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Suppressing Effects of *Feijoa sellowiana* Berg (Feijoa) on Cytokine Secretion by Intestinal Epithelium

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Intestinal mucosal immunity is very important to the body's defense system and can be affected by extrinsic factors such as bacteria and food components. In the present report we assessed the intestinal immunomodulatory activity of *Feijoa sellowiana* Berg (feijoa), a tropical fruit expected to become widely popular in Japan. We examined the effects of the aqueous extract from feijoa and the *in vitro*-digested feijoa on the secretion of IL-7 and TGF-β from Caco-2 cells that are used as *in vitro* models of intestinal epithelium. We found that both the aqueous extract and the *in vitro*-digested feijoa suppressed the secretion of TGF-β by Caco-2 cells. These results suggest that the continued intake of feijoa may induce a decrease in TGF-β concentrations in intestinal lamina propria, which may in turn cause suppressions of oral tolerance and disorders of mucosal homeostasis. Moreover, the polyphenols from feijoa appear to be involved in the suppressing effect of feijoa on TGF-β secretion by intestinal epithelium.

Keywords: *Feijoa sellowiana* Berg (feijoa), IL-7, TGF-β, Caco-2, polyphenol

Introduction

Intestinal mucosal immunity, which is very important to the body's defense system, is controlled by interactions between intestinal epithelium and various immune cells in the intestinal lamina propria. Cytokines secreted from these cells play major roles in these interactions. Intestinal epithelial cell-derived IL-7 serves as a potent regulatory factor for the proliferation of mucosal lymphocytes (Watanabe *et al.,* 1995) and is involved in the establishment of the pattern of cryptopatch and Peyer's patch formation (Laky *et al.,* 2000). IL-7 is also reported to mediate chronic inflammation in the colonic mucosa (Watanabe *et al.* 1998). TGF-β, which can be produced by all nucleated cells, including intestinal epithelial cells, is known to have multiple actions, such as effects on gastrointestinal mucosal integrity, wound repair, and neoplasia (Beck *et al.,* 2003, Engleet *et al.,* 1999). TGF-β is also reported to downregulate the immune functions of regulatory T cells (Weiner, 2001) and induce class switching of IgM^+ IgD^+ precursors to IgA^+ B cells (Zan *et al.,* 1998). Both IL-7 and TGF-β have key roles in maintaining mucosal immunity.

Recently, much attention has been paid to the bioactivities of plant foods as these activities are thought to be effective in the prevention of cancer and other chronic diseases. The effects of some vegetable extracts on immunomodulating activities have been reported (Kaku *et al.,* 1997, Fuke *et al.,* 2001, Manabe, 2003, Manabe *et al.,* 2003). *Feijoa sellowiana* Berg (feijoa), which is cultivated primarily in New Zealand and California, has been imported into Japan and is expected to become widely popular. It contains many volatile compounds including terpenes, tannins, and flavonoids, which are responsible for the strong feijoa-like character (Binder and Flath, Shaw *et al.* 1990). Vutto *et al.* (2000) reported that an aqueous extract from feijoa fruit showed both antibacterial and antioxidant properties. The acetonic extract and the 80% ethanol extract from feijoa fruit are also reported to have radical-scavenging activity (Ielpo *et al.* 2000, Isobe *et al.* 2003).

In the present research, we studied the effects of dietary feijoa on the immune responses of intestinal epithelial cells with an experimental system that uses a food sample digested by pepsin-bile extract/pancreatin and the human colonic adenocarcinoma cell line Caco-2, and that has been confirmed as a model appropriate for examination of intestinal immunity (Pinto *et al.* 1983, Manabe *et al.* 2003). We then examined the effects of *in vitro*-digested feijoa on IL-7 and TGF-β production of Caco-2 cells.

Materials and Methods

Plant material Feijoa fruits were collected from the botanical gardens in Miyazaki, Japan in 2003 and stored at -20℃ until use.

