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CEL-I, an Invertebrate *N*-Acetylgalactosamine-specific C-Type Lectin, Induces TNF-α and G-CSF Production by Mouse Macrophage Cell Line RAW264.7 Cells

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Our previous studies demonstrated that CEL-I, an *N*-acetylgalactosamine (GalNAc)-specific C-type lectin purified from the marine invertebrate *Cucumaria echinata* (Holothuroidea) showed potent cytotoxicity to several cell lines such as HeLa, MDCK and XC cells. In this study, we found that CEL-I induced increased secretion of tumour necrosis factor-α (TNF-α) and granulocyte colony stimulation factor (G-CSF) by mouse macrophage cell line RAW264.7 cells in a dose-dependent manner, whereas this cell line was highly resistant to CEL-I cytotoxicity. The cytokine-inducing activity of CEL-I was stronger than that of phytohaemagglutinin (PHA-L). A binding study using FITC-labelled CEL-I (F-CEL-I) indicated that the amount of bound F-CEL-I on RAW264.7 cells was greater than that of F-PHA-L, suggesting that the greater activity of CEL-I to induce cytokine secretion by RAW264.7 cells is partly due to the higher binding ability. Since the cell binding and cytokine-inducing activity of CEL-I were partly but significantly inhibited by the specific sugar (GalNAc), it is considered that the binding of CEL-I to cell-surface-specific saccharide moieties, which may be recognized by CEL-I with higher affinity than GalNAc, is essential for the induction of cytokine secretion. The secretion of TNF-α and G-CSF from CEL-I-treated RAW264.7 cells were almost completely prevented by brefeldin A (BFA), whereas increase in mRNA levels of these cytokines were not affected by BFA. Bio-Plex beads assay suggested that temporal increase in phosphorylation of extracellular-regulated kinase (ERK), c-jun NH_2-terminal kinase (JNK) and p38 MAP kinase occurred at relatively early time following CEL-I treatment. Furthermore, the secretion of TNF-α and G-CSF were inhibited by specific inhibitors for these MAP kinases. These results suggest that the intracellular signal transduction through the activation of MAP kinase system is involved in CEL-I-induced cytokine secretion.

Key words: C-type lectin, *Cucumaria echinata*, cytokines, granulocyte colony stimulating factor, macrophage cell line, tumour necrosis factor-α.

Abbreviations: BFA, brefeldin A; C-type lectin, Ca^{2+}-dependent lectin; DMEM, Dulbecco's modified Eagle's minimal essential medium; ERK, extracellular-regulated kinase; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; F-CEL-I, FITC-labelled CEL-I; FITC, fluorescein isothiocyanate; GalNAc, *N*-acetylgalactosamine; G-CSF, granulocyte colony stimulating factor; GlcNAc, *N*-acetylglucosamine; JNK, c-jun NH_2-terminal kinase; LPS, lipopolysaccharide; MAP kinase, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PHA-L, phytohaemagglutinin-L (*Phaseolus vulgaris* agglutinin); TNF, tumour necrosis factor; WGA, wheat germ agglutinin.

Lectins are proteins or glycoproteins with specific binding affinity for carbohydrate moiety of glycoproteins or glycolipids on cell surface. Thus, lectins are generally useful tools in studies of cell surface structure and in structural studies of oligosaccharides of glyconjugates (*1*). Many lectins also possess various biological activities *in vitro* and *in vivo*, and some lectins bind to specific carbohydrate receptors on cells, which can activate the receptors and thereby induce intracellular signalling cascades leading to alterations in cellular behaviour. If the target cells are involved in the innate immune system, the lectin binding can result in specific cellular responses, including cytokine secretion (*2*, *3*).

Lectins are ubiquitous in nature, and are found in plants, animals and micro-organisms. Some lectins are considered to play important roles in various tissues and body fluids as carbohydrate recognition molecules (*4*, *5*). In addition to the extensive studies on plant lectins, a number of animal lectins have been isolated from various organisms and studied along with their physiological functions (*6*, *7*). Animal lectins are generally classified as Ca^{2+}-dependent (C-type) or independent (S-type or galectin) (*4*, *8*, *9*). The C-type animal lectins

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have characteristic carbohydrate-recognition domain (CRD) consisting 120–130 amino acidic residues, which exhibits some degree of sequence homology between species. Several C-type lectins have been found in marine invertebrates (*10*–*13*). Hatakeyama *et al*. (*14*) have isolated four Ca^{2+}-dependent galactose/*N*-acetylgalactosamine (GalNAc)-specific lectins (CEL-I, II, III, and IV) from the marine invertebrate *Cucumaria echinata* (Holothuroidea). It has been demonstrated that one of these lectins, CEL-III, is a novel Ca^{2+}-dependent lectin that exhibits potent haemolytic activity and cytotoxicity, and membrane damage through the formation of ion-permeable pores in the plasma membrane is proposed to be the underlying toxic mechanism of CEL-III (*15*–*17*). On the other hand, CEL-I is the smallest Ca^{2+}-dependent lectin in *C. echinata* which is composed of two identical subunits of 16 kDa linked by a single disulphide bond. CEL-I shows very high specificity for GalNAc, and the binding affinity of CEL-I for GalNAc is estimated to be approximately 1000-fold higher than that for galactose (*14*, *18*). Furthermore, a recent study has demonstrated that CEL-I is highly cytotoxic to MDCK, HeLa, and XC cells, whereas CHO, L929, and RAW264.7 cells were relatively resistant to CEL-I cytotoxicity (*19*, *20*). Since neither haemolytic activity nor a pore-forming property of CEL-I have been found so far, the cytotoxic mechanism of CEL-I is thought to be different from that of CEL-III. Previous study demonstrated that the cytotoxicity of CEL-I to sensitive MDCK cells was accompanied by the dramatic cellular morphological change and disorder of membrane permeability. These results suggest that CEL-I causes the profound change in the plasma membrane integrity after cell surface binding (*20*).

