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Cytotoxicity of a GalNAc-Specific C-Type Lectin CEL-I toward Various Cell Lines

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We found that CEL-I was a potent cytotoxic lectin. MDCK, HeLa, and XC cells were highly sensitive to CEL-I cytotoxicity and killed in a dose-dependent manner, whereas CHO, L929, and RAW264.7 cells were relatively resistant to CEL-I, and no significant toxicity was observed up to 10 μg/ml. Among these cell lines, MDCK cells showed the highest susceptibility to CEL-I cytotoxicity. A binding study using FITC-labeled CEL-I (F-CEL-I) revealed that the amounts of bound F-CEL-I on the sensitive cell lines were evidently greater than those on the resistant cell lines, suggesting that the different susceptibility of the cell lines to CEL-I cytotoxicity is partly explained by different efficiencies of binding of CEL-I to these cell lines. Interestingly, the cytotoxicity of CEL-I toward MDCK cells was more potent than those of other lectins such as WGA, PHA-L, and Con A, even though these lectins were capable of binding to MDCK cells at comparable levels to CEL-I. Since the cytotoxicity of CEL-I was strongly inhibited by GalNAc, the binding to cell surface specific carbohydrates is essential for the CEL-I cytotoxicity. The trypan blue dye exclusion test indicated that CEL-I caused a disorder of plasma membrane integrity as a relatively early event. CEL-I failed to induce the release of carboxyfluorescein (CF) from CF-loaded MDCK cells as seen for pore-forming hemolytic isolectin CEL-III, suggesting that the primary cellular target of CEL-I may be the plasma membrane, but its action mechanism differs from that of CEL-III. Although CEL-I induced dramatic cellular morphological changes in MDCK cells, neither typical apoptotic nuclear morphological changes nor DNA fragmentation was observed in CEL-I–treated MDCK cells even after such cellular changes. Our results demonstrated that CEL-I showed a potent cytotoxic effect, especially on MDCK cells, by causing plasma membrane disorder without induction of apoptosis.

Key words: apoptosis, cellular morphological changes, C-type lectin, *Cucumaria echinata*, cytotoxicity.

Abbreviations: FITC, fluorescein isothiocyanate; CFDA, 6-carboxyfluorescein diacetate; CF, carboxyfluorescein; F-CEL-I, FITC-labeled CEL-I; WGA, wheat germ agglutinin; Con A, concanavalin A; PHA-L, phytohemagglutinin-L (*Phaseolus vulgaris* agglutinin); PBS, phosphate-buffered saline; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; MEM, minimal essential medium; FBS, fetal bovine serum; TBS, Tris-buffered saline; CLPs, C-type lectin proteins.

Lectins are a diverse group of proteins or glycoproteins that specifically bind to cell surface carbohydrates and induce various biological effects including agglutination of erythrocytes. They are ubiquitous in nature, and are found in plants, animals and microorganisms. Some lectins are considered to play important roles in various tissues and body fluids as carbohydrate recognition molecules (*1*, *2*). 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 (*3*, *4*). Animal lectins are generally classified into several groups (*1*) including the C-type (*5*) and galectin (*6*). Several C-type lectins have been found in marine invertebrates (*7–10*). Hatakeyama *et al*. 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) (*11*). It has been demonstrated that one of these lectins, CEL-III, is a novel Ca^{2+}-dependent lectin that exhibits potent hemolytic 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 (*12–14*). On the other hand, the smallest lectin, CEL-I, belonging to the C-type lectin family, is composed of two identical subunits of 16 kDa linked by a single disulfide bond. One of the characteristic features of CEL-I is its very high specificity for GalNAc; the binding affinity of CEL-I for GalNAc is estimated to be approximately 1,000-fold higher than that for galactose (*11*, *15*). Furthermore, a recent study has demonstrated that CEL-I is highly cytotoxic to HeLa cells, while recombinant CEL-I is much less toxic

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than the native lectin (*16*). Since neither hemolytic activity nor a pore-forming property of CEL-I has been found so far, the cytotoxic mechanism of CEL-I may differ from that of CEL-III. Although the majority of lectins can bind to and induce agglutination of target cells without a further deleterious effect such as cytolysis, some plant lectins such as wheat germ agglutinin (WGA), concanavalin A (Con A), phytohemagglutinin (PHA), and *Griffonia simplicifolia* 1-B_4 lectin ($GS1B_4$) have been shown to be cytotoxic to certain cell lines (*17*, *18*). Although it remains unclear how the binding of these cytotoxic lectins to cell surface carbohydrates leads to cell death, it has been proposed that causing cytolysis by triggering apoptosis, programmed cell death with activation of endogenous endonucleases leading to DNA fragmentation is a major cytotoxic mechanism for most of the cytotoxic lectins (*18–21*). Thus far, there have been no detail studies on the cytotoxic mechanism of C-type lectins such as CEL-I at the cellular level. Therefore, we studied the cytotoxic activity of CEL-I toward various cell lines, especially in terms of apoptosis induction.

Our results indicated that CEL-I is a highly cytotoxic lectin. Among the cell lines tested, MDCK cells showed the highest susceptibility to CEL-I toxicity, and at least against this cell line CEL-I showed extremely greater cytotoxicity than other toxic lectins such as WGA, PHA, and Con A. Our results also suggested that the underlying cytotoxic mechanism of CEL-I was not mediated by apoptosis.

MATERIALS AND METHODS

Materials—CEL-I and CEL-III were 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 (*11*, *12*). The purified proteins were stored at –83°C until use. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and phytohemagglutinin (PHA-L) were obtained from Sigma Chem. Co. (St. Louis, MO). Fluorescein isothiocyanate isomer I (FITC) was from Dojin Chemical Laboratories, Kumamoto, Japan. 6-Carboxyfluorescein diacetate (CFDA) was from Lambda Probes & Diagnostics, Austria. Concanavalin A (Con A) and wheat germ agglutinin (WGA) were purchased from Wako Pure Chemical Industry, Co., Ltd., Osaka, Japan.

