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Development of molecular immunoevaluation system for probiotics

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Research aims

There is currently growing interest in the worldwide studies on physiologically functional foods and feeds that can improve human and animal health. In Japan, the Food for Specified Health Uses, so-called FOSHU, have been authorized by the Ministry of Health, Labor and Welfare since 1993. To date, around 800 products have been approved as FOSHU in several categories. Among them, particularly many kinds of fermented products containing probiotics, which are expected to improve human intestinal microbial balance, are widely available in the market. However, immunologically functional foods containing probiotics have not been approved as FOSHU due to the lack of a unified evaluation standard for immunological activity even if there were many reports suggesting their immunological effects. In this study, we tried to develop a certain molecular immunoevaluation system for immunobiotics derived from probiotics. For this purpose, we established an evaluation system using the pattern recognition receptors (PRRs), which are known to play an essential role in innate immunity. In particular, we used porcine PRR instead of mouse PRR, since swine is phylogenetically more close to human than mice.

Methods

The complementary DNAs (cDNAs) encoding porcine PRR were cloned from gut-associated lymphoid tissues (GALT) of adult swine (1–3). The first-stranded cDNAs were synthesized from the total RNA from porcine GALT by using the primers whose sequences were designed from multiple alignments of the conserved sequences of human and mouse counterparts. The PCR products containing the complete open reading frames of porcine PRR were sequenced and characterized. Human embryonic kidney (HEK) 293 cells were transfected with the mammalian expression vectors encoding porcine PRR by lipofection method (1–3). Expression of porcine PRR was confirmed by reverse transcription-polymerase chain reaction, flow cytometry and confocal laser microscopy (1–3). The nuclear factor-κB (NF-κB) reporter assay was performed as described elsewhere (1–3). Briefly, the porcine PRR-expressing transfectants were transfected with pGLM-ENH-luci vector, which encodes the human NF-κB gene A1 and A2 sites. After stimulation with the indicated stimulants, luciferase assays were performed according to the manufacturer's protocol (Promega, Tokyo, Japan).

Results

In the present study, cDNAs encoding porcine nucleotide-binding oligomerization domain-1 (NOD1), NOD2, and radioprotective 105 (RP105)/MD-1 were isolated from porcine GALT (Table 1). Comparison of the ORF sequences of porcine PRRs with those of human and mouse counterparts indicated that porcine PRRs are more similar to human than to mouse (Table 1).

In order to develop the molecular immunoevaluation system for immunobiotics, we constructed porcine PRR-overexpressing HEK 293 cells. In the expression analysis, we confirmed the expression of PRR in the transfectants at the nucleotide and protein levels. Using the porcine NOD-expressing transfectans, we evaluated the immunological response to low-molecular peptidoglycan (PGN) fragments that are essential components of gram-positive bacteria such as immunobiotic lactic acid bacteria. When stimulated by muramyldipeptide (MDP; *N*-acetylmuramyl-L-alanyl-D-isoglutamine, MurNAc-L-Ala-D-isoGln), there was a significant increase in NF-κB dependent luciferase activity in the NOD2-expressing transfectans (Fig. 1). In the analysis of

Table 1. Characterization of porcine PRRs (NOD1, NOD2, RP105 and MD-1)

PRRs	Length of ORF		Homology to human		Homology to mouse		Accession
	nu*[1] (bp)	aa*[2]	nu (%)	aa (%)	nu (%)	aa (%)	
NOD1	2862	953	86.7	83.8	80.9	79.2	AB187219
NOD2	3039	1013	84.4	81.6	77.9	76.6	AB195466
RP105	1986	661	84.1	77.6	75.3	70	AB190767
MD-1	480	159	77.7	76.5	72.8	71.1	AB190766

