Inhibition of Monocyte Chemoattractant Protein-1 Expression in Cytokine-Treated Human Lung Epithelial Cells by Thiazolidinedione*

Atsuko Momoi, MD, PhD; Koji Murao, MD, PhD; Hitomi Imachi, MD, PhD; Toshihiko Ishida, MD, PhD; Wen Ming Cao, MD; Makoto Sato, MD, PhD; and Jiro Takahara, MD, PhD

Study objective: Several lung diseases are characterized by the presence of increased numbers of activated macrophages. The recruitment and activation of peripheral blood monocytes are potentially critical regulatory events for the control of pulmonary inflammation. The chemokine monocyte chemoattractant protein (MCP)-1 is a potent chemoattractant for monocytes. MCP-1 is produced by lung epithelial cells during the course of inflammatory lung diseases. In the present study, we examined the effects of a thiazolidinedione (TZD), which is used to improve the insulin resistance of individuals with diabetes mellitus, on MCP-1 expression in a human lung epithelial cell line, A549.

Measurements and results: In A549 cells, interleukin (IL)-1β and tumor necrosis factor (TNF)-α induced endogenous MCP-1 protein secretion and messenger RNA expression. The TZD inhibited the increase of MCP-1 secretion by IL-1β and TNF-α treatment. The TZD inhibited the expression of MCP-1 messenger RNA with IL-1β treatment, but not with TNF-α treatment. This observation was confirmed by the results of a monocyte chemotactic assay. The transcriptional activity of human MCP-1 promoter in A549 cells paralleled the endogenous messenger RNA expression by cytokines and TZD treatment.

Conclusions: Our findings indicated that the suppression of the expression of MCP-1 could be accomplished by TZD treatment, raising the possibility that TZD may be of therapeutic value in several lung diseases in which MCP-1 plays an important role.

(CHEST 2001; 120:1293–1300)

Key words: A549 cell line; interleukin-1β; monocyte chemoattractant protein-1; thiazolidinedione; tumor necrosis factor-α

Abbreviations: Dex = dexamethasone; ELISA = enzyme-linked immunosorbent assay; HUVEC = human umbilical vascular endothelial cell; IL = interleukin; MCP = monocyte chemoattractant protein; PCR = polymerase chain reaction; PPAR = peroxisome proliferator-activated receptor; RT = reverse transcription; TNF = tumor necrosis factor; TZD = thiazolidinedione

The alveolar macrophage, an important phagocyte of the pulmonary airspace and interstitium, is derived predominantly from peripheral blood monocytes and, to a limited extent, from local macrophage replication.1 The alveolar macrophage also functions as an important immune effector cell of the airspace via the generation of a vast array of bioactive mediators,2,3 including tumor necrosis factor (TNF)-α and interleukin (IL)-1β, cytokines that play a central role in mediating local pulmonary and systemic pathophysiology.4,5

Chemokines are a family of small molecular mass proteins (8 to 16 kd) that were originally classified based on the conservation of a four-
cysteine motif and on their ability to cause the directed migration of leukocytes in vitro. Monocytes have been shown to be selectively attracted to specific chemokines that predominantly belong to the C-C family of the chemotactant, which includes human monocyte chemotactant protein-1 (MCP-1). MCP-1 is a 76-amino acid chemokine that is thought to be the major chemotactic factor for monocytes. Several studies have reported that MCP-1 is one of the key factors initiating the inflammatory process of inflammatory lung diseases. MCP-1 is expressed by a variety of cell types, including monocytes, macrophages, endothelial cells, and alveolar epithelial cells, in response to several different stimuli, including IL-1β and TNF-α. A previous study reported that, using isolated rat type II epithelial cells, these cells produced MCP-1 and MCP-1 messenger RNA expression induced by exposure to the inflammatory cytokines IL-1β and TNF-α. In in vitro studies, increases in MCP-1 release have been found in patients with ARDS and chronic inflammatory diseases, including pulmonary sarcoidosis and idiopathic pulmonary fibrosis. These observations provide indirect evidence that MCP-1 is an important mediator of a variety of monocyte/macrophage-rich pathologic processes.

