Alteration in glycosaminoglycan metabolism and surface charge on human umbilical vein endothelial cells induced by cytokines, endotoxin and neutrophils

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Summary

There is increasing evidence that the glycosaminoglycan (GAG) component of the vascular endothelium is important in regulating vascular permeability, thromboresistance and cellular interactions. We have investigated the GAG metabolism of cultured human umbilical vein endothelial cells (HUVEC) in response to a range of inflammatory stimuli. Using both chemical measurement of cellular and supernatant GAGS and 35S labelling to identify newly synthesised GAGS, interleukin 1 (IL1), tumour necrosis factor (TNF) and interferon gamma (IFNy) were shown to influence sulphated GAG metabolism significantly. IL1 and TNF caused a marked increase in culture supernatant GAGS and a concomitant reduction in cell-associated GAGS. This was shown histochemically to be associated with a marked reduction and redistribution of endothelial surface anionic sites. The addition of neutrophils to HUVEC pretreated with Escherichia coli endotoxin, IL1 or TNF resulted in a further reduction in both cellular GAGS and surface anionic sites. These results suggest that changes in endothelial cell GAG metabolism during inflammation may contribute to the disturbance of vascular endothelial homeostasis associated with infectious and inflammatory states.

Key words: glycosaminoglycans, endothelium, inflammation, neutrophils, endotoxin, cytokines.

Introduction

The pathophysiology of serious sepsis is extremely complex. Three of the most important clinical features are capillary leakage, intravascular thrombosis and derangement of vascular tone, and this triad suggests that an alteration in the normal homeostatic properties of the vascular endothelium may be central to the pathogenesis of this condition (Tracey et al. 1986; Mercier et al. 1988; Levin, 1990).

Histochemical examination of vascular endothelial cells with cationic probes such as ruthenium red, Alcian blue and cationic ferritin, have shown the luminal surface to be highly negatively charged (Brenner et al. 1978; Brody et al. 1984; Kanwar, 1984; Rounds and Vaccaro, 1987). This property is largely attributed to the presence of the sulphated glycosaminoglycans (GAGS), heparan and dermatan sulphate, with a smaller contribution from sialoglycoproteins (Kanwar et al. 1981; Wight et al. 1986).

There is now evidence that these sulphated GAGS are important in maintaining vascular homeostasis by: (a) modulating antithrombotic and coagulant activity on the endothelial cell surface through specific interactions with antithrombin III and heparin cofactor II (Marcum and Rosenberg, 1984; Platt et al. 1990); (2) providing an electrostatic barrier to similarly charged cell surfaces, including those of platelets and red blood cells (Danon and Shutelsky, 1976; Springer, 1990); (3) binding important macromolecules such as lipoprotein lipase, fibroblast growth factor and superoxide dismutase to the cell surface (Gallagher et al. 1986; Karlsson and Marklund, 1988); and (4) regulating the vascular permeability of the endothelium to circulating molecules and particularly plasma proteins (Lindahl and Hook, 1978).

It is now well established that vascular permeability to macromolecules is dependent not only on molecular size, but also on electrostatic charge. Neutral proteins and dextrans are cleared more rapidly from the circulation than similarly sized anionic polymers, and the clearance of cationic proteins is greatly enhanced (Bennet et al. 1976; Bohrer et al. 1978). The importance of endothelial charge and GAGS in the regulation of vascular permeability has been demonstrated by enzymatic digestion of GAGS and their neutralisation by...
polycations, both of which increase the clearance of normally restricted molecules from the renal and systemic vasculature (Kelley and Cavallo, 1978; Kanwar et al. 1980; Vehaskari et al. 1984). Endothelial surface charge is particularly important in restricting albumin, the major protein responsible for plasma oncotic pressure, within the intravascular space. The clearance of albumin from the kidney is significantly less than that of similarly sized neutral molecules (Brenner et al. 1978; Kanwar, 1984) and indeed the fractional clearance of the cationic albumin exceeds that of native albumin by a factor of 300 (Purtell et al. 1979). Furthermore, experimental neutralisation of endothelial anionic sites, either in the glomerulus or in the systemic circulation, increases the clearance of native albumin to within the range of similarly sized neutral molecules (Hunsicker et al. 1981; Vehaskari et al. 1984). Patients with septic shock invariably have a profound capillary leak (Mercier et al. 1988), and may lose several times their circulating volume into the interstitial compartment. Albumin is the predominant protein lost and is therefore central to the process of capillary leakage of plasma.

