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Autonomous cure of damaged human intestinal epithelial cells by TLR2 and TLR4-dependent production of IL-22 in response to *Spirulina* polysaccharides

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ABSTRACT

In order to analyze the damage of human epithelial cells, we used human quasi-normal FPCK-1-1 cells derived from a colonic polyp in a patient with familial adenomatous polyposis as a monolayer, which is co-cultured with peptidoglycan (PGN)-stimulated THP-1 cells. Co-cultured FPCK-1-1 cells showed a decreased transepithelial electrical resistance (TER) and the lower level of claudin-2. When *Spirulina* complex polysaccharides were added one day before the start of the co-culture, there was no decrease of TER and claudin-2 (early phase damage). In contrast, when *Spirulina* complex polysaccharides were added to FPCK-1-1 cells after the level of TER had decreased, there was no recovery at the level of claudin-2, though the TER level recovered (late phase damage). The mucosa reconstitution is suggested to be involved in the recovery from the damaged status. Interestingly, autonomous recovery of FPCK-1-1 cells from both the early and late phase damage requires the production of IL-22, because anti-IL-22 antibodies inhibited recovery in these cases. Antibodies against either TLR2 or TLR4 inhibited the production of IL-22 from FPCK-1-1 colon epithelial cells, suggesting that signals through TLR2 and TLR4 are necessary for autonomous recovery of FPCK-1-1 colon epithelial cells by producing IL-22. In conclusion, we have established a useful model for the study of intestinal damage and recovery using human colon epithelial cells and our data suggest that damage to human colon epithelial cells can, at least in part, be recovered by the autonomous production of IL-22 in response to *Spirulina* complex polysaccharides.

1. Introduction

Inflammation of the intestine is caused by infectious, genetic, immunological, and environmental factors. Although the inflamed and injured area heals and is replaced by regenerated tissue, excess mucosal inflammation of the gut reportedly induces ulcerative colitis or Crohn's disease [1,2]. Continuous inflammation is associated with an increased risk for colon cancer and promotes the propagation, metastasis, and invasion of cancer cells. Therefore, regulation of inflammation is important for human health.

Although an in vitro model of inflammatory bowel disease has been reported, it is using human colon carcinoma line Caco-2 co-cultured with THP-1 stimulated with phorbol myristate acetate (PMA) [3]. Conditions promoted by this combination likely differ from events occurring in vivo. Frequently used Caco-2 cells are adenocarcinoma cells exhibiting hypertetraploidy with many translocations and loss of chromosome segments. In contrast, FPCK-1-1 cells used in this study have neither a point mutation on codon 12 of K-*ras* gene nor gene amplification of *myc*, c-H-*ras*, and/or c-K-*ras* genes [4].

Another important point is that we used bacterial polysaccharides instead of using chemicals such as PMA as a stimulant to THP-1 cells. Peptidoglycan (PGN) from *Staphylococcus aureus* (*S. aureus*) causes inflammation and organ injury by inducing TNF-α, IL-6, and IL-10 [5]. Intramural injection of peptidoglycan-polysaccharide (PG-PS) from *Streptococcus pyogenes* (*S. pyogenes*) into the distal colon produces a chronic granulomatous colitis in rats [6] and PGN is known to exist in human gut epithelial cells [7]. Compared to the responses in remissive

Abbreviations: PGN, peptidoglycan; CPS, complex polysaccharides; PS, polysaccharides; LPS, lipopolysaccharides; PMA, phorbol 12-myristate 13-acetate, 12-*o*-tetradecanoylphorbol 13-acetate.

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Crohn's disease, responses of peripheral blood mononuclear cells and in particular, lymph node mononuclear cells in active Crohn's disease to the eubacterial cell wall and streptococcal cell wall antigen from *S. pyogenes* were significantly higher [7].

We have studied anti-inflammatory polysaccharides from edible algae and found that TLR2 and TLR4 responsive polysaccharides are effective in dampening the inflammation [8]. Among the four algae tested, complex polysaccharides (CPS) extracted from *Spirulina* with phenol and polysaccharides extracted from *Petalonia binghamiae* with hot water were TLR2- and TLR4-responsive and showed an anti-inflammatory effect in delayed-type hypersensitivity assay without showing cytotoxicity [8,9]. *Spirulina* is a traditional food of some Mexican and African peoples. It is a planktonic blue-green algae, and also classified as a Gram-negative photosynthetic filamentous cyanobacterium found in warm water alkaline volcanic lakes. It has a highly unusual nutritional profile and has been utilized for its nutrients for decades [10]. How *Spirulina* CPS promotes anti-inflammatory activity is currently unknown except that it induces NFκB in either TLR-2 or TLR-4 dependent pathways [8].

IL-22 belongs to the IL-10 family of cytokines and has recently been shown to be preferentially expressed by the Th17 subset [11,12]. IL-22 targets immune pathways due to the restricted expression of IL-22 receptors on epithelial cells, keratinocytes, and hepatocytes but not acquired immune cells, including T or B cells [12,13]. IL-22 is expressed at barrier surfaces and engaged in border patrol by regulating the immunity, inflammation and tissue homeostasis [13]. Interestingly, recent studies have demonstrated that colonic IL-22 expression is induced in inflammatory bowel disease (IBD) [14], but this inducible IL-22 expression is significantly higher in Crohn's disease as compared with ulcerative colitis [14,15]. IL-22 can enhance extracellular signal-regulated kinase (ERK)-mediated expression of a proinflammatory cytokine, IL-8, by colonic epithelial cell lines in vitro [15]. Thus, a pathogenic role of IL-22 in Crohn's disease is proposed [14,15]. By contrast, a regulatory role of IL-22 in IBD has also been proposed due to its ability to dampen systemic inflammatory response [16–18] through the induction of lipopolysaccharide-binding protein. IL-22 contributes to goblet cell mucus restriction and rapid attenuation of local inflammation associated with Th1-mediated colitis. The role of IL-22 in IBD is complex and not completely characterized.

