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Activation of Nrf2-ARE signaling mitigates cyclophosphamide-induced myelosuppression

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HIGHLIGHTS

- Nrf2 deficiency deteriorated CTX-induced myelosuppression *in vitro*/*in vivo*.
- Nrf2 deficiency decreased antioxidant capacity of peripheral blood and bone marrow.
- Nrf2 deficiency impaired hematopoietic proliferating and colony formation ability.
- CDDO-Me Nrf2-dependently mitigated CTX-induced myelosuppression.

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ABSTRACT

Myelosuppression is the most common dose-limiting adverse effect of chemotherapies. In the present study, we investigated the involvement of nuclear erythroid 2-related factor 2 (Nrf2) in cyclophosphamide-induced myelosuppression in mice, and evaluated the potential of activating Nrf2 signaling as a preventive strategy. The whole blood from $Nrf2^{-/-}$ mice exhibited decreased antioxidant capacities, while the bone marrow cells, peripheral blood mononuclear cells and granulocytes from $Nrf2^{-/-}$ mice were more susceptible to acrolein-induced cytotoxicity than those from wild type mice. Single dosage of cyclophosphamide induced significantly severer acute myelosuppression in $Nrf2^{-/-}$ mice than in wild type mice. Furthermore, $Nrf2^{-/-}$ mice exhibited greater loss of peripheral blood nucleated cells and recovered slower from myelosuppression nadir upon multiple consecutive dosages of cyclophosphamide than wild type mice did. This was accompanied with decreased antioxidant and detoxifying gene expressions and impaired colony formation ability of $Nrf2^{-/-}$ bone marrow cells. More importantly, activation of Nrf2 signaling by CDDO-Me significantly alleviated cyclophosphamide-induced myelosuppression, while this alleviation was diminished in $Nrf2^{-/-}$ mice. In conclusion, the present study shows that Nrf2 plays a protective role in cyclophosphamide-induced myelosuppression and activation of Nrf2 is a promising strategy to prevent or treat chemotherapy-induced myelosuppression.

1. Introduction

Myelosuppression is the most common cause for chemotherapy dose reduction and treatment interval increase or even the termination of medication, and is potentially life threatening due to complications of neutropenia, anemia and thrombocytopenia (Carey, 2003). Hematopoietic growth factor such as granulocyte colony-stimulating factor (G-CSF) is the first and almost only choice for treatment of myelosuppression since more than 20 years ago (Miller and Steinbach, 2014). However, prophylactic

Abbreviations: Nrf2, nuclear factor erythroid 2-related factor 2; G-CSF, granulocyte colony-stimulating factor; HSCs, hematopoietic stem cells; HPCs, hematopoietic progenitor cells; 3LL, Lewis lung carcinoma; RBC, red blood cell; DPPH, 2, 2-diphenyl-1-picryl-hydrazyl; TOSC, total oxidant-scavenging capacities; TAOC, total anti-oxidant capacity; LDCL, luminol-dependent chemiluminescence; PBMC, peripheral blood mononuclear cells; SCF, stem cell factor; CDDO-Me, 2-cyano-3 12-dioxooleane-1, 9(11)-dien-28-oic acid methyl ester.

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application of G-CSF may have deleterious effects on the self-renewal ability of hematopoietic stem/progenitor cells (van Os et al., 2000). Furthermore, G-CSF could exacerbate long-term myelosuppression (Skedgel et al., 2016; Weycker et al., 2016). To the date, myelosuppression is still a serious concern of cytotoxic chemotherapies, and investigations on effective approaches to predict or prevent chemotherapy-induced myelosuppression are of great significance.

Chemotherapy-induced myelosuppression can be caused through various proposed mechanisms, in which oxidative or electrophilic stress has been consistently implied to play important roles (Grek et al., 2011). Nuclear erythroid 2-related factor 2 (Nrf2) is a pivotal transcription factor regulating many cellular processes including redox homeostasis, drug metabolism, and responses to oxidative and electrophilic stress (Hayes and Dinkova-Kostova, 2014). Nrf2 intrinsically regulates the survival, self-renewal, homing and differentiation of hematopoietic stem cells (HSCs) (Merchant et al., 2011; Tsai et al., 2013; Murakami et al., 2014). Moreover, pharmacological or genetic activation of Nrf2 mitigates ionizing radiation-induced myelosuppression and promotes hematopoietic reconstitution following bone marrow transplantation (Kim et al., 2014; Chute, 2014) A study on 5-Fluorouracil-mediated myelosuppression suggested loss of Nrf2 intensified 5-Fluorouracil-mediated decrease in the myeloid colony formation partially via down-regulating antioxidant proteins (Numazawa et al., 2011). In addition, several studies suggested the involvement of Nrf2 in chemotherapy-induced toxicities such as hepatotoxicity (Mahmoud and Al Dera, 2015), cardiotoxicity (Li et al., 2014), testicular damage (Maremanda et al., 2014), nephrotoxicity (Aleksunes et al., 2010), *etc*. However, the role of Nrf2 in cytotoxic chemotherapy-induced myelosuppression has not been clearly clarified, and it is largely unknown whether activation of Nrf2 can protect against chemotherapy-induced myelosuppression.

