Arch Pharm Res Vol 25, No 5, 669-674, 2002

Archives of Pharmacal Research
http://apr.psk.or.kr

Immunomodulating and Anticoagulant Activity of Glycosaminoglycans Derived from Porcine Testis

Yung Choon Yoo[1], Yeong Shik Kim[3], Kyung Sik Song[4], Eun Ho Moon[5], and Kyung Bok Lee[2]

[1]Department of Microbiology, [2]Department of Biochemistry, College of Medicine, Konyang University, Nonsan, Chungnam, Korea, [3]Natural Products Research Institute, Seoul National University, Seoul, Korea, [4]Department of Agricultural Chemistry, College of Agriculture, Kyungpook National University, Daegu, Kyungpook, Korea, and [5]General Education/Mathematics Myongji University, Yongin, Kyunggi, Korea

(Received August 8, 2002)

Glycosaminoglycans (GAGs) were isolated from the porcine testis, and their immuno-modulating and anticoagulant activity was investigated. From anion exchange chromatography (Dowex Macropolous Resin) used for further isolation of porcine testis GAGs (PT-GAGs), two fractions (PT-GAG-1.5 and PT-GAG-16) eluted by different salt concentration were obtained. In immunomodulating activity test, PT-GAG-1.5, but not PT-GAG-16, significantly enhanced the growth of murine peritoneal macrophages. In addition, treatment with PT-GAG-1.5 induced the production of cytokines, interleukin-1β (IL-1β), interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), from murine microphages. Unexpectedly, both of PT-GAGs had no effect on the growth of murine splenocytes. The anticoagulant activity of PT-GAG-1.5 and PT-GAG-16 was examined by activated partial thromboplastin time (aPTT) assay and thrombin time (TT) assay. Both of PT-kGAGs significantly increased the clotting times of aPTT and TT in a dose-dependent manner. The anticoagulant activity of PT-GAG-16 was found to be higher than that of PT-GAG-1.5. These results suggest that PT-GAGs possess biological activities such as immunomodulating activity and anticoagulant activity.

Key words: Porcine testis, Glycosaminoglycans, Immunomodulation, Anticoagulant

INTRODUCTION

The testes have an unusually thick connective tissue capsule, the *tunican alubuginea* (Michael *et al*, 1995). The glycosaminoglycans (GAGs) are major constituents of testis connective tissue. The proteoglycans in male reproductive tract have been characterized (Perin *et al.*, 1994), and tissue structure-specific distribution of GAGs in the human penis has been reported (Goulas *et al*, 2000). The analysis of these GAGs confirmed the presence of hyaluronic acid, heparan sulfate, and chondroitin 6-sulfate. Many reports indicate that they might be involved in various regulatory process during spermatogenesis, however, their biological roles are not currently well established.

GAGs are known to play a role as important regulators of many biological processes, such as cell growth, proliferation and recognition, extracellular matrix deposition, and morphogenesis (Papakonstantinou *et al*, 1998a; Rogerson *et al.*, 1995; Silbert *et al.*, 1995). It prompted us to isolate the GAGs from the porcine testis and investigate their biological activities. This paper reports the isolation of GAGs from the porcine testis and their immunomodulating and anticoagulant activity.

Correspondence to: Kyung Bok Lee, Department of Biochemistry, College of Medicine, Konyang University, Nonsan, Chungnam 320-711, Korea
E-mail: kyunglee@kytis.konyang.ac.kr

MATERILAS AND METHODS

Reagents

Porcine testes were obtained from Nokwon Meat Products Co. (Nonsan, Chungnam). Activated partial thromboplastin time (aPTT) reagent, thrombin time (TT) reagent, Dowex macropolous resin, protease (EC 3.4.24.31, *Streptomyces griseus*), chondroitinase ABC lyase (*Proteus vulgaris*), chondroitinase AC lyase (*Arthrobacter aurescens*), keratanase (*Pseudomonas sp*), and 1,9 Dimethylmethylene blue (DMMB) were purchased from Sigma (St. Louis, MO, USA). RPMI medium was purchased from GIBCO BRL (Grand Island, NY. USA). Coagulator (Amelung KC1A) was purchased from Sigma (St. Louis, MO).