Thiazolidinediones (TZDs), which are known to have potent enhancing effects on insulin sensitivity, have been developed for the treatment of non-insulin-dependent diabetes mellitus. TZDs can specifically and powerfully block the action of TNF-α to inhibit insulin signaling, suggesting one plausible mechanism for its action in improving insulin resistance. It also has been found that TZDs are a high-affinity ligand for the peroxisome proliferator-activated receptor (PPAR)-γ, which belongs to a nuclear receptor superfamily. Ricote et al have reported that PPAR-γ activators such as 15-deoxy-Δ12,14-prostaglandin J2 and TZDs can inhibit the production of several inflammatory cytokines, including IL-1β and TNF-α, by phorbol 12-myristate 13-acetate and interferon-γ-activated macrophages in vitro. In 1999, we reported that TZD inhibits the production of MCP-1 in cytokine-treated human umbilical vascular endothelial cells (HUVECs; Clonetics; San Diego, CA).

In the present study, we examined the effects of a TZD on the expression of MCP-1 in response to IL-1β and TNF-α in human lung epithelial cell lines. Our findings demonstrated that the expression of MCP-1 in IL-1-treated lung epithelial cells was inhibited by TZD at the transcriptional level.

Materials and Methods

The following materials were used in the study: TZD (Sankyo Pharmaceuticals; Tokyo, Japan); Wy14643 (Cayman; Ann Arbor, MI); dexamethasone (Dex) (Wako Pure Chemical Industries Ltd.; Osaka, Japan); and TNF-α and IL-1β (Research Biochemicals International; Natick, MA). All other reagents were of analytical grade.

Cell Culture

A human lung adenocarcinoma cell line (A549 cells) representative of distal respiratory epithelium was used in the study (American Type Culture Collection; Rockville, MD). A549 cells were cultured in RPMI-1640 medium (GIBCO BRL; Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Dainippon Pharmaceutical Co; Tokyo, Japan) in a humidified atmosphere containing 5% CO2 at 37°C. When confluent, the cells were washed twice and incubated with serum-free RPMI-1640 for 24 h before being stimulated with IL-1β or TNF-α. An hour after cytokine stimulation, the cells were treated with 10 μM TZD, 100 μM Wy14643, or 1 μM Dex for 12 h.

MCP-1 Enzyme-Linked Immunosorbent Assay

The levels of immunoreactive MCP-1 were quantified using a commercially available sandwich-type enzyme-linked immunosorbent assay (ELISA) (R&D Systems; Minneapolis, MN). ELISA plates were coated with a specific murine monoclonal antibody against human MCP-1. Dilutions of cell-free supernatants were added in duplicate, followed by the addition of a second horseradish peroxidase-conjugated goat polyclonal antibody against MCP-1. After washing to remove any unbound antibody-enzyme reagent, a substrate solution (a 1:1 solution of hydrogen peroxide and tetramethylbenzidine) was added to the wells. The color development was stopped with a 2-ns sulfuric acid bath, and the intensity of the color was measured at 540 nm on a spectrophotometer. This ELISA is sensitive to 2.5 pg/mL MCP-1, and it has an intra-assay coefficient of variation of < 0.5% and an interassay coefficient of variation of < 10%.

Chemotaxis Assay

Human monocytes from peripheral blood were separated on a discontinuous density Percoll column (American Pharmacia Biotech; Uppsala, Sweden). Monocyte chemotaxis experiments were performed using a 48-well chemotaxis chamber, as described previously. The numbers of monocytes that migrated through the filter were counted on 5 high-power fields (40 × 10) in duplicate. RPMI-1640 and human MCP-1 (100 mg/mL) were used as negative and positive controls, respectively.

