10-Hydroxydecanoic Acid Potentially Elicits Antigen-Specific IgA Responses

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The effective antigen (Ag) uptake by microfold cells (M-cells) is important for the induction of an efficient mucosal immune response. Here, we show that 10-hydroxydecanoic acid (10-HDAA) from royal jelly (RJ) potentially supports M-cell differentiation and induces effective antigen-specific mucosal immune responses in cynomolgus macaques. 10-HDAA increases the expression level of receptor activator of nuclear factor-kappaB (NF-κB) (RANK) in Caco-2 cells, which suggests that 10-HDAA potentially promotes the differentiation of Caco-2 cells into M-cells and increased transcytosis efficiency. This idea is supported by the following observations. Intranasal administration of 10-HDAA increased the number of M-cells in the epithelium overlying nasopharynx-associated lymphoid tissue (NALT) in macaques. Oral administration of 10-HDAA increased the number of M-cells in the follicle-associated epithelium (FAE) covering Peyer’s patches (PPs) and significantly increased the antigen-specific immunoglobulin A (IgA) level in macaques. These findings suggest that the exogenous honeybee-derived medium-chain fatty acid 10-HDAA potentially enhances antigen-specific immune responses.

Key words royal jelly; 10-hydroxydecanoic acid; microfold cell (M-cell); mucosal immune response

INTRODUCTION

A unique chemical feature of royal jelly (RJ) is the presence of medium-chain hydroxy fatty acids. 1) (E)-10-hydroxy-2-decenoic acid (10-HDA) is the major lipid component of RJ and a characteristic fatty acid of RJ not found in other foods. 10-Hydroxydecanoic acid (10-HDAA) is the second largest lipid component of RJ. 10-HDA has been reported to have anti-tumoral 2) and anti-bacterial 3) activities, while 10-HDA has been reported to have anti-inflammatory activity. 4) It is expected that these medium-chain hydroxy fatty acids may exhibit similar effects due to their similar structures. However, we individually need to examine in detail whether these fatty acids act on the target disease and show the expected effects. In fact, in our previous work, 5) ether extracts from bacterial alkaline protease-catalyzed hydrolysathe of RJ (pRJ) increased the expression level of a microfold cell (M-cell) marker, glycoprotein 2 (GP2), in an in vitro Caco-2 monolayer and pRJ enhanced the antigen-specific mucosal immunoglobulin A (IgA) response in vivo. Furthermore, the treatment of a Caco-2 monolayer for 3 d with 100 μM 10-HDAA but not 10-HDA increased the expression level of a gene related to M-cell differentiation (data not shown), suggesting that pRJ contains a nonproteinaceous factor involved in M-cell differentiation and 10-HDAA may induce efficient mucosal immune responses.

The mechanisms of the induction and differentiation of M-cells have been substantially characterized. M-cells arise from GPR49-positive epithelial intestinal crypt stem cells (SCs). 6) Their differentiation from the SCs is strongly affected by subepithelial stromal cells in gut-associated lymphoid tissues (GALTs). Indeed, receptor activator of nuclear factor-kappaB (NF-κB) ligand (RANK) expressed by stromal cells underneath follicle-associated epithelium (FAE) triggers M-cell differentiation. 7) Furthermore, the membrane-bound RANKL expressed by M-cell inducer cells (MCi cells) induces the differentiation of RANK-expressing enterocytes into M-cells. 8) Kanaya et al. showed that the downstream TRAF6-mediated nuclear factor-kappaB (NF-κB) signaling pathway of RANK/RANKL interaction (RANK-TRAF6-NF-κB axis) is essential for M-cell differentiation. 9) In addition to RANK/RANKL signaling, an important role of B-cells in inducing M-cell differentiation has been well established. 10) The RANKL-dependent release of CCL20 from enterocytes mediates the chemoattraction of CCR6-positive B-cells towards the FAE. 11) Interestingly, CCR6-deficient mice (CCR6 −/− mice) retain ANXA5-expressing immature M-cells but have a significantly decreased frequency of GP2-positive mature M-cells in the FAE, 12–14) suggesting that the blockade of CCL20-CCR6 signaling in CCR6 −/− mice does not affect the initial steps of M-cell differentiation but impedes their functional maturation. These findings intrinsically connect the induction of the differentiation of M-cells to the underlying IgA production in GALTs.

