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The immunopotentiating effects of shark-derived protein hydrolysate

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ABSTRACT

Objective: Peptides derived from natural sources can act as immunomodulating agents and prevent infections. The aim of this study was to investigate the immunopotentiating and protective effects of a shark-derived protein hydrolysate (SPH) against an enterotoxigenic Escherichia coli H10407 infection in a murine model.
Methods: Mice were fed an aqueous solution of SPH for 7 days before being inoculated with an experimental enterotoxigenic Escherichia coli H10407 infection. After euthanasia, small intestines were removed for histological study and the number of IgA and IgG producing cells was determined by direct immunofluorescence. Cytokines were measured in the serum and the intestinal fluid.
Results: The oral administration of SPH enhanced the gut barrier function via up-regulation of immunoglobulin A-producing cells and intestinal cytokines production, including interleukin-6 and tumor necrosis factor-α. The increase of transforming growth factor-β and interleukin-10 contribute to the down-regulation of uncontrolled-inflammatory reaction induced by *E. coli* infection. From these results, the anti-inflammatory properties of SPH may be caused by regulation and priming mechanisms of the immune system.
Conclusion: Enzymatic protein degradation confers immunomodulating and protective potentials to shark proteins and the resulted peptides could be used as an alternative therapy to reduce the risk of bacterial infections and inflammatory-related diseases.

Introduction

The immune response induced by bioactive peptides is thought to be one of the oldest forms of immunoregulation that has been conserved through evolution, as opioid-related peptides have been found in invertebrates [1]. These peptides are involved in antithrombotic, antioxidative, antibacterial, antifungal, and sensory physiological activities; they also improve the nutritional value of food [2]. Food-derived bioactive peptides are one source of these health-enhancing components. They can be released during gastrointestinal digestion or processing of a multitude of plant and animal proteins, especially milk, soy, or fish proteins [3]. Fish proteins are a particularly rich source of bioactive peptides [4–8]. Bioactive peptides have been isolated from fish tissues such as sardine muscle, tuna muscle, pacific hake [9], and bonito; many of these have antihypertensive properties because they inhibit the angiotensin I-converting enzyme. Marine-derived protein hydrolysates (MPH) are also sources of multifunctional peptides because antihypertensive biopeptides can enhance the activity of bradykinin, which stimulates macrophages, enhances lymphocyte migration, and increases the secretion of lymphokines [10–12].

Accordingly, an orally administered MPH from yeast-derived fermentations affected both the systemic (phagocytosis) and the small intestine mucosal (immunoglobulin [Ig]A) immune systems in a murine model [13,14]. Additionally, when analyzed in a variety of injury and repair models, some MPH have demonstrated biological activity and may offer a novel, less

JFM participated in the in vivo experiment and the drafting of the manuscript. JD conducted the experiments and revised the manuscript. GV conceived part of the study. CM conceived the study and drafted the manuscript. RA provided technical support. MB revised the manuscript. JFM, JD, GV, and CM have no conflicts of interest to report. RA and MB are affiliated with innoVactiv inc. They participated in the development of the shark-derived protein hydrolysate and the design of the experiment, but were not involved in obtaining the results of the animal experiments.

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expensive approach to preventing and treating the injurious effects of nonsteroidal anti-inflammatory drugs and other ulcerative conditions of the bowel [15]. Thus, it is clear that marine resources constitute a potential reservoir of powerful immunomodulators.

The immune regulatory response is a major cell event involved in the maintenance of immune homeostasis and the control of inflammation. Priming the immune response is key to this process and could increase the immunoregulatory response against mucosal infections, such as *Escherichia coli*. It is well known that priming the immune response induces a type of physiological inflammation that triggers immunoprotection in the mucosa-associated lymphoid tissue [16,17]. Shark is commonly used in traditional medicine in China with no scientific evidence so far. We hypothesized that peptides would exert a mild priming effect on the immune system by decreasing the expression of proinflammatory cytokines and helping to prevent exaggerated inflammatory responses when mice were challenged with *E. coli*. The aim of this study was to evaluate the effects of a shark-derived protein hydrolysate (SPH) on host immunity and to study their immunoprotection potential against enterotoxigenic *E. coli* (ETEC).

