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Structure–activity relationship of alginate oligosaccharides in the induction of cytokine production from RAW264.7 cells

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Abstract **Guluronate and mannuronate oligomers with various degree of polymerization were prepared from polyguluronate (PG) and polymannuronate (PM) with an alginate lyase from a *Pseudoalteromonas* sp., and their activities to induce cytokine secretion from mouse macrophage cell line RAW264.7 cells were examined. Enzymatically depolymerized unsaturated alginate oligomers induced tumor necrosis factor (TNF)-α secretion from RAW264.7 cells in a structure-depending manner, while the activities of saturated alginate oligomers prepared by acid hydrolysis were fairly low or only trace levels. These results suggest that unsaturated end-structure of alginate oligomers was important for the TNF-α-inducing activity. Among the unsaturated guluronate (G3–G9) and mannuronate (M3–M9) oligomers, G8 and M7 showed the most potent activity, respectively. Bio-Plex assay revealed that interleukin (IL)-1α, IL-1β, and IL-6 secretion from RAW264.7 cells were also induced by unsaturated alginate oligomers with similar structure–activity relationship profiles as seen in TNF-α, and the most potent activities were observed with G8 and M7. These results suggest that G8 and M7 may have the most suitable molecular size or entire structural conformation as stimulant for cytokine secretion. Since antibodies to Toll-like receptor (TLR)2 and TLR4 effectively inhibited the G8- and M7-induced production of TNF-α, these alginate oligomers may stimulate innate immunity through the pattern recognition receptors on macrophages similar to microbial products.**

Keywords: Alginate; RAW264.7 cells; TNF-α; TLR2; TLR4; IL-1; IL-6

1. Introduction

Alginate is naturally occurring acidic linear polysaccharide consisting of α-L-guluronate (G) and β-D-mannuronate (M); the residues are arranged in a block structure of a homopolymer (polyguluronate, PG or polymannuronate, PM) or heteropolymer (a mixed sequence of these residues), and these block structures are called G-blocks, M-blocks, and MG-blocks, respectively [1]. Alginates extracted from seaweed at industrial level are widely used in the food industry, biotechnology area, and for medical purpose. Since alginates have gel-forming properties as divalent cations such as calcium binds to the guluronate block, entrapment within spheres of a calcium alginate gel is a technique widely used for immobilization of living cells [2]. In fact, microencapsulation of hormone-producing cells into calcium alginate gel has been used for the treatment of diabetes mellitus and parathyroid disease [3,4]. In addition to the various biological activities of alginate polysaccharides with relatively high molecular weight, a number of physiological activities of enzymatically depolymerized alginate oligomers have also been reported [5,6]. Enzymatically depolymerized alginates promotes the growth of bifidobacteria, while alginates before depolymerization had no effect on the growth of the bacteria [7]. Alginate oligomers with an average molecular weight of 1800, prepared with bacterial alginate lyase, increase shoot elongation after germination of *komatsuna* (*Brassica rapa* var. *pervidis*) seeds [8]. In a similar way, alginate lyase–lysate promotes the elongation of barley roots [9].

Regarding the effects of alginate oligomers on mammalian cells, Kawada et al. have reported that alginate oligomers enhance the growth of human endothelial cells [10] and keratinocytes [11]. Recently, we also found that enzymatically depolymerized alginate oligomers induced the secretion of cytotoxic cytokine from human mononuclear cells [12]. Since the lyase–lysate or alginate oligomer mixtures used in these studies might contain various oligomers with undefined ratio, it was uncertain which oligomer is mainly responsible for the bioactivities. Although the underlying mechanisms of bioactivities of alginates are still unclear, it seems likely that the activities of alginate oligomers are strongly influenced by molecular size, composition (M/G ratio), MG sequence, and the entire molecular conformation.