RNA Isolation and Northern Blot Analysis

A single-step acid guanidinium thiocyanate-phenol-chloroform extraction technique was used to isolate total RNA from A549 cells treated with cytokines and/or TZD for 24 h. The separation of the RNA samples, the transfer to the membrane, and hybridization with human MCP-1 complementary DNA were described previously. The 18S-base pair complementary DNA of human MCP-1 was synthesized by a polymerase chain reaction (PCR) method using reverse transcription (RT) RNA, as previously described. Primers used for PCR were as follows: sense, 5'-
AATAGGAAGATCTCAGTGCA-3'; antisense, 5'-TCAAGTCTTCGGAGTTTGGG-3', corresponding to the published sequences.21 The probe used in the hybridization was radiolabeled with [32P]-deoxycytidine 5'-triphosphate (3,000 Ci/mmol) using a random priming kit (TaKaRa Biomedicals; Tokyo, Japan). Blots also were probed with human β-actin to assess equal loading of samples.22 After autoradiography at room temperature for 24 h, hybridization signals were detected using a bioimaging analyzer (BAS 1000; Fuji Photo Film; Tokyo, Japan).

Transfection of A549 and Luciferase Reporter Gene Assay

To confirm the transcriptional regulation by TZD of MCP-1 expression, we used a promoter construct of the MCP-1 gene. The reporter contains the human MCP-1 promoter lesion that was amplified by PCR and cloned into the luciferase reporter gene (pMCP-LUC), as previously described.17 Purified reporter plasmid was transfected into A549 (at 60% confluence) by conventional cationic liposome transfection methods (Lipofectamine; Life Technologies; Gaithersburg, MD). Two micrograms of Rous sarcoma virus-β-galactosidase was added to all transfections to monitor the efficiency of DNA uptake by A549 cells (24). All assays were corrected for β-galactosidase activity, and the total amounts of protein per reaction were identical. Transfected cells were maintained in control media containing 1 ng/mL IL-1β or 10 ng/mL TNF-α with or without TZD for 24 h. Transfected cells were harvested, and an aliquot of the cytoplasmic fraction was taken for the measurement of β-galactosidase activity.24 Twenty-microliter aliquots were taken for the luciferase assay, which was performed according to the manufacturer's instructions (ToyoInk; Tokyo, Japan).

Statistical Analysis

Statistical comparisons were made by one-way analysis of variance and Student’s t test, with p < 0.05 considered to be significant.

RESULTS

TZD Blocks the IL-1β-Mediated and TNF-α-Mediated Stimulation of MCP-1 Secretion in A549 Cells

Several studies have shown that IL-1β and TNF-α stimulate MCP-1 secretion and monocyte accumulation by normal epithelial cells and A549 cells.2,10 We also have reported that IL-1β and TNF-α stimulate not only MCP-1 secretion but also MCP-1 gene expression in HUVECs.17 As expected, TNF-α and IL-1β each stimulated MCP-1 secretion in a dose-dependent manner; the maximal effect was observed at 10 ng/mL TNF-α and 1 ng/mL IL-1β in A549 cells (data not shown). This stimulation of MCP-1 secretion in human lung epithelial cells was blocked by a glucocorticoid.25 We confirmed that a glucocorticoid inhibited MCP-1 secretion by A549 cells treated with TNF-α or IL-1β (Fig 1, top, A, and middle, B). When cells were treated with 10 μM TZD, it inhibited MCP-1 secretion by IL-1β-treated A549 cells at 8% compared with A549 cells treated...
with IL-1β only. TZD also inhibited MCP-1 secretion by TNF-α-treated A549 cells. This inhibitory effect of TZD was dose-dependent. Doses of TZD as low as 10 nm reduced MCP-1 release in 24-h cultures stimulated with 1 ng/mL of IL-1β (Fig 1, bottom, C). The concentration of TZD giving half-maximal inhibition was approximately 0.5 μM. In contrast, WY14643, which is an agent acting through the related PPAR-α, had no activity to inhibit MCP-1 secretion by cytokine-treated A549 cells.