FITC Labeling of CEL-I—FITC-labeled CEL-I (F-CEL-I) was prepared by essentially the same method as described previously (*14*). 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, pH7.4 (PBS), followed by dialysis against PBS. F-CEL-I retained the original hemagglutinating activity toward rabbit erythrocytes. FITC-labeled Con A (F-Con A), WGA (F-WGA), and PHA-L (F-PHA-L) were prepared in basically the same way.

Cell Culture—Vero (African green monkey kidney), MDCK (Madin-Darby canine kidney), XC (rat sarcoma), CHO (Chinese hamster ovary), HeLa (human epithelia carcinoma), and L929 (mouse fibrosarcoma) cells were cultured in α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 10 μg each of adenosine, guanosine, cytidine, and thymidine per ml of medium, penicillin (100 μg/ml), and streptomycin (100 μg/ml) as described (*14*). RAW264.7 (mouse monocyte) cells were cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 μg/ml), and streptomycin (100 μg/ml) as described (*22*). These media containing 10% FBS are referred to as the growth medium unless otherwise specified and were used throughout the experiment.

Measurement of Cytotoxicity of CEL-I—The cytotoxicity of CEL-I and other lectins was measured in terms of the inhibition of colony formation as described previously, with slight modification (*14*). In brief, 150–200 cells per well of a 48-well plate were cultured with varying concentrations of CEL-I in the growth medium for 5–7 days. The numbers of colonies formed were counted after staining with 1% methylene blue in 50% methanol. Clusters of 40 or more cells were considered as colonies. The colony forming efficiencies of MDCK, HeLa, XC, Vero, RAW264.7, L929, and CHO cells were nearly 90, 95, 75, 90, 80, 90, and 95%, respectively. The cytotoxicity of CEL-I and other lectins was also assessed by means of the MTT tetrazolium cytotoxicity assay. In brief, 2×10^4 cells per well of a 96-well plate were cultured with varying concentrations of lectins in the growth medium for 24 h, and then incubated with MTT for 2 h. After aspiration of the medium, dimethylsulfoxide was added to dissolve the MTT formazan reaction product and then the optical density was measured at 570 nm using a multiwell scanning spectrophotometer. To check the plasma membrane integrity of MDCK cells during the treatment with CEL-I, we employed the trypan blue dye exclusion test.

Binding of FITC-Labeled CEL-I—Cell monolayers (2×10^5 cells/well of 48-well plates) were incubated with 100 μg/ml of F-CEL-I at 37°C for 1 h in the growth medium. After removal of the medium by aspiration, the cells were washed three times with ice-cold PBS. The washed cells were solubilized in 1 ml of 20 mM Tris-HCl buffer, pH 8.5, containing 0.2% sodium dodecyl sulfate (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. In these binding experiments, the amounts of F-CEL-I associated with cells in the presence of 0.1 M GalNAc were considered as nonspecific binding. 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.

Assay for the Release of Carboxyfluorescein—Membrane integrity was measured by the previously described method (*14*) using the nonfluorescent, lipophilic, membrane-permeable ester derivative of 6-carboxyfluorescein (CF) 6-carboxyfluorescein diacetate (CFDA), which crosses the cell membrane and is hydrolyzed by intracellular esterases to afford the membrane-impermeable fluorophore CF (*23*). For loading with CFDA, cells grown

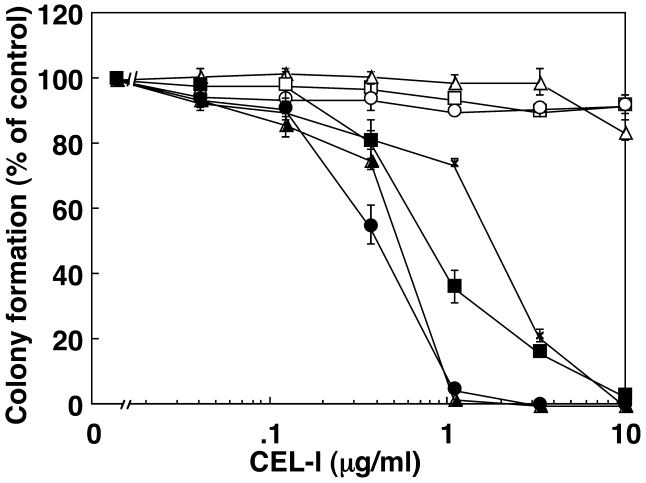

Fig. 1. **Dose-response curves of the cytotoxicity of CEL-I toward MDCK (solid circles), HeLa (solid triangles), XC (solid squares), Vero (crosses), RAW264.7 (open triangles), L929 (open circles), and CHO (open squares) cells.** The cytotoxicity of CEL-I was measured in terms of the inhibition of colony formation. Adherent cells (150–200 cells/well in 48-well plate) were cultured with varying concentrations of CEL-I in the growth medium at 37°C for 5–7 days, then the numbers of colonies formed were determined as described under "MATERIALS AND METHODS." Each point represents the average of triplicate measurements.

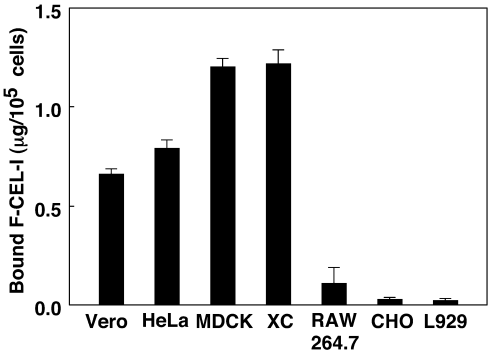

Fig. 2. **Binding of F-CEL-I to MDCK, HeLa, Vero, XC, RAW264.7, L929, and CHO cells.** Cell monolayers (2×10^5 cells/well in 48-well plates) were incubated with 100 μg/ml of F-CEL-I for 1 h at 37°C in the growth medium. After incubation, the cells were washed three times with PBS and then the amounts of cell-associated F-CEL-I were measured as described under "MATERIALS AND METHODS." The amounts of F-CEL-I associated with cells in the presence of 0.1 M GalNAc were considered as nonspecific binding and subtracted from each binding datum. The measurements were performed in triplicate.

in 48-well plates (2×10^5 cells/well) were incubated with 40 μg/ml of CFDA in Tris-buffered saline (TBS) (10 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl) at 37°C for 1 h. The loading solution was prepared just prior to use from a 10 mg/ml stock solution of CFDA in acetone stored at –20°C. After loading, cells were washed twice with PBS and then incubated with the indicated concentration of CEL-I or CEL-III in TBS in the presence or absence of 10 mM $CaCl_2$ at 37°C with or without 0.1 M GalNAc. After 1 h incubation, 0.2 ml aliquots of culture supernatants were collected and mixed with 0.5 ml of TBS, and then the fluorescence intensity at 520 nm with excitation at 490 nm was measured with a fluorescence spectrophotometer (Hitachi Model 650-40). The total cellular content of CF was determined after treatment with 0.2% SDS in TBS.

