Thiazolidinedione inhibits production of RANTES in a cytokine-treated human lung epithelial cell line

Atsuko Momoi, Koji Murao*, Hitomi Imachi, Yoshitaka Sayo, Hiroyuki Nakamura, Hitoshi Hosokawa, Makoto Sato, Jiro Fujita, Hiroki Okada, Toshihiko Ishida, Jiro Takahara

First Department of Internal Medicine, Kagawa Medical University, 1750-1, Miki-cho, Kita-gun, Kagawa 761-0793, Japan

Received 4 March 1999; received in revised form 27 April 1999

Abstract The chemokine RANTES is a potent chemoattractant for eosinophils. RANTES is produced by lung epithelial cells during eosinophil-rich inflammatory diseases such as asthma. In this study, we examined the effects of thiazolidinediones (TZD) on RANTES expression in a human lung epithelial cell line, A549. In A549 cells, interleukin-1β and tumor necrosis factor-α induced endogenous RANTES protein secretion, mRNA expression, and promoter activity. The TZD inhibited these effects. Our data indicate that the suppression of the expression of RANTES can be accomplished by TZD treatment, raising the possibility that TZD might be of therapeutic value in diseases such as asthma.

Key words: RANTES; Thiazolidinedione; Interleukin-1β; Tumor necrosis factor-α; Eosinophil

1. Introduction

Chemokines are a family of small molecular mass proteins (8–16 kDa) that are originally classified based on the conservation of a four-cysteine motif and on their ability to cause the directed migration of leukocytes in vitro [1]. RANTES (regulated upon activation normal T-cell expressed and secreted) is a member of a new class of chemokines [1,2] that play an important role in allergic inflammation. RANTES exerts potent and selective chemoattractant effects on cells which characteristically predominate in the allergic inflammatory cells, such as $CD45RO^+$ memory T lymphocytes [3], eosinophils [4], and mast cells [5]. In addition, RANTES is produced by epithelial cells during eosinophil-rich inflammatory diseases [6]. The bronchial asthma process is believed to involve the activation of T helper 2-type lymphocytes with the consequent accumulation of proinflammatory effector cells, particularly eosinophils [7,8]. It is believed that RANTES plays a significant role in the recruitment of eosinophils to the bronchial mucosa.

Thiazolidinediones (TZD), which are known to have potent enhancing effects on insulin sensitivity, have been developed for the treatment of non-insulin-dependent diabetes mellitus [9]. TZD can specifically and powerfully block the action of tumor necrosis factor (TNF)-α to inhibit insulin signaling, suggesting one plausible mechanism for its action in improving insulin resistance [10]. It has also been found that TZD are a high-affinity ligand for the peroxisome proliferator-activated receptor-γ (PPAR-γ), which belongs to a nuclear receptor superfamily [11]. Two groups recently reported that PPAR-γ activators such as 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2 (15d-PGJ_2) and TZD can inhibit the production of several inflammatory cytokines including interleukin (IL)-1β and TNF-α by phorbol 12-myristate 13-acetate and interferon-γ-activated macrophages in vitro [12,13].

In this study, we examined the effects of a TZD on expression of RANTES in response to IL-1β and TNF-α in human lung epithelial cells. Our results demonstrate that the expression of RANTES 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). Dexamethasone (Dex) was from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 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

A human lung adenocarcinoma cell line (A549 cells) representative of distal respiratory epithelium was obtained from American Type Culture Collection (Rockville, MD, USA). A549 cells were cultured in RPMI 1640 media (Gibco BRL, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Dainippon Pharmaceutical Co., Tokyo, Japan), in a humidified atmosphere containing 5% CO_2 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. One hour after cytokine stimulation, the cells were treated with 10 μM TZD, 100 μM Wy14643 or 1 μM Dex for 24 h.

