Stimulation of human keratinocyte growth by alginate oligosaccharides, a possible co-factor for epidermal growth factor in cell culture

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Abstract Oligosaccharides, involved in regulation of plant developmental and defensive processes, were tested to determine their ability to enhance proliferation of human keratinocytes. A mixture of alginate oligosaccharides remarkably stimulated keratinocyte growth and [^{3}H]thymidine uptake in the presence of epidermal growth factor (EGF). The activity was comparable to bovine pituitary extract, a common complement in keratinocyte culture, and additive on BPE-induced stimulation. The most effective oligosaccharide in the mixture was identified and its chemical structure was determined. These findings demonstrate a novel activity of alginate oligosaccharide(s) in keratinocyte growth and suggest a possible co-factor for EGF-dependent stimulation in medium for keratinocytes.

Key words: Alginate oligosaccharide; Keratinocyte; Proliferation; Epidermal growth factor; Bovine pituitary extract

1. Introduction

Materials from bovine tissues are widely utilized in biological experiments. In human keratinocyte cultures, bovine pituitary extract (BPE) is commonly used because of its recognized ability to promote proliferation of keratinocytes [1]. However, the incidence of bovine spongiform encephalopathy of cattle and other animal species, linked to the feeding of meat and bone meal prepared from scrapie-infected sheep, has extensively increased over the last few years [2]. Inoculation studies indicate that almost every mammalian species can be infected by the agent that produces spongiform encephalopathy [2], and humans are at a greater risk of being infected than previously thought [2,3]. Although relevance of scrapie to humans has not been determined, care should be exercised in handling medium containing BPE. In fact, it may be urgent to develop a substitute for BPE in keratinocyte culture.

Oligosaccharides have multiple roles in signal transduction systems that regulate plant developmental and defensive processes [4]. Therefore, we hypothesized that oligosaccharides might possibly be used as agents to stimulate keratinocyte growth. In the present study, we found a remarkable stimulatory effect of alginate oligosaccharides on keratinocyte growth. The alginate oligosaccharides were prepared from the alginic acid polysaccharides of seaweed by cleavage with an *Alteromonas sp.* lyase. The stimulatory effect of these oligosaccharides in the presence of epidermal growth factor (EGF) was similar to that of BPE.

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Abbreviations: BPE, bovine pituitary extract; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; HS, heparan sulfate; Δ, O-(4-deoxy-α-L-erythro-hex-4-enopyranosyluronic acid)-1→; -GulA-, →4)-O-(α-L-gulopyranosyluronic acid)-(1→; -GulA, →4)-O-β-L-gulopyranuronic acid; -ManA-, →4)-O-(β-D-mannopyranuronic acid)-(1→; -ManA, →4)-O-α-D-mannopyranuronic acid; -ManA′, →4)-O-β-D-mannuronic acid

2. Materials and methods

2.1. Preparation and fractionation of alginate oligosaccharides

Alginate lyase was prepared from the broth of *Alteromonas sp.* No. 1786 cultivated with 1% (w/v) sodium alginate (Wako Pure Chemical Co. Ltd., Osaka, Japan) as the carbon source. After the cells were removed by an ultrafiltration membrane (Romicon Inc., Woburn, MA), the filtrate was used for a crude enzyme solution. The crude enzyme solution was mixed with 0.5% (w/v) sodium alginate in 0.05 M sodium phosphate buffer, pH 7.5, and incubated at 40°C for 6 h with stirring. After the resulting reaction mixture was filtered, sodium and phosphate ions in the mixture were removed by an electrodialyzer (Micro Acilizer, Asahi Chemical Ind. Co. Ltd., Kawasaki, Japan) with anion and cation-exchange membranes having molecular size sieve of 300 Da (Aciplex Cartridge, Asahi Chemical Ind. Co. Ltd.). The mixture was lyophilized and was then used for the test. Fractionation of each component of the alginate oligosaccharide mixture was carried out by anion-exchange HPLC with a Cosmogel DEAE Glass Packed Column (Nacalai Tesque Inc., Kyoto, Japan).

