Available online at www.sciencedirect.com
SCIENCE DIRECT
Carbohydrate Polymers 58 (2004) 89–94
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Interaction of polysaccharides with interferon-gamma using an improved ELISA approach☆

Weiyun Feng[a], Luhang Zhao[a,*], Keyi Wang[b]

[a]Department of Biochemistry and Molecular Biology, Medical College of Zhejiang University, 353 Num Yanan Road, Hangzhou 310031, China
[b]Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academic of Sciences, Shanghai 200031, China

Received 14 February 2004; revised 23 April 2004; accepted 25 June 2004
Available online 7 August 2004

Abstract

Establishes an ELISA approach to study the interaction of polysaccharides with cytokine in vitro. Synthesizes and separates heparin–BSA complex, after identification of the complex via SDS-PAGE, the ELISA plate wells are coated with HBC, detecting the interaction of interferon-gamma (IFN-γ) with heparin–BSA complex. We have investigated recombinant human IFN-γ (rhIFN-γ) binds to heparin in a concentration-dependent manner. Free heparin and low molecular weight heparin (LMW heparin) competes for the binding of IFN-γ to heparin, the IC_{50} concentrations give 2.40 and 18.60 μg/ml, respectively. Other polysaccharides either with sulfate group or without sulfate group compete for the binding of IFN-γ to HBC with significant different ability. The results suggest that IFN-γ is a cytokine binding to some polysaccharides including heparin, chondroitin sulfate, fucoidan and carrageenans family with different affinity. ELISA is a simple, sensitive approach to detect the interaction of cytokine with polysaccharides.

Keywords: Heparin; Heparin–BSA; Interferon-gamma; Polysaccharides; ELISA

1. Introduction

Heparin is an acidic glycosaminoglycan (GAG), consists of D-glucosamine, L-iduronic acid, D-glucronic acid, N-sulfate, O-sulfate and N-acetyl group (Sakai, Ebina, & Ishida, 1986). Glucosamine is either N-acetylated or N-sulfated and may also be ester O-sulfated at C6. In addition, ester O-sulfate groups may occur in the uronic acid residues at the C2 position. These specific sulfate patterns are involved in the interaction of GAGs with other molecules (Talbot, Dark, Kirby et al., 1996). In vivo, heparin is located in the granules of mast cells. In addition to its traditional anticoagulant activity, heparin has been shown to inhibit various aspects of the inflammatory response such as lymphocyte trafficking and neutrophil chemotaxis (Seeds & Page, 2001), modulate various aspects of immune function via multiple mechanisms (Baram, Rashkovsky, Hershkoviz et al., 1997) and inhibit the metastasis of tumour cells via inhibiting the activities of endoglycosidase heparanase and angiogenesis (Lever & Page, 2002). In addition, heparin has been shown to potently inhibit HIV-1 replication (Bartolini, Di-Caro, Liverani et al., 2003) and affect cell growth, gene expression, modulate clinically relevant event such as diabetes and atherosclerosis (Arfors & Ley, 1993). Heparin is known to bind and modulate the activity of various proteins, including cell growth factors, cytokines, angiogenic factors, complement components, adhesion molecules (Baram et al., 1997). IFN-γ is a cytokine binding to heparin, heparin modulate some activities of IFN-γ, heparin inhibits the induction of class II MHC moleculars, a signature activity of IFN-γ, and antagonizes the up regulation of ICAM-1 in human umbilical vein endothelial cells (Talbot et al., 1996), inhibit the antiviral (Sakai et al., 1986) and antiparasitic (Arrand, Shaw, Mackett et al., 2000) effects of IFN-γ. Potent anticoagulant activity of heparin by the interaction

☆ This work was supported by grants from Natural Sciences Foundations of Zhejiang (302031).
* Corresponding author. Tel.: +571-872-17415; fax: +571-872-17415.
E-mail address: zhaoluhang@263.net (L. Zhao).

