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Research Article

Protective effect of wild ginseng cambial meristematic cells on D-galactosamine-induced hepatotoxicity in rats

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ABSTRACT

Background: *Panax ginseng* has a wide range of biological activities including anti-inflammatory, antioxidant, and immunomodulatory functions. Wild ginseng cambial meristematic cells (CMCs) were obtained from *P. ginseng* cambium. This study examined the protective mechanism of wild ginseng CMCs against D-galactosamine (GalN)-induced liver injury. GalN, a well-known hepatotoxicant, causes severe hepatocellular inflammatory damage and clinical features similar to those of human viral hepatitis in experimental animals.

Methods: Hepatotoxicity was induced in rats using GalN (700 mg/kg, i.p.). Wild ginseng CMCs was administered orally once a day for 2 wks, and then 2 h prior to and 6 h after GalN injection.

Results: Wild ginseng CMCs attenuated the increase in serum aminotransferase activity that occurs 24 h after GalN injection. Wild ginseng CMCs also attenuated the GalN-induced increase in serum tumor necrosis factor-α, interleukin-6 level, and hepatic cyclooxygenase-2 protein and mRNA expression. Wild ginseng CMCs augmented the increase in serum interleukin -10 and hepatic heme oxygenase-1 protein and mRNA expression that was induced by GalN, inhibited the increase in the nuclear level of nuclear factor-kappa B, and enhanced the increase in NF-E2-related factor 2.

Conclusion: Our findings suggest that wild ginseng CMCs protects liver against GalN-induced inflammation by suppressing proinflammatory mediators and enhancing production of anti-inflammatory mediators.

1. Introduction

Hepatitis is a serious clinical problem caused by exposure to different agents such as viruses, alcohol, and chemicals, and by autoimmune diseases. Hepatitis may occur with limited or no symptoms and often leads to anorexia, jaundice, and hepatic carcinoma [1]. Although extensive studies into the treatment of liver disease with several oral hepatoprotective agents have been carried out, few beneficial liver drugs are currently available in the clinic. D-Galactosamine (GalN) is a specific hepatotoxicant that depletes the uridine triphosphate pool and thereby inhibits macromolecule synthesis, inducing hepatotoxicity that resembles that of human viral and drug-induced hepatitis [2]. In GalN-induced acute liver injury, overproduction of reactive oxygen species from hepatocytes, infiltrated leukocytes, and activated Kupffer cells, accompanied by enhanced activity of the proinflammatory cytokine signaling pathway, contributes to liver damage [3]. Heme oxygenase (HO)-1 is an endogenous cytoprotective enzyme induced in response to cellular and environmental stresses. The critical role of HO-1 in the protection against *in vivo* and *in vitro* inflammatory disease models has been reported, and upregulation of HO-1 exerts an anti-inflammatory effect in experimental settings of hepatic ischemia/reperfusion and acute hepatitis [4].

Panax ginseng Meyer (Araliaceae) is a medicinal herb that has been used worldwide for > 5,000 yrs. *P. ginseng* has been widely used for treatment of inflammation, cardiovascular diseases,

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trauma, and bleeding caused by injury. Several investigators have reported the biologic role of *P. ginseng* in experimental models of liver diseases, including fatty liver disease, liver fibrosis, hepatic carcinoma, and chemical-induced liver injury [5]. Its biologic activities are primarily attributable to ginsenosides, which produce an array of pharmacologic responses [6]. Ginsenosides are located in the root, leaf, seed, and flower of *P. ginseng*, and have antitumor and immunomodulatory activity in various organs [7]. However, the yield of active components from natural sources can be highly variable depending on the source plant, location, season of harvest, and the prevailing environmental conditions [8]. In addition, a dramatic depletion of the plant population has occurred as a result of its consumption for medicinal purposes. The isolation of cambial meristematic cells (CMCs) from selected P. *ginseng* species may ensure a stable ginsenoside content and provide a sustainable supply for therapeutic applications [9]. However, there are no reports that demonstrate the biologic activity of natural compounds isolated from wild ginseng CMCs.

The objective of this study is to investigate the cytoprotective effects of wild ginseng CMCs against GalN-induced acute liver injury, particularly the modulation of inflammatory responses.

2. Materials and methods

2.1. Materials/chemicals

The followings are the materials and chemicals we used in this study: a 3-L bioreactor and a 20-L bioreactor (Samsung Science, Seoul, Korea), a 250-L bioreactor (Fermentec Co. Ltd, Cheongwon, Korea), 0.05% trifluoroacetic acid (Daejung Chemicals & Metals, Siheung, Korea), ginsenoside 20(*S*)-Rg3 and ginsenoside 20(S)-Rh2 (Chromadex, Irvine, CA, USA), mixture of ginsenoside Rk1 and Rg5 (Ambo Institute, Seoul, Korea), acetonitrile (Merck, Darmstadt, Germany), methanol (J.T. Baker, Center Valley, PA, USA), GalN and silymarin (Sigma-Aldrich, St. Louis, MO, USA), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) assay kits (IVDLab Co., Uiwang, Korea), tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-10 enzyme-linked immunosorbent assay kits (BD Science, San Diego, CA, USA), PRO-PREP and enhanced chemiluminescence detection system (iNtRON Biotechnology, Seongnam, Korea), NE-PER and BCA Protein Assay Kits (Thermo Fisher Scientific Inc., Rockford, IL, USA), polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA), Semi-Dry Trans-Blot Cell (Bio-Rad Laboratories, Hercules, CA, USA), RNAiso Plus and EcoDry cDNA Synthesis Premix (Takara Bio Inc., Shiga, Japan), Thermocycler and SYBR Green detection System (Roche Applied Science, Mannheim, Germany). The following antibodies were used in this study: cyclooxygenase-2 (COX-2; Cayman Chemicals, Ann Arbor, MI, USA), HO-1 (Enzo Life Sciences, Farmingdale, NY, USA), kelch-like ECH-associated protein 1 (Keap1) and lamin b1 (Abcam, Cambridge, MA, USA), nuclear factor-kappa B (NF-κB)/p65, p-cJun and NF-E2-related factor 2 (Nrf2; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β-actin (Sigma-Aldrich).

