Glycoconj J (2015) 32:153–159
DOI 10.1007/s10719-015-9588-4

ORIGINAL ARTICLE

Pectin from *Prunus domestica* L. induces proliferation of IEC-6 cells through the alteration of cell-surface heparan sulfate on differentiated Caco-2 cells in co-culture

Mitsutaka Nishida[1] · Kazuma Murata[2] · Kazuya Oshima[2] · Chihiro Itoh[2] · Kohji Kitaguchi[1,2] · Yoshihiro Kanamaru[1,2] · Tomio Yabe[1,2]

Received: 17 November 2014 / Revised: 15 March 2015 / Accepted: 2 April 2015 / Published online: 23 April 2015

Abstract Dietary fiber intake provides various physiological and metabolic effects for human health. Pectin, a water-soluble dietary fiber, induces morphological changes of the small intestine *in vivo*. However, the molecular mechanisms underlying pectin-derived morphological alterations have not been elucidated. Previously, we found that pectin purified from *Prunus domestica* L. altered the sulfated structure of cell-surface heparan sulfate (HS) on differentiated Caco-2 cells *via* fibronectin and α5β1 integrin. In this study, we investigated the biological significance of the effect of pectin on HS in differentiated Caco-2 cells. An *in vitro* intestinal epithelium model was constructed by co-culture of differentiated Caco-2 cells and rat IEC-6 cells, which were used as models of intestinal epithelium and intestinal crypt cells, respectively. We found that pectin-treated differentiated Caco-2 cells promoted growth of IEC-6 cells. Real-time RT-PCR analysis and western blotting showed that relative mRNA and protein expression levels of Wnt3a were upregulated by pectin treatment in differentiated Caco-2 cells. Analysis by surface plasmon resonance spectroscopy demonstrated that pectin-induced structural alteration of HS markedly decreased the interaction with Wnt3a. However, depression in the secretion of Wnt3a from Caco-2 cells by anti-Wnt3a antibody did not affect the proliferation of IEC-6 cells in co-culture system. These observations indicated that pectin altered the sulfated structure of cell-surface HS to promote secretion of Wnt3a from differentiated Caco-2 cells and Wnt3a indirectly stimulated the proliferation of IEC-6 cells.

Keywords Differentiated Caco-2 cell · Heparan sulfate · IEC-6 cell · Pectin · Wnt3a

Abbreviations

BSA	Bovine serum albumin
FBS	Fetal bovine serum
HS	Heparan sulfate
HSulf-2	Human HS 6-*O*-endosulfatase-2
QSulf-1	Quail HS 6-*O*-endosulfatase-1

Introduction

Dietary fiber intake has various physiological and metabolic effects on human health, including reduction of the risk of developing various diseases, such as hypertension, diabetes, obesity, and gastrointestinal disorders [1]. Moreover, higher consumption of dietary fiber has been shown to improve serum lipid concentration, lower blood pressure, improve blood glucose control in diabetes, help in weight loss, and it appears to improve immune function [1]. These physiological effects are presumably due to fermentation of dietary fiber by the colonic microflora with the production of short chain fatty acids [2]. On the other hand, dietary fiber intake provides direct (microflora-independent) effects on the gastrointestinal tract. Several studies have shown that pectin, which is a soluble dietary fiber, caused morphological changes and promoted crypt formation in the small intestine [3–5]. However, the mechanisms of pectin-induced morphological alteration of the small intestine have not been elucidated.

✉ Tomio Yabe
yabet@gifu-u.ac.jp

[1] United Graduate School of Agricultural Science, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

[2] Department of Applied Life Science, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

The intestinal epithelium of the small intestine is continuously renewed by cell generation and migration of stem cells located at the bottom of the crypts to the top of the villi [6]. Wnt proteins play a key role in development and homeostasis of the intestinal epithelium [7], and also heparan sulfate (HS) on intestinal epithelial cells plays a critical role in intestinal crypt homeostasis by regulating Wnt affinity [8].

HS is a sulfated polysaccharide that is ubiquitously present on the cell surface and in the extracellular matrix of all animal cells. The backbone of HS is synthesized by polymerization of alternating D-glucuronic acid and *N*-acetyl-D-glucosamine residues in the Golgi apparatus. Once the backbone is synthesized, HS is sulfated at the *N*, 6-*O*, and 3-*O* positions of glucosamine and 2-*O* position of uronic acid residues by multiple enzymes [9]. After HS is transported to the cell surface, HS 6-*O*-endosulfatases (Sulf1 and Sulf2) modify the sulfated structure of HS at the cell surface [10, 11]. Based on the sulfated structure synthesized by the enzymes, HS plays essential roles in various physiological activities as a receptor or co-receptor for different ligands, such as Wnt, Hedgehog, fibroblast growth factors, and transforming growth factor-β, and modulates the biological activities of these proteins [12, 13].

Our previous study showed that pectin purified from *Prunus domestica* L. caused structural alteration of HS by enhancing the expression of human 6-*O*-endosulftase-2 (HSulf-2) in differentiated Caco-2 cells [14]. In this study, we investigated the biological significance of pectin-induced structural changes of HS on differentiated Caco-2 cells using an *in vitro* intestinal epithelial model composed of differentiated Caco-2 cells and rat IEC-6 cells. As IEC-6 cell is a general model of intestinal crypt cells, our study provides further insights into pectin-derived morphological changes in the small intestine.