0144-8617/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2004.06.031

with antithrombin III is extensively used in medicine (Daubener, Nockemann, Gusche et al., 1995). However, the clinical application of heparin for the treatment of both cancer and various inflammatory is limited by its anticoagulant activity. So we want to know whether LMW heparin and some polysaccharides from algae can substitute heparin at some functions avoiding its side effects.

The carrageenans are a family of sulfated polysaccharides extracted 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 (Hoffman, 1993). Carrageenans differ in their patterns and degree of sulfation, which can be conveniently divided into three main types depending on the number and position of sulfate groups and the 3,6-anhydrogalactose concent. Carrageenans are extensively used as food additive in the food industry. It has been shown that carrageenans can exert some physiological effects including modulation of immune function (Vijayakumar & Muthukkaruppan, 1990), anti-viral activity (Girond, Renaudet, Deloince et al., 1991) and anti-metastatic activity (Coombe, Parish, Ramshaw et al., 1987). Recently carrageenans have been shown to interact with some growth factors including basic fibroblast growth factor (bFGF), transforming growth factor-β1 (TGF-β1), platelet-derived growth factor (PDGF) and modulate the binding to their receptors (Hoffman, 1993). In this report, in order to study the specification and affinity of the interaction of heparin with IFN-γ, we developed an ELISA approach of high sensitivity further evaluated whether carrageenans binding to IFN-γusing this indirect ELISA approach.

2. Materials and methods

2.1. Reagents and materials

Heparin-Na was purchased from Sino-American Biotechnology Company, Bovine serum albumin (BSA) and Greiner ELISA Microplates were purchased from Shanghai Sangon Biological Engineering Technology and Service Co Ltd. rhIFN-γ was purchased from PEPRO TECH EC LTD. Biotinylated Anti-human IFN-γ. Antibody was purchased from R&D Systems. Horseradish Peroxidase Labeled Streptavidin and TMB Microwell Peroxidase Substrate System were purchased from Kirkegaard and Perry Laboratories. BCA reagent Kit was purchased from Shenergy Biocolor Biological Science and Technology Company. Polysaccharides were all purchased from Sigma. LMW heparin was kindly provided by professor Wang Ke-Yi (Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academic of Sciences, Shanghai).

2.2. Synthesis of HBC

Heparin was end-conjugated to BSA using a method as described fully elsewhere (Najjam, Gibbs, Rider et al., 1997). The separation of the resulting complex from unconjugated reactants was achieved by gel-filtration, using a 1×90 cm column of Sepharose 4B. After concentration and desalting with Centriplus Centrifugal Filter Device (50,000 MW cut-off) from Millipore Corporation, the elution of high Mr heparin–BSA complex was identified by SDS-PAGE stained for GAGs with Azure A and for protein with Coomassie blue as described in full previously (Lee & Lander, 1991). BSA was treated with sodium cyanoborohydride under identical conditions, gel-filtration, SDS-PAGE and protein quantification as above.

2.3. Heparin binding ELISA

ELISA plate wells were coated either with heparin–BSA complex, 100 μl containing 0.5 μg BSA equivalent (BCA reagent kit) in 50 mmol/l Tris–HCl buffer, pH 7.4, containing 12.7 mmol/l EDTA; or with the same amount of mock-treated BSA, incubated overnight at 4 °C. After three times washing with phosphate-buffered saline (PBS), pH 7.4 containing 0.05%(V/V) Tween 20, the plate was blocked with 10 g/l BSA in PBS (250 μl/well) at 37 °C for 2 h. After three times washing, 100 μl different concentration rhIFN-γ dissolved in blocking buffer containing 0.05% Tween 20 was added per well, the plate was incubated at 37 °C for 2 h. After three times washing, 20 ng Biotinylated Anti-human IFN-γ antibody dissolved in blocking buffer containing 0.05% Tween 20 was added, incubated at 37 °C for 2 h. After three times washing, the Horseradish Peroxidase Labeled Streptavidin 100 μl/well, diluted 1/1000 in blocking buffer containing 0.05% Tween 20 was added at 37 °C for 30 min. After six times washing, Peroxidase activity was detected with 100 μl/well of TMB, 1 mol/l H_3PO_4 was added after 12 min. Absorbance values were read at 450 nm in 5 min.

