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A toxicogenomic approach for identifying biomarkers for myelosuppressive anemia in rats☆

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ABSTRACT

Myelosuppressive anemia is a serious side effect associated with several drugs. Thus, there is an increasing demand for sensitive biomarkers for the early detection of myelosuppressive anemia during toxicological studies. We applied a toxicogenomic approach to identify useful biomarker genes reflecting myelosuppressive anemia in the rat liver. Expression of the hemoglobin beta chain complex (*Hbb*), aminolevulinic acid synthase 2 (*Alas2*), and cell division cycle 25 homolog B (*Cdc25b*) genes changed as a result of anemia induced by the myelosuppressive agents linezolid, cisplatin, and carboplatin, suggesting that these genes may be suitable biomarkers. Moreover, evaluation of perfused and unperfused livers indicated that changes in the expression of these genes originate in circulating reticulocytes in the liver. Erythroid differentiation-associated changes in expression of the *Hbb*, *Alas2*, and *Cdc25b* genes were confirmed in vitro using Friend leukemia cells. In conclusion, our current research provides novel evidence that gene expression in circulating reticulocytes contained in the liver changes dramatically under myelosuppressive conditions. While further large-scale validation studies are needed, our results indicate that the genes we identified might be useful biomarkers for the sensitive detection of myelosuppressive anemia in rats.

1. Introduction

Myelosuppression is a common side effect of many chemotherapeutic agents as well as several antibacterial agents, and typically is the dose limiting factor (Wang et al., 2006). The clinical manifestations of myelosuppression include anemia, thrombocytopenia, and leucopenia. In severe cases of myelosuppression, complications may lead to death due to leucopenia-related infections and bleeding resulting from thrombocytopenia. Thus, the potential risks associated with myelosuppression must be considered during the nonclinical studies conducted prior to human clinical trials of new drugs. While there are various clinical symptoms of myelosuppression, the research described here focused on anemia caused by treatment with myelosuppressive compounds.

In nonclinical toxicity studies, myelosuppressive anemia is usually diagnosed by counting the number of red blood cells (RBCs) and reticulocytes (Retics), and measuring the concentrations of hemoglobin (HGB) and hematocrit (HCT). More detailed diagnosis may involve bone marrow examination. These clinical tests are useful for detecting clear evidence of myelotoxicity and anemia; however, they are of limited usefulness for the early detection of toxicity risks in single or short-term repeated dose toxicity studies. Therefore, it is generally hard to detect myelotoxic potential of novel drug candidates in the early stage of drug development. A widespread demand thus exists within the pharmaceutical industry for effective biomarkers that will enable early detection of myelosuppressive anemia throughout the drug discovery process, from the early phase of drug development to clinical trials. Furthermore, the application of translational biomarkers for monitoring prognostic changes associated with myelosuppressive anemia would facilitate safer clinical trials.

Toxicogenomics is considered one of the most powerful strategies for identifying genomic biomarkers predictive of drug-induced toxicity (Goodsaid, 2004; Merrick and Bruno, 2004; Tugwood et al., 2003; Uehara et al., 2010). Toxicogenomic approaches have been

Abbreviations: *Hbb*, hemoglobin beta chain complex; *Alas2*, aminolevulinic acid synthase 2; *Cdc25b*, cell division cycle 25 homolog B; RBCs, red blood cells; Retics, reticulocytes; HGB, hemoglobin; HCT, hematocrit; LZD, linezolid; HP-β-CD, hydroxypropyl-β-cyclodextrin; CDDP, cisplatin; CBDCA, carboplatin; NBMCs, nucleated bone marrow cells; H&E, hematoxylin and eosin; MAS, Microarray Analysis Suite; ANOVA, one-way analysis of variance; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase.

