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Effects of carrageenan on cell permeability, cytotoxicity, and cytokine gene expression in human intestinal and hepatic cell lines

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ABSTRACT

Carrageenan (CGN) is a common food additive used for its gelling and thickening properties. The present study was done to evaluate intestinal permeability, cytotoxicity, and CGN-mediated induction of proinflammatory cytokines. A standard Caco-2 absorption model showed no CGN permeability or cytotoxicity at concentrations of 100, 500, and 1000 μg/mL. In two human intestinal cell lines (HT-29 and HCT-8) CGN (0.1, 1.0, and 10.0 μg/mL) did not induce IL-8, IL-6, or MCP-1 (CCL2) or produce cellular toxicity after 24 h. The TLR4 agonist LPS produced weak induction of IL-8 in HT-29 cells and no induction in HCT-8 cells. The effects of κ-CGN (0.1, 1.0, and 10 μg/mL) on cellular oxidative stress was assessed in HT-29 cells using CM-H_2DCFDA as the probe. No effect on oxidative stress was observed after 24 h. In the human (HepG2) liver cell line, ʎ-CGN (0.1, 1.0, 10.0 and 100.0 μg/mL) had no effect on the expression of IL-8, IL-6, or MCP-1 (CCL2) after 24 h. In conclusion, CGN was not absorbed, and was not cytotoxic. It did not induce oxidative stress, and did not induce proinflammatory proteins.

1. Introduction

Carrageenan (CGN) is a polysaccharide isolated from several species of red seaweed from the family *Rhodophyceae*. This unique hydrocolloid consists of alternating galactose and 3,6-anhydrogalactose sugars linked by alternating α-1,3 and β-1,4 glycosidic linkages. Interspersed throughout the linear molecule are sulfate groups. The three commercial forms of CGN are lambda (ʎ) -, kappa (κ)-, and iota (ι). The number and placement of the sulfate groups determines the form and function of CGN (Blakemore and Harpell, 2010). CGN is not a single molecule with a single molecular weight (MW) associated with it. CGN consists of polysaccharide molecules of varying sizes and is therefore described as having a weight-average molecular weight (Mw) of 200,000–800,00 Da. This large polymer is non-nutritive and when ingested at high concentrations can have an effect similar to that of high fiber in the diet. In the digestive tract, CGN is highly resistant to microbial and enzymatic degradation and, therefore, passes through the gastrointestinal system without alteration (Uno et al., 2001; Weiner, 2014). Safety studies conducted over the last 15–20 years in which CGN was administered to test animals in the diet have not shown any adverse effects (Weiner, 2014). A recent study in which CGN was administered to piglets for 28 days in a simulated infant formula also did not reveal any adverse effects in the intestine or in systemic organ systems (Weiner et al., 2015). These studies support the approval of carrageenan as a permitted food additive. Most recently, the World Health Organization's Joint Expert Committee on Food Additives or JECFA (JECFA, 2015), reviewed carrageenan for use in infant formula and concluded that "*the use of carrageenan in infant formula or formula for special medical purposes at concentrations up to 1000 mg/L is not of concern*."

There have been a significant number of *in vitro* studies in which CGN has been reported to activate inflammatory signaling pathways resulting in an induction of proinflammatory cytokines (Bhattacharyya et al., 2008a, 2008b, 2010a, 2010b, 2013; Borthakur et al., 2007). These studies were primarily performed using a normal human intestinal epithelial cell line (NCM460, INCELL Corp., San Antonio, TX), but studies have also been reported by this group

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using two other cell lines; a human colorectal adenocarcinoma (HT-29) cell line and a human line (HCT-8) derived from a ileocecal colorectal adenocarcinoma. These studies reported that CGN induced IL-8, as well as other proinflammatory cytokines, in all of these cell types (Borthakur et al., 2012; Bhattacharyya et al., 2011). The authors posited two pathways by which CGN can induce inflammation; 1) direct binding to and activation of the Toll-Like-Receptor 4 (TLR4)-Bcl10 pathway and 2) induction of oxidative stress via an increase in H_2O_2. Choi et al. (2012) reported that exposure of HCT-8 cells to CGN increased IL-8 expression and activated NF-kB. However, Choi et al. (2012) did not elucidate upstream initiation events for activation of the signaling pathways leading to the increased expression of proinflammatory cytokines. In the same study it was reported that exposure to CGN weakens the tight junctions between Caco-2 cells in culture. It has also been suggested that CGN binding to TLR4 in a human hepatoma (HepG2) cell line and, subsequent downstream activation of kinases, can inhibit insulin signaling in the liver (Bhattacharyya et al., 2013). This work implies that CGN crosses the intestinal epithelium; is carried via portal circulation to the liver; binds to TLR4 and induces proinflammatory responses and inhibits insulin signaling. The *in vitro* studies described using human intestinal or hepatic cell lines do not support findings reported for animal safety studies in which CGN was administered in diet (Weiner, 2014). Mckim (2014) showed that commercial CGN is often not pure and this may not be reflected on the label. This work also demonstrated that when undegraded CGN, for which the Mw and purity had been verified, was evaluated in a cell model with enhanced TLR4 sensitivity that CGN does not induce TLR4 mediated signaling (McKim et al., 2015). Moreover, McKim et al. (2015) also showed that the presence of serum protein, which is used in most cell culture systems, binds CGN tightly leaving little, if any, free CGN to interact with cells.

