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Monocyte Chemotactic Protein 1 Upregulates IL-1β Expression in Human Monocytes

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Monocyte chemotactic protein-1 (MCP-1) chemoattracts and activates monocytes. The nature of the genes that are transcriptionally activated in the monocytes by MCP-1 is not well understood. To identify such genes, human blood monocytes were incubated with or without MCP-1 for periods of 1, 4, and 12 h and the RNA extracted from these monocytes was subjected to differential display. The differentially expressed transcripts were cloned and sequenced. Differential display showed that interleukin-1β (IL-1β) gene expression was upregulated by MCP-1 treatment of monocytes for 4 to 12 h. Quantitative PCR and ELISA assays showed that MCP-1 treatment caused elevation in the levels of IL-1β transcripts and protein, respectively. Immunoblot analysis showed that most of the protein was pro-IL-1β. Since IL-1β is known to induce MCP-1 synthesis, the present demonstration that MCP-1 induces IL-1β synthesis suggests that the induction of each other would amplify the biological effects of these cytokines during inflammation.

Key Words: **MCP-1; IL-1β; monocytes; differential display.**

Monocytes play an important role in host defense mechanisms. In most tissues, peripheral blood monocytes migrate in response to the gradient of monocyte chemotactic protein-1 (MCP-1). Once these cells interact with chemokines, they adhere to the vascular endothelium, diapedese into the subendothelial space, and traverse into the extracellular matrix where they mature into macrophages (1, 2). *In vitro,* MCP-1 shows chemotactic activity for monocytes, T cells, mast cells, and basophils. MCP-1 can be produced by a plethora of cells including endothelial cells, epithelial cells, fibroblasts, cardiomyocytes, leukocytes, and platelets (1). Its production is induced by cytokines indicative of injury and inflammation such as interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α), and by signals of infection such as lipopolysaccharide (LPS) (1, 3–7). MCP-1 expression has been shown to be induced under a variety of pathological conditions involving an inflammatory component such as atherosclerosis, congestive heart failure, rheumatoid arthritis, systemic lupus erythematosus, and idiopathic pulmonary fibrosis.

Once MCP-1 binds to its seven-transmembrane receptor, CCR2, a series of G-protein-coupled signaling events occur (8–11). Some of these events are involved in chemotaxis and others must lead to the induction of expression of specific genes for other biological functions possibly including activation and/or maturation of monocytes. The nature of the genes that are transcriptionally activated as a consequence of MCP-1 binding to monocytes is poorly understood. In this study, we explored this by performing mRNA differential display that revealed that IL-1β mRNA is among the transcripts upregulated in human monocytes as a result of exposure to MCP-1. We confirmed this finding by demonstrating increase in IL-1β transcripts by quantitative RT-PCR and increased production of pro-IL-1β protein by immunological methods. These results suggest that IL-1β and MCP-1 trigger the expression of each other to amplify their biological effects.

MATERIALS AND METHODS

Isolation of monocytes from blood. Monocytes were isolated from buffy coat from human blood (local Red Cross) using Ficoll-Paque followed by adherence separation. The adherent monocytes were

Abbreviations used: MCP-1, monocyte chemotactic protein-1; IL-1β, interleukin-1β; ELISA, enzyme-linked immunosandwich assay; PCR, polymerase chain reaction; RT, reverse transcription; LPS, lipopolysaccharide; ICE, interleukin-1β cleavage enzyme; NF-κB, nuclear factor-κB; CCR2, CC chemokine receptor 2; IL-IR1, interleukin receptor type 1; IL-1 RacP, interleukin-1 receptor accessory protein; TNFα, tumor necrosis factor α; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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incubated in RPMI-1640 with human sera (20%) in the presence or absence of MCP-1 (7 nM) for different periods of time.

mRNA differential display. Total RNA from monocytes, isolated by TRIzol method (Gibco BRL-Life Technologies), was treated with DNase I (Clontech), and purified with the QIAGEN RNeasy mini-kit. The mRNA differential display was performed using a Delta differential display kit (Clontech) according the manufacturer's instructions using pairwise combination of P and T primers [10 arbitrary P and 9 oligo(dT) T primers]. Example: P1 (ATTAACCCTCACTAAATGCTGGGGA) and T1 (CATTATGCTGAGTGATATCTTTTTTTTTAA). The PCR conditions: 94°C (5 min), 40°C (5 min), 68°C (5 min) for one cycle, 94°C (2 min), 40°C (5 min), 68°C (5 min) for two cycles and 25 cycles at 94°C (1 min), 60°C (1 min), 68°C (2 min). The PCR mixture (1.5 μl) was subjected to electrophoresis and transferred to Whatman paper, dried, and exposed to X-ray film at −70°C for 12–18 h. DNA from the gel segments was reamplified by PCR for 20 cycles at 94°C (1 min), 60°C (1 min), 68°C (1 min). These products were cloned into T/A-cloning vector (pCR 2.1-TOPO) from Invitrogen, and sequenced.