Similar to CEL-I, some plant lectins such as wheat germ agglutinin (WGA), concanavalin A (Con A), phytohaemagglutinin (PHA) and *Griffonia simplicifolia* 1-B_4 lectin (GS1B_4) have been shown to be cytotoxic to certain cell lines (*21*, *22*), whereas majority of lectins merely bind to and induce agglutination of target cells without further profound biological effects such as cytolysis. Some of these cytotoxic lectins such as PHA and WGA also show immunomodulatory and immunoadjuvant properties (*23*, *24*). To further clarify the biological activity of CEL-I, we examined the effects of CEL-I on mouse macrophage cell line RAW264.7 cells especially in terms of stimulation of cytokine secretion. As described above, RAW264.7 cells were resistant to CEL-I cytotoxicity, and this cell line has often been used for the analytical studies on lectin- or lipopolysaccharide-induced cytokine secretion (*25*, *26*). For the evaluation of the novel biological functions of CEL-I and the comparable study, this cell line is considered to be suitable. Our results indicated that CEL-I is capable to induce increased secretion of TNF-α and G-CSF by RAW264.7 cells in a dose-dependent manner. For the activity of CEL-I, the specific cell surface binding or plasma membrane attack rather than subsequent vesicle trafficking through the Golgi complex seems to be important. The involvement of MAP kinase system in such process was also suggested.

MATERIALS AND METHODS

Materials—CEL-I was purified from an aqueous extract of *C. echinata* by means of column chromatography on lactosyl-Cellulofine, GalNAc-Cellulofine and Sephadex G-75, essentially as reported previously (*14*, *15*). The purified CEL-I was stored at −83°C until use. Brefeldin A (BFA) and phytohaemagglutinin (PHA-L) were obtained from Sigma Chem. Co. (St Louis, MO, USA). Fluorescein isothiocyanate isomer I (FITC) was from Dojin Chemical Laboratories, Kumamoto, Japan. WGA was purchased from Wako Pure Chemical Industry, Co., Ltd, Osaka, Japan. PD98059, SB202190, and SP600125, which are specific inhibitor for extracellular-regulated kinase (ERK), p38 mitogen-activated protein (MAP) kinase and c-jun NH_2-terminal kinase (JNK), respectively, were obtained from Calbiochem (La Jolla, CA, USA).

FITC Labelling of CEL-I—FITC-labelled CEL-I (F-CEL-I) was prepared by essentially the same method as described previously (*17*). In brief, 2 mg of FITC was added to 1 ml of 0.5 M sodium bicarbonate buffer, pH 8.3, containing 10 mg of CEL-I and 0.1 M *N*-acetylgalactosamine (GalNAc). After stirring for 4 h at 4°C, the reaction mixture was applied to a column (1.5 × 10 cm) of Sephadex G-25 previously equilibrated with phosphate-buffered saline, pH 7.4 (PBS), followed by dialysis against PBS. F-CEL-I retained the original haemagglutinating activity towards rabbit erythrocytes. FITC-labelled WGA (F-WGA) and PHA-L (F-PHA-L) were prepared in basically the same way except for using GlcNAc as a specific sugar for WGA.

Cell Culture—RAW264.7 (mouse macrophage) cells were obtained from the American Type Culture Collection (Rockville, MD, USA), and cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 μg/ml) and streptomycin (100 μg/ml).

Detection of Cytokine in the Supernatant of CEL-I-treated RAW264.7 Cells—The levels of TNF-α and G-CSF in culture supernatants following CEL-I treatment were measured by sandwich enzyme-linked immunosorbent assay (ELISA) with two antibodies to two different epitopes on each cytokine molecule by similar methods as described previously (*27*, *28*). The ELISA procedure was performed according to the manufacturer's protocol. The cytokine concentrations were estimated from a reference to a standard curve for serial 2-fold dilution of murine recombinant cytokines.

RT-PCR—Adherent RAW264.7 cells in 12-well plates (1×10^6 cells/well) were pre-treated with or without 0.5 μg/ml of BFA for 10 min and then CEL-I (10 μg/ml) was added to each well. After 6 h incubation, total RNA was isolated from the cells with Trizol Reagent (Invitrogen). Total RNA (1 μg) was reverse transcribed with an oligo dT primer in a 10 μl reaction volume using PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa) according to the manufacturer's instructions. PCR was performed with 1 cycle of 70 s at 95°C, 30 cycles (TNF-α) or 25 cycles (G-CSF and β-actin) of 55 s at 93°C, 45 s at 61°C, 40 s at 72°C and 1 cycle of 100 s at 72°C, in a 25 μl reaction mixture containing 12.5 μl of GoTaq Green

Master Mix (Promega), 0.5 μl of forward and reverse primers (1 μM each), 0.5 μl of 1st strand cDNA and 11 μl of nuclease-free water. The primer set for G-CSF (forward, 5′-CTCAACTTTCTGCCCAGAGG-3′; reverse, 5′-AGCTGGCTTAGGCACTGTGT-3′) was designed based on the nucleotide sequence (accession number, NM_009971). The primer sets for TNF-α and β-actin, respectively, were synthesized as described by Chang *et al*. (*23*). Each PCR reaction (25 μl) was run on 2% agarose gels containing 0.1 μg/ml ethidium bromide, and the amplified products (286 bp for TNF-α, 337 bp for G-CSF and 840 bp for β-actin) were quantified with CS Analyser (ATTO).

