Oral Administration of EC-12 Increases the Baseline Gene Expression of Antiviral Cytokine Genes, IFN-γ and TNF-α, in Splenocytes and Mesenteric Lymph Node Cells of Weaning Piglets

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Weaning piglets are continuously exposed to various viruses. The antiviral effects of lactic acid bacteria (LAB) have been confirmed mainly in humans and mice, while few studies have been conducted in livestock. In this study, we evaluated the effect of oral administration of Enterococcus faecalis strain EC-12 (EC-12) on the gene expressions of antiviral cytokines in weaning piglets. Piglets were allocated to the EC-12-administered group (E group) and the no-treatment control group (C group). The small intestinal tissue, the mesenteric lymph node (MLN) cells and the splenocytes were collected from the piglets. The tissue and cells were co-cultured with a live vaccine of porcine reproductive respiratory syndrome virus, porcine epidemic diarrhea virus or EC-12. After the incubation, the gene expressions of IFN-γ and TNF-α in the tissue and cells were evaluated. The gene expressions of IFN-γ in the MLN cells and TNF-α in the splenocytes were significantly higher in the E group than in the C group. However, the increase in the gene expression of antiviral cytokines was observed independently of the antigen treatments. The results of the present study suggest that oral administration of EC-12 did not increase the response of immune cells to specific viral antigens but increased the baseline gene expression of antiviral cytokines.

Key words: antiviral cytokine, Enterococcus faecalis strain EC-12, ex vivo response, oral administration, piglet

INTRODUCTION

Weaning piglets are exposed to several stressful factors, including changes in food and housing environment. These factors cause various disadvantages for the growth of piglets: suppression of innate and acquired immunity [1, 2], morphological and functional alterations of the small intestine [3, 4], and increase of susceptibility to infections, particularly in the intestine and respiratory tract [5–7]. Respiratory pathogenic viruses, such as porcine reproductive and respiratory syndrome virus (PRRSV) and influenza virus, and enteropathogenic viruses, such as porcine epidemic diarrhea virus (PEDV) and rotavirus, are pandemic porcine pathogens that cause enormous economic losses to the pig industry [8]. Several vaccines have been released to control such viral infections; however, such infections persist worldwide [9, 10].

Lactic acid bacteria (LAB) are believed to be efficient antiviral materials. For instance, it has been shown that oral administration of heat-killed Lactobacillus pentosus strain b240 increased the production of mucosal immunoglobulin A (IgA), including virus-reactive ones that may provide effective clearance of pathogens [11, 12]. Furthermore, oral administration of L. casei strain Shirota and L. rhamnosus strain ATCC 53103 prevented influenza virus and rotavirus infection through activation of mucosal immune systems [13, 14]. Although the studies cited above on the antiviral effects of LAB were conducted mainly under an assumption of human use, the antiviral effects of LAB would also certainly be useful for livestock.

We previously demonstrated that oral administration of a cell preparation of Enterococcus faecalis strain EC-12 (EC-12) prevented rotavirus infection in weaning piglets [15]. In addition, EC-12 has the ability to stimulate the mucosal immune system, e.g., by enhancing production...
of several cytokines and secretion of intestinal secretory IgA [16–18]. All these immunostimulatory potentials of EC-12 suggest that EC-12 has antiviral potential.

Accordingly, the aim of this study was to assess whether oral administration of EC-12 in weaning piglets affects the expression of antiviral cytokines in immune cells.

For the evaluation of antiviral responsiveness, an ex vivo co-culture of tissue pieces or immune cells with viral antigens was performed in this study. One of two experimental groups was orally administrated EC-12, while nothing was administrated to another group, and the tissues/cells for ex vivo co-culturing were collected from the animals after dissection.