Nuclear Staining—The effects of CEL-I on nuclear morphology in MDCK cells were examined by staining with the DNA-binding fluorochrome bisbenzimide (Hoechst 33258) as described previously (*24*). In brief, cells (6×10^5 cells/dish) (35 mm) were incubated with CEL-I (100 μg/ml) in the growth medium for 1 h at 37°C. The cells were washed twice with PBS, and subsequently fixed with 1% glutaraldehyde for 30 min at room temperature, and then the cells were stained with Hoechst 33258 (40 μM) for 5 min at room temperature, and observed under a fluorescence microscope (Carl Zeiss Axiophot).

DNA Fragmentation Assay—Cell monolayers in dishes (35 mm) (6×10^5 cells/dish) were incubated with 0.1–100 μg/ml CEL-I or 10 ng/ml ricin in the growth medium for 18 h at 37°C. The cells were washed once with PBS and then lysed in 1 ml of ice-cold lysis buffer (0.5% Triton X-100, 10 mM Tris-HCl, pH 8.0, 10 mM EDTA). Samples were subsequently centrifuged for 30 min at $13,000 \times g$ to separate DNA fragments (supernatant) from intact DNA (pellet). The DNA contents of the supernatant and pellet fractions were determined using diphenylamine reagent as described previously (*24*).

Fluorescence Microscopy—Cells grown on glass coverslips to subconfluence were incubated with 100 μg/ml F-CEL-I in the growth medium in the presence or absence of 0.1 M GalNAc. After 30 min, the cells were washed twice with PBS or 0.1 M GalNAc in PBS, and then fixed with 10% formaldehyde in PBS for 15 min at room temperature. After washing with PBS, each coverslips with cells was mounted on a glass slide with 50% glycerol in PBS containing 2.6% 1,4-diazabicyclo[2,2,2]-octane and then viewed under a fluorescence microscope (Carl Zeiss Axiophot).

RESULTS

Cytotoxicity of CEL-I toward Various Cell Lines—The cytotoxicity of CEL-I was measured in terms of the inhibition of colony formation. Adherent cells were incubated with varying concentrations of CEL-I in the growth medium for 5–7 days. The numbers of colonies formed were determined after staining with 1% methylene blue. As shown in Fig. 1, the susceptibility of each cell line to CEL-I cytotoxicity was quite different among the cell lines tested. MDCK, HeLa, XC, and Vero cells were highly sensitive to CEL-I cytotoxicity, and these cell lines were killed by CEL-I in a dose-dependent manner, whereas CHO, L929, and RAW264.7 cells were relatively resistant to CEL-I cytotoxicity, and no significant toxic effect of CEL-I was observed up to 10 μg/ml. Differences in the susceptibility to CEL-I cytotoxicity among the cell lines, especially between MDCK and CHO cells, were also evident with the MTT assay (data not shown).

Binding of F-CEL-I to Various Cell Lines—To ascertain whether or not the differences in the susceptibility of these cell lines to CEL-I cytotoxicity is due to the differential efficiency of binding of CEL-I to these cell lines, we

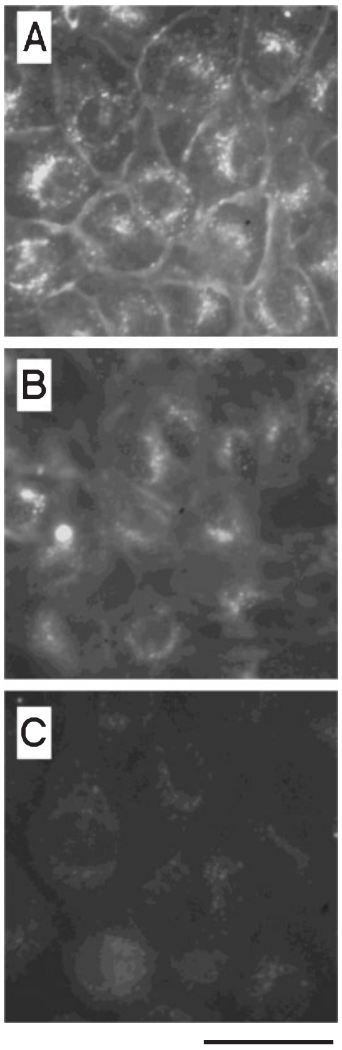

Fig. 3. **Fluorescence microphotographs of MDCK cells exposed to F-CEL-I in the presence or absence of 0.1 M GalNAc.** Adherent cells on coverslips were incubated 100 μg/ml F-CEL-I in the absence (A, B) or presence (C) of 0.1 M GalNAc in the growth medium for 1 h at 37°C. After washing with PBS alone (A) or PBS containing 0.1 M GalNAc (B, C), the cells were immediately subjected to microscopic observation. The bar indicates 20 nm.