Binding of FITC-Labelled CEL-I—Cell monolayers (2×10^5 cells/well of 48-well plates) were incubated with 1 nM of F-CEL-I at 37°C for 2 h in serum-free DMEM. After removal of the medium by aspiration, the cells were washed three times with ice-cold PBS. The washed cells were solubilized in 0.5 ml of 20 mM Tris–HCl buffer, pH 8.5, containing 0.1% sodium dodecyl sulphate (SDS). The fluorescence intensity of the solubilized cell lysate was measured with a fluorescence spectrophotometer (Hitachi Model 650-40) at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. Under these conditions no quenching of fluorescence due to binding to cells was observed. The amount of cell-associated fluorescent F-CEL-I was determined from the relationship between the concentration and fluorescence intensity of F-CEL-I. The amounts of other F-lectins bound on cells were measured in basically the same way except for the use of a specific saccharide for each lectin.

Bio-Plex Beads Assay for the Detection of Phosphorylated Mouse MAP Kinases—Bio-Plex™ phosphoprotein assay kits were obtained from Bio-Rad laboratory (Hercules, CA, USA) and used according to the recommended protocol. Adherent RAW264.7 cells in 96-well plates were incubated with 10 μg/ml of CEL-I in serum-free DMEM at 37°C for the indicated periods of time. After washing the cell with PBS, the lysis buffer provided in the Bio-Rad Cell Lysis Kit was added to each well, and cell lysate was prepared by the manufacturer's instruction. Each cell lysate was subjected to Bio-Plex™ phosphoprotein assay. Briefly, the filter plate was rinsed with 100 μl of phosphoprotein wash buffer, followed by adding 50 μl of antibody-conjugated beads, 25 μl of each cell lysate and 25 μl of phosphoprotein assay buffer. The plate was shaken overnight and incubated with biotinylated anti-phospho-antibodies, incubated with 50 μl of streptoavidin-labelled phycoerythrin, and read on the Bio-Plex™ system.

RESULTS

Effect of CEL-I on TNF-α and G-CSF Secretion by RAW264.7 Cells—When mouse macrophage cell line RAW264.7 cells were treated with CEL-I, increased secretions of TNF-α and G-CSF were induced in a dose-dependent manner (Fig. 1). These cytokines are known to be produced by macrophages in response to various stimuli, and cytokine production is one of the indicators of macrophage activation. Under the same conditions,

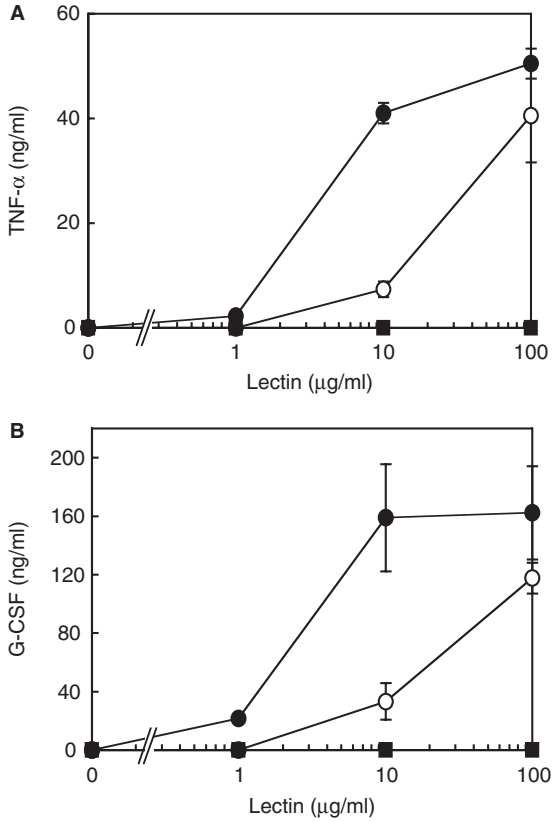

Fig. 1. **Production of TNF-α (A) and G-CSF (B) from RAW264.7 cells incubated with various concentrations of CEL-I (filled circle), PHA-L (open circle), and WGA (filled square).** Adherent cells (2×10^4 cells/well in 96-well plates) were incubated with the indicated concentration of each lectin in serum-free DMEM at 37°C. After 24 h, each cytokine level in the supernatant was measured by sandwich ELISA method as described under 'MATERIALS AND METHODS' section. Each point represents the average of triplicate measurements.

we also examined the effects of WGA and PHA-L as negative and positive lectins in terms of cytokine-inducing ability. As shown in Fig. 1, PHA-L activated RAW264.7 cells and induced the increased secretions of these cytokines, but no such activity of WGA was observed at least at the concentration range tested. Interestingly, the activity of CEL-I was much higher than that of PHA-L. The time course analysis of the productions of TNF-α and G-CSF in CEL-I-treated RAW264.7 cells (Fig. 2) indicated that there is a lag time before the initiation of cytokine secretion, and the lag time of G-CSF was longer than that of TNF-α. Furthermore, the level of TNF-α reached to the plateau after 9 h, and no further increase was observed, whereas the level of G-CSF continued to increase until 24 h. These results suggest that each cytokine is secreted with different kinetics by CEL-I-stimulated RAW264.7 cells.

