Inflammation in methotrexate-induced pulmonary toxicity occurs via the p38 MAPK pathway

Youn-Jung Kim, Mee Song, Jae-Chun Ryu*

Cellular and Molecular Toxicology Laboratory, Korea Institute of Science and Technology (KIST), P.O. Box 131, Cheongryang, Seoul, Republic of Korea

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ABSTRACT

Methotrexate (MTX) has been widely used for the treatment of inflammatory diseases and rheumatoid arthritis (RA), as well as a variety of tumors. However, MTX-induced toxicity is a serious and unpredictable side effect of this therapy and an important clinical problem. We used microarray analysis to examine MTX-induced gene expression in a human lung epithelial cell line (BEAS-2B) and identified 10 differentially expressed genes related to the p38 mitogen-activated protein kinase (MAPK) pathway, including IL-1β, MKK6, and MAPKAPK2. Differential gene expression was confirmed by real-time RT-PCR. To determine the functional significance of MTX-induced p38 MAPK activation, we used a p38 MAPK inhibitor (SB203580) to block the p38 MAPK cascade. We also used protein array technology to investigate the modulated expression of pro- and anti-inflammatory cytokines in BEAS-2B cells. MTX activated IL-1β expression and induced the phosphorylation of various proteins in the p38 MAPK cascade, including TAK1, MKK3/MKK6, p38 MAPK, MAPKAPK2, and HSP27. Finally, HSP27 activation may increase IL-8 secretion, resulting in a pulmonary inflammatory response such as pneumonitis. Although IL-1β and IL-8 expression increased, the expression of IL-4, IL-6, IL-12, TNF-α, MIP-1α, and MIP-1β decreased in a dose-dependent manner. These results suggest that the modulation of cytokine expression may play an important role in MTX-induced pulmonary toxicity.

1. Introduction

Since meeting the U.S. Food and Drug Administration requirements in 1953, MTX has been widely used to treat many kinds of cancers, rheumatoid arthritis (RA), psoriasis, immunological abnormalities, and systemic inflammation (Koyama et al., 2003; Turesson and Matteson, 2006). MTX has been prescribed to approximately 500,000 RA patients (Swierkot and Szechinski, 2006). However, MTX treatment is associated with a number of adverse reactions, including nonproductive cough, dyspnea, fever, pneumonitis, interstitial lung disease, and pulmonary fibrosis (Hsu et al., 2003). MTX-induced adverse effects occur in up to 60% of cases and are dose-dependent in nature (Yamauchi et al., 2004). Pulmonary toxicity occurs in 1–5% of patients taking MTX (Sikka, 2006).

MTX inhibits dihydrofolate reductase (DHFR), an enzyme of the folate synthesis metabolic pathway that catalyses the conversion of dihydrofolate to active tetrahydrofolate. Folic acid is indispensable for synthesizing the thymidine required for DNA synthesis. The affinity of MTX for DHFR is about 1000-fold that of folate for DHFR

(Phillips et al., 2003). Therefore, because MTX suppresses the synthesis of DNA, RNA, and proteins, this drug has an inhibitory effect on cells in S-phase.

Although low-dose MTX remains an effective treatment for RA, its beneficial effect may not be mediated through DHFR inhibition, but may instead be a side effect of suppressed T cell activation and the expression of intercellular adhesion molecules in T cells (Johnston et al., 2005).

MTX-induced pneumonitis stimulates the release of IL-8, MCP-1, G-CSF, and GM-CSF in airway epithelial (A549) cells. Thus, the mRNA expression for IL-8 and other cytokines was elevated in response to MTX (Turesson and Matteson, 2006). IL-8 levels were elevated in the bronchoalveolar lavage (BAL) fluid of patients with idiopathic interstitial pneumonia, sarcoidosis, and hypersensitivity pneumonitis (Fujimori et al., 2003). In addition, pro-inflammatory cytokines such as IL-1β and TNF-α induce the production of IL-8 (Yamauchi et al., 2004).

IL-8 production is induced by the phosphorylation of p38, a mitogen-activated protein kinase (MAPK) (Yamauchi et al., 2004). The three major MAPK signaling pathways are the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase or stress-activated protein kinase (JNK/SAPK), and p38 kinase cascades. In these MAPK signaling cascade, the phosphorylation of serine/threonine residues on MAPK kinase kinase (MAPKKK) activate

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* Corresponding author. Tel.: +82 2 958 5070; fax: +82 2 958 5059.
E-mail addresses: ryujc@kist.re.kr, duckyj@kist.re.kr (J.-C. Ryu).