2.3. RNA isolation and Northern blot analysis

A single-step acid guanidinium thiocyanate-phenol-chloroform extraction technique [14] was used to isolate total RNA from A549 cells treated with cytokines and/or TZD for 24 h. The separation of the RNA samples, transfer to membrane and hybridization with human RANTES cDNA were described previously [15]. The 197 bp cDNA of human RANTES was synthesized by a polymerase chain reaction (PCR) method using reverse transcribed RNA as previous described [15]. Primers used for PCR were as follows: sense: 5′-CGCTGTCATCCTCATTGCTA-3′, antisense: 5′-CACACACTTGGCGGTTCTT-3′. The probe used in the hybridization was radiolabeled with [^{32}P]dCTP (3000 Ci/mmol) using a random priming kit (TaKaRa Biomedicals, Tokyo, Japan). The membranes were also stained with methylene blue to assess the equal loading of samples [16]. After autoradiography at room temperature for 24 h, hybridization signals

*Corresponding author. Fax: (81) (878) 91-2147.
E-mail: mkoji@kms.ac.jp

Abbreviations: FBS, fetal bovine serum; RT-PCR, reverse transcription polymerase chain reaction; RANTES, regulated upon activation normal T-cell expressed and secreted; TZD, thiazolidinedione; PPAR-γ, peroxisome proliferator-activated receptor-γ; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2; Dex, dexamethasone; ELISA, enzyme-linked immunosorbent assay

0014-5793/99/$20.00
PII: S0014-5793(99)00678-X

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

2.4. Transfection of A549 cells and luciferase reporter gene assay

To confirm the transcriptional regulation by TZD of RANTES expression, we used a promoter construct of the RANTES gene. The reporter contains the human RANTES gene sequence spanning the region from −1014 to +2 of the published sequence [17]; it was amplified by PCR and cloned into the luciferase reporter gene (pRANTES-LUC). Purified reporter plasmid was transfected into A549 cells (at 60% confluence) by conventional cationic liposome transfection methods (Lipofectamine, Life Technologies, Gaithersburg, MD, USA). Two μg of Rous sarcoma virus-β-galactosidase was added to all transfections to monitor the efficiency of DNA uptake by A549 cells [18]. All assays were corrected for β-galactosidase activity and 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 or Wy14643 for 24 h. Transfected cells were harvested, and an aliquot of the cytoplasmic fraction was taken for the measurement of β-galactosidase activity [18]. Aliquots of 20 μl were taken for the luciferase assay, which was performed according to the manufacturer's instructions (ToyoInk, Tokyo, Japan).

2.5. RANTES enzyme-linked immunosorbent assay (ELISA)

The levels of immunoreactive RANTES were quantified using a commercially available sandwich-type ELISA (R&D Systems Inc., Minneapolis, MN, USA). This ELISA is sensitive to 2.5 pg/ml RANTES, and it has an intra-assay coefficient of variation of $< 0.5\%$ and an interassay coefficient of variation of $< 10\%$.

2.6. Chemotaxis assay

Eosinophils from allergic volunteers (peripheral blood eosinophil count 5–10%) were separated on a discontinuous density Percoll column (American Pharmacia Biotech, Uppsala, Sweden). Eosinophil chemotaxis experiments were performed using a 48 well chemotaxis chamber as described previously [19]. The numbers of eosinophils that migrated through the filter were counted on five high-power fields (40×10) in duplicate. RPMI 1640 and human eotaxin (100 ng/ml) were used as negative and positive controls, respectively.

2.7. 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 block the IL-1β- and TNF-α-mediated stimulation of RANTES secretion in A549 cells

Several studies have shown that IL-1β and TNF-α stimulate RANTES secretion and eosinophil accumulation by normal epithelial cells and A549 cells [20]. As expected, TNF-α and IL-1β each stimulated the RANTES 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 RANTES secretion in human lung epithelial cells was blocked by glucocorticoid [20,21]. We confirmed that glucocorticoid inhibited the RANTES secretion by A549 cells treated with TNF-α or IL-1β (Fig. 1A,B). When cells were pretreated with 10 μM TZD, it inhibited the RANTES secretion by IL-1β-treated A549 cells at 8% compared to A549 cells treated only with IL-1β. TZD also inhibited RANTES secretion by TNF-α-treated A549 cells. This inhibitory effect of TZD was dose-dependent. Doses of TZD as low as 10 nM reduced the RANTES release in 24 h cultures stimulated with 1 ng/ml of IL-1β(data not shown). 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 RANTES secretion by cytokine-treated A549 cells.

