).

The aim of the present study was to investigate how GM-CSF up-regulates the expression of MCP-1 in human monocytic U937 cells. Furthermore, we investigated whether atorvastatin would inhibit GM-CSF-induced MCP-1 expression as a potentially novel anti-inflammatory effect.

EXPERIMENTAL PROCEDURES

Chemicals—Mevalonate (Mev) and actinomycin D (AcD) were purchased from Sigma. Geranylgeranyltransferase inhibitor (GGTI-286), farnesyltransferase inhibitor (FTI-276), and Jak2 inhibitor (AG490) were from Merck. GM-CSF was from Peprotech (London, UK). The 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor atorvastatin (Atv) was kindly provided by Pfizer.

Cell Culture—Human monocytic U937 cells were obtained from the American Type Culture Collection (ATCC CRL-1593) and maintained in RPMI1640 medium (Invitrogen) with 5% fetal calf serum (ICN, Costa Mesa, CA).

MCP-1 in U937 Conditioned Medium—The U937 cells were seeded into 24-well plates at a density of 1 × 10⁶ cells/ml of RPMI1640 medium containing 0.5% fetal calf serum and were then stimulated with 1–10 ng/ml of GM-CSF for 24 h. MCP-1 in the culture supernatant was subsequently measured by an enzyme-linked immunosorbent assay according to the manufacturer’s instructions (R&D, Minneapolis, MN).

Northern Blot Analysis—U937 cells were incubated with GM-CSF (1–50 ng/ml) in the RPMI1640 medium with 0.5% fetal calf serum for 16 h. Then total RNAs were extracted by using Trizol reagent (Invitrogen) and fractionated on 1% agarose gel containing 6% formaldehyde (10 μg of total RNA/lane). After the RNAs were transferred onto a nylon membrane, the expression of MCP-1 mRNA was detected by hybridization with 32P-labeled MCP-1 probe, as previously described (9). Equal loading of RNA was confirmed by detecting β-actin as an internal control. The expression levels were measured by densitometry using the ImageJ program, free software available at the National Institutes of Health Web site.

Plasmid Construction—We cloned the human MCP-1 promoter region from the genomic DNA of U937 cells by PCR using the primer pairs 5′-GCTGGAGGCAGACTGCGAG-3′ and 5′-TCTAGATTCTCTTTAGCTGTT-3′, corresponding to nucleotide positions −932 to +60 relative to the transcription start site. The PCR products were cloned into pGhTl-basic vector (Promega, Madison, WI), and the sequence was confirmed. The 5′-serial and internal deletion mutants were generated by appropriate enzymatic digestion and ligation. The point mutations in the activator protein-1 distal site (AP-1d at −97 to −91) and the AP-1-proximal site (AP-1p at −69 to −63), NF-κB (at −89 to −80), and signal transducer and activator of transcription 5 (STAT5p; at −152 to −144; see the map in Fig. 24) were introduced by site-directed mutagenesis as follows: TACTCC to caACTCA for AP-1d, TGACTCC to caACTCC for AP-1p, GGAAGATCCCC to GGAAGATggg for NF-κB, and TTCCCTGGAA to TTCTTGtGtt for STAT5p. Jak2 (Janus kinase-2)- and Stat5a-expressing vectors were used for co-transfection studies (17).

To investigate the effects of the 3′-untranslated region (3′-UTR) of the MCP-1 gene (+361 to +732) on mRNA stability, the 3′-UTR was cloned into the MCP-1 luciferase construct (−538 construct in Fig. 24) at the end of the luciferase gene using the XbaI site. In this construct, thus, the luciferase gene is driven by the MCP-1 promoter, and the mRNA stability is regulated by the 3′-UTR. The AUUUA sequence in the 3′-UTR located at +496 to +500 was deleted by Spel/AflII digestion, followed by fill-in reaction by Klenow and ligation. This clone had the deletion from +477 to +538.

Transfection and Luciferase Assay—Luciferase assays were performed as previously described (18). Briefly, U937 cells (1.4 × 10⁶/cuvette) were transfected with 20 μg of reporter constructs along with 0.5 μg of β-galactosidase-expressing plasmid by electroporation (Gene Pulser II; Bio-Rad). After electroporation, the cells were seeded into a 24-well plate and incubated with or without GM-CSF (1–20 ng/ml) for 8 h. Then the cells were lysed, and luciferase activity was measured with a luminometer. For the co-transfection experiments, cells were electroporated with Jak2- and Stat5a-expressing plasmids (5 μg each per electroporation cuvette). To minimize variations in the transfection efficiency, we transfected cells in a single batch for each reporter plasmid and then divided the desired number of transfected cells to each well. Furthermore, co-transfection of β-galactosidase expression plasmid followed by measurement of β-galactosidase activity was used as internal controls to monitor the transfection efficiency. All luciferase assays were repeated at least three times, each in triplicate wells.

