Toxicology Letters 231 (2014) 1–8

Contents lists available at ScienceDirect

Toxicology Letters

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

Pro-apoptotic action of macrophage inhibitory cytokine 1 and counteraction of activating transcription factor 3 in carrageenan-exposed enterocytes

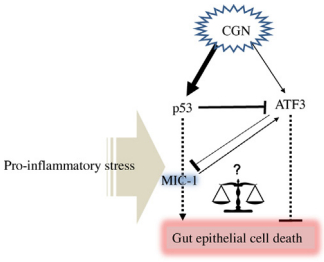
Hye Jin Choi [a,1], Hwi-Gon Kim [b,1], Juil Kim [a], Seong-Hwan Park [a], Jiyeon Park [a], Chang Gyu Oh [a], Kee Hun Do [a], Seung Joon Lee [a], Young Chul Park [c], Soon Cheol Ahn [c], Yong Sik Kim [d], Yuseok Moon [a,c,e,*]

[a] Laboratory of Mucosal Exposome and Biomodulation, Department of Biomedical Sciences, Pusan National University School of Medicine, Yangsan, South Korea
[b] Department of Obstetrics and Gynecology, Medical Research Institute, Pusan National University, Pusan, South Korea
[c] Department of Microbiology and Immunology and Medical Research Institute, Pusan National University, Pusan, South Korea
[d] Department of Pharmacology, Seoul National University College of Medicine, Seoul, South Korea
[e] Immunoregulatory Therapeutics Group in Brain Busan 21 Project, Busan, South Korea

HIGHLIGHTS

- Epithelial exposure to CGN triggers MIC-1 expression via p53-mediated pathway.
- ATF3 is critical in the cellular survival against MIC-1-mediated apoptosis.
- Counterbalance between MIC-1 and ATF3 determines the final cellular fate.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 16 January 2014
Received in revised form 28 August 2014
Accepted 29 August 2014
Available online 30 August 2014

Keywords:
CGN
MIC-1
ATF3
Epithelial apoptosis

ABSTRACT

Carrageenan (CGN), a widely used food additive, has been shown to injure the epithelial barrier in animal models. This type of damage is a clinical feature of inflammatory bowel disease (IBD) in humans. In the present study, the effects of CGN on pro-apoptotic responses associated with macrophage inhibitory cytokine 1 (MIC-1) regulation in human enterocytes were evaluated. CGN up-regulated the expression of MIC-1 that promoted epithelial cell apoptosis. Although MIC-1 induction was dependent on pro-apoptotic p53 protein, the pro-survival protein activating transcription factor 3 (ATF3) was negatively regulated by p53 expression. However, MIC-1 enhanced the expression of the pro-survival protein ATF3 in enterocytes exposed to CGN. Functionally, MIC-1-mediated epithelial cell apoptosis was counteracted by the pro-survival action of ATF3 in response to CGN exposure. These findings demonstrated that the counterbalance between MIC-1 and ATF3 is critical for deciding the fate of enterocytes under the food chemical stress.

1. Introduction

Carrageenan (CGN) is extracted from specific types of seaweed including *Gigartina*, *Chondrus*, and *Eucheuma*. This compound has been used to improve the texture of numerous

* Corresponding author at: Department of Biomedical Sciences, Pusan National University School of Medicine, Yangsan 626-870, South Korea. Tel.: +82 51 510 8094; fax: +82 55 382 8090.
E-mail address: moon@pnu.edu (Y. Moon).
[1] These authors contributed equally to the present study.

http://dx.doi.org/10.1016/j.toxlet.2014.08.022
0378-4274/

foods. Although the use of many food additives is generally considered a safe and well-accepted practice, studies have indicated that some additives including CGN pose health risks (Sharratt et al., 1971; Tobacman, 2001). The side effects of CGN and its derivatives have been widely studied since these compounds have been shown to trigger intestinal inflammation, ulceration, and neoplasm development in the gut mucosa of animal models. CGN-treated animals develop ulcerative colitis-like symptoms including loss of the haustral folds, mucosal granularity, crypt abscesses, lymphocytic infiltration, capillary congestion, pseudopolyps, and strictures (Fath et al., 1984; Moyana and Lalonde, 1991). Clinical features of experimental animals exposed to CGN are very similar to those of patients with inflammatory bowel disease (IBD). Ulcerative intestinal injuries caused by CGN are due to inflammatory infiltrates, epithelial barrier disruption, and uncontrolled mucosal immune activation (Sharratt et al., 1971; Tobacman, 2001).

As mediators of enterocyte cytotoxicity, macrophage inhibitory cytokine 1 (MIC-1), a pro-apoptotic cytokine, is a potent mediator of direct barrier disruption in enterocytes (Kim et al., 2002). Under normal resting conditions, there is little or no detectable expression of MIC-1 in epithelial cells in various organs such as the gastrointestinal tract, but MIC-1 expression is dramatically increased by inflammation, injury, and malignancy (Fairlie et al., 1999; Hsiao et al., 2000). For instance, numerous NSAIDs cause intestinal ulcerative injuries by inducing the expression of pro-apoptotic or growth-arresting proteins such as MIC-1 (also known as NSAID-activated gene-1) (Baek et al., 2005; Lee et al., 2006). MIC-1 is a cytokine belonging to the transforming growth factor-β superfamily and is also involved in epithelial tumor pathogenesis (Johnen et al., 2007; Matsumoto et al., 2006). During the early stages of tumorigenesis, elevated MIC-1 levels can lead to tumor cell apoptosis, inhibition of blood vessel formation, and tumor cell cycle arrest (Soto-Cerrato et al., 2007). The expression of MIC-1 can be induced either in a p53-dependent or -independent manner, and other diverse growth-regulatory signals are also involved in MIC-1 induction (Baek et al., 2003; Shim and Eling, 2005).

