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Comparative gene and protein expression analyses of a panel of cytokines in acute and chronic drug-induced liver injury in rats

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ABSTRACT

Drug-induced liver injury (DILI) is a significant safety issue associated with medication use, and is the major cause of failures in drug development and withdrawal in post marketing. Cytokines are signaling molecules produced and secreted by immune cells and play crucial roles in the progression of DILI. Although there are numerous reports of cytokine changes in several DILI models, a comprehensive analysis of cytokine expression changes in rat liver injury induced by various compounds has, to the best of our knowledge, not been performed. In the past several years, we have built a public, free, large-scale toxicogenomics database, called Open TG-GATEs, containing microarray data and toxicity data of the liver of rats treated with various hepatotoxic compounds. In this study, we measured the protein expression levels of a panel of 24 cytokines in frozen liver of rats treated with a total of 20 compounds, obtained in the original study that formed the basis of the Open TG-GATEs database and analyzed protein expression profiles combined with mRNA expression profiles to investigate the correlation between mRNA and protein expression levels. As a result, we demonstrated significant correlations between mRNA and protein expression changes for interleukin (IL)-1β, IL-1α, monocyte chemo-attractant protein (MCP)-1/*CC*-chemokine ligand *(Ccl)2*, vascular endothelial growth factor A (VEGF-A), and regulated upon activation normal T cell expressed and secreted (RANTES)/*Ccl5* in several different types of DILI. We also demonstrated that IL-1β protein and MCP-1/*Ccl2* mRNA were commonly up-regulated in the liver of rats treated with different classes of hepatotoxicants and exhibited the highest accuracy in the detection of hepatotoxicity. The results also demonstrate that hepatic mRNA changes do not always correlate with protein changes of cytokines in the liver. This is the first study to provide a comprehensive analysis of mRNA–protein correlations of factors involved in various types of DILI, as well as additional insights into the importance of understanding complex cytokine expression changes in assessing DILI.

Abbreviations: AAF, 2-acetamidofluorene; ANIT, α-naphthylisothiocyanate; AUC, area under the curve; CCL, CC-chemokine ligand; CCR2, CC chemokine receptor type 2; CSA, cyclosporin A; CSP, cisplatin; CXCL, CXC chemokine ligand; DEN, *N*-nitrosodiethylamine; DFNa, diclofenac sodium salt; DILI, drug-induced liver injury; DTL, dantrolene; EBU, ethambutol dihydrochloride; EE, ethynyl estradiol; ETH, ethionamide; FT, flutamide; GFZ, gemfibrozil; GMC, gentamicin sulphate; Gro-α, growth regulated oncogene alpha; HE, hematoxylin and eosin; HMGB1, high-mobility group protein B1; HSPs, heat shock proteins; ICAM-1, intercellular adhesion molecule-1; IFN-γ, interferon gamma; IL, interleukin; IM, indomethacin; IP-10, interferon gamma-induced protein 10; KC, keratinocyte chemo-attractant; MAS5, Microarray Analysis Suite 5.0; MCP, monocyte chemo-attractant protein; MCT, monocrotaline; MP, methapyrilene hydrochloride; NK cells, natural killer cells; PB, phenobarbital; RANTES, regulated upon activation normal T cell expressed and secreted; RIF, rifampicin; ROC, receiver operating characteristic; TAA, thioacetamide; TG-GATEs, Toxicogenomics Project-Genomics Assisted Toxicity Evaluation System developed by the TGP in Japan; TGP, Toxicogenomics Project; TGP2, Toxicogenomics Informatics Project in Japan; TLR, Toll-like receptor; TNF-α, tumor necrosis factor alpha; TNFSF11, tumor necrosis factor (ligand) superfamily, member 11; VA, vitamin A; VCAM-1, vascular cell adhesion molecule-1; VEGF-A, vascular endothelial growth factor A.

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1. Introduction

In drug development, drug dropouts or withdrawal from the market is a critical issue for pharmaceutical companies. It was reported that 56 drugs acquired a new black box warning or were withdrawn among 548 drugs approved in 1975–1999 (Lasser et al., 2002). According to Stevens and Baker (2009); 47 drugs were withdrawn from the market between 1975 and 2007. Among them, 15 were terminated due to hepatotoxicity (Stevens and Baker, 2009). It was also reported that drug-induced liver injury (DILI) accounts for up to 50% of acute liver failure cases (Lee et al., 2011). DILI is therefore the major causes for the failure in drug development and withdrawal in post marketing. It underscores the importance of toxicological property of putative drug candidates early in the discovery process. Thus, the tools to effectively detect potential hepatotoxicity in animals are helpful for novel drug discovery.

Cytokines are a category of signaling molecules produced by immune cells, such as T cells, dendritic cells and Kupffer cells/macrophages, which regulate immune responses, inflammatory responses, cell proliferation and differentiation, and cell death. Cytokines play crucial roles in the progression of DILI (Amanzada et al., 2014; Blazka et al., 1995). In acute liver injury, Kupffer cells/macrophages are known to secrete pro-inflammatory cytokines in response to RNA, DNA, C5a, high-mobility group protein B1 (HMGB-1) and heat shock proteins (HSP) released from necrotic hepatocytes (Zimmermann et al., 2012). Several key pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6, tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), monocyte chemo-attractant protein (MCP)-1/CC chemokine ligand (CCL) 2 and CXC chemokines, activate inflammatory responses and downstream signaling, via the induction of sinusoidal intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), resulting in the recruitment of neutrophils, monocytes, T cells and natural killer cells (NK cells) to the injured liver (Zimmermann et al., 2012). Thus, to fully understand the cytokine cascade involved in several types of DILI, comprehensive analyses of cytokines need to be performed. Although there are numerous reports of cytokine changes in several DILI models, a comprehensive analysis of cytokine expression changes in rat liver injury induced by various compounds has, to the best of our knowledge, not been performed.