Effect of Stat5a Knockdown by siRNA—The effect of Stat5a knockdown on the MCP-1 transcriptional activity was investigated by transfection of siRNA (Custom SMARTpool, Dharmacon, Amersham Biosciences). The siRNA for Stat5a knockdown was a mixture of four different synthetic siRNAs targeting the Stat5a mRNA (J-005169-11, -12, -13, and -14). For a negative control, nontargeting siRNA 5 (D-001210-05-05) was used. The U937 cells were transfected with 20 μg of luciferase reporter constructs along with 0.75 μg of siRNA by electroporation. The knockdown efficiency of the siRNA was monitored by the expression of Stat5a protein by Western blotting described below.

Nuclear Run-on Assay—U937 cells were incubated with GM-CSF (10 ng/ml for 3 h) in the presence or absence of Atv (5 μmol/liter) and collected in a Nonidet P-40 lysis buffer (10 mm Tris-HCl, pH 7.4, 10 mm NaCl, 3 mm MgCl₂, 0.5% Nonidet P-40). After a 10-min incubation on ice, the nuclei were isolated by centrifugation (1200 rpm for 5 min). The nuclei (5 × 10⁷) were washed twice with the Nonidet P-40 lysis buffer and resuspended in 100 μl of resuspension buffer (40% glycerol, 50 mm Tris-HCl, pH 8.0, 5 mm MgCl₂, 1 mm dithiothreitol). The nuclei (5 × 10⁷) were used to carry out run-on transcription in a reaction mixture (10 mm Tris-HCl, pH 8.0, 150 mm KCl, 5 mm MgCl₂, 100 mm sucrose, 10% glycerol, 0.5 mm dithiothreitol, 0.4 mm ATP, CTP, and GTP, and 10 μl of [α-³⁵S]UTP (>3000 μCi/mmoll) at 30°C for 30 min (200 μl/reaction). The genomic
The probes were biotin-labeled at their 3′(5′ site of the MCP-1 promoter region and the mutant STAT5 and the cells were treated with a lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 4 mM EDTA, 2 mM Na$_2$VO$_4$). The supernatant was subjected to SDS-PAGE (50 µg/lane) and Western blotting using anti-Jak2 (Upstate Biotechnology, Inc., Lake Placid, NY), phosphospecific Jak2 antibody (Upstate Biotechnology, Inc.), and phosphospecific Stat5a/b antibodies (Upstate Biotechnology). The lysates from the U937 cells treated with GM-CSF in the presence or absence of Jak2 inhibitor AG490 were also applied to Western blotting.

Electrophoretic Mobility Shift Assay (EMSA)—U937 cells overexpressing Jak2 and Stat5a were stimulated with GM-CSF (10 ng/ml for 15 min), and the cells were treated with a lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 4 mM EDTA, 2 mM Na$_2$VO$_4$). The supernatant was subjected to SDS-PAGE (50 µg/lane) and Western blotting using anti-Jak2 (Upstate Biotechnology, Inc., Lake Placid, NY), phosphospecific Jak2 (Chemicon, Temecula, CA), Stat5a (Chemicon), Stat5b (Chemicon), and phosphospecific Stat5a/b antibodies (Upstate Biotechnology). The lysates from the U937 cells treated with GM-CSF in the presence or absence of Jak2 inhibitor AG490 were also applied to Western blotting.

Electrophoretic Mobility Shift Assay (EMSA)—U937 cells were incubated with GM-CSF (20 ng/ml, 15 min), and nuclear extracts were prepared as previously described (19). Double-stranded oligonucleotide, including the STAT5p (−152 to −144 bp; 5′-CTTTTCTACTTCTTGAAATCCACAG-3′) site of the MCP-1 promoter region and the mutant STAT5 (5′-CTTTTCTACTTCTTGATCCACAG-3′) were used. The probes were biotin-labeled at their 3′-end by terminal deoxynucleotidyltransferase (Pierce). Nuclear extract (5 µg) and labeled probe (20 fmol) were incubated for 30 min at room temperature in a binding buffer (Lightshift Chemiluminescent EMSA kit; Pierce). The DNA-protein complexes were separated on a native 4% polyacrylamide gel and then electrophoretically transferred onto a positively charged nylon membrane. The biotin-labeled DNA was detected by a chemiluminescence method (Lightshift Chemiluminescent EMSA kit; Pierce). For antibody supershift experiments, 2 µg of antibodies specific for Stat5a and Stat5b (Chemicon) were incubated with the nuclear extract at 4 °C for 1 h prior to incubation with the labeled probes.