2.2. Structural analyses of alginate oligosaccharides

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a JEOL JNM-500 (Nihon Denshi Co. Ltd., Akishima, Japan) spectrometer with D_2O as the solvent. The chemical shifts were measured relative to an external $(CH_3)_3Si(CH_2)_3SO_3Na$ standard. Mass spectroscopy was performed with a quadruple mass spectrometer (API 300 LC/MS/MS, Takara Shuzo Co. Ltd., Seta, Japan).

2.3. Cell culture

Neonatal human foreskin keratinocytes (more than 5×10^5 cells) in EpiPack® (Clonetics, San Diego, CA) were used. Thawed cells were seeded in modified MCDB 153 [5] (Nissui Seiyaku Co. Ltd., Tokyo, Japan) that contained 150 μg/ml BPE, 5 μg/ml insulin, 0.4 μg/ml hydrocortisone, 10 μg/ml transferrin, 10 ng/ml EGF, and 0.09 mM Ca^{2+}. Cells were used at the second passage and seeded in six wells at a density of 1×10^4 cells/cm^2. The medium was changed to modified MCDB 153 with or without EGF and with or without alginate oligosaccharides (0.1–10 mg/ml) or BPE. For fractionated oligosaccharides, the medium was changed to modified MCDB 153 without BPE and with EGF, and with or without each oligosaccharide fraction (1.0 mg/ml).

2.4. Cell proliferation and [^{3}H]thymidine uptake

After 7 days, cells were detached, collected and counted by trypan blue exclusion on a hemocytometer. [^{3}H]Thymidine (0.5 μCi/well, Dupont/NEN Research Products, Boston, MA) was incubated along with keratinocytes in each well for 24 h. Cultured keratinocytes were

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detached and harvested with an automatic cell harvester (ACH-96, BioTec Co. Ltd., Tokyo). Radioactivity was counted in a liquid scintillation counter (LKB 1216 Packbeta, Wallac Oy, Turk, Finland). Experiments were triplicated and Student's t test for unpaired values was used to compare differences between means; differences were considered significant if $P \leq 0.05$.

3. Results and discussion

Human keratinocyte cultures examined at 7 days showed a dose-dependent stimulation of growth by alginate oligosaccharides (Fig. 1a,b) both in the presence and absence of EGF. There was no differentiation of keratinocytes throughout the study because calcium concentrations were always low. In the presence of EGF, cell numbers were greatly increased at alginate oligosaccharides concentrations between 0.1 and 10 mg/ml, whereas a stimulatory effect of EGF without alginate oligosaccharides was minimum. At an alginate oligosaccharides concentration of 10 mg/ml in the presence of EGF, cell numbers showed significantly more enhanced proliferation than they did in the presence of BPE ($P < 0.01$) (Fig. 1a). Moreover, in the presence of EGF, alginate oligosaccharides at 1.0 mg/ml showed a significant additional effect on BPE-induced proliferation ($P < 0.01$) (Fig. 1a). However, the stimulatory effects of alginate oligosaccharides all but disappeared in the absence of EGF. Alginate oligosaccharides induced a dose-dependent increase in [^{3}H]thymidine uptake at 7 days, indicating parallel results to the cell number studies (Fig. 2a,b).