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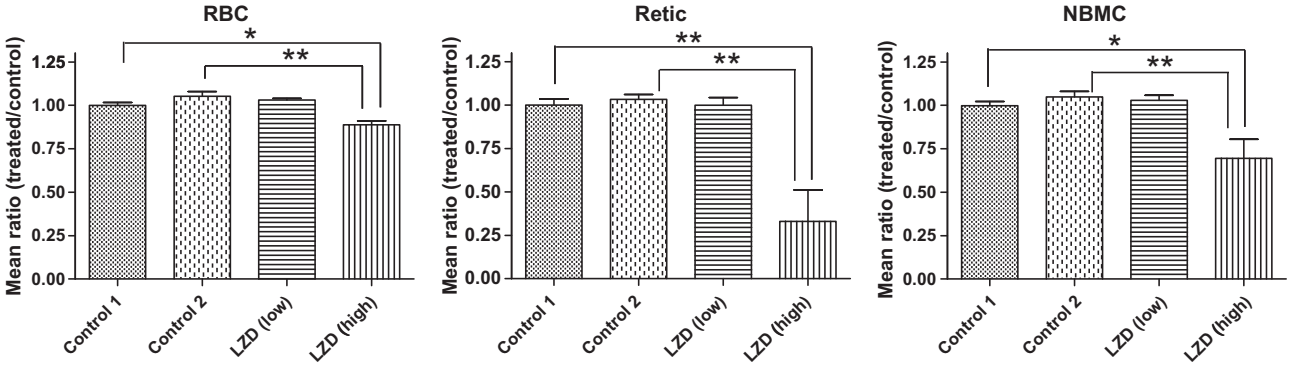

Fig. 1. Red blood cell (RBC) count, reticulocyte (Retic) count, and the number of nucleated bone marrow cells (NBMC) after a 2-week treatment with a low-dose (50 mg/kg, BID) or high-dose (150 mg/kg, BID) of LZD (Exp. 1). Control 1 and Control 2 groups were treated with vehicle alone (5% glucose solution and 5% hydroxypropyl-β-cyclodextrin in 5% glucose solution, respectively). Significant differences between groups were evaluated by Tukey's multiple comparison test (*$P < 0.05$; **$P < 0.01$). Data are expressed as mean ratio to the control group (5% glucose solution) ± S.E. ($n = 4$/group).

employed for the identification of genomic biomarkers for hepatotoxicity (Harrill et al., 2009; Kiyosawa et al., 2009; Uehara et al., 2008, 2010), nephrotoxicity (Kondo et al., 2009; Uehara et al., 2007a), and cardiac toxicity (Mori et al., 2010). In addition, Rokushima et al. (2007) identified biomarker genes related to drug-induced hemolytic anemia in rat liver. Their work focused on gene expression changes in the liver after treatment with various drugs that induce hemolytic anemia, and they successfully selected commonly regulated genes in the liver. However, little is known about gene expression profiles related to myelosuppressive anemia in the liver.

We hypothesized that comprehensive gene expression profiling might lead to the discovery of useful biomarker genes associated with myelosuppressive anemia in rats. Due to the abundance of rat microarray data, we selected the liver as a target organ for microarray analysis. Here, we report on the successful selection of three candidate biomarker genes that are differentially expressed in rat liver during anemia induced by several myelosuppressive agents: hemoglobin beta chain complex (*Hbb*), aminolevulinic acid synthase 2 (*Alas2*), and cell division cycle 25 homolog B (*Cdc25b*). Expression profiles of these selected genes were further confirmed using real time RT-PCR. We also compared the expression levels of these genes in the liver with or without perfusion of blood in order to clarify the origin of the expression changes. Finally, by evaluating gene expression changes throughout the erythroid differentiation process in Friend leukemia cells, we confirmed that changes in expression of *Hbb*, *Alas2*, and *Cdc25b* originate in circulating precursors of red blood cells (reticulocytes) in the liver. Our results provide novel evidence that gene expression in circulating precursors of red blood cells contained in the liver changes dramatically under myelosuppressive conditions. Moreover, while further large-scale validation studies are necessary, the genes we identified might be sensitive biomarkers for detecting myelosuppressive anemia in rats.