Binding of F-CEL-I on RAW264.7 Cells—To gain insight into the mechanism of potent cytokine-inducing activity of CEL-I, we examined the binding of F-CEL-I to RAW264.7 cells and compared to those of F-WGA and F-PHA-L. As shown in Fig. 3, the amount of CEL-I

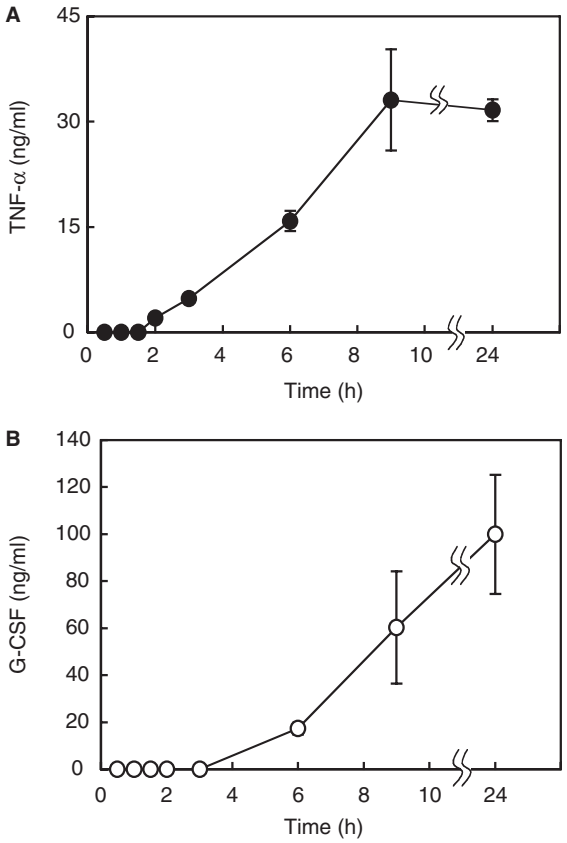

Fig. 2. **Time courses of the production of TNF-α (A) and G-CSF (B) from CEL-I-treated RAW264.7 cells.** Adherent cells (2×10^4 cells/well in 96-well plates) were incubated with 10 μg/ml of CEL-I for the indicated periods of time, and then the supernatant was withdrawn from each well and subjected to the measurement of cytokine levels by sandwich ELISA method as described under 'MATERIALS AND METHODS' section. Each point represents the average of triplicate measurements.

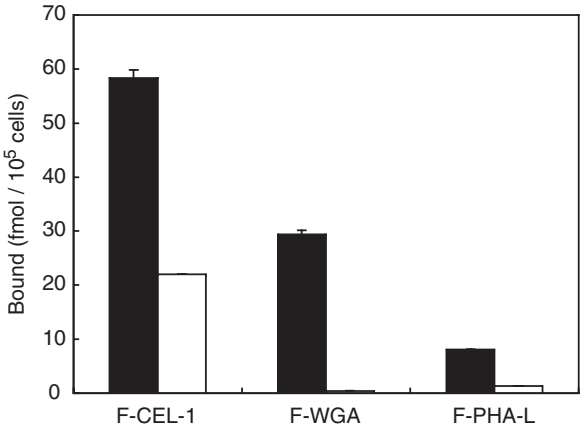

Fig. 3. **Binding of F-CEL-I, F-PHA-L and F-WGA to RAW264.7 cells in the presence (open column) or absence (filled column) of the specific sugar for each lectin.** Adherent cells (2×10^5 cells/well in 48-well plates) were incubated with 1 nM of each FITC-labelled lectin for 2 h at 37°C in serum-free DMEM. After incubation, the cells were washed three times with PBS and then the amount of cell-associated each F-lectin was measured as described under 'MATERIALS AND METHODS' section. As a specific sugar, 0.1 M GalNAc for CEL-I and PHA-L, and 0.1 M GlcNAc for WGA were used, respectively. Each column represents the average of triplicate measurements.

Table 1. **Effects of GalNAc and polymyxin B on CEL-I-induced cytokine secretion by RAW264.7 cells.**

Treatment	TNF-α (%)[a]	G-CSF (%)[a]
CEL-I	100	100
+GalNAc (0.1 M)	75	51
+Polymyxin B (2 μg/ml)	99	100
LPS[b]	100	100
+Polymyxin B (2 μg/ml)	3	3

[a]100% levels of TNF-α and G-CSF detected in the culture supernatant of CEL-I-treated RAW264.7 cells were 18.7 and 29.1 ng/ml, respectively, and in those of LPS-treated cells were 59.5 and 86.4 ng/ml, respectively. [b]To confirm the inhibitory effect of polymyxin B, the effects of the inhibitor on LPS-included cytokine secretion were examined at the same plates.

bound to RAW264.7 cells was much higher than those of F-WGA and F-PHA. In the presence of the specific monosaccharide for each lectin, namely 0.1 M GalNAc for CEL-I and PHA-L and 0.1 M GlcNAc for WGA, the bindings of F-WGA and F-PHA-L were almost completely inhibited, whereas only partial inhibition of F-CEL-I binding was observed.

Effects of GalNAc and Polymyxin B on CEL-I-induced Cytokine Secretion by RAW264.7 Cells—To confirm that the cytokine-inducing ability of CEL-I is due to the lectin activity rather than the contamination of trace amount of endotoxin in CEL-I sample, the effects of GalNAc, a specific sugar for CEL-I and polymyxin B, an inhibitor for LPS, were examined. As shown in Table 1, consistent with the results of binding studies, 0.1 M GalNAc showed partial but significant inhibitory effect on CEL-I-induced cytokine secretion, whereas polymyxin B did not reduce the production of TNF-α and G-CSF from CEL-I-treated RAW264.7 cells at the concentration at which almost complete inhibition of LPS-induced secretion of these cytokines was attained.

