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Fucoidan suppresses excessive phagocytic capacity of porcine peripheral blood polymorphonuclear cells by modulating production of tumor necrosis factor-alpha by lipopolysaccharide-stimulated peripheral blood mononuclear cells

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ABSTRACT

We examined the effect of fucoidan, an immune modulator, on the phagocytic capacity of porcine peripheral blood polymorphonuclear cells (PMNs) exposed to culture supernatant from lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells (PBMCs). For this purpose, we evaluated the phagocytic capacity of porcine PMNs by flow cytometry and measured levels of tumor necrosis factor-alpha (*TNF-α*) protein and mRNA in porcine PBMCs by enzyme-linked immunosorbent assay (ELISA) and real time-polymerase chain reaction (PCR), respectively. Fucoidan or LPS alone did not affect the phagocytic capacity of PMNs, but phagocytosis by these cells was increased by exposure to culture supernatant from PBMCs treated with fucoidan or LPS. In particular, the culture supernatant from PBMCs treated with LPS revealed excessive phagocytosis of PMNs. This excessive phagocytic capacity was diminished by co-treatment LPS with fucoidan. Production of *TNF-α* mRNA and protein increased upon treatment of PBMCs with either fucoidan or LPS, but this effect was also diminished by co-treatment LPS with fucoidan. The ability of culture supernatant from PBMCs treated with LPS and/or fucoidan to increase the phagocytic capacity of PMNs was inhibited by anti-recombinant porcine *TNF-α* polyclonal antibody. These results suggested that fucoidan suppresses the phagocytic capacity of PMNs by modulating *TNF-α* production by LPS-stimulated PBMCs.

1. Introduction

Phagocytes, including polymorphonuclear cells (PMNs), monocytes, and macrophages, defend the body by phagocytizing foreign particles, harmful pathogens, and dead cells (Lee et al. 2003). The phagocytic capacities of these phagocytes are transduced by intracellular signal cascades that triggers several cellular processes, including activation of phagocytosis mechanisms and production of inflammation-related cytokines (Aderem and Underhill 1999). When mononuclear cells such as monocytes, tissue macrophages, and lymphocytes are activated, they produce pro-inflammatory cytokines, which induce activation of PMNs and upregulation of PMNs functions, including generation of reactive oxygen species (ROS) and phagocytosis (Pechkovsky et al. 1996).

Tumor necrosis factor-alpha (*TNF-α*) is a key regulator of the inflammatory response. It is known that high levels of *TNF-α* are correlated with pathological responses in a variety of inflammatory diseases and inflammatory conditions (Grimble 1998). Excess production of *TNF-α* is also related to a number of toxic manifestations of infection (Oliff et al., 1987; Waage et al. 1989).

Fucoidan, which is found in the extracellular matrix of brown algae and brown seaweed, is polysaccharides containing fucose, mannose, galactose, and sulfate groups. These compounds of fucoidan possess diverse medicinal effect due to their ability to imitate patterns of sulfate substitution on glycosaminoglycans and sulfated glucans (Damonte et al. 2004; Li et al. 2008). Algal fucoidan has been widely examined in various biological contexts, including their anti-coagulant (Chandía and Matsuhiro, 2008), anti-angiogenic (Koyanagi et al. 2003), anti-proliferative (McCaffrey et al. 1992), anti-tumor (Alekseyenko et al. 2007), and anti-viral (Hayashi et al. 2008) properties.

The phagocytic capacities of macrophages are upregulated by fucoidan, and the activated cells produced markedly elevated levels of *TNF-α* (Choi et al. 2005). Although previous studies revealed that

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culture supernatant of fucoidan-stimulated canine peripheral blood mononuclear cells (PBMCs) boosts the phagocytic capacity of canine PMNs (Kim et al. 2011), no studies to date have evaluated the effect of fucoidan on the phagocytic capacity of PMNs under inflammatory conditions (Park et al. 2015; Park et al. 2017; Wang et al. 2018), which may be important in modulation of the inflammatory immune response.

In this study, we examined the modulating effect of fucoidan on the phagocytic capacity of porcine PMNs under inflammatory conditions. For this purpose, we administered fucoidan to porcine PMNs exposed to culture supernatant from lipopolysaccharide (LPS)-stimulated PBMCs.

2. Materials and methods

2.1. Chemicals and reagents

Fucoidan purified from *Focus vesiculosus*, LPS from *Escherichia coli* 0127:B8, Percoll, RPMI 1640 medium, and carboxylate-modified polystyrene-latex beads were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum was purchased from Gibco (Grand Island, NY, USA), and anti-recombinant porcine (rp) *TNF-α* polyclonal antibody (pAb) was purchased from RayBiotech (Norcross, GA, USA). Stock solution of fucoidan was prepared by dissolving the compound in phosphate buffered saline (PBS) to 20 mg/ml and filtering it through membrane filter (pore size, 0.45 μm) (Millipore, Bedford, MA, USA). The stock solution was stored in aliquots prior to use.