MAPK kinase (MAPKK) via the phosphorylation of tyrosine and threonine residues, which in turn induces the activation of MAPK (Ge et al., 2003).

Microarray tools have been widely used for comprehensive gene expression analysis, as well as single nucleotide polymorphism detection. Large-scale microarrays can be used to analyze simultaneous changes in thousands of genes and identify significant expression patterns (Sailendra et al., 2006), which can be used to identify functions and pathways affected by exposure to specific compounds, thus providing insight into toxic mechanisms.

Our objective was to elucidate the mechanism of MTX-induced pulmonary inflammation. We hypothesized that MTX promotes the phosphorylation of components of the p38 pathway, possibly TAK1, MKK3/MKK6, p38 MAPK, MAPKAPK2, and HSP27, thereby leading to an increase in IL-8 production. To evaluate this hypothesis, we used DNA microarray analysis to examine MTX-induced gene and protein expression. To identify the cytokines involved in MTX-induced pulmonary inflammation, we used protein arrays and ELISAs to examine the expression of pro- and anti-inflammatory cytokines at the cellular level.

2. Materials and methods

2.1. Cell culture

The human bronchial cell line BEAS-2B was purchased from Korean Cell Line Bank (KCLB, Seoul, Korea) and was maintained under a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The culture medium was 90% Dulbecco's modified Eagle's medium (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (GIBCO BRL), sodium bicarbonate (Sigma, St. Louis, MO, USA), sodium pyruvate (GIBCO BRL), and penicillin and streptomycin (GIBCO BRL). The medium was refreshed every 2–3 days.

2.2. Determination of cell viability

To examine cytotoxicity and effects on cell growth, the MTT cell proliferation assay was performed using the modifications described by Mosmann (1983). MTT [3-(4,5-dimethylthaizol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma] is a tetrazolium salt that is metabolized by living cells to form an insoluble purple salt, which can be quantified spectrophotometrically at 540 nm. In the cytotoxicity assay, BEAS-2B cells were seeded in 24-well cell culture plates (BD Falcon™; Mississauga, Canada) at a density of 3×10^4 cells/ml. After reaching 80% confluence, the cells were exposed to various concentrations of MTX (Sigma) for 48 h. After exposure, the cells were incubated for 3 h with 4 mg/ml MTT in phosphate-buffered saline (PBS) at 37 °C. To quench the reaction, the medium was removed, and dimethyl sulfoxide (DMSO; Sigma) was added. The absorbance of each sample was measured at 540 nm. Untreated samples were used as the negative control (100% viable). The 20% inhibitory concentration (IC_{20}) for cell proliferation was defined as the MTX concentration that reduced cell viability by 20% compared with the untreated control. The IC_{20} values were determined directly from semi-logarithmic dose–response curves. The MTT assay was performed in triplicate for each sample.

2.3. Chemical treatments

BEAS-2B cells were seeded in a 100-mm dish at a density of 1.24×10^6 cells/ml. After incubation for 24 h at 37 °C, the cells were treated with 0.144 μM MTX for 48 h. To examine the effect of MTX on IL-8 production via the p38 MAPK pathway, some cells were also pre-treated with the p38 MAPK inhibitor SB203580 (20 μM; Sigma) for 1 h before MTX treatment.

2.4. RNA extraction

Total RNA was extracted from the MTX-treated BEAS-2B cells using Trizol (Invitrogen, Carlsbad, CA, USA) and purified using an RNeasy mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Genomic DNA was isolated using an RNase-free DNase set (Qiagen) during RNA purification. Total RNA was quantified spectrophotometrically, and RNA quality was verified via agarose-gel electrophoresis.

2.5. Oligonucleotide microarray hybridization

Gene expression analysis was conducted using a 44-k whole human genome microarray (Agilent Technologies, Palo Alto, CA, USA). Labeling and hybridization were performed using a FairPlay microarray labeling kit (Stratagene, Glenville, VA, USA), followed by the coupling of Cy3 (controls) or Cy5 (treated samples) dye. Hybridization was performed in a hybridization oven at 62 °C for 12 h. After a series

