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Toll-like receptor 9 is expressed on follicle-associated epithelia containing M cells in swine Peyer's patches

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Abstract

The precise distribution and expression of Toll-like receptor (TLR) 9 in gut-associated lymphoid tissues (GALTs) has not been elucidated. In this study, we investigated the expression pattern of TLR9 in adult and neonatal swine GALTs by real-time quantitative PCR, western blot, confocal laser microscopy and flow cytometric analysis. The swine TLR9 gene was preferentially expressed in adult Peyer's patches (Pps) and mesenteric lymph nodes (MLNs), which contained approximately three times higher TLR9 than the spleen. Other tissues exhibited only weak expression of TLR9. In neonatal swine, elevated expression of TLR9 was detected only in MLNs. We firstly showed that highly expressive ($TLR9^+$) cells were formed in Pps and MLNs. In addition, $TLR9^+$ cells were present not only in immune cells such as dendritic cells and B cells but also in follicle-associated epithelia (FAE) including membranous cells (M cells) in Pps. These results suggest that Pps and MLNs provide the host defense with the ability to respond to a variety of bioactive oligonucleotides (ODNs) from bacteria at a conductive site of initial immune responses.

Keywords: FAE; M cells; MLNs; Peyer's patches; Swine; Toll-like receptor 9

1. Introduction

The Toll-like receptor (TLR) family responds to pathogen-associated molecular patterns (PAMPs) expressed by a diverse group of infectious microorganisms, thereby triggering the host's innate immune system [1–4]. TLR9, which is critically involved in the recognition of 5′-cytosine unmethylated CpG dinucleotides motifs [5] and also AT oligonucleotides [6], is expressed in plasmacytoid dendritic cells (pDC, $CD123^+$) but not in myeloid DC (mDC, $CD11c^+$) [7]. CpG DNA motifs induce high amounts of tumor necrosis factor-α (TNF-α) and interleukin-12 (IL-12), leading to Th-1 dominated immune responses [8].

Our previous studies have suggested that immunobiotic lactic acid bacteria (LAB) and their components exert health-promoting effects in the gut [9–11]. Gut mucosal surfaces form a mechanical barrier that separates the host from the external environment. In the gastrointestinal tract, epithelial cells exert an important role in the absorption, ion transport, secretion, and uptake of these functional molecules via M cells (membranous cells) in follicle-associated epithelia (FAE), and they may be involved in the first-line defense against luminal factors including pathogenic or non-pathogenic bacteria [12–14]. However, it is not known whether TLR9 is expressed in M cells. Therefore, in the present study, we investigated the expression of TLR9 in neonatal and adult swine GALTs, which are expected to be useful as a human model. We used real-time quantitative PCR to evaluate the expression of TLR9 mRNA in swine tissues. In addition, we investigated swine TLR9 (sTLR9) protein

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expression in swine GALTs by immunohistochemical analysis using a specific antibody that recognizes sTLR9 and cytokeratin 18 as a marker of M cells.

2. Materials and methods

2.1. Experimental tissues

Experimental tissues (heart, thymus, lung, spleen, liver, kidney, skeletal muscle, duodenum, jejunum, ileum, ileal Pps (adult only) and MLNs) were prepared from two neonatal (LWD, Hiruzu Co. Ltd., Miyagi, Japan) and three adult (LWD, 1-year-old) swine.

2.2. Real-time quantitative PCR

Total RNA was isolated from various swine tissues. All cDNAs were prepared by reverse transcription from 1 μg of total RNA using Oligo $d(T)_{18}$ primer (Invitrogen Co., Carlsbad, CA, USA). An equivalent volume of cDNA solution (5 μl) from each sample was used for the quantification of sTLR9-specific cDNA by real-time quantitative RT-PCR. Real-time quantitative RT-PCR reactions were performed on the LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics, Tokyo, Japan) using specific primers. The β-actin primers were designed based on the DNA sequence of swine β-actin (sβ-actin) [15,16]. Fragments were obtained by PCR amplification using the following primers: sTLR9: sense 5′-GTG GAA CTG TTT TGG CAT C-3′; antisense: 5′-CAC AGC ACT CTG AGC TTT GT-3′, sβ-actin: sense 5′-TGG CAT TGT CAT GGA CTC TG -3′; antisense 5′-AGG GGC GAT GAT CTT GAT CT-3′. The PCR cycling conditions were 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, 10 s at 60 °C and 5 s at 72 °C. The real time PCR for sTLR9 and sβ-actin were performed with the above primers separately. In the control tubes, poly(A) + RNA samples were used as templates to check for the presence of contaminating genomic DNA. The sensitivity for the reaction and amplifications of contaminant products such as primer dimers, indiscriminately detected by the SYBR green chemistry, were evaluated by amplifying the serial dilutions of the cDNA. Sequencing analysis showed that the amplified cDNA was identical to bases 2496–2694 of sTLR9.