MATERIALS AND METHODS

Animals and diet

Six (3 female and 3 male) 3-week-old crossbred (Large White x Landrace x Duroc) piglets obtained from a commercial farm were used in this study. The piglets were housed in individual pens at the experimental farm of Kyoto Prefectural University, Kyoto, Japan. They were allocated into two groups of the same mean body weight (5.7 kg). One group of piglets was administered EC-12 (E group), and the other was a control group (C group) (n=3, for both groups). The temperature was maintained with brooders at ca. 30°C (measured on the floor under the brooder). After introduction to the experimental facility, piglets were fed a commercial diet for weaning piglets (SDS No.1; Nippon Formula Feed, Yokohama, Japan) supplemented with or without EC-12. EC-12 was obtained and prepared as described in Tsukahara et al. [19]. The level of supplementation, 0.05% (w/w), was the same as that in previous studies [19]. This basal diet was free from any agents modifying intestinal microbiota, such as antimicrobials, prebiotics, and probiotics. The nutritional composition of this diet was shown previously [20]. The diet and water were given ad libitum throughout the study. The experiment lasted for 10 days.

Collection of samples

After 10 days, the animals were euthanized by exsanguination under general anesthesia with intraperitoneal injections of sodium pentobarbital (Somnopenyl, Kyoritsu, Tokyo, Japan). After making a midline incision, the pylorus and ileocecal junction were clipped with a silk string, and the entire small intestine was separated. Tissue approximately 5-cm long was collected from 10 cm above the ileocecal junction. Pieces of mesenteric lymph node (MLN) and spleen approximately 2 cm in length were also collected. All tissue samples were soaked in Hanks’ Balanced Salts Solution (HBSS, Nacalai Tesque, Kyoto, Japan) immediately after excision and stored at 4°C until sample preparation.

Sample preparation for ex vivo culture

Small intestine

A portion of the small intestine was longitudinally incised, and the luminal content was removed. After washing twice with HBSS, the tissue was stirred for 30 min in an ethylenediaminetetraacetic acid (EDTA) solution (4.5 mmol/l EDTA, 250 mmol/l NaCl; pH 7.5) at 37°C to remove intestinal epithelial cells. This EDTA-treated tissue was cut into 50-mg pieces, and the pieces (SI segments) were washed twice with a culture medium [RPMI medium (Sigma-Aldrich Japan, Tokyo, Japan) containing 10% fetal calf serum (FCS), penicillin (100 U/ml, Sigma-Aldrich Japan), streptomycin (100 µg/ml, Sigma-Aldrich Japan), and gentamicin (10 µg/ml, Sigma-Aldrich Japan)]. Each piece was transferred to a well of a 24-well plate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan), which was then filled with 1.5 mL of the culture medium. Caution was taken to assure that the serosal side of the pieces always touched the bottom of the well.

MLN cells

A portion of the MLN was further cut into small pieces in an HBSS. The pieces were gently compressed on 70 μm cell strainers (BD Biosciences Japan, Tokyo, Japan) to obtain single-cell suspensions, and the cell suspensions were centrifuged at 300 g for 10 min at ambient temperature. After removal of the supernatant, the cells were resuspended in 1 mL of the culture medium, and the viable cells were counted by trypan blue exclusion. Cell suspensions (200 µL) containing 1×10^5 cells were seeded into each well of a 96-well microplate (Sumitomo Bakelite).

Splenocytes

The cell suspension of splenocytes was prepared in the same manner as the MLN cell preparation with an additional erythrocyte removal step. Briefly, the cells were resuspended in an ACK lysing buffer (0.5 mol/l NH₄Cl, 10 mmol/l KHCO₃ and 0.1 mmol/l Na₂EDTA at pH 7.2) after the first centrifugation and incubated at room temperature for 10 min to remove erythrocytes. The remaining cells, splenocytes, were further washed twice with HBSS and finally resuspended in an adequate volume of the culture medium. Cell suspensions (200 µL) containing 1×10^5 cells were seeded into each well of a 96-well microplate (Sumitomo Bakelite).
Ex vivo co-culture

