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Structural features in carrageenan that interact with a heparin-binding hematopoietic growth factor and modulate its biological activity

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Abstract

The effects of carrageenans' structural features on its interaction with granulocyte colony-stimulating factor (G-CSF) and on the growth and differentiation of a G-CSF dependent leukemia cell line (NFS-60) were studied. λ, ι, and κ carrageenans, with decreasing contents of sulfation, bound to G-CSF with binding constants of $(6.2 \pm 0.6) \times 10^5\ M^{-1}$, $(7.4 \pm 0.5) \times 10^5\ M^{-1}$ and $(6.0 \pm 0.4) \times 10^5\ M^{-1}$, and with 27.7 ± 0.2, 17.4 ± 0.1 and 8.4 ± 0.1 binding sites, respectively. However, κ carrageenan oligosaccharide had no affinity for G-CSF. The three carrageenans significantly inhibited G-CSF-induced growth of NFS-60 cells. The high sulfate content λ carrageenan could also induce the maturation of the cells, but relatively low sulfate content ι and κ carrageenans could not. The results suggested that G-CSF–carrageenan bindings were dependent on carrageenans' sulfate contents and chain lengths, which could also affect the growth and differentiation of NFS-60 cells.
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Keywords: Carrageenan; Granulocyte colony-stimulating factor; Interaction; Capillary zone electrophoresis; Leukemia cells

1. Introduction

Glycosaminoglycans (GAG) are a protein-binding sulfated polysaccharide group that mediates fundamental biological mechanisms through interaction with proteins. Recently, there has a dramatic increase in the list of proteins known to interact with heparin/heparin sulfate (HS) GAG [1–3]. The biological activities of heparin/HS are strongly affected by binding to target proteins and the process can also affects the ultimate fate of the protein, that is, topographical destination, half life, and bioactivity [4]. The sulfate groups in heparin affect and control its biological activities and play a key role in heparin–protein interactions [5,6]. Therefore, other sulfated polysaccharides may bind to heparin-binding proteins, and modulate the biological activity of the proteins.

Carrageenans are a family of sulfated polysaccharides isolated from red algae. The backbone structure of carrageenans consists of the repeating disaccharide unit (1 → 4)-β-D-galactopyranosyl–(1 → 3)-α-D-galactopyranosyl, with 3,6-anhydrogalactose residues commonly present (Fig. 1). Carrageenans differ in their patterns and degree of sulfation, and this has marked effects on their physico-chemical properties [7,8]. λ, ι and κ carrageenans were found to be the most potent antagonists of basic fibroblast growth factor, platelet-derived growth factor and transforming growth factor β1, respectively [7]. Carrageenans also have other activities such as antiviral activity against dengue virus, hepatitis A virus and African swine fever virus [9–11]. These carrageenans are potentially useful drug candidates [12]. We previously found that granulocyte colony-stimulating factor (G-CSF) had affinity for heparins using capillary zone electrophoresis (CZE) and single molecules detection methods [13,14]. Therefore, carrageenans may bind to G-CSF and modulate G-CSF biological activity.

G-CSF is a hematopoietic growth factor that stimulates the proliferation and differentiation of hematopoietic progenitor cells, activates the function of mature neutrophils, and prevents apoptosis [15–18]. G-CSF is currently used clinically to reduce the risk of life-threatening infection in patients with neutropenia,

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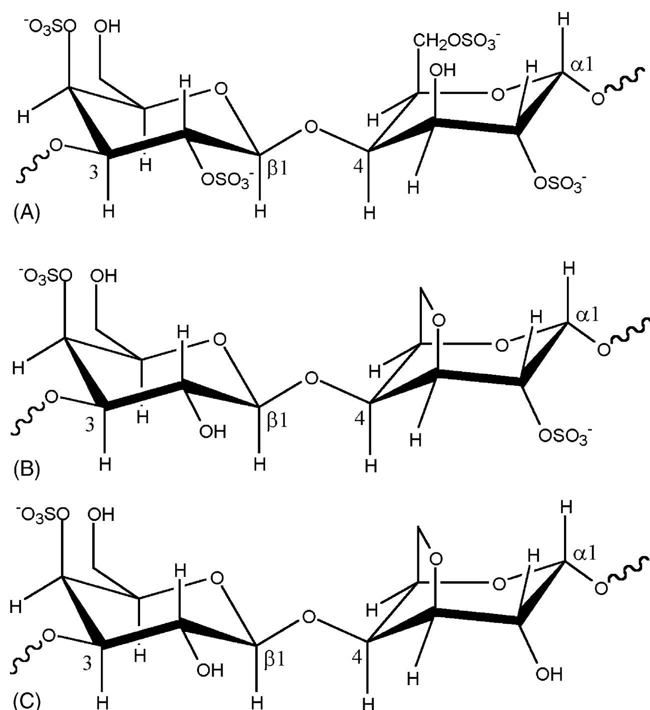