We have postulated that alteration in endothelial cell surface charge and GAG composition might underly the increased vascular permeability in septic shock, and such an alteration would also be expected to influence the thromboresistant properties of the endothelium. In view of the emerging evidence that cytokines and neutrophils are important mediators of the tissue and organ damage seen in overwhelming sepsis, we have studied the effect of cytokines, endotoxin and neutrophils on endothelial cell GAG metabolism and surface charge.

Materials and methods

Reagents

HRF1L and IL6 (interleukin) were donated by Dr S. Gillis, Immunex Corporation, Seattle, WA. HrFNy (interferon) was obtained from Biogen, Geneva, Switzerland, and HrTNF (tumour necrosis-factor) supplied by Dr. A. Gearing, NIBSC, South Mimms, GB. Lipopolysaccharide B (LPS) Escherichia coli 0128:B12, heparan sulphate (HS) from bovine kidney, dermatan sulphate (DS) from porcine skin, chondroitin 4- and 6-sulphate (CS) from bovine trachea and shark cartilage, respectively, protease E, chondroitinase ABC, heparinase II and neuraminidase type X were purchased from Sigma, South Mimms, GB. Alcian blue 8GX was from Imperial Chemical Industries Ltd., Blackley, Manchester, UK.

Endothelial culture

Endothelial cells were obtained from human umbilical veins by digestion with 0.1% collagenase as previously described (Jaffe et al. 1973). Cells were cultured in Dulbecco’s minimal essential medium (Gibco, Paisley, Scotland) with L-glutamine (1.6 mM), penicillin (80 i.u./ml), streptomycin (80 mg/ml) and 20% fetal calf serum (Gibco). Cells were grown to confluence in 25 cm² flasks (Nunc, Denmark) and passed by exposure to EDTA 10 mM (BDH, Essex, England) either to gelatinised 13 mm glass coverslips or to 6-well culture plates (Nunc). Human umbilical vein endothelial cells (HUVEC) used in all the experiments were always from the second passage. Cells were verified as endothelial by morphology, the presence of Von Willebrand factor and prostacyclin production.

Preparation of neutrophils

Venous blood from adult donors was collected into 3.8% trisodium citrate, divided into aliquots and placed in test tubes containing mono-poly resolving medium (Flow Laboratories, Herts, England). After centrifugation at 300 g for 30 minutes, a discrete band of neutrophils could be easily located and pipetted free from contaminating blood constituents. The neutrophils were washed twice in Hanks’ buffered salt solution, counted and reconstituted in culture medium. Morphological assessment of neutrophil purity and viability were estimated to be >93% and >95%, respectively.