RT-PCR. Total RNA, obtained as for mRNA differential display, was converted into first strand cDNA by SuperScript II RNase H Reverse Transcriptase (Gibco-BRL) and aliquots were subjected to PCR with the following primers for IL-1β: forward 5′-AAACAGATGAAGTGCTCCTTCCAG-3′; reverse 5′-TGGAGAACACCACTTGTTGCTCCA-3′ and for β-actin: forward 5′-GACTACCTCATGAAGATCCT-3′; reverse 5′-CCACATCTGGTGGAAGGTGG-3′.

Quantitative PCR. All real time PCR reactions were conducted in the Bio-Rad iCycler. For each 50 μl reaction mixture, the following components were assembled in a master mix: 45 μl 1.1× Platinum PCR Supermix (Life Technologies), 2 μl 50 mM $MgCl_2$, 0.15 μl 100 μM each of forward and reverse primers, 0.1 μl 100 μM probe (PE Biosystems), 1.0 μl DNA, 1.6 μl ddH_2O. Aliquots (50 μl) of the mix were dispensed into a 96-well thin-wall PCR plate, the plate was covered with a piece of optically clear sealing film and the plate was placed in the iCycler. PCR conditions were 3 min at 95°C, followed by 50 cycles of 95°C for 10 s and 63°C for 30 s. Optical data were collected during the 63°C step. Primer and probe sequence for β-actin were: forward: 5′-TGCGTGACATTAAGGAGAAG-3′, reverse: 5′-GCTCGTAGCTCTTCTCCA-3′, probe: 5′-6FAM-CACGGCTGCTTCCAGCTCCTC-TAMRA-3′. Primer and probe sequence for IL1-β were: forward: 5′-CATTGCTCAAGTGTCTGAAG-3′, reverse: 5′-CCTCATTGCCACTGTAATAAG-3′, probe: 5′-6FAM-CAGAAGTACCTGAGCTCGCCAGTG-TAMRA-3′. Amplification of genomic DNA was used to verify that the PCR conditions did not amplify any genomic DNA contamination.

Immunoblotting and ELISA. Cells were harvested, washed twice with PBS, lysed for 30 min at 4°C in lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% NP-40, 100 μg/ml PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin), and the cell lysate was centrifuged at 16,000*g* for 10 min. Aliquots of cell extract and cell culture media were diluted with PBS and analyzed for IL-1β by ELISA (R&D) and immunoblotting.

RESULTS AND DISCUSSION

Differential Display Showing Induction of IL-1β in Human Monocytes Resulting from Incubation with MCP-1

To explore induction of gene expression in human monocytes by MCP-1, a differential display approach was used. Total RNA extracted from human monocytes incubated with 7 nM of MCP-1 and from control (untreated) monocytes were reverse transcribed and first-strand cDNA was amplified by using a combination of arbitrary P primers and oligo(dT) T primers. Figure 1 shows the area of a differentially displayed gel where amplified products were obtained with P1 and T1 primer combination as indicated under Materials and Methods. Enhanced level of expression of a gene caused by treatment of monocytes with MCP-1 for 1, 4 and 12 h was indicated by the appearance of a product at 480 bp (Fig. 1). When the DNA from this band was recovered from the gel, re-amplified by PCR, and cloned, and six clones were sequenced, each showed the same sequence. A GenBank search revealed that this sequence was identical to that of IL-1β/pro-IL-1β cDNA (12, 13) beginning at position 671 and ending at 1054 to 1100 bp. The IL-β precursor is a 269 amino-acid protein which is cleaved into a 116 amino acid pro-segment and 153 amino acid mature peptide encoded by nucleotides from position 426 to 884 (12). With mRNA differential display, we found MCP-1 dependent synthesis of pro-IL-1β in human monocytes and this transcript level reached a maximum after several hours of treatment with physiological concentrations of MCP-1.

