INTRODUCTION

It has been known that there is a relationship between the development of pulmonary disease(s) and treatments with various drugs such as methotrexate (MTX), bleomycin (BLM), and amiodarone. However, the prediction of drug-induced pulmonary disease is extremely difficult because several factors must be taken into account: medical history, microviral infection, drug-drug interaction, and irradiation in addition to endogenous factors such as chemokines and prostanoids (Keane et al., 2005).

MTX is a folic acid antagonist and has been widely used at high dosages for the treatment of various malignancies such as leukemia, breast, head, neck and lung carcinomas, whereas it is used at low dosages for the treatment of rheumatoid arthritis (RA). In the clinical setting, severe pulmonary side effects have been observed when physicians administered to the patients at the low dose of MTX for a long-term period (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). Such complications have been observed with an acute or subacute onset of respiratory failure, often accompanied by cough and fever (Imokawa et al., 2000), and chest radiographs show diffuse interstitial opacity (Kremer et al., 1997). Generally, effective treatment for the patient with lung fibrosis is known to be withdrawal of MTX and/or additional corticosteroid administered as soon as possible to avoid progression of the disease. However, some patients may deteriorate; irreversible interstitial pneumonia and subsequent death have been reported. Such a lung toxicity is estimated to be approximately 0.5–14% with a low dose MTX treatment (St Clair et al., 1985; Kremer et al., 1986; Carson et al., 1987).

Induction of pulmonary fibrosis by methotrexate treatment in mice lung in vivo and in vitro

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ABSTRACT — Methotrexate (MTX) has been used as the first-line disease-modifying antirheumatic drug (DMARD) in patients with early progressive rheumatoid arthritis (RA). Several severe side effects such as myelosuppression, hepato-, nephro-, and pulmonary toxicities have been reported. However, the pathogenic mechanism of MTX-induced pulmonary fibrosis is still unknown. Here, we evaluated the morphological and biological changes of the pulmonary fibrosis in mice treated with MTX. Three, four and five weeks after consecutive administration of MTX (3 mg/kg/day), hydroxyproline content in the lung tissues increased significantly to about 2 times higher that of the control level. This result closely reflected to the results of hematoxylin and eosin (HE) and Azan stains. Immunohistochemical analysis revealed that MTX treatment resulted in a decrease of alveolar epithelial cells and an increase of fibroblast cells in the mouse lung tissues. When we examined the effects of MTX on primary mouse alveolar epithelial cell (MAEC) and mouse lung fibroblast (MLF) survival in vitro, the efficiency of MTX-induced cytotoxicity and apoptosis in MAEC was more sensitive than MLF cells. Thus, our results indicate that the administration of MTX by an oral route could induce a fibrotic response with cell dysfunction of the alveolar epithelium by which MTX-induced apoptosis. Our results thus suggest that MTX could induce alveolar epithelial cell injury and resulted in the loss of integrity of the alveolar-capillary barrier basement membranes followed by the recruitment and proliferation of myofibroblasts with the deposition of collagens.

Key words: Methotrexate, Pulmonary toxicity, Alveolar epithelial cell, Cytotoxicity, Apoptosis, Lung fibroblast

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et al., 1987; Gispen et al., 1987; Zisman et al., 2001). Especially, in RA patients who receive long-term MTX have a high risk for the die from lung fibrosis. Therefore, it is very important to elucidate the mechanism(s) and protect against the development of drug-induced pulmonary fibrosis.

Many papers concerning the drug-induced lung fibrosis animal models have been reported to date (Adamson, 1976; Satomi et al., 2007; Moeller et al., 2008; Lacerda et al., 2009). Fleischman et al. (1971) and McCullough et al. (1978) have reported that treatment with BLM resulted in the induction of lung fibrosis in canines and baboons, and BLM induced diffuse alveolar injury and subsequent pulmonary fibrosis (Blum et al., 1973; Rabinowits et al., 1990). Recently, Moeller et al. (2008) reported that BLM-induced lung fibrosis model is helpful to illustrate pathobiology in vivo and in vitro and to identify new targets for medication. Thus, animal models are useful tool to study drug-induced lung fibrosis; however, there is still limited data concerning the induction of lung fibrosis by the treatment of MTX in rodents.

Here, we investigated the induction of lung fibrosis in mice treated with MTX. Our results indicate that MTX administration is induces alveolar epithelial cell injury. In addition, we found that MTX-induced cytotoxicity and apoptosis in mouse alveolar epithelial cell (MAEC) was more sensitive than mouse lung fibroblast (MLF) cells. Thus, our findings would be a novel approach to understand of the molecular mechanism(s) of MTX-induced lung toxicity.