**FIGURE 1.** MCP-1 expression was dose-dependently increased by GM-CSF at protein, mRNA, and transcriptional levels. A, secretion of MCP-1 into the culture medium was increased by GM-CSF (1–10 ng/ml for 24 h; n = 4 each). B, Northern blotting analysis of MCP-1 mRNA expression. MCP-1 mRNA expression was induced by GM-CSF in a dose-dependent manner (1, 10, 20, and 50 ng/ml for 16 h). C, luciferase reporter constructs including −932 bp from the transcriptional initiation site of the MCP-1 gene (−932bp MCP-1-Luc) were used to transfect U937 cells. GM-CSF up-regulated the promoter activity in a dose-dependent manner (1, 10, and 20 ng/ml for 8 h). The pGL3, was used as the promoterless control plasmid. Three different experiments were performed in triplicate.

**RESULTS**

MCP-1 Expression by GM-CSF in the U937 Cells—GM-CSF stimulated the U937 cells to produce and secrete MCP-1 into the culture medium (Fig. 1A). The GM-CSF-induced MCP-1 production was accompanied by a dose-dependent increase in MCP-1 mRNA expression (Fig. 1B).

Transcriptional Assay of MCP-1 Gene—The vector constructs used were the human MCP-1 promoter region up to −932 bp, including AP-1, NF-κB, and STAT5 motifs, and its deletion mutants (Fig. 2A). GM-CSF increased the MCP-1 transcriptional activity from the −932 bp construct in a dose-dependent manner (1–20 ng/ml for 8 h) (Fig. 1C). When the −932 bp construct was deleted up to −538 bp, the basal activity was increased, thus indicating the presence of an unknown repressive region at −932 to −538 bp. The deletion constructs up to −415 and −292 showed decreased basal activity but still maintained the GM-CSF response. When the 5′-deletion construct up to −145 was used for transfection, the GM-CSF response was decreased, but the basal activity was still preserved (Fig. 2A). The −314/−292 internal deletion construct showed a similar basal activity and GM-CSF response in comparison with the parental −538 construct. In contrast, the −314/−145 and −292/−145 internal deletion constructs showed a decreased GM-CSF response (Fig. 2B).

Effects of 3′-UTR of MCP-1 Gene on Luciferase mRNA Stability—The MCP-1 luciferase construct including the 3′-UTR was transfected as described above. Afterward, the cells were incubated with GM-CSF (10 ng/ml) for 8 h and then incubated with AcD (10 µg/ml) in the absence or presence of Atv (5 µmol/ml). The total RNA was extracted at 0, 2, 3, and 6 h after the addition of AcD and subjected to Northern blotting to evaluate mRNA degradation.

**Statistical Analysis**—All quantitative data were presented as the mean ± S.D. Student’s t test was applied, and differences at p < 0.05 were considered significant.
ence of a GM-CSF-responsive motif(s) in the region spanning from −292 to −145.

Involvement of STAT5, but not AP-1 and NF-κB, in MCP-1 Transcription—Since the −292 to −145 region included a putative STAT5 motif at −152/−144, which is known as one of the GM-CSF-responsive motifs, a point mutation at the STAT5 (STAT5p) motif was introduced in the −538 construct. This point mutation reduced the GM-CSF response, whereas mutations in the AP-1 or NF-κB motifs still resulted in a 2-fold activation by GM-CSF (Fig. 3A).

GM-CSF activated the Jak2 and Stat5a of both constitutively expressed and exogenously overexpressed Jak2 and Stat5a which was demonstrated by the detection of phosphorylated Jak2 and Stat5 (since, however, the phosphospecific anti-Stat5 antibody recognizes both Stat5a and Stat5b proteins, the bands include both isoforms) (Fig. 3B). Co-expression of Jak2 and Stat5a with the −538 reporter construct exhibited increased transcriptional activity induced by GM-CSF (Fig. 3B). Furthermore, co-transfection of siRNA, which leads to knockdown of Stat5a but not Stat5b expression (Fig. 3C) resulted in reduced GM-CSF-induced transcriptional activity (Fig. 3E). Together with the results obtained from the luciferase assay using mutations of the STAT5 motif, these results suggest that the STAT5 located at −152/−144 (STAT5p) is a potential GM-CSF-responsive motif for Jak2- and Stat5a-mediated MCP-1 gene transcription in the U937 cells.

Effect of Jak2 Inhibitor AG490 on MCP-1 Expression—Since the involvement of the Jak2-Stat5 signaling pathway was indicated by the results of the luciferase assay, AG490, a specific Jak2 inhibitor that inhibits Jak2 activity to phosphorylate Stat5a, was introduced for the studies by enzyme-linked immunosorbent assay, Northern and Western blotting, and luciferase assay. AG490 pretreatment (10 μmol/liter for 1 h) decreased GM-CSF-stimulated (10 ng/ml) MCP-1 expression at both protein (Fig. 4A) and mRNA (Fig. 4B) levels. Furthermore, the AG490 treatment decreased the GM-CSF-induced Stat5 phosphorylation (Fig. 4C) and GM-CSF-induced MCP-1 promoter activity (Fig. 4D).