Methods

Animals and feeding procedures

Six- to 8-wk-old BALB/c mice weighing 18 to 20 g were obtained from Charles River (Montreal, Canada). Each experimental group included five mice that were housed together in plastic cages in a controlled atmosphere (temperature: 22 ± 2°C; humidity: 55 ± 2%) on a 12-h light–dark cycle. The experiment was repeated three times. The mice were treated in accordance with the guidelines of the Canadian Council on Animal Care and the experimental design was approved by the Animal Care Committee of the University of Ottawa.

An aqueous solution of SPH was administered by gavage (4.5 mg/kg) to the animals in lieu of water for 5 or 7 d consecutively. The control group was given water instead of SPH. Both groups were fed ad libitum with a conventional balanced diet (2018 Teklad Global 18% Protein Rodent diet, Harlan Laboratories Inc, Madison, WI, USA).

At the end of each feeding period, the animals were anesthetized with intraperitoneal injections of ketamine (100 mg/mL), xylazine (20 mg/mL), and acepromazine (10 mg/mL) and sacrificed by cervical dislocation to obtain the various tissues to be used in the immunologic studies.

Shark-derived protein hydrolysate

The SPH preparation studied, PeptiBal™, was provided by innoVactiv Inc. The product is a patent-pending blend of small peptides generated by a trypsin-chymotrypsin hydrolysis process of purified shark proteins. Protein extracts from shark flesh (Waitaki Biosciences, New Zealand) were used as raw material for the studies described here. Marine hydrolysates were produced using enzymatic digestion by trypsin from porcine pancreas (Sigma-Aldrich) and type II α-chymotrypsin from bovine pancreas (Sigma-Aldrich), using a concentration 5% (w/vol) of protein extract in water. The hydrolysis reaction was performed at pH 8.0 and 33°C to 37°C. The pH-stat technique was used to maintain the enzyme preparation at its optimal pH [18]. The enzymatic hydrolysate was ultrafiltered using a 10-kDa cutoff membrane to remove the protease and the non-hydrolyzed proteins. The retentate or reaction mixture was discarded, whereas permeate, so-called total hydrolysate was further characterized and used for evaluation of its functional properties. The enzymatic hydrolysate was clarified by filtration and centrifugation to remove insoluble material (i.e., non-hydrolysated protein). The filtrate was then ultrafiltered using two subsequent cutoff membranes to remove high-molecular-weight polypeptides including residual enzymes. After ultrafiltration, the retentate was discarded, whereas permeate, so-called total hydrolysate, was pasteurized to inactivate residual enzymes. Spray drying was performed on the pasteurized total hydrolysate.

SPH has been tested for endotoxins with the LAL Chromogenic Endotoxin Quantitation Kit by Pierce Protein (Thermo Fisher, Burlington, ON, Canada).

Bacterial translocation assay

The livers were removed in sterile conditions and homogenized using the Omni TH homogenizer with a 7-mm generator probe (Omni International, Kennesaw, GA, USA) in 5 mL 0.1% sterile peptone water. One mL of each liver homogenate was plated on MacConkey agar (for enterobacteria). Translocation was considered to have occurred when colonies were observed on the agar plates because the liver is an organ normally devoid of bacteria [19].

Immunofluorescence test to identify the B-cell population (IgA+ and IgG+ cells)

The small intestines were removed for histologic preparation using the Sainte-Marie technique for paraffin inclusion. The paraffin blocks were cut in 4-μm sections. The number of IgA-producing (IgA+) cells on histologic sections of the samples from the ileum region near Peyer's patch was determined using a direct immunofluorescence method. The number of IgG-producing (IgG+) cells on histologic sections of the small intestine was also determined. The immunofluorescence test was performed using fluorescein isothiocyanate (FITC)-conjugated goat (α-chain specific) polyclonal antimouse IgA or FITC-conjugated goat (γ-chain specific) polyclonal antimouse IgG (Sigma-Aldrich, St. Louis, MO, USA). The histologic sections were deparaffinized and rehydrated in a graded series of ethanol (from 95% to 40%). The deparaffinized histologic samples were incubated with the appropriate antibody dilution (1/100 for IgA or 1/50 for IgG) in phosphate-buffered saline (PBS) solution for 30 min at 37°C. The samples were then washed twice with PBS solution and examined using a fluorescent light microscope. Results were expressed as the number of IgA+ or IgG+ cells (positive: Fluorescent cell) per 10 fields (magnification: 1000×). The data represent the mean of three histologic sections from each animal for each feeding period.