To gain insight into the structure–activity relationship of alginate oligomers especially the cytokine inducing activity, it is important to use highly purified sample with defined structure. In this study, we prepared unsaturated mannuronate and guluronate oligomers with different degree of polymerization (DP) by digestion of purified PG and PM with an alginate lyase from a *Pseudoalteromonas* sp., and compared their

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Abbreviations: PG, polyguluronate; PM, polymannuronate; G, unsaturated guluronate oligomer; M, unsaturated mannuronate oligomer; sG, saturated guluronate oligomer; sM, saturated mannuronate oligomer; TLR, Toll-like receptor; FBS, fetal bovine serum; PBS, phosphate-buffered saline; TNF, tumor necrosis factor; IL, interleukin; LPS, lipopolysaccharide

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abilities to induce cytokine release from mouse macrophage cell line RAW264.7 cells.

2. Materials and methods

2.1. Preparation of polyguluronate and polymannuronate

Sodium alginate (1000-cps grade) was purchased from Nacalai Tesque Inc. (Kyoto, Japan). PG and PM (DP = 20–24) were prepared from sodium alginate by the method of Haug et al. [13]. The homogeneity of the prepared polyuronates was confirmed by the circular dichroic spectral analysis with a Jasco spectropolarimeter J500A coupled with a data processor, based on the method of Morris et al. [14].

2.2. Purification of alginate lyase

Alginate lyase was purified from the culture medium of *Pseudoalteromonas* sp. strain No. 272, as described previously [15]. The purified enzyme, which can recognize both PG and PM, produced unsaturated guluronate and mannurotate oligomers with various DP [15].

2.3. Preparation of enzymatically depolymerized unsaturated guluronate and mannuronate oligomers

PG or PM (10 g each) were dissolved in 100 ml of water and a portion (0.4 ml) of the enzyme solution (50 μg/ml) was added to PG or PM solution. After the reaction mixture was incubated at 30 °C for 2 h, the same volume of fresh enzyme solution was added again. This operation was repeated a total of four times. The enzyme reaction was stopped by the addition of HCl to reach pH 4.0 and the reaction mixture was left for 10 min before it was neutralized with NaOH. After filtration the solution through a membrane (pore size 0.45 μm), aliquot (20 ml) of the solution was applied to a column (8.8 × 95 cm) of Bio Gel P-6 previously equilibrated with 50 mM phosphate buffer, pH 7.5 and eluted with same buffer. The elution of unsaturated uronate oligomers was monitored by absorbance at 235 nm. Separated each peak was pooled and lyophilized. The DP was routinely measured by the method of Whitaker [16] by using Blue dextran 2000 and galacturonic acid for calibration of the column. Further purification and removing of phosphate from each oligosaccharide were conducted by rechromatography on Bio Gel P-6 and subsequent gel filtration on a column (2.5 × 95 cm) of Bio Gel P-2 as described previously [17]. By this procedure, phosphate-free trimer to nonamer of G (G3–G9) and of M (M3–M9) oligosaccharides were obtained. Before use, all alginate oligomers were filtered through an endotoxin-removing filter (Zetapor Dispo filter) purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). This filtration procedure could reduce even the bioactivity of 1 μg/ml of lipopolysaccharide (LPS) to negligible level.

2.4. Preparation of saturated guluronate and mannuronate oligomers

Saturated guluronate (sG3–sG9) and mannuronate oligomers (sM3–sM9) were prepared by acid hydrolysis of PG and PM as described previously [18]. In this procedure, acid hydrolyzed oligomers were neutralized with NaOH to remove possibly contaminated oligosaccharides with internal 1–4 and 1–5 lactones. The fast atom bombardment mass spectrometric analysis suggested that sG3–sG9 and sM3–sM9 obtained by this procedure were corresponding saturated oligomers with free carboxyl groups [18]. Each oligomer was purified by the same gel-filtration procedures as described above. Fig. 1 shows the schematic representation of chemical structures of saturated and unsaturated guluronate (G3–G9 and sG3–sG9) and mannuronate (M3–M9 and sM3–sM9) oligomers used in this study.