The SI segments, cells of the MLN, and splenocytes were co-cultured with either EC-12 or live viral vaccines. EC-12 was added to the culture to determine whether the immunocytes from the E-group, in which the piglets were primarily primed with EC-12, responded efficiently to EC-12 itself. EC-12 was suspended with sterilized PBS to 3 mg/ml. Live vaccines of PEDV (Nisseiken, Tokyo, Japan) and PRRSV (Boehringer Ingelheim Vetmedica Japan, Tokyo, Japan) were used at the original concentration without further dilutions. Thirty-three microliters of PBS, an EC-12 suspension, the PEDV vaccine or the PRRSV vaccine was added to the wells of a 24-well plate containing 50 mg of SI tissue. Five microliters of each additive was added to the wells of a 96-well plate containing the cells from the MLN or spleen. Taken together, 4 treatments on 3 sample types (SI segment, MLN cells and splenocytes) from two experimental groups (E and C) were set for the ex vivo co-culture experiment. Three wells were allocated to each treatment. Incubation was performed at 37°C for 48 hrs in 5% CO2 according to Ausiello et al. [21]. After incubation was terminated, the SI tissues were collected with sterile forceps. The cell suspensions were collected and centrifuged at 300 g for 10 min at ambient temperature. After removal of the supernatant, the cells and SI tissues were preserved in RNA-later (Sigma-Aldrich Japan) at 4°C for 24 hr and further stored at −20°C until use.

Cytokine expression analyses

The expression levels of cytokine mRNA in the SI segments, MLN cells and splenocytes were determined. Total RNA was extracted from the SI segments or cells using NucleoSpin® RNA II (MACHEREY-NAGEL, Duren, Germany). An on-column DNase treatment was performed using the RNase-Free DNase I included in the kit. RNA (150 ng) was subjected to reverse transcription using a ReverTra Ace-α kit (TOYOBO, Osaka, Japan) with an Oligo (dT) 20 primer. Real-time PCR was performed using a Rotor-Gene 6200 (Qiagen, Tokyo, Japan) with 5 µL of SYBR Premix EX Taq™ (Perfect Real Time; Takara Bio, Shiga, Japan), 0.6 µL cDNA and 0.4 µM of each primer. The thermal cycling profile was 5 min at 95°C followed by 40 cycles of 15 sec at 95°C, 5 sec at 60°C and 15 sec at 72°C. Melting curves were generated for each sample to verify the specificity of amplification.

Table 1. Real-time quantitative PCR primers used in this study

| Gene name | Sequence (5'-3') | Product size |
|-----------|-----------------|--------------|
| GAPDH     | F AGCAATGCTCTCCTGAC | 189 bp |
|           | R AAGCAGGGATGATGTTCTGG |     |
| IFN-γ     | F AGCAGTGGTAGTTGATCAAGCA | 248 bp |
|           | R TGCCAGGATGACAAAA |     |
| TNF-α     | F CCCCCTGTCCCATCCCCATT | 200 bp |

To prepare a standard curve, the plasmids containing the PCR product of each gene in pGEM-T Easy Vector (Promega, Tokyo, Japan) were generated and included in every run. The transcript copy number for each sample was calculated from the standard curve and normalized to the copy number for GAPDH.

Statistical analysis

Data were analyzed by randomized block design two-way ANOVA [diet (control diet and EC-12 supplemented diet) × treatment at incubation (Media+PBS, Media+EC-12, Media+PEDV and Media+PRRSV)]. Each individual was designated as an experimental block so that the time lags of dissection and sample preparation would not bias the results [22]. When significant differences were detected, the post-hoc Tukey-Kramer multiple comparison test was used. Differences among means were considered to be significant at p≤0.05. All data were analyzed using StatLight, which is an add-in application for Microsoft Excel (Redmond, WA, USA).

RESULTS

Gene expression of IFN-γ

The gene expression levels of IFN-γ in the SI segments, MLN cells, and splenocytes are shown in Fig. 1. No significant differences were detected between the E and C groups for the SI segments (p=0.23, Fig. 1a) and splenocytes (p=0.26, Fig. 1c). In the MLN cells, IFN-γ expression was significantly higher in the E group than in the C group (p=0.05, Fig. 1b), although the splenocytes from one piglet in the E group showed very low gene expression in the PBS, EC-12 and PRRSV vaccine treatments. On the other hand, none of the treatments (addition of EC-12, PEDV vaccine, and PRRSV vaccine in the culture) showed particularly noticeable effects on the gene expression of IFN-γ in the SI segments (p=0.21), MLN cells (p=0.66) and splenocytes (p=0.38).

Gene expression of TNF-α

The gene expression levels of TNF-α in the SI
segments, MLN cells, and splenocytes are shown in Fig. 2. No significant differences were observed between the E and C groups for the SI segments (p=0.08, Fig. 2a) and MLN cells (p=0.11, Fig. 2b).

