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Immune responses in the Japanese pufferfish (*Takifugu rubripes*) head kidney cells stimulated with particulate silica

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ABSTRACT

Studies on immune response to crystal silica in mammals indicate immune stimulation effect of environmental parameters including silica or asbestos, but there is no information on this aspect in lower vertebrates. Therefore, we examined expression of cytokine genes related to innate immunity in the Japanese pufferfish, *Fugu* (*Takifugu rubripes*) head kidney (HK) cells stimulated with particulate silica at 10 and 50 μg mL^{-1}. Expression of eleven cytokine genes was analyzed by the multiplex RT-PCR method (GenomeLab Genetic Analysis System, GeXPS; Beckman Coulter Inc.). Additionally, to confirm functionality of activated inflammatory immunity, we assessed phagocytic activity. Expression of NLR family genes as potential sensor molecules of inflammasome and inflammasome-associated genes (ASC and caspase-1) was also confirmed in HK cells by quantitative real-time PCR (qRT-PCR). As a result, an increased gene expression of pro-inflammatory cytokines (IL-6, IL-17A/F3, TNF-α, TNF-β and IFN-γ) and other cytokines (IL-4/13A, IL-4/13B, Type I-IFN) was recorded in particulate silica stimulated HK cells. Moreover, phagocytic activity showed a tendency to significantly increase in stimulated monocyte of HK cells after 6 h. Expression of NLR-C9 and NLR-C12 genes significantly increased in silica-stimulated HK cells. The particulate silica also significantly induced expression of inflammosome-associated genes, which may relate to the induced NLR-Cs.

1. Introduction

Environmental danger factors (*e.g.*, particulate silica, asbestos, and aluminum salt) and danger/damage-associated molecular patterns (DAMPs) including various endogenous danger signals stimulate innate immune system [1,2], and among these, particulate silica and aluminum salt have an effect to induce specific immunity through activation of the innate immunity. In mammals, experiments on immune response induced by the danger factors have been conducted to understand the relationship between danger signals and immune stimulation through environmental parameters including exogenous and endogenous elements such as silica, asbestos, hydroxyapatite, microbial toxins, cholesterol, ATP and other danger components. In addition, DAMPs of endogenous origin have a potential to induce an inflammatory reaction that is indispensable to an autoimmune response [3]. “Silica” or silicon dioxide (SiO_2) exists in the form of a crystalline material (*e.g.*, particulate silica) or the amorphous substance as a compound on the earth in soil, sand and rock.

The particulate silica exerts a stress and causes damage as an environmental nano-particle, which induces release of cytokine or growth factor from macrophages and epithelial cells. In mammals, silica and alum stimulate macrophages to produce the caspase-1-dependent cytokines, interleukin (IL)-1β and −18 by activating an inflammasome [4–7]. The inflammasome is comprised of three molecules, namely nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs; *i.e.* the sensor molecules including NLRP1, NLRP3, NLRP6, NLRP12 and NLRC4), apoptosis-associated speck-like protein containing a caspase activation and

Abbreviations: NLR, nucleotide binding oligomerization domain (NOD)-like receptor; ASC, apoptosis-associated speck-like protein containing a caspase activation and recruitment domain; NF-κB, nuclear factor-κB; multiplex RT-PCR, multiplex reverse transcription-polymerase chain reaction; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; TGF, transforming growth factor.

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Table 1
Oligonucleotide sequences used for qRT-PCR analysis

Gene	Accession number	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Product size (bp)
NLR-C1	ENSTRUG00000000100[a]	CACAGAAATACGCGGCTTCA	TGGGATCAGACACATGACGT	136
NLR-C2	ENSTRUG00000001210[a]	AGTCAACGTGCTCCTGATGA	TGTCAACACACGATGGAGGA	113
NLR-C3	ENSTRUG00000002199[a]	CAGATTCCTCCCTCGTGTGT	GGAGGCCTTGATGTGTGAGA	135
NLR-C4	ENSTRUG00000002379[a]	GGTATCGTCAGTTGAGGTGC	ACACACGAGGGAGGAATCTG	117
NLR-C5	ENSTRUG00000002476[a]	GCAGCCAATCAGATTCCTCCATCA	ATGATGTGGAGGCTCCTGGAGGCCTTGAG	161
NLR-C6	ENSTRUG00000002649[a]	GATGGCCTGGATGAAAGCAG	GGTGATCCAGATGAGAGCTG	144
NLR-C7	ENSTRUG00000004765[a]	TTTTATCAGAGACCAGCTGTGGTTC	GCTGGTTGTAGCTCAGTTTTAGAAT	90
NLR-C8	ENSTRUG00000005898[a]	GGAGAGCACTAATGAAATCTCTCA	GGATGGTTTCTGGGTGGTTCTCCA	143
NLR-C9	ENSTRUG00000006039[a]	TAAGGTTCAGTGACGAAGATCTGAG	AAGCTCCTCTCTCTCGTCCGTGGAG	153
NLR-C10	ENSTRUG00000008094[a]	ACAACACATCAGCAGAGGGA	TGAGGCCACGACTTCACAG	113
NLR-C11	ENSTRUG00000013076[a]	GAACCTGCGGCAGTACAAAA	GGGTTTGACGTCATTGCTGA	117
NLR-C12	ENSTRUG00000014811[a]	GTCCAGACCACAAAGGCGAA	GTCGGACTGATAGAAGACAA	150
NLR-C13	ENSTRUG00000010250[a]	ACAGGATCTTGACTCATGTCCT	AGATCCCACCTGTCTGTCCCTC	143
NOD2	NP_001035913.1[b]	CGACTAGGCCAGCTTGCCTTTA	GCGAAGAAACACTGCATGGTCAC	188
NLRC3	ENSTRUG00000015221[a]	GACGTGTTTGTGCGCTTTCTAGCG	GTACGAAGAGACACAACTGCACCT	164
NLRX1	XM_003976996[b]	CACATGTGCCACTCTGCATTGCC	GCATCAAAGAACTGTGGTGCTCC	167
ASC	NW_004071954[b]	GTGCTCTGATCAACAGGGTG	CAT GTT GGC AGA CCT CTG AT	109
Caspase-1	XM_003968404[b]	GACGTTTGAAGATGACCTCTTC	TGCAGGCCTGGATGATGATGAT	110
β-actin	U37499[b]	CCAGAAAGACAGCTACGTTGG	GCAACTCTCAGCTCGTTGTAG	147

