Role of Nrf2 in Methotrexate-Induced Epithelial–Mesenchymal Transition in Alveolar A549 Cells

Masashi Kawami,* Mikito Honda, Takuya Hara, Ryoko Yumoto, and Mikihisa Takano

Department of Pharmaceutics and Therapeutics, Graduate School of Biomedical and Health Sciences, Hiroshima University; 1–2–3 Kasumi, Minami-ku, Hiroshima 734–8553, Japan.

Received January 6, 2022; accepted May 16, 2022

Methotrexate (MTX) is known to induce serious lung diseases, such as pulmonary fibrosis. Although we demonstrated that MTX is associated with epithelial–mesenchymal transition (EMT), the underlying mechanism remains unclear. Nuclear factor erythroid 2-related factor 2 (Nrf2), an oxidative stress response regulator, is related to EMT induction. In the present study, we examined the association of Nrf2 with the MTX-induced EMT in the alveolar epithelial cell line A549. MTX treatment decreased the mRNA expression of heme oxidase-1 (HO-1), a target of Nrf2, which was inhibited by co-treatment with diethyl maleate (DEM), an Nrf2 activator. Additionally, the MTX-induced increase in reactive oxygen species (ROS) production was significantly suppressed by DEM. Furthermore, DEM decreased mRNA/protein expression levels of α-smooth muscle actin (SMA), a representative EMT marker, which were upregulated by MTX. Nuclear expression and localization of Nrf2 were suppressed by MTX treatment, which led to a decrease in Nrf2 activity. Finally, in Nrf2 knockdown cells, the MTX-induced enhancement of α-SMA mRNA/protein expression was not observed, indicating that downregulation of Nrf2 may play a critical role in the MTX-induced EMT in A549 cells. These results suggest that Nrf2-regulated transcriptional activity would be associated with the MTX-induced EMT induction.

Key words alveolar epithelial cell; diethyl maleate; epithelial–mesenchymal transition; methotrexate; nuclear factor erythroid 2-related factor 2

INTRODUCTION

Methotrexate (MTX) is widely used in the clinical treatment of cancers and inflammatory diseases. For example, MTX is administered at a high single dose of 1 g in patients with leukemia, while MTX at multiple lower doses of 15–25 mg per week is recommended for patients with rheumatoid arthritis. Conversely, MTX-induced lung injury frequently leads to serious lung diseases such as pulmonary fibrosis, and a study involving 123 patients with suspected MTX lung toxicity reported a mortality rate of 13%. However, to date, no effective preventive approaches have been developed against MTX-induced lung injury.

Drugs with potential toxic effects on the lungs can induce epithelial–mesenchymal transition (EMT), which is associated with pathophysiological state of the lung. Under physiological conditions, phenotypic conversion of epithelial cells to fibroblasts/myofibroblasts is fundamental to tissue development and homeostasis. Conversely, aberrant epithelial-mesenchymal crosstalk induces excessive deposition of the extracellular matrix by promoting the conversion of injured epithelial cells into collagen-producing myofibroblasts, a key component of the extracellular matrix. Therefore, it is important to recognize the underlying mechanism of EMT to develop preventive approaches against serious lung diseases such as pulmonary fibrosis.

So far, we have demonstrated that MTX induces EMT-associated phenotypic alterations in terms of morphology and gene expression in several types of cultured alveolar epithelial cells. Additionally, several cellular events, such as cell cycle arrest, and factors, such as p53 and transforming growth factor (TGF)-β1, were associated with the MTX-induced EMT in the cell lines. Alternatively, the inflammatory microenvironment around cancer cells should be a key factor in recognizing EMT-associated tumor invasion and metastasis, indicating a close relationship between EMT and inflammatory conditions. However, there is little information on the link between inflammatory factors and the MTX-induced EMT.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is associated with homeostatic responses to environmental or endogenous deviations in redox metabolism, proteostasis, and inflammation and is considered a promising therapeutic approach against various chronic diseases. In response to inflammatory irritation such as oxidative stress, Nrf2 dissociates from Kelch-like ECH-associated protein 1 (Keap1) and subsequently translocates into the nucleus, followed by generation of antioxidant responses via transcriptional regulation of the associated enzymes, such as heme oxygenase-1 (HO-1). Meanwhile, Nrf2 activity is closely related to EMT under in vivo conditions with pulmonary fibrosis, therefore, Nrf2 plays a significant role in the EMT-associated pathological state of pulmonary fibrosis.