MATERIALS AND METHODS

Chemicals
MTX was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). BLM was kindly provided by Nippon Kayaku (Tokyo, Japan). All 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. They 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.

MTX-induced pulmonary fibrosis animal models
MTX was dissolved in an appropriate volume of distilled water (DW) and injected orally at a single dose of 3, 10, and 15 mg/kg. Control animals received DW alone. On days 21, 28, and 35 after consecutive administration of MTX, mice were sacrificed by administrating an excess amount of pentobarbital sodium. Lung was fixed immediately with 4% paraformaldehyde or snap-frozen in liquid nitrogen and stored at -80°C until use.

Histological examination
After the treatment of MTX, the lung was carefully removed and dissected with scissors. Obtained lung tissues were fixed with 4% paraformaldehyde and embedded in paraffins and 4 μm sections were adhered to glass slides. For hematoxylin and eosin (HE) staining, Azan staining and immunohistochemical analyses, the lung sections were deparaffinized by passing through 100% xylene three times, and then through 100, 95, 80 and 70% alcohol for 10 min, respectively.

Immunohistochemical examination
Immunohistochemical analysis was performed by the streptavidine-biotin-horseradish peroxidase complex technique (DAKO Co., Ltd., Kyoto, Japan). Paraffin sections were rehydrated and incubated with 10 mM citrate buffer solution (pH 6.0) at 95°C for 40 min to eliminate endogenous peroxidase activity. After rinsing in 0.05 M Tris-buffered saline containing 0.1% Tween-20, sections were treated with primary antibody of human pan-cytokeratin (AE1/AE3; Nichirei Bioscience Inc., Tokyo, Japan), which recognizes mouse CK and α-SMA (DAKO Co., Ltd.) at 4°C for overnight. The sections were incubated with a secondary biotinylated goat polyclonal antibody against mouse immunoglobulin (DAKO Co., Ltd.) with horseradish peroxidase-labeled streptavidin for 30 min. This step was followed by incubation with diaminobenzidine and hydrogen peroxide. The sections were counterstained with hematoxylin and observed under light microscopy (BZ-8000, KEYENCE, Osaka, Japan).

Hydroxyproline assay
The amount of hydroxyproline content is used to calculate the total collagen content, and have been used to investigate the level and degree of fibrosis. The Sircol Collagen Assay has been introduced as a more convenient colorimetric assay for the quantification of collagen in tissues. Therefore, we examined the total lung collagen content was measured using the Sircol Soluble Collagen Assay (Biocolor, Newtownabbey, Northern Ireland). Briefly, the lung was ‘diced’ into small cubes using a
sharp scalpel and homogenized with 1 ml of 0.5 M acetic acid containing 1 mg pepsin for 10 mg tissue residue and slowly shaken overnight at room temperature. After centrifugation at 15,000 g for 60 min, 30 μl of each supernatant with 70 μl of 0.5 M acetic acid were assayed. One ml of Sircol dye reagent was added to each sample then slowly mixed at room temperature for 30 min. The collagen-dye complex was precipitated by centrifugation at 10,000 g for 10 min. The obtained pellet was dissolved in 1 ml of 0.5 M NaOH and a portion of the solution was transferred to a 96-well plate and measured with a spectrophotometer (540 nm) (Varioskan Flash, Thermo Fischer Scientific, MA, USA).

Preparation of lung alveolar epithelial cells and fibroblasts

Primary type II alveolar epithelial cells were isolated as described previously with a slight modification (Corti et al., 1996). 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 Earle’s medium (Sigma-Aldrich) containing 0.01% type-II DNase (Sigma-Aldrich) containing with 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. Cells were incubated with anti-CD32 antibodies (1 μg/10⁶ cells) (BD Pharmingen, Franklin Lakes, NJ, USA) and anti-CD-45 antibodies (1 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.

TUNEL staining

Alveolar epithelial cells and fibroblast were grown on 8 well chamber slides, and placed incubated in DMEM (0.1% FBS) with 1 μM MTX for 12 hr, 24 hr, and 48 hr. TUNEL staining was performed with In Situ Cell Death Detection kit, Fluorescein (Roche Diagnostics, Tokyo, Japan), following the manufacturer’s instructions. Stained cells were visualized using a BZ-8000 fluorescence microscope (KEYENCE Co., Ltd.). The numbers of TUNEL positive cells were counted in the whole field of each section using a microscope at × 200 magnification.