Glycosaminoglycan isolation

GAGS were isolated from both culture supernatants and endothelial cell monolayers. At specific times during the experiments (see below for details of sampling times) culture supernatants were removed and the monolayers washed twice with warm (37°C) Dulbecco’s phosphate buffered saline (PBS). The washings were added to the supernatants and then spun at 1200 g to remove any cellular debris. GAGS were released free from protein by incubation with Pronase (final concentration of 50 mg/ml) at 37°C for 20 hours. After centrifugation to remove any precipitate the supernatants were stored at −70°C so that an entire experiment could be simultaneously analysed. The cell layer was digested with Pronase (50 mg/ml) in PBS. After 5 minutes the cells had detached from the well and were counted in a haemocytometer. The cells were then digested for a further 20 hours at 37°C, spun at 1200 g and the supernatant was stored for further processing. To assess any degradation of GAGS during this processing, known quantities of GAG standards containing heparan, dermatan and chondroitin 4- and 6-sulphate were incubated with each new batch of Pronase and also with samples of culture supernatants and cells. These were treated identically to the test samples. GAGs were then isolated using a modification of the method of Whiteman (1973). All samples were incubated for 6 hours with 10 volumes of alcin blue 8GX solution (alcian blue, 0.05%, 50 mM sodium acetate buffer, pH 5.8 and 50 mM MgCl₂). The GAG/dye complex was isolated by centrifugation at 1000 g. After removal of the supernatant, the Alcian blue was dissociated from the GAGS with NaCl and methanol (final concentration 2.67 M and 33%, v/v) respectively, and the Alcian blue denatured with Na₂ CO₃ (final concentration 12.5 mM). The mixture was sonicated in a water bath sonicator for 2 minutes before removing the alcin blue by centrifugation at 10 000 g and precipitating the GAGS from the clear supernatant with 3 volumes of ethanol. After centrifugation to 10 000 g, the supernatant was removed, and the GAGs left to dry in air overnight. The sample was then dissolved in distilled water, spun at 10 000 g to remove any non-soluble material, before electrophoresis.

GAG characterisation

One-dimensional electrophoresis was performed by applying 0.7 μl of the GAG solution as a 3 mm band to a cellulose acetate sheet (Sartorius, Gottigen, W. Germany). After electrophoresis for 4 hours in 0.1 M barium acetate, pH 6.0, at 7.5 V/cm, the sheets were then developed in alcin blue solution for 30 minutes and destained in 5% acetic acid. The identity of the bands was established by comparison with the position of standards (HS, DS, CS), which were included in each run. To verify the identity of the bands, two-dimensional
Electrophoresis was undertaken after application of 1 μl of sample as a spot to a cellulose acetate sheet. Electrophoresis was performed first in pyridine/acetate buffer, pH 6.0, at 7.5 V/cm for 75 minutes and the cellulose acetate sheet was dried in air before electrophoresis at 90° to the first run in barium acetate buffer as described above. GAG standards were applied prior to the second run. Further confirmation of band identities was by electrophoresis of enzymatic digests of the sample with 0.5 unit/ml of chondroitinase ABC or with nitrous acid (made with equal volumes of 5% NaN₂O₂ and 33% acetic acid and then mixed 1:1 with the sample). Degraded GAGs did not appear on developed sheets.

**Enzyme specificity**

The specificities of the GAG-degrading enzymes were assessed by their activity on purified GAGs using cellulose acetate electrophoresis and by assaying their ability to inhibit the heparan sulphate stimulation of antithrombin III activity using an amidolytic assay system (Larson et al. 1978). Using cellulose acetate electrophoresis we found that the chondroitin and dermatan sulphate bands were both removed by chondroitinase ABC and that heparan sulphate was degraded by nitrous acid but not by heparinase II. This enzyme did, however, completely inhibit the heparan sulphate stimulation of antithrombin III activity assessed by using the amidolytic assay (data not shown).

**GAG quantitation**

Bands identified by reference to GAG standards were quantified using a laser densitometer (Pharmacia). Standard curves were constructed from the optical densities of band peaks, above background, from a range of known concentrations of GAG standards. Sample bands were then calculated as micrograms of heparan, dermatan or chondroitin sulphate.

Electrophoretic bands containing more than 0.5 microgram of GAG were too dense for densitometry. They were therefore cut out and dissolved in dimethylsulphoxide containing 0.5 g anhydrous sodium acetate, 1.27 g MgCl₂ and 1.56 ml acetic acid/250 ml as previously described (Vermelen et al. 1989) and their absorbance was read at 678 nm in a spectrophotometer (LKB). Standard curves were constructed from GAG standards of known concentrations, which were electrophoresed and treated as above. Exact quantities of individual GAGs could then be calculated from the sample bands.