Here, we describe a new model of repair from damage to colon epithelial cells using human colon epithelial FPCK-1-1 cells, which are derived from a tubular adenoma from a male familial polyposis coli patient [4]. FPCK-1-1 cells exhibit quasi-normal chromosomes and do not harbor the kinds of chromosomal abnormalities found in Caco-2 cells. We established the model by co-culturing FPCK-1-1 cells with PGN-stimulated monocytic leukemia THP-1 cells. We focused on the understanding of the mechanism of protective effects of algal polysaccharides such as *Spirulina* complex polysaccharides [8]. Especially, we focused on the recovery mechanism of damaged human colon epithelial cells. We also focused on regulation of potential effects of IL-22 in this damage model of intestinal epithelial cells, because it is reported that this cytokine promotes the proliferation, survival, and repair of epithelial cells in the skin, airway or intestine [13]. We found that following treatment with *Spirulina* CPS, TLR2 and TLR4 signaling had beneficial effects on FPCK-1-1 cells damaged by PGN-activated THP-1 cells. The beneficial effects were blocked by anti-IL-22 antibodies. We conclude that *Spirulina* CPS induces FPCK-1-1 cells to produce IL-22, suggesting that IL-22 functions in wound healing of colon epithelial cells in an autocrine manner.

2. Materials and methods

2.1. Reagents

Spirulina pacifica was generously given by Dr. G. Cysewski, Cyanotech Coop. (Kailua-Kona, Hawaii) and Mr. N. Miyaji, Toyo Koso Kagaku Co., Ltd., (Urayasu, Chiba, Japan). *S. pacifica* was selected from a strain of edible *Spirulina* (*Arthrospira*) *platensis* in 1984 and expresses different enzymes from parental strain. Complex polysaccharide (CPS) Westphal fraction (LPS fraction) was prepared from *S. pacifica* dried cells according to the method described [19]. Briefly, cells were washed by acetone, suspended in distilled water, and then extracted by an addition of 90% phenol-water with vigorous agitation at 68 °C. The crude preparation was dialyzed to remove phenol and then freeze-dried. The sample was dissolved in the water and sediment was eliminated by centrifugation. The molecular mass of the sample was in between 1000 and 20,000 by electrophoresis. By applying the matrix assisted laser desorption/ionization mass spectrometry analysis the value was estimated at 1280–28,300.

Other polysaccharides used include: Fucoidan prepared from *Fucus vesiculosus* (Sigma-Aldrich Corporation, St. Louis, MO), Mr: 841,388–1,113,156. Alginic acid is prepared from *Macrocystis pyrifera* (kelp) (Sigma-Aldrich Corporation), Mr: 420,100–420,700. *Escherichia coli* (055:B5) LPS (Sigma-Aldrich Corporation), Mr: 35,808–93,813. *P. binghamiae* polysaccharide (*Petalonia* PS, 66% ethanol precipitates from hot water extracts), Mr: 273,555–353,476. Peptidoglycan (PGN) from *S. aureus* was purchased from Fluka (Sigma-Aldrich production GmbH, Buchs, Switzerland).

2.2. Cytokines and enzyme linked immunosorbent assays (ELISA)

Recombinant human IL-6 and IL-22 were purchased from PeproTech Inc. (Rocky Hill, NJ). Recombinant human IL-1β and IL-23 were purchased from Human Zyme Inc. (Chicago, IL). IL-22 was measured using an ELISA kit from R&D Systems, Inc. (Minneapolis, MN). Recombinant human TNF-α was purchased from e-Bioscience (San Diego, CA). TNF-α was measured using an ELISA kit from e-Bioscience (San Diego, CA).

2.3. Antibodies

For Western blotting rabbit anti-human claudins-2, -4, and -8 sera were kindly provided by Dr. Mikio Furuse (Kobe University, Kobe, Japan). Anti-human beta-actin, TLR2, and TLR4 antibodies were purchased from BioLegend (San Diego, CA). Goat anti-human TNF-α, goat anti-human IL-22, goat anti-human IL-22R antibodies, and control goat IgG antibodies were obtained from R&D Systems, Inc. (Minneapolis, MN).

2.4. Cell lines and cell culture system

Caco-2 cells [20] were purchased from Riken BioResource Center, Tsukuba, Japan (RCB0988). As noted, the non-tumorigenic FPCK-1-1 cell line was derived from a patient with familial adenomatous polyposis [4]. Human monocytic leukemia THP-1 cells were purchased from Health Science Research Resources Bank, Japan Health Science Foundation, Osaka, Japan (JCRB0112). Caco-2 cells, FPCK-1-1 cells and THP-1 cells were maintained at 37 °C in 5% CO_2 in Dulbecco's-modified Eagle medium with high glucose (DMEM) supplemented with 8% fetal calf serum (FCS), 20 U/ml penicillin, 50 μg/ml kanamycin and subcultured on 1.1 cm^2, 0.4 μm pore Transwell permeable supports (polytetrafluoroethylene (PTFE) membrane) precoated with an equimolar mixture of types I and III collagen (3493, Corning, Ithaca, NY).

2.5. Co-culture system and the treatment of intestinal epithelial cells

2.5.1. Early phase damage model

Caco-2 cells or FPCK-1-1 cells were cultured at a density of 2×10^5 cells/insert on 12 well cell culture inserts described above to form a monolayer and early phase damage was examined as described [21]. THP-1 cells at a density of 1×10^5/well were cultured on 12-well culture