2.2. Isolation of porcine PBMCs and PMNs

Healthy 6-month-old crossbred pigs from a slaughterhouse (FarmStory, Cheongju, Korea) were used as blood donors. Porcine peripheral blood was drawn into a heparinized container from the jugular vein, diluted with an equal volume of PBS without calcium and magnesium, and overlaid on an equal volume of Percoll solution (specific gravity, 1.080; Sigma-Aldrich Co.). After centrifugation for 45 min at room temperature, the PBMCs at the interface between the plasma and Percoll solution were harvested and washed three times with PBS. To lyse remaining erythrocytes, the cells were treated with 0.83% NH_4Cl in Tris buffer (pH 7.2) for 5 min. PMNs were collected from the upper layer of erythrocytes after removal of the PBMC layer. To purify PMNs, the erythrocytes were allowed to sediment for 60 min in 1.5% dextran of molecular weight, 200,000, in PBS. The floating cells were gently harvested and pelleted by centrifugation for 5 min. The residual erythrocytes were treated with solution of 0.83% NH_4Cl. The resulting PBMCs and PMNs were washed with PBS. The viability of cells, as determined by exclusion of 0.5% Trypan blue (0.5%) dye, always were > 98%. The cells were resuspended in RPMI 1640 medium containing L-glutamine (2 mM) and $NaHCO_3$ supplemented with gentamicin (0.02 mg/ml) and 5% heat-inactivated FBS.

2.3. Culture supernatants

PBMCs adjusted to 3×10^6 cells/ml were incubated with fucoidan (200 μg/ml) and 0 or 0.5 μg/ml LPS. Control cells were treated with the corresponding volume of PBS. After incubation of 24 h at 37 °C in a 5% CO_2-humidified atmosphere, the supernatants were centrifuged at 900 *g* for 10 min, passed through a 0.2 μm membrane filter, and stored at −78 °C until used.

2.4. Viability assay

Porcine PBMCs and PMNs (3×10^6 cells/ml) were incubated with or without fucoidan (0–500 μg/ml) in the absence or presence of LPS (0–1.0 μg/ml) in 24-well plates for 24 h. Cell viability was determined by Trypan blue exclusion.

2.5. Measurement of phagocytic capacity of PMNs

PMNs (5×10^5 cells/ml/well) in each well of a 24-well plate received 100 μl of cells adjusted to 5×10^6 cells/ml were incubated for 12 h with fucoidan or the culture supernatant described above. Flow cytometric analysis of phagocytosis was performed as previously described with slight modification (Song et al. 2007). Briefly, twenty microliters of carboxylate-modified polystyrene-latex beads (5×10^8 beads/ml; bead size, 2.0 μm) were added to each well for the final 1 h of culture. PMNs incubated without carboxylate-modified polystyrene-latex beads served as negative controls. The cultured cells were gently harvested, centrifuged at 400 *g* for 3 min at 4 °C, and washed with PBS containing 3 mM EDTA. The cells were then fixed by resuspension in PBS containing 1% paraformaldehyde. Phagocytic capacity was measured on a FACS Calibur (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) flow cytometer using the CELLQuest software. The cells were gated by forward and side scatter characteristics, and carboxylate-modified polystyrene (yellow-green) fluorescence was monitored. The argon laser was set to emit an excitation wavelength of 488 nm, and emission was measured between 515 and 560 nm. Each sample comprised at least 10,000 cells. The results were expressed as percentages of absolute phagocytic capacity.

2.6. Measurement of TNF-α in the culture supernatant from PBMCs

Culture supernatants of PBMCs treated with or without fucoidan (200 μg/ml) and LPS (0.5 μg/ml) were collected after 24 h incubation. *TNF-α* levels in the culture supernatants were determined by direct sandwich enzyme-linked immunosorbent assay (ELISA) using the porcine *TNF-α* kit (RayBiotech). All samples including standards and controls prepared in in triplicate were assayed. Optical density at 450 nm was measured on an automate microplate reader (Elx808, Bio-Tek Instruments Inc., Winooski, VT, USA). *TNF-α* levels in the samples were quantified based on standard curves generated using purified porcine *TNF-α* at eight concentrations. The lower and upper detection limits were 14 and 10,000 pg/ml, respectively.

2.7. Real time-polymerase chain reaction (PCR)

Total RNA was extracted using the Trizol® reagent (Invitrogen, Carlsbad, CA, USA). The concentration of total RNA was measured at 260 nm absorbance. The complementary DNA (cDNA) which are synthesized from a single stranded RNA was prepared from total RNA (1 μg) with Moloney Murine Leukemia Virus RT (Invitrogen) and random 9-mer primers (Takara Bio, Otsu, Shiga, Japan). Reactions for quantification of *1A* and target genes were run for 40 cycles. PCR was performed in a 20 μl volume containing cDNA, 1 unit of Taq polymerase (iNtRON Biotechnology, Inc., Sungnam, Korea), deoxyribonucleotide triphosphate (2 mM), and specific primers (10 pmol). The fluorescence intensity of threshold for all sample was set manually. The number of reaction cycle at which the PCR products exceeded the threshold was defined as the threshold cycle (C_T) in the exponential phase of product amplification. Primer sequences were as follows: *TNF-α*, 5′-ACC TCA ACA AAG CTC TCA GC-3′ (sense) and 5′-CGG GAA AAC TCC AAG ATG CT-3′ (antisense); and *1A*, 5′-CAC CGT AGG AGG TCT AAC G-3′ (sense) and 5′-GTA TCG TCG AGG TAT TCC G-3′ (antisense). Data for each sample were analyzed by comparing C_T values at constant fluorescence intensity. The amount of transcript was inversely proportional to the observed C_T. Relative expression (R) was calculated with the following equation: $R = 2^{-\ [\Delta CT\ (sample) - \Delta CT\ (control)]}$. The expressions of target genes were quantified against of *1A* gene expression.

2.8. Statistical analyses

Statistical analyses were performed by GraphPad Prism 6 for Windows (GraphPad Software, La Jolla, CA, USA). One-way analysis of