Cyclophosphamide is one of the most frequently used alkylating cytotoxic chemotherapeutic agents, and myelosuppression is its dose-limiting adverse effect. (Salem et al., 2012). Oxidative stress is a major cause of cyclophosphamide-induced side effects, and antioxidants may ameliorate toxicities induced by cyclophosphamide (Patra et al., 2012; Alberts, 1999; Diaz-Montero et al., 2012). Acrolein, one major toxic metabolite of cyclophosphamide, is highly electrophilic and readily reacts with many biomolecules including cysteine residues (Li et al., 2010; Stevens and Maier, 2008). Acrolein has been reported to activate Nrf2 signaling, and activation of Nrf2 could protect against cyclophosphamide- or acrolein-induced toxicity (Mahmoud and Al Dera, 2015; Maremanda et al., 2014; Tripathi and Jena, 2010; Tirumalai et al., 2002; Sthijns et al., 2014). In the present study, cyclophosphamide was employed as a typical myelosuppressive chemotherapeutic agent. The involvement of Nrf2 in chemotherapy-induced myelosuppression and the potential cellular and molecular mechanisms were investigated by using Nrf2 deficient mice and a classic Nrf2 activator, 2-cyano-3, 12-dioxooleane-1, 9(11)-dien-28-oic acid methyl ester (CDDO-Me).

2. Materials and methods

2.1. Animals and treatments

Male C57BL/6J mice (Department of Laboratory Animal Science, Peking University Health Science Center, Beijing) and Nrf2 deficient (Nrf2-/-) mice of C57BL/6J background (kindly provided by Drs. J. D. Hayes at University of Dundee, Dundee, UK and M. Yamamoto at Tohoku University, Sendai, Miyagi, Japan) aging 6–8 weeks were housed on a 12 h light/dark cycle under controlled temperature and humidity, with access to standard diet and water ad libitum. All experimental protocols have been approved by Peking University Institutional Animal Care and Use Committee (IACUC).

Cyclophosphamide (Hengrui Medicine Co., Ltd., Jiangsu, China) was freshly dissolved in saline (0.9% NaCl) and administrated to mice intraperitoneally. CDDO-Me (Cayman Chemical, Ann Arbor, Michigan, USA) was freshly dissolved in phosphate-buffered saline (PBS) containing 10% DMSO and 10% Cremophor-EL (Aladdin Bio-Chem Technology Co., Ltd., Shanghai, China), and administrated intraperitoneally. Control groups received vehicles only.

For tumor-bearing mice model, mice were inoculated with 2×10^6 Lewis lung carcinoma cells (3LL, obtained from ATCC, Rockville, MD, USA) at axilla per mouse. When the tumor volume reached 200 mm^3, the mice were treated and sacrificed as indicated.

2.2. Measurement of reduced glutathione (GSH)

The reduced GSH levels in whole blood and plasma were measured using a kit following manufacturer's instructions (Beyotime Institute of Biotechnology, Jiangsu, China) according to Beutler's improved method with minor modifications (Beutler et al., 1963). Briefly, proteins were removed by using protein removal agent M. Reduced GSH was quantified by measuring the absorbance of 5-thio-2-nitrobenzoic acid (TNB) at 412 nm, which was produced from the reaction of reduced GSH with 5,5-dithiobis (2-nitrobenzoic) acid (DTNB). The protein content was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). Data were normalized as microgram of GSH per milligram of protein.

2.3. DPPH scavenging assay

2, 2-diphenyl-1-picryl-hydrazyl (DPPH), a purple-colored stable free radical, is reduced to the yellow-colored diphenylpicryl hydrazine upon antioxidant treatment. This assay is used to quantify the total oxidant-scavenging capacities of whole blood and plasma. Briefly, whole blood or plasma was diluted 5 fold in saline. 50 μL of diluted sample or saline was added to equal volume of 1 mmol/L solution of DPPH (Sigma-Aldrich, St Louis, MO, USA) in methanol. Two minutes later, 800 μL of methanol was added. The samples were vortexed and incubated for 5 min. The absorbance was read at 517 nm by a micro plate reader (Tecan Infinite M200 pro, Tecan Group Ltd., Switzerland). The DPPH scavenging capacity was calculated as follows: DPPH Scavenging Capacity = (Absorbance of Control − Absorbance of Sample)/protein content (mg).

2.4. Luminol-dependent chemiluminescence (LDCL) assay

The total antioxidant capacities of whole blood and plasma were further evaluated by a luminol-dependent chemiluminescence assay (Ginsburg et al., 2011) with minor modifications. This assay is based on the ability of antioxidant agents to quench the luminescence generated by a mixture of horseradish peroxidase (HRP) and BeyoECL reagents (BeyoECL Plus, Beyotime Institute of Biotechnology, Jiangsu, China) in a dose-dependent manner. BeyoECL reagents are constituted of agent A (containing luminol) and agent B (containing oxidants). Briefly, 1 μL of 5×10^5 U/μL HRP, 2 μL of whole blood or plasma and 5 μL of agent A were added to the plate well. 5 μL of agent B and 90 μL of Milli-Q water were added automatically. LDCL was measured by a micro plate reader (FlexStation3, USA).

2.5. Preparation of bone marrow cells and peripheral blood nucleated cells

Mice were sacrificed by cervical dislocation. The femurs and tibias were immediately harvested, and washed with sterile PBS.