Preparation of GAGs

The GAG was prepared by the following procedure (Papakonstantinou *et al*, 1998b). Wet porcine testes were cut into fine pieces using a surgical blade then freeze-dried. Freeze-dried sample (20 g) was defatted with 160 ml of 1:2 mixture (chloroform/methanol) for 2 h at room temperature. Samples were centrifuged at 3,000× g for 10 min. The pellets were taken and dissolved in 15 ml of ethanol then centrifuged at 3,000× g for 10 min. The resulting pellets were dissolved in 10 ml of 100 mM Tris-HCl (pH 8.0) containing 1 mM $CaCl_2$ and 1 ml of 30 mg/ml protease (*Streptomyces griseus*) was added. Digestion was performed for 72 h at 37°C. Then proteins were precipitated with 10% trichloroacetic acid. The precipitate was removed and the supernatant was treated with 0.05 M NaOH containing 1 M sodium borohydride at 45°C for 12 h. The reaction was terminated by the careful addition of 1 M acetic acid. Four volumes of ethanol containing of 0.1 volume of 3 M sodium acetate was added and maintained at -20°C overnight. The precipitate was recovered with centrifugation at 2000× g for 20 min. Uronic acid was estimated by the modified carbazole assay (Kosakai and Yosizawa, 1979) and protein content was determined by the method of Bradford (Bradford, 1976).

Fractionation of GAGs by anion exchange chromatography

The sample was dissolved in 2 ml of water and applied onto a column of Dowex Macropolous Resin MSA-1 (1.5 × 13 cm). After the column was washed with 40 ml water, stepwise elution was performed with 40 ml each of 1.5% NaCl and 16% NaCl . The effluents were collected in 2 ml fractions. GAG-containing fractions were identified by colorimetric determination of uronic acids, pooled and lyophilized. The fractionated GAGs were analyzed by agarose gel electrophoresis, as described previously (Wu *et al.*, 2000). Briefly, 100 μg of samples were applied to a 1% gel in TBE buffer (0.045 M Tris-borate, 0.001 M EDTA) and run at 80 V for 1 hr. The gel was visualized with 0.1% alcian blue (w/v) in 0.1% acetic acid (v/v).

Determination of GAG using DMMB assay

The DMMB dye binding assay for assessment of sulfated GAGs (chondroitin sulfate and keratin sulfate) was performed by a method described previously (Farndale *et al.*, 1986; Palmer *et al.*, 1995) with some modifications. The absorbance of the DMMB-GAG complexs was measured at 525 nm using a spectrophotometer (Beckmann Industries, Inc., Model 650, Irving, CA). The concentration of chondroitin sulfate and keratan sulfate was determined from the difference in absorbance after the addition of chondroitinase ABC lyase and keratanase.

Determination of hyaluronic acid

The concentration of hyaluronic acid in PT-GAGs was measured by a Hyaluronic Acid Determination Kit (READS Medical Products, Inc., USA) according to a manufacturers suggestion.

Determination of anti-coagulant activity

Activated partial thromboplastin time (APTT) assay: APTT test was performed on a Amelung KC 1A micro coagulation analyzer (St. Louis, MO, USA). $CaCl_2$ solution (35 mM) and aPTT reagent were pre-incubated at 37°C. Sample (50 ml) and human plasma (100 ml) were mixed well and 50 ml of above mixture was taken into bottom of cuvette and incubated for 2 min. APTT reagent (100 ml) was added and incubated for 3 min then 100 ml of 20 mM $CaCl_2$ was added and the clotting time was measured (Lee *et al.*1999; Song *et al.*, 1998).

TT assay: TT reagent and fibrinogen reference reagent were preincubated at 37°C. Sample 20 μl and TT reagent 30 μl were mixed well and incubated for 2 min. Fibrinogen reference 100 μl was added and the clotting time was measured.

