INTRODUCTION

Methotrexate (MTX) is a folic acid antagonist and has been used in the treatment of several malignancies, as well as for the treatment of rheumatoid arthritis (RA). Pulmonary toxicity is characterized by the presence of several symptoms such as dyspnea, dry cough, and fever, which have been reported when treating patients at a low dose MTX (St Clair et al., 1985; Kremer et al., 1986; Carson et al., 1987; Gispen et al., 1987; Kremer et al., 1997; Zisman et al., 2001). An acute or subacute onset of respiratory failure has been known to be a major complication associated with low-dose treatment of MTX (Imokawa et al., 2000). To avoid such respiratory failures, withdrawal of MTX or additional treatment of corticosteroid is effective; however, such respiratory failure has occurred in a few patients (approximately less than 14%) or in patients with long-term MTX treatment (St Clair et al., 1985; Kremer et al., 1986; Carson et al., 1987; Gispen et al., 1987; Zisman et al., 2001). The progressive fibrotic response in idiopathic pulmonary fibrosis (IPF) is associated with an epithelial-dependent fibroblast-activation process. The presence of fibroblasts and myofibroblasts in foci has been recognized as the central feature of IPF pathogenesis. Several investigators have suggested that myofibroblasts come from at least three possible origins, although the relative contribution of each of these pathways is currently unclear (Bucala et al., 1994; Kuwana et al., 2003; Hashimoto et al., 2004; Postlethwaite et al., 2004; Willis et al., 2005; Scotton and Chambers, 2007): proliferation of resident lung interstitial fibroblasts, generation of fibroblasts from epithelial cells through epithelial mesenchymal transition (EMT), and differentiation of circulating fibrocytes or mesenchymal progenitor cells. EMT is known as the morphological change of polarized epithelial cells from an epithelial phenotype to a mesenchymal phenotype in a process characterized by reduced cell-cell

Original Article

Involvement of epithelial-mesenchymal transition in methotrexate-induced pulmonary fibrosis

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ABSTRACT — Epithelial-mesenchymal transition (EMT) plays a pivotal event in the development of pulmonary fibrosis. We have previously reported that methotrexate (MTX)-induced alveolar epithelial cell injury followed by pulmonary fibrosis as a result of the recruitment and proliferation of myofibroblasts. However, there is no data concerning whether EMT occurs in MTX-induced pulmonary fibrosis. In the present study, therefore, we investigated the expression of EMT markers such as E-cadherin, α-SMA, and vimentin by immunofluorescence analysis in mouse lung tissues after administration of MTX. We found that vimentin and α-SMA-positive cells of the MTX-induced pulmonary fibrosis were increased; on the other hand, E-cadherin was decreased, indicating that epithelial cells act as the main source of mesenchymal expansion. These results exhibited the down-regulation of E-cadherin expression and the up-regulation of α-smooth muscle actin (α-SMA) in primary mouse alveolar epithelial cells (MAECs) and A549 cell lines. Additionally, MTX-induced A549 cells exhibited an EMT-like phenotype accompanied by the elevation of the expression of interleukin-6 (IL-6) and transforming growth factor (TGF)-β1, as well as an enhancement of migration. All of these findings suggest that MTX-induced pulmonary fibrosis occurs via EMT.

Key words: Methotrexate, Pulmonary toxicity, Epithelial mesenchymal transition, Alveolar epithelial cell