Effect of BFA on CEL-I-induced Cytokine Secretion by RAW264.7 Cells—As shown in Fig. 4, pre-incubation of RAW264.7 cells with BFA prior to the treatment with CEL-I and continuous presence of this reagent afterwards resulted in a significant inhibition of secretion of TNF-α and G-CSF. The effect of BFA was dose-dependent, and the levels of CEL-I-induced secretion of these two cytokines declined to the unstimulating control levels with 0.5 μg/ml of BFA.

Involvement of MAP Kinase System in the CEL-I-induced Cytokine Secretion—We analysed the kinetics of phosphorylation of p38, JNK, and ERK MAP kinases. In this study, we employed Bio-Plex beads assay kit for the detection of phosphorylated MAP kinases. This assay kit allow us to analyse the phosphorylation levels of ERK, p38, and JNK MAP kinase simultaneously even in small sample volume such as cell lysate prepared from one well of 96-well plates. Each cell lysate prepared from the RAW264.7 cells incubated with 10 μg/ml of CEL-I at 37°C for the indicated periods of time was subjected to Bio-Plex beads assay. As shown in Fig. 5,

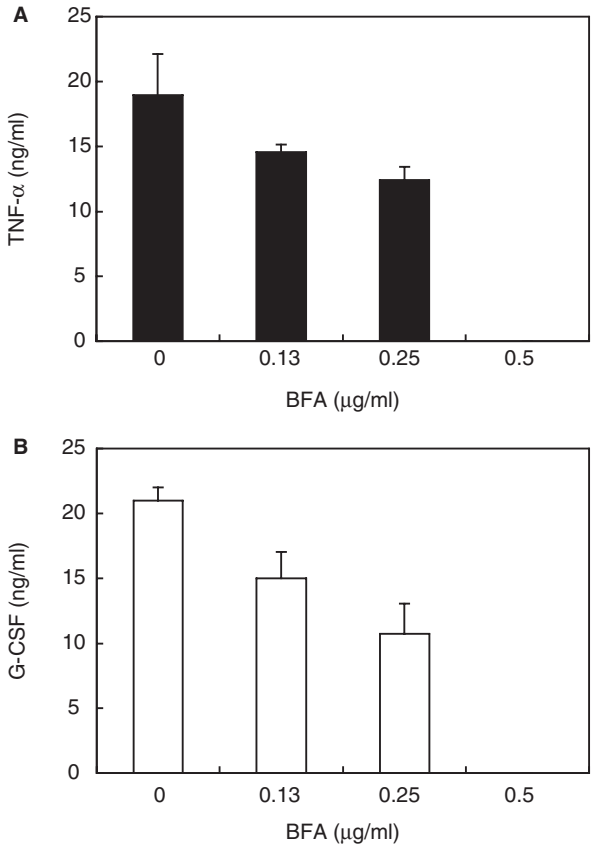

Fig. 4. **Effects of BFA on the secretion of TNF-α (A) and G-CSF (B) by CEL-I-treated RAW264.7 cells.** Adherent cells (2×10^4 cells/well in 96-well plates) were pre-incubated in the presence of indicated concentrations of BFA in serum-free DMEM at 37°C for 10 min, followed by the addition of CEL-I (final 10 μg/ml). After 6 h incubation at 37°C, TNF-α and G-CSF in the culture supernatants were measured by sandwich ELISA method as described under 'MATERIALS AND METHODS' section. Each point represents the average of triplicate measurements.

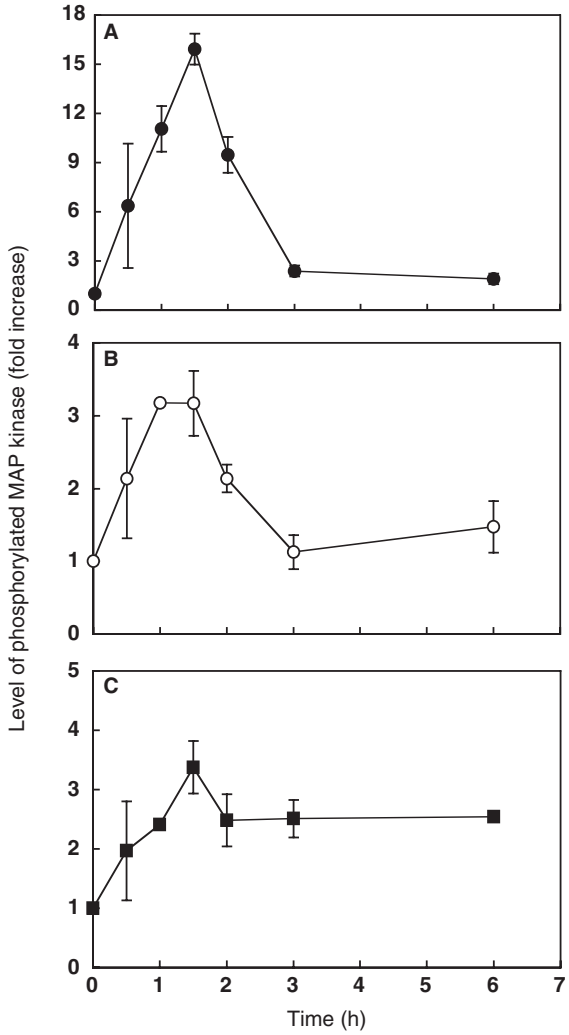

Fig. 5. **Kinetics of phosphorylation of ERK (A), p38 (B) and JNK (C) MAP kinases in CEL-I-treated RAW264.7 cells.** Adherent cells (3×10^4 cells/well in 96-well plates) were incubated with 10 μg/ml of CEL-I in serum-free DMEM at 37°C. At the indicated periods of time, the cells were harvested and lysed. Each cell lysate was subjected to Bio-Plex beads assay for detection of phosphorylated MAP kinases as described under 'MATERIALS AND METHODS' section. Each point represents the average of triplicate measurements.