Materials and methods

Purification of pectin from prune

Prunes, *Prunus domestica* L. cv. d'Agen, imported from the USA as material for a commercial prune extract (concentrated prune juice), were supplied by MIKI Corporation (Hyogo, Japan). Purification of pectin from prune was described previously [14].

In vitro co-culture experiments

Caco-2 cells and IEC-6 cells were purchased from American Type Culture Collection (Manassas, VA). They were cultured at 37 °C with 5 % CO_2 in Dulbecco's Modified Eagle's Medium (Nissui Pharmaceuticals, Tokyo, Japan) supplemented with 10 % fetal bovine serum (FBS), 1 % minimal essential medium non-essential amino acids, 80 units/mL of penicillin, 80 μg/mL of streptomycin, 0.06 % L-glutamine, and 0.19 % $NaHCO_3$.

Caco-2 cells were seeded in 6.4 mm i.d. cell culture inserts (polyethylene terephthalate membrane, 1.0 μm pore size; Corning, Tewksbury, MA) in 24-well plates at a density of 1.0×10^5 cells/well. The basolateral and apical compartments contained 0.8 and 0.3 mL of culture medium, respectively. Culture medium was replaced every 2 days. The cells were cultured for 21 days to be differentiated, except 24 days for the experiments of collection of HS from cell surface.

In the case of co-culture system, IEC-6 cells were seeded in 24-well plates at a density of 0.2×10^5 cells/well and incubated for 24 h. The cell culture inserts with differentiated Caco-2 cells were transferred to 24-well plates containing IEC-6 cells. Medium in the outer wells (for IEC-6 cells) was replaced with FBS-free medium and that in insert wells (for differentiated Caco-2 cells) was replaced with FBS-free medium containing 0, 0.05, or 0.10 mg/mL pectin and incubated for 24 h. IEC-6 cells were assayed for proliferation by incubation for 2 h with a WST-8 cell quantification kit (Dojindo, Kumamoto, Japan).

In the case of individual culture system, differentiated Caco-2 cells cultured on permeable membranes were incubated with FBS-free medium containing 0, 0.05, 0.10, or 0.20 mg/mL pectin for 24 h. IEC-6 cells were incubated with basolateral medium harvested from outer wells in a 24-well plate for 24 h and cell proliferation was assayed with a WST-8 cell quantification kit (Dojindo).

In the case of depression in the secretion of Wnt3a from Caco-2 cells by anti-Wnt3a antibody (ab81614; Abcam, Bristol, UK), differentiated Caco-2 cells cultured on permeable membranes were incubated with FBS-free medium containing 0.20 mg/mL pectin for 24 h. IEC-6 cells were incubated with basolateral medium harvested from outer wells in a 24-well plate containing 0, 1, 2, 5, or 10 μg/mL anti-Wnt3a antibody for 24 h and cell proliferation was assayed with a WST-8 cell quantification kit (Dojindo).

Effects of exogenous recombinant Wnt proteins on proliferation of IEC-6 cells

IEC-6 cells were seeded in 96-well plates at a density of 0.2×10^4 cells/well and incubated for 24 h. Medium was replaced with FBS-free medium containing 0, 0.75, 1.5, 3.0, or 6.0 nM of recombinant Wnt proteins (recombinant human Wnt3a, Wnt5a, and Wnt11; R&D Systems, Minneapolis, MN) and incubated for 24 h. IEC-6 cells were assayed for proliferation by incubation for 2 h with a WST-8 cell quantification kit (Dojindo).

Surface plasmon resonance (SPR) experiment

Caco-2 cells were cultured in 55-cm^2 dishes at a density of 6.0×10^5 cells/dish and incubated for more than 21 days to be differentiated. Differentiated Caco-2 cells were incubated with or without 0.1 mg/mL pectin for 24 h and cell-surface HS was

collected as described previously [14]. The HS collected from cells with and without pectin (0.1 mg/mL) stimulation was designated as "pHS" and "cHS", respectively. pHS and cHS were incubated with EZ-link sulfo-NHS-LC-Biotin (Thermo Fisher Scientific, Waltham, MA) for 1 h at room temperature. Each biotinylated HS was recovered as a precipitate by centrifugation at 18,000×*g* for 1 h at 4 °C twice and dissolved in water. Each purified biotinylated HS was mixed with HBS-EP buffer (GE Healthcare, Little Chalfont, UK) containing 2 M NaCl, and then coupled to a streptavidin-immobilized sensor chip (Sensor Chip SA; GE Healthcare) as described in the manufacturer's instructions. Real-time analysis of the interactions between HS and Wnt proteins was performed with a Biacore J SPR instrument (GE Healthcare). Each experiment was repeated at least three times.