INTRODUCTION

Methotrexate (MTX) is a folic acid antagonist and has been used in the treatment of several malignancies, as well as for the treatment of rheumatoid arthritis (RA). Pulmonary toxicity is characterized by the presence of several symptoms such as dyspnea, dry cough, and fever, which have been reported when treating patients at a low dose MTX (St Clair et al., 1985; Kremer et al., 1986; Carson et al., 1987; Gispen et al., 1987; Kremer et al., 1997; Zisman et al., 2001). An acute or subacute onset of respiratory failure has been known to be a major complication associated with low-dose treatment of MTX (Imokawa et al., 2000). To avoid such respiratory failures, withdrawal of MTX or additional treatment of corticosteroid is effective; however, such respiratory failure has occurred in a few patients (approximately less than 14%) or in patients with long-term MTX treatment (St Clair et al., 1985; Kremer et al., 1986; Carson et al., 1987; Gispen et al., 1987; Zisman et al., 2001). The progressive fibrotic response in idiopathic pulmonary fibrosis (IPF) is associated with an epithelial-dependent fibroblast-activation process. The presence of fibroblasts and myofibroblasts in foci has been recognized as the central feature of IPF pathogenesis. Several investigators have suggested that myofibroblasts come from at least three possible origins, although the relative contribution of each of these pathways is currently unclear (Bucala et al., 1994; Kuwana et al., 2003; Hashimoto et al., 2004; Postlethwaite et al., 2004; Willis et al., 2005; Scotton and Chambers, 2007): proliferation of resident lung interstitial fibroblasts, generation of fibroblasts from epithelial cells through epithelial mesenchymal transition (EMT), and differentiation of circulating fibrocytes or mesenchymal progenitor cells. EMT is known as the morphological change of polarized epithelial cells from an epithelial phenotype to a mesenchymal phenotype in a process characterized by reduced cell-cell

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junction, cytoskeletal rearrangement, increased cell motility, and synthesis of extracellular matrix. Several studies have reported that EMT may contribute to the development of fibroblast and myofibroblast accumulation in lung fibrosis (Willis et al., 2005). During EMT, the epithelial cells lose their epithelial phenotype, such as E-cadherin (a specific epithelial marker), acquire mesenchymal markers, including α-smooth muscle actin (α-SMA), vimentin, and fibronectin, and secrete matrix metalloproteinase (MMP). E-cadherin is a cell-cell adhesion molecule and the loss of its expression is a hallmark of EMT (Zeisberg et al., 2007; Willis and Borok, 2007). Additionally, the myofibroblast marker α-SMA has been shown to increase in cells that underwent transition. Kim et al. (2006) demonstrated that mice alveolar epithelial cells acquired a mesenchymal phenotype when exposed to TGF-β1. These results indicate that fibroblasts indeed can be derived from epithelial cells through EMT and may contribute to the development of pulmonary fibrosis. We have previously demonstrated that treatment of MTX resulted in a decrease of cytokeratin (alveolar epithelial cell marker) and an increase of α-SMA (myofibroblast marker) in fibrosis foci (Ohbayashi et al., 2010). However, there is still limited data concerning the involvement of EMT in MTX-induced pulmonary fibrosis.

The aim of this study is to elucidate the involvement of alveolar epithelial cells in MTX-induced pulmonary fibrosis via EMT. Our results indicate that activated fibroblasts and myofibroblast cells play a pivotal role in MTX-induced pulmonary fibrosis in vivo and in vitro.

MATERIALS AND METHODS

Chemicals

Methotrexate was obtained from Wako Pure Chemical Industries (Osaka, Japan). Recombinant human transforming growth factor (TGF)-β1 was purchased from R&D Systems (Minneapolis, MN, USA). Mouse anti-α-SMA and mouse anti-vimentin antibodies were from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-E-cadherin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-E-cadherin antibody was from BD Biosciences (Franklin Lakes, NJ, USA). Other chemicals not listed here were of the highest grade commercially available.

Animals

Male mice of C57BL/6J strain, weighing 20-25 g, were purchased from Saitama Animal Supply, Co. (Saitama, Japan) and used throughout this study. The mice were fed a commercial solid diet (MF, Oriental Yeast, Tokyo, Japan) and water ad libitum. Lighting was operated on a 12 hr light/dark cycle, and temperature was maintained at 24 ± 2°C. All animal experiments in the present study were approved by the Committee on Ethics regarding Animal Experiments of Showa University.

Cell cultures

A549 cells were purchased from American Tissue Culture Collection (Rockville, MD, USA), and derived from a human alveolar epithelial cell carcinoma. A549 cells were cultured in 35 mm glass bottom dish (Matsunami glass, Tokyo, Japan) and 24-well flat-bottom tissue culture plates with RPMI-1640 containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin in an incubator supplemented with 5% CO2 at 37°C.