the phosphorylated forms of all these three MAP kinases were detected. These reactions were temporal and the optimal phosphorylations of three MAP kinases were observed for 90 min. This tendency was especially obvious in the case of ERK kinase. At the peak levels, the increase in the level of phosphorylation of ERK was predominantly higher than those of others as compared to the control level of each kinase without CEL-I treatment. In agreement with these results, the MAP kinase inhibitor, PD98059, SB202190 and SP600125, which are specific inhibitor for ERK, p38 and JNK MAP kinase, respectively, showed the inhibitory effects on cytokine secretion from CEL-I-treated RAW264.7 cells with different extents (Fig. 6).

Effects of BFA on the TNF-α and G-CSF mRNA Expression in CEL-I-treated RAW264.7—As shown in Fig. 7, CEL-I induced the increases in the mRNA levels of TNF-α and G-CSF, and this activity was also dose dependent. These results are comparable to the effects of CEL-I on TNF-α and G-CSF protein secretion (Fig. 1). On the other hand, no significant inhibitory effects of BFA on mRNA expression levels of these cytokines were observed.

DISCUSSION

In the present study, GalNAc-specific C-type lectin CEL-I was found to induce increased secretion of TNF-α and G-CSF by mouse macrophage cell line RAW264.7 cells in a dose-dependent manner. At 10 μg/ml of CEL-I, the maximum activity was nearly induced. Macrophage is generally known to release multiple cytokines simultaneously responding to exogenous stimuli such as LPS and some lectins. In our previous study, we found that RAW264.7 cells secrete relatively large amounts of G-CSF and TNF-α as compared to other cytokines

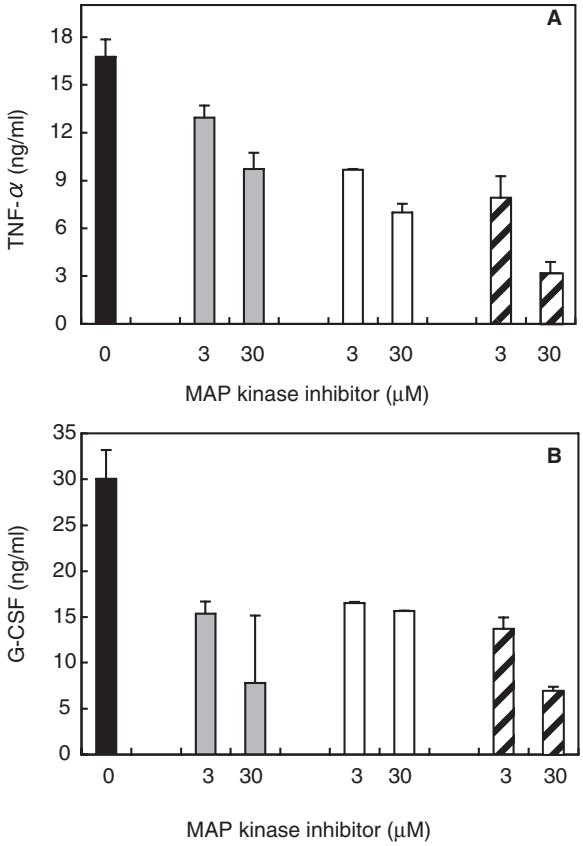

Fig. 6. **Effects of MAP kinase inhibitors on the secretion of TNF-α (A) and G-CSF (B) by CEL-I-treated RAW264.7 cells.** Adherent cells (2×10^4 cells/well in 96-well plates) were pre-incubated in the presence of 3 or 30 μM of ERK (shaded column), p38 (open column) or JNK (hatched column) MAP kinase inhibitor in serum-free DMEM at 37°C for 1 h, followed by the addition of CEL-I (final 10 μg/ml). After 6 h incubation at 37°C, TNF-α and G-CSF in the culture supernatants were measured by sandwich ELISA method as described under 'MATERIALS AND METHODS' section. Control values without inhibitor; (Filled column). Each point represents the average of triplicate measurements.