2.5. Cell culture

RAW264.7 cells (mouse macrophage cell line) were obtained from the American Type Culture Collection (Rockville, MD, USA), and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 μg/ml), and streptomycin (100 μg/ml) as described previously [19]. Monolayers of RAW264.7 cells in 96-well plates (2×10^4 cells/well) were cultured with each alginate oligosaccharide sample in the growth medium. After 24 h, the supernatant was withdrawn from each well and assayed for tumor necrosis factor (TNF)-α by enzyme-linked immunosorbent assay (ELISA) method or Bio-Plex system (Bio Rad, Tokyo, Japan).

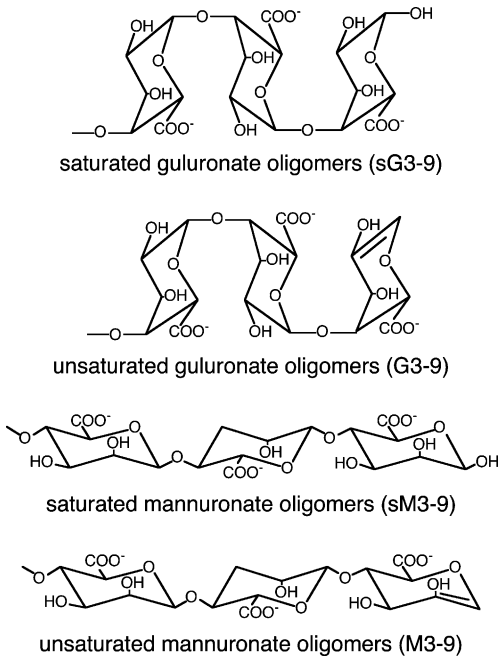

Fig. 1. Schematic representation of chemical structures of saturated (sG3–sG9 and sM3–sM9) and unsaturated (G3–G9 and M3–M9) guluronate and mannuronate oligomers used in this study.

2.6. ELISA assay for TNF-α

The concentration of TNF-α in culture supernatant was determined by a sandwich ELISA as described previously [20]. In brief, 96-well Maxisorp immunoplates (Nunc, Roskilde, Denmark) were coated with anti-mouse TNF-α monoclonal antibody (PIERCE Biotechnology, Inc., Rockford, IL, USA) in phosphate-buffered saline (PBS). After blocking with 4% bovine serum albumin (BSA) in PBS, 100 μl of each culture supernatant was added to the well in triplicate and incubated at room temperature for 60 min, and then exposed to anti-mouse TNF-α polyclonal antibody (PIERCE Biotechnology, Inc., Rockford, IL, USA). The plate was developed by peroxidase-conjugated antibodies/substrate system (TMB microwell peroxidase substrate system; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA).

2.7. Bio-Plex assay for IL-1α, IL-1β, and IL-6

Bio-Plex mouse cytokine assay for simultaneous quantitation of interleukin (IL)-1α, IL-1β, and IL-6 was employed according to the recommended procedure. In brief, the premixed standards were reconstituted in 0.5 ml of culture medium, generating a stock concentration of 50 000 pg/ml for each cytokine. The standard stock was serially diluted in the same culture medium to generate 8 points for the standard curve. The assay was performed in a 96-well filtration plate supplied with the assay kit. Premixed beads (50 μl) coated with target capture antibodies were transferred to each well of the filter plate and washed twice with Bio-Plex wash buffer. Premixed standards or samples (50 μl) were added to each well containing washed beads. The plate was shaken for 30 s and then incubated at room temperature for 30 min with low-speed shaking. After incubation and washing, premixed detection antibodies (50 μl) were added to each well. The incubation was terminated after shaking for 10 min at room temperature. After washing three times, the beads were resuspended in 125 μl of Bio-Plex assay buffer. Beads were read on the Bio-Plex suspension array system, and the data were analyzed using Bio-Plex Manager™ software with 5PL curve fitting.

