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Research paper

Fucoidan directly regulates the chemotaxis of canine peripheral blood polymorphonuclear cells by activating F-actin polymerization

Soo-Hyun Kim, Ji-Houn Kang, Mhan-Pyo Yang*

Laboratory of Veterinary Internal Medicine, Department of Veterinary Medicine, College of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk 361-763, Republic of Korea

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ABSTRACT

Fucoidan was recently shown to enhance innate immune functions. The objective of this study was to examine the direct stimulatory effect of fucoidan on the chemotactic activity of canine peripheral blood polymorphonuclear cells (PMNs). The chemotactic activity of PMNs was evaluated in a modified Boyden chamber assay and total cellular filamentous (F)-actin levels were measured using a flow cytometer. The chemotactic response of PMNs was increased by exposure to recombinant canine (rc) interleukin (IL)-8. *In vitro* treatment with fucoidan increased the chemotactic activity of PMNs in response to rcIL-8 compared with that of untreated PMNs, and also stimulated total cellular F-actin polymerization. The increased chemotactic activity of fucoidan-treated PMNs was suppressed by cytochalasin D, an inhibitor of F-actin polymerization. These results suggest that fucoidan directly regulates PMN chemotaxis, and that this effect is associated with an increase in actin polymerization.

1. Introduction

Polymorphonuclear cells (PMNs) are primary mediators of the innate immune response to invading microorganisms. They are dynamic, motile cells that have a unique capacity to cross the endothelial barrier, enter tissues, and phagocytose (thereby eliminating) pathogens and cell debris (Nathan, 2002; Wagner and Roth, 2000).

The migration of neutrophils is guided by a number of chemotactic factors, including the bacterial product formyl-Met-Leu-Phe (fMLP), and host-derived products such as leukotriene B_4 (LTB_4) (Foxman et al., 1997, 1999) and interleukin (IL)-8 (Baggiolini, 1998; Baggiolini et al., 1997). Chemotaxis of PMNs is a complex process that involves extracellular and intracellular signaling, regulation of the cytoskeleton, and interactions between cells and the extracellular matrix (ECM) (Horwitz and Parsons, 1999; Sánchez-Madrid and del Pozo, 1999). IL-8 plays an important role in neutrophil recruitment, lysosomal degranulation, and the oxidative burst (Navarini-Meury and Conrad, 2009).

The "crawling" of PMNs is associated with changing cytoskeletal dynamics, including F-actin polymerization and rearrangement, F-actin-enriched pseudopodia formation, and myosin II assembly (Chung et al., 2001; May and Machesky, 2001; Torres and Coates, 1999). Actin is a major cytoplasmic component of the neutrophil cytoskeleton and exists in two main states: a globular monomeric form (G-actin) and a filamentous helical polymer (F-actin) (Reisler and Egelman, 2007; van Eeden et al., 1999). Signaling pathways induced by chemotactic stimuli cause G-actin to polymerize. Thereby forming F-actin (Chung et al., 2001; Howard and Meyer, 1984).

Fucoidan, which is found in the extracellular matrix of brown algae, is a cell wall polysaccharide composed of variable amounts of fucose, uronic acid, galactose, xylose, and sulfates (Matsubara et al., 2001). Fucoidan is reported to possess diverse biological activities of

* Corresponding author. Tel.: +82 43 261 2539; fax: +82 43 261 3224.
E-mail address: mpyang@chungbuk.ac.kr (M.-P. Yang).

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potential medicinal value. Such activities include anticoagulant (Grauffel et al., 1989), anti-angiogenic (Koyanagi et al., 2003), anti-thrombotic (Chandía and Matsuhiro, 2008), antitumor (Alekseyenko et al., 2007; Riou et al., 1996), antiviral (Damonte et al., 2004; Hayashi et al., 2008), and anti-adipogenic effects (Yokota et al., 2009). It also has inflammatory modulatory effects (Cumashi et al., 2007) and stimulates both humoral and cell-mediated immune responses under *in vitro* and *in vivo* conditions (Hayashi et al., 2008). It was recently suggested that fucoidan has an immunoenhancing effect on the phagocytic capacity and oxidative burst activity (OBA) of canine PMNs, which is mediated by tumor necrosis factor (TNF)-α production by peripheral blood mononuclear cells (PBMCs) (Kim et al., 2011). We also recently observed that *in vitro* treatment with fucoidan stimulated the chemotactic activity of canine PMNs *via* IL-8 produced by PBMCs (Jeon et al., 2012). However, it remains unclear whether fucoidan directly influences the cytoskeleton of neutrophils. The objective of this study was to determine whether *in vitro* treatment with fucoidan directly regulates the chemotactic activity of canine PMNs, and if so, whether this effect is associated with changes in F-actin polymerization.