Effect of TZD on Monocyte Chemotaxis in A549 Cells

TZD inhibition of MCP-1 secretion induced by IL-1β and TNF-α in A549 cells was associated with decreased chemotactic activity. It has been reported that IL-1β and TNF-α increase monocytic chemotactic activity in bronchial epithelial cells and bronchial cell lines.2,10 Figure 2 shows that IL-1β and TNF-α stimulated the monocytic chemotactic activity in A549 cells. This increase in monocytic chemotactic activity was inhibited by TZD in A549 cells, indicating that the inhibition of MCP-1 secretion of both TZD-treated and cytokine-treated A549 cells reflected the monocytic chemotactic activity in A549 cells. In contrast, WY14643 showed no activity that inhibited monocyte chemotactic activity.

Gene Expression of PPAR-γ in A549 Cells

PPAR-γ is one of the transcriptional factors thought to be the functional receptor for the TZDs.15 PPAR-γ is expressed at the highest levels in adipose tissue, although it is also expressed in other tissues at lower levels. To examine PPAR-γ gene expression in A549 cells, we employed RT-PCR analysis with messenger RNA from A549 cells and HUVECs. Although a previous study26 reported that PPAR-γ is expressed in HUVECs, PPAR-γ messenger RNA was detected in A549 cells at the same level found in HUVECs (Fig 3). In contrast, PPAR-α, which is one of the transcriptional factors, is thought to be the functional receptor for WY14643. We could detect the gene expression of PPAR-α in HUVECs but not in A549 cells. These findings confirmed that WY14643 has no effect on MCP-1 secretion and expression in A549 cells.

![Figure 2](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/21968/ on 04/04/2017)
Using a human MCP-1-specific probe, we performed Northern blot analyses and detected a signal of the expected size.21 As shown in Figure 4, both IL-1β and TNF-α stimulated the expression of MCP-1 in A549 cells, a result that agrees with previous reports.2,10 TZD suppressed the induction of the MCP-1 messenger RNA in A549 cells treated with IL-1β but not with TNF-α. In contrast, TZD had no effects on the steady-state expression of MCP-1 in the cells. It had no effect on cell viability, as determined by the cell number and cell morphology (data not shown).

**Discussion**

In the present study, we examined the effects of TZD on MCP-1 expression in response to cytokines in the human lung epithelial cell line A549. Interest in this topic stems from the clinical observation that the secretion of a monocyte-specific chemoattractant by cytokine-activated macrophages and epithelial cells provides part of the basis for the accumulation of monocytes/macrophages at sites of inflammatory lung diseases.6 The accumulation of monocytes/macrophages is thought to play a key role in the inflammatory response of several pulmonary diseases, including sarcoidosis, idiopathic pulmonary fibrosis, asthma, and infected lung diseases.12,27–29

The alveolar macrophage, an important phagocyte of the pulmonary airspace and interstitium, is predominantly derived from differentiated peripheral blood monocytes and, to a limited extent, from local macrophage replication.3 In addition to the produc-
tion of inflammatory cell chemotaxins, alveolar macrophages secrete TNF-α and IL-1β, cytokines that play a central role in mediating local pulmonary and systemic pathophysiology. O’Brien et al. reported that alveolar epithelial cells have the capacity to direct alveolar macrophage migration within the alveolar space through the local elaboration of soluble factors, including RANTES, granulocyte macrophage-colony-stimulating factor, and MCP-1. MCP-1 is a major chemotactic factor for monocytes in vitro. Gunn et al. showed that MCP-1 is chemotactic for monocytes and lymphocytes in vivo using MCP-1 transgenic mice and that MCP-1 expression alone does not cause inflammatory activation of cells but leads to an enhanced inflammatory response after treatment with other stimuli. Primary cultures of type II alveolar epithelial cells express MCP-1 protein and messenger RNA. The expression of MCP-1 messenger RNA by type II epithelial cells in culture is induced by the inflammatory cytokines IL-1β and TNF-α. Paine et al. have reported that these cells have the potential to play both stimulatory and suppressive roles in inflammatory and immune interactions.

