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A novel *in vitro* co-culture model comprised of Caco-2/RBL-2H3 cells to evaluate anti-allergic effects of food factors through the intestine

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ABSTRACT

The prevalence of type I allergic diseases such as food allergy and allergic rhinitis has increased. Therefore, many studies have focused on food factors with anti-allergic activities in recent years. In order to investigate the effect of food factors on mast cell activation, a RBL-2H3 cell monoculture system has been widely used, in which various food factors have been reported to inhibit degranulation of RBL-2H3 cells. However, some orally administered food factors do not interact directly with immune cells but do so indirectly through intestinal epithelial cells. In this report, we established a novel *in vitro* co-culture model to evaluate anti-allergic effects of orally administered food factors. The co-culture system, comprised of Caco-2 cells (apical component) and RBL-2H3 cells (basolateral component), was able to evaluate the effects of two flavonoids that are known to have the inhibitory effects on mast cell degranulation. Moreover, we evaluated the anti-allergic effects of *Enterococcus faecalis* strains that are not absorbed through the intestine. We identified two strains of lactic acid bacteria that had inhibitory effects on mast cell degranulation using this co-culture system and possessed anti-allergic properties in a passive cutaneous anaphylaxis model mouse. This novel *in vitro* co-culture model was applicable for finding food factors with anti-allergic effects and might be useful for examining its anti-allergic mechanisms.

1. Introduction

There are four types of allergic diseases, and type I allergic disease is a major global public health concern. Type I allergic disease, including food allergy, allergic rhinitis, asthma, and atopic dermatitis, is induced by certain allergens (antigens) such as food, dust, and pollen (Galli et al., 2008). However, the fundamental approaches of therapy for type I allergic diseases have not been established.

The cascade of type I allergy is as follows. First, allergens are captured by antigen-presenting cells (APCs), especially dendritic cells (DCs), and internalized by APCs *via* phagocytosis, pinocytosis, or endocytosis (Gould and Sutton, 2008). The degraded allergens are presented by major histocompatibility complex class II (MHC II) to T cell receptors (TCRs) on naïve $CD4^+$ T cells. The activated $CD4^+$ T cells are divided into two T helper cells (Th1 or Th2), which are classified by the type of cytokines they produce (Mosmann et al., 1986, Mosmann and Sad, 1996). Allergen-specific Th2 cells secrete Th2 cytokines such as interleukin (IL)-4 and IL-13, which enhance class switching of immunoglobulin from IgM to allergen-specific IgE in B cells (Takhar et al., 2005). B cells produce many allergen-specific IgE antibodies, which bind to the high affinity IgE receptor I (FcεRI) on the surface of mast cells (Galli and Tsai, 2013). Upon second exposure to an allergen recognized by allergen-specific IgE on FcεRI, cross-linking of IgE-FcεRI with the allergen induces the activation of mast cells, leading to degranulation and the release of inflammatory mediators including histamine, β-hexosaminidase, lipid mediators, Th2 cytokines, and chemokines (Izawa et al., 2012). It is considered that allergic symptoms are mitigated by inhibiting mast cell activation.

Recent studies have demonstrated a relationship between gut microbiota and allergic disease (Björkstén et al., 1999). Lactic acid bacteria (LAB) such as *Enterococcus*, *Lactobacillus*, and *Lactococcus* are a group of Gram-positive, anaerobic bacteria that are an important component of the indigenous microbiota. Recently, many reports have revealed that LAB mitigate allergic symptoms. Indeed, it has been reported that several *Lactobacillus* strains, including *L. brevis* SBC8803, *L. casei* Shirota, and *L. plantarum* K37, could inhibit the serum levels of antigen-specific IgE in mouse models (Segawa et al., 2008; Shida et al., 2002; Liu et al., 2014). Furthermore, studies on *Enterococcus faecalis* FK-23 have shown that it could inhibit allergen-induced peritoneal accumulation of eosinophils and active cutaneous anaphylaxis in a mouse model (Shimada et al., 2003, 2004). Many clinical trials revealed that several *Lactobacillus* strains, including *L. rhamnosus* GG, *L. acidophilus* L-92, and *L. casei* Shirota, were effective in the prevention of early atopic disease in children and of allergic symptoms in patients sensitive to Japanese cedar pollen (Kalliomäki et al., 2001; Ishida et al., 2005; Tamura et al., 2007). Therefore, many studies have focused on the