2. Materials and methods

2.1. Reagents

Fucoidan, purified from *Focus vesiculosus*, was purchased from Sigma–Aldrich (St. Louis, MO, USA). The stock solution was prepared by dissolving fucoidan to a final concentration of 10 mg/ml in phosphate-buffered saline (PBS) and passing it through a 0.2 μm membrane filter (Millipore Co., Bedford, MA, USA). Fetal bovine serum (FBS) (Sigma–Aldrich), recombinant canine (rc)IL-8 (R&D Systems Inc., Minneapolis, MN, USA), and cytochalasin D (Sigma–Aldrich) were also used in this study.

2.2. PMNs isolation

Five clinically healthy male Beagle dogs (mean age, 1 year) were used as blood donors. All dogs were housed separately in cages with a 12 h light/dark cycle, fed a commercial diet (ProPlan; Purina Korea, Seoul, Korea), and provided with tap water. All experimental procedures were approved by the ethics committee of the Chungbuk National University. To evaluate PMN function, the cells were isolated by density gradient centrifugation immediately after blood sample collection as described previously (Jeon et al., 2012; Paek et al., 2010). Briefly, heparinized blood samples were overlaid on a Histopaque solution at a 1:1 ratio (specific gravity, 1.077; Sigma–Aldrich). After centrifugation at 400 × *g* for 45 min at room temperature, the PMNs were collected from the upper layer of sedimented erythrocytes. To purify the PMNs, erythrocytes were allowed to sediment for 60 min in a PBS solution containing 1.5% dextran (molecular weight, 200,000; Wako Pure Chemical Industries Ltd., Osaka, Japan). The floating cells were then gently collected and pelleted by centrifugation at 400 × *g* for 5 min. The residual erythrocytes were lysed by a brief treatment with 0.83% NH_4Cl in a tri(hydroxymethyl)-aminomethane-base buffer (pH 7.2) for 5 min. The purity of PMNs in the final cell suspension was verified to be >96%, as determined by Wright–Giemsa staining analysis of a blood film obtained by use of cytocentrifugation. The resulting PMNs were suspended in RPMI 1640 medium (Sigma–Aldrich) supplemented with 2 mM L-glutamine and 0.02 mg gentamicin/ml.

2.3. Chemotaxis assay

The chemotactic activity of PMNs was determined in terms of the distance migrated through nitrocellulose membrane filters in a modified Boyden chamber assay as previously described (Son et al., 2006; Watanabe et al., 1985). Briefly, the chemotaxis chamber (Neuro Probe, MD, USA) and FBS-free RPMI 1640 medium were prewarmed for 2 h at 37 °C. The lower chamber was filled with 200 μl of FBS-free RPMI 1640 medium containing rcIL-8 or fucoidan as the chemoattractant. A nitrocellulose filter (120 μm thick with a 3.0 μm pore size; Millipore Corporation, Bedford, MA) was placed on top of the well in the lower compartment. Then, 200 μl of a PMN suspension (2×10^6 cells/ml) containing fucoidan or fucoidan plus cytochalasin D (an inhibitor of F-actin polymerization) in a minimal volume (<1% of the medium) of DMSO as the solvent was placed in the upper compartment. The same amount of DMSO (vehicle-only control) was added to the control wells. DMSO was also added to the lower compartment to equalize the osmotic pressure between the compartments. The chambers were incubated for 45 min at 37 °C in a 5% CO_2-humidified atmosphere. After incubation, the membrane filters were immediately removed, fixed in ethyl alcohol, dried, stained with hematoxylin, decolorized in ethyl alcohol, and mounted on a slide glass. The distance that the cells migrated through the nitrocellulose filter toward the other side was measured by a distance between migrated and non-migrated cells under a bright field microscope at 400× magnification. Each experiment was performed in triplicate and five randomly-selected fields per filter were examined. The chemotactic responsiveness of the input cells was evaluated as the absolute distance (μm/45 min) migrated by the PMNs in response to the chemoattractant.

2.4. Determination of total cellular F-actin content

Total cellular F-actin levels were measured as described previously (Kang et al., 2009; Kang and Yang, 2008). Isolated PMNs were placed in 24-well plates (1×10^6 cells/ml/well) and incubated with either fucoidan and/or rcIL-8 or fucoidan plus cytochalasin D for 40 min at 37 °C in a 5% CO_2 humidified atmosphere. The cultured cells were gently harvested, centrifuged at 400 × *g* for 3 min at 4 °C, and washed three times with PBS solution containing 3 mM EDTA. The cells were fixed with fixation buffer (BD Cytofix; Becton Dickinson Bioscience, San Jose, CA, USA) at 4 °C according to the manufacturer's instructions, washed three times, and stained in the dark for 15 min at 37 °C with 165 nM FITC-labeled phalloidin/100 μg/ml lysophophatidylcholine (Sigma–Aldrich). The cells were then washed and analyzed within 30 min using a