In this study, we show that 10-HDAA potentially supports M-cell differentiation and is useful for the induction of effective antigen-specific mucosal immune responses.

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MATERIALS AND METHODS

Cell Cultures  Caco-2 and Raji-B cells were obtained from RIKEN BioResource Research Center.

Induction of Expression of RANK by 10-HDAA  A Caco-2 monolayer was cultured in the presence of 100 µM or 1 mM 10-HDAA (Merck KGaA, Darmstadt, Germany; purity: 99.3%) for 6h and further cultured in MEM/20% fetal calf serum (FCS) + 0.1 mM nonessential amino acids for more 18h. The monolayer was then treated with 1 mM ethylenediaminetetraacetic acid (EDTA)·2Na/phosphate buffered saline (PBS) (−). Finally, the cells (5.0 × 10^6) were stained with anti-RANK antibody (Ab) and isotype control Ab (Cell Signaling Technology, Inc., Danvers, MA, U.S.A.) and Alexa 488-conjugated goat anti-rabbit IgG and then subjected to flow cytometry. Furthermore, the membrane fraction (15 µg) of 100 µM 10-HDAA-treated cells was prepared using a ProteoExtract® Subcellular Proteome Extraction Kit (Merck KGaA) and subjected to Western blot analysis with anti-RANK Ab (Cell Signaling Technology, Inc.).

In Vitro Human M-Cell Model  The M-cell model was constructed using cocultures of Caco-2 cells and Raji-B cells in accordance with the method of Kernéis et al. with slight modification. We seeded Caco-2 cells (1 × 10^6 cells) on the lower surface of Transwell inserts and cultured them for 16h. The inserts were then transferred to a Transwell device with the epithelial cells facing the lower chamber of the plates. Caco-2 cells were cultured for 21 d. Raji-B cells (1 × 10^6 cells) were added to the upper chamber facing the basolateral side of Caco-2 cells. The cultures were maintained for 5 d with or without 100 µM 10-HDAA in the lower chamber. To examine GP2 expression, the Caco-2 monolayers were fixed with cold methanol, blocked with 5% skim milk/PBS (−), and stained with an anti-GP2 Ab (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) labeled with Alexa555 using a Zenon mouse IgG labeling kit. Slides were then incubated with 4′,6-diamidino-2-phenylindole (DAPI). Finally, the monolayers were analyzed with a Keyence BIORÉVO BZ-9000. To further investigate whether 10-HDAA enhances transcytosis, the monolayers including M-cells or Caco-2 monolayers were incubated with Fluospheres® carboxylate-modified microspheres (LifeTechnologies Corporation, Carlsbad, CA, U.S.A.). The microspheres were quantified using an ARVO multimicroplate reader.

Animals  Purpose-bred male cynomolgus macaques (Macaca fascicularis) obtained from a supplier in China (3–6 years old) were used for this study. This study (permission no. A29-037) was approved and conducted in accordance with the guidelines of the Animal Care and Use Committee of Kumamoto University.

Collection of Nasopharynx-Associated Lymphoid Tissue (NALT) and Peyer’s Patches (PPs)  The part of NALT including the site of 10-HDAA inoculation was collected. After washing the collected part, the blocks of this part were i) fixed in 10% neutral buffered formalin, trimmed, embedded in paraffin, and sectioned for in situ hybridization; or ii) embedded in the OCT compound (Sakura Finetechical, Tokyo, Japan) for immunofluorescence staining. On the other hand, the part of the ileum including PPs near the site where an enteric capsule, QUALI-V-S (Qualicaps Co., Ltd., Nara, Japan), containing Blue Dextran 2000 collapsed in a control experiment was collected. After washing the collected part of the ileum, 2 cm^2 blocks of PPs were fixed in ice-cold 3% glutaraldehyde/0.1 M sucrose/PBS (−) (pH 7.4).