Regarding the relationship between MTX and Nrf2, MTX inhibits the transcriptional activity of Nrf2 in HEK293 cells. Therefore, we hypothesized that the MTX-induced EMT is associated with Nrf2 activity. However, few studies have focused on the role of Nrf2 in the MTX-induced EMT in alveolar epithelial cells. The present study aimed to clarify the involvement of Nrf2 in the MTX-induced EMT in the alveolar epithelial cell line A549.
MATERIALS AND METHODS

**Chemicals** MTX and diethyl maleate (DEM), an Nrf2 activator, were purchased from Wako Pure Chemical Corporation (Osaka, Japan). All other chemicals used in the experiments were of analytical grade and were of the highest purity commercially available.

**Cell Culture** A549 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin in an atmosphere containing 5% CO₂ and 95% air at 37°C. The cells were subcultured every 7 d with replacement of fresh medium every 2–3 d.

**Drug Treatment** A549 cells were seeded on 12-well plates at a density of 1 × 10⁵ cells/well and cultured for 24 h. The cells were treated with 0.3 µM MTX in the absence or presence of 100 µM DEM, an Nrf2 activator, and/or 50 µM Heme and Sn-protoporphyrin (SnPP), an HO-1 inhibitor, for 24 h.

**Small Interfering RNA (siRNA) Transfection** A549 cells were transfected with MISSION siRNA (Merck KGaA, Darmstadt, Germany) for Nrf2 using Lipofectamine® 2000 (Invitrogen, Waltham, MA, U.S.A.). The siRNA sense and antisense sequences were 5'−CAA ACA GAAUGGUCCUAAAdT−3' and antisense 5'−CAA ACA GAAUGGUCCUAAAd-3', respectively.

**mRNA Real-Time PCR Analysis** Total RNA extraction, reverse transcription of the total RNA into cDNA, and real-time PCR analysis were performed as previously reported. The primer sequences were as follows: EMT markers, sense 5'−GCTGTTCCTCCATCCATTGT−3' and antisense 5'−TTTGCTCTGTTCTCGTAC−3'; and N-cadherin, sense 5'−AATCAGTGGCGGAGATCCTA−3' and antisense 5'−CCCTGGAATGGAATGGC−3'; and vimentin, sense 5'−TCAGAGAGAGGAGCGCCAGGA−3' and antisense 5'−CAGAAAAAGCATCTCTTGTCC−3'; HO-1, a transcription target of Nrf2, sense 5'−TGACCCATGACACCAAGGAC−3' and antisense 5'−GTGTAAGGCCCATCGGAGA−3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, sense 5'−CCACCTAGGCAAATCC−3' and antisense 5'−TGGATTTCTTACGACA−3'.

**Flow Cytometry for Reactive Oxygen Species (ROS) Detection** After drug treatment, the cells were washed with phosphate-buffered saline (PBS; 137 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4), and incubated with dihydroethyldium (DHE; 10 µM) in serum-free DMEM medium for 1 h at 37°C. Then, the cells were detached by trypsin treatment, and intracellular DHE was detected by flow cytometry using Guava® easyCyte™ 8HT (Merck Millipore, Darmstadt, Germany) at an excitation wavelength of 488 nm and an emission wavelength of 583 nm. Data were obtained as median values in each histogram, and results were expressed as % of the control.