EMSA for GM-CSF-induced Stat5 Binding—To confirm that the MCP-1 gene expression was mediated through the Stat5

FIGURE 2. Luciferase assay using 5′-serial and internal deletions of MCP-1 promoter constructs. The −932 bp construct includes two STAT5s (STAT5d at −306/−298 and STAT5p at −152/−144), two AP-1s (AP-1d at −97/−91 and AP-1p at −69/−63), and 1 NF-κB motif (κB at −89/−80). A, the 5′-deletion up to −145 bp resulted in decreased GM-CSF response (10 ng/ml for 8 h). Three different experiments were performed in triplicate. B, when the internal deletions excluding −314/−145 and −292/−145 were introduced, the transcriptional up-regulation by GM-CSF (10 ng/ml for 8 h) was abolished. Three different experiments were performed in triplicate.
binding to the STAT5 motif, we subjected the nuclear extracts from GM-CSF-stimulated U937 cells to EMSA (Fig. 5). The nonstimulated control extract showed four DNA-protein complexes (NS) with the wild-type (wt) STAT5p probe (lane 3), whereas the extract from the GM-CSF-stimulated cells showed another slower migrating complex (lane 4, closed arrowhead). When the wild-type cold competitor was used (50-fold excess), most of the complexes were abolished (lane 5). In contrast, mutant (mt) cold competitor did not compete with the slower migrating complex (lane 6), indicating that the slower migrating complex (closed arrowhead) was specific for the STAT5 motif. In support of this interpretation, the mutant probe did not detect the slower migrating complex (lane 7). When the extract was incubated with normal rabbit serum (NRS), the specific complexes were still detected (lane 10). In contrast, the specific complex was supershifted by anti-Stat5a antibody (open arrowhead in lane 8). The incubation with anit-Stat5b antibody showed, however, only a minor shifted band (open arrowhead in lane 9). Therefore, the supershift assay indicated that the specific complex contained, at least, the Stat5a transcriptional factor as a major component.

**Atorvastatin Effects on MCP-1 Expression in Protein and mRNA Levels**—Since Atv suppresses the production of mevalonate, farnesylpyrophosphate, and geranylgeranylpipholipidate, it inhibits farnesyl- and geranylgeranylation of cellular proteins, such as RhoA and Ras. Thus, mevalonate rescues from the Atv effects, and geranylgeranylation transferase inhibitor (GGTI) or farnesyltransferase inhibitor (FTI) mimics those. Atv (2.5 and 5 μmol/liter) reduced the MCP-1 content in the culture medium, and mevalonate (50 μmol/liter) partially rescued the MCP-1 expression (Fig. 6A). GM-CSF-induced MCP-1 mRNA expression was also reduced by the Atv treatment (2.5–10 μmol/liter; Fig. 6B). Furthermore, the reduced MCP-1 mRNA expression was partially recovered by the addition of mevalonate (50 μmol/liter). GGTI (10 μmol/liter) mimicked the effect of Atv, whereas FTI (10 μmol/liter) showed less of an inhibitory effect (Fig. 6C).

**Atorvastatin Did Not Affect the MCP-1 Promoter Activity but Did Affect mRNA Stability**—Atv treatment, however, did not inhibit the GM-CSF-induced MCP-1 transcription even at 10 μmol/liter concentration when the −538 construct was transfected (Fig. 7A). Furthermore, a nuclear run-on assay showed that Atv (5 μmol/liter) did not reduce the de novo synthesis of MCP-1 mRNA (114 ± 19% of GM-CSF treatment alone; not significant) (Fig. 7B). These results indicated that some post-transcriptional mechanism(s) for the reduction of MCP-1 mRNA expression is present, so we next investigated MCP-1 mRNA stability by using AcD. After a 16-h stimulation with GM-CSF, U937 cells were incubated with AcD to stop mRNA synthesis. Then total RNAs were harvested at 2, 3, and 6 h after AcD treatment to investigate the RNA stability in either the presence or absence of Atv (5 μmol/liter). When the cells were incubated with Atv, the MCP-1 mRNA half-life was significantly decreased compared with that with AcD treatment alone (half-life of 11.5 versus 8.1 h; Fig. 7B), indicating that Atv increased the destabilization of MCP-1 mRNA.