2.8. Effects of antibodies against TLR2 and TLR4 on TNF-α production induced by G8 and M7

Monoclonal antibody to mouse Toll-like receptor (TLR)2 (cat. no. HM1054) and TLR4 (cat. no. HM1029) purchased from Hycult biotechnology (Uden, The Netherlands) were used. Adherent RAW264.7 cells in 96-well plates (2×10^4 cells/well) were preincubated with 10 μg/ml of each antibody in the growth medium for 1 h at 37 °C. After removing the medium, the cells were washed with PBS, and then the fresh growth medium containing 1000 μg/ml of G8 or M7 were added. After 24 h incubation at 37 °C, the supernatant of each well was withdrawn and subjected for ELISA assay for TNF-α as described above.

3. Results

3.1. Effect of various alginate oligomers on TNF-α secretion by mouse macrophage cell line RAW264.7 cells

We first examined the cytotoxic activity of alginate oligomers on RAW 264.7 cells by MTT assay. Pretreatment of RAW264.7 cells for 24 h with saturated (sG3–sG9 and sM3–sM9) and unsaturated (G3–G9 and M3–M9) oligomers up to 1000 μg/ml did not affect the cell viability (data not shown).

In control RAW264.7 cells in the absence of oligosaccharides, only small amounts of TNF-α was secreted into the medium (31 ± 3.3 pg/ml). Pretreatment of RAW264.7 cells with PG and PM for 24 h did not result in significant increase in the secretion of TNF-α into the medium. However, in the presence of enzymatically depolymerized unsaturated oligomers, the secreted levels of TNF-α markedly increased depending on the oligomer structures. As shown in Fig. 2, similar structure–activity relationship profiles between guluronate oligomers and mannuronate oligomers were observed, although the maximum effect was found with G8 among guluronate oligomers and with M7 among mannuronate oligomers, respectively. On the other hand, the effects of saturated alginate oligomers, which were prepared by acid hydrolysis, on TNF-α secretion by RAW264.7 cells were fairly lower or only trace levels as compared to those of unsaturated oligomers (Fig. 3).

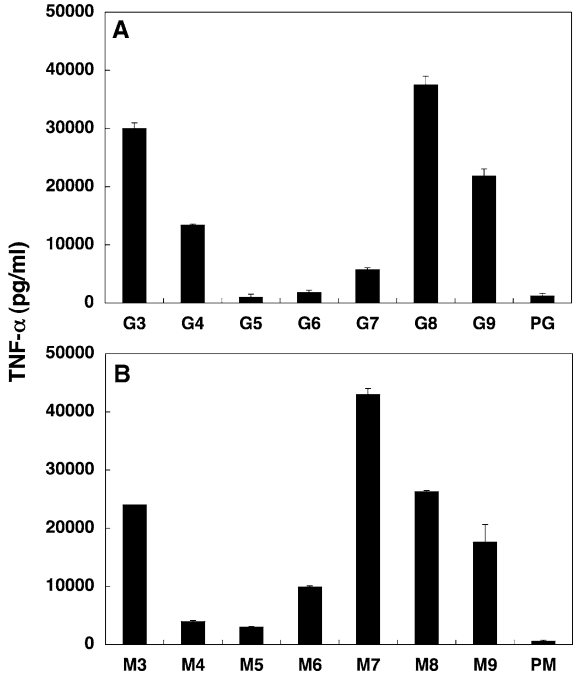

Fig. 2. Effects of unsaturated guluronate (G3–G9) (A) and mannuronate (M3–M9) (B) oligomers with various DP on the production of TNF-α from RAW264.7 cells. Cells in 96-well plates (2×10^4 cells/well) were cultured in the growth medium in the presence of each purified alginate oligomer at a concentration of 1000 μg/ml at 37 °C. After 24 h, the supernatant was withdrawn from each well, and the amounts of TNF-α was measured by ELISA as described in the text. The effects of PM and PG were measured by the same way. Each value represents an average of triplicate measurements and each bar indicates the standard deviation.