The MLN cells from one piglet in the E group, the same piglet in which splenocytes showed very low expression of the IFN-γ gene, exhibited negligible expression of the TNF-α gene in the PBS, EC-12, and PRRSV vaccine treatments. However, the MLN cells from all piglets in the C group also showed very low expressions of TNF-α in all treatments, while two piglets in the E group showed notable expressions in most treatments. The difference in the TNF-α gene expression of splenocytes was significant between the E and C groups (p=0.05).

None of the treatments had a significant effect on the gene expression of TNF-α in the SI segments (p=0.22), MLN cells (p=0.59) and splenocytes (p=0.83).

DISCUSSION

Weaning piglets are continuously exposed to infections due to their immature immunity. PRRSV, PEDV, porcine circovirus (PCV) 2, and rotavirus are frequently detected on Japanese farms [23, 24].

In this study, we focused TNF-α and IFN-γ as antiviral cytokines because they are likely to be induced by LAB rather than IFN-α and IFN-β, the other major antiviral cytokines [25, 26]. TNF-α induces a striking increase in the number of intra-alveolar neutrophils and their phagocytic capacity against various viruses [27]. In addition, TNF-α induces apoptosis in cells infected with a virus [28]. IFN-γ plays a role in the inhibitory effect on PRRSV replication in macrophages and induces an antiviral state in uninfected neighboring cells [29, 30]. Furthermore, synergy with TNF-α dramatically enhances the antiviral effects of IFN-γ [31].

In this experiment, the effect of EC-12 on antiviral
cytokines was evaluated to assess a part of the mechanisms involved in its health promotive effect seen in weaning piglets.

The gene expression of IFN-γ was significantly high in the MLN cells collected from the E group of piglets (Fig. 1). The MLN is the major site for T cell stimulation. Immature antigen-presenting cells (APCs), such as dendritic cells (DCs), sample the antigens in the tissue of the intestine, most likely at Peyer’s patches, and then migrate to the MLN to activate T cells [32]. According to this context, APCs that sampled EC-12 might migrate to the MLN and induce T cell activation. The increase in IFN-γ expression in the MLN cells from the E group is explained by this T cell activation.

In addition to IFN-γ, TNF-α also showed significantly high expression in the splenocytes of E group (Fig. 2). Miyasaka (2004) reported that T cells, once activated in the MLN, further migrate to the spleen [33]. The activated T cells, expressing the CD40 ligand (CD40L), induce macrophages expressing CD40 to produce TNF-α [34]. The high TNF-α gene expression observed in the splenocytes of the E group can be explained by this interaction between the activated T cells from the MLN and the splenic macrophages. This sequential immune response occurred in the MLN and consequently in the spleen is a possible reason for why the increase in gene expression of IFN-γ and TNF-α was differentially induced by EC-12 administration in the specific tissues.

However, none of the antigens added to the culture significantly altered the gene expression of IFN-γ and TNF-α in any of the tissues evaluated in this study (Figs. 1 and 2). This suggests the possibility that orally administrated EC-12 stimulated the immune systems in a nonspecific manner. Indeed, the splenocytes and MLN cells from the E group showed higher gene expression of IFN-γ and TNF-α than the cells from the C group even when cultured with PBS (control treatment: Figs. 1 and 2). Although the precise reason for why antigen addition did not alter the gene expression of antiviral cytokine genes remains to be clarified, the incubation time set in this study might not be necessarily appropriate to detect a sharp response to the viral antigens.

An increase in the baseline gene expression of IFN-γ and TNF-α genes should contribute to the antiviral effect of EC-12 by inducing apoptosis of infected cells and enhancing the innate immune response [35]. Indeed, we previously demonstrated that oral administration of EC-12 prevented the diarrhea induced by rotavirus infections in suckling and weaning piglets [15].

In conclusion, the findings in this study suggest that oral administration of EC-12 raised the basal gene expression of the antiviral cytokine mucosal (MLN) immune system and subsequently the systemic (spleen) immune system. This may explain why oral administration of EC-12 exhibits a wide range of probiotic-like effects, including antibacterial and antiviral effects, in pigs and other mammals [15–17].

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