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therapeutic and preventive effects of LAB in allergic diseases. However, the exact mechanisms of the interaction of LAB and allergic diseases are not yet well understood.

In order to investigate the effect of food factors on the activation of mast cells, especially degranulation, an IgE-dependent passive cutaneous anaphylaxis (PCA) mouse model has been used as a type I allergy model (Bouike et al., 2011). However, *in vivo* experiments take a long time to produce results and need a large amount of test samples and animals. Hence, a rat basophilic leukemia cell line, RBL-2H3 cell monoculture system has been widely used as a good tool for investigating the anti-allergic effect of food factors (Yamada et al., 2007). RBL-2H3 cells display phenotypic characteristics of mucosal-type mast cells and express several hundred thousand FcεRI on their surface. After stimulation with an antigen, RBL-2H3 cells release β-hexosaminidase, which is used as a marker of mast cell degranulation. Recently, various food factors have been reported to inhibit degranulation of RBL-2H3 cells (Han et al., 2013), but almost all of them were applied directly to RBL-2H3 cells.

Bouike et al. demonstrated that oral administration of *Agaricus blazei* Murill (ABME) suppressed allergic responses in an allergy model mouse, and the active compound contained in ABME indirectly inhibited allergic responses through a human intestinal epithelial cell line, Caco-2 cells, *in vitro* (Bouike et al., 2011). These results suggested that intestinal epithelial cells (IECs) stimulated by ABME might affect immune cells. IECs play an important role in antigen invasion through the body orally, and in the regulation of the intestinal immune responses (Lu and Walker, 2001). However, there are few *in vitro* experimental methods that evaluate the anti-allergic effects of food factors *via* IECs.

Flavonoids are the plant secondary metabolites found abundantly in plants and they have a variety of biological effects, including anti-allergic effects (Singh et al., 2011; Lee et al., 2010; Trinh et al., 2010). We have established *in vitro* model using Caco-2 and RAW264.7 cells (Tanoue et al., 2008). We demonstrated that lentinan which is a polysaccharides containing *Lentinus edodes* possessed anti-inflammatory effects using this model (Nishitani et al., 2013a). In this report, it was made clear the mechanism which lentinan affected Caco-2 cells to cause endocytosis to decrease TNFR1 contents in basolateral sides. Thus, it was suggested that co-culture system is very useful to investigate the crosstalk between different cells. However, no co-culture model exists to measure food factors possessing anti-allergy activity on orally administration. In this study, an *in vitro* co-culture model to evaluate anti-allergic effects of food factors was established, in which human IECs, Caco-2 cells, were placed in a Transwell on the apical side and rat basophilic leukemia cell line, RBL-2H3 cells, were placed on the basolateral side. When RBL-2H3 cells were stimulated with anti-dinitrophenyl (DNP) IgE and DNP-albumin, β-hexosaminidase, which is an index of allergy activity, was released from them. We investigated whether treatment with luteolin or kaempferol, which are absorbed through the small intestine, suppressed β-hexosaminidase release by degranulation of RBL-2H3 cells in this co-culture system. Moreover, this study examined the effects of three strains of LAB that are not absorbed by the small intestine in this *in vitro* co-culture model and a conventional *in vivo* model.