Cell viability assay

Cytotoxicity was determined by LIVE/DEAD® Viability/Cytotoxicity Kit (Invitrogen), following the manufacturer’s instructions. Alveolar epithelial and fibroblast cells were grown on 24 well culture plates until sub-confluent monolayer, and incubated in DMEM (1% FBS) for 24 hr; then cultured with 0.01, 0.1, 1, 10, and 100 μM MTX for 72 hr. To evaluate the viability of the cultured alveolar epithelial cells, fibroblast cells were stained by incubation in 250 μl/well of PBS containing 2 μM of calcine-AM and 4 μM of ethidium homodimer (EthD-1) (LIVE/DEAD® Viability/Cytotoxicity Kit, Invitrogen) for 30 min at room temperature. The living cells stained with calcine-AM (485 nm) and the dead cells stained with EthD-1 (530 nm). Stained cells were measured by the fluorescence microplate reader (Varioskan Flash, Thermo Fischer Scientific).

Statistical analysis

Data are represented as the mean ± S.D. 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

Dose-response effect of MTX treatment on blood toxicity

The dose-response effect of MTX on blood toxicity was investigated. Mice were treated with MTX at doses of 3, 10 and 15 mg/kg for 28 consecutive days. Because myelosuppresiveness and infections are a dose-limiting factor for the patients treated with MTX, we evaluated the blood toxicity. As shown in Table 1, we did not observe
any toxic effect after MTX treatment at a dose of 3 mg/kg. However, at a higher dose of MTX (> 10 mg/kg), the body weight and survival duration tended to decrease (data not shown). On the other hand, white blood cells (WBC), red blood cells (RBC), hemoglobin (Hb) and hematocrit (Hct) level were decreased in a dose-dependent manner. At 3 mg/kg dose of MTX did not affect myelosuppression. On the basis of these findings, therefore, we concluded that 3 mg/kg dose of MTX is a suitable for subsequent study.

Morphological and biochemical changes in MTX-treated mice
We subsequently examined whether time-dependent pulmonary toxicity is observed. Mice were treated MTX with a dose of 3 mg/kg once a day for 21, 28, and 35 consecutive days. As shown in Fig. 1, occasional alveolar septa seemed to increase slightly when compared with the control animals (day 35, Fig. 1, panels a vs d). On days 28 and 35, randomly selected areas of alveolar septa were the hyperplasia of interstitial tissue (Fig. 1, panels c and d, respectively). To confirm these findings, the lung sections were subsequently stained by the Azan staining method. As illustrated in Fig. 1 (panels e-h), the increase of collagen content in the alveolar wall and fibrosis area. The collagen in the alveolar septa is specifically localized in surrounding the fibrosis lesion. Such observations were histologically quite similar to BLM-treated mice lung tissues. In addition, we found that collagen was accumulated on day 35. Interstitial hyperplasia was observed around the peripheral vascular of the lungs after MTX treatment. Control mice lung sections (Fig. 1, panel e) did not show a significant increase of collagen content in the alveolar wall. Based on these findings, we next examined the hydroxyproline content in lung tissues. A series of mice samples obtained from 21-, 28-, and 35-day exposures of MTX revealed a statistically significant increase of hydroxyproline content. The hydroxyproline content of the whole lung after administered with MTX, was significantly increased to about 143.2 ± 4.8%, 129.2 ± 5.1%, and 188.4 ± 10.3% on days 21, 28, and 35, respectively (Fig. 2).

Effects of MTX on alveolar epithelial and fibroblast cells of the lung in mice
Since pathological characteristics of fibrosis have been well established by immunohistochemical analysis, we employed anti-cytokeratin as alveolar epithelial cell marker for pathological analysis. Of interest was that the expression level of cytokeratin decreased significantly when 3 mg/kg dose of MTX for 35 consecutive days (Fig. 3A, panels b and d). On the contrary, the level of immunoreactive α-SMA protein which is a fibroblast cell marker increased (Fig. 3B, panels b and d), indicating that MTX could induce lung fibrosis and resulted in a decrease of alveolar epithelial cells and proliferation of myofibroblasts in fibroblastic foci.