2.4. Soluble saccharides competitive binding assays

The same ELISA approach, but 1.5 ng rhIFN-γ was preincubated at room temperature for 30 min in the presence and absence of different concentration soluble saccharides in block buffer containing 0.05% Tween 20 before addition to blocked wells. Then the plate was added orderly biotinylated anti-IFN-γ antibody, Horseradish Peroxidase Labeled Streptavidin, TMB and 1 mol/l H_3PO_4, at the end absorbance values were read at 450 nm.

3. Results

3.1. Synthesis and characterizations of heparin–BSA complex

Free heparin, unlike polypeptides, adsorb poorly onto plastic surfaces. Therefore, in order to study interaction between heparin and cytokine using ELISA assay, heparin–BSA complex were synthesized by conjugating heparin to BSA in the presence of sodium cyanoborohydride, heparin chains adsorb onto the plate via BSA. The reaction products were resolved by gel filtration on Sepharose 4B. The fractions isolated from the leading edge of the gel filtration peak reveal the presence of a high *Mrs* components by SDS-PAGE, the high *Mrs* components enter the resolving gels but migrate slower than free heparin and BSA. Subsequent elution solutions are seen to contain smaller amounts of this material, but also appreciable quantities of unconjugated heparin and BSA. Since this latter material has high *Mrs* and stain for both protein and GAGs, we take them to be HBC. We began ELISA experiments using the fractions containing heparin–BSA complex without free heparin and BSA.

3.2. ELISA binding of IFN-γ

rhIFN-γ bind to wells coated with heparin–BSA complex, as described in Section 2; and as control, rhIFN-γ bind to wells coated with mock-treated BSA (Fig. 1). The binding of IFN-γ to HBC is readily detectable at as little as 0.25 ng IFN-γ/well, increases in a dose-dependent manner, and saturates at around 2 ng/well. Although some dose-dependent binding of IFN-γ also occurs in control wells, coated with cyanoborohydride-treated BSA in place of heparin–BSA complex, the background binding is considerably lower.

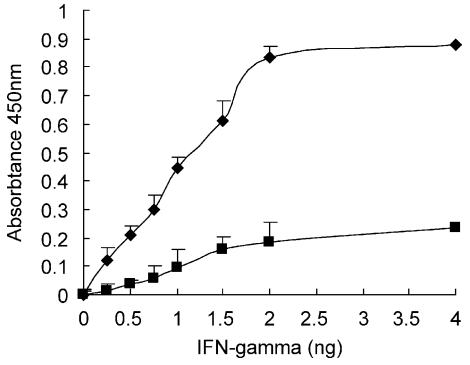

Fig. 1. ELISA of IFN-γ binding to the heparin–BSA complex. Increasing concentrations of IFN-γ were added to wells coated with either 5 μg/ml protein equivalent of heparin–BSA complex(♦) or the same amount of mock-treated BSA (■). Absorbance values were read after 5 min ending the substrate reaction. Dose-dependent binding of IFN-γ to heparin–BSA complex, saturates at around 2 ng. The curves are typical of three independent experiments performed under identical routine conditions. Each absorbance value is the mean of triplicate wells shown ± SD.