3.2. Bio-Plex assay for the effects of unsaturated alginate oligomers on IL-1α, IL-1β, and IL-6 secretion by mouse macrophage cell line RAW264.7 cells

Since it has been reported that alginate polysaccharides stimulate human monocytes to produce IL-1 and IL-6 in addition to TNF-α [21], we examined the effects of unsaturated alginate oligomers on these cytokines secretion from RAW264.7 cells by Bio-Plex assay system. Bio-Plex assay is a recently developed new suspension bead array technology that allows simultaneous measurement of multiple cytokine targets from a single sample in a microplate well [22]. Similar to the results of ELISA, significantly high levels of TNF-α were detected in the cultured medium of RAW264.7 cells in the presence of some of unsaturated oligomers, and the highest activities of G8 and M7 to induce TNF-α secretion were confirmed by this assay method (data not shown). As shown in Fig. 4, the secretion of IL-6 was also induced by unsaturated oligomers, whereas no significant effects of PG and PM were observed in this cytokine. The structure–activity relationship profiles of IL-6 were similar to those of TNF-α, although the induced levels of IL-6 were lower than those of TNF-α, and the activity of M7 was clearly higher than G8. Although the detected levels of IL-1α and IL-1β were fairly lower than those of TNF-α, the

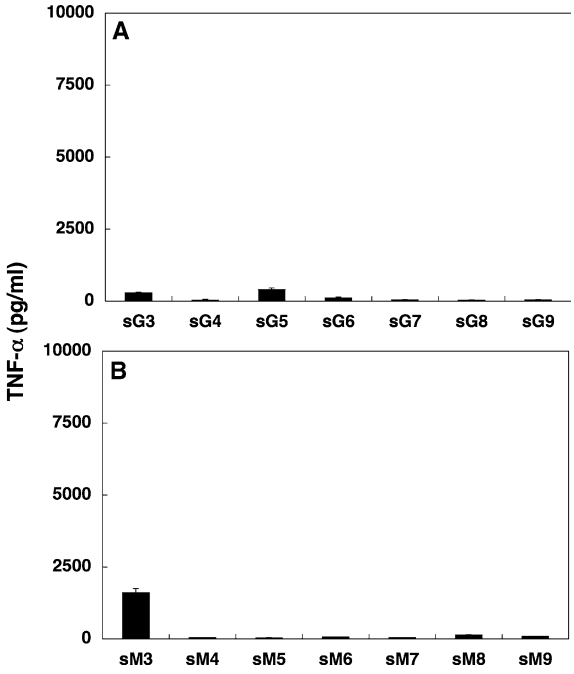

Fig. 3. Effects of saturated guluronate (sG3–sG9) (A) and mannuronate (sM3–sM9) (B) oligomers with various DP on the production of TNF-α from RAW264.7 cells. The TNF-α inducing activities of saturated oligomers were measured as described in the legend to Fig. 2.

