Carrageenan inhibits granzyme A-induced detachment of and interleukin-8 release from alveolar epithelial A549 cells

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Abstract Granzyme A (GrA) is a lymphocyte serine protease that is believed to enter virus-infected cells and growing tumors and induce apoptosis. We found recently that recombinant rat GrA (rGrA) promotes detachment of and interleukin (IL)-8 release from alveolar epithelial A549 cells and suggested that this protease is involved in the pathogenesis of certain inflammatory lung diseases. In the present study, we found that λ -carrageenan (a sulfated oligosaccharide constituting the cell walls of seaweeds) potently inhibits rGrA-induced detachment and IL-8 release of A549 cells. This sulfated oligosaccharide might be useful for suppressing the development of inflammatory lung diseases in which GrA is thought to be involved.

Keywords A549 cells · Carrageenan · Detachment · Granzyme A · Interleukin-8 release

Abbreviations

ECM Extracellular matrix
GrA Granzyme A
IL Interleukin
rGrA Recombinant rat GrA

SFM Serum-free Dulbecco's modified eagle medium supplemented with 0.1% w/v BSA

Introduction

Granzyme A (GrA) is a serine protease produced in cytotoxic lymphocytes, which has trypsin-like activity (Kam et al. 2000). This protease has been believed to participate in the killing of abnormal cells such as virus-infected cells or growing tumor via induction of apoptosis (Kam et al. 2000; Chowdhury and Lieberman 2008). On the other hand, elevated level of GrA was found in patients with hypersensitive pneumonitis and chronic obstructive pulmonary disease, both of which are characterized by loss of alveolar wall structures, neutrophil accumulation, and chronic inflammation (Tremblay et al. 2000; Vernooy et al. 2007). We recently reported that a recombinant rat GrA (rGrA) causes detachment of and promotes the release of interleukin (IL)-8 (a potent neutrophil chemoattractant) from a human alveolar type II epithelial cell line A549 (Yoshikawa et al. 2008). These findings suggest that GrA, besides its role in the killing of abnormal cells, also contributes to the pathogenesis of certain chronic inflammatory lung diseases. Previous studies indicated that sulfated oligosaccharides such as heparin modulate GrA activity in vitro (Simon et al. 1990; Hirayasu et al. 2005). In the present study, we investigated the effect

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of sulfated oligosaccharides on rGrA-induced detachment and IL-8 release in A549 cells.

Materials and methods

Materials

λ -Carrageenan (from *Gigartina aciculata* and *Gigartina pistillata*) and bovine serum albumin (BSA) (protease free) were purchased from Sigma (St Louis, MO, USA). Chondroitin sulfate A was purchased from Nacalai Tesque (Kyoto, Japan). All other reagents used are of analytical grade.

Preparation of rGrA

rGrA was produced in *Pichia pastoris* as described previously (Tsuzuki et al. 2003). Purified rGrA was subjected to gel filtration in serum-free Dulbecco's modified eagle medium containing 0.1% w/v BSA (SFM). After titration (Hirayasu et al. 2008), the purified enzyme was sterile filtered and used in the experiments.

Treatment of A549 cells with test substances, detachment assay, and determination of IL-8 concentration in the conditioned media

A549 cells were maintained as described previously (Yoshikawa et al. 2008). Cells trypsinized were seeded into 48-well microtiter plates at a density of 1×10^4 cells/well and incubated for 48 h to be grown to 90% confluence. The cells were washed with phosphate buffered saline (PBS; 8 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , 136 mM NaCl, 2.7 mM KCl, pH 7.4), exposed to 100 μl of SFM or SFM containing test substances, and incubated for 24 h. After light-microscopic observation, cell detachment and IL-8 assays were conducted as follows: the conditioned media were harvested. The cells that remained attached were washed twice with PBS, and then lysed with 50 μl of 0.5N NaOH solution. Detached cells included in the conditioned media were precipitated by centrifugation at $5,000 \times g$ for 2 s at 22 °C, washed twice with PBS, and lysed with 50 μl of 0.5 N NaOH. These lysates were centrifuged at $10,000 \times g$ for 10 min at 4 °C to remove insoluble debris. The protein contents in the

resulting supernatants were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) with BSA as the standard. Cell detachment was expressed as the percentage of protein contents obtained from the detached cells, in the total protein contents (contents from the detached cells and from cells that remained attached). Concentration of IL-8 in the conditioned media was determined using Human IL-8 chemiluminescent ELISA (Pierce).

