1. Introduction

Transforming growth factor-β (TGF-β) plays a pivotal role in the fibrogenic action involved in the induction of connective tissue growth factor (CTGF), extracellular matrix and fibroblast transformation. Smad3 mediates TGF-β signaling related to the fibrotic response. In human lung fibroblasts or bronchial smooth muscle cells, we demonstrated that an increase in the intracellular glutathione level suppressed TGF-β1-induced phosphorylation of Smad3, while inhibiting TGF-β1-induced expressions of CTGF, collagen type1, fibronectin and transformation into myofibroblasts, which are characterized by the expression of α-smooth muscle actin. These data indicate that the intracellular glutathione redox status regulates TGF-β-induced fibrogenic effects through Smad3 activation.

Glutathione redox regulates TGF-β-induced fibrogenic effects through Smad3 activation

Akihiro Ono, Mitsuyoshi Utsugi, Ken Masubuchi, Tamotsu Ishizuka, Tadayoshi Kawata, Yasuo Shimizu, Takeshi Hisada, Junji Hamuro, Masatomo Moria, Kunio Dobashic

*Address correspondence to:
Akihiro Ono: Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine, 3-39-15, Showa-machi, Maebashi, Gunma 371-8511, Japan
Mitsuyoshi Utsugi: Department of Microbiology and Immunology, Keio University Medical School, Shinjuku, Tokyo, Japan

Corresponding author. Fax: +81 27 220 8136.

*Corresponding author. Fax: +81 27 220 8136.

E-mail address: mutsugi@med.gunma-u.ac.jp (M. Utsugi).

**Abbreviations:**
α-SMA, α-smooth muscle actin; BSMC, bronchial smooth muscle cells; CT, threshold cycle; CTGF, connective tissue growth factor; DEX, dexamethasone; ECM, extracellular matrix; GCE, γ-glutamylcystein ethyl ester; GSH, reduced glutathione; GSSG, oxidized glutathione; HFL-1, human lung fibroblasts; RANTES, regulated-on-activation, normal T-cell-expressed and-secreted chemokine; TGF-β, transforming growth factor-β.


© 2008 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
Glutathione is the most abundant tripeptide in almost all cells and it is a major intracellular redox buffer [15]. Glutathione redox has been reported to not only have an anti-oxidative action but also regulate many kinds of cellular biological functions. We have previously reported that the intracellular glutathione redox status in antigen presenting cells regulates the Th1/Th2 balance through the production of interleukin-12 [16,17]. However, the role of glutathione redox in the pathogenesis of fibrotic lung disease has not yet been determined. Therefore, the present study was designed to investigate the effect of glutathione redox on TGF-β-induced fibrogenic action in human lung fibroblasts and airway smooth muscle cells using γ-GCE which is a precursor of reduced glutathione (GSH); it was kindly donated by Teijin Pharma Limited (Tokyo, Japan). DEX was purchased from Sigma (St. Louis, MO).

2. Materials and methods

2.1. Reagents

Recombinant human TGF-β1 was obtained from R&D Systems (Minneapolis, MN). γ-GCE is a hydrophilic chemical compound of diamino-acid that is the precursor of GSH; it was kindly donated by Teijin Pharma Limited (Tokyo, Japan). DEX was purchased from Sigma (St. Louis, MO).

2.2. Cell culture

Human lung fibroblasts (HFL-1) were purchased from the American Type Culture Collection (Rockville, MD) and cultured as previously described [18]. Human bronchial smooth muscle cells (BSMC) were purchased from Clonetics (San Diego, CA) and cultured as previously described [19]. Before reaching confluence, the cells were washed and cultured overnight in fresh medium without fetal bovine serum. After pretreatment with or without γ-GCE or DEX, the cells were stimulated with 1 ng/ml TGF-β.