**In vitro labelling of GAGS with ³⁵S**

To ascertain the newly synthesised component of both cellular and supernatant sulphated GAGs, 20 μCi and ³⁵S as H₂SO₄ (ICN, U.K., sp. activity 43 Ci/g SO₄) was added to culture medium and then extracted, electrophoresed and quantified. Recoveries were 58% ± 6% of CS, 56% ± 4% of DS and 24% ± 5% of HS (mean ± s.e.m. from 15 samples). Pronase digestion had no effect on these results, indicating that there was no detectable enzymic degradation of the GAGS during processing.

**GAG recovery**

To assess the efficiency of individual GAG extraction, GAG standards were added to culture medium and then extracted, electrophoresed and quantified. Recoveries were 58% ± 6% of CS, 56% ± 4% of DS and 24% ± 5% of HS (mean ± s.e.m. from 15 samples). Pronase digestion had no effect on these results, indicating that there was no detectable enzymic degradation of the GAGS during processing.

**Analysis**

Data presented from unstimulated cultures were expressed either as micrograms GAG/10⁶ cells or as cts min⁻¹/10⁶ cells after correction for extraction efficiencies. To allow comparison of data derived from the various methods of quantification utilised in this study, results from experiments involving cytokines, LPS and neutrophils were corrected for cell numbers and then expressed as a percentage of the values obtained from the internal control.

**Visualisation of endothelial anionic sites**

To ascertain the correlation between cellular GAGs and surface charge, we developed a morphological light-microscopic technique based upon the charged interaction of a cationic probe with endothelial anionic sites, which has
previously been used for ultrastructural studies (Skutelsky and Roth, 1986). Endothelial cells were grown to confluence on gelatinised coverslips and treated with cytokines, endotoxin and neutrophils as described above. At similar time points to those used for the biochemical GAG estimations, coverslips were washed in warm (37°C) PBS and then fixed in cold (−20°C) methanol. Anionic sites were visualised with a poly-L-lysine probe conjugated to 5 nm gold particles (Biocell Research Laboratories, Cardiff, UK). The probe was applied to the cells for 60 minutes, washed off with deionised water and developed with a silver enhancer (Biocell) for 15 minutes at room temperature (Volker et al. 1991). Binding was pH dependent, but was found to be most specific for glycosaminoglycans at pH 1.2. Addition of 25 mmol MgCl₂ increased the GAG specificity. The cells were counterstained in Meyer's haematoxylin for 1 minute and mounting in an Aquamount (BDH, Essex, England).

The charge-dependent nature of the binding was demonstrated by inhibition of binding by other cationic molecules including poly-L-lysine and DEAE-dextran. Staining was not inhibited by neutral dextran. In order to establish the identity of endothelial anionic sites, live cultures were incubated with 10 units/ml of heparinase II, 0.5 unit/ml of chondroitinase ABC and 0.5 unit/ml of neuraminidase for 4 hours and fixed cultures were treated with nitrous acid for 2 hours. These were then stained as described above.

Results

GAG metabolism in unstimulated cultures

During growth of freshly passaged cells to confluence, there was a progressive increase in GAGS, both released into the culture supernatant and associated with endothelium. Fig. 1 shows a typical electrophoretic profile of supernatant GAGS at 48 hours. Fig. 2 depicts a representative experiment in which the GAG content of supernatants and cells for each 24 hour epoch over a 96 hour period. HS, DS and CS were detected in culture supernatants at 24 hours, with maximal levels occurring at confluence (24-48 hours) and then declining steadily over the following 48 hours (Fig. 2A). Cell layer-associated GAGS constituted less than 25% of released GAGS, (Fig. 2B); however, the time course of detectable HS and DS was similar to that seen in culture supernatants. CS was rarely recovered from the endothelial cell monolayer.