of washes (2× SSC/0.1% SDS for 2 min at 58 °C, 1× SSC for 3 min at room temperature, 0.2× SSC for 2 min at room temperature), the slides were dried by centrifugation at 800 rpm for 3 min at room temperature. The hybridized slides were scanned using a GenePix 4000B microarray scanner (Axon Instruments, Union City, CA, USA), and the images were analyzed using GenePix 4.1 software (Axon Instruments) to obtain gene expression ratios. The fluorescence intensity of each spot was calculated by local median background subtraction. We then used the robust scatter-plot smoother LOWESS function to perform intensity-dependent normalization of gene expression. Scatter-plot analysis was performed using Microsoft Excel 2000 (Microsoft Corp., Redmond, WA, USA). A significance analysis of microarray (SAM) was performed for genes with significant changes in expression (Tusher et al., 2001). To determine whether changes in expression were statistically significant, a q-value was calculated for each gene, using the permutation procedure. For each permutation, two-sample t statistics were computed for each gene. Genes were considered to be differentially expressed when the logarithmic gene expression ratios in three independent hybridizations were greater than 0.5 or less than −0.5 (2.0-fold difference in expression level) and when the q-value was <5.

2.6. Quantitative real-time RT-PCR

Messenger RNA expression levels for the genes of interest were analyzed via quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) using a Bio-Rad iCycler system (Bio-Rad, Hercules, CA, USA). Total RNA was reverse-transcribed into cDNA using an Omniscript RT kit (Qiagen). Primer specificity was tested by running a regular PCR for 40 cycles (95 °C for 20 s and 60 °C for 1 min), followed by agarose-gel electrophoresis. Real-time RT-PCR was performed using a SYBR supermix kit (Bio-Rad). Samples were subjected to 45 cycles of 95 °C for 20 s and 60 °C for 1 min. PCR efficiency was determined by running serial dilutions of template cDNA, and melting curve data were collected to assure PCR specificity. Each cDNA sample was analyzed in triplicate, and the corresponding no-RT mRNA sample was included as a negative control. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer was included in every plate as an internal loading control. The mRNA level of each sample for each gene was normalized against that of GAPDH mRNA. The relative mRNA level was determined as $2^{[(Ct/GAPDH - Ct/gene of interest)]}$. All data are presented as the mean ± standard deviation (SD) of three separate experiments. The primers used for the quantitative real-time RT-PCR are listed in Table 1.

2.7. Cell lysates and Western blotting

BEAS-2B cells from each treatment group were washed in PBS and lysed via gentle agitation in ice-cold lysis buffer [20 mM Tris buffer (pH 8.0), 137 mM NaCl, 2 mM EDTA, 10% (v/v) NP-40] containing freshly added protease inhibitor tablets (Roche Applied Science, Mannheim, Germany) and phosphatase inhibitor (Sigma), as reported previously Ohhashi et al., 2001). Lysates were clarified by centrifugation at 12,000 rpm for 10 min at 4 °C. Total protein in the supernatant was quantified using the BCA assay (Pierce, Bonn, Germany), which was performed in triplicate for each sample.

Proteins were separated via SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Bio-Rad). The membranes were

Table 1
Quantitative real-time RT-PCR primers used to validate the differentially expressed genes identified via DNA microarray analysis.

Gene	Accession no.		Primer sequence (5′→3′)
DUSP10	BC031405	F	AAGAGGCTTTTGAGTTCATTGAG
		R	CAAGTAAGCGATGACGATGG
DUSP14	BC000370	F	AATCTCATGCCTTTGGCACCTTGG
		R	ACCTTCCCTAAGCATCTGTCTGGT
IL1A	BC013142	F	AGGCATCCTCCACAATAGCAGACA
		R	TCGGACCAATTACTGGCTCAAGGT
FAS	AB209361	F	AGCTTGGTCTAGAGTGAAAA
		R	GAGGCAGAATCATGAGATAT
DUSP1	AK127679	F	GGCCCCGAGAACAGACAAA
		R	GTGCCCACTTCCATGACCAT
IL1B	BC008678	F	AAGCTGAGGAAGATGCTG
		R	ATCTACACTCTCCAGCTG
MKK6	BC012009	F	AAGCGGATCCGAGCCACAGTAAAT
		R	ACAGTCCACCGTCCTCATGGAAAT
MAPK14	NM_001315	F	GCCGAGCTGTTGACTGGAAG
		R	GGAGGTCCCTGCTTTCAAAGG
MAPKAPK2	BC036060	F	TCATGTACATCCTGCTGTGTGGGT
		R	TCATTCTCTGGGTGGGCCTCTGTTT
HSP27	BM557864	F	GCGTGTCCCTGGATGTCAAC
		R	ATCTCCACCACGCCATCCT
IL8	AI742278	F	GAATGGGTTTGCTAGAATGTGATA
		R	CAGACTAGGGGTTGCCAGATTTAAC
GAPDH	NM_002046	F	TGCACCACCAACTGCTTAGC
		R	GGCATGGACTGTGGTCATGA