**Immunostaining for Actin Filaments and Observation by Fluorescence Microscopy** After drug treatment, the cells seeded on a 35 mm glass-bottomed dish were washed with PBS and fixed with 3.7% paraformaldehyde for 10 min. Then, the cells were permeabilized with 0.1% Triton X-100 for 4 min and incubated with BODIPY™ FL phallacidin (4U/mL) (Invitrogen, Grand Island, NY, U.S.A.) in PBS buffer containing 1% bovine serum albumin (BSA). For 20 min, then the cells were incubated with Hoechst 33342 stain (10 µM) (Wako Pure Chemical Corporation) for 30 min and finally treated with Vectashield mounting medium (Vector Laboratories, Inc., CA, U.S.A.). The cells were washed with PBS three times after each step. The fluorescence intensity was detected using fluorescence microscope (Olympus IXplore IX73, Olympus Corporation, Tokyo, Japan).

**Immunostaining for Nrf2 and Observation by Fluorescence Microscopy** After drug treatment, the cells seeded on a 35 mm glass-bottomed dish were washed with PBS buffer and fixed with 3.7% paraformaldehyde for 10 min. Then, the cells were permeabilized with 0.1% Triton X-100 for 10 min and blocked with 0.1% bovine albumin mixed in PBS for 1 h. Immunoreaction with primary antibody against Nrf2 (1:250 dilution, GeneTex, San Antonio, TX, U.S.A.) for 2 h, and subsequently with secondary fluorescein isothiocyanate-labeled anti-rabbit antibody (1:500 dilution) was performed for 1 h. The stained cells were incubated with Hoechst 33342 stain (10 µM) for 30 min and finally treated with Vectashield mounting medium. The cells were washed with PBS three times after each step. The fluorescence intensity was detected using fluorescence microscope.

**Extraction of Nuclear Fraction** Nuclear protein extracts were prepared as previously reported. Briefly, the treated cells were washed with PBS, scraped, and collected by centrifugation at 500 × g for 5 min at 4°C. Cell lysis was performed in ice-cold hypotonic lysis buffer (10 mM N-(2-hydroxyethyl) piperazine-N’-2-ethanesulfonic acid (HEPES) [pH 7.9], 10 mM KCl, 1 mM ethylene diamine tetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 0.5 mM NaN₃, 0.5% (v/v) NP-40, 10% (v/v) glycerol, 1 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride (PMSF)). Nuclear and cytoplasmic fractions were separated by centrifugation at 1000 × g for 5 min at 4°C. The membrane pellet was then resuspended in ice-cold hypertonic lysis buffer (20 mM HEPES [pH 7.9], 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, 500 mM Na₃VO₄, 20% (v/v) glycerol, 1 mM dithiothreitol, and 1 mM PMSF) and incubated with shaking for 15 min at 4°C. Soluble compounds were then isolated by centrifugation at 12000 × g for 5 min at 4°C. The resulting supernatant (nuclear fraction) was stored at −80°C until further analysis.

**Western Blot Analysis** Western blot analysis was performed as previously reported. Briefly, samples of the cell lysate or nuclear fractions were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels and were transferred onto a polyvinylidene difluoride (PVDF) membrane. After blotting, the membranes were exposed to 0.5% skim milk for 1 h, and immunoreaction with primary antibodies against α-SMA (1:500 dilution, GeneTex), Nrf2 (1:1000 dilution, GeneTex), GAPDH (1:5000 dilution, Sigma-Aldrich, St. Louis, MO, U.S.A.), Lamin A+C (1:3000 dilution, GeneTex), and alpha Tubulin 4a (1:5000 dilution, GeneTex) was performed for 2 h. The membrane was washed three times in Tris-buffered saline-Tween (TBS-T;20.5 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.5), and then incubated for 1 h with horseradish peroxidase-labeled secondary antibodies (1:5000 dilution, GE Healthcare, Milwaukee, WI, U.S.A.). After washing three times with TBS-T, the antibody complexes were visualized using Immobilon Western FHR (Merck, Darmstadt, Germany).
Statistical Analysis  Data are expressed as the mean ± standard error of the mean (S.E.M.). Statistical analysis was performed using Student's t-test or one-way ANOVA followed by the Tukey–Kramer test for multiple comparisons. Statistical significance was set at $p < 0.05$.

