Thiazolidinedione inhibits the production of monocyte chemoattractant protein-1 in cytokine-treated human vascular endothelial cells

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Abstract The chemokine monocyte chemoattractant protein-1 is a potent chemoattractant for monocytes. Monocyte chemoattractant protein-1 is produced by vascular endothelial cells during inflammatory diseases such as atherosclerosis. In this study, we examined the effects of a thiazolidinedione on monocyte chemoattractant protein-1 expression in human vascular endothelial cells. In human vascular endothelial cells, interleukin-1β and tumor necrosis factor-α induced endogenous monocyte chemoattractant protein-1 protein secretion, mRNA expression and promoter activity. The thiazolidinedione inhibited these effects. In summary, our results indicated that the suppression of the expression of monocyte chemoattractant protein-1 can be accomplished by thiazolidinedione treatment, raising the possibility that thiazolidinedione may be of therapeutic value in the treatment of diseases such as atherosclerosis.

Key words: Monocyte chemoattractant protein-1; Thiazolidinedione; Interleukin-1β; Tumor necrosis factor-α; Human vascular endothelial cell

1. Introduction

Chemokines are a family of small molecular mass proteins (8–16 kDa) that was originally classified based on the conservation of a four cysteine motif and on the ability to cause the directed migration of leukocytes in vitro [1]. The recruitment of monocytes and lymphocytes into sites of atherosclerotic lesions is mediated, in part, by chemotactic cytokines or chemokines [2]. Monocytes have been shown to be selectively attracted to specific chemokines that predominantly belong to the C-C family of chemoattractants which includes human monocyte chemoattractant protein-1 (MCP-1). MCP-1 is a 76 amino acid chemokine thought to be the major chemotactic factor for monocytes [3]. Several reports indicated that MCP-1 is one of the key factors initiating the inflammatory process of atherogenesis [2–4]. MCP-1 is expressed by a variety of cell types including monocytes, smooth muscle cells and human vascular endothelial cells (HUVECs) in response to several different stimuli including interleukin (IL)-1β and tumor necrosis factor (TNF)-α [5]. Recently, MCP-1 was found in macrophage-rich areas of atherosclerotic lesions [4,6].

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 [7]. 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 [8]. It has also been found that TZDs are a high affinity ligand for the peroxisome proliferator-activated receptor-γ (PPAR-γ), which belongs to a nuclear receptor superfamily [9]. Two groups recently reported that PPAR-γ activators such as 15-deoxy-$\Delta^{12,14}$ 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 [10,11].

In this study, we examined the effects of a TZD on expression of MCP-1 in response to IL-1β and TNF-α in HUVECs. Our results demonstrated that the expression of MCP-1 was inhibited by the TZD at the transcriptional level.

2. Materials and methods

2.1. Materials

The TZD was provided by Sankyo Pharmaceuticals (Tokyo, Japan). Wy14643 was from Cayman (Ann Arbor, MI, USA). TNF-α and IL-1β were obtained from Research Biochemicals International (Natick, MA, USA). All other reagents were of analytical grade.

2.2. Cell culture

HUVECs were purchased from Clonetics (San Diego, CA, USA) and used between passages 1–6. HUVECs were maintained in M199 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Dainippon Pharmaceutical, Tokyo, Japan), 100 U/ml penicillin and 100 mg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. When 80% confluent, the cells were washed twice and incubated with serum-free M199 for 12 h before being stimulated with IL-1β or TNF-α. An hour after cytokine stimulation, cells were treated with TZD or 100 μM Wy14643 for 12 or 24 h.

2.3. RNA isolation and Northern blot analysis

A single-step acid guanidinium thiocyanate-phenol-chloroform extraction technique [12] was used to isolate total RNA from HUVECs treated with cytokines and/or TZD for 24 h. The separation of the RNA samples, transfer to a membrane and hybridization with human MCP-1 cDNA were described previously [13]. The 183 bp cDNA of human MCP-1 was synthesized by a polymerase chain reaction (PCR) method using reverse transcribed RNA as previously described [14]. Primers used for PCR were as follows, sense: 5′-AATAGGAAGATCTCAGTGCA-3′, antisense: 5′-TCAAGTCTTCGGAGTTTGGG-3′ corresponding to the published sequences [15]. The probe used in the hybridization was radiolabelled with [^{32}P]dCTP (3000 Ci/mmol) using a random priming kit (TaKaRa Biomedicals, Tokyo, Japan). Blots were also probed with human β-actin to assess equal loading of samples [16]. After autoradiography at room temperature for 24 h,

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Abbreviations: PCR, polymerase chain reaction; MCP-1, monocyte chemoattractant protein-1; TZD, thiazolidinediones; PPAR-γ, peroxisome proliferator-activated receptor-γ; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; ELISA, enzyme-linked immunosorbent assay

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hybridization signals were detected using a Bioimaging Analyzer (BAS 1000, Fuji Photo Film, Tokyo, Japan).