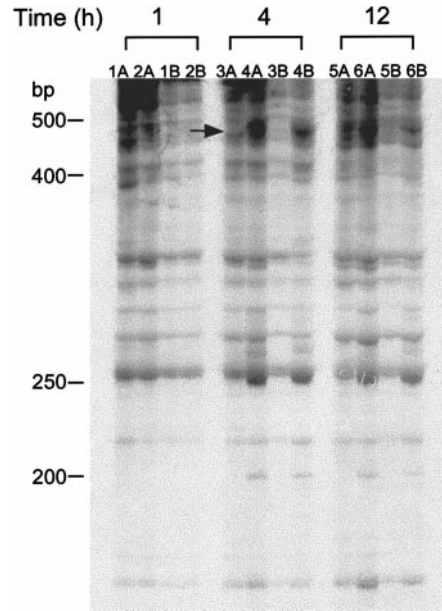

FIG. 1. A differential-display gel showing the amplified products obtained with P1 and T1 primer combination. (A) 1:10 dilution of cDNA, (B) 1:40 dilution of cDNA. 1, 3, 5: templates from untreated monocytes; 2, 4, 6: from MCP-1-treated monocytes; MCP-1 treatment periods are shown on top. Arrow, the band representing IL-1β transcript fragment.

Effect of MCP-1 Treatment on the Level of IL-1β Transcripts in Human Monocytes

We performed RT-PCR and real time PCR to further test whether MCP-1 treatment caused an increase in IL-1β transcript level. We found that 18 cycles of PCR under our conditions most clearly demonstrated the

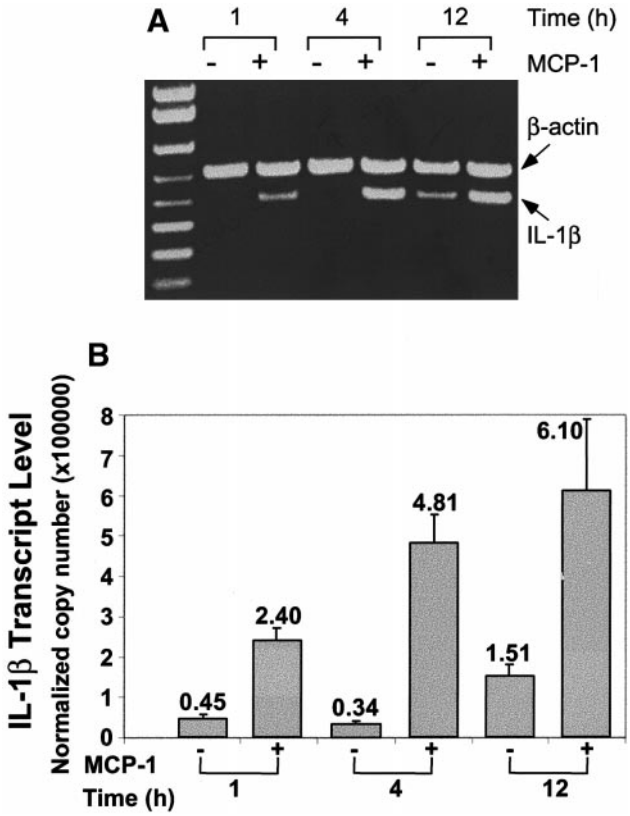

FIG. 2. (A) RT-PCR products of IL-1β and β-actin transcripts from human monocytes treated with 7 nM MCP-1. Equal amounts of RNA were used for first-strand cDNA synthesis. (B) MCP induced changes in the levels of IL-1β transcripts measured by real-time PCR. Standard curves derived from plasmids were used to quantitate starting copy number in the unknown samples. The data were normalized to the β-actin controls. A one-tailed, paired Student's t test showed significance at all treatment times (1 h, $P < 0.01$; 4 and 12 h, $P < 0.05$). Data are displayed as means + SEM.

enhanced level of IL-1β transcript resulting from MCP-1 treatment of monocytes (Fig. 2). This increase was discernible at 1, maximal after 4 h, and remained approximately at this level for 12 h of MCP-1 treatment; β-actin transcript level remained unchanged (Fig. 2A).

IL-1β transcript levels were measured by real-time PCR using the probe and primers indicated under Materials and Methods with β-actin transcript as a control (Fig. 2B). Increase in IL-β transcript level was obvious (5-fold) after 1 h of treatment with MCP-1. Further increase by 4 h (14-fold) and less marked increase (4-fold) during the next 12 h were observed.