RT-PCR and real-time RT-PCR

Total RNA from differentiated Caco-2 cells was prepared with RNAiso Plus (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Aliquots of 1 μg of total RNA were treated with RNase-free DNase I (Toyobo, Osaka, Japan) and reverse-transcribed with oligo(dT)20 primer (Toyobo) and ReverTra Ace (Toyobo). The primer sequences used were as follows: for Wnt3a (326 bp), 5′-CAGGAACTACGTGG AGATCATG-3′ (F), 5′-CCATCCCACCAAACTCGATG TC-3′ (R); for Wnt11 (594 bp), 5′- GTAAGTGCCATGGG GTGTCT -3′ (F), 5′- GCTTCCAAGTGAAGGCAAAG -3′ (R); and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 457 bp), 5′-CATCACCATCTTCCAGGAGC-3′ (F), 5′-GTTCAGCTCTGGGATGACCT-3′ (R). PCR was carried out in a final volume of 15 μL containing each primer at 0.2 μM, PCR buffer for KOD-plus-NEO (Toyobo), 1 mM $MgSO_4$, 200 μM dNTP, and 0.15 U KOD-Plus-Neo (Toyobo) for 35 cycles of denaturation at 98 °C for 10 s, annealing at 58 °C for 15 s, and extension at 72 °C for 5 s.

To investigate the effects of pectin on each mRNA expression, the cells in 12-well plates were incubated with 0.1 mg/mL pectin for 1 h. After the medium was replaced with fresh medium, the cells were incubated for several hours. Relative expression of each mRNA was analyzed by real-time RT-PCR performed using THUNDERBIRD SYBR qPCR Mix (Toyobo) with ABI Step One Plus (Applied Biosystems, Foster City, CA). Real-time RT-PCR was performed with hot start at 95 °C for 60 s, followed by 40 cycles of denaturation for 15 s at 95 °C, annealing for 15 s at 58 °C, and extension for 10 s at 72 °C.

Immunoblotting analysis

Differentiated Caco-2 cells cultured in 6-well plates at a cell density of 1.0×10^5 cells/well were incubated with 0.1 mg/mL pectin for 24 h. The cells were washed with ice-cold PBS, homogenized in cold cell lysis buffer containing 25 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 5 % glycerol, and Protease Inhibitor Cocktail (Nacalai Tesque, Kyoto, Japan), and then centrifuged (800×*g*, 10 min, 4 °C). The supernatant was centrifuged (20,000×*g*, 60 min, 4 °C) and collected. Protein concentration was measured using a BCA Protein Assay Kit (Thermo Fisher Scientific). Samples were mixed with an equal volume of SDS sample buffer containing 62.5 mM Tris (pH 6.8), 2 % SDS, 10 % glycerol, 0.72 M 2-mercaptoethanol, and 0.1 % bromophenol blue, and then boiled for 3 min. Samples were separated by electrophoresis through 12 % polyacrylamide gels and transferred onto Immobilon-P transfer membranes (Merck Millipore). Membranes were blocked with 1 % BSA in Tris-buffered saline (pH 7.4) containing 0.1 % Tween 20. Wnt3a was detected by enhanced chemiluminescence (Immobilon Western Chemiluminescent HRP Substrate; Merck Millipore), using a primary antibody to Wnt3a (ab81614; Abcam) and anti-mouse horseradish peroxidase-linked secondary antibody (NA931; GE Healthcare). Beta-actin in the applied sample was detected by anti-actin antibody (sc-8432; Santa Cruz Biotechnology, Dallas, TX) for normalization of the protein levels. Recombinant human Wnt3a (R&D Systems) was loaded as a positive control.

Statistical analysis

Differences were evaluated by Tukey's test. Results are expressed as means±standard deviation (SD). In all analyses, $P<0.05$ was taken to indicate statistical significance.

Results

Effects of pectin on proliferation of IEC-6 cells in a co-culture system

In co-culture system, proliferation of IEC-6 cells in the outer well was investigated. The number of IEC-6 cells increased in a pectin concentration-dependent manner (Fig. 1a). This result suggested that pectin-treated Caco-2 cells secreted growth factors that promoted the growth of IEC-6 cells.

To investigate whether growth factors released by Caco-2 cells into the basolateral medium caused growth of IEC-6 cells, the effects of Caco-2 cell basolateral medium on IEC-6 cells were evaluated. In individual culture system, as shown in Fig. 1b, basolateral medium collected from pectin-stimulated Caco-2 cells promoted growth of IEC6 cells in a pectin dose-dependent manner. However, basolateral medium collected from cells without pectin administration failed to increase the number of IEC-6 cells. Administration of pectin to IEC-6 cells did not cause cellular proliferation (87.3 % for 0.1 mg/mL pectin), suggesting that the growth of IEC-6 cells was not