RESULTS

Effect of DEM on MTX-Induced Changes in Nrf2-Related Events and EMT in A549 Cells  Nrf2 regulates the transcription activity of several antioxidant genes, such as $HO-1$. In contrast, MTX affects the gene expression levels of antioxidants, followed by an increase in ROS production. Therefore, the effect of DEM, an Nrf2 activator, on MTX-induced alterations in mRNA expression of $HO-1$, an antioxidant gene, and ROS production was examined. DEM significantly up-regulated the mRNA expression of HO-1 and also ameliorated the MTX-induced decrease in HO-1 expression (Fig. 1A). In addition, the MTX-induced increase in ROS production was suppressed by co-treatment with DEM (Fig. 1B).

Morphological change of the cells into spindle-shape and the remodeling of the actin filaments are significant indicators to evaluate EMT. The morphology and actin filament under the treatment of A549 cells with MTX in the absence or presence of DEM were observed by phase-contrast microscope and fluorescence microscope, respectively. As shown in Fig. 2, MTX induced morphological changes of the cells and remodeling of actin filaments, which were suppressed by the co-treatment with DEM.

Additionally, we analyzed mRNA expression levels of several mesenchymal markers such as $\alpha$-SMA, N-cadherin, and vimentin. All the mRNA expressions were increased by MTX treatment, which were significantly canceled by the co-treatment with DEM (Fig. 3A). $\alpha$-SMA is a representative mesenchymal marker for EMT, and we demonstrated that...
MTX increased α-SMA expression in several types of alveolar epithelial cell lines. Accordingly, we further analyzed protein expression of α-SMA, and similar results to the case of mRNA expression was obtained (Fig. 3B).

Mechanism Underlying MTX-Induced Decrease in Nrf2 Activity in A549 Cells

The effect of MTX on the translocation of Nrf2 into the nucleus was examined by evaluating the nuclear expression of Nrf2. As shown in Fig. 4A, MTX suppressed the nuclear expression of Nrf2 in A549 cells. Additionally, fluorescence microscopy analysis revealed that MTX reduced the nuclear expression of Nrf2, while DEM increased it (Fig. 4B), indicating that the inhibitory effect of DEM on the MTX-induced EMT may be associated with the translocation of Nrf2 into the nucleus.

Effect of Nrf2 Knockdown on MTX-Induced EMT in A549 Cells

To elucidate the contribution of HO-1 function to MTX-associated changes in A549 cells, the effect of SnPP, an HO-1 inhibitor, on the MTX-induced ROS production and EMT was examined. ROS production enhanced by MTX was suppressed by co-treatment with DEM, which was partially inhibited by SnPP (Fig. 5A). However, SnPP did not affect the inhibitory effect of DEM on the MTX-induced upregulation of α-SMA at the mRNA and protein levels (Fig. 5B). These results suggest that downregulation of Nrf2, but not HO-1, may play an important role in the MTX-induced EMT in A549 cells.

Role of HO-1 Activity in MTX-Induced ROS Production and EMT in A549 Cells

To elucidate the contribution of HO-1 function to MTX-associated changes in A549 cells, we established HO-1 knockdown cells by introducing siRNA specific for HO-1 into the cells and found that HO-1 protein expression levels were drastically decreased in HO-1 knockdown cells (Fig. 6A). Additionally, the mRNA expression level of α-SMA in the HO-1 knockdown cells was significantly lower than that in the siCont cells (Fig. 6B), indicating that the transcriptional function of HO-1 was almost suppressed regardless of the treatment conditions. HO-1 knockdown led to increases in mRNA/protein expression levels of α-SMA, but further upregulation of α-SMA expression by MTX was not observed in HO-1 knockdown cells (Fig. 6C). These results indicate that downregulation of Nrf2 may contribute to the MTX-induced EMT in A549 cells.
DISCUSSION