Although endothelial cells synthesize MCP-1 in response to an exogenous stimulus such as lipopolysaccharide, fibroblasts and epithelial cells require a host-generated signal (e.g., TNF or IL-1) before MCP-1 expression can occur. Thus, alveolar epithelial cells may amplify inflammatory responses by expressing MCP-1 after stimulation with IL-1β or TNF-α that is released by alveolar macrophages or might themselves initiate the recruitment and activation of these macrophages in direct response to the lipopolysaccharide. Excess alveolar epithelial cell-derived MCP-1, either spontaneously secreted or induced in response to inflammatory cytokines, might contribute to the pathogenesis of immunologically mediated lung diseases, such as sarcoidosis or diffuse interstitial fibrosis, in which large numbers of activated macrophages are found within the alveolar space. If MCP-1 has a causal role in these diseases, then the suppression of alveolar epithelial cell-derived MCP-1 messenger RNA expression by TZD may contribute to the therapeutic effect in selected patients.

We have reported that IL-1β and TNF-α stimulated RANTES protein secretion, messenger RNA expression, and promoter activity. The TZD inhibits these effects. Kurokawa et al. reported that PPAR-γ activators such as 15-deoxy-Δ12,14-prostaglandin J2 and TZD can inhibit the production of several inflammatory cytokines, including IL-1β and TNF-α, by phorbol 12-myristate 13-acetate and interferon-γ-activated macrophages in vitro. Taken together with the present study, these findings suggest that synthetic PPAR-γ ligands may have therapeutic applications in diseases in which activated macrophages play prominent pathogenic roles.

When A549 cells were exposed to the cytokine IL-1β or TNF-α, the cytokine stimulated not only MCP-1 messenger RNA expression and protein secretion but also MCP-1 promoter activity. Although the exact mechanism of the cytokine-mediated in-

Figure 5. The effect of TZD on MCP-1 promoter activity in A549 cells. A549 cells were transfected with 10 μg pMCP-LUC and were treated with 1 ng/mL IL-1β (top, A) or 10 ng/mL TNF-α (bottom, B) and/or 10 μM TZD, and 100 μM Wy14643 for 24 h prior to cell harvesting. All assays were corrected for β-galactosidase activity, and the total amount of protein per reaction were identical. The results are expressed as relative luciferase activities compared to control cells arbitrarily set at 100. Each data point shows the mean and SEM (n = 4) of separate transfections. * = significance at p < 0.01. See the legend for Figure 1 for any other abbreviations not used in the text.
duction of MCP-1 expression is unknown, the present findings indicate that the inhibition of cytokine-mediated MCP-1 expression by TZD was partially regulated at the transcriptional level. The promotor region of the human MCP-1 gene has been cloned, sequenced, and shown to contain putative consensus binding sites for a variety of transcription factors. Although the precise mechanism underlying the inhibition of the cytokine-induced MCP-1 expression by TZD is unknown, the activation of PPAR-γ by a TZD may modulate the activation of several transcriptional factors in response to cytokines. In this study, TZD inhibited the MCP-1 promoter activity induced by IL-1β, but not that induced by TNF-α. This result indicates that there might be different MCP-1 activation mechanisms in response to different cytokines. Further studies are necessary to determine the transcriptional regulation of the MCP-1 gene by TZD and cytokines.

In summary, we examined the effects of TZD on MCP-1 expression in response to IL-1β and TNF-α in A549 cells. The findings indicate that the suppression of MCP-1 expression can be accomplished by TZD treatment, suggesting that TZD has a therapeutical role in the treatment of several lung diseases in which MCP-1 plays an important role.

ACKNOWLEDGMENT: We acknowledge Mss. R. Kubo and K. Yamaji for their excellent technical assistance.

REFERENCES
28 Smith RE, Strieter RM, Zhang K, et al. A role for C-C