Since the AUUUA Sequence in the 3′-UTR of the MCP-1 gene is suggested to regulate the mRNA stability of cytokines and chemokines (20), next we addressed the 3′-UTR effects on the stability of the luciferase gene by measuring the luciferase
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FIGURE 4. AG490 effects on MCP-1 expression and Jak2/Stat5a activation. The U937 cells were preincubated with Jak2 inhibitor AG490 (10 μM/liter for 1 h) and then incubated with GM-CSF (10 ng/ml) for 24 h for the enzyme-linked immunosorbent assay (A), 16 h for Northern blotting (B), 15 min for Western blotting (C), and 8 h for the luciferase assay (D). GM-CSF-induced MCP-1 expression was down-regulated by Jak2 inhibitor AG490 in protein (n = 4 each) (A) and mRNA levels (B). C, Western blotting analysis of total Stat5a and phosphorylated Stat5. GM-CSF-induced phosphorylation of the Stat5 detected by phospspecific antibody (phospho in lane 2) was down-regulated by Jak2 inhibitor AG490 (phospho in lane 3). Since the phospespecific anti-Stat5 antibody recognizes both Stat5a and Stat5b proteins, the bands include both isoforms. The total expression levels of the Stat5a were not changed (total). D, AG490 reduced the GM-CSF-induced MCP-1 promoter activity from −538 reporter plasmid.

FIGURE 5. Electrophoretic mobility shift assay with STAT5 motif located at −152/−144 (STAT5p). The STAT5p probe (5′-CTTCTACTCCCTGGAAATCCACAG-3′) detected a specific DNA-protein complex from the nuclear extracts of GM-CSF-stimulated (20 ng/ml for 15 min) U937 cells (lane 4, closed arrowheads). The specific band was competed by wild-type cold competitor (lane 5) but not by the mutant competitor (lane 6). The mutant probe (5′-CTTCTACTCCCTGGATTATCCACAG-3′) did not detect the specific band (lane 7). Incubation with anti-Stat5a and Stat5b antibodies generated supershift bands (open arrowheads in lanes 8 and 9, respectively). wt, wild type probe; mt, mutant probe; Comp., cold competitor; NRS, normal rabbit serum.

activity (Fig. 8). When the 3′-UTR was absent, the activity was decreased to ~60% of initial activity after 6 h of ACD treatment (Fig. 8A). The addition of 3′-UTR at the end of the luciferase gene increased the activity (Fig. 8B), which was significantly decreased by the treatment with Atv. When the AUUUA sequence was deleted from the 3′-UTR, the activity was decreased in comparison with AUUUA(+) constructs (Fig. 8B) and Atv-mediated suppression of the luciferase activity was no longer detected (Fig. 8C). However, in comparison with the 3′-UTR(−) construct (Fig. 8A), the AUUUA-deleted constructs still showed higher activities. These suggested that the 3′-UTR increased the mRNA stability in a AUUUA sequence-dependent manner and that Atv decreased the stability also via a AUUUA-dependent mechanism(s), at least in part.

DISCUSSION

GM-CSF is a hematopoietic growth factor and a proinflammatory cytokine produced by inflammatory cells, including T-lymphocytes, monocytes/macrophages, and vascular endothelial and smooth muscle cells (21, 22). Previously, we demonstrated that GM-CSF has multiple effects, enhancing monocyte migration via RhoA/integrin activation and via expression of MMPs and MCP-1 (7, 9). In the present study, we show a molecular mechanism by which GM-CSF induced MCP-1 expression in U937 monocytic cells. GM-CSF increased MCP-1 transcription, mRNA expression, and MCP-1 production mediated through the Jak2-Stat5 signaling pathway and STAT5 motif but not via the AP-1 or NF-κB pathway. Furthermore, Atv inhibited GM-CSF-induced MCP-1 expression via destabilization of MCP-1 mRNA. Therefore, the findings presented here provide evidence that GM-CSF is an important regulator of monocytic MCP-1 expression and, moreover, suggest that the inhibitory effects of Atv on MCP-1 expression, which are independent of its lipid-lowering effect, may decrease the inflammatory reaction in atherosclerotic lesions.

Among the cells constituting the vascular system, human monocytic cell lines (THP-1 and U937), human umbilical vein endothelial cells, and vascular smooth muscle cells have been used for investigating the regulation of MCP-1 expression. For example, bacterial lipopolysaccharide has been reported to stimulate THP-1 cells to enhance MCP-1 expression, whereas IL-1β, IL-4, IL-6, IFN-γ, and tumor necrosis factor-α were shown to increase MCP-1 expression in U937 cells (23, 24). It has also been reported that vascular endothelial growth factor and IL-1β enhance MCP-1 expression in human umbilical vein endothelial cells (25, 26) and that tumor necrosis factor-α increases MCP-1 in rat vascular smooth muscle cells (27). Some of these studies have described the molecular mechanisms involved in the MCP-1 expression. Namely, lipopolysaccharide-induced MCP-1 up-regulation is mediated by NF-κB activation in THP-1 cells (23). In human umbilical vein endothelial cells, vascular endothelial growth factor- and IL-1β-induced expression of MCP-1 is up-regulated by the AP-1 and NF-κB signaling pathways, respectively (25, 26). Thus, many proinflammatory cytokines are able to up-regulate the MCP-1 expression in the vascular cells, at least in part, via AP-1 or NF-κB signaling pathways. In the present study, we studied the effects of GM-CSF, one of the proinflammatory cytokines, and
found that GM-CSF up-regulated MCP-1 expression in human monocyotic U937 cells. However, neither AP-1 nor NF-κB played a critical role in the GM-CSF-induced MCP-1 expression.