MTX is a unique anticancer drug included in several types of therapies such as chemotherapy and anti-inflammatory therapy in the clinical setting. Dihydrofolate reductase (DHFR) is a well-known pharmacological target of MTX, and its inhibition by MTX suppresses tumor growth. Conversely, our previous report suggested that DHFR was not involved in the MTX-induced EMT in DHFR knockdown A549 cells, indicating that factors other than DHFR are associated with the MTX-induced EMT. Additionally, we demonstrated that no significant correlation was observed between the MTX-induced EMT and cytotoxic effects, indicating that a mechanism different from the pharmacological effect of MTX may be associated with the MTX-induced EMT. According to this hypothesis, searching for a specific therapeutic target against the MTX-induced EMT would facilitate the establishment of a novel MTX treatment strategy without EMT-related adverse drug reactions such as lung injury. In the present study, we focused on Nrf2 as a potential target against the MTX-induced EMT in A549 cells.

We found that MTX decreased Nrf2 activity in A549 cells, with the observations that DEM, an Nrf2 activator, canceled the MTX-induced increase in ROS production and decreased the mRNA expression level of HO-1, a transcriptional target of Nrf2. Additionally, DEM suppressed the MTX-induced increase in mRNA and protein expression levels of α-SMA, a representative EMT marker gene, in A549 cells. These findings strongly indicate that the MTX-induced EMT is mediated by Nrf2 inactivation. As no investigations have shown the relationship between the MTX-induced EMT and Nrf2 activity in alveolar epithelial cell lines, our findings may provide novel insights into the mechanism of drug-induced EMT and lung injury.

We also found that MTX suppressed the translocation of Nrf2 into the nucleus, which was canceled by DEM, indicating that the MTX-induced decrease in Nrf2 activity may be due to a suppressive effect of MTX on the translocation of Nrf2 into the nucleus. Nrf2, a bZIP transcription factor, protects cells from oxidative stress via transcriptional regulation of antioxidant enzymes, including HO-1. Keap1 suppresses Nrf2 activity by inducing proteasomal degradation, suggesting that Keap1 plays an essential role in Nrf2 activity. When oxidative stress or Nrf2 activators inactivate Keap1, Nrf2 dissociates from Keap1, which allows the entry of Nrf2 into the nucleus. Therefore, we also examined the effect of MTX on the mRNA expression level of Keap1 in A549 cells. However, MTX treatment for 24h had no effect on Keap1 expression (data not shown), suggesting that MTX inhibits the Nrf2 ac-

![Fig. 4. Effect of MTX on Localization of Nrf2 Protein in A549 Cells](image)

The cells were treated with 0.3µM MTX in the absence or presence of 100µM DEM for 24h. (A) The protein expression level of Nrf2 in nuclear fractions of the treated cells was evaluated by Western blot analysis. Dashed lines indicate control levels. Each value represents the mean ± S.E.M. (n = 3). *p < 0.05, **p < 0.01; significantly different from Cont. (B) The localization of Nrf2 in the treated cells was evaluated by immunostaining of Nrf2 with FITC (green)-labeled antibody. Blue staining shows the nucleus in the cells. The scale bar means 10µm.

![Fig. 5. Involvement of HO-1 Function in the Inhibitory Effect of DEM on the MTX-Induced EMT in A549 Cells](image)

The cells were treated with 0.3µM MTX in the absence or presence of 100µM DEM and/or 10µM SnPP for 24h. (A) ROS production was evaluated by detecting fluorescence intensity of DHE using flow cytometry. Dashed lines indicate control levels. (B) The mRNA expression level of HO-1 was evaluated by real-time PCR analysis. Each value represents the mean ± S.E.M. (n = 3). *p < 0.05, **p < 0.01; significantly different from Cont. †p < 0.05, ††p < 0.01; significantly different from MTX. ##p < 0.01; significantly different from MTX + DEM.
activity via factors other than Keap1. Further studies are needed to clarify the detailed mechanism underlying the MTX-induced decrease in Nrf2 activity.