Materials Porcine pepsin (975 units/mg of protein), pancreatin (4x, USP), and bile extract were purchased from Sigma (St. Louis, MO, USA). Cellmatrix type I-A purchased from Nitta Gelatin (Osaka, Japan) was used for

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the collagen treatment of plates.

Cell culture Caco-2 cells (ATCC HTB-37) were seeded at a density of 1×10^5 cells/cm^2 in collagen-treated 12-well plates (Greiner, Germany). To achieve full differentiation, the cells were cultured as described by Kanzato *et al.* (2001). Specifically, Caco-2 cells were cultured for 9–12 days postconfluence in a medium consisting of DMEM (Nissui Pharmaceuticals, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; JRH, KS, USA) and 1% non-essential amino acids (Invitrogen Corp., Carlsbad, CA, USA); the medium was replaced every 1–2 days. Cells were also cultured in Transwell devices (Corning Coster, Acton, MA, USA). Caco-2 cells were seeded at a density of $1x10^5$ cells/0.5 ml/well in an apparatus with a 12-mm diameter semipermeable membrane (0.4 μm pore size) and cultured to achieve full differentiation.

Preparation of the aqueous extract from feijoa Feijoa fruits were ground with two-fold (weighted) phosphate-buffered saline (PBS) on ice. After centrifugation (28,000 $\times$g for 15 min at 4℃), the supernatant was sterilized by filtration through a 0.22-μm membrane and then stored at −80℃ until use as the aqueous extract. The concentration of feijoa substances was 29.4 mg dry weight /ml extract.

Preparation of the sample digested in vitro The *in vitro* digestion procedure was conducted as described by Manabe *et al.* (2003). Briefly, the homogenized feijoa fruits were acidified (pH 2.0) before adding pepsin to a final concentration of 1.25 mg/ml and incubated at 37℃ in a water bath with shaking for 1 hr. The pH of the gastric digest was then increased to 6.0 with sodium bicarbonate, and bile extract and pancreatin were added to the digest to give final concentrations of 1.85 mg/ml and 0.3 mg/ml, respectively. The pH value was then increased to 7.0 with 1 mol/L sodium hydroxide, and 6-ml aliquots of the partially digested feijoa were transferred to dialysis tubing (15,000 molecular weight cutoff, Spectra/Por 2.1, Spectrum Medical, Rancho Domingez, CA, USA) and dialyzed against 25 ml of serum-free DMEM medium at 37℃ for 2 hr. The dialysate was sterilized by filtration through a 0.22-μm membrane and then stored at −80℃ until used as the *in vitro*-digested feijoa. The concentration of *in vitro*-digested feijoa was 0.317 mg dry weight/ml extract. A control solution was prepared by the same procedure but without feijoa.

Preparation of the crude polyphenol fraction from feijoa Feijoa fruits (100 g) were ground with 80% ethanol. After filtration through No. 2 filter paper, the filtrate was evaporated *in vacuo* to remove ethanol. This concentrate was applied onto a Diaion HP2MG column (Mitsubishi Kasei Co., Tokyo, Japan) and washed with distilled water. The crude polyphenol fraction was eluted with 80% ethanol and evaporated *in vacuo* to remove ethanol. After lyophilization, 0.5 g of crude polyphenol (equivalent to 200 mg of gallic acid) was maintained at −20℃ prior to dissolution in ethanol for use as the crude polyphenol fraction from feijoa.

Determination of crude polyphenol The content of crude polyphenol was determined by the method of Folin - Ciocaerou. Each sample was measured in triplicate, with data presented as the weight equivalent to gallic acid/ml (mg GAE/ml).

Cytokine assays Levels of IL-7 and TGF-β in the supernatant of Caco-2 cells that had been cultured for 48 hr in a medium containing the feijoa sample, were determined using ELISA developed by R & D Systems (Minneapolis, MN, USA) and Pharmingen (OptEIA Set; San Diego, CA, USA), respectively.

Lactate dehydrogenase (LDH) assay LDH concentration in the medium was determined enzymatically using an LDH-Cytotoxic Test kit (Wako Pure Chemical, Osaka, Japan). Cytotoxicity to Caco-2 cells was expressed as the relative amount of LDH released into the medium to the total amount of LDH, this latter amount established by the sum of intracellular LDH and LDH released from the control cells.