As shown in Fig. 2C and D, there was little ³⁵S incorporation into GAGS isolated from both supernatants and cell layer during the first 24 hours of culture. There was then a marked increase in ³⁵S incorporation into HS and DS at 48 hours, followed by a progressive decrease over the next 2 days. Radio-labelled CS was only detected in culture supernatants. Synthesis was rapid over the initial 48 hours, and then declined over the subsequent 48 hours. Comparison of Fig. 2A, B and C, D indicates that GAGS detected in the first 24 hours were predominantly synthesised prior to subculture, whereas those detected after 24 hours were mostly newly synthesised.

GAG release, content and synthesis were extremely sensitive to a number of variables including the type of tissue culture medium, culture flask, batch of serum, umbilical cords and as shown in Fig. 2 by proliferation and age of the culture. Subsequent experiments were therefore always performed on confluent monolayers and were internally controlled using identical cells and conditions.

Effect of cytokines on GAG metabolism

Addition of TNF and IL1 to the culture medium induced an increase in total GAGS detected in supernatants at 12, 24 and 48 hours. Quantification of individual GAG components established that CS was most affected, with smaller increases in DS and HS (Table 1). In contrast, there was a small, but consistent, decrease in cell-associated GAGS. Measurement of newly synthesised GAGS, by ³⁵S incorporation (Table 2) indicated that CS production was increased throughout the experiment, whereas there was a biphasic alteration in HS and DS synthesis. ³⁵S-labelled HS and DS were reduced relative to the control at 12 hours, but increased incorporation was observed at 24 and 48 hours. Cell-layer-associated ³⁵S-labelled GAG was depressed at all three time points.

IFN gamma did not influence GAG metabolism in the first 24 hours, but after 48 hours all three classes of GAGS had increased on both cells and in the supernatant. Table 2 indicates that this was the result of stimulated GAG synthesis.
Endothelial GAG modulation in inflammation

Fig. 2. Time course of GAG production by cultured endothelial cells. A typical time course of GAGS isolated, as described above, from supernatants (A) and endothelial cell monolayers (B) showing the pattern of HS, DS, and CS in 24 hour epochs for 96 hours. Newly synthesised GAGS, as assessed by $^{35}$S incorporation into isolated GAGS, are shown for the supernatant (C) and cell layer (D). GAG synthesis was not very marked until 24 hours of culture, whereas GAGS detected after 48 hours, were predominantly newly synthesised.

LPS and IL6 had no demonstrable effect on endothelial GAG metabolism (data not shown).

The effect of neutrophils together with cytokines on GAG metabolism

In the absence of cytokines or endotoxin, co-cultivation of neutrophils and EC resulted in a modest increase in supernatant GAGS at 4 hours with a 23% reduction in cell layer-associated GAGS (Table 3). After 48 hours a significant elevation in supernatant GAGS was observed, together with a marked loss of cell layer-associated GAGS. Pretreatment of HUVEC with IL1 and TNF reduced cellular GAGS by over 60% at 4 hours with minimal change in the supernatant. The combination of LPS and neutrophils reduced both supernatant and cellular GAGS by 4 hours. After 48 hours of IL1, TNF and LPS incubation, GAGS were undetectable from the cells, but were increased in concentration in culture supernatants (Table 3). This increase in release of GAGS was predominantly due to increased biosynthesis, as shown by the results of $^{35}$S incorporation (Table 4). Release and synthesis of GAGS were not detected from the neutrophils that had been added to empty wells.

Histochemical detection of anionic sites

Using the poly-L-lysine gold probe to detect endothelial anionic sites, unstimulated cells were found to have an extensive fibrillar network of poly-L-lysine binding sites extending across and between the cultured cells, with a fine punctate array of sites located on the cellular membrane overlying the endothelial cytoplasm (Fig. 3a). Acellular regions of the culture, where cells had detached, were also densely stained with cationic gold, whereas there was minimal staining of gelatinised coverslips that had not been exposed to HUVEC (not
class of GAG at 12, 24 and 48 hours. The results are expressed as a percentage of unstimulated cultures. At all time points TNF caused a significant increase in released HS (heparan sulphate), DS (dermatan sulphate) and CS (chondroitin sulphate) when compared with the unstimulated cultures. The results were similar with IL1, IFN and IL1 failed to influence either supernatant or cellular GAGS until 48 hours of incubation, by which time all classes of cellular and supernatant GAGS were elevated. *P<0.05, †P<0.01.

