CYTOKINE GENE EXPRESSION AND PRODUCTION BY HUMAN LPS-STIMULATED MONONUCLEAR CELLS ARE INHIBITED BY SULFATED

Journal of Thrombosis and Haemostasis, **2**: 1657–1662

ORIGINAL ARTICLE

Cytokine gene expression and production by human LPS-stimulated mononuclear cells are inhibited by sulfated heparin-like semi-synthetic derivatives

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To cite this article: Gori AM, Attanasio M, Gazzini A, Rossi L, Lucarini L, Miletti S, Chini J, Manoni M, Abbate R, Gensini GF. Cytokine gene expression and production by human LPS-stimulated mononuclear cells are inhibited by sulfated heparin-like semi-synthetic derivatives. *J Thromb Haemost* 2004; **2**: 1657–62.

Summary. *Background*: The K5 polysaccharide obtained from *Escherichia coli* strain 010:K5:H4 is a polymer of the disaccharidic unit formed by D-glucuronic acid and N-acetylglucosamine. This structure is akin to N-acetylheparosan, the precursory polymer of heparin and of heparan sulfate. This structural affinity with N-acetylated heparin and with de-sulfated heparin makes the K5 polysaccharide extremely useful for the preparation of sulfated heparin-like semi-synthetic derivatives. It has been demonstrated that heparins are able to inhibit tissue factor and cytokine production and expression by human monocytes. *Objective*: The aim of this study was to evaluate the effects of four different heparin-like semi-synthetic derivatives on inflammatory cytokine production and expression by human mononuclear cells. *Results*: The simultaneous addition of lipopolysaccharide (LPS; 0.2 and 10 μg mL^{-1}) and the K5 polysaccharide did not inhibit interleukin (IL)-1β, IL-6 or tumor necrosis factor (TNF)-α production by stimulated mononuclear cells. IL-1β, IL-6 and TNF-α concentrations in supernatants of LPS-stimulated mononuclear cells were not influenced by the addition of N,O-sulfated K5 polysaccharide (K5-N, OS) and epimerized N-sulfated K5 polysaccharide (K5 NS epi) at 5 and 10 μg mL^{-1}, whereas the addition of epimerized N,O-sulfated K5 polysaccharide (K5-N, OS epi) (5 and 10 μg mL^{-1}) and O-sulfated K5 polysaccharide (K5-OS) (5 and 10 μg mL^{-1}) to LPS-stimulated cells caused a significant dose-dependent inhibition of IL-1β, IL-6 and TNF-α. All sulfated heparin-like semi-synthetic derivatives did not influence the IL-10 production by LPS-stimulated mononuclear cells. In LPS-stimulated cells (0.2 and 10 μg mL^{-1}), K5-OS or K5-N, OS epi at 5 and 10 μg mL^{-1} markedly decreased TNF-α mRNA expression. *Conclusions*: These results indicate that the sulfated heparin-like semi-synthetic derivatives K5-OS and K5-N, OS epi are able to inhibit both expression and production of inflammatory cytokines, whereas they do not influence the anti-inflammatory cytokine IL-10, suggesting a potential role for these products as modulators of inflammatory reactions.

Keywords: heparin derivatives, interleukin 10, proinflammatory cytokines, TNF-alpha mRNA expression.

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Received 9 November 2003, accepted 24 March 2004

Introduction

Heparin, a highly sulfated proteoglycan, has several biological actions independent of its well-known anticoagulant activity, including the ability to modulate extracellular matrix synthesis, cellular proliferation, angiogenesis, and particularly inflammation [1–6]. Mononuclear phagocytes and neutrophils are actively involved in inflammatory processes and synthesize and release a number of cytokines such as proinflammatory interleukins and, therefore, modulate the immune response through both T and B lymphocytes and the activation of accessory cells [7]. Lipopolysaccharide (LPS), a major component of the outer surface of Gram-negative bacteria, is a potent modulator of the host immune response and exhibits a variety of biological effects [8, 9]. A short exposure to LPS is sufficient to activate monocytes and macrophages to synthesize and release cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, and IL-8, which may induce an inflammatory state [10]. Interestingly, heparins have been shown to inhibit proinflammatory cytokine production by LPS- or interferon-γ-stimulated human monocytes even when heparin is added after stimulation [11] and actively reduce the

