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Porcine Toll-like receptors: Recognition of *Salmonella enterica* serovar Choleraesuis and influence of polymorphisms

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ABSTRACT

Salmonella enterica serovar Choleraesuis (SC) is a highly invasive pathogen that causes enteric and septicemic diseases in pigs. Although there have been some reports on gene expression profiles in the course of infection with SC in pigs, little is known about the genes involved in the infection. By measuring activation, as represented by nuclear factor-κB activity, after stimulation by the pathogen, we showed the involvement of Toll-like receptor (TLR) 5 and the TLR2–TLR1 heterodimer in the recognition of SC. We previously found single nucleotide polymorphisms (SNPs) in the TLRs of various pig populations. Here we demonstrated that the polymorphisms resulting in amino acid changes $TLR5^{R148L}$, $TLR5^{P402L}$, and $TLR2^{V703M}$ attenuated the responses to SC by the cells transfected with the TLR genes. Each of these three SNPs was differently restricted in distribution among breeds. These results suggest that there are differences in resistance to salmonellosis among breeds; these differences may be of great importance for the pig industry in terms of breeding and vaccine development.

1. Introduction

Toll-like receptors (TLRs) play crucial roles in innate immunity by recognizing conserved microbial components, and they have profound effects on adaptive immunity. TLRs are type I transmembrane glycoproteins characterized by extracellular domains containing variable numbers of leucine-rich-repeat (LRR) motifs, a single transmembrane domain, and a cytoplasmic signaling region composed mainly of a Toll/interleukin-1 receptor (TIR) domain (Akira and Takeda, 2004; Matsushima et al., 2007). TLRs are divided into two groups: those that recognize molecules such as proteins and lipids from bacteria and are expressed on the surfaces of immune cells (TLR1, TLR2, TLR4, TLR5, and TLR6); and those that sense nucleic acids from pathogens and are localized in intracellular compartments (TLR3, TLR7, TLR8, and TLR9) (Akira and Takeda, 2004). Polymorphisms in TLRs are associated with resistance and susceptibility to various diseases (Lazarus et al., 2002; Schröder and Schumann, 2005). In human TLR4, the cosegregating polymorphisms D299G and T399I have been found at a higher frequency among people hyporesponsive to inhaled lipopolysaccharide (LPS) than in a control population (Arbour et al., 2000). D299G polymorphism is also associated with increased risk of Crohn's disease, ulcerative colitis, or severe sepsis following burn injury (Barber et al., 2004; Franchimont et al., 2004). The R677W and R753Q polymorphisms in human TLR2 are associated with diseases resulting from infection with mycobacteria; R677W is associated with lepromatous leprosy caused by *Mycobacterium leprae* and R753Q is associated with tuberculosis caused by *Mycobacterium tuberculosis* (Bochud et al., 2003; Kang and Chae, 2001; Ogus et al., 2004). In human TLR5, the presence of a stop codon (R392stop) abolishes the ability to recognize flagellin and is associated with pneumonia caused by *Legionella pneumophila* (Hawn et al., 2003).

In our previous studies of single nucleotide polymorphisms (SNPs) in porcine *TLR* genes, we found that most of the nonsynonymous SNPs in the coding sequences of genes encoding TLRs expressed on the cell surface were present in the extracellular region involved in pathogen recognition, rather than the intracellular region (Morozumi and Uenishi, 2009; Shinkai et al., 2006;

Abbreviations: ELAM-1, endothelial leukocyte adhesion molecule-1; LRR, leucine-rich repeat; PRR, pattern recognition receptor; SC, *Salmonella enterica* serovar Choleraesuis; ST, *Salmonella enterica* serovar Typhimurium; TIR, Toll/interleukin-1 receptor; TLR, Toll-like receptor.

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Uenishi and Shinkai, 2009). This discovery prompted us to examine the influences of the resulting amino acid alterations on recognition of the pathogens that cause serious damage in the pig industry.

Salmonella spp. are ubiquitously present in nature and have been recovered from nearly all vertebrates (Edwards et al., 2002). Among more than 2500 different serovars, *Salmonella enterica* serovar Choleraesuis (SC) has a narrow host range and infects predominantly pigs and occasionally humans, whereas other serovars, such as *Salmonella enterica* serovar Typhimurium (ST) have broad host ranges (Chiu et al., 2004). SC can cause enterocolitis, pneumonia, septicemia, and hepatitis in pigs, and it is more serious than ST infection, which usually causes only enterocolitis. Human systemic infections caused by SC are considered to be acquired from pigs (Chiu et al., 2006).

Here, we examined porcine TLRs recognizing SC and demonstrated the involvement of TLR5 and the TLR2–TLR1 heterodimer. Furthermore, we identified some SNPs in *TLR5* and *TLR2* genes that affected induction of the response of the cells tranfected with the *TLR* genes after stimulation by the ligands and showed that each of these important SNPs existed in different porcine breeds. These results suggest the possibility of genetic improvement of disease resistance to salmonellosis in pigs.

2. Materials and methods

2.1. Cells and reagents

Human embryonic kidney (HEK) 293 cells (American Type Culture Collection [ATCC] CRL-1573) were maintained at 37 °C in Dulbecco's modified Eagle medium (Gibco/Invitrogen, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco/Invitrogen) in a 5% CO_2 incubator. Synthetic bacterial lipoprotein Pam_3CSK_4 and flagellin purified from ST were purchased from InvivoGen (San Diego, CA, USA). SC (ATCC 7001) cultured in LB medium to a concentration of 1×10^9 colony forming units (cfu)/ml were killed in 70% ethanol and suspended in phosphate-buffered saline.