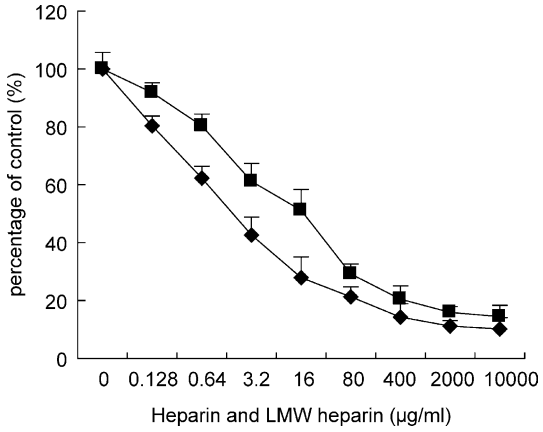

Fig. 2. Heparin and LMW heparin competitive binding to IFN-γ. Competition with soluble heparin and LMW heparin. rhIFN-γ (1.5 ng in 100 μl 0.05% Tween 20 block buffer) was preincubated for 30 min at room temperature in the absence or presence of increasing concentration soluble heparin (♦) or LMW heparin (■) before addition to wells coated with heparin–BSA complex. Results are expressed as the percentage of the binding of IFN-γ to heparin–BSA complex as shown in graph.

3.3. Heparin and LMW heparin competitive binding assays

To confirm that the strong binding of rhIFN-γ to heparin–BSA complex is indeed due to the presence of the heparin chains, rhIFN-γ was preincubated with increasing different concentrations of heparin and LMW heparin before adding to wells coated with heparin–BSA complex. As shown in Fig. 2, soluble heparin and LMW heparin result in increasing inhibition for IFN-γ binding to HBC, the IC_{50} concentrations of heparin and LMW heparin give 2.4 and 18.6 μg/ml, respectively using POMS software. The ability of soluble heparin to compete for binding establishes that heparin chains are involved in the retention of IFN-γ by the complex.

3.4. Inhibition of heparin binding to IFN-γ by GAGs

To conform whether other GAGs influence the binding of IFN-γ to heparin, 1.5 ng rhIFN-γ was preincubated with increasing concentrations of different type of GAGs, the mixtures were added to wells coated with heparin–BSA complex. As shown in Fig. 3, GAGs tested inhibited IFN-γ binding to heparin–BSA complex with significant different ability. Comparing to heparin, chondroitin sulfate A (CS-A), chondroitin sulfate C (CS-C) and hyaluronic acid are poor competitor.

3.5. Influence of sulfate groups of saccharides on the binding of heparin to IFN-γ

To deep conform the important role of the sulfate group of saccharides in the interaction of saccharides with IFN-γ, 1.5 ng rhIFN-γ was preincubated with increasing concentrations of dextran sulfate, dextran, glucosamine sulfate and acetylglucosamine, followed by adding the mixtures to

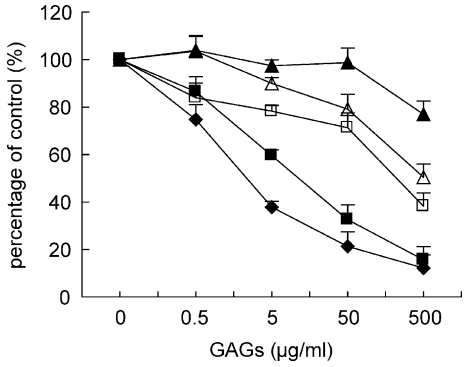

Fig. 3. Specificity of IFN-γ binding to soluble GAGs. Competition with soluble GAGs. rhIFN-γ (1.5 ng in 100 μl 0.05% Tween 20 block buffer) was preincubated for 30 min at room temperature in the absence (control) or presence of increasing concentration GAGs including heparin (♦), LMW heparin (■), CS-A (▲), CS-C (△) hyaluronic acid (□) before addition to wells coated with heparin–BSA complex. Results are expressed as the percentage of the binding of IFN-γ to heparin–BSA complex as shown in graph.