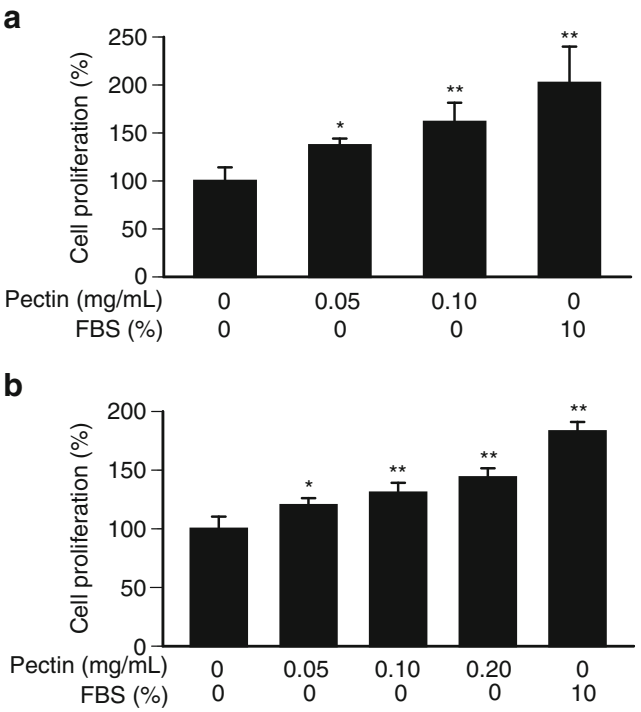

Fig. 1 Effects of pectin on proliferation of IEC-6 cells in the co-culture system. **a** Caco-2 cells were cultured for 21 days on permeable membranes and then IEC-6 cells were cultured in basolateral wells for 24 h. Differentiated Caco-2 cells were treated with either pectin-containing medium or control medium for 24 h. **b** Caco-2 cells were cultured for 21 days on permeable membranes and incubated with or without pectin for 24 h. IEC-6 cells were incubated with basolateral medium harvested from outer wells in a 24-well plate for 24 h. Cell proliferation was determined by the WST-8 method, and the percentage changes relative to control are presented. The values are shown as means ±S.D. of seven independent experiments. Statistical analyses were performed by Tukey's test. *: $P<0.05$, **: $P<0.01$

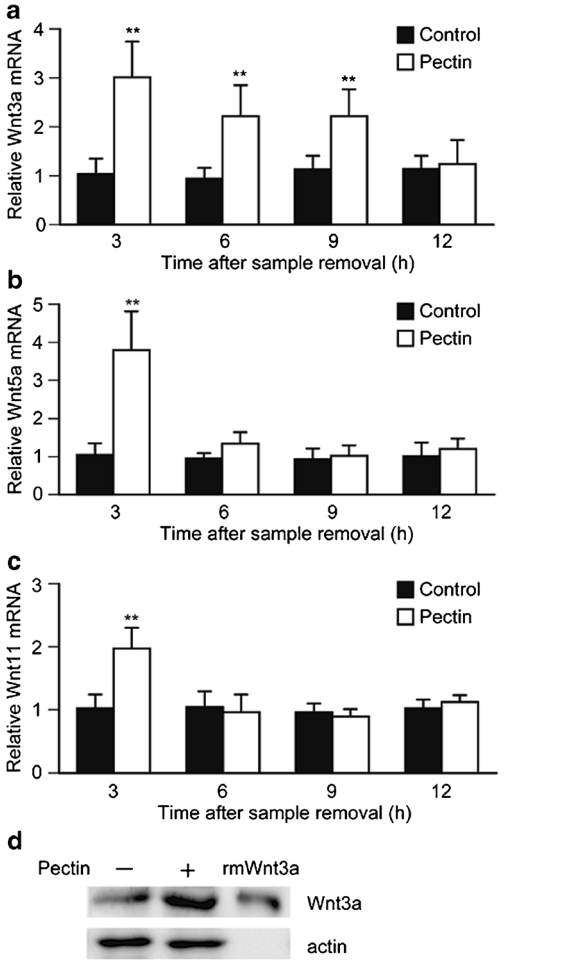

Fig. 2 Effects of pectin on the expression of Wnt proteins in differentiated Caco-2 cells. Differentiated Caco-2 cells were incubated with 0.1 mg/mL pectin for 1 h, and then cultured with fresh medium for the indicated times. Transcription levels of Wnt3a (**a**), Wnt5a (**b**), and Wnt11 (**c**) with or without pectin treatment were determined by real-time RT-PCR and normalized relative to GAPDH expression. The values are shown as means±S.D. of at least three independent experiments. Each value was compared with each control and statistical analyses were performed by Tukey's test. **: $P<0.01$. **d** Differentiated Caco-2 cells were incubated with 0.1 mg/mL pectin for 1 h. Whole-cell lysates were subjected to western blotting analysis to detect Wnt3a expression. Recombinant Wnt3a (rmWnt3a) was loaded as a positive control

induced by pectin that passed through the permeable membrane. These results indicated that pectin purified from *P. domestica* L. caused the secretion of some growth factors from differentiated Caco-2 cells into the basolateral medium that induced the proliferation of IEC-6 cells.

Effects on the expression of Wnt proteins in Caco-2 cells after pectin administration

The effects of pectin on the mRNA expression of Wnt3a, Wnt5a, and Wnt11 in differentiated Caco-2 cells were evaluated by quantitative RT-PCR. These genes were expressed in differentiated Caco-2 cells by RT-PCR. Real-time RT-PCR analysis indicated that pectin treatment enhanced mRNA expression of Wnt3a (Fig. 2a), Wnt5a (Fig. 2b), and Wnt11 (Fig. 2c), and its effect on Wnt3a was maintained for up to 9 h after stimulation (Fig. 2a). Moreover, immunoblotting analysis showed that pectin promoted Wnt3a expression in differentiated Caco-2 cells (Fig. 2d). However, Wnt11 was not detected with or without pectin administration (data not shown).