Statistical analysis

Differences in values were determined by the 2-tailed unpaired *t*-test or the alternate Welch test using Instat statistical software (GraphPad, San Diego, CA, USA). A *p*-value of <0.05 was considered to be statistically significant.

Results and discussion

We found recently that rGrA causes morphological changes and detachment of and promotes release of IL-8 from A549 cells with a threshold of 100–150 nM (Yoshikawa et al. 2008). In the present study, cells were treated with 300 nM rGrA, because the maximal effects were observed at this concentration (Yoshikawa et al. 2008). Cell rounding (or cell extension), increase in detachment, and increase in IL-8 release were confirmed in the present study when incubated for 24 h with 300 nM rGrA (Fig. 1; Table 1). We also showed previously that inclusion of sulfated oligosaccharides (λ -carrageenan, fucoidan, and heparin) enhanced rGrA-catalyzed hydrolysis of *N* α -benzyloxy-L-lysine thiobenzyl ester, a synthetic substrate commonly used for determination of GrA activity in vitro (Hirayasu et al. 2005). Of these, λ -carrageenan [(1,3)- β -D-galactose-2-sulfate-(1,4)- α -D-galactose-2,6-disulfate-polymer, which is found in seaweeds] showed the highest potency (Hirayasu et al. 2005). On the other hand, chondroitin sulfate A [a polymer of (1,4)- β -D-glucuronic acid-(1,3)- β -D-N-acetyl-galactosamine 6-sulfate, which is widely distributed in animal and plant tissues] had no effects (Hirayasu et al. 2005). For this reason, we examined the effects of λ -carrageenan and chondroitin sulfate A on the rGrA-mediated morphological changes, detachment, and IL-8 release of A549 cells. Neither λ -carrageenan alone nor chondroitin sulfate

Fig. 1 λ -Carrageenan but not chondroitin sulfate A inhibits morphological changes of A549 cells induced by rGrA. A549 cells were exposed to SFM (None), SFM containing 5 $\mu\text{g/ml}$ λ -carrageenan (Carrageenan), and SFM containing 5 $\mu\text{g/ml}$ chondroitin sulfate A (Chondroitin), without (GrA–, upper photographs) and with (GrA+ , bottom photographs) 300 nM rGrA. After 24-h incubation, cells were analyzed by light-microscopic observation and photographed. Bar = 25 μm

A alone caused morphological changes (Fig. 1) and promoted detachment and IL-8 release (Table 1) in A549 cells. λ -Carrageenan inhibited the effects of rGrA in a dose-dependent manner (Table 1). For instance, λ -carrageenan abolished rGrA-induced morphological changes, detachment, and IL-8 release when included at the concentration of 5 $\mu\text{g/ml}$ (Fig. 1; Table 1). On the other hand, chondroitin sulfate A showed no inhibitory effects at any concentrations tested (Fig. 1; Table 1).

Sulfated oligosaccharides, including λ -carrageenan, were shown to enhance the activity of GrA toward the hydrolysis of smaller synthetic substrates (Simon et al. 1990; Hirayasu et al. 2005). It is therefore unlikely that λ -carrageenan inhibits GrA activity to ameliorate the detachment of A549 cells. So far, certain extracellular matrix (ECM) components such as collagen type IV, fibronectin, and heparan sulfate proteoglycans have been known to be potential substrates for GrA (Simon et al. 1991; 1998; Kam et al. 2000). On the other hand, laminin (a constituent of ECM) is resistant to GrA (Nakamura et al. 1993). Also, we reported that rGrA-induced detachment occurs in A549 cells plated with collagen type IV or fibronectin but not in those plated with laminin and suggested that GrA reduces the cell adhesion through cleavage of ECM (Yoshikawa et al. 2008). GrA was also shown previously to bind with heparan sulfate proteoglycans in the basement membranes prepared from kidney, a bond that was