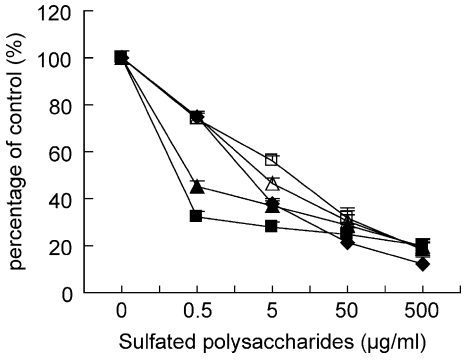

Fig. 5. Specificity of sulphated polysaccharides extracted from algae bind to IFN-γ. Competition with some polysaccharides extracted from algae. rhIFN-γ (1.5 ng in 100 μl 0.05% Tween 20 block buffer) was preincubated for 30 min at room temperature in the absence or presence of increasing concentration heparin (♦), fucoidan (■) carrageenan-λ (▲), carrageenan-κ (□), carrageenan-ι (△) before addition to wells coated with heparin–BSA complex. Results are expressed as the percentage of the binding of IFN-γ to heparin–BSA complex as shown in graph.

wells coated with heparin–BSA complex. As shown in Fig. 4, sulfate dextran, a highly sulphated polysaccharides strongly inhibited the binding of IFN-γ to heparin–BSA complex, it suggests that sulfate dextran binds to IFN-γ with high affinity.

3.6. Influence of sulfated polysaccharides extracted from algae to IFN-γ binding to HBC

Some sulfated polysaccharides extracted from alage give multiple biological functions. To confirm whether sulphated polysaccharides extracted from algae bind to IFN-γ, 1.5 ng rhIFN-γ was preincubated with increasing concentrations fucoidan, carrageenan-λ, carrageenan-κ and carrageenan-ι, followed by addition the mixture to wells coated with heparin–BSA complex. As shown in Fig. 5, all sulfated polysaccharides tested inhibit the binding of IFN-γ to heparin–BSA complex. The results suggest that sulfated polysaccharides extracted from algae tested bind to IFN-γ with high affinity.

In order to compare the different affinity of IFN-γ binding to these polysaccharides, the IC_{50} concentrations of polysaccharides inhibit the binding of IFN-γ to heparin–BSA complex and the sugar component of polysaccharides are displayed in Table 1.

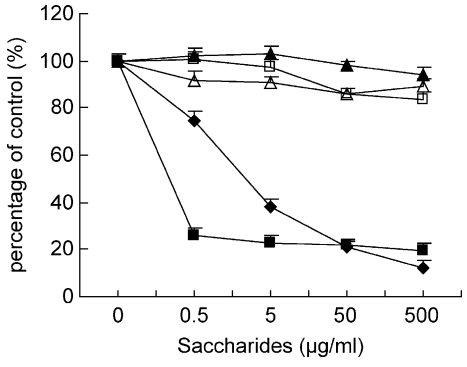

Fig. 4. Specificity of IFN-γ binding to some saccharides with or without sulfate group. Competition with some saccharides. rhIFN-γ (1.5 ng in 100 μl 0.05% Tween 20 block buffer) was preincubated for 30 min at room temperature in the absence or presence of increasing concentration heparin (♦), sulfate dextran (■), dextran (▲), glucosamine sulfate (□), acetylglucosamine (△) before addition to wells coated with heparin–BSA complex. Sulfate dextran strongly inhibited IFN-γ binding to HBC but dextran, glucosamine sulfate, acetylglucosamine did not inhibit the binding. Results are expressed as the percentage of the binding of IFN-γ to heparin–BSA complex as shown in graph.

4. Discussion

In the present study, we have synthesized heparin–BSA complex and established an ELISA approach to detect the interaction of polysaccharides with cytokines. We first demonstrated IFN-γ binding to heparin with high affinity using ELISA approach. The binding was high reproducible detected at 0.25 ng IFN-γ, and increases in a dose-dependent manner. This shows ELISA is high sensitive to detect the binding of polysaccharides with cytokines. It provides a new method to study the interaction of GAGs with cytokines in vitro.