Scanning Electron Microscopy (SEM)  The tissue samples were rinsed in PBS (−) with 0.1 M sucrose (pH 7.4) and postfixed with 1% osmium tetroxide in 0.1 M PBS (−) at 4°C for 2h. All the samples were dehydrated with 50:50, 70:30, 80:20, 90:10, and 95:5 ethanol/water mixtures and 100% ethanol for 10 min each and rinsed three times with 100% ethanol for further dehydration. The samples were critical-point-dried by flooding with liquid carbon dioxide at 5°C for 20 min. The temperature was then raised to the critical point (JCPD-5, JEOL). Samples were sputter-coated with gold (JFC-1100E, JEOL) and examined by SEM with a JEOL JSM-5200 at an accelerating voltage of 15 kV.

In Situ Hybridization  DNA fragments encoding macaque GP2 were amplified by PCR from PP FAE-derived cDNA. The following sets of primers were used: GP2, sense, 5′-GAG TGA GGA TGT CGG AGA CCT GTG-3′, antisense, 5′-CTCCG AA ATG TTCT CCT GCA GGC AC-3′; and sense, 5′-GTA ATACGACTCTATAGGGGAT GAG GAT GTC GAA GAC CTG-3′, antisense, 5′-GTAATACGACTCTACTAGGGCT CAG AAT GTT CCT GCA GGC AC-3′ (T7 promoter sequences are shown in italics). Digoxigenin-labeled sense and anti-sense RNA probes were transcribed in vitro from T7 promoter-conjugated PCR products with a DIG RNA labeling mix. Paraffin-embedded tissue sections prepared from NALT were deparaffinized and hybridized with 1.2 µg/mL digoxigenin-labeled RNA probes. Specific binding was detected by incubation with an alkaline phosphatase-conjugated anti-digoxigenin Ab (Roche Diagnostics, Basel, Switzerland) and revealed by incubation with a purple alkaline phosphatase substrate overnight.

Immunofluorescence Staining  Frozen sections derived from macaques were fixed in cold acetone, blocked with 1% nonfat skim milk in PBS (−), and stained with an anti-GP2 Ab (Abcam, Cambridge, U.K.) labeled with Alexa555 using a Zenon mouse IgG labeling kit. Slides were then incubated with 4′,6-diamidino-2-phenylindole (DAPI). Finally, the monolayers were analyzed with a Keyence BIORÉVO BZ-9000. To further investigate whether 10-HDAA enhances transcytosis, the monolayers including M-cells or Caco-2 monolayers were incubated with Fluospheres® carboxylate-modified microspheres (LifeTechnologies Corporation, Carlsbad, CA, U.S.A.). The microspheres were quantified using an ARVO multimicroplate reader.

Immunization Schedule  All of the macaques were housed in individual cages and maintained in accordance with the rules and guidelines of the National Institute for Infectious Diseases for experimental animal welfare. Three 3- to 6-year-old macaques (Nos. 1–3) were orally administered with enteric-coated capsules QUALI-V-S containing feticuin (3 mg; Sigma-Aldrich Co., LLC, St. Louis, MO, U.S.A.), an inactivated poliovirus type III antigen for complement fixation test [10^6 50% cell culture infectious dose (CCID50)]; Denka Seiken Co., Ltd., Tokyo, Japan], and an inactivated native influenza A H1N1 virus (A/California/7/2009/NYM C X-179A) antigen (220 HA units; Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.). Another three macaques (Nos. 4–6) were immunized with the enteric-coated capsule including the above-mentioned Ags with 10-HDAA (2.4 mg) on Days 1, 4, 8, 11, 15, and 18. Furthermore, when the 10-HDAA-containing Ags were not
orally administered, only 10-HDAA was orally administered with the enteric capsule. Fecal pellets were obtained at 0, 12, and 21 d postinitial immunization.