2. Materials and methods

2.1. Chemicals

Linezolid (LZD) powder was purified from Zybox® (Pfizer, New York, NY, USA) in our institute. Hydroxypropyl-β-cyclodextrin (HP-β-CD, Tokyo Chemical Industry, Tokyo, Japan) was used as the solubilizing agent for preparing the LZD dosing solution. HP-β-CD was dissolved in 5% glucose solution (Otsuka Pharmaceutical Factory, Tokushima, Japan) at a concentration of 5% and used as the vehicle for the LZD dosing solution. Cisplatin (CDDP) and carboplatin (CBDCA) were purchased from Sequoia Research Products (Pangbourne, UK) and Kemprotec (Middlesbrough, UK), respectively. These compounds were dissolved in 5% glucose solution before use (Otsuka Pharmaceutical Factory). All other chemicals used in this study were of the highest purity available.

2.2. Animals and experimental conditions

Male Crl:CD (SD) rats were purchased from Charles River (Kanagawa, Japan). The animals were housed under a 12-h light/dark cycle (lights on 8 AM to 8 PM). All animals were allowed free access to food (autoclaved CRF-1, CLEA Japan Inc., Tokyo, Japan) and water (sterilized city water supplied via an automatic water supply system). Test compounds were administered when animals were 6–9 weeks old. In experiment (Exp.) 1, rats ($n = 4$/group) were intravenously treated with LZD (50 or 150 mg/kg) or vehicle alone (5% glucose solution or 5% HP-β-CD in 5% glucose solution) at the rate of 5 ml/kg/min (15 ml/kg) twice a day (BID) for 2 weeks. Rats were sacrificed the day after the last dosing. In Exp. 2, rats ($n = 3$/group) were intravenously injected with a single bolus dose of CDDP (4 mg/kg), CBDCA (32 mg/kg), or vehicle alone (5% glucose solution) at a dosing volume of 10 ml/kg. All animals were sacrificed 24 h or 4 days after a single treatment. In Exp. 3, rats ($n = 5$–6/group) were treated with a single dose of CDDP (4 mg/kg) or vehicle alone and sacrificed 24 h after dosing. Before necropsy, circulating blood involved in the liver was removed from half the animals in each group by perfusion with RNAlater® (Ambion, Austin, TX, USA). Pentobarbital sodium was used for anesthesia prior to necropsy in all in vivo experiments. All experimental procedures were conducted only after approval from the Institutional Animal Care and Use Committee of Shionogi Research Laboratories.

2.3. Hematology

In Exps. 1 and 2, blood samples were collected from the vena cava while the animals were under anesthesia before being sacrificed. Blood was then placed into two vacuum blood-collecting tubes containing 1 ml of EDTA-2K (Terumo Corporation, Tokyo, Japan). The following blood parameters were examined using an ADVIA 120 Hematology System (Siemens, New York, NY, USA): RBCs, Retics, HGB, and HCT.

2.4. Bone marrow analysis

In Exps. 1 and 2, the number of nucleated bone marrow cells (NBMCs) in the femur (left) was determined as described previously (Uehara et al., 2007b). Briefly, after the femur was removed and freed of soft tissue attachments, the epiphyseal cap was detached from the proximal end and the extreme distal tip of each epicondyle was cut-off. A suspension of bone marrow cells was prepared by injecting 1.5% acetic acid solution into the femur to lyse erythrocytes and flush out the cells. Nucleated cells contained in the suspension were counted using an automatic blood cell analyzer (T540, Beckman Coulter Inc., Fullerton, CA, USA), and the number of NBMCs per unit weight of femur bone marrow ($\times 10^6$/mg) was calculated.

2.5. Histopathological examination

In Exp. 1, liver tissues were stored in 10% neutral buffered formalin, dehydrated in alcohol, and then embedded in paraffin for light microscopy. Samples of right femur were decalcified in a mixture of 10% formic acid and formalin before histological specimens were prepared. Paraffin sections were stained with hematoxylin and eosin (H&E) using a routine method. Histopathological changes were graded by a pathologist according to the following criteria: ± = very slight; + = slight; 2+ = moderate; 3+ = severe. All specimens were re-examined by another pathologist for confirmation of results.

2.6. RNA extraction

In all in vivo experiments, the liver (left lateral) was quickly removed following exsanguination and stored in RNAlater® at −80 °C until further processed. Liver samples were then thawed and homogenized in QIAzol Lysis Reagent (Qiagen,