*[1] nu=nucleotides, *[2] aa=amino acids

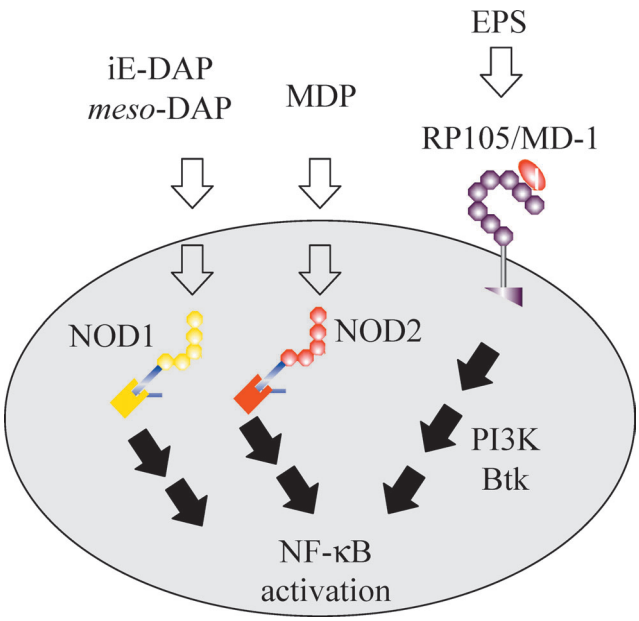

Fig. 1. Schematic representation of the recognition of immunobiotics and immunogenics by NOD1, NOD2 and RP105/MD-1.

NOD1-expressing transfectans, NF-κB was significantly activated by the stimulation with γ-D-glutamyl-*meso*-diaminopimelic acid (iE-DAP). Furthermore, we observed that *meso*-diaminopimelic acid (*meso*-DAP) has an ability to induce the immune activation via NOD1-mediated signaling pathway (Fig. 1).

We have reported that the extracellular phosphopolysaccharides (EPS) produced by *Lactococcus lactis* subsp. *cremoris* (*Lc. cremoris*) exerts immunological functions such as antitumor and immunostimulatory activities (4,5). Because the precise mechanisms by which the PRR contribute to the recognition of EPS have not been elucidated, we examined the involvement of RP105/MD-1 in cellular immune activation by EPS. In the NF-κB-dependent luciferase assay, EPS from *Lc. cremoris* strongly induced immune activation via RP105/MD-1 (Fig. 1). In addition, we observed that RP105/MD-1-mediated immune activaiton was involved in phosphatidylinositol 3-kinase and Bruton's tyrosine kinase pathway (Fig. 1). The results suggest that EPS from *Lc. cremoris* is a potent immunoregulator recognized by RP105/MD-1 complex.

Conclusion

Studies on the immunological functions of immunobiotic lactic acid bacteria and immunogenics have contributed to their worldwide application as functional foods, supplements and feeds (6,7). With respect to the health-promoting effects of immunobiotics, we believe that several structural factors of these immunobiotics are recognized by PRRs on innate immune cells in the GALT. In the present study, porcine PRRs were shown to be more close to human than mouse counterparts, supporting the notion that swine may be a good model for human intestinal immune system as compared with mouse. The clarification of the immune system mediated by porcine PRRs in the swine GALT is important to develop a model system for studies on human mucosal immune system.

In this study, we constructed the porcine NOD1-, NOD2-, or RP105/MD-1-expressing transfectants, and identified some immunoregulatory factors as novel immunogenics on the basis of the relationship between PRRs and their ligands. In our previous studies, we have cloned several porcine Toll-like receptor family members and analyzed the specificity of their ligands from immunobiotic lactic acid bacteria (8–13). Together with our previous and present studies, we successfully developed various kinds of porcine PRR-expressing transfectants as novel molecular immunoevaluation system, which are useful for understanding the immunoregulatory effects of individual immunogenics, and screening high potential immunobiotic lactic acid bacteria strains via PRR signaling. Our system may also provide important findings at the molecular level on PRR signal transduction pathways and recognition mechanisms for the specific ligands. Screening of immunobiotic lactic acid bacteria strains with certain immunogenics, and better understanding of intestinal immunoregulation should help in the development of not only immunologically functional foods but also oral vaccines that specifically target intestinal immune responses.

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