2. Materials and methods

2.1. Reagents

Eagle's Minimum Essential Medium (MEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan). Dulbecco's Modified Eagle's Medium (DMEM, High Glucose) with glutamine, streptomycin, and Evans blue were purchased from Wako Pure Chemical Industries (Osaka, Japan). Anti-dinitrophenyl (DNP) IgE, DNP-albumin, and *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Biological Industries (Beit, Israel). Trypsin and MEM non-essential amino acids (NEAA) were purchased from Gibco BRL (Grand Island, NY, USA). DNP-bovine serum albumin (DNP-BSA) was purchased from Cosmo Bio (Tokyo, Japan). Penicillin was purchased from MP Biomedicals (Aurora, OH, USA). Luteolin was purchased from Sarsynthese (Merignac, France). Kaempferol was purchased from Extrasynthese (Genay, France). Anti-Toll-like receptor (TLR) 1, anti-TLR2, and anti-TLR6 antibodies were purchased from Hycult Biotech (NL, Netherlands). IgG1 Isotype Control was purchased from Bay bioscience (Hyogo, Japan).

2.2. Bacterial strain

E. faecalis IC-1 and FK-23 were purchased from Chichiyasu Pharmaceutical Co., Ltd. (Hiroshima, Japan) and Nichinich Pharmaceutical Co., Ltd. (Iga, Japan), respectively as a commercial product which was killed by heat. A commercial product of a heat killed preparation of *E. faecalis* EC-12 (Combi Corporation, Saitama, Japan) was used.

2.3. Mice

Female 7-week-old BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). The mice were housed in an air-conditioned animal room at 25 ± 1 °C and acclimated for 7 days before experiments. The mice were maintained in filter-top cages in specific pathogen-free conditions with free access to laboratory chow and water. All animal experiments were approved and carried out in accordance with the Animal Experiment Ethnics Committee of Kobe University.

2.4. Cell culture

Rat basophilic leukemia cell line, RBL-2H3 cells, were cultured in dishes in Eagle's MEM supplemented with 10% (*v*/v) heat-inactivated FBS (57 °C, 30 min), 100 μg/ml streptomycin, 100 U/ml penicillin, and 2 mM L-glutamine. Cell cultures were incubated at 37 °C in a 5% CO_2 incubator. Passage numbers 14–32 were used. Human intestinal epithelial cell line, Caco-2 cells, were cultured in a 75 cm^2 plastic flask in DMEM (high glucose) supplemented with 10% FBS, 1% MEM-NEAA, 100 μg/ml streptomycin, and 100 U/ml penicillin, and incubated at 37 °C in a 5% CO_2 incubator. Passage numbers 48–64 were used. When either cell line reached 80% confluence, cells were recovered from the culture dish or flask by trypsin digestion after washing with phosphate-buffered saline (PBS). The cells were replated in a new dish or flask.

2.5. Transepithelial electrical resistance measurement

The integrity of the Caco-2 monolayer was evaluated by measuring the transepithelial electrical resistance (TER) value. Tight junctions serve as barriers to paracellular diffusion, and TER reflects the tightness of the junctions between epithelial cells (Hidalgo et al., 1989). The monolayer cells were gently rinsed with Hank's Balanced Salts Solution (HBSS: 137 mM NaCl, 5.36 mM KCl, 1.26 mM $CaCl_2$, 0.55 mM $MgSO_4$, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 2.92 mM NaH_2PO_4) for 30 min in a CO_2 incubator. The integrity of the cell monolayers was evaluated by measuring TER using Millicell-ERS equipment (Millipore, Eschborn, Germany).

2.6. β-Hexosaminidase assay

To evaluate anti-allergy effects, an *in vitro* assay using the RBL-2H3 mono-culture system was performed in accordance with a previous study (Yamada et al., 2007). RBL-2H3 cells (4.0×10^5 cells/ml) were plated at 500 μl/well in a 24-well tissue culture plate in Eagle's MEM and were sensitized overnight with 1 μg/ml anti-DNP IgE in a 5% CO_2 incubator at 37 °C. The cells were washed three times with Siraganian buffer (SB; 119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$,

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