2.3. Measurement of intracellular GSH concentrations

HFL-1 cells and BSMC cultured subconfluently in six well plates were treated with 0–10 mM of γ-GCE for each time period. The cells in each well were thawed in 200 μl lysis buffer (0.1% triton-X, 0.1 M sodium phosphate, 5 mM EDTA, pH 7.5) and homogenized. The lysates were acidified with 30 μl of 0.1 N HCl, and protein was precipitated with 30 μl of 50% sulfosalicylic acid. The GSH levels were determined as previously described [16].

2.4. Western blotting

Western blotting was performed as previously described [20]. The primary antibody selectively recognizing phosphorylated form of Smad3 (anti-phospho-Smad3, Ser423/425) and phosphorylation state-independent anti-Smad3 antibody to determine the amounts of precipitated Smad3 were obtained from Cell Signaling Technology (Beverly, MA).

2.5. Real-time PCR analysis

The total RNA from HFL-1 or BSMC cells was treated by DNase using an RNeasy Mini Kit and RNase-Free DNase Set (QIAGEN, Valencia, CA), and converted to cDNA using a High-Capacity cDNA Archive kit (Applied Biosystems, Foster, CA). The transcription
levels of CTGF, α-SMA, collagen type1, and fibronectin mRNAs were detected using real-time PCR (7500 Real-Time PCR System; PE Applied Biosystems) with the TaqMan Universal PCR Master Mix (PE Applied Biosystems). The primers and the probes of the target genes were TaqMan Assay-on-Demand Gene Expression Assays (PE Applied Biosystems): CTGF (Hs00170014_m1), α-SMA (Hs00426835_g1), collagen type1 (Hs01028956_m1), fibronectin (Hs01565277_m1) and, as a control, glyceraldehyde-3-phosphate dehydrogenase (Hs99999905_m1). The threshold cycle (CT) was recorded for each sample to calculate the relative expression using ΔCT method.

2.6. Statistical analysis

Data were compared using either Student’s t-test or one-way ANOVA. A P value below 0.05 was considered statistically significant. Furthermore if statistical significance was reached in the ANOVA analysis, post hoc test using Bonferroni/Dunn test was performed and a P value below 0.05/m (in which m is the number of comparisons) was considered to be statistically significant.

3. Results and discussion

3.1. γ-GCE increased intracellular GSH levels

The effect of γ-GCE, the precursor of GSH, on intracellular GSH content was determined. γ-GCE can pass through the biomembrane [21], thus subsequently increasing the intracellular GSH level. Treatment with γ-GCE for 4 h significantly increased intracellular GSH levels in HFL-1 cells (Fig. 1A, P < 0.05) and BSMC (Fig. 1B, P < 0.05). In both cell types, the maximal intracellular GSH concentration occurred 4 h after adding γ-GCE. In BSMC, there was no significant difference in the intracellular GSH levels between 5 and 10 mM treatment with γ-GCE (data not shown).

3.2. γ-GCE suppressed TGF-β1-induced Smad3 phosphorylation

Smad3 is thought to be a critical signaling molecule in the fibrogenic effects induced by TGF-β. Therefore, the effect of γ-GCE on TGF-β1-induced Smad3 phosphorylation was assessed. HFL-1 cells were pretreated with γ-GCE for 4 h before the stimulation of TGF-β1 for 40 min. In contrast to a recent study that GSH did not affect Smad3 phosphorylation in murine embryonic fibroblasts [22], TGF-β1-induced Smad3 phosphorylation was suppressed by γ-GCE pretreatment in a dose-dependent manner (Fig. 2A) in HFL-1 cells. A densitometric analysis showed that both 5 and 10 mM γ-GCE significantly inhibited TGF-β1-induced Smad3 phosphorylation (Fig. 2C). Similarly, in the BSMC, TGF-β1-induced Smad3 phosphorylation was suppressed by pretreatment of 5 mM γ-GCE (Fig. 2B), and a densitometric analysis demonstrated 5 mM γ-GCE to significantly inhibit TGF-β1-induced Smad3 phosphorylation (Fig. 2D). These results suggest that an increased intracellular GSH level can attenuate TGF-β1-induced Smad3 activation.