Determination of the activity of aminopeptidase-N (AP-N) The activity of AP-N was determined using ala 7-amino 4-methylcoumarin as the substrate. Released methylcoumarin was assayed with a fluorescence plate reader (excitation, 355 nm; emission, 442 nm, Dainippon Pharmaceutical, Osaka, Japan).

Results and Discussion

Effects of feijoa on cytokine secretion by intestinal epithelial cells We first examined the effect of the aqueous extract from feijoa on cytokine secretion by Caco-2 cells, an intestinal Epithelial Cell model. When the aqueous extract was diluted 1/10 with medium and added to the monolayer of Caco-2 cell, the monolayer peeled off and was severely damaged. The aqueous extract diluted with PBS was also added to the Caco-2 monolayer (control). The amount of LDH released from cells to which two fold-diluted aqueous extracts were added, at 1/10 dilution with medium, was 6–9% of total cellular LDH. As this was not significantly different from that of cells to which PBS (control) was added, the aqueous extracts diluted to this extent or higher did not show cytotoxicity (Table 1). Although IL-7 secretion was not suppressed by the diluted aqueous extracts (Fig. 1A), significantly less TGF-β was secreted by Caco-2 cells cultured with the diluted extracts than by Caco-2 cells cultured with PBS (control) ($p<0.001$) (Fig. 1B). This suppressing effect was enhanced as more concentrated aqueous extract was applied. These results indicated that feijoa contained a substance that suppressed TGF-β secretion.

To clarify the effect of dietary feijoa on cytokine secretion by intestinal epithelial cells, we measured IL-7 and TGF-β secreted by Caco-2 cells cultured in the *in vitro*-digested feijoa with 10% FBS. Though the *in vitro*-digested feijoa suppressed TGF-β secretion, it showed no effect on IL-7 secretion (Fig. 2A, B). As the *in vitro*-digested feijoa did not show cytotoxicity either (Table 1), these results suggest that the suppressing effect of the aqueous extract from feijoa on TGF-β secretion was retained after the *in vitro* digestion of feijoa and that dietary feijoa suppresses TGF-β secretion by intestinal epithelial cells.

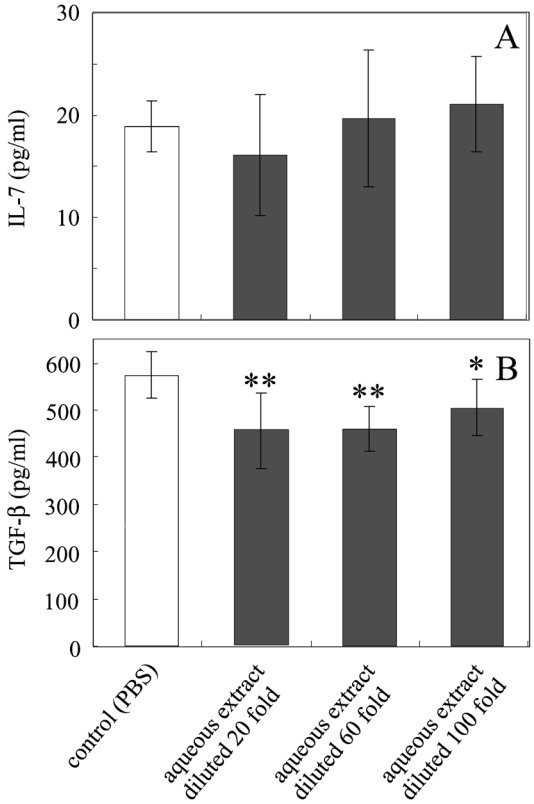

Fig. 1. Aqueous extract from feijoa suppressed the secretion of TGF-β by Caco-2 cells.
A: IL-7, B: TGF-β. Caco-2 cells were cultured with medium containing the 20 fold, 60 fold and 100 fold-diluted aqueous extract at a volume 1/10 that of medium for 48 hr. Each value is the mean±SD (n=6). Statistical difference was analyzed by Student's t-test and asterisks denote significant difference from the control (*$P<0.05$, **$p<0.01$).