Fig. 1. The structure of (A) λ, (B) ι and (C) κ carrageenans.

particularly after intense cytotoxic chemotherapy, radiotherapy and bone marrow transplantation [16,17]. The specific binding of G-CSF to its receptor on murine myeloblastic cell line NFS-60 acts as a primer of cellular proliferation [19]. NFS-60 cells remained in the promyelocytic state in the presence of G-CSF [20]. The combination of G-CSF and all-*trans* retinoic acid could induce the differentiation of leukemia cells [21], but the effects of the combination of G-CSF and carrageenan on the proliferation and differentiation of NFS-60 cells have not been examined before.

In this study, we investigate the interactions between G-CSF and carrageenans with different chain lengths and sulfate contents for the first time. Furthermore, the function of the carrageenans in treatment of leukemia cells was also studied. This work may help with understanding the structure–function relationship of carrageenans, the mechanism of carrageenans in regulating cell growth and maturation, and the potential applications of carrageenans in leukemia treatment.

2. Experimental

2.1. Chemicals and reagents

RPMI-1640, tetrazolium salt, fetal calf serum (FCS), and carrageenans (λ, ι and κ) (*M*: 100 kDa) were purchased from Sigma (USA). rhG-CSF (*M*: 18,987 Da, 360 μg/mL in 10 mM NaAc-HAc buffer containing 5% mannitol, pH 4.0) were kindly provided by Qilu Pharmaceutical Factory (Jinan, China). κ carrageenan oligosaccharide (*M*: 1 kDa) was obtained from acid hydrolysis of κ carrageenan. A 4% κ carrageenan was hydrolyzed by 2% H_2SO_4 for 4 h at 80 °C with stirring, then cooled and neutralized by NaOH. The SO_4^{2-} was removed by adding $Ba(OH)_2$ and the upper solution was freezed. The G-CSF dependent murine myeloblastic leukemic cell line NFS-60 was obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Cells were maintained in RPMI 1640 medium containing 10% FCS and 10 ng/mL G-CSF at 37 °C in 5% CO_2, replacing with freshly prepared medium every 3 days. Cell populations were observed using an inverted microscope (Olympus CK40, Tokyo, Japan). Other chemicals were all analytical grade. Redistilled water was used throughout this work. For CZE analysis and cell culture studies, reagents were dissolved and diluted by NaAc-HAc buffer and RPMI-1640 medium, respectively.

2.2. Interactions between G-CSF and carrageenans by CZE

The interactions between G-CSF and carrageenans were studied using a P/ACE MDQ system (Beckman, Fullerton, USA) with a photodiode array detector and an uncoated fused silica capillary (Yongnian Optical Fibre Corp., China) with an internal diameter of 50 μm. The total and effective lengths of the capillary were 31.2 cm and 21 cm, respectively. Data were collected and processed with the Beckman System Software. The temperature of the capillary was kept at 25 °C. Before each measurement, the capillary was rinsed with the running electrolyte (50 mM phosphate buffer, pH 7.0) for 3 min at 20 psi (1 psi = 6894.76 Pa). Samples containing the mixture of G-CSF and carrageenans were injected after 40 min incubation at the anodic end using pressure injection with 0.5 psi for 4 s and detected at the cathodic end at a wavelength of 210 nm. The running voltage was 8.0 kV and the current during runs was between 61 μA and 64 μA. After each run, the capillary was flushed consecutively with 1 mol/L HCl for 2 min, water for 3 min, 1 mol/L NaOH for 2 min, and finally with water again for 3 min at 20 psi.