Effect of Wnt proteins on proliferation of IEC-6 cells

The enhanced expression of Wnt3a in differentiated Caco-2 cells after pectin stimulation (Fig. 2d) allowed us to investigate whether Wnt proteins stimulate proliferation of IEC-6 cells. To determine whether the effects of pectin-treated basolateral medium on proliferation of IEC6 cells (Fig. 1b) was due to the secretion of Wnt3a, we conducted cell proliferation assay using recombinant Wnt3a (rmWnt3a), recombinant Wnt5a (rmWnt5a), and recombinant Wnt11 (rmWnt11) on IEC-6 cells

cultured in 96-well plates. As shown in Fig. 3a and c, treatment with rmWnt3a and rmWnt11 induced proliferation of IEC-6 cells in a concentration-dependent manner. However, rmWnt5a did not show cellular growth activity (Fig. 3b).

Molecular interaction between Wnts and HS on the surface of differentiated Caco-2 cells

The effects of pectin-induced structural alteration of HS on the affinity for Wnts were investigated using the Biacore system. Cell-surface HS collected from the cells without or with pectin treatment was designated as "cHS" or "pHS," respectively, and immobilized on the sensor chip. The immobilization levels of cHS and pHS on the surface of the sensor chip were 849 and 874 resonance units, corresponding to 1.06 and 1.09 ng of each HS, respectively, indicating immobilization of approximately the same amount of HS. Wnt3a bound to the cHS-immobilized sensor chip in a concentration-dependent manner and the stable interaction was maintained after sample injection (Fig. 4a, left). In addition, the molecular interactions of Wnt3a against pHS were decreased (Fig. 4a, right). Wnt11 also bound to the cHS immobilized sensor chip in a concentration dependent manner, whereas its interaction was gradually decreased after 120 s (Fig. 4c, left). In addition, the molecular interactions of Wnt11 against pHS were dramatically decreased (Fig. 4c, right). These results suggested that pectin-treatment altered the specific sulfated structure of HS, which was recognized by Wnt3a or Wnt11, on the differentiated Caco-2 cells.

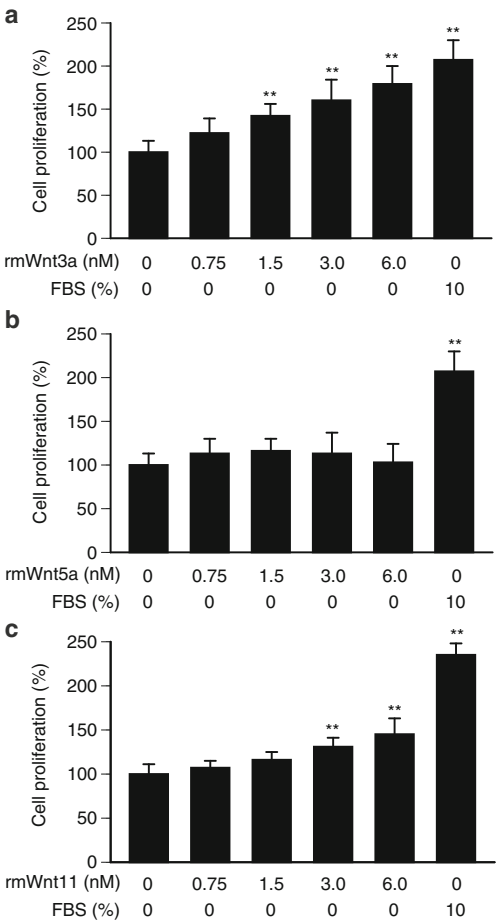

Fig. 3 Effects of exogenous recombinant Wnt3a, Wnt5a, and Wnt11 on proliferation of IEC-6 cells. Proliferation of IEC-6 cells treated with recombinant (rm) Wnt3a (**a**), rmWnt5a (**b**), and rmWnt11 (**c**) for 24 h was measured using the WST-8 assays. Data are shown as percentages of control. The values are shown as means±S.D. of seven independent experiments. Statistical analyses were performed by Tukey's test. **: $P<0.01$

Depression in the secretion of Wnt3a from Caco-2 cells by anti-Wnt3a antibody

In order to confirm that the secretion of Wnt3a from Caco-2 cells induced by pectin directly promoted proliferation of IEC-