Next, we confirmed whether feijoa suppressed TGF-β secretion to the basolateral side of intestinal epithelial cells. After the Caco-2 cells were cultured with the *in vitro*-digested feijoa in the upper chamber of Transwell units for 48 hr, the IL-7 and TGF-β levels were measured for the supernatant in the lower chamber. As shown in Fig. 2C and D, the *in vitro*-digested feijoa suppressed TGF-β secretion to the basolateral side of Caco-2 cells. This result suggests that the dietary feijoa would affect the subepithelial immune responses in the intestinal lamina propria.

The activity of AP-N located in the apical membrane of Caco-2 cells was examined after the cells were cultured with feijoa samples. The AP-N activity of Caco-2 cells was not affected by the addition of feijoa (Fig. 3), indicating that feijoa did not induce cell damage.

As feijoa inhibited the secretion of TGF-β which plays an important role in the induction of oral tolerance (Weiner, 2001), dietary feijoa may prevent oral tolerance. Although TGF-β was reported to be associated in the mechanisms regulating IgA production in the gut (Kawasaki *et al.,* 1983, Zan *et al.,* 1998), intestinal epithelial cell derived TGF-β was found to have no effect on IgA secretion (Goodrich and McGee, 1999). However, Bulut *et al.* (2004) reported that the glucagon-like peptide2-induced improvement of intestinal wound healing is mediated by TGF-β in the intestine. Moreover, IL-10 release from subepithelial monocyte was shown to be induced by intestinal derived mediators such as TGF-β, which regulate mucosal immune homeostasis toward commensal bacteria (Haller *et.al,* 2002, Kucharzik *et. al,* 1997). Therefore, the continued intake of feijoa appears to obstruct oral tolerance and the regulation of mucosal homeostasis.

Contribution of polyphenols in feijoa to the suppression of cytokine secretion by intestinal epithelial cells Attention is currently being focused on the wide distribution of plant polyphenols because various biological functions of polyphenols found in plant foods have been identified. The flavon 3-ol monomers and their related oligomers the

Table 1. The cytotoxicity of feijoa.

	released LDH/total LDH(%)
control (PBS)[a]	8.52± 0.89
aqueous extract diluted 2 fold[a]	7.78± 1.25
control (digested without feijoa)	8.06± 1.00
digested feijoa	7.89± 0.51
control (ethanol)	10.01± 0.42
polyphenol 25μg/ml	9.82± 0.33

Each value is the mean of 6 replicates±SD.
[a]added to Caco-2 cells at a volume 1/10 that of the medium

polycyanidines, which are abundant in cocoa, were shown to have the potential to modulate the production of several immunomodulatory cytokines, including IL-1β, IL-2, IL-4 and TGF-β (Mao *et al.* 2000, Mao *et al.* 2003). Feijoa contains polyphenols such as flavonoids and tannins (Binder and Flath, 1989, Shaw *et al.,* 1990, Isobe *et al.,* 2003). Because Caco-2 cells cultured with *in vitro*-digested feijoa were colored brown (data not shown), the contents of crude polyphenol in the aqueous extract from feijoa and the *in vitro*-digested feijoa were measured. Polyphenols derived from feijoa were present at 0.619 mg GAE/ml and 0.034 mg GAE/ml, respectively (Table 2).