To elucidate whether HO-1, a transcriptional target of Nrf2, is involved in the MTX-induced EMT in A549 cells, we further examined the contribution of HO-1 to MTX-induced alterations in ROS production and α-SMA mRNA expression. The suppressive effect of DEM on MTX-stimulated ROS production was inhibited by co-treatment with SnPP, an HO-1 inhibitor. The suppression of HO-1 functions as a ROS scavenger in MTX-treated cells. However, SnPP did not affect the inhibitory effect of DEM on the MTX-induced increase in mRNA expression of α-SMA, suggesting that HO-1 function may not be associated with the MTX-induced EMT in A549 cells. Notably, the upregulation of α-SMA under Nrf2 knockdown conditions was not further increased by MTX treatment. These findings suggest that downregulation of Nrf2 may be closely associated with the MTX-induced EMT in A549 cells. Considering MTX-induced decrease in mRNA expression of HO-1 as well as α-SMA was not observed under siNrf2 treatment condition, knockdown of Nrf2 may compete with MTX-induced suppression of Nrf2 translocation into nucleus in A549 cells, which further

Fig. 6. Effect of Nrf2 Knockdown on MTX-Induced EMT in A549 Cells

The cells were transfected with Nrf2 siRNA using Lipofectamine® 2000. After 24h, the cells were treated with 0.3 µM MTX in the absence or presence of 100 µM DEM for 24h. (A) The protein expression level of Nrf2 in the treated cells was evaluated by Western blot analysis. Dashed lines indicate control levels. Each value represents the mean ± S.E.M. (n = 3). *p < 0.05; significantly different from Cont. (B, C) The mRNA expression levels of HO-1 (B) and α-SMA (C) were evaluated by real-time PCR analysis. Each value represents the mean ± S.E.M. (n = 3). *p < 0.05, **p < 0.01; significantly different from Cont. †p < 0.05; significantly different from MTX in siCont. condition.
assumes that downregulation of Nrf2 by siRNA as well as inhibition against Nrf2 nucleus translocation would be critical factor in MTX-induced EMT. Conversely, we previously showed that several anticancer drugs such as BLM, MTX, and PTX induce cell cycle arrest, which is a common event in EMT induction. In contrast, the BLM-induced decrease in mRNA expression of HO-1 and the inhibitory effect of DEM on the BLM-induced increase in mRNA expression of α-SMA were less observed compared with the case of MTX (Supplementary Fig. 2). Therefore, downregulation of Nrf2 may be a specific factor in the MTX-induced EMT in A549 cells.

Recently, Nrf2 was reported to be involved in a hybrid EMT state in cells. Hybrid EMT, which is currently highlighted as a novel concept regarding the cell state, acquires both epithelial and mesenchymal phenotypes and promotes cancer progression. Accumulating evidence suggests that aberrant Nrf2 activity in various types of cancers, including lung cancer, leads to poor prognosis due to the Nrf2-mediated progression, metastasis, and resistance to chemotherapy and radiotherapy, in contrast to the cytoprotective effect of Nrf2 in normal cells. These findings indicate that Nrf2 positively regulates EMT. In contrast, the present results and other reports suggest that Nrf2 suppresses EMT. Although it is difficult to recognize the net contribution of Nrf2 to the EMT process, our findings provide basic knowledge on the role of Nrf2 in drug-induced EMT.

In conclusion, we found that the MTX-induced EMT was associated with the loss of Nrf2 activity in A549 cells, as evidenced by the decrease in the mRNA expression level of HO-1, a transcriptional target of Nrf2, and in the translocation of Nrf2 into the nucleus following MTX treatment. Additionally, knockdown of Nrf2 induced an increase in mRNA expression of α-SMA, an EMT marker gene, without MTX treatment, indicating that Nrf2 may be directly involved in the MTX-induced EMT. These findings would help in understanding the mechanism underlying the MTX-induced EMT in A549 cells and contribute to the establishment of a novel preventive approach against MTX-induced lung injury.

Acknowledgments The present study was supported in part by the Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JP18H02586, JP18K06749, and JP19K16447). A part of this work was carried out at the National Science Center for Basic Research and Development, Hiroshima University.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials This article contains supplementary materials.

REFERENCES

22) Ojima T, Kawami M, Yumoto R, Takano M. Differential mecha-