[a] Ensembl genome browser ID.
[b] GenBank database accession number.

recruitment domain (ASC) and pro-caspase-1, and it functions for recognition of some of the pathogen associated molecular patterns (PAMPs), and finally cleaves pro-IL-1β and −18 to mature them in the cytoplasmic area [8]. However, the NLR orthologs as the sensors for inflammasome is still unknown in vertebrates other than mammals. Since the diversity of NLR family in teleosts is very different from that of mammals [9,10], it is difficult to find the mammalian counterparts of the inflammasome sensors such as NLRP1, NLRP3, NLRP6, NLRP12 and NLRC4 in fish.

To date, more than 100 cytokine molecules have been identified in human. These cytokines are known to play an important role in immune response of host innate defense mechanism [11]. Cytokines include ILs, tumor necrosis factors (TNFs), transforming growth factor (TGF), chemokines and interferons (IFNs) produced by different cells including macrophages, lymphocytes, granulocytes, dendritic cells (DCs) and epithelial cells, and have pro-inflammatory, anti-inflammatory and pathogen-killing properties [12]. Cytokines play an important role in the immune system by binding to specific receptors at the cell membrane, setting off a cascade that leads to induction, enhancement or inhibition of a number of cytokine-regulatory genes in the nucleus [13]. However, comprehensive gene expression patterns of cytokines after stimulation with the environmental factors such as silica nanoparticles are still not clear in lower vertebrates including teleosts, and it is important to understand the effects of environmental substances as danger components to the host innate immunity.

In the present study, we focused on understanding expression of pro-inflammatory cytokines, NLR family and inflammasome-associated genes in the Japanese pufferfish, *Fugu* (*Takifugu rubripes*) head kidney (HK) cells stimulated with particulate silica *in vitro* using the multiplex RT-PCR and quantitative real-time PCR (qRT-PCR). Additionally, to confirm functionality of the inflammatory response induced by silica, we also assessed phagocytic activity in the particulate silica-stimulated HK cells.

2. Materials and methods

2.1. Experimental fish, HK cells stimulation by silica and RNA extraction

Fugu (300 ± 5 g; n = 3) were obtained from the local fish farm (Matsumoto Fisheries Farm, Miyazaki, Japan) and the fish were first acclimatized in an aerated saltwater tank at 20 °C and fed a commercial diet (Sango, Higashimaru Co., Ltd., Kagoshima, Japan) for a week under a natural photoperiod prior to their use in this study. This experiment was conducted according to the relevant national and international guidelines, 'Act on Welfare and Management of Animals' (Ministry of the Environment, Japan). Isolation of HK and cell preparation were performed as per the previous protocol [14]. Viable cells in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS) and 1% streptomycin/penicillin (Invitrogen) were adjusted to 1×10^7 cells mL^{-1} after enumeration using trypan blue stain (Life Technologies, NY, USA) and seeded in wells of a 24-well plate (Nunc A/S, Roskilde, Denmark). The cells were treated with particulate silica (Junsei Chemical Co., LTD, Tokyo, Japan) at 10 or 50 μg mL^{-1} final concentrations and incubated for 1, 6, 12, 24 and 36 h at 25 °C. A control (cells without stimulation) was maintained for each time point. Each treatment and control had three replicates. Incubated cells were harvested at the time points mentioned above, submerged in RNAlater solution (Ambion, Austin, TX, USA) for overnight and finally stored at −80 °C prior to RNA extraction.

Total RNA was extracted from the stimulated HK cells using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. RNA samples were treated with a recombinant DNase I (RNase-free) for removal of genomic DNA, as per the manufacture's protocol (Takara Bio Inc., Shiga, Japan). All RNA samples were checked quantitative and qualitatively in a NanoDrop spectrometer, ND-1000 (Thermo Scientific, Wilmington, DE, USA).

2.2. Multiplex RT-PCR and capillary electrophoresis

In this study, a multiplex reverse transcription-polymerase chain reaction (RT-PCR) assay (GenomeLab Genetic Analysis System, GeXPS; Beckman Coulter, Inc., Brea, CA, USA) was used to analyze expression of 19 cytokine genes simultaneously from a single reaction tube. RNA samples of the stimulated HK cells (1, 6, 12 and 24 h) were used in the assay. Primer design (19 cytokine plex) and multiplex analysis were conducted using the multiplex assay panel described previously [15]. The multiplex RT-PCR was performed using 19 cytokine universal primers adjusted to amplify