The production of survival factors can also be induced by the mucosal insults and confers protection to maintain the epithelial integrity [79]. Pro-apoptotic MIC-1 expression has been reported to be related to co-occurring survival related proteins such as activating transcription factor 3 (ATF3) protein (Lee et al., 2010; Piyanuch et al., 2007). ATF3 is a transcription factor belonging to the ATF/cyclic AMP response element-binding family and contains a basic region/leucine zipper DNA-binding motif specific for the cyclic AMP response element consensus sequence [80]. In most cases, expression of this protein is induced by external stress such as that caused by ischemic injuries, exposure to mutagens, carcinogens, and mitogenic cytokines or endoplasmic reticulum (ER) stresses associated with abnormal protein processing (Jiang et al., 2004; Yin et al., 2008). One example of the growth stimulatory role of ATF3 is when genetic re-programming of the epithelium is initiated in response to external skin insults and the expression of ATF3 is induced as a wound healing-associated mitogenic mediator (Harper et al., 2005). Mechanisms underlying the crosstalk between stress-induced apoptosis and ATF3 are still unclear in terms of epithelial homeostasis, a critical factor of mucosal diseases.

Maintenance of mucosal epithelial integrity is crucial in the defense against microbial infection and exposure to harmful luminal antigens. In the present study, the decisions between survival and death were evaluated in gut epithelial cells exposed to toxic CGN which was particularly associated with MIC-1 and ATF3 proteins. Investigating the network between MIC-1 and ATF3 would provide new molecular insights into prevention of adverse effects of CGN.

2. Materials and methods

2.1. Cell culture conditions and reagents

HCT-8 human epithelial cells and CMT-93 mouse colon cancer cells were purchased from the American Type Culture Collection (Rockville, MD, USA). HCT-116 cells containing (+/+) or lacking (−/−) wild-type p53 were generously provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD) to address the effects of genetic ablation of p53 on expression of p53-regulated genes including MIC-1. HCT-8 and HCT-116 intestinal epithelial cells are frequently used as a model of mucosal inflammatory diseases and inflammation-linked cancers (Bansal et al., 2010; Law et al., 2014; Thebault et al., 2006). The cells were maintained in RPMI 1640 medium (Welgene, Daegu, South Korea) for HCT-8 and HCT-116 and DMEM (Welgene) for CMT-93 supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Welgene), 50 units/mL penicillin and 50 μg/mL streptomycin (Welgene) in a 5% CO_2 humidified incubator at 37 °C. Cell number was assessed by exclusion of trypan blue dye (Sigma–Aldrich Chemical Company, St. Louis, MO, USA) using a hemacytometer. All experiments of cellular exposure were performed by using the complete media containing 10% FBS.

2.2. Isolation of primary mouse intestinal epithelial cells

Intestinal epithelial cells were isolation from specific pathogen-free male C57BL/6 mice, six weeks old, which were obtained from Hyochang Science (Daegu, South Korea). The mice were kept under standard conditions including a 12 h light–dark cycle and free access to drinking water and standard diet. The proximal 10 cm of the small intestine, comprising duodenum and proximal jejunum was excised, flushed with 50 mL of cold PBS. Small intestine was longitudinally opened and washed in cold HBSS (Ca^{2+}, Mg^{2+}-free) several times for 1 m until complete removal of stool by inverting 50 mL conical tube. The intestine was cut into 0.5 mm fragments in 20 mL HBSS containing 5% FBS, 25 mM HEPES, 100 units/mL penicillin and 100 μg/mL streptomycin in 50 mL conical tube and incubated in shaking incubator at 37 °C for 40 m. Supernatant was collected in new 50 mL conical tube. Collected cells were passed through the cell strainer or cotton gauze. The isolated cells were re-suspended in 1 mL of RPMI 1640 complete media. To remove enriched lymphocyte populations, a discontinuous density gradient was devised using Percoll (Santacruz biotechnology, TX, USA), diluted in growth medium. The gradient was prepared in 50-mL conical tubes, layering from the bottom: 10 mL 25% (vol/vol) Percoll and 10 mL 40% (v/v) Percoll. Cells in 1 mL RPMI 1640 complete media were placed on the top of this gradient, and the tube was centrifuged at room temperature at 600 g for 10 min. Cells from the interface between layers were then collected, re-suspended in 100% FBS, and washed twice in PBS twice. Cells re-suspended in RPMI 1640 complete media were counted and immediately used in CGN-exposure experiments.

2.3. Western blot analysis

Protein expression levels were compared using Western immunoblot analysis. The cells were washed with ice-cold phosphate buffer, lysed in boiling lysis buffer [1% (w/v) sodium dodecyl sulfate (SDS), 1.0 mM sodium ortho-vanadate, and 10 mM Tris, pH 7.4], and sonicated for 5 s. Fifty microgram of protein were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred onto polyvinylidene fluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Each blot was blocked for 1 h with 5% skim milk in Tris-buffered saline plus 0.05% Tween-20 (TBST) and