In addition, the effect of the polyphenol fraction from feijoa on cytokine secretion by Caco-2 cells was examined. Figure 4 shows that the crude polyphenol fraction from feijoa suppressed the secretion of TGF-β by Caco-2 cells. The suppressing effect was found to increase as the concentration of polyphenol increased. These results indicate that the intensities of the suppressing effects of the aqueous extract from feijoa and the *in vitro*-digested feijoa are equivalent to the intensities of the effects of polyphenol contained in these samples (Table 2, Fig. 1, Fig. 2, Fig. 4). The release of LDH and the activity of AP-N of Caco-2 cells with the crude polyphenol fraction were not significantly different from those of the control cells (with added ethanol) (Table 1, Fig. 3). We could not confirm that the polyphenols completely account for the suppressing effects of the aqueous extract from feijoa and the *in vitro*-digested feijoa on TGF-β secretion by Caco-2 cells because we did not examined the variety of polyphenols in each sample. However, these results suggest that the polyphenol from feijoa seemed to be involved in the suppressing effect.

Our results in this *in vitro* model suggest the possibility that the continued intake of feijoa would induce a decrease in the concentrations of TGF-β in intestinal lamina propria, which may cause suppression of oral tolerance and disorders of mucosal homeostasis.

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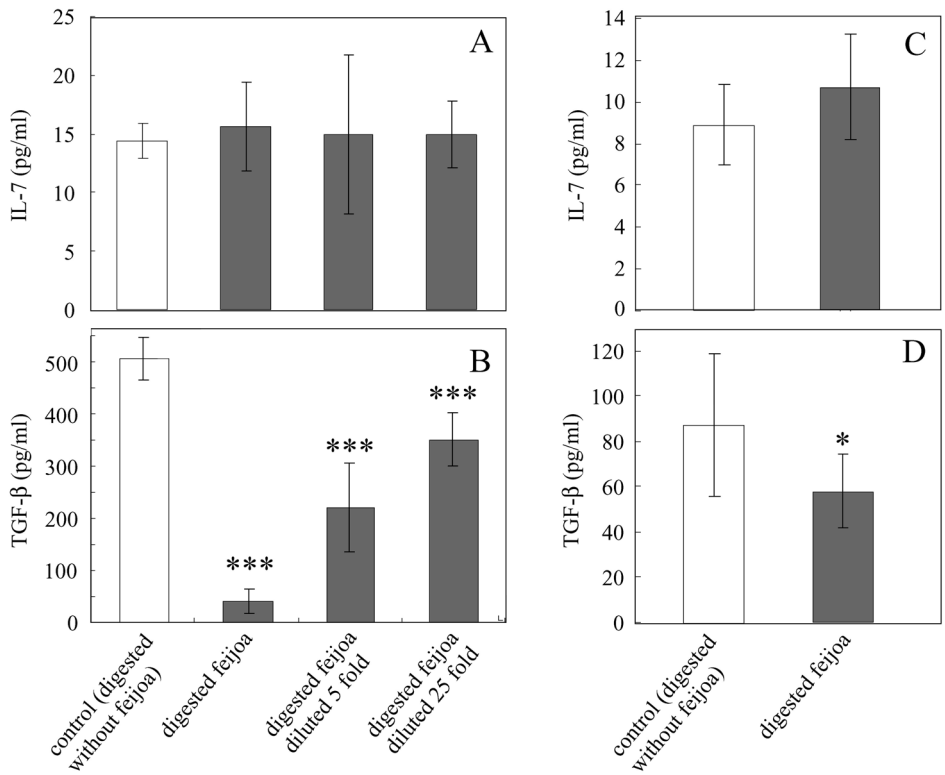

Fig. 2. The *in vitro* digested feijoa suppressed the secretion of TGF-β by Caco-2 cells.
Caco-2 cells were cultured with the *in vitro* digested feijoa with 10% FBS for 48 hr. A, B: the IL-7 (A) and the TGF-β (B) contents were measured in the supernatant of Caco-2 cells cultured in 12-well plates. C, D: the IL-7 (C) and TGF-β (D) contents were measured in the supernatant in the lower chamber of 12-Transwell diveces, of which upper chamber Caco-2 cells were cultured in. Each value is the mean±SD (n =6). Statistical difference was analyzed by Student's t-test and asterisks denote significant difference from the control (*P<0.05, ***P<0.001).