Table 1. Dose-response effect of MTX on bone-marrow suppression in mice

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>WBC (x10^9/l)</th>
<th>RBC (x10^12/l)</th>
<th>Hb (g/dl)</th>
<th>Hct (%)</th>
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<tr>
<td>Control</td>
<td>3.24 ± 0.42</td>
<td>8.58 ± 0.13</td>
<td>12.50 ± 0.32</td>
<td>40.30 ± 0.86</td>
</tr>
<tr>
<td>3</td>
<td>3.09 ± 0.31</td>
<td>7.75 ± 0.29</td>
<td>11.20 ± 0.34</td>
<td>37.30 ± 1.12</td>
</tr>
<tr>
<td>10</td>
<td>2.45 ± 0.38</td>
<td>7.19 ± 0.70</td>
<td>10.00 ± 1.16</td>
<td>33.70 ± 3.51</td>
</tr>
<tr>
<td>15</td>
<td>1.09 ± 0.41*</td>
<td>4.32 ± 1.12*</td>
<td>5.48 ± 1.74*</td>
<td>19.70 ± 5.61*</td>
</tr>
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Male mice were administered MTX orally at a dose of 3 mg/kg for 21, 28, and 35 consecutive days, and blood samples were collected and checked using VetScan HM2™ (Abaxis, Union City, CA, USA) after the final treatment. After high-dose MTX (15 mg/kg) administration, WBC, RBC, Hb and Ht were significantly decreased. Control mice were treated with an appropriate volume of DW alone. Each value represents the mean ± S.E.M. of three to nine mice. *Significantly different from controls at *p < 0.05. WBC, white blood cells; RBC, red blood cells; Hb, hemoglobin; Ht, hematocrit.

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positive cells on MAEC at 12 hr (11.5 ± 1.75%), 24 hr (27.7 ± 3.17%), and 48 hr (25.2 ± 5.65%). However, treatment of MTX did not show a significant increase of TUNEL positive cells in MLF.

**DISCUSSION**

In the present study, we evaluated the morphological and biological changes of the pulmonary fibrosis in mice treated with MTX. Our results suggest that the oral route administration of MTX could be induced fibrotic response with cell dysfunction and will be mediated by apoptosis of the alveolar epithelial cells.

Of the administration route and strain of mice producing lung fibrosis similar to human disease, Chua et al. (2005) recently reported that is intravenous (i.v.) or intraperitoneal administration more suitable than the intratracheal instillation. On the other hand, Harrison and Lazo (1987) have reported that the induction of lung fibrosis by BLM in C57BL/6J mice is more susceptible than the BALB/C mice strain. Taking these findings into consideration, therefore, we choose oral route for the establishment of MTX-induced lung fibrosis model animals.

On the basis of these findings, therefore, we employed the C57BL/6J strain and choose oral route for the establishment of MTX-induced lung fibrosis model animals.

Firstly, we investigated the dose-response effect of MTX on blood toxicity and body weight change. We found that MTX (3 mg/kg) dose regimen is a non-toxic dose under our experimental condition. The patholog-

**Fig. 1.** Histopathological examination in C57BL/6J mice lung treated with MTX. Paraformaldehyde-fixed lung tissues were stained with HE (a-d) or AZAN stain (e-h). Representative photographs indicate mice left lung sections after peroral administration of DW for 35 consecutive days (a, d), respectively, and of MTX for 21 (b, f), 28 (c, g), and 35 days (d, h), respectively. MTX, MTX; Br, bronchi; V, vessel. Scale bar represents 500 μm in each photograph.
Fig. 2. Time-dependent changes of the lung hydroxyproline content by MTX administration in C57BL/6J mice. Mice were treated with MTX at the dose of 3 mg/kg/day for 21, 28 and 35 consecutive days and hydroxyproline content was measured. Hydroxyproline content was significantly increased in a time-dependent manner. Each value represents the mean ± S.D. of at least 6-10 mice. *Significantly different from controls at *p < 0.05.

Fig. 3. Immunohistochemical analysis of MTX-induced C57BL/6J mice lung. Mice were treated with MTX at a dose of 3 mg/kg/day for 35 days. Four-micrometer sections were incubated with polyclonal anti-cytokeratin (A) and α-SMA antibodies (B). Lung tissues were obtained from mice on day 35 after oral administration of MTX (A, panels b and d; B, panels b and d). Control mice were administered with an appropriate volume of DW alone (A, panels a and c; B, panels a and c). The expression of cytokeratin and α-SMA were stained with DAB (brown) and nuclei were stained with hematoxylin solution (blue). Scale bar represents 200 µm in each photograph.
ical response to lung fibrosis by drugs has been well documented (Allen and Spiteri, 2002; White et al., 2003; Moeller et al., 2008). We subsequently examined whether time-dependent pulmonary toxicity is observed in mice treated with MTX 3 mg/kg once a day for 21, 28, and 35 consecutive days. Although collagen is the most predominant component of the lung matrix composing 10-20% of the dry weight of lung tissues (Crystal et al., 1975), we observed that MTX induced accumulation of collagen content on the alveolar wall. Interestingly, these findings are in good agreement with several other studies that exhibited an enhancement of collagen accumulation after exposure to toxic compounds, such as silica and BLM (Crystal et al., 1975; Reinhart et al., 1996; Suzuki et al., 1996; Chung et al., 2001). Taking these published papers and our results into consideration, we subsequently measured the hydroxyproline content. As expected, a series of mice samples from 21, 28, and 35 days of exposure to MTX revealed a statistically significant increase of hydroxyproline content in the lung. The extent of hydroxyproline content became greater as time passed after MTX administration, indicating that the difference in the histological image associated with MTX-treated mice may reflect a significant difference in hydroxyproline content. Thus, our findings are very similar to BLM-treated mice. These observations suggest that MTX-induced pulmonary fibrosis would be mediated by recruitment of inflammatory cells in the fibrosis area around the peripheral vascular.