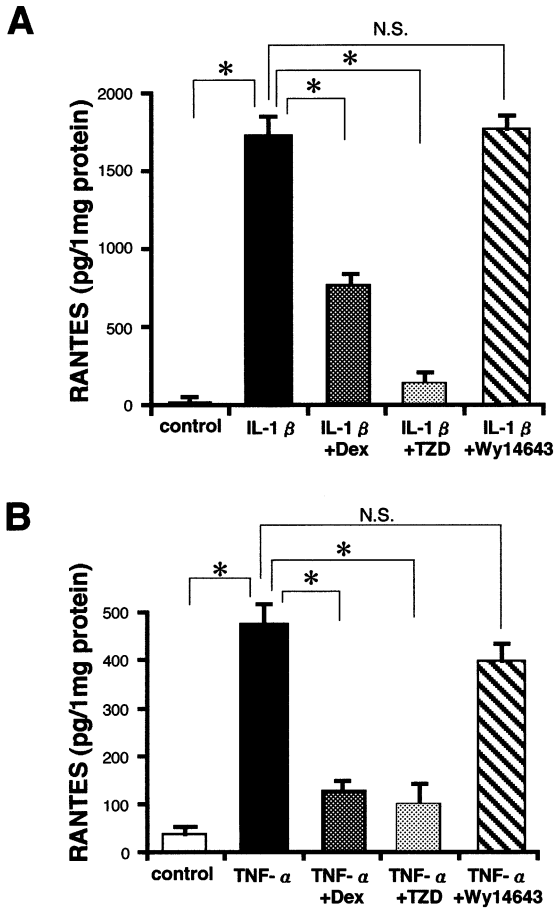

Fig. 1. Inhibition of cytokine-induced RANTES protein secretion by TZD in A549 cells. Effects of TZD, Wy14643, and Dex on the induction of RANTES by IL-1β (A) and TNF-α (B). A549 cells were co-incubated with either 1 μM Dex, 10 μM TZD or 100 μM Wy14643 in medium containing 1 ng/ml IL-1β or 10 ng/ml TNF-α. RANTES release into the medium was measured by an ELISA. The asterisk denotes a significant difference ($P < 0.05$); N.S., no significant difference. Error bars show S.E.M. of three determinations.

3.2. Effect of TZD on eosinophil chemotaxis in A549 cells

TZD inhibition of RANTES 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 eosinophilic chemotactic activity in bronchial epithelial cells and bronchial cell lines [20,21]. Fig. 2 shows that IL-1β and TNF-α stimulated the eosinophilic chemotactic activity in A549 cells. This increase in eosinophilic chemotactic activity was inhibited by TZD in A549 cells, indicating that the inhibition of RANTES secretion of both TZD- and cytokine-treated A549 cells reflected the eosinophilic chemotactic activity in A549 cells.

3.3. Effect of TZD on RANTES mRNA in A549 cells

Using a human RANTES-specific probe, we performed Northern blot analyses, and detected a signal transcript of 1.2 kb, as previously reported [22]. As shown in Fig. 3, both IL-1β and TNF-α stimulated the expression of RANTES in A549 cells, in agreement with previous reports [20,21]. TZD suppressed the induction of the RANTES mRNA in A549 cells treated with IL-1β but not TNF-α. In contrast, TZD had no effects on the steady-state expression of

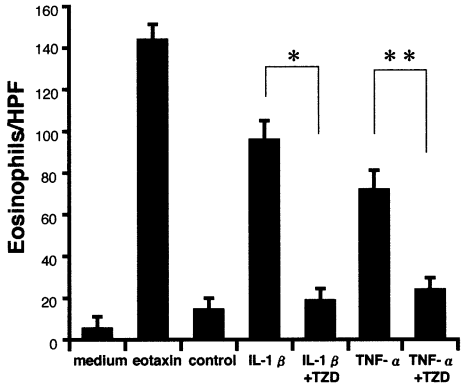

Fig. 2. Eosinophil chemotactic activity in supernatant of A549 cells. Culture medium alone was used as a negative control and eotaxin (100 ng/ml) was used as a positive control. Supernatants were obtained from 24 h cultured cells in the presence of TNF-α or IL-1β with/without 10 μM TZD. S.E.M. of three or four separate experiments is shown. 1, medium; 2, eotaxin; 3, control; 4, IL-1β; 5, IL-1β+TZD; 6, TNF-α; 7, TNF-α+TZD. HPF: high-power field. The asterisk and double asterisk denote significant differences ($P < 0.01$ and $P < 0.05$, respectively).