Preparation of lung alveolar epithelial and fibroblast cells

Primary type II alveolar epithelial cells were isolated as described previously (Ohbayashi et al., 2013). Briefly, 1 ml dispase (Goudo-shusei, Tokyo, Japan) and 0.45 ml of low-melting-point agarose were instilled intratracheally into mice lungs. Lungs were then removed and placed on ice for 2 min, followed by incubation at room temperature for 45 min. The lung tissue was then transferred to a Petri dish and was teased from the large airways with forceps, minced and placed in Dulbecco’s modified Eargle’s medium (Sigma-Aldrich) containing 0.01% type-II DNase (Sigma-Aldrich). After gentle swirling, the suspension was successively filtered through 132-, 45-, and 25-μm nylon mesh, and centrifuged at 1,000 rpm for 5 min. Cells were incubated with anti-CD32 antibodies (1 μg/106 cells) (BD Biosciences) and anti-CD-45 antibodies (1 μg/106 cells) (BD Biosciences) at 4°C, for 20 min, centrifuged, and resuspended in DMEM (1 x 105 cells), followed by the addition of Dynabeads® Sheep anti-Rat IgG (Life Technologies Corporation, Carlsbad, CA, USA). After slowly shaken for 30 min at 4°C, a magnetic bead separator was used to bind magnetic particles. Unbound cells were collected, dispersed with DMEM supplemented with 10% FBS and then plated on a Petri dish for 4 hr to remove mesenchymal cells adhered to the dish bottom. The floating cells (non-adherent cells) were collected and then plated on fibronectin (Sigma-Aldrich) -coated plastic dishes or 24-well plates.

Primary cell culture of lung fibroblasts was achieved using the protocol described previously (Ohbayashi et al., 2010). Briefly, the lungs removed from mice were minced into pieces of 2-3 mm³, agitated, and digested enzy-
matically for 80 min at 37°C. The digestion buffer was composed of collagenase type I (1 mg/lung) and DNase (250 μg/lung) (Sigma-Aldrich) supplemented in PBS. After filtration, released cells were centrifuged, washed, and then placed in 6-cm plastic culture dishes and incubated in 1 ml DMEM supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml) and, 10% FBS at 37°C in 5% CO₂.

Quantitative real-time polymerase chain reaction (Real-time RT-PCR)

Real-time RT-PCR was performed as described previously (Ohbayashi et al., 2013) with some modifications. Total RNA was extracted from A549 cells using a ReliaPrep™ RNA Cell Miniprep System (Promega, Madison, WI, USA), according to the manufacturer’s protocol. Approximately 1 μg of total RNA was used to synthesize cDNA using a SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies Corporation, Carlsbad, CA, USA). Real-time RT-PCR was performed in a 20 μl volume using 1 μl (100 ng) of the first-strand cDNA template, 1 μl of 20x TaqMan Gene Expression Assay Mix, 10 μl of the 2x TaqMan Universal PCR Master Mix (Life Technologies Corporation) and 8.0 μl of water (Life Technologies Corporation). Probes were designed by Life Technologies Corporation, and supplied as TaqMan MGB probe. Assay IDs were Hs99999901_s1, Hs00985639_m1, Hs00981833_m1, Hs01023894_m1, Hs00426835_g1 for [18S (Eukaryotic 18S rRNA) ], [IL6 (interleukin 6) ], [TGFB1 (transforming growth factor-beta 1) ], [CDH1 (E-cadherin) ], [ACTA2 (α-SMA) ], [SNAIL (Snail)], [ZEB1 (ZEB1)], [VIM (vimentin)] respectively. Expression of 18S was used as internal controls to normalize each mRNA expression. All real-time RT-PCR reactions were performed on the eppendorf realplex® Mastercycler ep gradient S (Eppendorf, Hamburg, Germany) and analyzed using Eppendorf Realplex 1.5 software.