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Table 1. Alterations in supernatant and cell-associated GAGS in response to cytokines

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Total GAG</th>
<th>HS</th>
<th>DS</th>
<th>CS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>12 hours</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL1</td>
<td>189 (175 - 206)*</td>
<td>164 (130 - 168)*</td>
<td>147 (135 - 155)</td>
<td>271 (260 - 295)†</td>
</tr>
<tr>
<td>TNF</td>
<td>183 (168 - 196)†</td>
<td>174 (165 - 187)†</td>
<td>140 (128 - 149)*</td>
<td>236 (211 - 251)†</td>
</tr>
<tr>
<td>IFN</td>
<td>101 (93 - 108)</td>
<td>105 (100 - 110)</td>
<td>98 (91 - 106)</td>
<td>85 (71 - 85)*</td>
</tr>
<tr>
<td><strong>24 hours</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL1</td>
<td>210 (192 - 227)†</td>
<td>158 (145 - 170)*</td>
<td>162 (142 - 181)*</td>
<td>312 (290 - 330)†</td>
</tr>
<tr>
<td>TNF</td>
<td>196 (189 - 213)†</td>
<td>126 (115 - 133)*</td>
<td>134 (121 - 145)*</td>
<td>338 (316 - 360)*</td>
</tr>
<tr>
<td>IFN</td>
<td>98 (89 - 117)</td>
<td>92 (85 - 115)</td>
<td>92 (81 - 117)</td>
<td>104 (99 - 109)</td>
</tr>
<tr>
<td><strong>48 hours</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL1</td>
<td>162 (146 - 187)*</td>
<td>140 (130 - 149)*</td>
<td>145 (129 - 156)*</td>
<td>202 (180 - 225)*</td>
</tr>
<tr>
<td>TNF</td>
<td>166 (148 - 183)*</td>
<td>154 (142 - 170)*</td>
<td>148 (131 - 163)*</td>
<td>197 (171 - 218)*</td>
</tr>
<tr>
<td>IFN</td>
<td>174 (171 - 193)*</td>
<td>145 (132 - 165)*</td>
<td>152 (135 - 170)*</td>
<td>228 (183 - 269)*</td>
</tr>
</tbody>
</table>

After incubation with IL1, TNF and IFN, GAGs were isolated from both supernatants and cells at 12, 24 and 48 hours and the individual components quantified. The values shown represent the mean and range of three experiments and are expressed as a percentage of unstimulated cultures. At all time points TNF caused a significant increase in released HS (heparan sulphate), DS (dermatan sulphate) and CS (chondroitin sulphate) when compared with the unstimulated cultures. The results were similar with IL1. This was accompanied by a small but consistent decrease in cell-associated GAGS. In contrast, IFN failed to influence either supernatant or cellular GAGS until 48 hours of incubation, by which time all classes of cellular and supernatant GAGS were elevated. *P<0.05, †P<0.01.

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Table 2. 35S incorporation into endothelial GAGS following cytokine stimulation