Collection of peritoneal macrophages and splenocytes

BALB/c mice (6-8 weeks old) were injected intraperitoneally with 1 ml of 2% thioglycollate (Difco, USA) and peritoneal exudative cells were harvested 4 days after injection by the method described previously (Watanabe *et al.*, 1999). Briefly, the cells suspended in 0.5 ml of RPMI-1640 containing 5% FBS were plated into 96-well or 48-well tissue culture plate. After 2 hr-incubation, adherent macrophages (viability >98%) were obtained by washing the plate with PBS to remove nonadherent cells. Splenocytes were aseptically isolated from the spleen of BALB/c mice.

Cell proliferation assay

Splenocytes and peritoneal macrophages were suspended in RPMI-1640 complete media containing 5% FBS, 2 mM L-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 15 mM HEPES, and 50 μM 2-mercaptoethanol. Splenocytes (5×10^5/100 ml/well) were plated into 96-well culture plate and cultured in a humidified 5% CO_2 incubator at 37°C for 48 hr in the presence of PT-GAG-1.5 or PT-GAG-16 (50 μg/ml) with or without either 10 μg/ml of lipopolysaccharide (LPS) or 10 μg/ml of concanavallin A (Con A). Peritoneal macrophages (2×10^5/100 μl/well) were plated into 96-well plate and co-incubated with PT-GAG-1.5 or PT-GAG-16 (50 μg/ml) for 48 hr. The proliferation of splenocytes and macrophages was measured spectrometrically by using Cell Counting Kit (Dojindo, Japan) under the manufacturers instruction.

Cytokine production from peritoneal macrophages

Peritoneal macrophages (5×10^5/500 μl/well) were co-incubated with 50 μg/ml of PT-GAG-1.5 or PT-GAG-16 in 48-well plate for 48 hr. The concentration of various cytokines such as IL-1β, IFN-γ and TNF-α in the culture supernatants was measured by ELISA kits (Endogen, Cambridge, MA).

Statistical analysis

The statistical significance of difference between groups was determined by applying Student's two-tailed *t* test.

RESULTS AND DISCUSSION

Preparation of PT-GAGs

The GAG component of the porcine testis was isolated by proteolysis of defatted tissue and fractionated by Dowex Macropolous ion-exchange chromatography. As shown in Fig. 1, minor GAG component (PT-GAG-1.5) was eluted with 1.5% NaCl and major GAG component (PT-GAG-16) was eluted as unsymmetrical peak with 16% NaCl. Uronic acid content of fractionated GAG was determined by carbazole assay in order to estimate efficiency of isolation process and the yield is presented in Table 1. The GAGs eluted from the Dowex Macropolous Resin MSA-1 with 1.5% NaCl and 16% NaCl were found equivalent to 33.0 μg and 309.2 μg of uronic acids per g dry tissue, respectively (Table 1). GAG proportion of each fractionated GAG was determined by DMMB dye binding assay (Table 2). PT-GAG-1.5 and PT-GAG-16 contained 274.8 and 484.2 μg/mg of chondroitin sulfate (CS), respectively. PT-GAG 16, but not PT-GAG-1.5, contained 248.1 μg/mg of keratan sulfate (KS). The total amount of sulfated GAG minus both CS and KS was attributed to non-chondroitin sulfate GAG /non-keratan sulfate GAG. Hyaluronic acid was found in both PT-GAG-1.5 and PT-GAG-16 at the concentration of 4.0 and 5.9 μg/mg, respectively. When the fractionated GAGs were subjected to agarose gel electrophoresis, they were visualized with 0.1% alcian blue (data not shown).