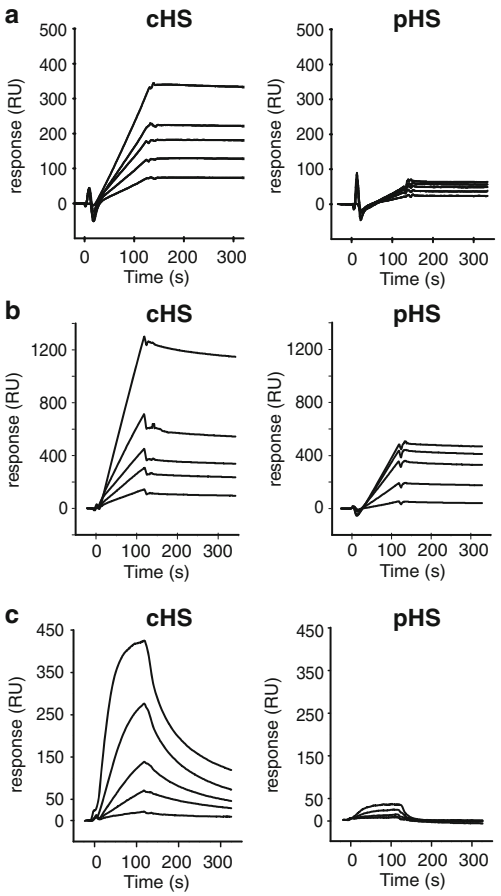

Fig. 4 Molecular interactions between Wnt proteins and HS collected from normal and pectin-treated Caco-2 cells. Differentiated Caco-2 cells were incubated with or without 0.1 mg/mL pectin for 24 h and cell-surface HS was collected. cHS (HS from non-treated cells) and pHS (HS from pectin-stimulated cells) were immobilized on the sensor chip. The molecular interaction was analyzed by five different concentrations of Wnt3a (**a**; 50, 75, 100, 125, or 150 nM), Wnt5a (**b**; 50, 75, 100, 125, or 150 nM), or Wnt11 (**c**; 1, 5, 20, 50, or 100 nM) with the cHS- (*left*) or pHS- (*right*) immobilized sensor chip. The samples were injected for 2 min, and then the dissociation phase began. Results are representative of three separate experiments

6 cells, the secretion was depressed by anti-Wnt3a antibody. Anti-Wnt3a antibody did not inhibit the proliferation of IEC-6 cells by itself even at high concentrations in the medium (Fig. 5, black bar). Addition of anti-Wnt3a antibody into the medium including secreted factors from the Caco-2 cells treated with pectin did not affect the proliferation of IEC-6 cells at all (Fig. 5, white bar). These results suggested that the Wnt3a secreted from Caco-2 cells indirectly stimulated the proliferation of IEC-6 cells.

Discussion

The present study demonstrated the involvement of cell-surface HS in the cellular proliferation effect of pectin from *P. domestica* L. on IEC-6 cells *via* differentiated Caco-2 cells. Although previous reports suggested that pectin intake caused morphological changes in the small intestine *in vivo* [3–5], the detailed mechanisms underlying the effect of pectin on structural alteration of the small intestine have not been elucidated. We hypothesized that the direct interaction of pectin with intestinal epithelial cells promoted the proliferation of cryptal cells, thereby inducing morphological changes. To address this hypothesis, we created an *in vitro* model of intestinal epithelium by incubating differentiated Caco-2 cells, which express morphological and functional characteristics of mature small-intestinal enterocytes, on a permeable membrane and IEC-6 cells were cultured in a basolateral well as a model

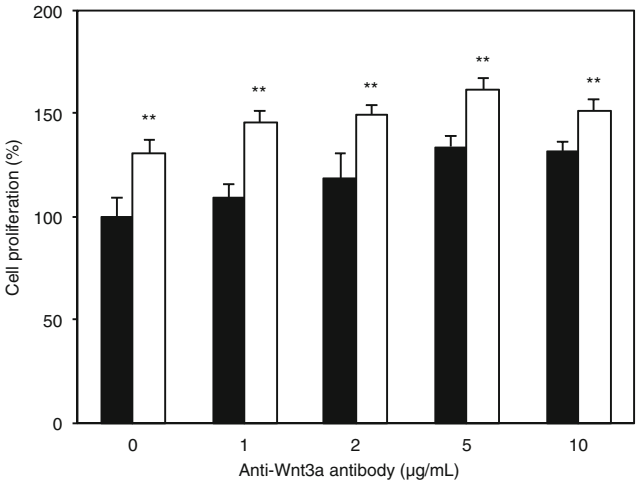

Fig. 5 Depression in the secretion of Wnt3a from Caco-2 cells by anti-Wnt3a antibody. Caco-2 cells were cultured for 21 days on permeable membranes and incubated without (*black bar*) or with (*white bar*) 0.2 mg/mL pectin for 24 h. IEC-6 cells were incubated with basolateral medium harvested from outer wells in a 24-well plate containing 0, 1, 2, 5, or 10 μg/mL anti-Wnt3a antibody for 24 h. Cell proliferation was determined by the WST-8 method, and the percentage changes relative to control (cultured without pectin) are presented. The values are shown as means±S.D. of six independent experiments. Student's *t*-test was used to determine significant difference between with and without stimulation of pectin. **: $P<0.01$

of intestinal crypt cells. In this co-culture system, differentiated Caco-2 cells cultured with pectin from *P. domestica* L. promoted proliferation of IEC-6 cells, suggesting that differentiated Caco-2 cells secreted some growth factors into the basolateral medium in response to pectin, thereby inducing an increase in number of IEC-6 cells.