Previous reports, including our own, demonstrated GM-CSF stimulation of MCP-1 expression in monocyte/macrophage (8, 9, 28). These studies, however, did not address the molecular mechanism(s) by which GM-CSF enhances monocyotic MCP-1 expression. In general, GM-CSF binding to its receptor leads to activation of several kinases and transcriptional factors, such as Jak2, Ras, Raf, Erk, Stat5, and c-Fos, which consequently regulate gene expression (29). In the case of monocytes/macrophages, Jak2-Stat5 activation is essential for GM-CSF signaling, and GM-CSF-induced Jak2-Stat5 activation is required for gene expression, monocyte/macrophage differentiation, phagocytosis, and macrophage proliferation (30–32). In addition, our current findings clearly demonstrated that GM-CSF stimulation resulted in the activation of the Jak2-Stat5 signaling pathway and that the STAT5 motif was critical for MCP-1 gene transcription in U937 monocyotic cells. Therefore, the activation of Jak2-Stat5 signaling induced by GM-CSF might be associated with the critical functions of monocytes/macrophages. The knockdown experiments by siRNA targeting Stat5a (Fig. 3, D and E), however, did not show a complete suppression of the MCP-1 transcriptional activity, indicating the presence of further unknown mechanism(s). Since the phosphospecific anti-Stat5 antibody used in this study recognizes both Stat5a and Stat5b proteins, GM-CSF-mediated activation of Stat5 includes both isoforms (Figs. 3B and 4C). Thus, together with the results obtained from EMSA (Fig. 5), the Stat5b protein might be involved in the GM-CSF-induced MCP-1 expression.

The adhesion of monocytes to the endothelium and consequent

FIGURE 6. Atv effects on GM-CSF-induced MCP-1 expression. The U937 cells were treated as follows: preincubation with Atv (2.5 and 5 μmol/liter) and/or mevastatin (Mev; 50 μmol/liter) for 1 h and incubation with GM-CSF (10 ng/ml) for 24 h or for enzyme-linked immunosorbent assay (A); preincubation with Atv (2.5, 5, and 10 μmol/liter) for 1 h and incubation with GM-CSF (10 ng/ml) for 16 h for Northern blotting (B); preincubation with Atv (5 μmol/liter), Mev (50 μmol/liter), GGTI or FTI (10 μmol/liter) each for 1 h, followed by incubation with GM-CSF (10 ng/ml) for 16 h for Northern blotting (C). A, Atv inhibited GM-CSF-induced MCP-1 expression at the protein level, and Mev partially reversed the Atv inhibitory effect (n = 4 each). *, p < 0.05; **, p < 0.01. B, Atv decreased GM-CSF-induced MCP-1 mRNA expression in a dose-dependent manner. C, the Atv-mediated down-regulation of GM-CSF-induced MCP-1 mRNA expression was partially reversed by Mev as well as by GGTI or FTI.

FIGURE 7. Atv effects on MCP-1 transcriptional activity and mRNA stability. A, Atv pretreatment (2.5, 5, and 10 μmol/liter for 1 h) did not reduce the GM-CSF-induced (10 ng/ml) MCP-1 transcriptional activity (−538 bp construct). B, nuclear run-on assay after GM-CSF (10 ng/ml) and Atv (5 μmol/liter) treatment. Radioactive de novo synthesized mRNA was hybridized to MCP-1, vector, or β-actin CDNA. The de novo synthesis of MCP-1 mRNA was increased by GM-CSF (253 ± 36% of control; p < 0.001) but not affected by Atv (289 ± 48% of control; p > 0.01). Treatment with α-amanitin (+Amm) inhibited the mRNA synthesis. MCP-1 mRNA stability after AcD (10 μmol/liter) treatment was monitored by Northern blotting (n = 3). In the presence of Atv (5 μmol/liter), the degradation of MCP-1 mRNA was enhanced (closed circle) in comparison with that in the absence of Atv (open square). The expression of MCP-1 mRNA was normalized by that of β-actin and presented as -fold increase over the MCP-1 mRNA expression before AcD treatment (time 0 h). #, p < 0.05; ##, p < 0.01 in Atv(−) versus Atv(+) at each time. A representative autoradiogram is shown.