RANTES in the cells. It had no effect on cell viability, as determined by the cell number and cell morphology (data not shown).

3.4. Effects of TZD on human RANTES promoter in A549 cells treated with IL-1β

The response of pRANTES-LUC to 1 ng/ml of IL-1β in A549 cells is shown in Fig. 4A. Consistent with the observed changes in the level of endogenous RANTES mRNA, 1 ng/ml IL-1β stimulated promoter activity. In the presence of both IL-1β and TZD, TZD inhibited the IL-1β-induced RANTES promoter activity in A549 cells. In contrast, TZD had no effect of the TNF-α-induced RANTES promoter activity in A549 cells (Fig. 4B). These findings indicate that the activity of the human RANTES promoter in A549 cells following

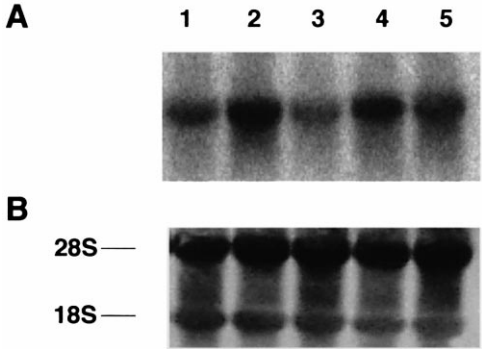

Fig. 3. TZD inhibits RANTES mRNA expression in cytokine-treated A549 cells. 10 μg of total RNA isolated from A549 cells treated for 24 h with 1 ng/ml IL-1β or 10 ng/ml TNF-α with/without 10 μM TZD was separated by agarose gel electrophoresis and transferred to a membrane. The blots were hybridized with the coding region of human RANTES [^{32}P]cDNA (A) and methylene blue staining of 28S and 18S ribosomal RNA, demonstrating the similarity of the quantity of RNA in each lane (B). Lane 1, control; lane 2, IL-1β; lane 3, IL-1β+TZD; lane 4, TNF-α; lane 5, TNF-α+TZD. An identical experiment independently performed gave similar results.

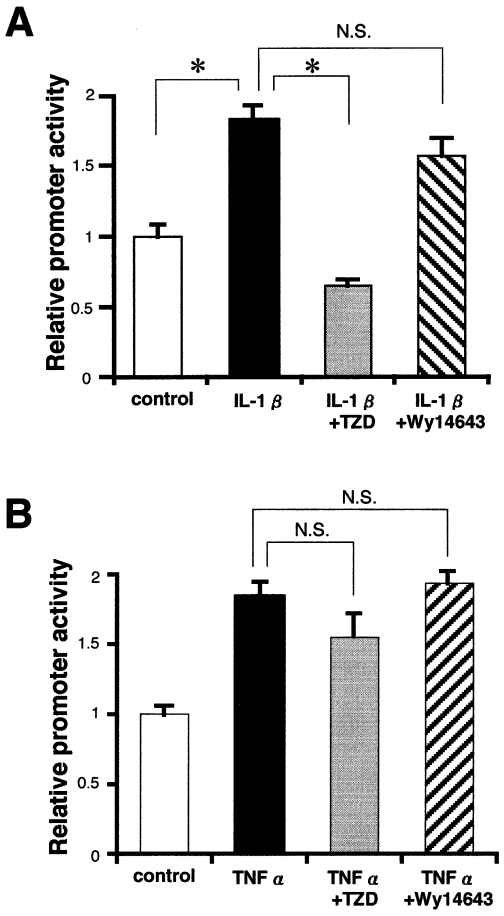

Fig. 4. Effect of TZD on RANTES promoter activity in A549 cells. A549 cells were transfected with 10 μg of pRANTES-LUC and treated with 1 ng/ml IL-1β (A) or 10 ng/ml TNF-α (B) and/or 10 μM TZD, 100 μM Wy14643 for 24 h prior to cell harvest. All assays were corrected for β-galactosidase activity and total 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. The asterisk denotes a significant difference ($P < 0.01$); N.S., no significant difference.

exposure to IL-1β and TZD reflected the endogenous expression of RANTES mRNA.