Binding studies often involve a proof of bindings, an identification of binding sites and an estimation of the quantitative parameters [22]. Scatchard analysis is a common way to linearize the binding data, and the model can be expressed in the following equation:

$$\frac{r}{C_f} = -Kr + nK$$

where *r* is the ratio of the concentrations of the bound ligand (or receptor) to the total receptor (or ligand) and C_f is the unbound ligand (or receptor) concentration. *K* is the apparent binding constant and *n* is the number of binding sites [23]. In this study, *r* is the concentration ratio of the bound G-CSF to the total carrageenans and C_f is the unbound G-CSF concentration.

2.3. Proliferation assays

Cell proliferation was studied by colorimetric cell proliferation assay, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and by counting total cells using Sysmex SE-9000 hematology analyzer (Sysmex, Japan). NFS-60 cells resuspended in RPMI-1640 complete medium were aliquotted at 1.0×10^4 cells/well and 2.0×10^4 cells/well to 96-well and 24-well plates, respectively. G-CSF was pre-incubated

with carrageenans for 40 min at room temperature and then the mixture was added to wells in triplicate. The final concentrations of G-CSF and carrageenans were 10 ng/mL and 0.1 mg/mL in a total volume of 0.2 mL/well and 2.0 mL/well for 96-well and 24-well, respectively. For MTT assay, cells were cultured in 96-well tissue culture plate for 1–4 days and assayed on an enzyme-linked immunosorbent detection system (Wellscan MK3, Labsystems Dragon, Helsinki, Finland) at 570 nm. Cell survival treated with carrageenans plus G-CSF was quantified and expressed as a percentage of values for cells treated with G-CSF alone. Total cells were counted after 3 and 4 days of culture in 24-well plates.

2.4. Differentiation analysis

Differentiation was assessed on the basis of cell morphology and expression of cell surface antigens. Morphological changes were examined on the cytospin slides stained with Wright–Giemsa and then taken photos by a Carl Zeiss Polarizing Microscope (Axioskop 40 Pol, Germany) after 3 days of culture. Cell surface antigens were determined after 3 and 4 days of culture by immunofluorescence staining with monoclonal antibodies FITC anti-mouse CD11b, PE anti-mouse CD18, PE anti-mouse, CD45 (BioLegend, San Diego, USA) and FITC anti-mouse CD34 (eBioscience, San Diego, USA) followed by flow cytometry (FACSCalibur, Becton-Dickinson, USA).

2.5. Cell cycle analysis

Cell cycle status was determined by the analysis of propidium iodide (PI)-stained cells. Cells were suspended in 0.5 mL DNA-prcp stain (RNase, PI) (Beckman, Fullerton, USA) and incubated in darkness at 4 °C for 30 min. The percentage of cells in different phases of the cell cycle was determined by flow cytometry.

2.6. Statistical analysis

Data are presented as mean ± S.D. The effects of carrageenans on the proliferation of NFS-60 cells were evaluated by using the *t*-test (two populations) of software Origin 6.1. Values of $p < 0.05$ were considered to indicate statistical significance.

3. Results and discussion

3.1. Effects of incubation time on G-CSF–carrageenan interaction

CE may be conveniently used to determine the minimum time for equilibration of an analyte–ligand mixture simply by performing repeated injections and determining the time at which the amount of free analyte is not changed anymore [22]. In this study, the effects of incubation time on G-CSF–carrageenan interaction were investigated by repeatedly injecting a mixture of a fixed concentration of G-CSF and λ carrageenan at different incubation time and recording the electropherograms (Fig. 2A). The addition of λ carrageenan to the sample makes the G-CSF peak broadened and then a new peak appeared just behind free G-CSF peak. Because carrageenan has no signal with UV detection, only G-CSF and G-CSF–carrageenan complex can be detected. With the increase of incubation time, the free G-CSF peak decreases and the new peak increases. At about 50 min of incubation, the two peaks nearly keep constant. Since λ carrageenan is a polysulfated polysaccharide, the G-CSF–λ carrageenan complex would migrate slowly than free G-CSF. Therefore, the new peak must be the complex and 50 min was chosen as the incubation time.