Western blot analysis

Cells were lysed in a RIPA buffer solution (Cell Signaling Technology, Danvers, MA, USA), followed by SDS gel-electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 and then treated with diluted (1:1,000) monoclonal antibodies specific for E-cadherin, β-catenin, Snail, β-actin (Cell Signaling Technology, Danvers, MA, USA) and with diluted (1:1,000) polyclonal antibodies specific for vimentin and α-SMA at 4°C with gentle shaking, overnight. Immunoreactive proteins were visualized using horseradish peroxidase-conjugated secondary antibodies corresponding to each primary antibody and enhanced Clarity™ Western ECL Substrate (Bio-Rad Laboratories). Images and analysis were taken with ChemiDoc™ XRS+ with Image Lab™ software (Bio-Rad Laboratories). Positive immunoreactive bands were quantified using densitometry and compared with the expression of human β-actin.

Immunohistochemical analysis

Paraffin sections were rehydrated and incubated with 10 mM citrate buffer solution (pH 6.0) at 95°C for 30 min to eliminate endogenous peroxidase activity. Nonspecific sites were blocked by incubation of 3% BSA in 0.05 M Tris-buffered saline containing 0.05% Tween-20 at room temperature (RT) for 1 hr. The sections were treated with primary antibody of rabbit anti-E-cadherin diluted 1:100 at 4°C overnight. The sections were followed by incubating with a secondary antibody of goat anti-rabbit Alexa 488 (1:2,000) at RT for 30 min. and for 1.5 hr in a working solution of M.O.M.™ Mouse Ig Blocking Reagent (Vector Laboratories, Burlingame, CA, USA). Sections were then washed for 5 min at RT and then for 5 min in a working solution of M.O.M.™ diluent. The sections were incubated with primary antibody of mouse anti-α SMA at RT for 2 hr. A working solution of M.O.M.™ Biotinylated Anti-Mouse IgG Reagent (Vector Laboratories, Burlingame, CA, USA) (1:2,500 dilution) was applied at RT for 10 min, and then incubated with a 1:500 dilution of rohdamin 600 avidin D for 5 min at RT. Then the sections were stained with 4,6-diamidino-2-phenylindole (DAPI) for 2 min at RT. Fluorescent images were taken by KEYENCE BZ-9000 fluorescence microscope (KEYENCE, Osaka, Japan).

Immunocytochemical analysis

A549 cells were cultured in a 35-mm glass bottom dish (Matsunami glass, Tokyo, Japan). For the detection of E-cadherin or α-SMA, cells were fixed with 3.7% paraformaldehyde/PBS at RT for 20 min. Nonspecific binding of the antibodies was prevented by blocking with 3% BSA/PBS containing 0.01% Tween-20 at RT for 1 hr. The cells were incubated with the primary antibodies for 1 hr at RT and washed. The bound primary antibodies were detected using Alexa Flour secondary antibodies (Life Technologies Corporation). Finally, the cells were washed with PBS and incubated with DAPI (Sigma-Aldrich). Fluorescent images were taken with a KEYENCE BZ-9000 fluorescence microscope (KEYENCE).
Wound closure assay
After A549 cells were confluent, a wound was made by scratching with a tip in the middle of the culture in 3-cm plastic culture dishes. Cells were then treated with 1 μM MTX or TGF-β1 5 ng/ml (Chen et al., 2012; Wang et al., 2013). The cell-free area was measured before and after 24 hr of incubation using a KEYENCE BZ-9000 fluorescence microscope (BZ-9000, KEYENCE).

Statistical analysis
Data are represented as the mean and standard error of the mean. The experimental data were analyzed by one-way ANOVA followed by Tukey’s multiple comparison test. A p-value less than 0.05 was considered significant (p < 0.05).