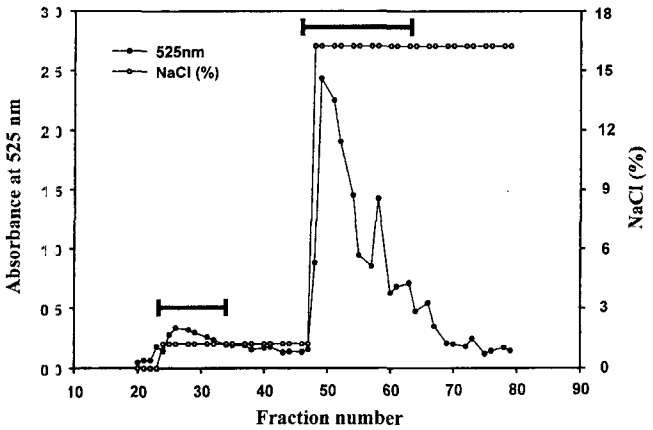

Fig. 1. Purification of GAG derived from porcine testis by Dowex macropolous anion-exchange chromatography. After the column was washed with water, stepwise elution was performed with 1.5% NaCl and 16% NaCl. The effluents were collected in 2 ml fractions and monitored by uronic acid assay (filled circles). The NaCl concentration applied to the column is indicated by the open circles.

Regulatory effect of PT-GAGs on the function of immune-related cells

In order to investigate the activity of PT-GAGs to regulate the function of immune-related cells, we first examined the effect of PT-GAG-1.5 and PT-GAG-16 on the growth of splenocytes and peritoneal macrophages. As depicted in Fig. 2-A, both of PT-GAGs did not affect the growth of normal spleen cells as well as mitogen-stimulated cells. On the other hand, in experiment for the effect on other immune-related cells, PT-GAG-1.5 significantly enhanced the proliferation of peritoneal macrophages (Fig. 2-B), indicating that PT-GAG-1.5 up-regulates macrophage function. In contrast, PT-GAG-16 eluted with higher NaCl concentration than PT-GAG-1.5 had no effect on the proliferation of macrophages.

It is well recognized that macrophage is multi-functional mediator to regulate various immunological functions such as antigen-presentation to T cells, phagocytosis of microorganisms and killing of tumor cells, and cytokines such as IL-1β, IFN-γ and TNF-α secreted from activated ma-

Table 1. Purification of glycosaminoglycans from porcine testis

Elution from the Dowex column	Yield[a]	Protein content[b] (mg/g of dried tissue)	Uronic acid content[c] (mg/g of dried tissue)
1.5% NaCl fraction	4.4 mg	4.4	33.0
16% NaCl fraction	36 mg	20.5	309.2

[a]average yield based on three different preparations; expressed as the weight of elutions isolated from 20 g of freeze-dried tissue
[b]measured by the Bradford method as described in Materials and Methods
[c]measured by the carbazole method as described in Materials and Methods

Table 2. Composition of GAG preparations from porcine testis

	Samples	
	PT-GAG-1.5 (μg/mg)[a]	PT-GAG-16 (μg/mg)
Total GAG[b]	279.6	854.3
CS[c]	274.8	484.2
KS[c]	0	248.1
HA[d]	4.0	5.9

[a]Values represent average based on three experiments.
[b]Total GAG was measured by DMMB dye assay.
[c]The concentration of chondroitin sulfate and keratan sulfate were analyzed in each sample following sequential chondrotinase ABC lyase or keratanase digestion of the sample.
[d]The amount of hyalurnoic acid was determined by a commercial kit.