3.3. The effect of γ-GCE on TGF-β1-induced Smad3 activation is dependent on the intracellular GSH level

To further investigate the relationship between the intracellular GSH level and TGF-β1-induced Smad3 activation, the time...
course of the effect of γ-GCE on the intracellular GSH level and on TGF-β1-induced Smad3 activation was assessed. In HFL-1 cells, the intracellular GSH level was significantly increased at 4 h; then, 16 h after adding γ-GCE, the intracellular GSH level returned to near basal levels (Fig. 3A). The TGF-β1-induced Smad3 phosphorylation was inhibited by γ-GCE pretreatment for 4 h as the results in Fig. 2, but γ-GCE had no inhibitory effect after 16 h of pretreatment (Fig. 3C). Similarly, in BSMC, γ-GCE treatment for 36 h restored both the intracellular GSH level and TGF-β1-induced Smad3 phosphorylation to the levels that were seen in cells without γ-GCE treatment (Fig. 3B and D). A densitometric analysis showed a significant difference in the levels of Smad3 phosphorylation between no treatment and treatment groups for 4 h with γ-GCE in both cells, while no significant difference was observed between no treatment and treatment groups for 16 h (HFL-1) or 36 h (BSMC) with γ-GCE (Fig. 3E and F). These findings indicate that the change of the intracellular GSH level, specifically glutathione redox status, is closely associated with the levels of TGF-β1-induced Smad3 phosphorylation. In addition, the recovery of Smad3 activation after γ-GCE treatment in the current study suggested that γ-GCE did not have any harmful effect on these cells.

3.4. γ-GCE suppressed TGF-β1-induced CTGF, collagen type1 and fibronectin expression, and transformation of fibroblasts into myofibroblasts

CTGF and ECM expressions and transformation of fibroblasts appeared to be involved in Smad pathway. Therefore, the effect of γ-GCE on TGF-β1-induced CTGF, collagen type1, fibronectin and α-SMA expression was studied. A real-time PCR analysis demonstrated that TGF-β potently enhanced the mRNA expressions of CTGF, Collagen type1, fibronectin and α-SMA in HFL-1 (Fig. 4A, C, D and E) or BSMC (Fig. 4B). However, the pretreatment of γ-GCE significantly suppressed TGF-β1-induced CTGF mRNA expression in HFL-1 cells (Fig. 4A) and BSMC (Fig. 4B). Furthermore, in HFL-1 cells, γ-GCE markedly and significantly suppressed TGF-β1-induced mRNA expressions of collagen type1 (Fig. 4C), fibronectin (Fig. 4D) and α-SMA (Fig. 4E). These results indicate that the increase in the intracellular GSH level by γ-GCE markedly suppressed the expressions of TGF-β-induced CTGF, collagen type1, fibronectin and α-SMA through the inhibition of Smad3 activation. CTGF has been reported to induce collagen type1 and fibronectin in a number of cell types, and it probably mediates some of the TGF-β1-induced ECM production in human cells [6]. We also considered

Fig. 4. Effects of γ-GCE on TGF-β1-induced CTGF, collagen type1, fibronectin and α-SMA expression. HFL-1 cells (A, C, D and E) and BSMC (B) were treated with or without γ-GCE for 4 h and then were stimulated by TGF-β1 for 8 h (A) or 12 h (B) or 24 h (C, D and E). Total RNA was extracted and the mRNA expression of CTGF (A and B), collagen type1 (C), fibronectin (D) or α-SMA (E) was evaluated using a real-time PCR analysis. The results are expressed as ratio (%) of the control value. The controls were TGF-β1 stimulated cells untreated with γ-GCE. Values represent means ± S.E.M. of five (A, C, D and E) or four (B) experiments. *P < 0.05 compared with control cells.
that γ-GCE might influence the expression of collagen type1 and fibronectin in the context of the inhibition of CTGF expression. These findings in the present study suggest that the glutathione redox status has the potential to regulate TGF-β-induced fibrogenic effects through its action on fibroblasts and smooth muscle cells.