Table 1 Effects of different heparin-like semi-synthetic derivatives on cytokine production (ng per 10^7 cells) by lipopolysaccharide (LPS) (0.2 μg mL^{-1})-stimulated mononuclear cells

	IL-1β production	IL-6 production	TNF-α production
LPS (0.2 μg mL^{-1}) + RPMI medium	0.93 ± 0.35	1.09 ± 0.53	0.99 ± 0.98
LPS (0.2 μg/ml) + K5 10 μg mL^{-1}	0.92 ± 0.35	1.08 ± 0.54	0.97 ± 0.96
LPS (10 μg mL^{-1}) + K5-OS (5 μg mL^{-1})	0.69 ± 0.29*	0.98 ± 0.50*	0.81 ± 0.89*
LPS (0.2 μg mL^{-1}) + K5-OS (10 μg mL^{-1})	0.60 ± 0.29**	0.69 ± 0.44**	0.61 ± 0.38**
LPS (0.2 μg mL^{-1}) + K5-N, OS (5 μg mL^{-1})	0.92 ± 0.36	1.07 ± 0.53	0.98 ± 0.97
LPS (0.2 μg mL^{-1}) + K5-N, OS (10 μg mL^{-1})	0.91 ± 0.34	1.06 ± 0.18	0.98 ± 0.98
LPS (0.2 μg mL^{-1}) + K5-NS Epi (5 μg mL^{-1})	0.92 ± 0.36	1.08 ± 0.53	0.98 ± 0.95
LPS (0.2 μg mL^{-1}) + K5-NS Epi (10 μg mL^{-1})	0.90 ± 0.33	1.07 ± 0.53	0.99 ± 0.98
LPS (0.2 μg mL^{-1}) + K5-N, OS Epi (5 μg mL^{-1})	0.71 ± 0.28*	0.85 ± 0.42§	0.72 ± 0.65*
LPS (0.2 μg mL^{-1}) + K5-N, OS Epi (10 μg mL^{-1})	0.56 ± 0.29**	0.61 ± 0.29**	0.53 ± 0.66**

*$P < 0.05$ vs. LPS; §$P < 0.01$ vs. LPS; **$P < 0.001$ vs. LPS.

Table 2 Effects of different heparin-like semi-synthetic derivatives on cytokine production (ng per 10^7 cells) by lipopolysaccharide (LPS) (10 μg mL^{-1})-stimulated mononuclear cells

	IL-1β production	IL-6 production	TNF-α production
LPS (10 μg mL^{-1}) + RPMI medium	5.61 ± 1.77	9.61 ± 4.35	7.88 ± 4.69
LPS (10 μg mL^{-1}) + K5 10 μg mL^{-1}	5.59 ± 1.57	9.51 ± 4.34	8.10 ± 4.84
LPS (10 μg mL^{-1}) + K5-OS (5 μg mL^{-1})	4.37 ± 1.39*	8.82 ± 4.01*	6.12 ± 3.68*
LPS (10 μg mL^{-1}) + K5-OS (10 μg mL^{-1})	3.61 ± 1.29**	6.41 ± 3.46**	4.96 ± 2.90**
LPS (10 μg mL^{-1}) + K5-N, OS (5 μg mL^{-1})	5.68 ± 1.74	9.62 ± 4.46	8.07 ± 4.64
LPS (10 μg mL^{-1}) + K5-N, OS (10 μg mL^{-1})	5.69 ± 1.62	9.17 ± 3.97	7.78 ± 4.74
LPS (10 μg mL^{-1}) + K5-NS Epi (5 μg mL^{-1})	5.63 ± 1.77	9.68 ± 4.21	7.79 ± 4.61
LPS (10 μg mL^{-1}) + K5-NS Epi (10 μg mL^{-1})	5.71 ± 2.15	9.42 ± 4.19	7.85 ± 4.57
LPS (10 μg mL^{-1}) + K5-N, OS Epi (5 μg mL^{-1})	4.24 ± 1.39*	7.60 ± 3.52§	6.10 ± 3.13*
LPS (10 μg mL^{-1}) + K5-N, OS Epi (10 μg mL^{-1})	3.41 ± 1.51**	5.62 ± 2.59**	4.91 ± 3.35**

*$P < 0.05$ vs. LPS; §$P < 0.01$ vs. LPS; **$P < 0.001$ vs. LPS.