FIGURE 8. 3′-UTR effects on luciferase mRNA stability. The 3′-UTR was cloned into the MCP-1 luciferase construct (−538) at the end of the luciferase gene to regulate the luciferase mRNA stability. After transfection, the cells were incubated with GM-CSF (10 ng/ml, 8 h) and then treated with AcD (10 μmol/liter) with and without Atv (5 μmol/liter). After 6 h of AcD treatment, the luciferase activity was measured. A, 3′-UTR(−); B, 3′-UTR(+); C, 3′-UTR(+)ΔUUA−deletion construct. The luciferase activity was presented as percentage of activity in comparison with before AcD treatment (time 0 h). Three different experiments were performed in triplicate. Open circle, Atv(−); closed circle, Atv(+); a, p < 0.001 versus 3′-UTR(−)Atv(−) in A; b, p < 0.001 versus 3′-UTR(+)/Atv(−) in B. *, p < 0.01 versus 3′-UTR(−)/Atv(−) in A. ns, not significant.
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inflammatory response are an initial process in the early stage of atherogenesis, and the expression of proinflammatory cytokines by vascular cells modifies the vascular microenvironment (5, 33, 34). For example, it has been reported that monocytendothelial cell interaction induces GM-CSF and MCP-1 synthesis in both cells (35, 36). Since both GM-CSF and MCP-1 activate lymphocyte function-associated antigen-1 and very late antigen-4 on U937 monocytes via RhoA activation (9), monocytendothelial interaction might be enhanced by GM-CSF and further induce the cytokine expression. Although the molecular mechanisms underlying the adhesive interaction enhancing GM-CSF and MCP-1 expression are currently unknown, expression of GM-CSF and MCP-1 might be closely related to the initiation of inflammatory reactions. Therefore, GM-CSF-induced transcriptional up-regulation of the MCP-1 gene by monocytes in the vascular microenvironement is quite possible for the initiation of atherosclerosis. Interestingly, it has been reported that cervastatin and atorvastatin reduce U937 monocyte adhesion to endothelial cells via down-regulation of lymphocyte function-associated antigen-1 and very late antigen-4 expression and via RhoA inactivation (37, 38). In addition, recent studies reported that GM-CSF-induced RhoA activation is inhibited by statins (cervastatin and simvastatin) (39). Thus, statins could inhibit both GM-CSF-induced monocytic MCP-1 expression and integrin/RhoA activation, which action would consequently result in down-regulation of monocytic recruitment in the subendothelial microenvironment.

Just as simvastatin reduces tumor necrosis factor-α-induced MCP-1 expression in human macrophages (40), we demonstrated that Atv treatment decreased GM-CSF-induced MCP-1 mRNA expression in U937 monocytes. The luciferase assay using 3′-UTR-including constructs suggests a possibility that the 3′-UTR increases mRNA stability and that AUUUA sequence is responsive to Atv. In rat vascular smooth muscle cell, platelet-derived growth factor increases MCP-1 mRNA expression via both increased transcription and mRNA stability (41, 42), and dexamethasone decreases the mRNA stability via the AUUUA-independent pathway (43).

Since the Atv effects were partially reversed by mevalonate and mimicked by GGTI or FTI, Atv may regulate the MCP-1 mRNA half-life by inhibiting geranylgeranylation or farnesylation of proteins. In agreement with the present study, Atv has also been reported to decrease mRNA stability of angiotensin AT1 receptor (half-life of 6 versus 2.5 h) in vascular smooth muscle cells via inhibition of geranylgeranylation (44). In contrast, statins up-regulate endothelial nitric-oxide synthase expression by causing an increase in mRNA stability (half-life of 14 versus 27 h) via blocking of the geranylgeranylation of RhoA (45). The exact mechanism(s) of Atv-mediated mRNA stabilization and destabilization is still unknown.

In conclusion, our results indicate that GM-CSF enhanced monocytic MCP-1 expression at the transcription level via activation of the Jak2-Stat5 signaling pathway and that Atv decreased the stability of MCP-1 mRNA, potentially via an iso-prenoid pathway. Thus, these results provide further evidence that statins have anti-inflammatory effects independent of their cholesterol-lowering effects. In addition to the inhibitory effects of statins on RhoA and integrins (36–38), the novel regulatory mechanism by which statins reduce monocytic MCP-1 expression, thereby negatively affecting atherogenesis, would be a new therapeutic target for cardiovascular diseases.