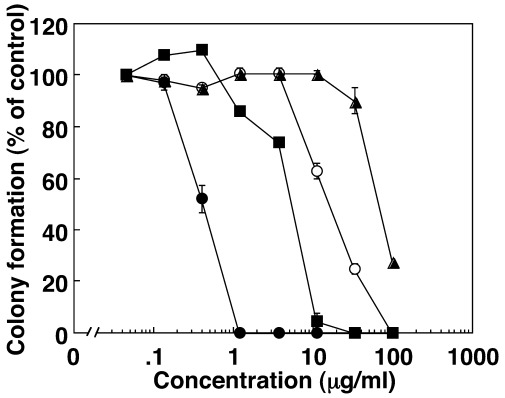

Fig. 4. **Comparison of the cytotoxicity of CEL-I (solid circles), Con A (solid triangles), PHA (open circles), and WGA (solid squares) toward MDCK cells.** Adherent cells (150–200 cells/well in 48-well plates) were cultured with varying concentrations of each lectin in the growth medium at 37°C for 5 days, and then the numbers of colonies formed were determined as described under "MATERIALS AND METHODS." Each point represents the average of triplicate measurements.

examined the binding of F-CEL-I to these cell lines. On preliminary time course analysis, the binding of F-CEL-I (100 μg/ml) on MDCK cells was found to have nearly reached a plateau level after 1 h incubation at 37°C in the growth medium. Thus, the amount of bound F-CEL-I on each cell line was measured under these conditions. During the binding experiments, no significant loss of cells was observed for any of the cell lines. As shown in Fig. 2, the amounts of CEL-I bound to these cells also differed noticeably. Interestingly, the amounts of CEL-I bound to MDCK, XC, HeLa, and Vero cells, which are highly sensitive to CEL-I, were significantly higher than those to CHO, L929, and RAW264.7 cells, which are resistant to CEL-I cytotoxicity. For instance, the amount of F-CEL-I bound to MDCK cells was about 40-fold greater than that to CHO cells. Thus, differences in the susceptibility to CEL-I cytotoxicity between cell lines may be partly explained by the different receptor levels for CEL-I in these cell lines. The intensive binding of F-CEL-I on MDCK cells was also confirmed by fluorescence microscopic observation (Fig. 3). After washing with 0.1 M GalNAc to remove cell surface bound F-CEL-I, a significant level of fluorescent activity was observed in the perinuclear cytoplasm (Fig. 3B), whereas the binding of F-CEL-I was markedly prevented when 0.1 M GalNAc and F-CEL-I were added at the same time (Fig. 3C). These results suggest that some of the cell surface bound CEL-I *via* the specific carbohydrates may be internalized into the cytoplasm through receptor-mediated endocytosis.

Comparison of Cytotoxicity and Binding of CEL-I and Other Lectins—It has been demonstrated that some lectins such as phytohemagglutinin (PHA-L), concanavalin A (Con A), and wheat germ agglutinin (WGA) exhibit cytotoxic effects on certain cell lines (*17–21*). We compared the cytotoxic potency of CEL-I with those of these lectins by colony formation assaying with MDCK cells. As can be seen in Fig. 4, CEL-I showed the strongest cytotoxicity among the lectins tested. The LD_{50} values of CEL-I, WGA, PHA-L, and Con A were estimated to be 0.43, 5.4, 16.2, and 67.0 μg/ml, respectively. Although PHA-L is known to recognize GalNAc similar to CEL-I, its LD_{50} was more than 35-fold higher than that of CEL-I. Based on the results in Figs. 1 and 2, it is reasonable to consider that one of the factors that determine the susceptibility of the cells to CEL-I-toxicity is the level of the lectin binding to the cell surface carbohydrates. To determine if this is the case for other lectins, we measured the amounts of lectins bound to MDCK cells in the absence or presence of their specific saccharides. Since the binding of these lectins to MDCK cells was strongly inhibited by their specific saccharides, it appears that the most of the binding of these lectins was mediated by the specific carbohydrates on the cell surface (Fig. 5). The amounts of lectins bound to MDCK cells showed the following order, CEL-I > WGA > Con A > PHA-L. Based on the amount of specific binding obtained on subtracting nonspecific binding from total binding, the numbers of each lectin molecule

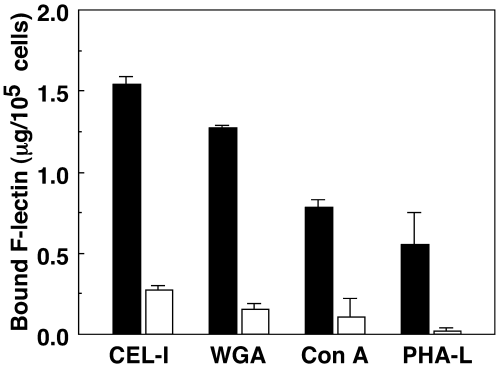

Fig. 5. **Binding of FITC-labeled CEL-I, WGA, Con A, and PHA on MDCK cells.** Cell monolayers (2×10^5 cells/well in 48-well plates) were incubated with 100 μg/ml of each FITC-labeled lectin for 1 h at 37°C in the growth medium in the presence (open columns) or absence (closed columns) of 0.1 M specific saccharide for each lectin. GalNAc, for CEL-I and PHA, mannose/glucose, for Con A, and GlcNAc, for WGA, were used as specific saccharides, respectively. After the incubation, the cells were washed three times with PBS and then the amounts of cell-associated F-CEL-I were determined as described under "MATERIALS AND METHODS." The measurements were performed in triplicate.

bound per cell were estimated to be 2.40, 1.90, 0.39, and 0.26×10^8 for CEL-I, WGA, Con A, and PHA-L, respectively. This order was not necessarily well correlated with the order of the toxic potential of these lectins, and differences in the levels of binding of the lectins to MDCK cells seem to be too small to explain the differences in the cytotoxic effects. For instance, the LD_{50} value of Con A was more than 150-fold higher than that of CEL-I, even though Con A could bind to MDCK cells at nearly 15% the level in the case of CEL-I. These results suggest that binding of lectins to cell surface carbohydrates is a prerequisite process, but that a subsequent action mechanism that may be specific for each lectin is more important for lectin-mediated cytotoxicity.