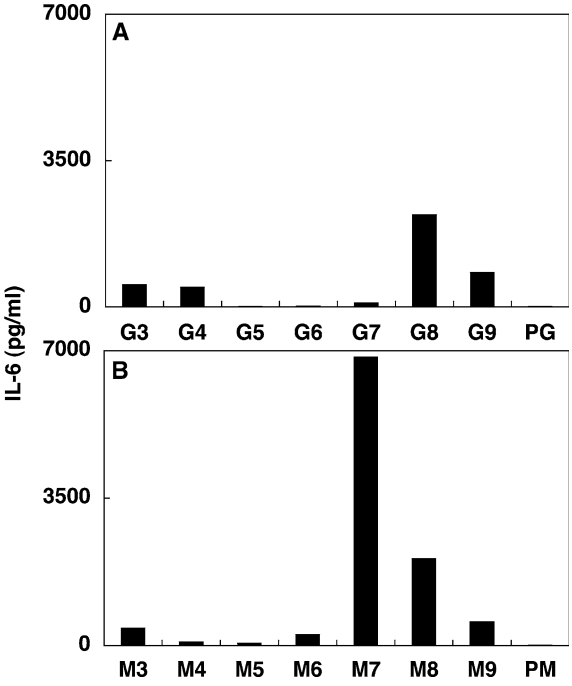

Fig. 4. Effects of unsaturated guluronate (G3–G9) (A) and mannuronate (M3–M9) (B) oligomers with various DP on the production of IL-6 from RAW264.7 cells. Cells in 96-well plates (2×10^4 cells/well) were treated with each oligomer as described in the legend to Fig. 2, and then the culture supernatants were subjected to Bio-Plex assay to determine IL-6 levels as described in the text. Each value represents an average of duplicate measurements. Differences between duplicate measurements for each value were within 5%.

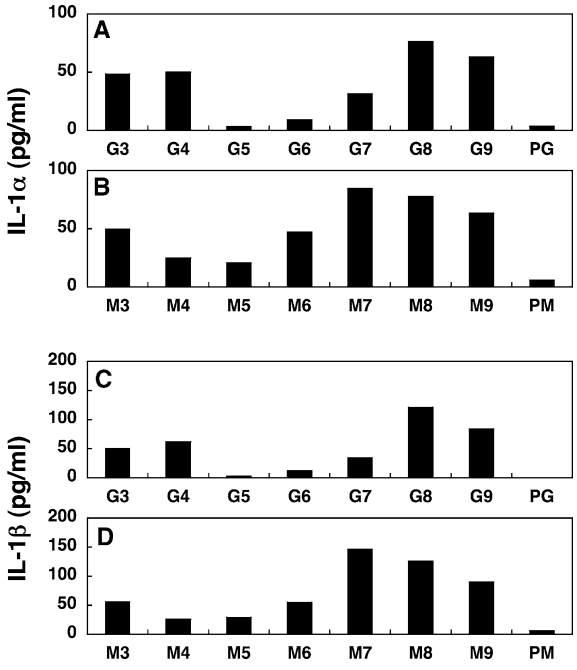

Fig. 5. Effects of unsaturated guluronate (G3–G9) (A,C) and mannuronate (M3–M9) (B,D) oligomers with various DP on the production of IL-1α (A,B) and IL-1β (C,D) from RAW264.7 cells. Cells in 96-well plates (2×10^4 cells/well) were treated with each oligomer as described in the legend to Fig. 2, and then the culture supernatants were subjected to Bio-Plex assay to determine IL-1α and IL-1β levels as described in the text. Each value represents an average of duplicate measurements. Differences between duplicate measurements for each value were within 5%.

patterns of the relationship between molecular structures of oligomers and the activities to induce secretion of IL-1α and IL-1β were similar to those of TNF-α (Fig. 5).

3.3. Effect of blockage of TLR2 and TLR4 on G8- and M7-induced TNF-α secretion by RAW264.7 cells

To examine the possible involvement of TLR2 and TLR4 in G8- and M7-induced TNF-α secretion from RAW264.7 cells, the specific blocking antibodies were used. As shown in Fig. 6, both antibodies to TLR2 and TLR4 inhibited the activities of G8 and M7 to induce TNF-α secretion with similar efficiencies. On the other hand, LPS-induced TNF-α secretion was differently affected by these antibodies, and the influence of antibodies toTLR4 was greater than that of antibodies toTLR2 (Fig. 6).