of 10 μg mL^{-1}: 36.92 ± 11.9% for IL-1β, 38.75 ± 14.61% for IL-6, and 36.30 ± 17.1% for TNF-α. The inhibitory effect on IL-1β, IL-6 and TNF-α of K5-N, OS epi at 5 μg mL^{-1} was 23.5 ± 14.7% for IL-1β, 22.61 ± 9.7% for IL-6 and 23.75 ± 13.23% for TNF-α. K5-N, OS epi at 10 μg mL^{-1} inhibited 41.3 ± 16.80% of IL-1β, 43.36 ± 12.37 % of IL-6 and 42.50 ± 18.50 % of TNF-α production.

The simultaneous addition of LPS 10 μg mL^{-1} and the K5 polysaccharide (starting material) (10 μg mL^{-1}), K5-N, OS and K5-NS epi at 5 and 10 μg mL^{-1} to cellular suspensions did not cause any significant inhibition of IL-1β, IL-6 or TNF-α production (Table 2), whereas the addition of K5-OS (5 and 10 μg mL^{-1}) and K5-N, OS epi (5 and 10 μg mL^{-1}) to LPS-stimulated cells (10 μg mL^{-1}) caused a significant ($P < 0.05$) decrease of IL-1β, IL-6 and TNF-α production (Table 2).

The addition to cellular suspensions of all sulfated heparin-like semi-synthetic derivatives did not cause a significant inhibition of the production of the anti-inflammatory cytokine IL-10 by LPS-stimulated mononuclear cells (0.2 μg mL^{-1}). Mononuclear cells stimulated by 0.2 μg mL^{-1} of LPS produced 16.1 ± 8.1 pg mL^{-1} of IL-10. LPS (0.2 μg mL^{-1})-stimulated cells, treated with K5-N,OS, or K5-N OS epi, or K5-OS or K5-NS epi, at 10 μg mL^{-1} produced 16.5 ± 8.3, 16.3 ± 8.3, 16.7 ± 9.5, 16.8 ± 8.9 pg mL^{-1} of IL-10 respectively. Mononuclear cells stimulated with 10 μg mL^{-1} of LPS produced 96.2 ± 63.0 pg mL^{-1} of IL-10. The simultaneous addition of the heparin derivatives at 10 μg mL^{-1} to LPS (10 μg mL^{-1})-stimulated mononuclear cells did not significantly influence IL-10 production (K5-N,OS 99.1 ± 64.9; K5-N OS epi 98.7 ± 63.8; K5-OS 97.9 ± 66.2; or K5-NS epi 98.7 ± 63.8 pg mL^{-1}).

The addition of either K5-OS or K5-N, OS epi at 5 and 10 μg mL^{-1} in LPS (0.2 μg mL^{-1})-stimulated mononuclear cells resulted in a dose-dependent inhibition of TNF-α mRNA expression: K5-OS at 5 and 10 μg mL^{-1} inhibited 40.9% (from 10.0% to 74.0%) and 58.8 % (from 12.0% to 86.0%) of TNF-α mRNA expression, respectively. K5-N, OS epi at 5 and 10 μg mL^{-1} inhibited 68.0% (from 10.0% to 100.0%), and 94.3 % (from 46.0% to 100.0%) of TNF-α mRNA expression. Similar results were obtained when mononuclear cells were stimulated with 10 μg mL^{-1} of LPS: 5 μg mL^{-1} K5-OS inhibited 38.1% (from 10.0% to 68.0%), 10 μg mL^{-1} K5-OS 64.2% (from 12.0% to 88.0%), 5 μg mL^{-1} K5-N, OS epi 63.0% (from 9.0% to 100.0%), and 10 μg mL^{-1} K5-N, OS epi 92.3 % (from 46.0% to 100.0%) of TNF-α mRNA expression by LPS (10 μg $m:^{-1}$)-stimulated cells.

No inhibition of TNF-α mRNA expression was observed when the other sulfated polysaccharide derivatives K5-N,OS and K5-NS epi at 5 and 10 μg mL^{-1} were added after 3 h stimulation (data not shown).