In the 1990s, a sensitive technique developed for the study of the potential interaction of cytokine with heparin and related polysaccharides is affinity coelectrophoresis (ACE) (Lee & Lander, 1991). Using this method, the binding of heparin to FGF-2, GROα, IL-7, IL-8, PF-4 was demonstrated (Najjam et al., 1997). Our ELISA demonstrates a sensitivity at least as high as ACE, and through the use of soluble competitors is well able to make sure the specificity of cytokine/GAG interactions. It also has the advantage that binding may be studied at the physiological pH and ionic strength conditions. Moreover, the ELISA approach avoids the radiological hazards and reagent

Table 1
IC_{50} concentrations of polysaccharides inhibit IFN-γ binding to heparin–BSA complex and the sugar component of these polysaccharides

Compound	Disaccharide unit	IC_{50} (μg/ml)
Heparin	→4)-α-L-IdoA*p*-(2 so_3^-)-(1→4)-α-D-GlcA*p*[2,6(so_3^-)$_2$]- (1→	2.40
LMW heparin	→4)-α-*l*-IdoA*p*-(2 so_3^-)-(1→4)-α-D-GlcA*p*[2,6(so_3^-)$_2$]- (1→	18.60
CS-A	→4)-β-GlcA-(1→3)-β-GalNAc(4 so_3^-)-(1→	>500
CS-C	→4)-β-GlcA-(1→3)-β-GalNAc(6so_3^-)-(1→	>500
Hyaluronic acid	→4)-β-D-GlcA-(1→3-β-D-GlcNAc-(1→	247.13
Fucoidan	→3)-α-L-Fuc(2so_3^-)-(1→4)-α-L-Fuc(2so_3^-)-(1→	0.0177
Carrageenan-λ	→3)-β-D-galactopyranosy(2so_3^-)-(1→4)-α-D-galactose-[2,6(so_3^-)$_2$]-(1→	0.17
Carrageenan-κ	→3)-β-D-galactopyranosy(4so_3^-)-(1→4)-α-D-galactose-(1→	8.33
Carrageenan-ι	→3)-β-D-galactopyranosy(4so_3^-)-(1→4)-α-D-galactose-(2so_3^-)-(1→	6.16

The IC_{50} concentrations of polysaccharides were obtained using POMS software. Dextran sulfate has strongly inhibited IFN-γ binding to heparin–BSA complex, IC_{50} concentrations of it has not been shown.

shelf-life limitations associated with the radio-iodination of heparin required for ACE. Recently, capillary electrophoresis (CE) is also used to study interactions of glycans/proteoglycans with growth factors (Militsopoulou, Lamari, & Karamanos, 2003). CE is a useful tool to study the interactions of proteoglycans with growth factors, but it has the disadvantage that failing to provide the physiological pH and ionic strength conditions as ELISA. In addition, as an advantage, ELISA allows cytokine binding to many different polysaccharides at the same conditions.

We found that the binding of IFN-γ to heparin–BSA complex was competed out by soluble heparin and LMW heparin, the IC_{50} concentrations give 2.40 and 18.60 μg/ml, respectively. Heparin is a mixture with different length polysaccharide chains; the complicated structure of heparin decides its complicated functions. Specifical polysaccharide chains have the specifical functions such as high affinity interaction between heparin and antithrombin III, requires a pentasaccharides sequence (Najjam, Mulloy, Theze et al., 1998). LMW heparin is the degraded products of heparin, though without extensive application like heparin, LMW heparin has been shown to have higher bioavailability, longer half life and reduced risk of bleeding compared with heparin (Lever & Page, 2002). LMW heparin have received particular attention in clinic, due to reduced anticoagulant effects without significant reduction of its anti-inflammatory property (Beltran, Garrido, Glaria et al., 1999). LMW heparin binding to IFN-γ suggests LMW heparin interaction with some proteins selectively exerts some activities of heparin reducing the side effects. It is significant for exploitation and application LMW heparin in medicine.