Effects of Various Reagents and Cell-Density on the Cytotoxicity of CEL-I toward MDCK and Vero Cells—Brefeldin A (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 (*25*). It has been reported that BFA prevents the intoxication of Vero cells and other cell lines by plant toxic lectin ricin and modeccin, but has no effect on the cytotoxicity of diphtheria toxin (*26*). On the other hand, NH_4Cl, which elevates the intravesicular pH, inhibits the cytotoxicity of diphtheria toxin and modeccin (*27*). To gain an insight into the potent cytotoxic mechanism of CEL-I, especially the underlying post-binding mechanisms, we examined the effects of BFA and NH_4Cl on CEL-I cytotoxicity in addition to GalNAc. Since MDCK cells were known to be a naturally BFA resistant cell line, we used Vero cells instead of MDCK cells when checking the effect of BFA. As shown in Fig. 6, A and B, the cytotoxicity of CEL-I toward MDCK and Vero cells was strongly inhibited in the presence of 0.1 M GalNAc, but *N*-acetylglucosamine (GlcNAc) had no effect, as expected. On the other hand, neither BFA nor NH_4Cl had an inhibitory effect on the cytotoxicity of CEL-I. These results suggest that the

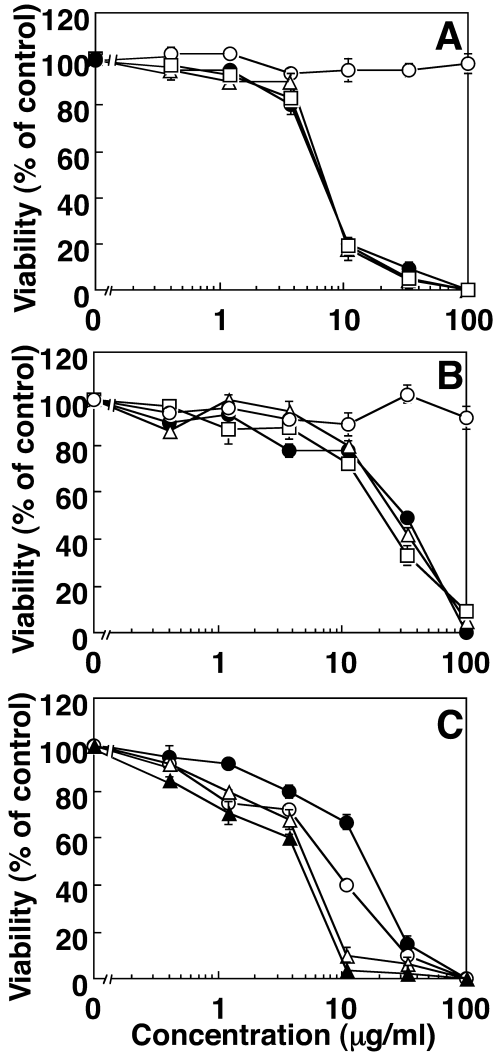

Fig. 6. **Effects of BFA, NH_4Cl, GlcNAc, GalNAc, and cell density on the cytotoxicity of CEL-I toward MDCK and Vero cells.** (A) MDCK cells (2×10^4 cells/well in 96-well plates) were preincubated in the absence (solid circles) or presence of 0.1 M GlcNAc (open triangles), 20 mM NH_4Cl (open squares), or 0.1 M GalNAc (open circles) in the growth medium at 37°C for 1 h, followed by the addition of varying concentrations of CEL-I. (B) Vero cells (2×10^4 cells/well in 96-well plates) were preincubated in the absence (solid circles) or presence of with 1 μg/ml BFA (open triangles), 20 mM NH_4Cl (open squares), or 0.1 M GalNAc (open circles) in the growth medium at 37°C for 1 h, followed by the addition of varying concentrations of CEL-I. (C) To MDCK cells in 96-well plates at 1×10^4 cells/well (solid triangles), 2×10^4 cells/well (open triangles), 4×10^4 cells/well (open circles), or 8×10^4 cells/well (solid circles), varying concentrations of CEL-I were added. All these cells were incubated for 18 h at 37°C, and then the viability of the cells was examined by MTT assaying as described under "MATERIALS AND METHODS." The measurements were performed in triplicate.

binding to a specific carbohydrate receptor on the cell surface is essential, but that at least the Golgi/endoplasmic reticulum-mediated vesicular system and intravesicular acidic environment are not required for CEL-I cytotoxicity. As shown in Fig. 6C, the cytotoxicity of CEL-I was also influenced by the cell density of MDCK cells, more potent cytotoxicity being observed at a lower cell density.

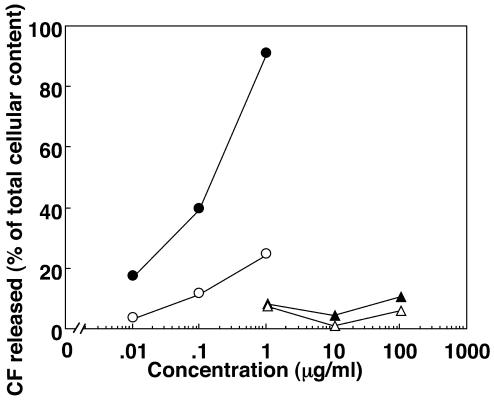

Fig. 7. **Comparison of the abilities of CEL-I and CEL-III to induce CF release from CF-loaded MDCK cells.** CF-loaded MDCK cells were incubated with the indicated concentrations of CEL-I (triangles) or CEL-III (circles) in 10 mM Tris-HCl, pH 7.5 buffer containing 0.15 M NaCl in the presence (solid circles and triangles) or absence (open circles and triangles) of 10 mM $CaCl_2$ for 1 h. The CF released from the cells was measured as described under "MATERIALS AND METHODS." The measurements were performed in duplicate.

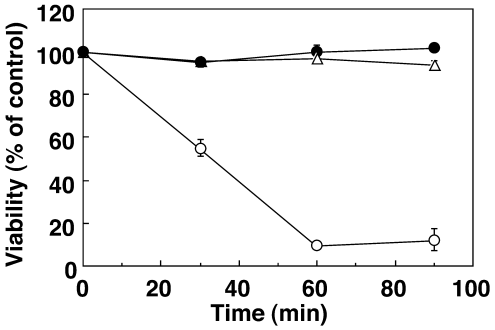

Fig. 8. **Time course analysis of the cytotoxicity of CEL-I toward MDCK cells as examined by the trypan blue dye exclusion test.** MDCK cells (2×10^4 cells/well in 96-well plates) were incubated with 100 μg/ml of CEL-I in the presence (open triangles) or absence (open circles) of 0.1 M GalNAc in the growth medium at 37°C for the indicated periods of time, and then the cells were subjected to the trypan blue dye exclusion test. (solid circles) Control cells (without CEL-I). The measurements were performed in triplicate.