Discussion

Our results demonstrate that sulfated heparin-like semi-synthetic derivatives and in particular the K5-OS and K5-N, OS epi are able to inhibit in a dose-dependent manner both expression and production of inflammatory cytokines, whereas they do not influence anti-inflammatory IL-10 production by LPS-stimulated human mononuclear cells, suggesting a potential role for these compounds as anti-inflammatory agents.

During immune and inflammatory processes the proinflammatory cytokines IL-1β, IL-6 and TNF-α increase many times their levels in the circulation or locally. The synthesis of several proinflammatory cytokines is inhibited by the anti-inflammatory cytokine IL-10, which is an important immunoregulatory cytokine produced by B and T lymphocytes and monocytes and macrophages [22].

Heparin is a glycosaminoglycan composed of alterning D-glucosamine and uronic acid (L-iduronic or D-glucuronid acid) residues that are heterogeneous in size and degree of sulfation. It is well known that the anticoagulant properties of heparin depend on the presence of a specific pentasaccharide sequence with high affinity to antithrombin, which enhances its inhibitory action against serine protease. Heparin, in addition to its well-known anticoagulant properties, is endowed with inhibitory activities on proinflammatory cytokines, as demonstrated in *in-vitro* studies, in which cytokine production and expression by stimulated monocytes [11, 12] decreased in the presence of unfractionated heparin. After the development of synthetic oligosaccharides, *in-vitro* and *in-vivo* studies have documented that heparan sulphate and heparin derivatives are able to bind to and regulate the metabolism of several growth factors, as well as to L-, and P-selectin [4, 6, 15, 23, 24].

In our study, the inhibitory effect of two semi-synthetic O-sulfated heparin derivatives (K5-OS and K5-N, OS epi) on cytokine production and expression is likely to be specific, as this effect is associated with a particular chemical and enzymatic modification of these molecules. In fact, the chemical modifications of the starting material (K5 polysaccharide) in terms of N-acetylation and O-sulfation enable this molecule to inhibit cytokine production and expression differently from that observed with K5-N, OS and K5-NS epi molecules, which do not contain N-acetyl groups, suggesting that N-acetylation is required for anti-inflammatory activity of the O-sulfated molecules. These data are in accordance with different studies, in which N-acetyl heparin was found to specifically bind and inhibit growth factors as well as complement components [5,25,26].

Other structural modifications, regarding the degree of sulfation and the localization of the sulphate groups, ameliorate the capability of heparin derivatives to interact with cells involved in the inflammatory processes [26–29]. In a recent report, it was also demonstrated that the interaction of heparin with P- and L-selectins is critically related to the presence of the 6-O-sulphate group and in addition the 2-O,3-O desulfation of heparin generated a potent, non-anticoagulant, anti-inflammatory activity, suggesting that appropriate sulfation of heparins plays a critical role in selectin recognition and binding [23].

In our study, the N and O-sulfation of the starting material (K5), which determines the formation of K5-N, OS polysaccharide, seems not to influence its anti-inflammatory activities (K5-N, OS does not decrease cytokine production and expression). On the other hand, the enzymatic modification of K5-N, OS by C5-epimerase produces a conformational change in the molecule and enables its anti-inflammatory activity, suggesting that, in addition to N-acetylation, epimerization is also necessary for cytokine inhibition. The possible role of the heparin derivatives rich in sulfated and epimerized regions is underlined by a recent study in which it was found that the L- and P-binding fragments include a more heavily sulfated and epimerized region [30].

A direct effect on TNF-α mRNA and cytokine proteins and an interference of the heparin-like molecules with the experimental procedures used for cytokine determination and mRNA quantification can be ruled out, as the addition of these molecules at the end of incubation did not modify cytokine production and TNF-α mRNA expression.

As the mechanisms possibly responsible for cytokine inhibition are concerned, different aspects have to be considered. First, the ability of heparin molecules to bind electrostatically to cell membranes of different cells and to internalize into the cytosolic compartment causes the inhibition of proinflammatory nuclear transcription factor activation by preventing the translocation of NF-κB from the cytoplasm to the the nucleus. Second, inhibition at a transductional level should be considered. However, our experiments cannot rule out that heparin-like molecules affect the stability of mRNA.