Sample Collection and Processing Fecal pellets (3 g) were collected in tubes, then immersed in a 100% ethanol/dry ice cooling bath and frozen. The fecal pellets were resuspended in a sample buffer [0.1% sodium azide, 1 mmol/L EDTA·2Na, 0.05% Tween 20, 5% nonfat skim milk, and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) in PBS (pH 7.2)]. The suspensions [(20% w/w)] were centrifuged at 13000 × g for 5 min to remove fecal solids. The processed fecal Ab samples were subjected to enzyme-linked immunosorbent assay (ELISA).

ELISA of Anti-fetuin, Polio Virus, Influenza Virus Abs Each well of a flat-bottom 96-well MaxiSorp microplate was i) coated with 50 µL of antigen buffer [50 mmol/L Tris–HCl (pH 8.0), 10 mmol/L MgCl₂, 0.1% Tween 80] containing fetuin (100 µg/mL) and incubated at 37°C for 3 h, ii) coated with 50 µL of antigen buffer containing the inactivated polio virus type III antigen (2.7 × 10⁶ CCID₅₀/mL) and incubated overnight, or iii) coated with 50 µL of antigen buffer containing the inactivated native influenza A H1N1 virus (A/California/7/2009/NYMC X-179A) antigen (440 HA unit/mL) and incubated overnight. Subsequently, 200 µL of blocking buffer containing 1% nonfat skim milk and 0.01% sodium azide in Milli-Q water was added to each well and incubated at 4°C overnight. A fecal Ab sample (50 µL) diluted to 1/10 in PBS (−) was added to each well. Finally, 50 µL of peroxidase-conjugated goat anti-mouse IgG (diluted 1/5000; Alpha Diagnostic Intl., Inc., San Antonio, TX, U.S.A.) was added to each well and incubated for 45 min. Fifty microliters of 3,3′,5,5′-tetramethylbenzidine solution (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was added to each well and incubated. Absorbance was measured at 450/630 nm using a microplate reader.

RESULTS

Stimulation with 10-HDAA Increased the Expression Level of RANK, Which Was Associated with the Differentiation of Functional M-Cells Since RANK/RANKL interaction is essential for in vivo M-cell differentiation, we investigated whether 10-HDAA increased the expression level of RANK. The treatment of the Caco-2 monolayer for 6 h with 10-HDAA increased the expression level of RANK (Fig. 1A). In the in vitro M-cell model, the number of GP2-positive cells increased after coculturing the Caco-2 monolayer with Raji-B cells, and further increased when stimulated simultaneously with 10-HDAA (Fig. 1B). In addition, the simultaneous stimulation with Raji-B cells and 10-HDAA markedly increased transcytosis efficiency (Fig. 1C). Interestingly, RANKL is expressed on Raji-B cells (Sup-

![Fig. 1. Stimulation with 10-HDAA Increased the Expression Level of RANK and Induced Functional M-Cells](image-url)
Therefore, these results suggest that M-cell differentiation was promoted by the increased interaction of RANK on the Caco-2 monolayer with RANKL expressed on Raji-B cells. These findings support the 10-HDAA-dependent M-cell differentiation via the RANKL/RANK-mediated pathway and suggest that the differentiation of functional M-cells could be induced in vivo by administering 10-HDAA from the luminal side to the mucosa.

**Intranasal Administration of 10-HDAA Increased the Number of M-Cells in the Epithelium Covering the Luminal Surface of NALT**