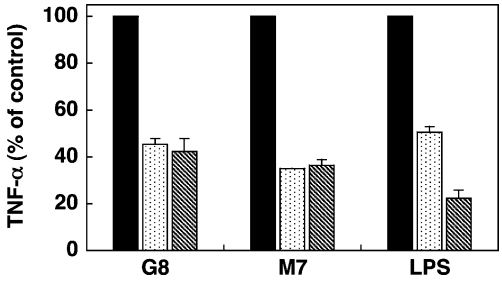

Fig. 6. Effects of anti-TLR2 and anti-TLR4 antibodies on the production of TNF-α by RAW264.7 cells stimulated with alginate oligomers (G8 and M7) and LPS. Cells in 96-well plates (2×10^4 cells/well) were pretreated with 10 μg/ml of anti-TLR2 (□) or anti-TLR4 (▨) antibody for 1 h at 37 °C. After washing with PBS, alginate oligomers (G8 or M7 at final concentration of 1000 μg/ml) or LPS (at final concentration of 1 ng/ml) were subsequently added, followed by incubation for further 24 h. Supernatants were analyzed for TNF-α levels as described in the legend to Fig. 2. Each value represents an average of triplicate measurements and each bar indicates the standard deviation. ■, control without antibody.

4. Discussion

Macrophages can be stimulated by various agents such as LPS to produce TNF-α, IL-1, and IL-6 [21]. These cytokines play an important role in various immune responses and in inflammatory reactions [23]. Apart from LPS, a wide variety of polysaccharides of microbial and plant origins are also known to stimulate immune system including activation of macrophage functions [24]. In particular, β-D-glucans from fungi have been reported to induce secretion of inflammatory cytokines from macrophages [25,26], and some of these polysaccharides are known as biological response modifiers with apparent anti-tumor activity [27]. Alginate as uronic acid polysaccharide family has also been reported to have anti-tumor activity in Sarcoma 180 in vivo [5]. Alginate is found mainly as the structural components of marine seaweed but also as an extracellular mucus polysaccharide produced by bacteria such as *Pseudomonas aeruginosa* [28]. Alginate extracted from seaweed is manufacturing at industrial level

and widely used in the food industry, biotechnology, and for medical purpose. Previous studies have shown that alginates stimulate human monocytes to produce cytokines [21,29]. In addition to polysaccharides themselves, it has been reported that depolymerized oligosaccharides derived from bioactive polysaccharides such as β-glucan, xylogucan, chitin, and pectin can be potent signaling molecules [30]. Especially, the effects of α-(1-4)-oligogalacturonic acid with varying of DP on growth regulation and defense mechanism in plants have been extensively studied [31]. Similar to oligogalacturonic acids, several studies have suggested that oligosaccharides derived from alginates also act as plant-signaling molecules. Alginate oligomers with an average molecular weight of 1800, prepared with bacterial alginate lyase, increase shoot elongation after germination of *komatsuna* (*Brassica rapa* var. *pervidis*) seeds [8]. Iwasaki et al. [32] have reported that an alginate oligosaccharide mixture had promoting activity toward the root growth of lettuce seedlings, and the tri-, tetra-, penta-, and hexa-saccharides had especially strong activity. More recently, we have found that penta-guluronate (G5) had the highest root growth-promoting activity on carrot and rice among the various alginate oligomers tested [17]. These findings suggest that there is the most effective structure or molecular size of alginate oligomer depending on each assay system in which the specific activities of oligomers are evaluated.

In our previous study, we found that enzymatically depolymerized alginate oligomers caused cytotoxic cytokine production from human mononuclear cells, while the original PG and PM had no effects before depolymerization [12]. In the present study, we could confirm that PG and PM prepared by the same procedure had no effect in terms of induction of TNF-α production from RAW264.7 cells. We also found that unsaturated guluronate and mannuronate oligomers prepared from PG and PM by enzymatic digestion were highly active inducers for cytokine production by RAW264.7 cells, whereas saturated guluronate and mannuronate oligomers prepared from PG and PM by acid hydrolysis were fairly less effective. These results suggest that unsaturated terminal structure with double bond is important for the bioactivity of alginate oligomers regardless the molecular size or structure (G or M). Probably, both plant and mammalian system have a common recognition mechanism which may be able to distinguish saturated and unsaturated terminal structure of alginate oligomer (Fig. 1).