2.4. Transfection of HUVECs 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 gene sequence spanning the region from −515 to +44 as the published sequence [17], was amplified by PCR and cloned into the luciferase reporter gene (pMCP-LUC). Purified reporter plasmid was transfected into HUVECs (at 60% confluence) by conventional cationic liposome transfection methods (Lipofectamine, Life Technologies, Gaithersburg, MD, USA). 2 μg of Rous sarcoma virus β-galactosidase was added to all transfections to monitor the efficiency of DNA uptake by HUVECs [18]. 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 [18]. 20 μl aliquots were taken for the luciferase assay, which was performed according to the manufacturer's instructions (ToyoInk, Tokyo, Japan).

2.5. MCP-1 enzyme-linked immunosorbent assay (ELISA)

The levels of immunoreactive MCP-1 were quantified using a commercially available sandwich type ELISA (R and D Systems, Minneapolis, MN, USA). ELISA plates were coated with a specific murine monoclonal antibody (mAb) 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 Ab against MCP-1. After washing to remove any unbound Ab-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 2 N sulfuric acid 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 inter-assay coefficient of variation of $<10\%$.

2.6. Statistical analysis

Statistical comparisons were made by one-way analysis of variance and Student's *t*-test, with $P<0.05$ considered significant.

3. Results

3.1. TZD blocks the IL-1β- and TNF-α-mediated stimulation of MCP-1 secretion in HUVECs

Several studies have shown that IL-1β and TNF-α stimulate MCP-1 secretion by HUVECs [6,19]. As expected, TNF-α and IL-1β each stimulated the MCP-1 secretion in a time-dependent manner (Fig. 1A). The maximal effect was observed at 10 ng/ml TNF-α and 1 ng/ml IL-1β in HUVECs (data not shown). When cells were pretreated with 10 μM TZD, the MCP-1 secretion by IL-1β-treated HUVECs was inhibited. TZD also inhibited MCP-1 secretion by TNF-α-treated HUVECs. This inhibitory effect of TZD was dose-dependent. Doses of TZD as low as 10 nM reduced the MCP-1 release in 24 h cultures stimulated with 1 ng/ml IL-1β and 10 ng/ml TNF-α (Fig. 1B). In contrast, Wy14643, which acts through the related PPAR-γ, had no activity to inhibit MCP-1 secretion by cytokine-treated HUVECs.

3.2. The effect of TZD on MCP-1 mRNA in HUVECs

Using a human MCP-1-specific probe, we performed Northern blot analysis and detected a signal as the expected size [15]. As shown in Fig. 2, both IL-1β and TNF-α stimulated the expression of MCP-1 in HUVECs, consistent with previous reports [6,19]. TZD suppressed the induction of the MCP-1 mRNA in HUVECs treated with IL-1β and TNF-α. In contrast, TZD had no effect on the steady-state expression of β-actin in the cells. It had no effect on the cell viability, as determined by the cell number and cell morphology (data not shown).