Increased IL-1β Protein Levels in Human Monocytes Treated with MCP-1

To test whether the enhanced IL-1β mRNA level was reflected in the level of IL-1β protein in human monocytes, we performed immunoblotting and ELISA assays of the culture media and cell extracts. Figure 3 shows a time course of increase of pro-IL-1β levels in human monocyte extracts. We observed much higher levels of pro-IL-1β in MCP-1-treated monocytes as compared to control cells. We did not detect mature IL-1β in the monocyte extract. The medium containing the secreted form of IL-1β also showed only the unprocessed form. Quantitative analysis of IL-1β with antibodies that detect both the pro- and the mature forms of IL-1β confirmed induction of IL-1β by MCP-1 treatment of monocytes. Time-dependent accumulation of IL-1β in the culture media was observed and higher levels of this cytokine were found in MCP-1-treated monocytes when compared to controls (Fig. 4). Both Western blot analysis and ELISA assay showed that at every time point MCP-1-treated cells had higher IL-1β protein levels when compared to controls. This finding is consistent with the elevated mRNA levels observed in MCP-1-treated monocytes. This cytokine accumulated in the monocytes during the early period of MCP-1 treatment and subsequently was released into the medium.

It is well established that inflammatory cytokines such as IL-1β stimulate expression of MCP-1 that is produced by many different cell types including endothelial cells, fibroblasts, and monocytes. MCP-1 also plays a pivotal role in monocyte migration and activation (14). Migration is an early response that probably does not require expression of new genes. Activation, on the other hand, probably requires expression of genes triggered by the MCP-1 induced signaling. The nature of the genes that might be transcriptionally activated as a result of MCP-1 treatment of its target cells remains unclear. In this paper, we demonstrate that MCP-1 treatment of human monocytes causes increased expression of IL-1β gene. Quantitative real time PCR and immunological assays show the increase in IL-1β transcript and protein levels, respectively, caused by MCP-1 treatment of the monocytes. This direct demonstration of induction of IL-1β gene by MCP-1 treatment is consistent with the following observations. A D10 proliferation assay of lysates of MCP-1 treated monocytes suggested that MCP-1 might have caused an increase in IL-1β (15). Treatment of sensitized rabbits with anti-MCP-1 antiserum showed a trend towards decreased levels of IL-1β in the lung tissue (16).

The sequence of events involved in processing and release of the 31-kDa pro-IL-β to the mature 17-kDa form has not been established. It has been postulated that IL-1β is produced by monocytes and macrophages and released by a normal secretory apparatus only after proteolytic processing. However, some recent evidence suggests that the primary translation product (31 kDa) and 28- and 3-kDa fragments as well as the 17-kDa mature form can be released (17). Our immunoblots show that the unprocessed IL-1β is produced

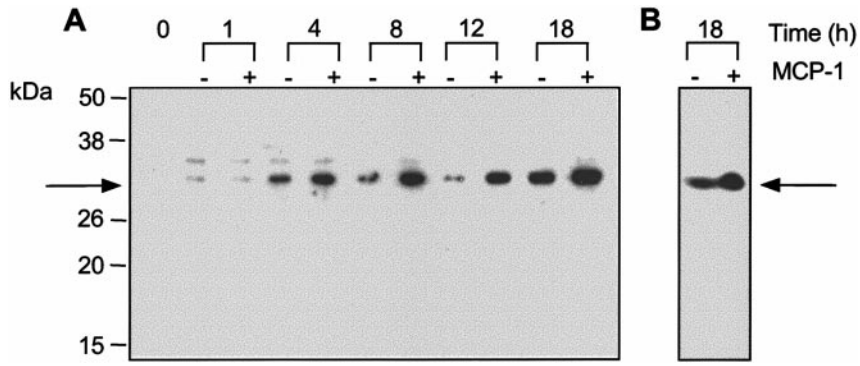

FIG. 3. Immunoblot of IL-1β in cell extract (A) and culture media (B) of human monocytes incubated in the absence or presence of 7 nM MCP-1 for different periods of time. 5–20 μg of protein from cell extract and 30 μl of the culture media were subjected to 15% SDS-PAGE, transferred onto an Immobilon-P membrane and treated with rabbit anti-human IL-1β polyclonal antibody (1:10,000 dilution) (Upstate Biotechnology). After incubation with HRP-conjugated secondary antibody (1:100,000 dilution) (Sigma), the membrane was immersed in Super Signal West Dura Extended Duration Substrate (Pierce) and exposed to a film. Bands at 31 kDa represent pro-IL-1β, shown by an arrow.