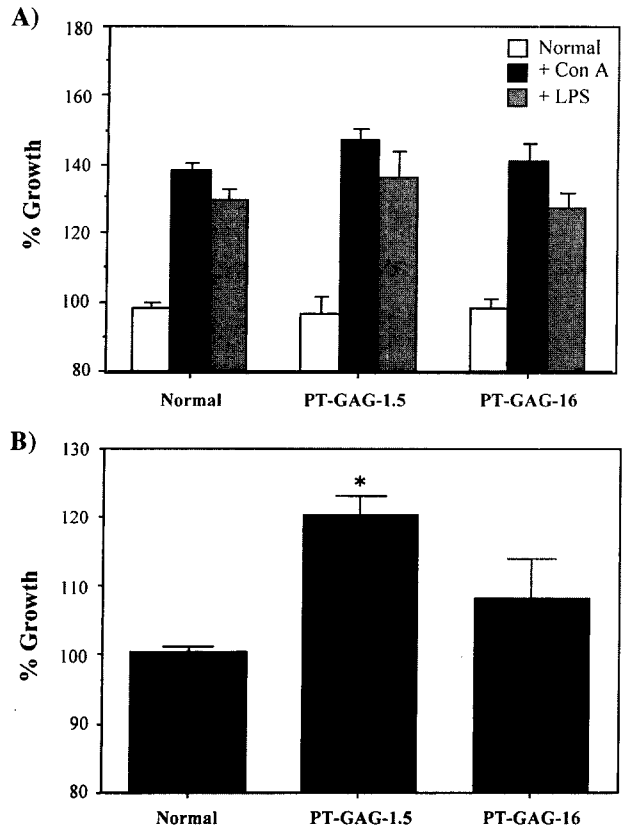

Fig. 2. Effect of PT-GAGs on the proliferation of murine splenocytes and peritoneal macrophages. (A) Splenocytes were co-incubated with PT-GAGs (50 mg/ml) in the presence or absence of the indicated mitogens for 48 hr. (B) Peritoneal macrophages were treated with PT-GAGs (50 mg/ml) for 48 hr. After incubation, the proliferation of these cells was measured using Cell Counting Kit. *p<0.05, compared with untreated control (by Student's two-tailed *t* test).

crophages play important roles in the immuno-modulating actions of the cells. So, these cytokines are thought to be hallmarks of activated macrophages. Since PT-GAG-1.5 was shown to augment the proliferation of peritoneal macrophages, we next tried to address if the enhanced proliferation of macrophages by PT-GAG-1.5 was associated with potential induction of cytokines. The activity of PT-GAG-1.5 to induce cytokines from macrophages was assayed by quantifying the level of IL-1β, IFN-γ and TNF-α in culture supernatants of peritoneal macrophages treated with this GAG. When peritoneal macrophages were co-incubated with or without PT-GAGs, the cells treated with PT-GAG-1.5 exhibited the higher level of three types of cytokines than that of the untreated cells (Fig. 3). However, as expected, PT-GAG-16 failed to induce cytokine production from macrophages. This suggests that PT-GAG-1.5 is a potent immunomodulator to stimulate macrophages to secrete cytokines.

Anticoagulant activity of PT-GAGs

The anticoagulant activity of PT-GAGs was examined

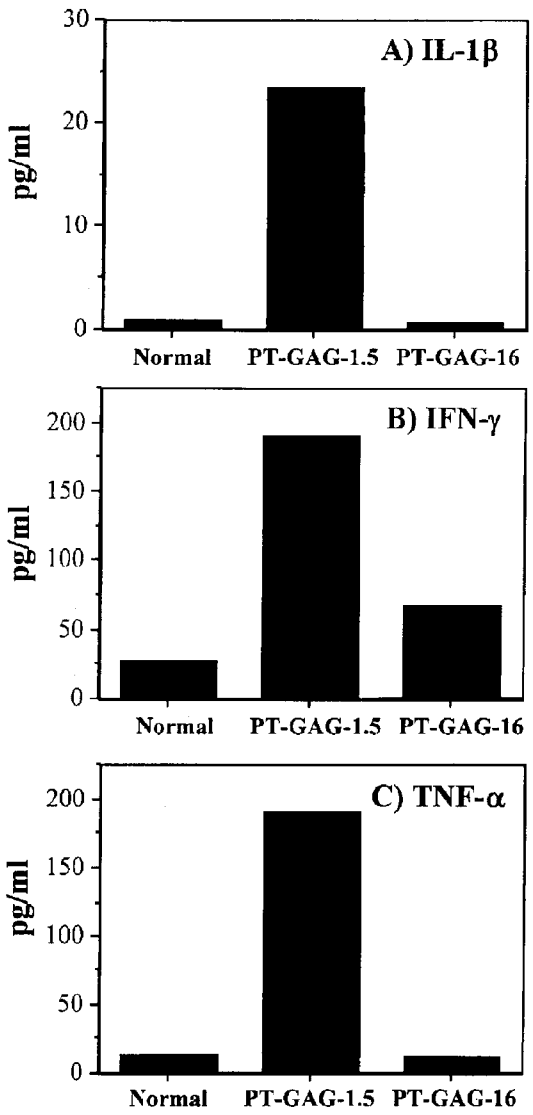

Fig. 3. Effect of PT-GAG-1.5 on the production of cytokines from macrophages. Peritoneal macrophages were treated with PT-GAGs for 48 hr, and the supernatants of the cultures were used for cytokine detection. The data is the representative of three different experiments.