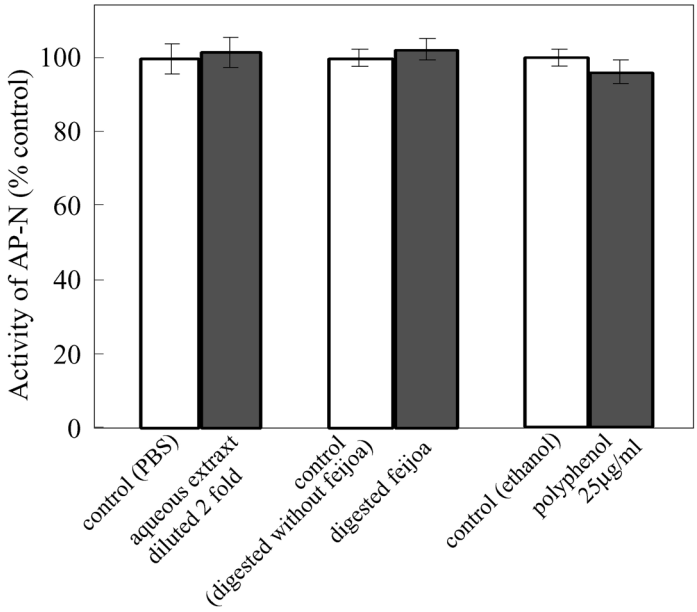

Fig. 3. Feijoa did not affect the AP-N activity in the apical membrane of Caco-2 cells.
□: control, ■: feijoa sample. After Caco-2 cells were cultured with feijoa samples for 48 hr, the AP-N activity in the apical membrane of Caco-2 cells was measured. The amounts of AP-N activity of Caco-2 cells added feijoa samples are presented relative to the value of AP-N activity of the control cells. Each value is the mean±SD (n=6).

Table 2. Crude polyphenol content of the samples prepared from feijoa.

	total (mg GAE[a]/ml)	derived from feijoa[b] (mg GAE[a]/ml)
control (PBS)	0.000	-
aqueous extract	0.619	0.619
control (digested without feijoa)	0.050	-
digested feijoa	0.084	0.034

Each sample was measured in triplicate.

[a]GAE, Gallic acid equivalent.

[b]polyphenol content derived from feijoa = total polyphenol $_{\text{each feijoa sample - control}}$

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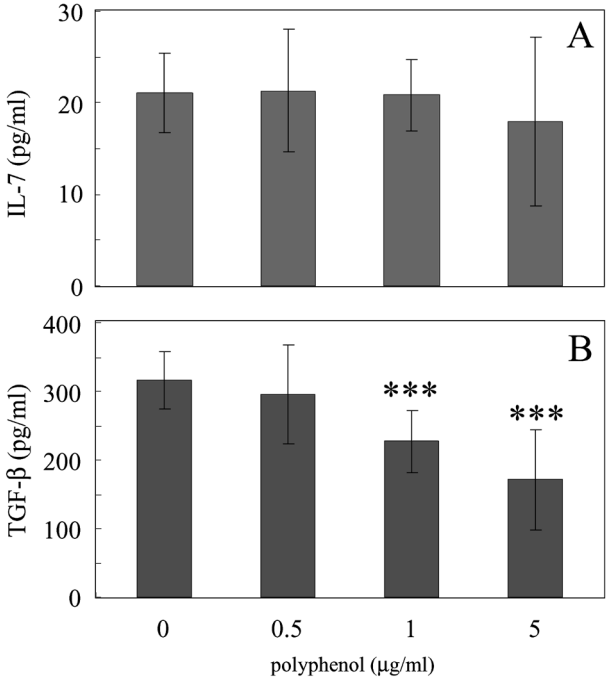

Fig. 4. The polyphenols extracted from feijoa suppressed the secretion of TGF-β by Caco-2 cells.
A: IL-7, B: TGF-β. Caco-2 cells were cultured with crude polyphenol fraction extracted from feijoa for 48 hr. Each value is the mean±SD (n=6). The statistical difference was analyzed by Student's t-test. Statistical difference was analyzed by Student's t-test and asterisks denote significant difference from the control (***P<001).

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