2.2. Expression vectors

The coding regions of the porcine *TLR1*, *TLR2*, *TLR5*, and *TLR6* genes were amplified by PCR of the genomic DNA from Berkshire (*TLR1* and *TLR5*) and Large White (*TLR2* and *TLR6*) breed pigs with primers comprised of the most common nucleotide sequences in the European pigs (*TLR1*, GenBank ID: AB208695; *TLR2*, GenBank ID: AB208696; *TLR5*, GenBank ID: AB208697; *TLR6*, GenBank ID: AB208698) (Shinkai et al., 2006). PCR amplification was conducted by using PfuTurbo DNA Polymerase (Stratagene, La Jolla, CA, USA). Reference expression vectors were constructed by inserting the coding regions into pEF6/V5-His TOPO (Invitrogen, Carlsbad, CA, USA), as previously described (Bochud et al., 2003). Expression vectors encoding mutant proteins were produced by introducing each of the 20 SNPs in *TLR1*, 10 SNPs in *TLR2*, and 12 SNPs in *TLR5* into the above reference vectors by using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) in accordance with the manufacturer's instructions. A nuclear factor-κB (NF-κB)-dependent endothelial leukocyte adhesion molecule-1 (ELAM-1) firefly luciferase construct was generated by cloning five NF-κB binding sites (GGGACTTTCC × 5) (Schindler and Baichwal, 1994), followed by a fragment (−130 to +26 bp) of the human ELAM-1 promoter, into the luciferase reporter vector GL4.10 (Promega, Madison, WI, USA). Thymidine kinase *Renilla* luciferase vector (pGL4.74) for use as a control was purchased from Promega.

2.3. Luciferase reporter assay

HEK293 cells were transiently cotransfected, using FuGENE 6 Transfection Reagent (Roche Diagnostics, Mannheim, Germany), at a density of 6×10^4 cells per well in a 96-well plate format, with 100 ng of one of the *TLR* expression vectors, 100 ng NF-κB-dependent ELAM-1 firefly luciferase construct, and 20 ng thymidine kinase *Renilla* luciferase vector. In the case of the cotransfection of TLR2 and its heterodimer partner, TLR1 or TLR6, 50 ng of each of the vectors was used for transfection. After 24 h, the cells were stimulated by the ligands for 5 h. Luciferase activity was measured by using a Dual-Glo Luciferase Assay System (Promega). After the firefly luciferase units had been divided by the *Renilla* units, the values of stimulated cells were divided by those of unstimulated cells to show the degree of induction of NF-κB. All experiments were performed in triplicate wells three times to confirm reproducibility.

2.4. RT-PCR

Total RNA from HEK293 cells was extracted by using Isogen (Nippon Gene, Tokyo, Japan), and then treated with DNase I to eliminate DNA contamination. Human spleen total RNA was purchased from Ambion (Austin, TX, USA). cDNA was synthesized by using PowerScript Reverse Transcriptase (Clontech, Palo Alto, CA, USA). PCR was performed by using Advantage 2 Polymerase Mix (Clontech) and primers for amplification of human *TLR* genes (Kadowaki et al., 2001) and the human glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*), as follows: forward, 5′-GGAAGGTGAAGGTCGGAGTCAACGG-3′; and reverse, 5′-CCTGGAAGATGGTGATGGGATTTCC-3′. The PCR was conducted with 30 cycles consisting of 95 °C for 30 s and 68 °C for 1 min after incubation at 95 °C for 1 min.

2.5. Genotyping

Pig genomic DNA was extracted by using blood or tissue samples. We collected samples from 144 unrelated (i.e., at least two generations of kinship apart from each other) European commercial pigs (36 each of the Berkshire, Duroc, Landrace, and Large White breeds). We also used 24 Chinese pigs (12 each of the Jinhua and Meishan breeds) and 12 each of Clawn miniature pigs and Japanese wild boars. Genomic DNA from these samples was purified by a standard protocol based on phenol–chloroform extraction (Sambrook and Russell, 2001). SNPs were genotyped by sequencing the PCR products, including the corresponding nucleotides, as previously described (Shinkai et al., 2006).

2.6. Sequence alignment

Comparison of amino acid sequences of TLR5 of vertebrates was conducted by using ClustalX2 (Larkin et al., 2007). Amino acid alignment for prediction of 3D models of porcine TLR5 was done manually so that the typically conserved residues and topologically equivalent residues in LRRs were aligned.

2.7. Prediction of 3D models of porcine TLR5

The 3D structure of the ectodomain of porcine TLR5 was constructed by using MODELLER Release 9v8 (Šali and Blundell, 1993). The crystal structures of a human apo-TLR3-ectodomain (PDB ID: 1ZIW, 2A0Z), a mouse apo-TLR3-ectodomain (PDB ID: 3CIG), two mouse TLR3-ectodomains complexed with double-stranded RNA (PDB ID: 3CIY), a mouse TLR4-ectodomain complexed with MD-2 (PDB ID: 2Z64), and a human $(TLR4–MD-2–LPS)_2$ duplex-heterodimer (PDB ID: 3FXI) were used as templates (Bell et al., 2005; Choe et al., 2005; Kim et al., 2007; Liu et al., 2008; Park