RESULTS

Immunohistochemical analyses for the expression of E-cadherin and α-SMA in mouse lung
We have previously reported that hyperplasia of interstitial tissues was observed in the alveolar septa and the increase of collagen content in the alveolar wall and fibrosis area after consecutive administration of MTX for 35 days (Ohbayashi et al., 2010). In addition, we have also revealed that the E-cadherin (epithelial cell marker) is significantly detected, whereas α-SMA (mesenchymal marker) is faintly observed in the alveolar septa and, which is restricted to vascular smooth muscle cells in control mice. On the contrary, the level of E-cadherin was decreased and α-SMA was increased in MTX-treated mice, indicating that MTX could induce pulmonary fibrosis resulting in a decrease of alveolar epithelial cells and proliferation of myofibroblasts in fibrosis foci (Ohbayashi et al., 2010). To elucidate, based on these findings, we examined the expression of EMT markers, vimentin, in mouse lung using immunohistochemical analysis. As shown in Figs. 1a and b, the expression level of vimentin was significantly increased in fibrosis foci with 3 mg/kg/day dose of MTX for 21 consecutive days (Fig. 1b). We subsequently examined whether expression of mesenchymal markers such as down-regulated E-cadherin and up-regulated α-SMA as well as their co-localization are observed in mouse lung using immunofluorescence analysis (Figs. 1c-j). As illustrated in Fig. 1, immunofluorescence analyses revealed a decrease of E-cadherin and an increase of α-SMA induced with MTX 3 mg/kg for 35 days when compared with control mice (Figs. 1c-f). Furthermore, we observed co-localization with both E-cadherin and α-SMA positive cells in MTX-induced pulmonary fibrosis foci (Figs. 1i and j) suggesting that MTX promoted α-SMA expression and suppressed E-cadherin during MTX-induced pulmonary fibrosis.

Immunocytochemical analyses for the expression of E-cadherin and α-SMA in mouse alveolar epithelial cells
On the basis of our previous data, we subsequently investigated whether MTX-induced morphological change via EMT is associated with regulation of E-cadherin and α-SMA in mouse alveolar epithelial cells (MAECs). MAECs were treated with MTX (1 μM) or with TGF-β1 (5 ng/ml), and the morphological characteristics using the expression of EMT markers such as E-cadherin, α-SMA were examined by immunocytochemical analysis (Fig. 2). However, we found that MAECs exhibit classic cobblestone morphology (Fig. 2a). After stimulation by 1 μM MTX treatment (72 hr), MAECs acquired a more fibroblast-like, elongated and narrower, spindle-shaped morphology, with low cell-cell contact (Fig. 2d) and high expression of α-SMA with reduction of E-cadherin (Figs. 2e and f), similar to the result of TGF-β1-simulated cells (Figs. 2g-h). These results indicate that MTX could induce an EMT-like phenotype in MAEC cells.

Dose-dependent changes of the expression of EMT markers by MTX in A549 cell lines
To elucidate the involvement of EMT in human alveolar epithelial cells induced by MTX in vitro, we used A549 cell lines. As shown in Fig. 3, A549 cells exhibited an EMT in response to MTX 1 μM for 72 hr, converting from their epithelial phenotype to fibroblast-like morphologies, with low cell-cell contact and high primary expression of the mesenchymal marker α-SMA (Figs. 3c and f) with reduction of E-cadherin (Figs. 3b and e), similar to the results in MAECs. We subsequently examined whether the expression of EMT markers are exhibited in a dose-dependent manner. A549 cells were incubated with 0.03, 0.1, 0.3, 1 and 3 μM MTX for 72 hr. Western blot analyses revealed that the dose-dependent expression of E-cadherin was significantly decreased, and the expression of α-SMA was increased (Figs. 4a-c). In addition, real-time PCR analyses stratified MTX-induced down-regulation of E-cadherin and up-regulation of α-SMA mRNA for 72 hr (Figs. 4d and e). Thus, the EMT markers showed significant changes with MTX treatment at 1 μM.