ELISA of serum samples

Blood was collected from anesthetized mice by cardiac puncture. The blood was coagulated by allowing it to rest for 30 min at room temperature and then was centrifuged for 10 min at 1000 *g*. The serum was stored at −20°C until it was analyzed. The level of transforming growth factor (TGF)-β in the serum was analyzed by enzyme-linked immunosorbent assay (ELISA; eBioscience, Inc., San Diego, CA, USA). The serum level of IgA was analyzed by DAS-ELISA using affinity-purified goat antimouse IgA antibodies (Sigma Chemical Co., St Louis, MI, USA).

Cytokine assay

At the end of each treatment period, the small intestines were removed and processed for histologic preparation as described previously. Interleukin (IL)-4, IL-6, IL-10, tumor necrosis factor (TNF)-α, cytotoxic T-lymphocyte antigen 4 (CTLA-4), and interferon (IFN)-γ were analyzed using an indirect immunofluorescence method. The histologic sections were deparaffinized and rehydrated in a graded series of ethanol (from 95% to 40%) and then incubated for 30 min in a 1% blocking solution of bovine serum albumin (Jackson Immuno Research, West Grove, PA, USA) at room temperature. The histologic sections were then incubated for 60 min at 37 °C with affinity purified rabbit antimouse IL-4, IL-6, IL-10, TNF-α, IFN-γ (Peprotech, Inc., Rocky Hill, NJ, USA) or CTLA-4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) polyclonal antibodies. After incubation, the samples were washed twice with PBS solution, and finally, the sections were treated for 45 min at 37°C with a diluted FITC-conjugated goat antirabbit antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). The data represent the mean of three histologic sections from each animal for each feeding period.

Primary culture of mouse small intestine epithelial cells

The primary cultures of the enterocytes were prepared as described previously [20], with slight modifications as described in this section. At the end of each period of orally administering the test substances (SPH or water) the animals were euthanized as previously described. The small intestines were removed and placed in a digestion buffer of Hanks' balanced salt solution (Sigma-Aldrich, St. Louis, MO, USA) with 2% glucose (Sigma-Aldrich), 100 U/mL penicillin (Sigma-Aldrich), and 0.1 mg/mL streptomycin (Sigma-Aldrich). The intestines were flushed six times with 10 mL of the digestion buffer. They were then cut into 2- to 3-mm fragments and digested in 20 mL of digestion buffer supplemented with 300 U/mL Type XI collagenase (Sigma-Aldrich) and 0.1 mg/mL dispase (Gibco, Grand Island, NY, USA) at 25°C at 150 *g* for 45 min. Digestion was stopped by adding 20 mL of Dulbecco's modified Eagle medium (DMEM) without phenol red (Gibco), supplemented with 10% heat-inactivated fetal bovine serum (ATCC, Manassas, VA, USA), 10 ng/mL epidermal growth factor (U.S. Biological, Swampscott, MA, USA), insulin-transferrin-selenium-A (2.50 μg/mL, 0.55 μg/mL, and 1.68 pg/mL, respectively) from a 100× ready-to-use solution (Gibco), 100 U/mL penicillin (Sigma-Aldrich) and 0.1 mg/mL streptomycin (Sigma-Aldrich). Large fragments were removed by allowing them to settle for 2 min at the bottom of the flask. The supernatant was centrifuged for 3 min at 300 *g*. The pellet was washed twice with the DMEM solution and then resuspended in the same culture medium at a concentration of 4×10^5 to 6×10^5 organoids (single cells or intestinal epithelial cells [IEC]) per mL. The IEC suspensions were then transferred to 96-well cell culture plates (200 μL/well) and