With the availability of advanced technologies enabling high-throughput comprehensive measurement, recent research strategies for biomarker discovery and mechanistic analysis have been adapted to systems toxicology—the combination of traditional toxicology methods with new tools for integrating high-throughput transcriptomic, proteomic, and metabolomic data. In recent years, the field of toxicogenomics has been advancing, and some progress in safety and risk assessment of candidate pharmaceutical and chemical compounds has been made using this approach (Afshari et al., 2011; Cui and Paules, 2010; Guengerich, 2011). Owing to Japanese collaboration efforts in the Toxicogenomics Project (TGP), we have built a large-scale toxicogenomics database called Open TG-GATEs (Toxicogenomics Project-Genomics Assisted Toxicity Evaluation System developed by the TGP in Japan; http://toxico.nibio.go.jp/english/) (Uehara et al., 2010). In this project, we tested 170 compounds, mainly medicinal drugs. Male Sprague Dawley rats were used for the *in vivo* experiments, which involved both single (3, 6, 9, and 24 h)- and repeated-dose (4, 8, 15, and 29 day) experimental designs at three dose levels. As part of the collaborative efforts of the Toxicogenomics Informatics Project in Japan (TGP2), we comprehensively measured cytokine protein levels in frozen rat liver, which had been obtained for the original TGP study. For cytokine protein measurements, a multiplex immunoassay that utilizes distinctly colored bead sets created by fluorescent dyes at distinct ratios and coated with antibodies against multiple analytes (Borg et al., 2011; Kulp et al., 2012; Paquet et al., 2012) was used. In the present study, we hypothesized that we can define liver injury more precisely by the measurement of protein levels of cytokines in addition to mRNAs. Firstly, we analyzed protein expression profiles of a panel of cytokines in the rat livers combined with mRNA expression profiles to investigate the correlation between mRNA and protein expression levels for each cytokine. Then, we comprehensively examined changes in cytokine expression involved in various types of DILI using hierarchical clustering analysis based on mRNA and protein expression profiles. Finally, we assessed the usefulness of each cytokine mRNA and protein as a biomarker to detect liver injury. This study is the first to provide a comprehensive analysis of the mRNA–protein correlation of cytokines involved in various types of DILI and additional insights into the importance of understanding complex cytokine expression changes in assessing DILI.

2. Materials & methods

2.1. Chemicals

Among a total of 170 compounds in the Open TG-GATEs database, 20 of the compounds used in this study are listed in Table 1 with their doses, administration routes and vehicles. In this database, information about various different types of hepatotoxicants are available, and their mechanisms involved in the hepatotoxicity are varied. Since molecular mechanisms involved in the regulation of cytokine production can be considered varied, we have selected various different types of hepatotoxic and some additional non-hepatotoxic compounds based on their known mechanisms of actions, histopathology, and chemical classes. Histopathological findings in the liver of each treated group are also summarized in Table 1. Based on their liver histopathological profiles, the compounds analyzed were largely divided into the following two classes: hepatotoxic (10 compounds: 2-acetamidofluorene (AAF), α-naphthylisothiocyanate (ANIT), *N*-nitrosodiethylamine (DEN), ethambutol dihydrochloride (EBU), ethynyl estradiol (EE), ethionamide (ETH), indomethacin (IM), monocrotaline (MCT), methapyrilene hydrochloride (MP) and thioacetamide (TAA)) or non-hepatotoxic (10 compounds: cyclosporine A (CSA), cisplatin (CSP), diclofenac sodium salt (DFNa), dantrolene (DTL), flutamide (FT), gemfibrozil (GFZ), gentamicin sulphate (GMC), phenobarbital (PB), rifampicin (RIF) and vitamin A (VA)).

2.2. Animals and experimental design

Gene expression data and histopathological data obtained from the Open TG-GATEs database were used for this study. For cytokine profiling, multiplex immunoassays were performed with frozen liver samples stored in the TGP. Briefly, 6-week-old male Sprague Dawley rats (Charles River Japan Inc., Kanagawa, Japan; five animals per group) were used in the study. Rats received single (3, 6, 9, and 24 h) or repeated (4, 8, 15, and 29 day) doses. For cytokine profiling, we used samples collected at 6 and 24 h for the single dose study. For the repeated dose study, time-dependent cytokine changes were investigated at early and late time points. We selected samples collected at 4 day and one additional later time point where no animal deaths occurred: 8 day (DEN), 15 day (ETH, EBU, MCT, IM, CSP and VA), and 29 day (remaining compounds). The animals were euthanized by exsanguination from the abdominal aorta under ether anesthesia after blood sampling, and liver samples were obtained from the left lateral lobe immediately after the euthanization. For examination with light microscopy, liver samples were fixed in 10% neutral buffered