Methotrexate-induced up-regulation of IL-6 and TGF-β1 mRNA
To clarify the mechanism of MTX-induced EMT in
Fig. 1. Distribution and colocalization of EMT markers (vimentin, E-cadherin and α-SMA) in MTX-induced C57BL/6J mice lung. Lung tissues were obtained from mice after oral administration of MTX 3 mg/kg/day for 21 (b) and 35 days (d, f, h and j). Control mice were administrated with an appropriate volume of distilled water (DW) alone for 21 (a) and 35 days (c, e, g and i). Four-micrometer sections were incubated with monoclonal anti-vimentin antibody. The expression of EMT markers vimentin was stained with DAB (brown) and nuclei was stained with hematoxylin solution (blue). Vimentin-positive cells were stained by brown (b). Immunohistochemical analyses for the distribution of E-cadherin and α-SMA in MTX-induced mice lung (c-j). E-cadherin-positive cells were stained by green (c and d). α-SMA-positive cells were stained by red (e and f). DAPI-positive nuclei appear in blue (g and h). E-cadherin-positive cells and α-SMA-positive cells were co-expressed in fibroblastic foci (yellow, allows in j). Scale bar represents 200 μm (a and b) and 20 μm (c-j) in each photograph. MTX, methotrexate.
A549 cells, we subsequently examined MTX-induced up-regulation of IL-6 and TGF-β1 using real-time RT-PCR analysis. Real-time RT-PCR analyses showed that MTX induced up-regulation of both IL-6 and TGF-β1; however, untreated cells did not increase the expression of IL-6 and TGF-β1 mRNAs. At 48 hr later, however, treatment of A549 cells with MTX significantly increased the expression of IL-6 and TGF-β1 to about 59- and 2-fold, respectively (Figs. 5a and b). Exogenous TGF-β1 induced more significantly increased expressions of IL-6 and TGF-β1 to about 48- and 3-fold, respectively, than that of MTX-treated cells (Figs. 5a and b).

Methotrexate enhances cellular migration in A549 cells

We finally examined the ability for migration after MTX treatment using a wound healing assay compared with the TGF-β1 treatment. Liu reported that TGF-β1 (5 ng/ml)-induced increases in cell adhesion, migration and invasion in A549 without promoting cell proliferation (Liu et al., 2013; Wang et al., 2012). Confluent cells were scratched on a plate and the wounded closure area (migrated cells) was evaluated using image analysis. As shown in Fig. 6, after stimulation by 1 μM MTX treatment (24 hr), the wound was significantly closed from the edge of both sides, whereas non-treated cells maintained

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**Fig. 2.** Cell morphology and the expression of E-cadherin / α-SMA in MTX-treated MAECs. MAECs (mouse alveolar epithelial cells) were cultured in 1% FBS media (a-c), with MTX 1 μM (d-f) or TGF-β1 5 ng/ml (g-i) for 72 hr. Phase contrast microscopy reveals elongated, fibroblast-like cells after MTX 1 μM and TGF-β1 5 ng/ml stimulation for 72 hr, compared with a cuboidal cobble stone appearance for the control MAECs (a, d and g). MAECs were fixed and stained for epithelial cell markers E-cadherin (red: b, e and h) and EMT markers α-SMA (green: c, f and i). MTX treatment for 72 hr results in a decrease in E-cadherin (e) and an increase in α-SMA (f). Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI) (blue). Results shown are typical photograph representative of at least three independent experiments. Scale bar represents 100 μm. MTX, methotrexate; TGF-β1, transforming growth factor-β1.
the wound (Fig. 6a). A similar experiment was examined by TGF-β1 treatment and showed that approximately 2-3 times cells migrated following treatment with MTX or TGF-β1 for 24 hr compared with non-treated cells (Fig. 6b). Of interest was that, when TGF-β1 was treated with A549 cells, the enhancement of migration was significantly more than MTX-induced migration. Thus, we found that there is an apparent difference in levels of migration activity in MTX-induced and TGF-β1-induced cells.

