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Comparison of the activities of various alginates to induce TNF-α secretion in RAW264.7 cells

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Abstract We compared the abilities of alginate polymers having different molecular sizes and compositions to induce the secretion of tumor necrosis factor (TNF)-α in RAW264.7 cells. The molecular sizes and α-L-guluronate/β-D-mannuronate (M/G) ratios of highly purified alginate polymers used in this study were 9000–38 000 and 1.50–3.17, respectively. Among the alginates tested, I-S, which had the highest molecular weight, showed the most potent TNF-α-inducing activity. The M/G ratio also seemed to influence this activity, and, among alginates with similar molecular sizes, alginates with a higher M/G ratio tended to show higher activity. Interestingly, the enzymatic depolymerization of I-S with bacterial alginate lyase resulted in a dramatic increase in the TNF-α-inducing activity. Such an effect of enzymatic digestion was also observed in a relatively low-molecular-weight alginate (ULV-3), which originally had very low TNF-α-inducing activity. Furthermore, the inhibition profiles of the TNF-α-inducing activity of enzymatically digested I-S shown by three specific mitogen-activated protein (MAP) kinase inhibitors differed from those of intact I-S. These results suggest that the underlying mechanism of the TNF-α-inducing activity of enzymatically depolymerized alginate oligomers is not necessarily the same as that of original alginate polymer.

Key words Alginate · RAW264.7 cells · Macrophage · TNF-α · Alginate lyase

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Alginate, extracted from brown algae, is an 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 or polymannuronate) or a heteropolymer (a mixed sequence of these residues), and these block structures are called G-blocks, M-blocks, and MG-blocks, respectively.[1] Alginates have gel-forming properties, as divalent cations such as calcium bind to the guluronate block. Entrapment within the spheres of a calcium alginate gel is a widely used technique for the immobilization of living cells.[2] Microencapsulation of hormone-producing cells into calcium alginate gel has been used for the treatment of diabetes mellitus and parathyroid disease.[3,4] On the other hand, a number of physiological activities of alginate and its enzymatically depolymerized oligomers have been reported, including antitumor activities of alginates in experimental animals.[5,6] Kawada et al. have reported that alginate oligomers enhance the growth of human endothelial cells[7] and keratinocytes.[8] Enzymatically depolymerized alginates promoted the growth of bifidobacteria, while alginates before depolymerization had no effect on the growth of the bacteria.[9] Alginate oligomers with an average molecular weight of 1800, prepared with bacterial alginate lyase, increase shoot elongation after the germination of *komatsuna* (*Brassica rapa* var. *pervidis*) seeds.[10] In a similar way, alginate lyase-lysate promotes the elongation of barley roots.[11] More recently, it has been reported that pentaguluronate prepared from alginate significantly promoted the growth of rice and carrot roots,[12] and short polymers of M- and MG-blocks stimulated monocytes to produce tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6, whereas G-blocks did not induce cytokine production.[13] However, to our knowledge, there is no available information regarding how structural aspects, such as molecular size, composition (M/G ratio), MG sequence, and molecular conformation influence the biological activities of alginate extracted from brown algae.

To examine the structure-activity relationship of alginates, especially their cytokine-inducing activity, a simple assay system using an appropriate cell line is required,

rather than an assay using a primary culture of monocytes, which may be contaminated with trace amounts of other kinds of cells. In our previous study, we found that the cells of the macrophage cell line RAW264.7 secreted TNF-α in response to cytotoxic stress.[14] Thus, in this study, we examined the abilities of alginates of varying molecular sizes and M/G ratios to induce TNF-α release in RAW264.7 cells. We also examined the effects of enzymatic depolymerization on the activity of the alginate samples.

Highly purified sodium alginates (food and medical usage grade) with varying viscosities were obtained from Kimika (Tokyo, Japan) and used without further purification. Other sodium alginates were purchased from Nacalai Tesque (Kyoto, Japan) and Sigma Chemical (St. Louis, MO, USA). The ratio of mannuronate residues to guluronate residues (M/G ratio) of each alginate was estimated by circular dichroism analysis.[15] The molecular weight of each alginate was estimated by a combination of the measurement of total uronic acid content[16] and reducing endo-sugar content.[17]

Alginate lyase was purified from the culture medium of *Pseudoalteromonas* sp. strain No. 272, as described previously.[18] The purified enzyme, which recognizes both polyguluronate and polymannuronate, produces unsaturated oligomers.[18] Enzymatic digestion of alginates was conducted by basically the same procedure as that described previously.[19] In brief, alginate samples (1%) in aqueous solution were incubated with alginate lyase (final concentration, 1 μg/ml) at 40°C for 3–7 days. The enzymatic reaction was stopped by heating the solution in boiling water for 10 min. Gel-filtration analysis of the enzymatically digested alginates was conducted with a Waters HPLC alliance system (2695 Separations Module and 2996 photodiode Array Detector; Milford, MA, USA), using a column of Superdex Peptide 10/300 GL (Amersham Biosciences Piscataway, NJ, USA). Before use, all alginate samples were filtered through an endotoxin-removing filter (Zetapor Dispo filter) purchased from Wako Pure Chemical Industries (Osaka, Japan). Our preliminary study demonstrated that this filter was capable of attaining almost complete remove of lipopolysaccharide (LPS) from aqueous solution containing 10 μg/ml of LPS (data not shown).