3.2. The interaction between G-CSF and λ carrageenan

Fig. 2B shows electropherograms for G-CSF alone and mixtures containing G-CSF and λ carrageenan. Each electropherogram contains a fixed concentration of G-CSF at 4.39 μM, but an increased concentration of λ carrageenan from 0.025 μM up to 0.3 μM. Upon the increase of λ carrageenan concentration, the G-CSF peak decreased, and a new peak behind G-CSF appeared corresponding to G-CSF–λ carrageenan complex, which increased due to the increase of the λ carrageenan concentration. To determine the binding parameters of G-CSF with λ carrageenan, the standard curve of G-CSF was obtained. The peak heights of G-CSF were found to be proportional to the concentrations of G-CSF within the range of 1.90–5.69 μM. The relationship between peak height and G-CSF concentration was expressed as $y = 1415.9x - 256.72$ ($n = 6$), with a correlation coefficient equal to 0.999. The free concentrations of G-CSF in G-CSF–carrageenan bindings were calculated from the calibration plot, and then values of C_f and r were obtained. Using Scatchard plot (Fig. 3), the binding constant and the number of binding sites of λ carrageenan with G-CSF were determined to be $(6.2 \pm 0.6) \times 10^5\ M^{-1}$ and 27.7 ± 0.2, respectively.

3.3. Effects of sulfate contents and chain lengths of carrageenan on its interaction with G-CSF

κ and ι carrageenans, with increasing sulfate contents but below that of λ carrageenan, were employed to study the effect of sulfate contents of carrageenan on G-CSF–carrageenan interaction. It showed similar results with G-CSF–λ carrageenan binding (data not shown). From Scatchard plot (Fig. 3), the binding constants of G-CSF with ι and κ carrageenans were determined to be $(7.4 \pm 0.5) \times 10^5\ M^{-1}$ and $(6.0 \pm 0.4) \times 10^5\ M^{-1}$, and the number of binding sites were 17.4 ± 0.1 and 8.4 ± 0.1, respectively. From Fig. 3, it can be seen that λ, ι and κ carrageenans interact with G-CSF with a similar binding constant (around $6.0 \times 10^5\ M^{-1}$) for 27.7 ± 0.2, 17.4 ± 0.1 and 8.4 ± 0.1 binding sites on the three carrageenans. The number of binding sites of the three carrageenans increased with the increase of their sulfate contents, which suggested that the sulfate contents of carrageenans affected G-CSF–carrageenan interaction.

The effect of carrageenan's size on its interaction with G-CSF was also investigated (Fig. 2C). The peaks of G-CSF and κ carrageenan oligosaccharide are nearly unchanged before and after their incubation. Even if the concentration of κ carrageenan oligosaccharide is increased nearly 10 times, the peak of G-CSF remains constant. Therefore, G-CSF could not interact with κ