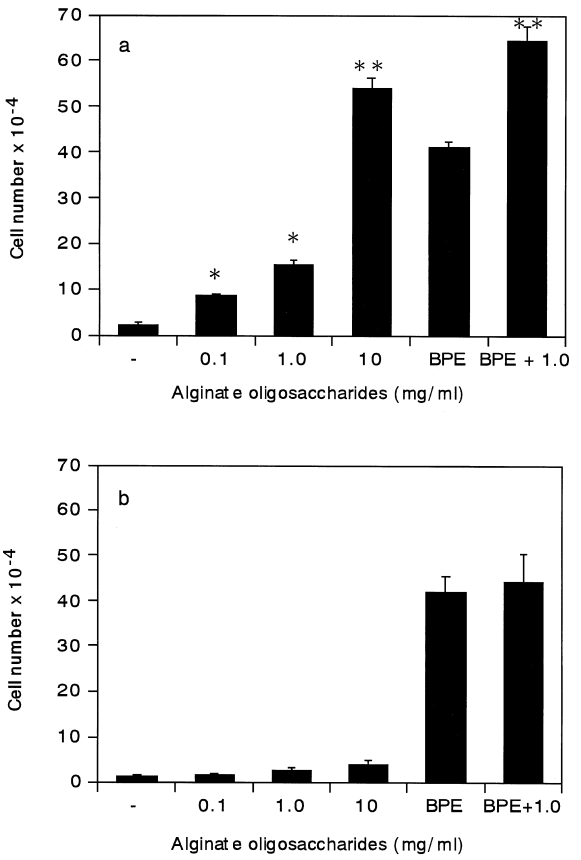

Fig. 1. Stimulation of keratinocyte proliferation by alginate oligosaccharides after 7 days. Cells were cultured in modified MCDB 153 with (a) or without (b) EGF, and with or without alginate oligosaccharides and/or BPE. Stars and double stars express significant P values ($^*P < 0.02$; $^{**}P < 0.01$) compared with alginate oligosaccharides (−) and BPE, respectively. Alginate oligosaccharides stimulated cell proliferation in medium with EGF to an extent higher than BPE and showed an additional effect on BPE-induced cell growth. In contrast, the activity of alginate oligosaccharides was present but markedly reduced in media without EGF.

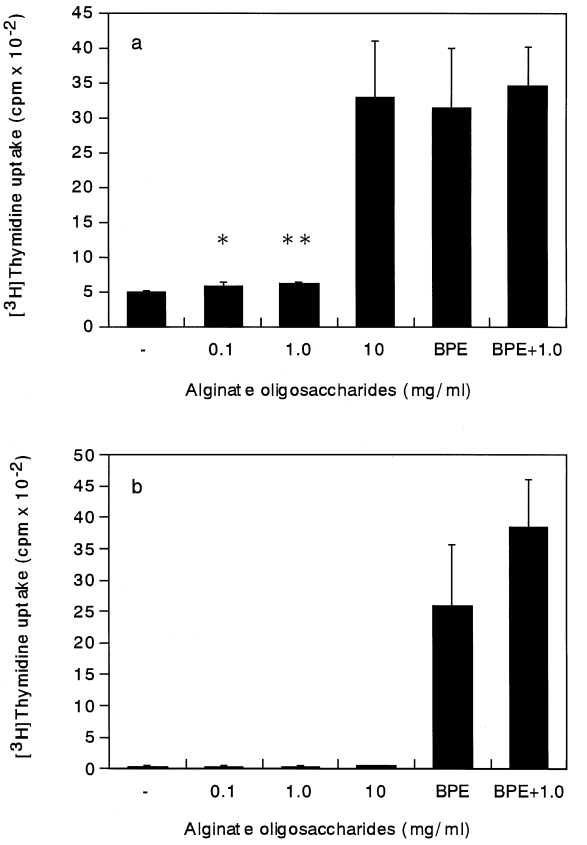

Fig. 2. Stimulation of [^{3}H]thymidine uptake in keratinocytes by alginate oligosaccharides after 7 days. Cells were cultured in modified MCDB 153 with (a) or without (b) EGF, and with or without alginate oligosaccharides and/or BPE. Cell were incubated with [^{3}H]thymidine (0.5 μCi/well) for 24 h and harvested. Star and double star express significant P values ($^*P < 0.02$; $^{**}P < 0.01$) compared with alginate oligosaccharides (−). Alginate oligosaccharides stimulated DNA synthesis in medium with EGF comparable to BPE, whereas the activity of alginate oligosaccharides was not discernable in media without EGF.

