Chemico-Biological Interactions 206 (2013) 143–152

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Helicobacter pylori infection combined with DENA revealed altered expression of p53 and 14-3-3 isoforms in *Gulo*$^{-/-}$ mice ☆

Arulkumar Nagappan [a,1], Hyeon Soo Park [a,1], Kwang Il Park [b], Gyeong Eun Hong [a], Silvia Yumnam [a], Ho Jeong Lee [a], Mun Ki Kim [a], Eun Hee Kim [c], Won Sup Lee [d], Wang Jae Lee [e], Myung Je Cho [f], Woo Kon Lee [f], Chung Kil Won [a], Jae Hyeon Cho [a], Gon Sup Kim [a,*]

[a] Research Institute of Life Science and College of Veterinary Medicine, Gyeongsang National University, 900 Gajwadong, Jinju, Gyeongnam 660-701, Republic of Korea
[b] Department of Biological Science, Center for Colon Cancer Research, University of South Carolina, Columbia, SC 29208, USA
[c] Department of Nursing Science, International University of Korea, Jinju 660-759, Republic of Korea
[d] Department of Internal Medicine, Institute of Health Sciences, Gyeongsang National University School of Medicine, Jinju, Republic of Korea
[e] Department of Anatomy, Seoul National University College of Medicine, Seoul, Republic of Korea
[f] Department of Microbiology, Gyeongsang National University School of Medicine, Jinju, Republic of Korea

ARTICLE INFO

Article history:
Received 21 June 2013
Received in revised form 22 August 2013
Accepted 1 September 2013
Available online 11 September 2013

Keywords:
Gulo$^{-/-}$ mice
Gastric cancer
Helicobacter pylori
Diethylnitrosamine
Proteome analysis
14-3-3 Isoforms

ABSTRACT

Unlike most other mammals, human bodies do not have the ability to synthesize vitamin C inside of their own bodies. Therefore, humans must obtain vitamin C through daily diet. *Gulo*$^{-/-}$ mice strain is known with deficiency, in which vitamin C intake can be controlled by diet like human, and would be valuable for investigating the molecular mechanism of various diseases. In the present study, we established *Gulo*$^{-/-}$ mice model and investigated the differentially expressed proteins in stomach tissue of *Gulo*$^{-/-}$ mice after *Helicobacter pylori*-infected, and followed by DENA, using immunohistochemistry and proteomic approach. The results of immunohistochemistry analysis of stomach tissue showed that the tumor suppressor, p53 protein, expression was significantly decreased ($p < 0.05$) but not messenger RNA (mRNA) transcriptional level, and 14-3-3ε, 14-3-3δ, Ki-67 and cleaved caspase 3 expressions were significantly increased ($p < 0.05$) by *H. Pylori* infection, and followed by DENA treatment in *Gulo*$^{-/-}$ mice. Moreover, knockdown of 14-3-3 isoforms (14-3-3ε, 14-3-3σ, 14-3-3ζ and 14-3-3η) were significantly increased sub-G1 phase (characteristics of apoptosis) in AGS cells and, phenotypic changes like cell shrinkage, density and cleaved nuclei were also observed. Proteome analyses showed that 14-3-3σ, 14-3-3η, and tropomyosin alpha-1 chain were down-regulated, and Hspd1 protein and HSC70 were up-regulated after *H. Pylori*-infection, and followed by DENA. The combined results of immunohistochemistry and proteomic analysis suggest that *H. pylori* altered the p53 and 14-3-3 isoforms expression and DENA further enhanced the *H. pylori* effect, which might be involved in carcinogenesis and metastasis of gastric cancer on *Gulo*$^{-/-}$ mice.