Differences in the potencies of the induction of TNF-α production from RAW264.7 cells were observed depending on the structures of alginate oligomers tested. Interestingly, similar patterns of structure–activity relationship profiles between mannuronate oligomer and guluronate oligomer groups were observed although G8 and M7 showed the highest activity among each guluronate and mannuronate oligomer group, respectively. Previous structural studies have proposed that mannuranate and guluronate adopt different chair conformations as shown in Fig. 1 [33]. Furthermore, X-ray diffraction analysis of the partial hydrolysis products of alginate has suggested that mannuronate block predominately form an extended ribbon structure, whereas guluronate block forms a buckled chain [34,35]. Thus, such differences in entire conformation between G8 and M7 may result in the fact that the most effective guluronate oligomer was one residue longer than mannuronate oligomer. Since quite similar levels of TNF-α production were induced by G8 and M7, both oligomers may stimulate RAW264.7 cells with equal potency through a common recognition site on the cells.

Since the maximum activities of G8 and M7 were also observed in inductions of IL-1α, IL-1β, and IL-6 production, these cytokines may be induced simultaneously once stimulated by G8 and M7. Interestingly, G3 and M3, the smallest oligomers among the oligomers tested, also showed relatively high cytokine inducing activities even higher than other larger oligomers. The reason for this is uncertain now, but the finding may provide a clue to understand the recognition site or mechanism on RAW264.7 cells by alginate oligomers. Unfortunately, we could not prepare salt-free monomer or dimmer alginate oligomers due to the limitation of the ability of gel-filtration. Further efforts are required to determine the minimum alginate oligomer size in terms of cytokine inducing ability.

Regarding the initial host defense against bacterial infection, it has been proposed that the recognition of microbial products such as LPS by the host defense system is mediated by members of the TLRs family [36]. TLRs are evolutionary conserved pattern recognition receptors that discriminate between self and non-self by recognition of pathogen-associated molecular patterns with no apparent structural similarity [36]. TLRs play important roles in signal transduction for the initiation of mammalian immunity responses including cytokine production [37]. In addition to bacterial products, TLRs seem to be responsible for the polysaccharides-mediated stimulation processes. For instance, it has been shown that two uronic acid-containing polysaccharides with different origins induce cell activation through TLR4 [38,39]. Furthermore, it has recently reported that TLR2 and TLR4 are involved in the stimulation of cytokine production from human monocytes by mannuronate polymer isolated from mucoid produced by *Pseudomonas aeruginosa* [40]. The activities of G8 and M7 to induce TNF-α production from RAW264.7 cells were also inhibited by anti-TLR2 and anti-TLR4 antibodies with similar extent, whereas LPS-induced TNF-α production was more effectively inhibited by anti-TLR4 than anti-TLR2 (Fig. 6). Therefore, these results suggest that alginate oligomers with molecular weight less than 2000 are also recognized by innate immune system through TLRs, although the way of recognition might be somehow different from bacterial products.

Many polysaccharides including alginate polymer have interesting potentials for treatment of various infections diseases and cancer immune therapy [41,42]. However, the actual application of those polysaccharides into in vivo system is limited due to their high molecular weight. Depolymerization may be one possible strategy to overcome such drawback of polysaccharides if the resulted oligomers can retain the specific bioactivity. In the case of active alginate oligomers found in this study, especially G8 and M7, gastrointestinal absorption after oral administration may be possible. G3 and M3 may be better promising candidates for orally administrative agents, although these oligomers are slightly less active. The study designed for checking such possibility in vivo system is now in progress.

In conclusion, this is the first report demonstrating that alginate oligomers with defined chemical structures have cytokine-inducing activities in a structure-dependent manner. Highly purified sets of alginate oligomers obtained in this study are interesting agents not only as potential therapeutic agents but also as a tool especially for analyzing pattern recognition

mechanism. Further study of structure–activity relationship of alginate oligomers may provide insight into the TLR-mediated recognition mechanism as well as signal transduction system leading to the cytokine production.

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