BPE contains some factors that contribute to the proliferation of keratinocytes, e.g. EGF, basic fibroblast growth factor (bFGF), insulin-like growth factors, and prolactin [6–10]. Some pituitary-derived growth promoting factors, ethanolamine, phosphoethanolamine and/or inositol are used for synthesis of phospholipid, a constituent of cell membrane, and partly represent the activity of BPE [11,12], but the role of BPE in keratinocyte culture is still unresolved. Oligosaccharides are known to be potent signaling molecules that regulate growth and development in plants [4,13]. Signal transduction is conducted via oligosaccharide-specific high-affinity receptors on cell membrane [13]. The phosphorylation is enhanced by oligosaccharides and occurs at a threonine residue(s), indicating the involvement of a membrane-associated protein kinase of the serine/threonine type [14,15]. Oligosaccharides also have the capacity to control auxins, plant growth-regulatory hormones [16,17]. Alginate oligosaccharide(s) induce

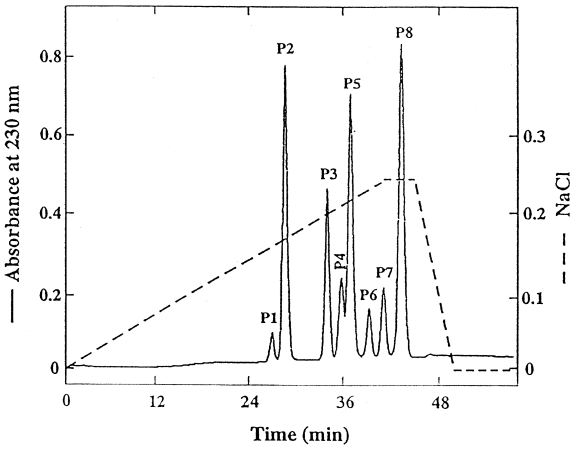

Fig. 3. Elution profile of alginate oligosaccharides on anion-exchange HPLC. The solution of alginate oligosaccharide mixture was applied to a Cosmogel DEAE Glass Packed Column (Nacalai Tesque Inc., 30×150 mm) equilibrated with H_2O. The oligosaccharides were fractionated by three-step elutions: step 1, a linear gradient of NaCl concentration from 0 to 0.25 M (0–40 min); step 2, a constant elution of 0.25 M NaCl (40–46 min); step 3, a gradient of NaCl concentration from 0.25 to 0 M (46–50 min). The flow rate was 5 ml/min. The oligosaccharides were monitored by measuring the absorbance at 230 nm.

marked keratinocyte proliferation in the presence of EGF but there is little increase in growth without EGF, indicating that the activity of alginate oligosaccharides differs from that of EGF. Thus, we postulate that alginate oligosaccharides may be involved in binding to keratinocytes, may participate in signaling transduction (including phosphorylation), and may affect cell kinetics and/or may stimulate proliferative activity of EGF and other factors in the culture. Heparan sulfate (HS) or heparin is known to have affinity to growth factors, including EGF [18] and bFGFs [19]. HS or heparin, of which oligosaccharide fragments have specific affinity for bFGF [20], promotes the binding of bFGF to its high affinity receptor in signal transduction and enhances the mitogenic activity of bFGF [21,22]. Alginate oligosaccharides may have properties similar to HS or heparin and could also interact with EGF to activate the proliferation of keratinocytes.