by aPTT and TT assay. As shown in Fig. 4 and Fig. 5, both of PT-GAG-1.5 and PT-GAG-16 elicited prolongation in clotting time in a dose-dependent manner. In aPTT and TT analysis, PT-GAG-1.5 and PT-GAG-16 at the concentration of at 50 μg/tube displayed approximately 1.5- and 2.5-times prolongation effect on blood coagulation, respectively (Fig. 4). Interestingly, PT-GAG-16 which had no effect on immune-related cells showed higher anticoagulant activity than that of PT-GAG-1.5.

The present study demonstrates that the porcine testis contains at least two biologically active glycosaminoglycans, PT-GAG-1.5 and PT-GAG-16, and that these GAGs may be promising candidates for the development of immunomodulator or anticoagulant. Further study on active

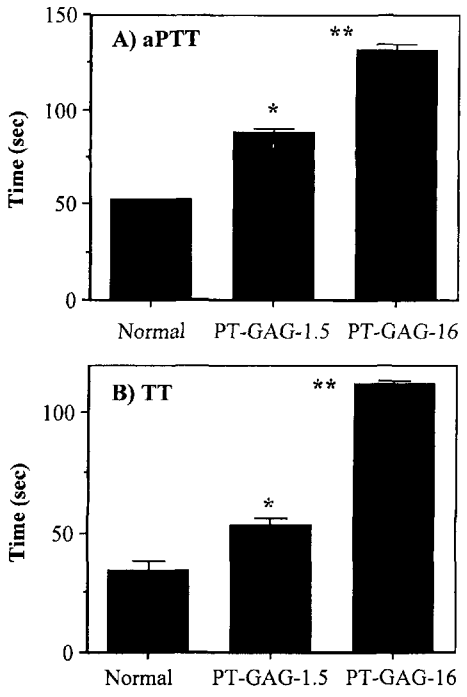

Fig. 4. Anticoagulant activity of PT-GAGs. The clotting times were measured by aPTT (A) and TT assay (B) as described in materials and Methods. *p<0.05; **p<0.01, compared with untreated control (by Student's two-tailed *t* test).

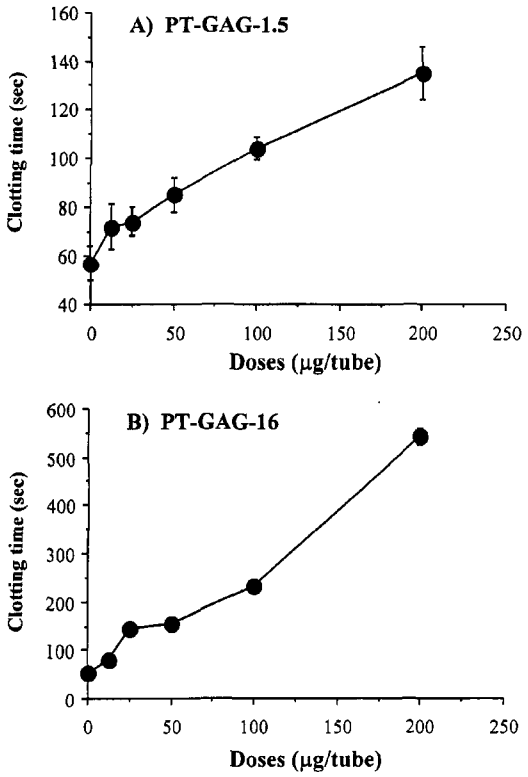

Fig. 5. Dose-dependency of anticoagulant activity of PT-GAGs. The clotting times were measured by aPTT assay as decribed in Fig. 4.

molecules and biochemical mechanisms related to biological activities of PT-GAGs is underway in our laboratory.

ACKNOWLEDGEMENTS

This work was supported by the Grant for University-Enterprise Joint Research Program of Chungnam Province in Korea. We are very thanks Mr. M. H. Yoon, Nokwon Meat Products Co. for his kind gift of porcine testis and skillful assistance.