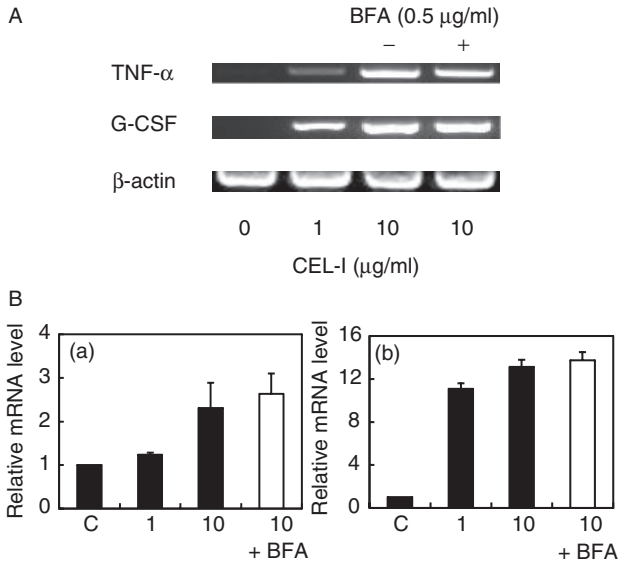

Fig. 7. **Effects of BFA on mRNA expression levels of TNF-α (A) and G-CSF (B) in CEL-I-treated RAW264.7 cells.** Adherent cells (1×10^6 cells/well in 12-well plates) were preincubated in the absence (filled column) or presence (open column) of 0.5 μg/ml BFA in serum-free DMEM at 37°C for 10 min, followed by the addition of CEL-I (final 1 or 10 μg/ml). After 6 h incubation at 37°C, TNF-α and G-CSF mRNA levels were examined by RT-PCR as described under 'MATERIALS AND METHODS' section. (A) Representative results of agarose gel electrophoresis from several experiments are shown. **(B)** Intensity of each band for TNF-α (A) or for G-CSF (B) was determined with densitometry and mRNA levels normalized to β-actin mRNA were expressed as relative to the control (*1*).

responding to certain stimulation (*27*, *28*). Thus, we analysed these two cytokines as major cytokines supposed to be secreted from this cell line following CEL-I treatment. Although TNF-α has been extensively studied as a major cytokine secreted from macrophages, there are no detailed studies on G-CSF as a potential cytokine secreted form macrophages except for our previous studies (*27*, *28*), and the biological functions of these cytokines are quite different. From the standpoint of the evaluation of the ability of CEL-I to induce the secretion of multiple cytokines with different features, it is interesting to analyse TNF-α and G-CSF simultaneously. The time course analysis indicated that there was 1.5–3 h lag time before the initiation of secretion of these cytokines. The lag time for TNF-α secretion was shorter than that of G-CSF, suggesting that the pathways of these cytokines leading to the eventual secretion may be somehow different. Although the exact secretion mechanisms of these cytokines are still unclear now, it is considered that CEL-I is an interesting lectin capable to induce secretion of multiple cytokines by macrophage. Furthermore, the activity of CEL-I was much higher than that of PHA-L, a well-known cytokine-inducing lectin (*23*). Since the binding studies using FITC-labelled CEL-I (F-CEL-I) indicated that the amount of CEL-I bound to RAW264.7 cells was greater than that of F-PHA-L, the superior binding ability of CEL-I to RAW264.7 cells may partly account for the higher cytokine-inducing activity of CEL-I, even though both lectins are GalNAc-specific lectins. Probably, the actual binding sites on the cell surface might have fairly more complex oligosaccharide structure that is recognized by CEL-I with much higher affinity to GalNAc. This speculation may also explain the reason for the results that even high concentration of GalNAc (0.1 M) could not attain the complete inhibition of the cell binding and cytokine induction by CEL-I. Since polymyxin B, an LPS inhibitor, showed no inhibitory effect on CEL-I-induced cytokine secretion, it seems likely that the activity of CEL-I is derived from its specific lectin function rather than due to trace amount of contamination of endotoxin. Regarding the immunomodulatory properties of lectins, however, it has been reported that heat denatured *Abrus* agglutinin, which lost any sugar-binding activity, is still capable to stimulate murine peritoneal macrophages to release TNF-α and interleukin-1 (*29*, *30*). Although it is uncertain now whether or not this is the case for CEL-I,

we cannot completely rule out the possibility that still unknown functional sites on CEL-I molecule apart from carbohydrate-recognition site is involved in the cytokine-inducing activity. Further detailed study for the structure–activity relationship of CEL-I may provide an answer to this question.

It has been reported that biological activities of several lectins are elicited after internalization and subsequent intracellular vesicle trafficking that can be inhibited by BFA (*31*). BFA, a fungal antibiotic that profoundly affects the structure and function of the Golgi apparatus, interferes with intracellular vesicle trafficking during both retrograde and anterograde transport, and therefore, inhibits protein secretion (*32–34*). It has been reported that BFA inhibited the release of TNF-α from the stimulated rat mast cell line (*35*). Similar to this report, the secretions of both TNF-α and G-CSF by CEL-I-treated RAW264.7 cells were inhibited by BFA. Given the well-characterized properties of BFA, it is uncertain whether this inhibitory effect of BFA is due to the direct inhibition of cytokine secretion process or the result of the prevention of vesicle trafficking of internalized CEL-I following cell-surface binding. To distinguish these possibilities, therefore, we examined the effects of BFA on mRNA expression levels of the cytokines. The results clearly indicated that CEL-I induced the significant increase in the mRNA levels of these cytokines as well as protein levels (Fig. 7). However, BFA did not block the increase in the mRNA levels of both TNF-α and G-CSF in CEL-I-treated RAW264.7 cells. Thus, it seems obvious that CEL-I is capable to stimulate gene expression of these cytokines. These results also suggest that the vesicle trafficking of CEL-I molecule through the Golgi complex following cell surface binding may not be involved in CEL-I-induced gene-expression of the cytokines. Although the exact mechanism of biological activities of CEL-I is still unclear, several lines of evidence suggested that the primary target of CEL-I is the plasma membrane (*20*). Perhaps, fairly strong interaction between CEL-I and the specific receptors may perturb the membrane integrity that in turn leads to exert cytotoxicity or cytokine-induction depending on cell types. When CEL-I attacked RAW264.7 cells, CEL-I probably activated the specific receptor linked with intracellular signal transduction pathway that may be involved in cytokine secretion. In the case of RAW264.7 cells, the binding or attack of CEL-I on the plasma membrane may result in sufficient impact to induce cytokine secretion rather than the lethal change as seen in CEL-I-sensitive cell lines. Regarding the target molecules of some lectins on the plasma membrane, it has been demonstrated that several C-type lectins isolated from snake venom show anti-integrin activity (*36*, *37*). Integrins, a family of cell surface adhesion receptors, play a central role in the dynamic interaction between the extracellular matrix and the cytoskeleton. Thus, it is considered that integrins transmit molecular signals regarding the cellular environment, which influences cell shape, survival, proliferation, and gene transcription (*38*). For instance, it has been reported that lebectin, a C-type lectin isolated from *Macrovipera lebetina* venom, inhibited the proliferation of human tumour cells as well as integrin-mediated adhesion, migration, and invasion of tumour cells (*36*). Since several structural similarities between lebectin and CEL-I have been seen (*20*), one can speculate that integrin itself or integrin-like molecules may be potential targets for CEL-I. Further studies are required to identify such specific receptors or target molecules for CEL-I.