Recently, Kuwano (2007) reported that alveolar epithelial damage is an important initial event in pulmonary fibrosis. The report indicates that when the degree of lung injury is mild, damaged tissues will normally be repaired, whereas excess cell death may lead to irreparable lung damage and pulmonary fibrosis (Kuwano, 2007). However, the difference between alveolar epithelial cells and lung fibroblast concerning MTX cytotoxicity is still unknown. Therefore, we subsequently examined the effects of MTX on cell survival in alveolar epithelial cells and lung fibroblasts in vivo and in vitro. We found that,
in vivo studies, MTX could induce pulmonary fibrosis and resulted in a decrease of alveolar epithelial cells and proliferation of myofibroblasts. The results suggest that MTX induces the cytotoxicity of epithelial cells and subsequently proliferation of myofibroblasts in fibrosis area. Moreover, the number of apoptotic cells was increased in primary alveolar epithelial cells by MTX treatment (1 μM). Thus, we found that cell injury of primary alveolar epithelial cells would be more sensitive than the fibroblasts cells.

Imokawa et al. (2000) have reported that interstitial inflammation, fibrosis, giant cells, and tissue eosinophils increased intra-alveolar macrophages in patients with MTX pneumonitis. Our findings in MTX-induced model mice exhibited similarity interstitial inflammation, fibrosis, diffuse alveolar damage, and accumulation of collagen. Their results together with our finding into consideration, our model is similar to the patients with MTX pneumonitis.

The mechanism(s) of the development of lung fibrosis by BLM is proposed by Keane et al. (2005) and Moeller et al. (2008). Helene et al. (1999) and Keane et al. (2005) have suggested that the development of lung injury by BLM is T-cell independent. On the other hand, the development of fibrotic lesions is dependent on the release of chemokines, specifically CCL2 or CCL12, from the injured lung (Keane et al., 2005). Transforming growth factor-β1 (TGF-β1) and prostanoids are also reported to be critically involved in the development of BLM-induced pulmonary fibrosis (Zhao et al., 2002; Keane et al., 2005). From these findings, it would be interesting to investigate the effect of MTX on the expression of TGF-β1, the release of chemokines, and the production of anti-fibrotic prostanooids in this lung fibrosis model mouse. In this respect, further study is needed.

It has been well known that several drug transporters play a pivotal role in the transport of organic solutes across the biological membrane, and resulted in the increase and/or decrease of the rate of drug-induced side effects (Rizman and Burckhardt, 2007). Hornscha et al. (2000) have revealed that transport of MTX into mammalian cells can occur via drug transporter-mediated pathways. With regard to the carrier-mediated transport of MTX, other investigators and we have reported that organic anion transporter kidney 1 (OAT-K1[Slc21a4/Slco1a3_v1]) (Saito et al., 1996), mouse organic anion transporter 2 (mOat2[Slc22a7]) (Kobayashi et al., 2002), and mouse reduced folate carrier 1 (mRfc1[Slc19a1]) transports MTX (Dixon et al., 1994). In addition, mRfc1[Slc19a1] is known to be an uptake transporter that expressed in MAEC; therefore, at this moment, we hypothesis that mRfc1[Slc19a1] is one of an important molecule for involving the development of the lung fibrosis. It would be interesting to elucidate whether the carrier proteins are involved in the transport of MTX in MAEC.

In conclusion, we established MTX-induced lung fibrosis model mouse. We found that histological changes were observed in the mouse lung after MTX treatment. Our results suggest that MTX could induce alveolar epithelial cell injury and resulted in the loss of integrity of the alveolar-capillary barrier basement membranes followed by the recruitment and proliferation of myofibroblasts with the deposition of collagens. Thus, our model may provide a particular advantage for further study in elucidating the mechanism(s) of pulmonary fibrosis. Our results would lead us to a new strategy to maximize the therapeutic efficacy and to minimize toxicity with low dose treatment of MTX.

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