and released by monocytes. Neither the shorter forms nor the mature IL-1β were detected in the MCP-1-treated monocytes. It has been reported that secondary stimuli such as ATP and monovalent cations enhance processing and secretion of IL-1β (18, 19). Maturation of the IL-1β processing enzyme (caspase-1, ICE), present in monocytic cells as an inactive 45-kDa precursor (20), is enhanced by treatment with ATP (21). Extracellular ATP was reported to increase post-translational processing of pro-IL-1β via a surface P2Z-type purine receptors (22, 23).

The molecular basis of MCP-1 induction of IL-1β production is not clear although it is likely that such

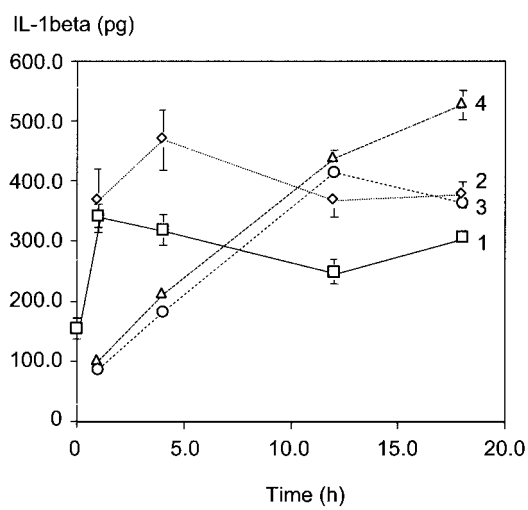

FIG. 4. Total amount of pro-IL-1β in cell extract and cell culture media of monocytes incubated in the absence and presence of 7 nM MCP-1 as determined by ELISA. After different periods of time cultured media were collected, and cells were harvested and lysed. Equal amounts of cell lysate and equal volumes of culture media were analyzed and total amounts of IL-1β were calculated. 1, control cell extract; 2, extract from MCP-1 treated cells; 3, control media; 4, media from MCP-1 treated cells.

induction involves some products of MCP-1-induced signaling. The cellular effects of MCP-1, mediated by its binding to chemokine receptor 2 (CCR2), involves stimulation of the formation of the lipid products of phosphatidyl inositol signaling pathways (24) and protein kinase C (PKC) (25). IL-1β synthesis in human monocytes induced by Epstein–Barr virus is known to involve the same phosphatidyl inositol signaling pathways and PKC (26). This process involves activation of nuclear factor κB (NF-κB) (26, 27). Thus, MCP-1 binding can activate IL-1β synthesis via NF-κB.

IL-1β, in turn, is known to stimulate MCP-1 synthesis (1, 3–7). IL-1β signals through the type 1 interleukin receptor (IL-1RI) and its co-receptor accessory protein (IL-1 RacP) (28) and activates NF-κB (29). Using Gene Discovery Array (GDA, Genome Systems) we found enhanced production of IL-1RAcP and IL-1RI precursor in monocytes treated with MCP-1 for 4 h (data not shown). IL-1β itself is a major inducer of NF-κB (30–32). The promoter of the MCP-1 gene contains sites for NF-κB and thus, IL-1β causes induction of MCP-1 synthesis (33, 34). Thus, mutual transcriptional activation of MCP-1 and IL-1β probably involves NF-κB.

Like IL-1β, MCP-1 is involved in many inflammatory, immune-mediated, and infectious human diseases such as rheumatoid arthritis, sepsis, inflammatory bowel disease, and atherosclerosis to name a few. IL-1β is known to increase MCP-1 levels, and our results provide direct evidence that MCP-1 induces expression of IL-1β. Thus, these two cytokines have positive feedback loops to enhance the production of each other to amplify their biological consequences and amplification probably occurs in certain pathological conditions. Both of these cytokines are found to be elevated in the serum of patients with heart failure (35). MCP-1 is also known to play a very important role in atherogenesis as it attracts monocytes to the damaged

vessel wall. MCP-1 has been detected in atherosclerotic lesions (36, 37) and elevated levels of MCP are found in the serum of patients with heart failure (38). By elucidating the mechanisms involved in the interplay between these cytokines, it might be possible to devise novel therapies directed at these pathways.

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