RAW264.7 cells (a 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.[20] Monolayers of RAW264.7 cells in 96-well plates (2×10^4 cells/well) were cultured with each alginate sample in the growth medium. After 24 h, the supernatant was withdrawn from each well and assayed for TNF-α.

The concentration of TNF-α in the culture supernatant was estimated by a sandwich enzyme-linked immunosorbent assay (ELISA). For the ELISA system, 96-well Maxisorp immunoplates (Nunc, Roskilde, Denmark) were coated with anti-mouse TNF-α monoclonal antibody (Pierce Biotechnology, Rockford, IL, USA) in phosphate-buffered saline (PBS). Uncoated binding sites in the wells were blocked with 4% bovine serum albumin (BSA; Seikagakukogyo, Tokyo, Japan) in PBS. After washing, 100 μl of each culture supernatant was added to the well, in duplicate, and the plates were incubated at room temperature for 60 min, and then exposed to anti-mouse TNF-α polyclonal antibody (Pierce Biotechnology). The plate was developed using peroxidase-linked species-specific whole anti-rabbit antibody (from donkey; Amersham Biosciences, Tokyo, Japan) and peroxidase substrate (TMB microwell peroxidase substrate system; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Recombinant mouse TNF-α (Genzyme/Techne, Minneapolis, MN, USA) was used to construct a standard curve. Specific p38 (SB 203580), ERK (PD 98059), and JNK (SP 600125) mitogen-activated protein (MAP) kinase inhibitors were obtained from Calbiochem-Novabiochem (Darmstadt, Germany). The effects of these inhibitors were examined at a final concentration of 5 μM. Statistical analysis of data was conducted with the *t*-test for two correlated samples, and $P < 0.05$ was considered to be significant.

As shown in Table 1, I-S, which had the largest molecular size among the alginate polymers tested, showed the highest activity to induce the secretion of TNF-α in RAW264.7 cells. The activity of I-S was concentration-dependent, and a significant level of TNF-α (300 pg/ml) was induced even at 100 μg/ml (Fig. 1). I-3, with a molecular weight above 30 000, also showed relatively high activity. On the other hand, ULV-3 and Sigma alginate (A 2158), with molecular weights of less than 10 000, had no significant activity. These results suggest that there is a tendency for the higher molecular sizes to show higher activity. Other alginate samples (I-5, I-1, IL-2, Sigma- A 2033, and Nacalai) had similar molecular weights (21 000–25 000), but their activities were

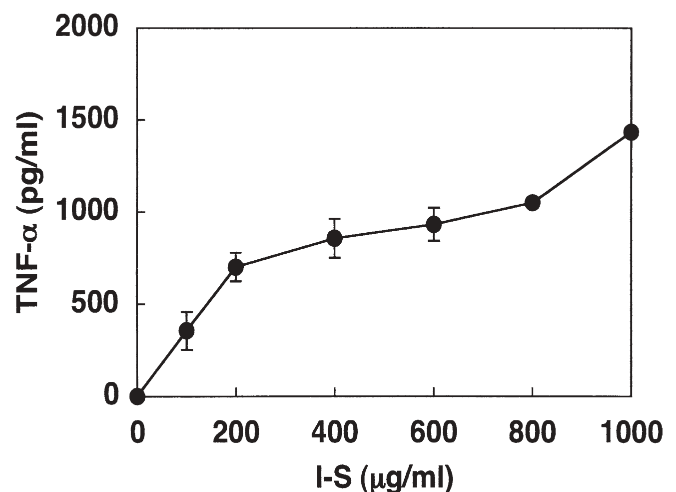

Fig. 1. Concentration-dependent activity of high-molecular-weight alginate polymer (*I-S*) to induce tumor necrosis factor-α (*TNF-α*) secretion in RAW264.7 cells. RAW264.7 cells in 96-well plates (2×10^4 cells/well) were incubated with varying concentrations (0–1000 μg/ml) of I-S in RPMI 1640 medium containing 10% fetal bovine serum (FBS) at 37°C. After 24 h, the amounts of TNF-α in the cell-free supernatants were measured by enzyme-linked immunosorbent assay (ELISA), as described in the text. *Each value* represents the average of triplicate measurements, and *each bar* indicates the SD

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