3.5. All results, including the Smad3 phosphorylation, CTGF expression, collagen type1 expression, fibronectin expression, α-SMA expression, were not suppressed by DEX

Corticosteroids are often used systemically for the treatment of fibrotic lung diseases and severe asthma. DEX is frequently used to evaluate the effect of corticosteroids in vitro. The anti-inflammatory effects of corticosteroids have been described in many reports. We ascertained the marked inhibitory effect of pretreatment with 10 μM DEX on tumor necrosis factor-α induced regulated-on-activation, normal T-cell-expressed and-secreted chemokine (RANTES) secretion in BSMC as previously reported (data not shown)[23]. On the other hand, pretreatment with 10 μM DEX did not change TGF-β-induced Smad3 phosphorylation in HFL-1 cells (Fig. 5A) and in BSMC (Fig. 5B) in contrast to the strong inhibitory effect of γ-GCE on Smad3 phosphorylation activated by TGF-β1. Moreover, DEX enhanced TGF-β1-induced CTGF mRNA expression in HFL-1 cells and BSMC (Fig. 5C and D), and a significant difference was
observed between the treatment with and without DEX in HFL-1 cells (Fig. 5C). In addition, DEX did not affect TGF-β1-induced the mRNA expressions of collagen type1 (Fig. 5E), fibronectin (Fig. 5F), and α-SMA (Fig. 5G) in HFL-1 cells. In contrast, γ-GCE decreased the expressions of CTGF, collagen type1, fibronectin, and α-SMA mRNA (Fig. 5C–G) as the result of Fig. 4. Some reports have shown that corticosteroid treatment either does not inhibit ECM deposition [24] or it appears to accelerate ECM deposition [25]. In addition, corticosteroids have been reported to not suppress either the thickening of the airway smooth muscle layer or subepithelial collagen deposition in an asthma model mouse [26], thus suggesting our results to be compatible with those findings. These findings indicated that fibrotic changes by TGF-β1 are resistant to corticosteroid treatment, while these are notably suppressed by γ-GCE.

Glutathione is the most prevalent cellular thiol and consists of reduced glutathione (GSH) and oxidized glutathione (GSSG). The relative levels of GSH and GSSG influence the redox status and are regulated by the redox reaction, the oxidation of the thiol group and the reduction of the disulfide bond [15]. Glutathione is able to regulate various cellular functions by facilitating reversible constructive changes of target proteins or enzymes through the redox reaction [27,28], that is by "redox regulation" [28]. In fact, GSH, which is a major reductant of cellular protein disulfides, can regulate the activity of redox-sensitive enzymes by reducing a wide variety of disulfides [29]. Therefore, it is possible that Smad3 or other upstream molecules might constructively change under high intracellular GSH levels, therefore suppressing the activation of Smad3 by TGF-β. However, further study is required to clarify the specific mechanisms that are involved.

In conclusion, we herein demonstrated that glutathione redox is involved in TGF-β1-induced fibrogenic effects, such as CTGF, collagen type1, and fibronectin expression and the transformation of fibroblasts into myofibroblasts, during the mediation of Smad3 activation. This is the first report to describe that glutathione redox may potentially be able to regulate TGF-β1-induced fibroproliferative effects. This study suggests that glutathione redox could control TGF-β1-induced pathologic fibrosis in the human pulmonary interstitium and airway.

Acknowledgements

The authors thank Yoko Koike (Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine) for the much valuable discussion and Hiroyuki Mochizuki (Department of Pediatrics and Developmental Medicine, Gunma University Graduate School of Medicine) for the technical support. This work was supported in part by Grant 19790680 (to M.U.) from the Ministry of Education, Culture, Sport, Science and Technology, Japan.

References