1. Introduction

Globally, gastric cancer (stomach cancer) is the second leading cause of cancer-related death, next to lung cancer, and approximately 989,600 new cases are diagnosed and 738,000 deaths every year [1]. Currently, the diagnosis is based on clinical, endoscopic, and histological criteria of gastric cancer patients; moreover, it is very difficult while examining many patients. The alternative possibility is to use laboratory animals, like mouse and rat for such studies. Vitamin C (ascorbic acid) is an excellent antioxidant, and an essential nutrient of most living tissues [2]. Humans must obtain vitamin C through daily diet due to a lack of L-gulono-g-lactone oxidase (GULO), gene encoding the key enzyme in ascorbic acid biosynthesis of most mammalian species. Even though humans overcome ascorbic acid deficiency by diet, its requirements vary greatly among the individuals for optimal health. The most common laboratory animals, including the mouse and rat possess a functional GULO gene that can readily synthesize ascorbic acid when destitute of this vitamin in the diet. In such case, ascorbic acid deficiency animal would be an appropriate and prominent model for human disease studies. Recently, *Gulo*$^{-/-}$ mice model have been used for *in vivo* experiment [3] and these *Gulo*$^{-/-}$ mice strain is known with deficiency, in which vitamin C intake can be

* Corresponding author. Tel.: +82 55 772 2346; fax: +82 55 772 2349.
E-mail address: gonskim@gnu.ac.kr (G.S. Kim).

[1] These two authors contributed equally to this study.

0009-2797/$ - see front matter © 2013 The Authors. Published by Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.cbi.2013.09.002

controlled by diet like human, would be valuable for investigating the molecular mechanism of various diseases.

Helicobacter pylori infection is a major cause of gastric cancer [4] and it is known to be an important pathogen associated with various severe gastric diseases, including peptic ulcers, chronic gastritis, and gastric cancer [5]. Epidemiological studies have reported that *H. pylori* infection is a risk factor for gastric carcinoma and vitamin C deficiency humans linked to more severe *H. pylori*-associated gastritis and a gastric cancer risk is also higher [6,7]. However, the mechanism by which *H. pylori* promotes the gastric epithelial cells to become cancerous is not fully understood. Moreover, Nitrosamines are the most important carcinogens that are involved in development of esophageal squamous-cell cancer (SCC) and gastric adenocarcinoma [8]. Diethylnitrosamine (DENA) is a well-known N-nitrous compound that provokes esophageal cancer in laboratory animals [9,10]. It has been first reported that diethylnitrosamine (DENA) used for the induction of esophageal and gastric tumors in hamsters [11]. Presently, only limited studies are available regarding DENA effects on gastric cancer. Hence, the combined effects of *H. pylori*-infection and DENA treatment in laboratory animals like *Gulo*$^{-/-}$ mice will provide new leads to understand the mechanism between gastric cancer, *H. pylori* and DENA.

With recent advances in proteomics, protein biomarker discovery is now a major area of proteome research. Profiling methods like matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF/MS) mass spectrometry analyses of animal tissues and fluids are promising techniques for biomarker discovery. In present study, we established *in vivo Gulo*$^{-/-}$ mice model like humans (it cannot synthesis vitamin C) and to identify the proteins which are differentially expressed in *H. pylori*-infected alone and/or combined with DENA that may play important roles in the pathological mechanism of *H. pylori*-induced gastric diseases and enhancement effect of DENA. To our knowledge, this is the first study using proteomics approach to profile protein changes in *Gulo*$^{-/-}$ mice model after *H. pylori* infection alone and/or combined with DENA.

2. Materials and methods

2.1. Chemical and reagents

Materials and chemicals used for electrophoresis were obtained from BioRad (Hercules, CA, USA). Antibodies 14-3-3ε and 14-3-3δ were obtained from Bioworld Technology Inc. (St. Louis Park, MN, USA), and p53 was purchased from Millipore (Billerica, MA, USA). RPMI 1640 medium was purchased from Hyclone (Logan, UT, USA). Fetal bovine serum (FBS) and antibiotics (streptomycin/penicillin) were purchased from Gibco (BRL Life Technologies, Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), and RNase A were obtained from Sigma–Aldrich (St. Louis, MO, USA). The 14-3-3ε siRNA (h), 14-3-3σ siRNA (h), 14-3-3ζ siRNA (h) and 14-3-3η siRNA (h) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Vitamin C (Ascorbic acid) was provided by Animal Resources Research Bank (ABRB). All other chemicals used in this study were purchased from AMRESCO (Solon, OH, USA) and Sigma–Aldrich (St. Louis, MO, USA). All the chemicals used were of the highest grade available commercially.