Our results show that GAGs and some sulfated polysaccharides including fucoidan and carrageenan family bind to IFN-γ with high affinity. Though how GAGs interaction with cytokine is not completely clear, heparin and sulfated polysaccharides binding to IFN-γ with high affinity indicated that the presence of anionic groups was absolutely essential for optimal binding, since unsulfated hyaluronic acid and dextran had an insignificant binding to IFN-γ. Nevertheless, charge densities could not be ascribed to differences in binding capacities since CS-A and CS-C with high sulfated group had an insignificant binding to IFN-γ. Glucosamine sulfate, acetylglucosamine failing to bind to IFN-γ demonstrated a peculiar structure for polysaccharides is also necessary for binding. Sulfated polysaccharides extracted from algae have been shown to possess various functions such as anti human immunodeficiency virus (HIV) (Witvrouw & De Clercq, 1997), all three types of carrageenans tested in this study strongly inhibit IFN-γ binding to HBC. This result suggests that carrageenan family binds to IFN-γ, and the binding decreases the interaction of heparin with IFN-γ. The possible mechanism is that the carrageenans may interact with the heparin binding domain on IFN-γ or carrageenans binding to IFN-γ change the conformation of cytokine failing to binding to heparin. Carrageenan family interact selectively with some growth factors such as PDGF, TGF-β1 and bFGF high affinity (Hoffman, 1993), and PDGF, TGF-β1, bFGF also bind to heparin with high affinity (Lever & Page, 2002). However, there are no reports of either TGF-α or IGF-1 binding to heparin, and neither of these growth factors bind to carrageenans. The result suggests that the interaction of sulphated polysaccharides with some proteins might not be absolutely specific. Here, we first demonstrated that carrageenan family binds to IFN-γ with high affinity, the affinity of carrageenan family binding to IFN-γ is carrageenan-λ > carrageenan-ι > carrageenan-κ. The structure of the three forms of carrageenan differ only in the number of sulfate groups per disaccharides: kappa has one, iota has two and lambda has three (Langendorff, Cuvelier, Michon et al., 2000). The affinity of carrageenans binding to IFN-γ is consistent with the number of their sulfate groups, carrageenan-λ with three sulphate groups have the most potent affinity binding to IFN-γ. Koyanagi et al. reported that oversulfation of fucoidan enhances its anti-angiogenic and antitumor activities (Koyanagi, Tanigawa, Nakagawa et al., 2003). It suggests that based on the same polysaccharides structure, more sulfation results in stronger interaction with some proteins. All results suggest that maybe like heparin, carrageenans can bind to IFN-γ, and exert some analogous biological functions to modulating the activities of cytokine. These sulfate polysaccharides extracted from algae are inexpensive, water soluble

and substantive. The results suggest it is significant of exploitation drug from algae even other marine organism.

We first demonstrate that carrageenan polysaccharides bind to IFN-γ using an improved ELISA approach. ELISA approach is a simple, effective method to study interaction of polysaccharides with cytokines in vitro. The influence of polysaccharides on IFN-γ biology activities need further study.

Acknowledgements

This work was supported by grants natural science foundation from Zhejiang. We thank professor Wang Ke-Yi (Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academic of Sciences, Shanghai) for providing LMW heparin.

References

Arfors, E. K., & Ley, K. (1993). Sulfated polysaccharides in inflammation. *The Journal of Laboratory and Clinical Medicine*, *121*(2), 201–202.

Arrand, J. R., Shaw, D., Mackett, M., & Salek-Ardakani, S. (2000). Heparin and heparan sulfate bind interlekin-10 and modulate its activity. *Blood*, *96*(5), 1879–1888.

Baram, D., Rashkovsky, M., Hershkoiz, R., Drucker, I., Reshef, T., Ben-Shitrit, S., & Mekori, Y. A. (1997). Inhibitory effects of low weight heparin on mediator release by mast cells preferential inhibition of cytokine production and mast cell-dependent cutaneous inflammation. *Clinical and Experimental Immunology*, *110*(3), 485–491.

Bartolini, B., Di-Caro, A., Cavallaro, R. A., Liverani, L., & Mascellani, G. (2003). Susceptibility to highly sulphated glycosaminoglycans of human immunodeficiency virus type 1 replication in peripheral blood lymaphocytes and monocyte-derived macrophages cell culture. *Antiviral Research*, *58*(2), 139–147.

