Modulatory Roles of Interferon-γ through Indoleamine 2, 3-dioxygenase Induction in Innate Immune Response of Dental Pulp Cells

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Abstract

Introduction: Marked infiltration of inflammatory cells such as activated T cells producing interferon-γ (IFN-γ) is observed in severe pulpitis. However, the roles of IFN-γ in the innate immune response of dental pulp have not been reported. Indoleamine 2, 3-dioxygenase (IDO) is a regulator of immune responses, and the IDO expression is induced by IFN-γ in many cells whose expression in dental pulp is unknown. The purpose of this study was to determine the role of IFN-γ in the immune response through microbial pattern recognition receptors (PRRs) such as Toll-like receptors or nucleotide-binding oligomerization domain–like receptors on the production of proinflammatory cytokines such as CXCL10 and interleukin (IL)-6 and the expression of IDO in cultured human dental pulp cells (HDPCs). **Methods:** HDPCs were established from explant cultures of healthy pulp tissues. CXCL10 and IL-6 production was determined using enzyme-linked immunosorbent assay. Confirmation of IDO localization in dental pulp tissues was examined using immunohistochemistry. IDO expression in HDPCs was analyzed by immunoblot. **Results:** IFN-γ significantly up-regulated CXCL10 and IL-6 production in the HDPCs stimulated with ligands for PRRs in a concentration-dependent manner. The expression of IDO was detected in inflamed pulp tissue. In addition, IFN-γ in combination with the PRR ligands enhanced IDO expression in HDPCs compared with IFN-γ alone. Moreover, CXCL10 production in IFN-γ–stimulated HDPCs was inhibited by an IDO inhibitor. **Conclusions:** This study showed the synergistic effects by IFN-γ on cytokine production and IDO expression in HDPCs, suggesting that IFN-γ may modulate the innate immune response of dental pulp. *(J Endod 2014;40:1382–1387)*

Key Words

CXCL10, indoleamine 2, 3-dioxygenase, interleukin6, nucleotide-binding oligomerization domain, pattern recognition receptors, pulpitis, Toll-like receptors

With advanced dental caries, the infiltration of lymphocytes and the production of various inflammatory mediators become apparent, and, thus, the inflammatory reaction in dental pulp develops into irreversible status (1, 2). T cells are most likely the predominant lymphocyte population in inflamed dental pulp tissue (1, 3); many T helper 1 cells are distributed in pulpitis tissue with both shallow and deep carious lesions (4). Moreover, it is reported that the amount of interferon gamma (IFN-γ) produced from activated T helper 1 cells was predominantly higher than interleukin (IL)-4 produced from T helper 2 cells in a rat pulpitis model (5). We also reported that the T cells expressing CXCR3, a receptor of CXCL10, which is a member of the IFN-inducible non-ELR CXC chemokine family and is participating in the pathogenesis of various diseases (6–8), were detected in the inflamed pulp tissues (9). Generally, the initial sensing of microbial pathogens is mediated by pattern recognition receptors (PRRs) for pathogen-associated molecular patterns. Microbial PRRs, such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)–like receptors are essential for the mammalian innate immune response. Among TLRs, TLR2 recognizes a wide range of pathogen-associated molecular patterns including peptidoglycan (PGN) of gram-positive bacteria, whereas TLR4 is involved in the recognition of most bacterial lipopolysaccharides (LPSs). On the other hand, NOD exists in cytoplasm, and 2 types of NODs are known (10). NOD1 mainly recognizes the diaminopimelic acid structure of PGN from gram-negative bacteria (11), and NOD2 is reported to recognize the structure muramildipeptide (MDP), a minimal motif that is present in all PGNs (12). We previously reported that dental pulp cells, as a major cell type in the dental pulp, express several types of TLRs and NODs, stimulating the production of CXCL10 and proinflammatory cytokines such as IL-6 and suggesting that these receptors play important roles in the enhancement of pulpitis (13). However, the role of IFN-γ in the innate immune response of dental pulp cells has not been reported.

Indoleamine 2, 3-dioxygenase (IDO) is known as an enzyme that catalyzes the first and rate-limiting step of the conversion of tryptophan, the least available essential amino acid, to kynurenine as the main tryptophan metabolite. IDO expression is found in many tissues such as the human lung, small intestine, and placenta and is up-regulated when the tissue is under infection and inflammation (14, 15). In infection, IDO activity was first described as a mechanism for stopping the growth of microorganisms (16). Thereafter, it is reported that IDO can prevent exacerbation by the regulation of T-cell activity in the inflammatory disorder model (17, 18). Moreover, IDO expression is inducible in different cell types such as dendritic cells, macrophages, and fibroblasts (19–21) upon exposure to IFN-γ, which is a potent inducer of IDO, and dendritic cells and macrophages expressing IDO can suppress T-cell responses and promote tolerance (20). In addition, the enzyme activity of IDO in infected cells or tumor cells goes up

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100 times by IFN-γ stimulation (22, 23). Taken together, these observations show that IDO can have an immunoregulatory role. Regarding dental pulp inflammation, IDO may also contribute to the pathogenesis of pulpitis in relation to IFN-γ produced within dental pulp tissues. However, there are no reports concerning IDO in dental pulp disease. The purpose of this study was to determine the role of IFN-γ in the innate immune response on proinflammatory cytokines such as CXCL10 and IL-6 productions and IDO expression in cultured human dental pulp cells (HDPCs) stimulated with PRR ligands.