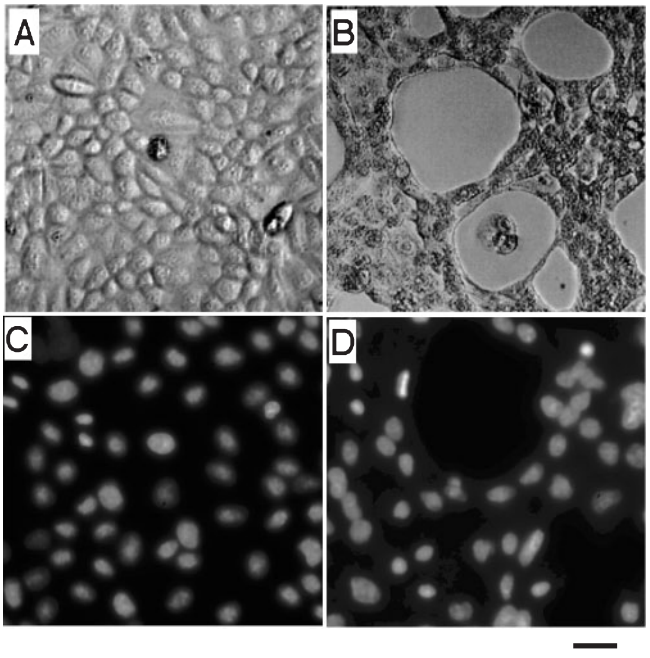

Fig. 9. **Effects of CEL-I on the cellular and nuclear morphology of MDCK cells.** MDCK cells (6×10^5 cells/dish) were incubated with 100 μg/ml of CEL-I in the growth medium for 1 h at 37°C. Phase contrast micrographs of control (A) and CEL-I–treated cells (B). Another set of cells was fixed and stained with 40 μM Hoechst 33258, and then observed under a fluorescence microscope. (C) Control cells, (D) CEL-I–treated cells. The bar indicates 20 μm.

CF Release from MDCK Cells Treated with CEL-I or CEL-III—It has been reported that CEL-III, an isolectin of CEL-I, exhibits strong hemolytic activity and cytotoxicity, and that these activities of CEL-III are manifested through damage to the cell membrane through pore-formation in a Ca^{2+}-dependent manner (*12*, *14*). In fact, it has been shown that CEL-III induced the release of ATP from rabbit erythrocytes even when osmotically protected by dextran 8 (*12*). Furthermore, CEL-III was found to cause marked membrane damage to sensitive cells, as judged from the release of CF from CF-loaded cells that had been preincubated with CFDA. To examine the effect of CEL-I on the integrity of the plasma membrane, the CF release assay was conducted on MDCK cells. In accordance with a previous report (*14*), CEL-III induced CF release from CF-loaded MDCK in a dose-dependent manner, and the activity of CEL-III was significantly potentiated in the presence of Ca^{2+}, whereas no CF release activity of CEL-I was observed even in an extremely higher concentration range (Fig. 7). Therefore, these results suggest that the cytotoxic mechanism of CEL-I differs from that of CEL-III, in that the cytotoxicity is attributed to the damage to the plasma membrane through ion-permeable pore-formation.

Effect of CEL-I on the Plasma Membrane Integrity of MDCK Cells, as Judged with the Trypan Blue Dye Exclusion Test—The trypan blue dye exclusion test is a simple and common assay method for examining the viability of cultured cells, based on a change in membrane permeability as to trypan blue dye. After the addition of 100 μg/ml CEL-I to MDCK cells, the number of cells stained with trypan blue increased very rapidly, and nearly 90% of the cells were stained after 1 h incubation (Fig. 8). Since this effect of CEL-I was inhibited in the presence of 0.1 M GalNAc, it seems likely that the binding of CEL-I through the cell surface–specific carbohydrates may cause changes in the plasma membrane integrity (Fig. 8). In contrast to CEL-I, ricin, that exhibits cytotoxicity through enzymatic inactivation of ribosomes, required a longer incubation time to cause cell death, as shown by the trypan blue dye exclusion test, and there were no stained cells during a few hours ricin-treatment (data not shown). These results suggest that CEL-I may affect the plasma membrane in terms of a permeability change as to trypan blue in a relatively early time period.

Effects of CEL-I on Cellular and Nuclear Morphology of MDCK Cells—During exposure to CEL-I, especially at 100 μg/ml, dramatic cellular morphological changes of MDCK cells were observed, and many empty areas on the

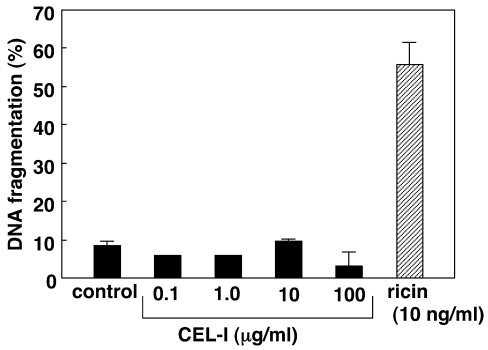

Fig. 10. **Detection of DNA fragmentation in MDCK cells treated with ricin or CEL-I on diphenyamine assaying.** MDCK cells (6×10^5 cells/dish) were incubated with varying concentrations of CEL-I (0.1, 1.0, 10, and 100 μg/ml) or 10 ng/ml ricin in the growth medium at 37°C for 18 h. DNA fragmentation in each type of treated cells was assayed with diphenylamine as described under "MATERIALS AND METHODS." The measurements were performed in triplicate.

plate appeared concomitant with the shrinkage of many cells (Fig. 9B). However, no nuclear morphological changes such as typical apoptotic nuclear condensation or fragmented nuclei were observed even after the characteristic cellular morphological changes had been induced by CEL-I (Fig. 9D). Furthermore, no significant DNA fragmentation was detected in CEL-I–treated MDCK cells even after prolonged incubation (18 h), as observed on diphenylamine assaying with a wide range of concentrations (0.1–100 μg/ml), whereas ricin (10 ng/ml), a potent apoptosis inducer, caused marked DNA fragmentation in MDCK cells (Fig. 10).