2.3. Anti-swine TLR9 polyclonal antibody

Swine TLR9 protein was analyzed using GENETYX-SV/RC (11.0.3.1) in order to predict the secondary structure and antigenicity, and the amino acid residue CPKDHPKLHSDTFSHLS (269–285 of sTLR9 including epitope (KDHPKLH)) in the extracellular domain was subsequently chosen for peptide synthesis. The synthetic polypeptide was used for antibody production as an antigen at a dose of 0.3 mg/rabbit. After two boosters at monthly intervals, rabbits were bled and the anti-sera were collected followed by purification by peptide affinity chromatography. High antibody titers for sTLR9 were obtained, which were detected by a direct ELISA analysis (data not shown).

2.4. Western blot analysis

Intestinal organs (duodenum, jejunum, ileum, Pps and MLNs) were isolated from adult swine, suspended in phosphate-buffered saline (PBS), homogenized, and sonicated. The homogenates were suspended in lysis buffer (CelLytic™-M, SIGMA, Tokyo, Japan) containing protease inhibitor cocktail (SIGMA). The lysates were centrifuged at 17,000 × *g* for 20 min at 4 °C and the supernatants were precleared with goat anti-rabbit IgG antibody coupled to agarose at 4 °C for 4 h (SIGMA). After the treatments, the supernatants were incubated with the anti sTLR9 antibody at 4 °C for 16 h, and then with anti-rabbit IgG antibody coupled to agarose at 4 °C for 4 h. After washing, immunoprecipitated proteins were eluted by boiling in a SDS-PAGE loading buffer (Wako, Sendai, Japan) and then separated by 8% (v/v) SDS-PAGE and transferred to poly vinylidene difluoride (PVDF) membrane (Hybond-P, Amersham, UK). The membrane was probed with the anti-sTLR9 antibody, developed with alkaline phosphatase-conjugated anti-rabbit IgG and enhanced with chemifluorescence (ECF Substrate, Amersham).

2.5. Immunohistochemical analysis

Fresh tissues were washed with PBS and fixed in Zamboni's solution (0.2% saturated picric acid and 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) [17] for 16 h at 4 °C. Fixed tissues were washed for 48 h with several changes of 0.1% arabic gum in 0.1 M Phosphate buffer (PB**)** containing sucrose (8% once and 16% once, each for 24 h), immersed in TISSUE TEK®O.C.T. compound (Sakura Finetechnical, Tokyo, Japan) and quickly frozen in a dry ice/acetone bath. Cryostat sections (5 μm thickness) were prepared from the frozen tissues, mounted on poly-L-Lysine coated glass slides, and washed in PBS containing 0.02% Tween 20 (PBST). The sections were incubated for 20 min with 1% bovine serum albumin and 1.5% normal goat serum (Vector Laboratories, USA) in PBST at room temperature (RT) to prevent non-specific binding of antibodies. After removal of the blocking solutions, anti-swine TLR9 polyclonal antibody (1:1600 dilution in 0.1 M PBS) and anti-cytokeratin 18 mAb (SIGMA; 1:1000 dilution in 0.1 M PBS) were applied as primary antibodies for 60 min at RT. After washing three times in PBST, Alexa488-conjugated goat anti-rabbit IgG and Alexa660-conjugated goat anti-mouse IgG (Molecular Probes Inc., Eugene, OR, USA; 1:1000 dilution in 0.1 M PBS) were applied as secondary antibodies for 30 min at RT. While shielding from direct light exposure, the sections were washed three times in PBST and stained with SYTOX orange (Molecular Probes Inc., Eugene, OR; 1:200,000 dilution in 0.1 M PBS) for 15 min to detect nuclei. Finally, the specimens were washed three times in PBST, immersed in Perma fluor aqueous mountant (Immunon™, Pittsburgh, PA,