To gain insight into the intracellular signalling pathway leading to the cytokine secretion in CEL-I-treated RAW264.7 cells, we analysed the phosphorylation of MAP kinase family. Regarding this point, it has been demonstrated that protein phosphorylation mediated by tyrosine kinase and serine/threonine kinases are correlated with the production of TNF-α and other cytokines in macrophages activated by LPS (*39*). In addition, it has recently been reported that tyrosine kinases and MAP kinases are involved in the production of TNF-α and IL-1β by murine peritoneal macrophages treated with PHA (*40*). These findings prompted us to investigate whether or not MAP kinase system is also involved in the CEL-I-induced cytokine secretion. In this study, we employed Bio-Plex beads assay that allow us to detect phosphorylated p38, JNK and ERK MAP kinases simultaneously even in one sample prepared from a well of 96-well plate. The temporal phosphorylation of these three MAP kinases following CEL-I treatment was detected. Among these MAP kinases, the highest degree of phosphorylation of ERK was observed, and optimum phosphorylation of these three MAP kinases were observed by 90 min. Since it has been demonstrated that MAP kinase family has been associated with the various intracellular events such as proliferation, differentiation and even apoptotic death in a number of different cell types, CEL-I may activate RAW264.7 cells through MAP kinase pathway linked with the activation of transcription factors responsible for the expression of TNF-α and G-CSF genes. The significant inhibitory effects of these MAP kinase-specific inhibitors on the secretion of TNF-α and G-CSF from CEL-I-treated RAW264.7 cells also support this notion.

In our previous study, we found that CEL-I showed cytotoxicity to several cell lines. Among the cell lines tested, MDCK cells showed the highest susceptibility to CEL-I cytotoxicity. The cytotoxicity of CEL-I to this cell line was even more stronger than other lectins such as WGA, PHA-L and Con A which are known to show cytotoxic effects to various cell types. CEL-I also showed significant cytotoxicity to HeLa, XC and Vero cells. In contrast to these sensitive cell lines, RAW264.7 cells were highly resistant to CEL-I, and no significant toxic effect of CEL-I was observed even after 7 days culture with 10 μg/m of CEL-I under the usual growth conditions (*20*). Furthermore, the cytotoxicity of CEL-I was not affected by BFA and NH_4Cl, which are known to prevent the intoxication of Vero cells by toxic lectin, ricin and bacterial protein toxin, diphtheria toxin, respectively (*33*). Based on these findings together with the results obtained in this study, it seems likely that the biological activities of CEL-I may be exerted via the binding to the specific receptors without involvement of intracellular events. It is uncertain whether RAW264.7 cells have a single receptor or multiple receptors for CEL-I. Namely, it is possible that the receptor involved in the cytotoxicity

is different from that involved in induction of cytokine production. Another possibility is that the presence of intracellular secondary receptor or target molecule for CEL-I, may differ from GalNAc-containing carbohydrate receptor located on the cell surface. CEL-I is a relatively small lectin with homodimer structure, and the particular biological function at molecular level has not been discovered except for the specific carbohydrate recognition activity. Under these circumstances, an identification of the naturally occurring ligands for CEL-I seems to be one strategy for understanding the biological activities of CEL-I. Our recent preliminary study on the saccharide specificity of CEL-I indicated that CEL-I has relatively high affinity to the oligosaccharide chains with GalNAcβ1-4Gal or GalNAcβ1-3Gal structures that were seen in glycolipids such as ganglioside GM2 or globoside Gb4 (unpublished data). Therefore, there is a possibility that actual sites recognized by CEL-I on the cell surface might be glycolipids rather than glycoproteins. Recently, Hatakeyama *et al*. (*19*) constructed recombinant CEL-I (rCEL-I). Unexpectedly rCEL-I showed decreased Ca^{2+}-binding affinity and lower cytotoxic activity toward HeLa cells than the native CEL-I, while rCEL-I exhibited similar carbohydrate-binding specificity to native CEL-I. Thus, further comparative study of native CEL-I and rCEL-I in terms of the structure–function relationship may provide an insight into the unique biological activities of CEL-I including induction of cytokine production observed in this study.

In conclusion, we found that CEL-I is a potent cytokine-inducing lectin, and the results suggested that intracellular signal transduction through the activation of MAP kinase system is responsible for the activity.

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