We examined whether the number of M-cells is increased by administering 10-HDAA to the nasal cavity of macaques. Sections for H&E staining, SEM, *in situ* hybridization, and immunofluorescence staining were prepared from NALT (Fig. 2A). The epithelium mounting over the dome of lymphoid nodules in NALT (Fig. 2B, regions a and b) derived from the control macaque showed the absence of goblet cells, the reduced number of cell layers, and the stratified squamous epithelial cells. In region c, “G” indicates a goblet cell. C. SEM analysis of respiratory M-cells. D. *In situ* hybridization demonstrates the expression of GP2 mRNA in FAE (arrow head) in NALT. E. Immunofluorescence analysis of GP2 revealed respiratory M-cells. F. In NALT of a macaque nasally administered with 10-HDAA, the expression level of GP2 was significantly higher than that in a control macaque (arrow head). The significance of difference (unpaired-Student’s *t*-test) is indicated as follows: **, *p* < 0.01. The mean values (open circle) of at least five independent GP2-positive areas per follicle are shown. (Color figure can be accessed in the online version.)
and the presence of cilia. In contrast, goblet cells existed in a part slightly displaced from the lymphoid nodules with the pseudostratified ciliated columnar epithelium (Fig. 2B, region c). SEM demonstrated that the respiratory M-cells present on the side of the ciliated epithelium (CE) show the characteristic feature of typical M-cells, namely, having a depressed surface with short and irregular microvilli (Fig. 2C). Furthermore, in situ hybridization and immunofluorescence staining revealed that GP2 mRNA and GP2 protein were expressed in the epithelium overlying the NALT follicles (Figs. 2D, E). However, although there is a possibility that the induction of GP2-positive M-cells may be affected by the rearing environment, they were not easily detected in the control cynomolgus macaque. In the case of the 10-HDAA-administered macaque, 100 μL of 10-HDAA (100 μM) was intranasally administered to it once a day for 3 d with an intranasal drug delivery device (Fine Atomizer Nasal) and NALT samples were collected on Day 4. As shown in Fig. 2F, the number of GP2-positive M-cells significantly increased in the epithelium overlying the follicles of NALT. These findings suggest that 10-HDAA may prompt the differentiation of respiratory M-cells in vivo.

Oral Administration of 10-HDAA Increased the Number of M-Cells in FAE Covering the Luminal Surface of GALT To further confirm the capability of 10-HDAA to prompt M-cell differentiation in vivo, we orally administered 10-HDAA to macaques. First, an enteric capsule containing Blue dextran 2000 was orally administered to a macaque to identify the part of the intestinal tract exposed to 10-HDAA. As a result, we confirmed that the capsule collapsed at 3h post-oral administration in the vicinity of the terminal ileum, where many Peyer’s patches (PPs) were observed (Fig. 3A right panel). Therefore, we investigated whether the expression
level of GP2 is elevated in FAE covering the dome of PPs. As shown in Figs. 3B, C, in the control macaque, the fluorescence of GP2 derived from typical M-cells was confirmed in FAE, and the frequency of M-cells in FAE was higher than that in NALT, suggesting that the frequency of exposure to commensal bacteria and other microorganisms was affected. In contrast, 10-HDAA (2.4 mg) was orally administered to a macaque once a day for 20 d with an enteric capsule. We used the previously reported protocol to demonstrate the mucosal immunomodulatory properties of pRJ via stimulation of the effective uptake of Ags through M-cells, and PPs were collected on Day 21. The number of GP2-positive M-cells significantly increased in FAE overlying the follicles of PPs (Figs. 3D–F). Furthermore, although the behavior of GP2 after its transcytosis is still unclarified, a significant amount of the fluorescence derived from GP2 was transferred from FAE to the subepithelial dome (SED) region (Figs. 3D, E, G). Terahara et al., Kobayashi et al., and de Lau et al. also showed that mouse GP2 moved from FAE to SED (Fig. 3B in Ref. 15; Fig. 5C in Ref. 16; Fig. 4 in Ref. 17, respectively), suggesting that luminal antigens were incorporated into the SED region along with GP2 via transcytosis across M-cells. These findings suggest that 10-HDAA may prompt the differentiation of M-cells in PPs in vivo.