2.2. Experimental design and sample collection

We followed Animal Science guidelines for animal experimentation. *Gulo*$^{-/-}$ mice were kindly provided by Prof. Wang Jae Lee (Department of Anatomy, Seoul National University College of Medicine). *Gulo*$^{-/-}$ breeding pairs were originally obtained from the Mutant Mouse Regional Resource Centers, University of California at Davis. Genotypes of the off springs (Ed note: offspring) were evaluated by PCR as recommended [12]. Female *Gulo*$^{-/-}$ and C57BL/6 mice at 6–7 weeks of age were used, and they were maintained in specific pathogen free condition in the animal facility at the Gyeongsang National University School of Medicine with the animal experiments protocol reviewed and approved by Ethics Committee of the Gyeongsang National University. *Gulo*$^{-/-}$ mice were supplemented with 1.0 g/L of vitamin C in drinking water to prevent the death by vitamin C deficiency [13]. *Gulo*$^{-/-}$ mouse was divided into three groups; the control, *H. Pylori* infected group and *H. Pylori* infected followed by DENA treatment. In all three groups, vitamin C was supplemented (20 mg/animal/day, PO) and only group 3 was treated with DENA (10 mg/L, PO). A schematic representation of whole experimental procedure showed in Fig. 1. After 48 weeks, sacrificed all animals, stomach tissues were collected. Samples were stored at −70 °C until analysis.

2.3. Immunohistochemistry

We performed immunohistochemistry to evaluate the expression of p53, 14-3-3ε, 14-3-3δ, Ki-67 and cleaved caspase 3 in *Gulo*$^{-/-}$ mice mouse stomach tissue of all three groups, as mentioned in the experimental design. Collected samples were immersed in 4% prarformaldehyde for 48 h, and then processed for paraffin wax histology. In addition, 5 and 10 μm thick paraffin embedded lung slices were collected. To estimate histological changes, Hematoxylin–eosin (H–E) staining was carried out. Immunohistochemistry for p53, 14-3-3ε, 14-3-3δ, Ki-67 and cleaved caspase 3 were performed using rabbit monoclonal antibodies. Immunoreactivity was visualized with an avidin–biotin peroxidase reaction (PK-4001, Vectastain ABC Kit). The peroxidase reaction was developed using a 3, 3′-diaminobenzidine tetrahydrochloride (D-5905, Sigma). The sections were counterstained with hematoxylin before being mounted.

2.4. Real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated from *Gulo*$^{-/-}$ mice stomach tissues of control, *H. pylori* infected group and *H. pylori* infected combined

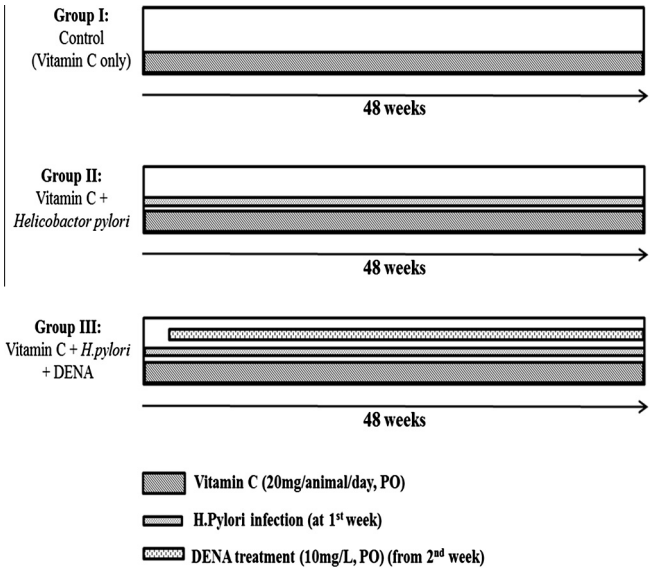

Fig. 1. A schematic representation of experimental procedure followed in *Gulo*$^{-/-}$ mice.