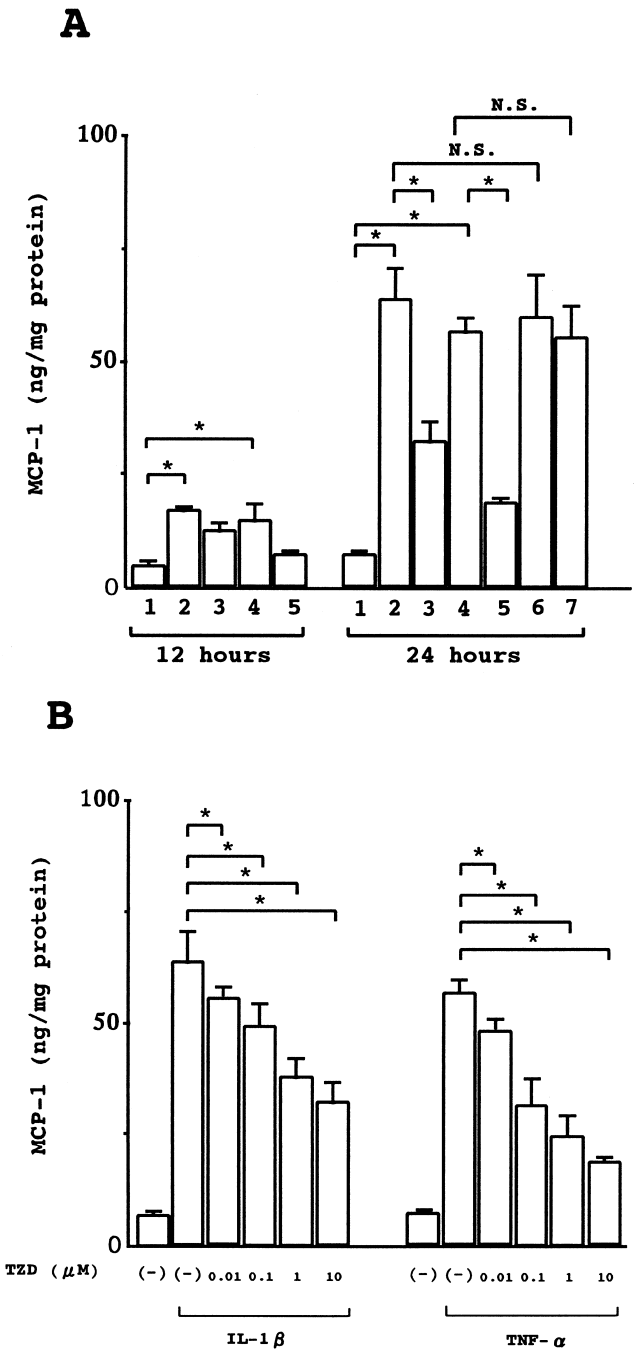

Fig. 1. Inhibition of cytokines-induced MCP-1 protein secretion by TZD in HUVECs. (A) Effects of TZDs and Wy14643 on the induction of MCP-1 by IL-1β and TNF-α. HUVECs were co-incubated with either 10 μM TZD or 100 μM Wy14643 in medium containing 1 ng/ml IL-1β or 10 ng/ml TNF-α for 12 or 24 h. MCP-1 release into the medium was measured by an ELISA. Lane 1, control; lane 2, IL-1β; lane 3, IL-1β and TZD; lane 4, TNF-α; lane 5, TNF-α and TZD; lane 6, IL-1β and Wy14643; lane 7, TNF-α and Wy14643. (B) Dose-response of TZD on MCP-1 release. HUVECs were co-incubated with IL-1β (1 ng/ml) or TNF-α (10 ng/ml) and various doses of TZD in combination for 24 h. The asterisk and N.S. denote a significant difference ($P<0.05$) and not a significant difference, respectively. The error bars show S.E.M. of four determinations.

3.3. Effects of TZD on the human MCP-1 promoter in HUVECs treated with IL-1β and TNF-α

The response of pMCP-LUC to 1 ng/ml of IL-1β and 10 ng/ml of TNF-α in HUVECs is shown in Fig. 3. Consistent with the observed changes in the level of endogenous MCP-1 mRNA, 1 ng/ml IL-1β stimulated promoter activity. In the presence of both IL-1β and TZD, TZD inhibited the IL-1β-induced MCP-1 promoter activity in HUVECs. In contrast, TZD had a less, but still a significant, effect on the TNF-α-induced MCP-1 promoter activity in HUVECs. These findings indicate that the activity of the human MCP-1 promoter in HUVECs following exposure to the cytokines and TZD reflected the endogenous expression of MCP-1 mRNA.

4. Discussion

In this study, we examined the effects of TZD on MCP-1 expression in response to cytokines in HUVECs. Interest in this topic stems from the clinical observation that the secretion of a monocyte-specific chemoattractant by cytokine-activated endothelial cells provides part of the basis for the accumulation of monocytes at sites of vascular injury and inflammation such as atherosclerosis [6,19]. MCP-1 is chemotactic for monocytes but not for neutrophils and seems to be the major chemotactic molecule generated within the vessel wall such as macrophage-rich areas of the atherosclerotic lesion [1].