2.2. Plant cell culture

Wild ginseng CMCs were obtained from the cambium of *P. ginseng* Meyer (wild ginseng; native to Kangwon province, Korea, for $>$ 50 yrs; appraised by the Korea Association of Wild Ginseng Appraiser) [10]. Wild ginseng CMCs were gradually scaled up from 250-mL flasks to a 3-L bioreactor, a 20-L bioreactor, and a 250-L bioreactor. The bioreactors were all the airlift type. The working volume of the 250-L bioreactor was 210 L, 84% of the total volume. Wild ginseng CMCs were cultured in two stages: (1) a proliferation stage to obtain biomass in the 250-L bioreactor and (2) a production stage to obtain secondary metabolites such as ginsenosides. In general, plant growth regulators, such as 2,4-dichlorophenoxyacetic acid or naphthaleneacetic acid, are used to induce cell division. However, to obtain biomass under the proliferation culture condition without using growth regulators, wild ginseng CMCs underwent a cell habituation process for 1 yr without growth regulators [9]. Wild ginseng CMCs obtained through this process were maintained for 37 mo. Culture conditions for the proliferation stage to obtain biomass of wild ginseng CMCs were Murashige and Skoog medium (MS medium) containing 3% sucrose, pH 5.8, cell inoculum of 2.09 g/L [dry cell weight (DCW)], and aeration rate of 0.038 vvm (air volume/media volume/min), 0.052 vvm, and 0.066 vvm for Days 1–5, Days 5–10, and Days 11–13 (Dwyer Instruments, Michigan City, IN, USA), respectively. When proliferation was complete, wild ginseng CMCs were subcultured every 13 days. Culture conditions for the production stage to obtain secondary metabolites from wild ginseng CMCs were 1/2 MS media containing 3% brown sugar, cell inoculum of 3.94 g/L (DCW), and 100μM methyl jasmonate. The air flow rate was 0.06 vvm, and the cells were cultured for 7 d. Proliferation and production culture were performed in the same room in the dark at a temperature of 21 $\pm$ 1°C. After proliferation and production culture, biomass and major ginsenosides (Rb1, Rb2, Rc, Rd) of wild ginseng CMCs were obtained. The cells were then heat-treated in an extractor at 95°C for 48 h to obtain rare ginsenosides. During the heat treatment, major ginsenosides were converted to Rg3, Rh2, Rg5, and Rk1. The biomass was harvested and freeze-dried.

2.3. HPLC analysis (chromatographic analysis of wild ginseng CMCs)

An Agilent HPLC 1260 DAD system (Agilent Technologies, Santa Clara, CA, USA) and Agilent Zorbax Eclipse plus C18 column (4.6 $\times$ 100 mm, 3.5 μm, Agilent Technologies) were used for the analysis of rare ginsenosides in wild ginseng CMCs. The detection wavelength was 203 nm, the temperature of the column was 30°C, and the mobile phase was 0.05% trifluoroacetic acid in water and 0.05% trifluoroacetic acid in acetonitrile with a flow rate of 1 mL/min. Standards used for the analysis were ginsenoside 20(*S*)-Rg3, ginsenoside 20(*S*)-Rh2, and a mixture of ginsenoside Rk1 and Rg5. Solvents used for the analysis were acetonitrile, methanol, and trifluoroacetic acid. Each standard was weighed and the concentration was adjusted to 0.5 mg/mL using methanol. Freeze-dried wild ginseng CMCs were ground to a fine powder, and 80% of methanol solution was added to 0.5 g of ground wild ginseng CMCs to a volume of 10 mL. This solution was extracted for 2 h under ultrasonic waves, centrifuged, and filtered through a 0.2-μm syringe filter (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) for HPLC analysis.

2.4. Treatment of animals

Male Sprague–Dawley rats weighing 250–300 g (Orient Bio, Seongnam, Korea) were fasted overnight but given access to water *ad libitum*. All animal experiments were approved by the Animal Care Committee of Sungkyunkwan University School of Pharmacy, Suwon, Korea (SUSP14-04) and performed in accordance with the guidelines of National Institutes of Health (NIH publication No. 86-23, revised 1985). To generate GalN-induced hepatitis model, rats were injected intraperitoneally with 700 mg/kg of GalN dissolved in phosphate-buffered saline. The dose of GalN was selected according to previous reports [11,12]. There was no mortality in each experimental group, and all animals were shown to induce hepatotoxicity after GalN administration. Wild ginseng CMCs were suspended in distilled water and administered orally once a day for 2 wks and then 2 h prior to and 6 h after GalN injection. Animals were randomly assigned among the following seven groups ($n = 10$