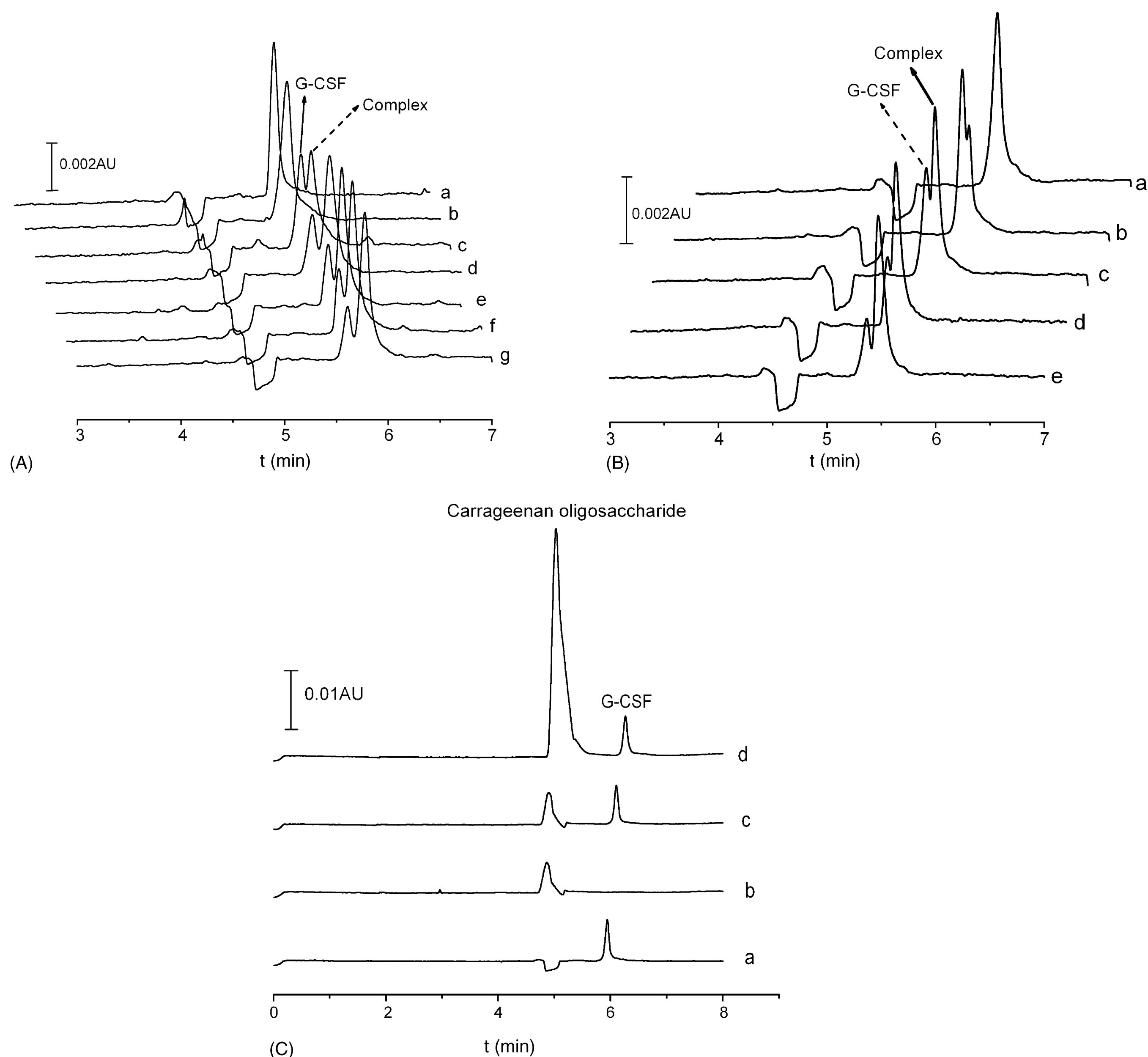

Fig. 2. Electropherograms of (A) 4.39 μM G-CSF mixed with a fixed concentration of λ carrageenan at different incubation time: (a) 0 min, (b) 30 min, (c) 40 min, (d) 50 min, (e) 70 min, (f) 80 min and (g) 120 min. (B) 4.39 μM G-CSF mixed with various concentrations of λ carrageenan: (a) 0.025 μM, (b) 0.05 μM, (c) 0.1 μM, (d) 0.2 μM and (e) 0.3 μM. (C) G-CSF and κ carrageenan oligosaccharide: (a) 3.16 μM G-CSF, (b) 1.5 mM κ carrageenan oligosaccharide, (c) 3.16 μM G-CSF + 1.5 mM κ-carrageenan oligosaccharide, (d) 3.16 μM G-CSF + 10 mM κ carrageenan oligosaccharide. Experimental conditions were the same as in Section 2.

carrageenan oligosaccharide, which indicated that the size of saccharide chain affected the G-CSF binding.

3.4. Effects of carrageenans on the leukemia NFS-60 cells

Firstly, the proliferation effects of three G-CSF binding carrageenans on the G-CSF responsive cell line, NFS-60, were exploited by MTT assay and cell counting. The dose response curves were determined (Fig. 4A). Cells were cultured with 10 ng/mL G-CSF and different concentrations of carrageenans for 3 days, and then determined by MTT assay. When the concentration of carrageenans is at 0.1 mg/mL, they all significantly inhibit the growth of the cells ($p < 0.001$). λ carrageenan showed the most significant inhibiting effects at all concentrations used in Fig. 4A. Cell counting showed similar results (data not shown). Therefore, the concentrations of carrageenans were set as 0.1 mg/mL in the following experiments.