In conclusion, we have demonstrated that specific structural modifications [chemical (N-acetylation) and enzymatic modification (C5 epimerization)] of O-sulfated heparin-like molecules enable the inhibition of proinflammatory cytokines, without affecting the anti-inflammatory cytokine IL-10, suggesting a potential role for these molecules as anti-inflammatory agents. Heparin is known to have inhibitory effects on multiple components of the inflammation cascade, including integrins, cytokines, neutrophil-derived elastases and complement activation, but its use in clinical practice as an anti-inflammatory drug is restricted by the potential for bleeding.

The heparin-like molecules developed for this study show anti-inflammatory activities and low anticoagulant activities, so encouraging continuation of the development of specific structural modification of K5 selectively sulfated K5 derivatives in order to obtain inhibitors that might interfere with inflammatory processes.

Contribution of authors

Study design: A.M.G., R.A., G.F.G.

Laboratory investigation:

Preparation of heparin-like semi-synthetic derivatives: J.C., S.M., M.M.

Isolation of mononuclear cells and detection of cytokine production and statistical analysis: A.M.G., A.G.

RNA extraction and TNF-α mRNA expression analysis: M.A., L.R., L.L.

Writing up: A.M.G., R.A., G.F.G.

References

1 Guyton Jr, Rosenberg RD, Clowes AW, Karnovsky MJ. Inhibition of rat arterial smooth muscle cell proliferation by heparin. *In vivo* studies with anticoagulant and nonanticoagulant heparin. *Circ Res* 1980; **46**: 625–34.

2 Folkman J. Regulation of angiogenesis: a new function of heparin. *Biochem Pharmacol* 1985; **34**: 905–9.

3 Au YPT, Montgomery KF, Clowes AW. Heparin inhibits collagenase gene expression mediated by phorbol ester-responsive element in primate arterial smooth muscle cells. *Circ Res* 1992; **70**: 1062–9.

4 Nelson RM, Cecconi O, Roberts WG, Aruffo A, Linhardt RJ, Bevilacqua MP. Heparin oligosaccharides bind L- and P-selectin and inhibit acute inflammation. *Blood* 1993; **82**: 3253–58.

5 Weiler JM, Edens RE, Linhardt RJ, Kapelanski DP. Heparin and modified heparin inhibit complement activation *in vivo*. *J Immunol* 1992; **148**: 3210–15.

6 Xie X, Rivier AS, Zakrzewicz A, Bernmoulin M, Zeng XL, Wessel HP, Schapira M, Spertini O. Inhibition of selectin-mediated cell adhesion and prevention of acute inflammation by nonanticoagulant sulfated saccharides. *J Biol Chem* 2000; **275**: 34818–25.

7 Yang KA, Hilli HR. Functional biology of the granulocyte monocyte series. In: Bick RL, ed. *Hematolgy: Clinical and Laboratory Practice*. St Louis: Mosby, 1993; 1077–92.

8 Cavaillon JM, Haeffner-Cavaillon N. Structure–function relationships to core oligosaccharide. In: Morrison DC, Ryan JL, eds. *Bacterial Endotoxic Lipopolysaccharide*. Boca Raton, FL: CRC, 1992.

9 Ingalls RR, Heine H, Lien E, Yoshimura A, Golenbock DT. Lipopolysaccharide recognition, CD14, and lipopolysaccharide receptors. *Bacterial Sepsis Septic Shock* 1999; **13**: 341–53.

10 Gallay P, Jongeneel CV, Barras C, Burnier M, Baumgartner JD, Glauser MP, Heumann D. Short time exposure to lipopolysaccharide is sufficient to activate human monocytes. *J Immunol* 1993; **150**: 5086–93.

11 Attanasio M, Gori AM, Giusti B, Pepe C, Comeglio P, Brunelli T, Prisco D, Abbate R, Gensini GF, Neri Serneri GG. Cytokine gene expression in human LPS- and IFNγ-stimulated mononuclear cells is inhibited by heparin. *Thromb Haemost* 1998; **79**: 959–62.

12 Myrvang-Hogasen AK, Abrahamsen TG. Heparin suppresses lipopolysaccharide-induced monocyte production of several cytokines, but simultaneously stimulates C3 production. *Thromb Res* 1995; **80**: 179–84.

13 Wang JG, Mu JS, Zhu HS, Geng JG. N-desulfated non-anticoagulant heparin inhibits leukocyte adhesion and transmigration *in vitro* and attenuates acute peritonitis and ischemia and reperfusion injury *in vivo*. *Inflamm Res* 2002; **51**: 435–43.