4. Discussion

In this study, we examined the effects of TZD on RANTES expression in response to cytokines in a human lung cell line, A549. Interest in this topic stems from the clinical observation that eosinophils accumulate in patients with asthma [7,8]. RANTES is known to directly stimulate eosinophil migration and to up-regulate the expression of the adhesion molecule Mac-1 on eosinophils [23]. A clinical study showed that RANTES is a major eosinophil chemoattractant in the bronchoalveolar lavage fluid of asthmatic subjects [24].

When A549 cells were exposed to the cytokines IL-1β or TNF-α, the cytokines stimulated not only RANTES mRNA expression and protein secretion but also RANTES promoter activity. Previous studies showed that TNF-α and IL-1β increase the RANTES mRNA expression and release [20,21]. TNF-α or other cytokines released by alveolar macrophages

may stimulate lung epithelial cells to release RANTES, which in turn recruits eosinophils together with T lymphocytes and monocytes into the lung [1]. In this study, TZD inhibited not only the IL-1β-induced RANTES mRNA expression and protein secretion but also RANTES promoter activity. TZD also inhibited the TNF-α-induced RANTES secretion but not the transcription of the RANTES gene. The reason for this discrepancy between RANTES secretion and transcription in response to TZD in TNF-α-treated A549 cells is unknown. Although the exact mechanism of the cytokine-mediated induction of RANTES expression is unknown, our present results indicate that the inhibition of cytokine-mediated RANTES expression by TZD was partially regulated at the transcriptional level. The answer to these questions will be clarified when the precise mechanism by which TNF-α and IL-1β stimulate RANTES expression in lung epithelial cells is known. Not only RANTES but also several factors such as eotaxin and granulocyte/macrophage colony-stimulating factor have previously been shown to have chemotactic activity for eosinophils and to be expressed in human bronchial epithelial cells [25], and therefore the effect of TZD on expression of these factors deserves further investigations.

The 5′-flanking region of the RANTES gene contains a large number of potential binding sites for transcriptional factors [26]. PPAR-γ, which is one of the transcriptional factors, is thought to be the functional receptor for the TZD [11,27]. A previous report indicated that 15d-PGJ_2 and PPAR-γ ligand inhibit STAT, NF-κB and AP-1 activity in a PPAR-γ-dependent manner [12]. PPAR-γ is expressed at highest levels in adipose tissue, although it is also expressed in other tissues at much lower levels. We have found that PPAR-γ mRNA was expressed at a considerable level in A549 cells by a reverse transcription-PCR method (data not shown). We analyzed the promoter region of the RANTES gene [26] and identified a sequence (−638 to −625) with homology to the PPAR-γ consensus sequence. The DNA motif in RANTES shares 84% homology with a similar PPAR-γ consensus sequence found in the CD36 promoter sequence [28]. Although the precise mechanism underlying the inhibition of the cytokine-induced RANTES 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. Further examinations are necessary to determine the transcriptional regulation of the RANTES gene by TZD and cytokines.

Glucocorticoids are potent agents for the treatment of asthma, but the clinical use of glucocorticoids for asthma is limited by their severe side effects. Our results indicate that TZD inhibits RANTES. Therefore, TZD might be useful drugs for the treatment of asthmatic patients. Understanding the pathways leading to the activation of TZD might expose new targets for the development of disease-modifying antiasthmatic drugs; however, this hypothesis requires further examinations using normal bronchial epithelial cells.

In summary, we examined the effects of TZD on RANTES expression in response to IL-1β and TNF-α in human lung epithelial cells. The results indicate that the suppression of RANTES expression can be accompanied by TZD treatment, raising the possibility that TZD may have therapeutic value in human diseases such as asthma in which RANTES plays an important role.

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