DISCUSSION

Our results demonstrate that CEL-I is highly cytotoxic toward various cell lines, depending on the cell line. Among the cell lines tested, higher susceptibility to CEL-I cytotoxicity was observed for MDCK, HeLa, XC, and Vero cells, whereas CHO, L929, and RAW264.7 cells were relatively resistant. The LD_{50} value of CEL-I with the most sensitive MDCK cells was 0.43 μg/ml, whereas no significant toxic effect of CEL-I was observed on CHO, L929, and RAW264.7 cells up to 10 μg/ml (Fig. 1). MTT assaying also indicated that MDCK cells were the most susceptible to CEL-I cytotoxicity among the cell lines tested, while CHO, L929, and RAW264.7 cells were highly resistant (data not shown). The IC_{50} of MDCK cells estimated by MTT assaying was 6.5 μg/ml, which is 15-fold higher than the LD_{50} obtained on colony forming assaying. The difference may be due to the different assay conditions, such as the different cell density and incubation time. In the MTT assay, stronger cytotoxicity of CEL-I was observed at lower cell density (Fig. 6C). Thus, it seems likely that cell density is a more important factor determining the susceptibility of cells to CEL-I cytotoxicity, especially for MDCK cells, which are known to be polarized under confluent culture conditions. Probably the cell surface features of MDCK cells differ between two assay conditions, and such differences may be attributed to inconsistent dose dependency in two assay systems.

Since lectin-specific sugar GalNAc, but not GlcNAc, effectively inhibited the CEL-I cytotoxicity (Fig. 6, A and B), it appears that the binding of CEL-I to specific carbohydrate chains on the cell-surface is a prerequisite step for the cytotoxicity. Binding studies using FITC-labeled CEL-I (F-CEL-I) indicated that the amounts of CEL-I bound to relatively sensitive MDCK, HeLa, XC, and Vero cells were significantly greater than those to CHO, RAW264.7, and L929 cells (Fig. 2). Thus, the binding efficiency of CEL-I partly accounts for the differences in the susceptibility to CEL-I cytotoxicity among these cell lines. To further clarify the relationship between the binding efficiency and the cytotoxic activity of lectins, we compared the cytotoxicity and binding ability of CEL-I as to MDCK cells with those of other cytotoxic lectins. Based on the LD_{50} values estimated from the dose-response curves, it was found that the cytotoxic activities showed the following order, CEL-I > WGA > PHA-L > Con A (Fig. 4). Regarding the cytotoxic potential of lectins, it has been reported that WGA was cytotoxic toward nine human pancreatic carcinoma cell lines tested (IC_{50} at 2.5–5 μg/ml), and its effect was extensively stronger than those of Con A and PHA (*21*) for all these cell lines. In agreement with these findings, our results also indicated that WGA showed more potent cytotoxicity toward MDCK cells than Con A and PHA-L, the estimated LD_{50} for WGA was 5.4 μg/ml. This value was in good agreement with the IC_{50} obtained for pancreatic carcinoma cell lines, as described above. Interestingly, CEL-I (LD_{50} at 0.43 μg/ml) showed a nearly 10-fold stronger cytotoxic effect on MDCK cells than WGA. Since the numbers of

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CEL-I          --NQCPTDWEAEGD-HCYRFFNTLTTWENAHHECVSYSCST----LNVRSDLVSVHS
CEL-IV         CLTSCPPLWTGFNG-KCFRLFHNHLNFDNAENACRQFGLASCSGDELATGHLASIHS 46%
SJL-1          --CSCYPDWMAIPGSGCYKYVDTPKTFEEAESTCRHFSPGPYG-----FGHLASVHS 44%
Echinoidin     --GCCPTFWTSFGS-NCYRFFAVSLTWAEGEQFCQSFSVPSRG-DIDSIGHLVSIHS 56%
Lebectin       ---SXPSDWSSHE--EXYYVFRLFXTTWEAEXFXTQQVNGG---------XLN---- 38%
IX/X-BP-a      ---DCLSGWSSYEG-HCYKAFEKYKTWEDAERVCTEQAKGA---------HLVSIES 57%
IX/X-BP-b      ---DCPSDWSSYEG-HCYKPFSEPKNWADAENFCTQQHAGG---------HLVSFQS 45%
Botrocetin-b   ---DCPPDWSSYEG-HCYRFFKEWMHWDDAEEFCTEQQTGA---------HLVSFQS 53%
Botrocetin-a   ---DCPSGWSSYEG-NCYKFFQQKMNWADAERFCSEQAKGG---------HLVSIKI 52%
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Fig. 11. **Comparison of the N-terminal sequence of CEL-I with those of marine C-type lectins and C-type lectin-like proteins from venom.** The positions of conserved amino acid residues are highlighted: black, identical; gray, similar. Sequence alignment was performed using the CLUSTAL W program (*34*), and the results are displayed using BOXSHADE. CEL-IV, *Cucumaria echinata* lectin-IV; SJL-1, *Stichopus japonicus* (sea cucumber) lectin-1; Echinoidin, *Anthocidaris crassispina* (sea urchin) lectin; Lebectin, C-type lectin protein from *Macrovipera lebetina* venom; IX/X-BP-a, *Trimeresurus flavoviridis* (Habu snake) coagulation factor IX/factor X–binding protein A chain; IX/X-BP-b, *Trimeresurus flavoviridis* coagulation factor IX/factor X–binding protein B chain; Botrocetin-β, *Bothrops jararaca* (jararaca snake) platelet coagglutinin β chain; Botrocetin-α, *Bothrops jararaca* platelet coagglutinin α chain. The percentage of sequence similarity with CEL-I is indicated at the right.