Secondly, the differentiation effects of carrageenans on NFS-60 cells were studied by Wright–Giemsa staining. When cultured with G-CSF alone for 3 days, nearly all cells retained the morphology of promyelocytes (data not shown), which agree with previous study [20]. When cultured with G-CSF plus λ carrageenan for 3 days, most of the cells had a lobulated nucleus to the stage of metamyelocytes. The treatment with ι or κ

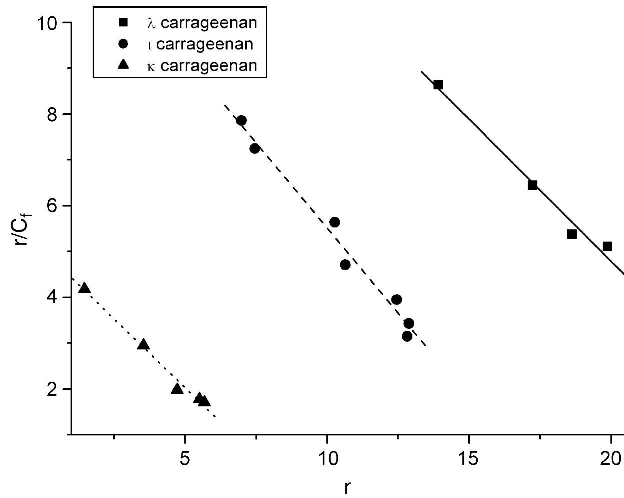

Fig. 3. Scatchard plots of the interactions between G-CSF and λ (■), ι (●), or κ (▲) carrageens. Experimental conditions were the same as in Fig. 2.

carrageenans did not result in significant changes in cell morphology. The results indicated that λ carrageenan induced the differentiation of the leukemia cells.

The expression of cell surface antigens was also determined by flow cytometry. Results showed that the expression of CD18 and/or CD45 enhanced after the stimulation of G-CSF plus the three carrageenans after 4 days culture (Fig. 4B). The expression of CD34 decreased to a different extent after treatment with G-CSF and the carrageenans. The expressions of CD18 and CD45 are the marker of cell maturation [21,24]. However, CD34 is the identification of primitive hematopoietic progenitor cells [25]. These results indicated that NFS-60 cells retained the responsiveness to λ carrageenan which efficiently induced the differentiation of NFS-60 cells. However, ι and κ carrageenans could not effectively induce the cell differentiation.

Thirdly, cell populations in different cycles were determined by flow cytometry after treatment with carrageenans and G-CSF. The combination of G-CSF and λ carrageenan increased the pop-

Fig. 4. Effects of carrageenans on the proliferation (A), differentiation (B) and cell cycle profile (C) of NFS-60 cells. Cells were stimulated with 10 ng/mL G-CSF alone ("0" or "Control") or in the combination of 10 ng/mL G-CSF and various concentrations (A) or 0.1 mg/mL (B and C) of carrageenans.

ulation of G0-G1 phase and G2-M phase of NFS-60 cells after 3 days of culture (Fig. 4C). As a result, the population of S phase decreased. The population of S phase also decreased to a different extent after treatment with G-CSF plus ι or κ carrageenans. The results explained the inhibiting effects of these carrageenans on the cells by MTT assay.

4. Conclusions

The interactions between G-CSF and carrageenans with different sulfate contents and chain lengths were investigated and the binding parameters were determined by CZE. Results indicated that the interactions were dependent on the sulfate contents and chain lengths of carrageenans. Furthermore, the carrageenans were also used to stimulate NFS-60 cells and a series of biological tests were carried out using biological methods. The three carrageenans significantly inhibited the G-CSF-induced growth of NFS-60 cells. λ carrageenan also effectively induced the differentiation of the leukemia cells, but ι and κ carrageenans could not induce the differentiation. From this study, it was observed that λ carrageenan, which has a higher sulfation content and higher number of binding sites in its interaction with G-CSF, had a stronger effects on growth inhibiting and differentiation inducing of NFS-60 cells than ι and κ carrageenans. Since G-CSF could induce the proliferation of NFS-60 cells through specific binding to G-CSF receptors on the cells [19], the interaction of carrageenans with G-CSF may influence the binding of G-CSF to G-CSF receptors. Therefore, giving rise to the biological effects observed, a possible mechanism is that the interactions between G-CSF and carrageenans might influence the growth or differentiation of NFS-60 cells. However, a much more complicated mechanism may exist and not just act through the interaction of carrageenan with G-CSF. In addition, the sulfate contents of carrageenans could also affect the growth and differentiation of the cells. The finally identified agents might be the leading compounds for new drug development.

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