REFERENCES

1. Ross, R. (1999) *N. Engl. J. Med.* **340**, 115–126
2. Tanimoto, A., Sasaguri, Y., and Ohtsu, H. (2006) *Trends Cardiovasc. Med.* **16**, 280–284
3. Hansson, G. K. (1997) *Curr. Opin. Lipidol.* **8**, 301–311
4. Ross, R. (1993) *Nature* **362**, 801–808
5. Butcher, E. C. (1991) *Cell 67*, 1033–1036
6. Goetzl, E. J., Banda, M. J., and Leppert, D. (1996) *J. Immunol.* **156**, 1–4
7. Wu, L., Tanimoto, A., Murata, Y., Fan, J., Sasaguri, Y., and Watanabe, T. (2001) *Biochem. Biophys. Res. Commun.* **285**, 300–307
8. Jost, M. M., Ninci, E., Meder, B., Kempf, C., van Royen, N., Hua, J., Berger, B., Hoefler, L., Modolell, M., and Buschmann, I. (2003) *FASEB J.* **17**, 2281–2283
9. Kohno, Y., Tanimoto, A., Cirathaworn, C., Shimajiri, S., Tawara, A., and Sasaguri, Y. (2004) *Pathol. Int.* **54**, 693–702
10. Ni, W., Egashira, K., Kitamoto, S., Kataoka, C., Koyanagi, M., Inoue, S., Imaizumi, K., Akiyama, C., Nishida, K., and Takeshita, A. (2001) *Circulation* **103**, 2096–2101
11. Boring, L., Gosling, J., Cleary, M., and Charo, I. F. (1998) *Nature* **394**, 894–897
12. Gu, L., Okada, Y., Clinton, S. K., Gerard, C., Sukhova, G. K., Libby, P., and Rolls, B. J. (1998) *Mol. Cell 2*, 275–281
13. Caballo, P., and Yeh, E. T. (2005) *Curr. Opin. Cardiol.* **20**, 541–546
14. Mason, R. P., Walter, M. F., Day, C. A., and Jacob, R. F. (2005) *Am. J. Cardiol.* **96**, 11F–23F
15. Kinlay, S., Schwartz, G. G., Olsson, A. G., Riffai, N., Leslie, S. J., Sasiela, W. J., Szarek, M., Libby, P., and Ganz, P. (2003) *Circulation* **108**, 1560–1566
16. Ascer, E., Bertolami, M. C., Venturinelli, M. L., Baccheri, V., Souza, J., Nicolau, J. C., Ramires, J. A., and Serrano, C. V., Jr. (2004) *Atherosclerosis* **177**, 161–166
17. Ilaria, R. L., Jr., Hawley, R. G., and van Etten, R. A. (1999) *Blood* **93**, 4154–4166
18. Murata, Y., Tanimoto, A., Wang, K. Y., Tsutsui, M., Sasaguri, Y., Corte, F. D., and Matsushita, H. (2005) *Arterioscler. Thromb. Vasc. Biol.* **25**, 430–435
19. Kao, C. Y., Tanimoto, A., Arima, N., Sasaguri, Y., and Padmanabhan, R. (1999) *J. Biol. Chem.* **274**, 23043–23051
20. Tebo, I., Der, S., Frewel, M., Khabar, K. S. A., and Williams, B. R. G. (2003) *J. Biol. Chem.* **278**, 12085–12093
21. Gasson, J. (1991) *Blood 77*, 1131–1145
22. Plenz, G., Koenig, C., Severs, N. J., and Robenek, H. (1997) *Arterioscler. Thromb. Vasc. Biol.* **17**, 2489–2499
23. Ueda, A., Ishigatsubo, Y., Okubo, T., and Yoshihura, T. (1997) *J. Biol. Chem.* **272**, 31092–31099
24. Biswas, P., Delfanti, F., Bernacconi, S., Mengozzi, M., Kota, M., Polenta-rutti, N., Mantovani, A., Lazzarin, A., Sozzani, S., and Poli, G. (1998) *Blood* **91**, 258–265
25. Yamada, M., Kim, S., Egashira, K., Takeya, M., Ikeda, T., Mimura, O., and Iwao, H. (2001) *Arterioscler. Thromb. Vasc. Biol.* **23**, 1996–2001
26. Choi, E. K., Park, H. J., Ma, J. S., Lee, H. C., Kang, H. C., Kim, B. G., and Kang, I. C. (1999) *FASEB J.* **13**, 2281–2283
27. Chen, Y. M., Chiang, W. C., Lin, S. L., Wu, K. D., Tsai, T. J., and Hsieh, B. S. (2004) *J. Pharmacol. Exp. Ther.* **309**, 978–986
28. Arjun, D. L., Nibbs, R. J., Jamieson, T., de Bono, J. S., and Graham, G. J. (1999) *Exp. Hematol.* **27**, 1735–1745
29. Kwon, E. M., Raines, M. A., Blenis, J., and Sakamoto, K. M. (2000) *Blood* **95**, 2552–2558
30. Feldman, G. M., Rosenthal, L. A., Liu, X., Hayes, M. P., Wynshaw-Boris, A., Leonard, W. L., Henninghausen, L., and Finbloom, D. S. (1997) *Blood* **90**, 1768–1776
31. Lehtonen, A., Matikainen, S., Miettinen, M., and Julkunen, I. (2002) *J. Leukocyte Biol.* **71**, 511–519
32. Warby, T. J., Crowe, S. M., and Jaworowski, A. (2003) *J. Virol.* **77**, 12630–12638
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