Because *in vitro* mechanism-based effects of CGN are being used to predict adverse effects in humans, it is important to carefully evaluate the *in vitro* models, methods, and the mechanisms proposed. Developing an understanding of the *in vitro* data reported using well characterized CGN is essential to understanding any potential human risk posed by CGN in food.

Therefore, the aim of the present work was to systematically evaluate the effects of three commercial forms (lambda, kappa, and iota) of CGN on intestinal absorption, TLR4 mediated proinflammatory signaling pathways, oxidative stress and cytotoxicity using Caco-2, HT-29, HCT-8 and HepG2 cell lines.

2. Materials and methods

2.1. Chemicals

Routine buffers, salts, and solvents were purchased from Sigma-Aldrich Chemical (St. Louis, MO) and were of the highest grade possible. Phosphate buffered saline (PBS, Cat#10010-023), McCoy's modified 5A and RPMI-1640 cell culture media, 5-(and 6)-chloromethyl-2′-7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) (Cat#C6827), and certified fetal bovine serum (FBS, Cat#16000-044) were from Life Technologies (Carlsbad, CA). Hanks Balanced Salt Solution (HBSS) and HEPES were from Gibco/Invitrogen sold by Life Technologies (Carlsbad, CA). A food-blend CGN consisting of κ- and ʎ-CGN (Lot# 90303011), ʎ-CGN (Lot# G1375-142) and poligeenan (Lot# ZP703) were provided by FMC Biopolymer (Philadelphia, PA). ι-CGN (Cat# C1138-100, Lot# SLBB2304V), 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT, Cat# M2003), lipopolysaccharide (LPS, from Escherichia Coli 0111:B4, TLR4 ligand tested Cat# L3024), human tumor necrosis factor alpha (TNF-α, Cat# SRP3177), ranitidine, pindolol, and TAK242 were from Sigma-Aldrich (St. Louis, MO). κ-CGN (Cat#22048-25 GF, Lot# 1432063V) was from Fluka Chemical Corp. Horse serum was from American Type Culture Collection (ATCC, Cat# 61373986).

2.2. Characterization of carrageenan and preparation of stock solutions

Each form of CGN was characterized by The National Food Laboratory, CA, Celtic Colloids Inc., ME, and Malvern Instruments, Herrenberg, Germany for composition, purity, molecular weight profile, and physical properties. The details of this characterization were described previously (McKim et al., 2015). For the HT-29, HCT-8, and HepG2 experiments, stock solutions (1 mg/mL) of CGN were prepared by adding CGN to sterile phosphate buffered saline with calcium and magnesium (PBS, pH 7.4). In the Caco-2 permeability experiments, CGN was prepared in Hanks balanced salt solution (HBSS) at pH 7.4. The presence of the buffer cations insures that CGN will remain in a random conformation. Solutions were prepared under sterile conditions. Aliquots of the stock solutions were added to an appropriate volume of complete media. Each dosing solution was pre-warmed to 37 °C and vortexed for 1 min prior to adding 100 μL aliquots to cells cultured in a 96-well culture plate. Stock solutions and dosing solutions were prepared fresh for each experimental day.

2.3. Cell lines

Three human intestinal cell lines were used in this study. These cell lines were selected because they were used in previous reports to evaluate the effects of CGN on various cellular pathways and biochemical effects (Choi et al., 2012; Bhattacharyya et al., 2014). The HT-29 (ATCC HTB-38) cell line is a colon epithelial cell derived from a colorectal adenocarcinoma. Cells were cultured in McCoy's Modified 5A media supplemented with 10% FBS (v/v). The HCT-8 cell line (ATCC CCL-244) was derived from a human ileocecal colorectal adenocarcinoma. These cells were cultured in RPMI-1640 media supplemented with 10% (v/v) horse serum as recommended by ATCC. The Caco-2 (ATCC HTB-37) cells were also derived from a human colorectal adenocarcinoma. The cells were cultured in low glucose Minimum Essential Medium (MEM) supplemented with 1 mM Pyruvate and 20% FBS.

The human hepatoma cell line (HepG2, ATCC CRL-8065) was cultured in Eagles Minimum Essential Medium (MEM) with or without 10% FBS. The incubations without FBS were included to evaluate the effects of protein binding on CGN mediated effects. All cells were cultured in a humidified incubator at 37 °C with 5% CO_2.

2.4. CGN Caco-2 cell permeability assay

Caco-2 cells were seeded onto 24-well plates with *trans*-well inserts (1 μm pore size) at a density of 20,000 cells per well in 0.4 mL of culture medium (low glucose minimum essential medium, Gibco 11095) with 20% FBS and allowed to grow for 14–21 days. In this study, cells were considered acceptable on day 16 as determined by *trans*-epithelial electrical resistance (TEER) values that were greater than 300 Ω (Breeman and Li, 2005). TEER values for this study were 600 ± 50 Ω on the day of dosing. Two preparations of CGN were used in this experiment, 1) Food Blend CGN consisting of both ʎ- and κ-CGN, and 2) ʎ-CGN. Ranitidine and Pindolol (10 μM) were included as low and high permeability control substances, respectively. Stock solutions were prepared in Hanks Balanced Salt Solution (HBSS) pH 7.4 with 25 mM HEPES. Both CGN samples were prepared at 100, 500, and 1000 μg/mL final concentrations in HBSS. These concentrations of CGN were selected to be in excess of concentrations previously reported. Prior to