14 Kouretas PC, Kim YD, Cahill PA, Myers AK, To LN, Wang Y-N, Sitzmann JV, Hannan RL. Nonanticoagulant heparin prevents coronary endothelial dysfunction after brief ischemia-reperfusion injury in the dog. *Circulation* 1999; **99**: 1062–8.

15 Nelson RM, Cecconi O, Roberts WG, Aruffo A, Linhardt RJ, Bevilacqua MP. Heparin oligosaccharides bind L- and P-selectin and inhibit acute inflammation. *Blood* 1993; **82**: 3253–8.

16 Thourani VH, Brar SS, Kennedy TP, Thornto LR, Watts JA, Ronson RS, Zhao ZQ, Sturrock AL, Hoidal JR, Vinten-Johansen J. Nonanticoagulant heparin inhibits NF-kappaB activation and attenuates myocardial reperfusion injury. *Am J Physiol Heart Circ Physiol* 2000; **48**: H2084–H2093.

17 Anastase-Ravion S, Carreno MP, Blondin C, Ravion O, Champion J, Chaubet F, Haeffner-Cavaillon N, Letourneur D. Heparin-like polymers modulate proinflammatory cytokine production by lipopolysaccharide-stimulated human monocytes. *J Biomed Mater Res* 2002; **60**: 375–83.

18 Boyum A. Isolation of lymphocytes, granulocytes and macrophages. *Scand J Immunol* 1976; **5**: 9–15.

19 Manzoni M, Bergomi S, Cavazzoni V. Extracellular K5 polysaccharide of *Escherichia coli*: production and characterization. *J Bioactive Compat Polym* 1993; **8**: 251–7.

20 Casu B, Gennaro U. A conductimetric method for the determination of sulphate and carboxyl groups in heparin and other mucopolysaccharides. *Carbohyd Res* 1975; **39**: 168–76.

21 Chomczynski P, Sacchi N. Single step-method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Ann Biochem* 1987; **162**: 156–9.

22 Waal MR, Abrams J, Bennet B, Figdor CG, Vries JE. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med* 1991; **174**: 1209–20.

23 Wang L, Brown JR, Varki A, Esko JD. Heparin's anti-inflammatory effects require glucosamine 6 O-sulphation and are mediated by blockade of L- and P-selectins. *J Clin Invest* 2002; **110**: 127–36.

24 Tangelder GJ, Arfors KE. Inhibition of leukocyte rolling in venules by protamine and sulfated polysaccharides. *Blood* 1991; **7**: 1565–71.

25 Barzu T, Lormeau JC, Petitou M, Michelson S, Choay J. Heparin-derived oligosaccharides: affinity for acid fibroblast growth factor and effect on its growth-promoting activity for human endothelial cells. *J Cell Physiol* 1989; **140**: 538–48.

26 Baskin P, Doctrow S, Klagsbrun M, Svahn CM, Folkman J, Vlodavsky I. Basic fibroblast growth factor binds to subendothelial extracellular matrix and is released by heparinase and heparin-like molecules. *Biochemistry* 1989; **28**: 1737–43.

27 Matzner Y, Marx G, Drexler R, Eldor A. The inhibitory effect of heparin and related glycosaminoglycans on neutrophil chemotaxis. *Thromb Haemost* 1984; **52**: 134–7.

28 Castellot JJ Jr, Choay J, Lormeau JC, Petitou M, Sache E, Karnovsky MJ. Structural determinants of the capacity of heparin to inhibit the proliferation of vascular smooth muscle cells. Evidence for a pentasaccharide sequence that contains a 3-O sulphate group. *J Cell Biol* 1986; **102**: 1979–84.

29 Fryer A, Huang YC, Rao G, Jacoby D, Mancilla E, Whorton R, Piantadossi CA, Kemmedy T, Hoidal J. Selective O-desulfation produces nonanticoagulant heparin that retains pharmacological activity in the lung. *J Pharmacol Exp Ther* 1997; **282**: 208–19.

30 Koening A, Norgard-Sumnicht K, Linhardt R, Varki A. Differential interactions of heparin and heparan sulphate glycosaminoglycans with the selectins. Implications for the use of unfractionated and low molecular weight heparins as therapeutic agents. *J Clin Invest* 1997; **101**: 877–89.