Our previous study showed that pectin from *P. domestica* L. enhanced the mRNA expression of HSulf-2 and altered the sulfated structure of HS in differentiated Caco-2 cells [14]. However, the biological significance of pectin-induced structural alteration of HS remains to be elucidated. QSulf-1, which is a quail homologue of HSulf-2, can function in a cell autonomous manner to remodel the sulfation pattern of cell-surface HS and promote Wnt signaling [15]. In this study, the mRNA expression of Wnt3a, Wnt5a, and Wnt11 were confirmed in differentiated Caco-2 cells and all expressions were enhanced by pectin-treatment. Wnt3a and Wnt11 are known to promote proliferation and migration of IEC-6 cells [16, 17]. On the other hand, Gross *et al.* reported that Wnt5a was limitedly secreted into apical well in polarized Caco-2 cells [18]. Since Wnt5a controls cell polarity [19], pectin may increase the polarity of Caco-2 cells by inducing Wnt5a expression. Hence, we focused on Wnt3a and Wnt11, which can be secreted from Caco-2 cells into basolateral well and promote cellular proliferation of IEC-6 cells. However, immunoblotting analysis revealed that pectin increased Wnt3a expression in differentiated Caco-2 cells, whereas Wnt11 expression was not confirmed with or without pectin-administration. Therefore, Wnt3a is one of promising candidates, which are secreted from differentiated Caco-2 cells into the basolateral well and directly or indirectly promote proliferation of IEC-6 cells by the pectin administration.

Ai *et al.* suggested that QSulf-1 remodeled the sulfated structure of HS to convert HS to a low-affinity binding state for Wnts [15]. Wnt3a bound strongly to HS from normal differentiated Caco-2 cells in a dose-dependent manner (Fig. 4a). On the other hand, structure-remodeled HS showed markedly decreased binding to Wnt3a (Fig. 4a). As cell-surface HS is mainly expressed on the basolateral side of polarized Caco-2 cells [20] and is predominantly located on the basement surface in the developing intestine [21], these results suggested that pectin promoted the secretion of Wnt3a from differentiated Caco-2 cells by changing the sulfated structure of HS to reduce its interaction with Wnt3a. In order to consider that HS participates in this promotion, cell proliferation assay should be performed using the conditioned medium of the differentiated Caco-2 cells with or without treatment by heparitinase and/or heparanase. However, the role of HS is not only to concentrate Wnt molecules at the cell surface but also to prevent them from aggregating in the extracellular environment [22]. Furthermore, secretion of Wnt proteins is usually inefficient and attempts to characterize Wnt proteins are hampered by their high degree of insolubility [23].

Therefore, since it is vital for exerting the effects of Wnt proteins to express HS on the cell surface, we predicted that the treatment by heparitinase and/or heparanase for HS on the differentiated Caco-2 cells was biologically meaningless. Further studies are needed to evaluate the changing of the sulfated structure of HS to reduce its interaction with Wnt proteins. In addition, depression in the secretion of Wnt3a from Caco-2 cells by anti-Wnt3a antibody did not affect the proliferation of IEC-6 cells in co-culture system (Fig. 5). This means that pectin alters the sulfated structure of cell-surface HS to promote secretion of Wnt3a from differentiated Caco-2 cells and then the secreted Wnt3a indirectly stimulates the proliferation of IEC-6 cells. Since the results indicate that the medium from the basolateral side of Caco-2 cells does not include Wnt3a and does include other growth factors, our results define some potential for the role of Wnt3a as autocrine Wnt signaling to secrete growth factors from Caco-2 cells for the proliferation of IEC-6 cells.

In conclusion, we demonstrated that pectin-treated Caco-2 cells promoted the secretion of Wnt3a to the basolateral side by lowering the interaction with cell-surface HS and Wnt3a indirectly stimulated the proliferation of IEC-6 cells *in vitro*. Wnt3a is mainly expressed in Paneth cells, which reside next to intestinal stem cells [24]. As transit-amplifying cells in crypts migrate along the crypt-villus axis, pectin may promote the morphological changes of the small intestine by regulating the affinity between Wnt3a and HS in crypts. This study provided fundamental insights into the mechanisms of pectin-induced morphological changes in the small intestine *in vivo*.

Acknowledgments We thank the members of the Division of Genomics Research, Life Science Research Center, Gifu University, for their support in conducting the experiments and thank Miki Corporation (Hyogo, Japan) for providing the concentrated prune juice. This work was supported by Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research (C) Grant Number 23580163.