REFERENCES

Bradford M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 72, 248-254 (1976)

Farndale, R. W., Buttle, D. J. and Barrett A. J., Improved quantitation and discrimination of sulphated glycosaminoiglycans by use of dimethylmethylene blue *Biochemica et Biophysica Acta*, 883, 173-177 (1986)

Galliher, P. M., Cooney, C. L, Langer, P. and Linhardt, R. J., Heparinase production by *Flavobacterium heparinum*. *Appl. Environ. Microbiol.*, 41, 360-365 (1981)

Goulas A., Papakonstantinou, E., Karakiulakis, G., Mirtsou, Fridani, V., Kalinderis, A., and Hatzichristou, D. G., Tissue structure-specific distribution of glycos- aminoglycans in the human penis *Int. J. Biochem. Cell Biol.*, 32, 975-982 (2000)

Kosakai M., and Yosizawa Z., A partial modification of the carbazole method of Bitter and Muir for quantitation of hexuronic acids. *Anal. Biochem.*, 93, 295-298. (1979)

Lee, H. J. Kim, J. H., Lee, C. H., Kim, J. S., Kwak, S. T., Lee, K. B., Song, K. S., Choi, B. W., and Lee, B. H., Inhibitory activities of sea weeds on prolyl endopeptidase, tyrosinase and coagulation. *Kor. J. Pharmacogn.*, 30, 231-237 (1999).

Lee, K. B., Kim, J. S., Kwak, S. T., Sim, W. B., Kwak, J. H., and Kim, Y. S., Isolation and identification of chondroitin sulfates from the mud snail. *Arch. Pharm. Res.*, 21, 555-558 (1998)

Micahel H. R., Kynn, J. R., and Gordon, I. K., *Histology; A text and Atlas*. Williams &Wilkins, Baltimore (1995)

Palmer, J. L., Bertone A. L. and McClain H. Assessment of glycosaminoglycan concentration in equine synovial fluid as a marker of joint disease. *Can. J. Vet. Res.*, 59, 205-212 (1995)

Papakonstaninou, E., Karakiulakis, G., Eickelberg, O., Perruchoud, A. P., Block, L. H. and Roth, M., A 340 KDa hyaluronic acid secreted by human vascular smooth muscle cells regulates the proliferation and migration. *Glycobiol.*, 8, 821-830 (1998a)

Papakonstaninou, E., Roth, M., Block, L. H. Mirtsou-Fidani, V., Argiriadis, P., and Karakiulakis, G., The differential distribution of hyaluronic acid in the layers of human atheromatic aortas is associated with vascular smooth muscle cell proliferation and migration. *Atherosclerosis*, 138, 79-89

(1998b)

Perin , J. P., Alliel, P. M., Jolles P. and Bonnet, F., Proteoglycans in male product tract. *EXS*, 70, 191-197 (1994)

Rogerson, S. J., Chaiyaroj, S. C., Reeder, J. C. and Brown, G. V., Chondroitin sulfate A is a cell surface receptor for *Plasmodium falciparum* infected erythrocytes. *J. Exp. Med.*, 182, 15-20. (1995)

Silbert, J. E. and Sugumaran, G., Intracellular membranes in the synthesis, transport, and metabolism of proteoglycans. *Biochim. Biophys. Acta*, 1241, 371-384 (1995).

Watanabe, R., Yoo, Y.C., Hata, K., Mitobe, M., Koike. Y., Nishizawa, M., Garcia, D.M., Nobuchi, Y., Imagawa, H., Yamada, H. and Azuma, I., Inhibitory effect of trehalose dimycolate (TDM) and its stereoisometric derivatives, trehalose dicorynomycolates (TDCMs), with low toxicity on lung metastasis of tumor cells in mice. *Vaccine*, 17, 1484-1492 (1999)

Wu, S. J., Chun, M. W., Shin, K. H., Toida, T., Park, Y., Linhardt, R. J., and Kim, Y.S., Chemical sulfonation and anticoagulant activity of acharan sulfate. Thromb. Res., 92, 273-281 (1998)