Oral Administration of 10-HDAA Significantly Increased the Antigen-Specific IgA Levels in Macaques
To examine the capability of 10-HDAA to induce efficient mucosal immune responses, three macaques were immunized with enteric coated capsules containing fetuin (3 mg), inactivated influenza virus (220 HA units), and poliovirus antigens (10^6 TCID50) with 10-HDAA (2.4 mg) by oral administration according to the time schedule shown in Fig. 4A. Another three macaques were immunized with enteric-coated capsules containing only the mixture of Ags. Interestingly, fetuin-specific IgA in stool samples was induced in all the macaques immunized with Ags with 10-HDAA at least by Day 12 (Fig. 4B lower), although no fetuin-specific IgA induction was observed on Day 20 in macaque No. 4. In contrast, the immunization of all the macaques with only Ags did not effectively induce fetuin-specific IgA (Fig. 4B upper). Next, we investigated whether the effective induction of IgA against viral antigens, such as the poliovirus and influenza virus. Poliovirus- and influenzavirus-specific IgAs were induced in all the macaques immunized with Ags with 10-HDAA at the earliest by Day 12 (Figs. 4C, D lower panels). In contrast, IgA induction against the poliovirus antigens and inactivated influenza virus was observed only in control macaque No. 2 on Day 20 (Figs. 4C, D upper panels). As expected, no induction of IgAs against the poliovirus antigens and inactivated influenza virus was observed on Day 20 in macaque No. 4. These findings suggest that 10-HDAA enhances antigen-specific IgA production.

DISCUSSION
The first in vitro M-cell model was developed by Kernéis et al. using Caco-2 cells. In this study, we found that Caco-2 cells express RANK and Raji-B cells express RANKL (Fig. 1A, Supplementary Fig. 1), suggesting that RANK/RANKL interaction is involved in M-cell differentiation in this model. Raji-B cells are not the original MCI cells, as revealed by Nagashima et al., but in this model, Raji-B cells are considered to be responsible for the function of MCI cells. In addition, the Caco-2 monolayer incubated with 10-HDAA for 6h increased the expression level of RANK on the cell surface.
of GP2 in FAE of PPs shifted from FAE to SED. The GP2 RANK expression. Furthermore, we frequently observed that number of M-cells by the increased 10-HDAA-dependent pRJ, fetuin-specific IgA was detected in the feces obtained influenza virus were also added. In previous studies with studies, inactivated antigens derived from the poliovirus and specific IgA responses, 10-HDAA was orally administered the frequency with which luminal antigens are transcytosed (Fig. 3). These findings indicate that 10-HDAA increases tion of antigen-specific IgA by pRJ containing 10-HDAA ex-

\(\text{Fig. 2F)}\). To evaluate the potential clinical applications of 10-HDAA, it is necessary to further investigate whether a single intranasal administration of 10-HDAA can induce GP2-positive M-cells of NALT. Next, we orally administered 10-HDAA to GALT and found that the number of M-cells in FAE of GALT also increased (Figs. 3D, E). Moreover, in the FAE of macaque No. 5, which was administered with 10-HDAA with the antigen, we observed an increase in the RANK expression level on the basolateral side of FAE (Supplementary Fig. 2). These suggest that the previous induc-

To investigate whether 10-HDAA increases antigen-specific IgA responses, 10-HDAA was orally administered to macaques with Ags. In addition to fetuin used in previous studies, inactivated antigens derived from the poliovirus and influenza virus were also added. In previous studies with pRJ, fetuin-specific IgA was detected in the feces obtained on Day 20. However, in this study, the enhanced production of IgA against fetuin was confirmed on Day 12. Furthermore, the production of IgA against the inactivated poliovirus and influenza virus Ags was also observed on Day 12. Although the IgA Ab titer in macaque No. 4 on Day 21 decreased, the increase in IgA titer in macaques Nos. 4–6 was faster than that in control macaques Nos. 1–3. These findings indicate that 10-HDAA in pRJ contributed to the induction of antigen-specific IgA. Taken together, this study is the first to show the potential of medium-chain fatty acids derived from honeybees in promoting the induction of M-cell differentiation.

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Conflict of Interest Regarding conflict of interest, one author (S.M.) has received a research grant from the Yama-
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pany, Inc. Other authors report no conflict of interest related to this study.

Supplementary Materials The online version of this article contains supplementary materials.

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