References

1. Anderson, J.W., Baird, P., Davis Jr., R.H., Ferreri, S., Knudtson, M., Koraym, A., Waters, V., Williams, C.L.: Health benefits of dietary fiber. Nutr. Rev. **67**, 188–205 (2009)
2. Brown, L., Rosner, B., Willett, W.W., Sacks, F.M.: Cholesterol-lowering effects of dietary fiber: a meta-analysis. Am. J. Clin. Nutr. **69**, 30–42 (1999)
3. Tasman-Jones, C., Owen, R.L., Jones, A.L.: Semipurified dietary fiber and small-bowel morphology in rats. Dig. Dis. Sci. **27**, 519–524 (1982)
4. McCullogh, J.S., Ratcliffe, B., Mandir, N., Carr, K.E., Goodlad, R.A.: Dietary fibre and intestinal microflora: effects on intestinal morphometry and crypt branching. Gut **42**, 799–806 (1998)
5. Langhout, D.J., Schutte, J.B., van Leeuwen, P., Wiebenga, J., Tamminga, S.: Effect of dietary high- and low-methylated citrus pectin on the activity of the ileal microflora and morphology of the small intestinal wall of broiler chicks. Br. Poult. Sci. **40**, 340–347 (1999)
6. Babyatsky, M.W., Podolsky, D.K.: Growth and development of the gastrointestinal tract. In: Yamada, T. (ed.) Textbook of gastroenterology, pp. 547–584. Lippincott Williams & Willkins, Philadelphia (1999)
7. Crosnier, C., Stamataki, D., Lewis, J.: Organizing cell renewal in the intestine: stem cells, signals and combinatorial control. Nat. Rev. Genet. **7**, 349–359 (2006)
8. Yamamoto, S., Nakase, H., Matsuura, M., Honzawa, Y., Matsumura, K., Uza, N., Yamaguchi, Y., Mizoguchi, E., Chiba, T.: Heparan sulfate on intestinal epithelial cells plays a critical role in intestinal crypt homeostasis via Wnt/β-catenin signaling. Am. J. Physiol. Gastrointest. Liver Physiol. **305**, G241–G249 (2013)
9. Habuchi, O.: Diversity and functions of glycosaminoglycan sulfotransferases. Biochim. Biophys. Acta **1474**, 115–127 (2000)
10. Lamanna, W.C., Frese, M.A., Balleininger, M., Dierks, T.: Sulf loss influences N-, 2-O-, and 6-O-sulfation of multiple heparan sulfate proteoglycans and modulates fibroblast growth factor signaling. J. Biol. Chem. **283**, 27724–27735 (2008)
11. Frese, M.A., Milz, F., Dick, M., Lamanna, W.C., Dierks, T.: Characterization of the human sulfatase Sulf1 and its high affinity heparin/heparan sulfate interaction domain. J. Biol. Chem. **284**, 28033–28044 (2009)
12. Bernfield, M., Götte, M., Park, P.W., Reizes, O., Fitzgerald, M.L., Lincecum, J., Zako, M.: Functions of cell surface heparan sulfate proteoglycans. Annu. Rev. Biochem. **68**, 729–777 (1999)
13. Bishop, J.R., Schuksz, M., Esko, J.D.: Heparan sulphate proteoglycans fine-tune mammalian physiology. Nature **446**, 1030–1037 (2007)
14. Nishida, M., Murata, K., Kanamaru, Y., Yabe, T.: Pectin of *Prunus domestica* L. alters sulfated structure of cell-surface heparan sulfate in differentiated Caco-2 cells through stimulation of heparan sulfate 6-*O*-endosulfatase-2. Biosci. Biotechnol. Biochem. **78**, 635–643 (2014)
15. Ai, X., Do, A.T., Lozynska, O., Kusche-Gullberg, M., Lindahl, U., Emerson Jr., C.P.: QSulf1 remodels the 6-O sulfation states of cell surface heparan sulfate proteoglycans to promote Wnt signaling. J. Cell Biol. **162**, 341–351 (2003)
16. Liu, L., Rao, J.N., Zou, T., Xiao, L., Smith, A., Zhuang, R., Turner, D.J., Wang, J.Y.: Activation of Wnt3a signaling stimulates intestinal epithelial repair by promoting c-Myc-regulated gene expression. Am. J. Physiol. Cell Physiol. **302**, C277–C285 (2012)
17. Ouko, L., Ziegler, T.R., Gu, L.H., Eisenberg, L.M., Yang, V.W.: Wnt11 signaling promotes proliferation, transformation, and migration of IEC6 intestinal epithelial cells. J. Biol. Chem. **279**, 26707–26715 (2004)
18. Gross, J.C., Chaudhary, V., Bartscherer, K., Boutros, M.: Active Wnt proteins are secreted on exosomes. Nat. Cell Biol. **14**, 1036–1045 (2012)
19. Witze, E.S., Litman, E.S., Argast, G.M., Moon, R.T., Ahn, N.G.: Wnt5a control of cell polarity and directional movement by polarized redistribution of adhesion receptors. Science **320**, 365–369 (2008)
20. Mertens, G., van der Schueren, B., van den Berghe, H., David, G.: Heparan sulfate expression in polarized epithelial cells: the apical sorting of glypican (GPI-anchored proteoglycan) is inversely related to its heparan sulfate content. J. Cell Biol. **132**, 487–497 (1996)
21. Simon-Assmann, P., Bouziges, F., Vigny, M., Kedinger, M.: Origin and deposition of basement membrane heparan sulfate proteoglycan in the developing intestine. J. Cell Biol. **109**, 1837–1848 (1989)
22. Fuerer, C., Habib, S.J., Nusse, R.: A study on the interactions between heparan sulfate proteoglycans and Wnt proteins. Dev. Dyn. **239**, 184–190 (2010)
23. Willert, K., Brown, J.D., Danenberg, E., Duncan, A.W., Weissman, I.L., Reya, T., Yates III, J.R., Nusse, R.: Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature **423**, 448–452 (2003)
24. Sato, T., van Es, J.H., Snippert, H.J., Stange, D.E., Vries, R.G., van den Born, M., Barker, N., Shroyer, N.F., van de Wetering, M., Clevers, H.: Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. Nature **469**, 415–418 (2011)