Materials and Methods

Reagents

Pam3CSK4 and γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) were purchased from InvivoGen (San Diego, CA). *Escherichia coli* LPS and MDP were purchased from Sigma-Aldrich (Walkersville, MD). IFN-γ was purchased from Roche (Mannheim, Germany).

Human Dental Pulp Tissue Samples

Inflamed dental pulp tissue samples were obtained from 5 carious third molars with irreversible pulpitis caused by carious exposure of the pulp and showing spontaneous pain and/or lingering pain in response to a cold and/or heat stimulus. Clinically, healthy dental pulp tissue samples were obtained from 7 noncarious teeth extracted for orthodontic reasons. These tissue samples were obtained under informed consent at Tokushima University Hospital, Tokushima, Japan. The medical histories of all patients in this study were noncontributory. Inflamed pulps were used for immunohistochemical analysis, and healthy pulps were used for cell culture ($n = 4$) and immunohistochemistry ($n = 3$). The study was performed with the approval from and compliance of the Tokushima University Ethical Committee (No. 329).

Cell Culture

HDPCs were established from explant cultures of healthy pulp tissues as previously described (9). In brief, minced pieces of pulp tissues were seeded into 35-mm culture dishes and cultured in Dulbecco modified Eagle medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS), 1 mmol/L sodium pyruvate (Gibco), and 100 U/mL penicillin/100 μg/mL streptomycin (Gibco) at 37°C in a humidified atmosphere of 5% CO_2. Confluent primary cultures were harvested and subcultured. The HDPCs obtained in this manner were used for experiments at passages 5–10.

Treatment of Dental Pulp Cells with PRR-specific Ligands and/or Interferon-γ

HDPCs were seeded in wells of a 96-well tissue culture plate and incubated until a confluent monolayer developed (1×10^4 cells/well). The media were then replaced with DMEM containing 2% FBS for 24 hours. To determine the effects of IFN-γ on cytokine production in HDPCs stimulated with PRR ligands, the HDPCs were incubated with Pam3CSK4 (TLR2 ligand), LPS (TLR4 ligand), iE-DAP (NOD1 ligand), and MDP (NOD2 ligand) with or without IFN-γ in 2% FBS-containing DMEM for the designated periods of times (ie, 12 or 24 hours). After incubation, culture supernatants were collected and used to determine the quantities of CXCL10 and IL-6.

Enzyme-linked Immunosorbent Assay

The concentrations of CXCL10 and IL-6 in the cell culture supernatants were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Duo Set ELISA Developing System; R&D Systems, Minneapolis, MN).

Immunohistochemistry

Formalin-fixed, paraffin-embedded 6-μm-thick serial tissue sections were treated with 3% H_2O_2 for 5 minutes to eliminate intrinsic peroxide. After the blocking procedure with Protein Block Serum-free (Dako, Carpinteria, CA), sections were treated with rabbit polyclonal antihuman IDO antibodies (Alexis Biochemicals, San Diego, CA) overnight at 4°C. Sections were incubated with Envision + Dual Link System–horseradish peroxidase (Dako) for 30 minutes. The reaction was visualized by 3,3′-diaminobenzidine tetrahydrochloride (Dako). Sections were counterstained with hematoxylin and mounted with Entellan Neu (Merck KGaA, Darmstadt, Germany). Normal rabbit immunoglobulin (Dako) was used as the negative control.

IDO Expression in Dental Pulp Cells

HDPCs seeded into a 6-well tissue culture plate (2×10^5 cells/well) were stimulated with Pam3CSK4 (10 ng/mL), LPS (0.1 μg/mL), iE-DAP (10 μg/mL), and MDP (10 μg/mL) with or without IFN-γ (0.1 ng/mL) for 24 hours. The stimulated HDPCs were collected with RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA). The protein concentrations in lysates were quantified with a bicinchoninic acid protein assay kit (Sigma-Aldrich). An equal amount of protein underwent 5%–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by electrotransfer to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was first incubated with mouse monoclonal antihuman IDO antibodies (Millipore). After washing, the membrane was reacted with horseradish peroxidase–conjugated secondary antibody (Dako). Protein bands were finally visualized on x-ray film using the ECL system (GE Healthcare, Buckinghamshire, England). An equal loading of gels was confirmed by immunoblot with antiactin antibody (Sigma-Aldrich) as an internal control.

Analysis of the Effects of IDO Inhibitor on Dental Pulp Cells Stimulated with IFN-γ

HDPCs seeded into a 96-well tissue culture plate (1×10^4 cells/well) were exposed with 1-methyl-DL-tryptophan (MT) (an inhibitor of IDO) with or without IFN-γ for 24 hours. Thereafter, the concentrations of CXCL10 in the cell culture supernatants were determined using ELISA.

Statistical Analysis

All statistical analyses were performed with the unpaired Student *t* test. Differences were considered significant when the probability value was less than 5% ($P < .05$).

Results

Effects of IFN-γ on Proinflammatory Cytokine Production in HDPCs Stimulated with PRR Ligands

To determine the role of IFN-γ in the innate immune response, HDPCs were stimulated with PRR ligands with or without IFN-γ for the designated periods of time, and the concentration of CXCL10 and IL-6 in the supernatant was measured using ELISA. In IFN-γ–stimulated HDPCs, CXCL10 but not IL-6 production was up-regulated in a concentration-dependent manner. Both Pam3CSK4 and LPS induced the production of CXCL10 and IL-6. Moreover, in combination with Pam3CSK4 or LPS, IFN-γ synergistically induced not only CXCL10 but also IL-6 production (Fig. 1*A–D*). In contrast, neither iE-DAP nor