**DISCUSSION**

In the present study, we investigated the involvement of EMT in MTX-induced pulmonary fibrosis in vivo and in vitro. Our results suggest that an oral route administration of MTX could induce fibrotic response through EMT, a part of pulmonary fibrosis. Interestingly, we also observed that the morphological and biological changes through EMT in MTX-treated alveolar epithelial cells are quite similar to those of human pulmonary fibrosis. All of these findings lead us to conclude that, at least partly, EMT may play a pivotal role in the progression of MTX-
Fig. 4. Dose-dependent of changes of the expression of EMT markers induced by MTX in A549 cell lines. A549 cells were analyzed by Western blot methods for epithelial cell markers E-cadherin (a and b) and mesenchymal markers α-SMA (a and c). Representative western blotting with quantitative analyses demonstrating a slightly decrease in the E-cadherin with acquisition of α-SMA in MTX stimulated A549 cells with concentration-dependent. Results are expressed as ratio of E-cadherin or α-SMA to GAPDH and are given as the mean ± S.E.M. of three independent experiments. *p-value < 0.05 vs control. A549 cells were analyzed by real-time RT-PCR methods for epithelial cell markers mRNA E-cadherin (d) and mesenchymal markers mRNA α-SMA (e). MTX 1 μM and TGF-β1 5 ng/ml treatment for 72 hr decrease the mRNA expression of E-cadherin, whereas increase the level of α-SMA mRNA expression. Results are expressed as ratio of E-cadherin or α-SMA to 18S and are given as the mean ± S.E.M. of three independent experiments. *p < 0.05 vs control. #p < 0.05 vs MTX-treated. MTX, methotrexate; TGF-β1, transforming growth factor-β1.
Kuwano reported that alveolar epithelial damage is an important initial event in the development of pulmonary fibrosis (2007). Their report indicates that when the degree of lung injury is mild, damaged tissues will be normally repaired, whereas excess cell death may lead to irreversible lung damage and pulmonary fibrosis (Kuwano, 2007). However, the difference between alveolar epithelial cells and lung fibroblasts concerning MTX cytotoxicity is still unknown. We observed that MTX could induce pulmonary fibrosis and result in a decrease of alveolar epithelial cells and proliferation of myofibroblasts. Our results suggest that MTX induces cytotoxicity of epithelial cells and subsequent proliferation of myofibroblasts in fibrosis. Thus, we found that alveolar epithelial cells would be more sensitive to cell injury than the fibroblast cells.

We next investigated the involvement of EMT in MTX-induced fibrosis model mice. Several studies support the notion that TGF-β1 is an important molecule in pulmonary fibrogenesis promoting alveolar epithelial cell transition to form mesenchymal cells with a myofibroblast-like phenotype (Desmouliere,
pulmonary fibrosis and in patients with IPF (Aumiller et al., 2013). Yoshida et al. reported that MTX-induced pneumonitis accumulates polymorphonuclear neutrophil leukocytes (PMNs) in BALF (Yoshida et al., 1999). Sampson revealed that PMNs are able to produce a wide range of products such as cytokines (IL-6, TGF-β), proteases, reactive oxygen intermediates (Sampson, 2000). Thus, the knowledge of these cytokines during a process of drug-induced fibrosis gradually accumulated; therefore, we subsequently tested whether MTX-induced up-regulation of IL-6 and TGF-β1 in A549 cells. Interestingly, at 48 hr later, cells treated with MTX showed significant increases of the expressions of both IL-6 and TGF-β1. Thus, up-regulation of the expression of cytokines IL-6 and TGF-β1 may be a pivotal role for MTX-induced pulmonary fibrosis.

We finally investigated the effects of pathological responses by the treatment of MTX (Willis and Borok, 2007; Xu et al., 2009). Our study revealed that the migration activity of MTX-treated A549 cells is significantly less than of TGF-β1-induced cell migration. This may suggest that population of cells showing EMT is larger in TGF stimulation condition compared to MTX treatment. Taking these facts and previous paper into consideration, we considered the following three speculations: first, cell injury and inflammation are induced by MTX treatment; second, after cell injury and inflammation, EMT is produced; and third, neither the first nor the second speculation is valid. We hypothesized that the balance of these steps will contribute to the development of MTX-induced pulmonary fibrosis. In this respect, further study is needed.

In conclusion, we describe the mechanism of MTX-induced lung fibrosis. We found that EMT is, at least in part, an important step for the development of MTX-induced lung fibrosis; specifically, IL-6 and TGF-β1 may play a pivotal role for inducing lung fibrosis by MTX. Our results, therefore, are expected to facilitate research on new drug discovery for preventing drug-induced fibrosis and to provide clues in the search for still unidentified mechanisms of lung fibrosis.

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