lectin molecules bound per cell were estimated to be 2.4, 1.9, 0.39, and 0.26 × 10^8 for CEL-I, WGA, Con A, and PHA-L, respectively, the greater binding efficiency of CEL-I is a factor responsible for the potent cytotoxicity of CEL-I. However, the differences in binding efficiency seem to be too small to explain the differences in the cytotoxic activity of these lectins. For instance, the LD_{50} value of Con A for MDCK cells was more than 150-fold as compared to that for CEL-I, while Con A could bind for MDCK cells at nearly 15% of the level in the case of CEL-I (Figs. 2 and 4). Furthermore, despite that the amount of Con A bound to MDCK cells was greater than that of PHA-L, Con A was less toxic than PHA-L to this cell line (Fig. 4). Similar to our results, inconsistencies between the lectin-binding level and cytotoxicity have been reported for other lectins. BL6-8 melanoma cells, which lack the binding-site for *Griffonia simplicifolia* 1-G_4 ($GS1B_4$) lectin and are resistant to the cytotoxicity of this lectin, were transfected with an α1,3 galactosyltransferase gene. The transfected cells expressed a high level of the lectin-binding site, but remained resistant to the cytotoxicity of $GS1B_4$ lectin (*18*). From the results of analysis of the relationship between the degree of lectin-binding and the cytotoxicity of three WGA isolectins toward normal lymphocytes and cultured leukemic cell lines, an inverse correlation rather than a positive one was found between cytotoxicity and the degree of lectin-binding at statistically significant levels (*28*). These findings together with our results suggest that binding of lectins to cell surface carbohydrates is only an initial step, and that a post-binding action mechanism that may be specific for each lectin is more important for lectin-mediated cytotoxicity. Such a possibility that some intracellular mechanisms are involved in the regulation of lectin-mediated cytotoxicity has been proposed (*18*). Although the underlying post-binding mechanism of CEL-I remains unclear, one possible factor involved in the potent cytotoxicity of CEL-I could be the extremely high affinity of CEL-I for GalNAc. Regarding this point, it has been reported that a defined subset of lymphocytes were selectively killed by GalNAc-specific mistletoe lectin (ML III), which consists of an A-chain with ribosome inactivating activity and a B-chain with lectin activity, while galactose-specific isolectin (ML I) was less effective. This selective killing is not sufficiently explained by protein synthesis inhibition alone, since this subset was not affected by other ribosome-inactivating proteins such as the lectin from *Ricinus communis* (RCA_{120}), the lectin from *Abrus precatorus* (APA), abrin A, and inhibitors of RNA, DNA and/or protein synthesis such as actinomycin D, mitomycin C, and cycloheximide (*29*). These findings suggest that lectin-binding to specific receptors containing GalNAc may be the most important step in the ML III–mediated cytotoxic process for a particular subset of lymphocytes.

The CEL-I–induced cellular morphological changes concomitant with an increase in the number of trypan blue stainable cells as relatively early events caused by this lectin, and the inability of BFA and NH_4Cl to prevent the CEL-I cytotoxicity suggest that the plasma membrane may be the primary target of CEL-I rather than intracellular targets. Although how the binding of CEL-I to cell surface carbohydrates with a GalNAc moiety leads to cellular morphological changes accompanied by a disorder of membrane permeability, and eventual cell death is unknown, it has been demonstrated that several C-type lectin proteins (CLPs) isolated from snake venom exhibit anti-integrin activity (*30*, *31*). Integrins, a family of cell surface adhesion receptors, play a central role in the dynamic interaction between the extracellular matrix and the cytoskeleton. Therefore, it is considered that integrins transmit molecular signals regarding the cellular environment, which influences cell shape, survival, proliferation and gene transcription (*32*). In fact, recently it was reported that lebectin, a novel C-type lectin isolated from *Macrovipera lebetina* venom, potently inhibited the proliferation of human tumor cells as well as integrin-mediated adhesion, migration and invasion of tumor cells (*30*). Although further studies are required to determine the exact target molecules of CEL-I and its cytotoxic mechanism, one can speculate that CEL-I may interact with integrin-like molecules, as seen for lebectin, and such an interaction may in turn lead to dramatic cellular morphological changes and eventual cell death. This notion may be supported by the fact that there are several structural similarities between CEL-I and lebectin. Lebectin is composed of two identical subunits (16 kDa) and its entire molecular mass is 32 kDa, which is similar to that of CEL-I. Fig. 11 shows a comparison of the N-terminal amino acid sequence of CEL-I with those of other C-type lectins from marine invertebrates and several CLPs from venom. A relatively high degree of sequence similarity with these invertebrate C-type lectins was observed. Interestingly, this region of CEL-I even showed a slightly higher degree of similarity with some venom-derived CLPs, especially with IX/X-BP-b, the similarity being 57%. This value was higher than that for isolectin CEL-IV (46%). Since a single crystal of CEL-I had been obtained (*33*) and an X-ray crystallographic study has been performed recently (*35*), a comparative study of the tertiary structures of CEL-I and CLPs could provide further information concerning the structure-function relationship of these proteins in the near future.

It has been demonstrated that some cytotoxic plant lectins such as Con A, WGA, ricin, and *Griffonia simplicifolia* 1-B_4 lectin induce apoptosis. However, this is not the case for CEL-I cytotoxicity. No typical apoptotic nuclear morphological changes had been observed in CEL-I–treated MDCK cells even after cellular morphological changes had been significantly induced by CEL-I. Furthermore, DNA fragmentation, as measured by diphenylamine assaying, was not induced by CEL-I over a wide range of concentrations (0.1–100 μg/ml), whereas ricin, a potent apoptotic inducer, caused significant DNA fragmentation in MDCK cells. These results may support the idea that the cytotoxic effect of CEL-I starts with the membrane attack, as described above. Although at present we can not rule out the possibility of the involvement of internalized CEL-I, which was suggested on fluorescence observation with F-CEL-I (Fig. 3), we prefer the idea that the unique CEL-I–mediated membrane damage, as suggested by morphological changes and trypan blue staining, was a direct cause of the CEL-I cytotoxicity, since these changes were observed at a relatively early time after the addition of CEL-I.

Recently, Hatakeyama *et al*. constructed recombinant CEL-I (rCEL-I) (*16*). 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 cytotoxic mechanism of CEL-I, which may differ from apoptosis-mediated cytotoxicity, as proposed for other cytotoxic lectins so far.

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