The expression of MCP-1 is observed in a variety of cell types including monocytes, vascular smooth muscle cells and HUVECs in response to several different stimuli including IL-1β and TNF-α [5,6,19]. In addition to inducing chemoattraction by means of MCP-1, IL-1βand TNF-α also cause an increased adhesion of monocytes to the endothelial cell surface, probably by including the expression of specific endothelial monocyte adhesion molecules [20]. Recently, Boring et al. generated mice that lack CCR2, the receptor for MCP-1, and crossed them with apolipoprotein (apo) E-null mice which develop severe atherosclerosis, showing that the selective absence of CCR2 decreases atherosclerotic lesion formation markedly in apoE−/− mice [21]. These results provide strong evidence for a direct effect of MCP-1 in macrophage recruitment and atherogenesis.

When HUVECs were exposed to the cytokine IL-1β or TNF-α, the cytokine stimulated not only MCP-1 mRNA expression and protein secretion but also MCP-1 promoter activity. Although the exact mechanism of the cytokine-mediated induction of MCP-1 expression is unknown, our present results indicate that the inhibition of cytokine-mediated MCP-1 expression by TZDs was partially regulated at the transcriptional level. The promoter 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 [17]. IL-1β-induced MCP-1 gene expression in human endothelial cells depends on the cooperative action of NF-kB and AP-1 [22]. PPAR-γ, one of the transcriptional factors, is thought to be the functional receptor for the TZDs [9], since the activation of PPAR-γ by a TZD may modulate the activation of several transcriptional factors in response to cytokines. Further examinations are necessary to determine the transcriptional regulation of the MCP-1 gene by TZD and cytokines.

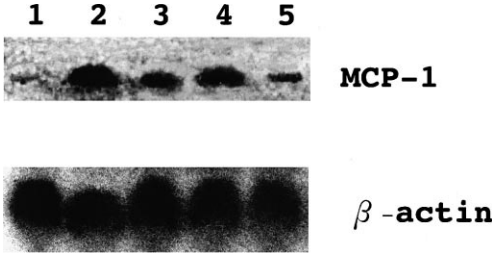

Fig. 2. TZD inhibits MCP-1 mRNA expression in cytokine-treated HUVECs. 10 μg of total RNA isolated from HUVECs treated for 24 h with 1 ng/ml IL-1β or 10 ng/ml TNF-α with/without 10 μM TZD was separated by agarose gel electrophoresis and transferred to a membrane. The blots were hybridized with the encoding region of human MCP-1 [^{32}P]cDNA and human β-actin [^{32}P]cDNA that was used as a control for equal RNA loading and transfer. Lane 1, control; lane 2, IL-1β; lane 3, IL-1β and TZD; lane 4, TNF-α; lane 5, TNF-α and TZD. An identical experiment independently performed gave similar results.

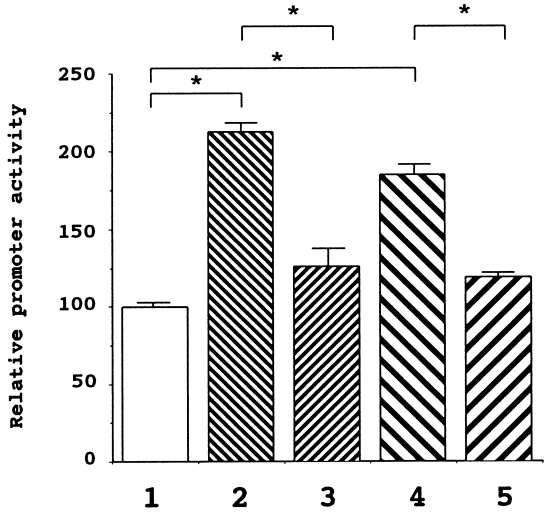

Fig. 3. The effect of TZD on MCP-1 promoter activity in HUVECs. HUVECs were transfected with 10 μg of pMCP-LUC and treated with 1 ng/ml IL-1β (A) or 10 ng/ml TNF-α(B) and/or 10 μM TZD for 24 h prior to cell harvest. All assays were corrected for β-galactosidase activity and total amounts of protein per 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 S.E.M. ($n=4$) of separate transfections. Lane 1, control; lane 2, IL-1β; lane 3, IL-1β and TZD; lane 4, TNF-α; lane 5, TNF-α and TZD. The asterisk denotes a significant difference ($P<0.05$).

In summary, we examined the effects of TZD on MCP-1 expression in response to IL-1β and TNF-α in HUVECs. The results indicate that the suppression of the MCP-1 expression can be accomplished by TZD treatment, suggesting that TZD has a therapeutic value in the treatment of human diseases such as atherosclerosis in which MCP-1 plays an important role.

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