Beltran, A. E., Concepcion, F., Manzanares, D., Garrido, G., Glaria, L. A., & Rojas, A. (1999). Heparin and low molecular weight heparin decrease nitric oxide production by human polymorphonuclear cells. *Archives of Medical Research*, *32*(2), 116–119.

Coombe, D. R., Parish, C. R., & Ramshaw, I. A. (1987). Analysis of the inhibition of tumour metastasis by sulphated polysaccharides. *International Journal of Cancer*, *39*(1), 82–88.

Daubener, W., Nockemann, S., Gusche, M., & Hadding, U. (1995). Heparin inhibits the antiparasitic and immune modulatory effects of human recombinant interferon-γ. *European Journal of Immunology*, *25*(3), 688–692.

Girond, S., Crance, J. M., Van Cuyck-Gandre, H., Renaudet, J., & Deloince, R. (1991). Antiviral activity of carrageenan on hepatitis A virus replication in cell culture. *Research in Virology*, *142*(4), 261–270.

Hoffman, R. (1993). Carrageenans inhibit growth-factor binding. *The Biochemical Journal*, *289*(2), 331–334.

Koyanagi, S., Tanigawa, N., Nakagawa, H., Soeda, S., & Shimeno, H. (2003). Oversulfation of fucoidan enhances its anti-angiogenic and antitumor activities. *Biochemical Pharmacology*, *2*(15), 173–179.

Langendorff, V., Cuvelier, G., Michon, C., Launay, B., & Parker, A. (2000). Effects of carrageenan type on the behaviour of carrageenan/milk mixtures. *Food Hydrocolloids*, *14*, 273–280.

Lee, M. K., & Lander, A. D. (1991). Analysis of affinity and structural selectivity in the binding of proteins toglycosaminoglycans: development of a sensitive electrophoretic approach. *Proceedings of the National Academy of Science USA*, *88*(7), 2768–2772.

Lever, R., & Page, C. P. (2002). Novel drug development opportunities for heparin. *Nature Reviews. Drug Discovery*, *1*(2), 140–148.

Militsopoulou, M., Lamari, F., & Karamanos, N. K. (2003). Capillary electrophoresis: a tool for studying interactions of glycans/proteoglycans with growth factors. *Journal of Pharmaceutical and Biomedical Analysis*, *32*(4), 823–828.

Najjam, S., Mulloy, B., Theze, J., Gordon, M., Gibbs, R., & Rider, CC. (1998). Further characterization of the binding of human recombinant interleukin 2 to heparin and identification of putative binding sites. *Glycobiology*, *8*(5), 509–516.

Najjam, S., Gibbs, R. V., Gordon, M. Y., & Rider, C. C. (1997). Characterization of human recombinant interlekin-2 binding to heparin and heparan sulfate using an ELISA approach. *Cytokine*, *9*(12), 1013–1022.

Sakai, A., Ebina, T., & Ishida, N. (1986). Inhibition of murine L cell interferin action by heparin. *Archives of Virology*, *90*(1–2), 73–85.

Seeds, E. A., & Page, C. P. (2001). Heparin inhibits allergen-induced eosinophil infiltration into guinea-pig lung via a mechanism unrelated to its anticoagulant activity. *Pulmonary Pharmacology and Therapeutics*, *14*(2), 111–119.

Talbot, D., Dark, J. H., Kirby, J. A., Rix, D. A., & Douglas, M. S. (1996). Role of glycosaminoglycans (GAGs) in regulation of the immunogenicity of human vascular endothelial cells. *Clinical and Experimental Immunology*, *104*(1), 60–65.

Vijayakumar, R. K., & Muthukkaruppan, V. R. (1990). Immunoragulatory processes induced by carrageenan in BALB/C mice. *Immunological Investigations*, *19*(2), 163–183.

Witvrouw, M., & De Clercq, E. (1997). Sulfated polysaccharides extracted from sea algae as potential antiviral drugs. *General Pharmacology*, *29*(4), 497–511.