completely inhibited in the presence of sulfated oligosaccharides such as fucoidan (Vettel et al. 1993). Taken together, we conclude that certain sulfated polysaccharides (including λ -carrageenan), via their ability to bind with GrA, enhances the hydrolysis of smaller synthetic substrates mediated by the protease, but interrupts the binding between GrA and heparan sulfate proteoglycans, the binding of which is prerequisite for the cleavage of ECM proteins. It is unclear why chondroitin sulfate A showed no effects. The spatial positioning of the negatively charged groups and the stereostructure of sulfated polysaccharides might be inappropriate for interruption of binding between GrA and heparan sulfate proteoglycans. We reported recently that rGrA-induced release of IL-8 from A549 cells might be due to microtubule disruption resulting from the detachment (Yoshikawa et al. 2008). Hence, the effect of λ -carrageenan on IL-8 release may be accounted for by its ability to inhibit the detachment of the cells.

In summary, we found that λ -carrageenan inhibits rGrA-promoted detachment of and IL-8 release from A549 cells. This sulfated oligosaccharide may be of use for suppressing the pathogenesis of certain chronic inflammatory lung diseases in which GrA is thought to be involved. However, the oral administration of λ -carrageenan is known to induce experimental enterocolitis (Moyana and Lalonde 1990; Pricolo et al. 1996). The oligosaccharide

Table 1 Effects of λ -carrageenan and chondroitin sulfate A on the detachment and IL-8 release in the untreated or rGrA-treated A549 cells

Treatments	Cell detachment (%)	IL-8 release (ng/ml)
None	10 $\pm$ 1.2	6 $\pm$ 0.6
+Carrageenan (0.05 μ g/ml)	9 $\pm$ 1.7	7 $\pm$ 1.2
+Carrageenan (0.5 μ g/ml)	10 $\pm$ 1.2	6 $\pm$ 0.5
+Carrageenan (5 μ g/ml)	8 $\pm$ 1.6	6 $\pm$ 0.6
+Chondroitin (0.05 μ g/ml)	11 $\pm$ 1.2	5 $\pm$ 0.6
+Chondroitin (0.5 μ g/ml)	12 $\pm$ 2.3	6 $\pm$ 1.3
+Chondroitin (5 μ g/ml)	10 $\pm$ 1.5	6 $\pm$ 0.5
Granzyme A	36 $\pm$ 5.8*	13 $\pm$ 1.2*
+Carrageenan (0.05 μ g/ml)	37 $\pm$ 5.2*	12 $\pm$ 1.7*
+Carrageenan (0.5 μ g/ml)	20 $\pm$ 3.5**	10 $\pm$ 0.5**
+Carrageenan (5 μ g/ml)	11 $\pm$ 1.7***	6 $\pm$ 0.6***
+Chondroitin (0.05 μ g/ml)	37 $\pm$ 5.3*	13 $\pm$ 1.7*
+Chondroitin (0.5 μ g/ml)	35 $\pm$ 5.7*	12 $\pm$ 1.2*
+Chondroitin (5 μ g/ml)	36 $\pm$ 3.6*	13 $\pm$ 1.3*

Cell detachment and IL-8 assays were performed as described in the text. λ -Carrageenan and chondroitin sulfate A are indicated as Carrageenan and Chondroitin, respectively. The concentrations of each sulfated oligosaccharide are indicated in parenthesis. The values shown are means $\pm$ SE of three separate experiments performed in duplicate. Differences in values were determined by 2-tailed unpaired *t*-test (or alternate Welch test, when appropriate). * $p < 0.05$ versus A549 cells incubated with SFM alone (None); ** and ***, respectively, $0.05 < p < 0.1$ and $p < 0.05$ versus A549 cells incubated with 300 nM rGrA (Granzyme A)

should be administered by inhalation or intravenously at a low dose when used for clinical purposes (Murakami et al. 2002). Further studies are required to assess the utility of λ -carrageenan for suppressing lung inflammation.

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