In order to identify the alginate oligosaccharide(s) having the proliferative activity, we isolated the oligosaccharides and tested their activity. The total alginate oligosaccharide mixture was separated into eight peaks (P1 through P8) by chromatography; the elution profile of each oligosaccharide on the anion-exchange HPLC is shown in Fig. 3. MS and high-resolution NMR techniques were employed to determine the structure of each purified oligosaccharide. The general approach involved an investigation of molecular ions in MS and assignment of peaks in the ^{13}C- and ^{1}H-NMR spectra, from which the chain lengths, relative stereochemistry, and anomeric configuration of the constituent glycosyl residues were identified. On the basis of physicochemical evidence, the structures for the alginate-derived oligosaccharide could be identified as follows: P1, Δ-GulA; P2, Δ-ManA (ManA′); P3, Δ-GulA-GulA; P4, Δ-ManA-GulA; P5, Δ-ManA-ManA (ManA′); P6, Δ-GulA-GulA-GulA; P7, Δ-ManA-GulA-GulA; P8, Δ-ManA-GulA-ManA (ManA′). In NMR spectra, P2, P5, and P8 gave two kinds of H-1 signal, δ 5.21-5.25 ($J_{1,2}$ 2.45-3.05 Hz) and δ 4.91-4.92 ($J_{1,2}\sim0$ Hz), which indicated →4)-O-α-D-mannopyranuronic acid (-ManA) and the β anomer (ManA′) residues, respectively. The ratio of α to β anomer was estimated to be about 4:1 by reference to the corresponding anomeric proton signals. In NMR spectra of P7 and P8, several minor peaks in addition to those assigned for above structures were observed. Therefore, it was presumed that P7 and P8 were the mixture of several oligosaccharides. P4 shows the most proliferative effect on both cell number and [^{3}H]thymidine uptake among the eight fractionated alginate oligosaccharides (Fig. 4a,b) and an effect comparable to BPE. P6, P7, and P1 also showed less proliferation than P4, while the other fractions did not.

The alginate oligosaccharides showing proliferative effects have gulonic acid in the reduced terminus. The peripheral guluronic acid may be effective because of an affinity to receptor on the keratinocyte or EGF and may cause more enhanced stimulation. P3 also has a gulonic acid as the reduced-terminal sugar, but this oligosaccharide did not enhance keratinocyte growth. Thus, the relationship between chemical structure and the promotional ability of alginate oligosaccharides in cell growth is not clear at present. In sum, our findings indicate that alginate oligosaccharides enhance the growth of human keratinocytes as a co-factor for EGF-dependent stimulation and may therefore be used as a substitute for BPE in keratinocyte cultures. An investigation dealing with the effect

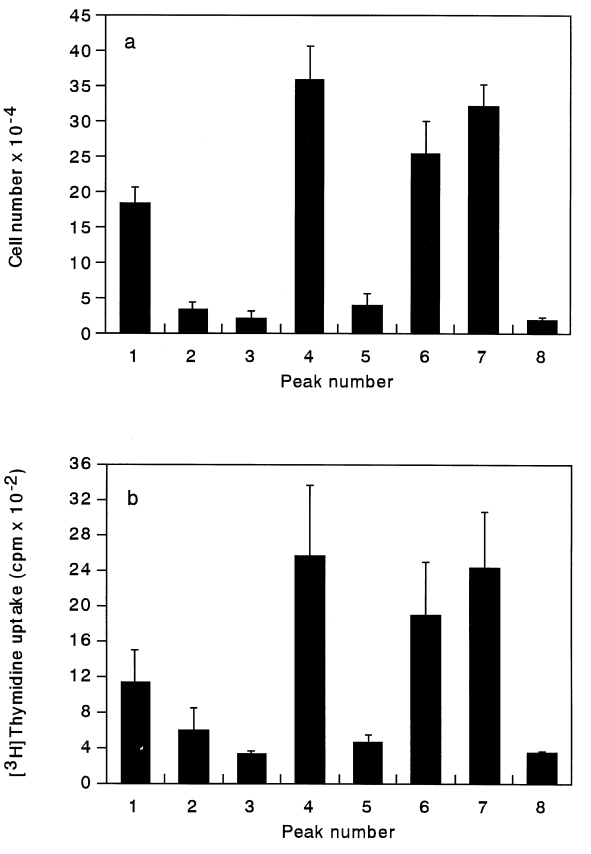

Fig. 4. Stimulation of cell number (a) and [^{3}H]thymidine uptake (b) in keratinocytes by each purified alginate oligosaccharide after 7 days. Cells were cultured in modified MCDB 153 with EGF and without BPE, and with each purified alginate oligosaccharide at a concentration of 1.0 mg/ml. P4 showed the most proliferative effects on both cell number and [^{3}H]thymidine uptake among the alginate oligosaccharides tested, and its effects were comparable to that of BPE. P1, P6, and P7 also showed proliferation, although less than P4, while other fractions did not.

of alginate oligosaccharides on cell kinetics and affinity to EGF is currently under way.

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