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Total GAG</th>
<th>HS</th>
<th>DS</th>
<th>CS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>12 hours</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL1</td>
<td>111 (96 - 125)</td>
<td>55 (44 - 65)*</td>
<td>65 (51 - 81)</td>
<td>212 (192 - 229)†</td>
</tr>
<tr>
<td>TNF</td>
<td>105 (99 - 113)</td>
<td>85 (77 - 93)</td>
<td>87 (81 - 92)</td>
<td>144 (125 - 167)</td>
</tr>
<tr>
<td>IFN</td>
<td>99 (97 - 107)</td>
<td>90 (89 - 100)</td>
<td>100 (91 - 115)</td>
<td>102 (100 - 109)</td>
</tr>
<tr>
<td><strong>24 hours</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL1</td>
<td>207 (187 - 225)†</td>
<td>122 (112 - 134)</td>
<td>146 (137 - 159)*</td>
<td>454 (431 - 491)†</td>
</tr>
<tr>
<td>TNF</td>
<td>149 (129 - 171)</td>
<td>109 (98 - 120)</td>
<td>122 (110 - 130)</td>
<td>478 (430 - 515)†</td>
</tr>
<tr>
<td>IFN</td>
<td>102 (99 - 114)</td>
<td>101 (92 - 110)</td>
<td>98 (86 - 115)</td>
<td>106 (93 - 120)</td>
</tr>
<tr>
<td><strong>48 hours</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL1</td>
<td>166 (145 - 171)*</td>
<td>145 (129 - 161)*</td>
<td>142 (134 - 158)*</td>
<td>236 (199 - 272)*</td>
</tr>
<tr>
<td>TNF</td>
<td>148 (139 - 156)*</td>
<td>135 (119 - 154)</td>
<td>131 (119 - 141)*</td>
<td>172 (151 - 203)*</td>
</tr>
<tr>
<td>IFN</td>
<td>169 (154 - 189)*</td>
<td>148 (129 - 164)*</td>
<td>155 (141 - 173)*</td>
<td>213 (195 - 244)*</td>
</tr>
</tbody>
</table>

Endothelial cultures were incubated in the presence of 35S and following the addition of the cytokines IL1, TNF and IFN the degree of 35S incorporation was determined for each class of GAG at 12, 24 and 48 hours. The results are expressed as a percentage of the unstimulated cultures. The mean and range of three experiments are shown. *P<0.05, †P<0.01.
Table 3. The influence of cytokines, endotoxin and neutrophils on endothelial GAG metabolism

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Total GAG</th>
<th>HS</th>
<th>DS</th>
<th>CS</th>
<th>Total GAG</th>
<th>HS</th>
<th>DS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 hours</td>
<td></td>
<td></td>
<td></td>
<td>48 hours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL1+neutrophils</td>
<td>109 (95 - 120)</td>
<td>103 (91 - 114)</td>
<td>91 (87 - 95)</td>
<td>120 (105 - 135)</td>
<td>55 (46 - 62)*</td>
<td>58 (48 - 64)*</td>
<td>51 (43 - 59)*</td>
</tr>
<tr>
<td>TNF+neutrophils</td>
<td>96 (91 - 102)</td>
<td>91 (86 - 99)</td>
<td>88 (83 - 94)</td>
<td>112 (104 - 121)</td>
<td>59 (53 - 70)*</td>
<td>65 (59 - 72)*</td>
<td>52 (48 - 67)*</td>
</tr>
<tr>
<td>LPS+neutrophils</td>
<td>63 (51 - 69)*</td>
<td>63 (53 - 71)*</td>
<td>60 (48 - 71)*</td>
<td>88 (71 - 106)</td>
<td>52 (40 - 63)*</td>
<td>57 (44 - 66)*</td>
<td>46 (36 - 59)*</td>
</tr>
<tr>
<td>Neutrophils alone</td>
<td>109 (106 - 113)</td>
<td>93 (87 - 98)</td>
<td>101 (88 - 112)</td>
<td>120 (112 - 131)</td>
<td>77 (68 - 87)</td>
<td>85 (74 - 91)</td>
<td>74 (61 - 89)</td>
</tr>
<tr>
<td></td>
<td>48 hours</td>
<td></td>
<td></td>
<td></td>
<td>351 (269 - 405)</td>
<td>241 (207 - 271)*</td>
<td>212 (173 - 214)*</td>
</tr>
<tr>
<td>IL1+neutrophils</td>
<td>173 (159 - 189)*</td>
<td>191 (162 - 215)*</td>
<td>162 (139 - 187)*</td>
<td>148 (131 - 163)*</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>TNF+neutrophils</td>
<td>178 (171 - 193)†</td>
<td>212 (184 - 243)*</td>
<td>142 (122 - 158)</td>
<td>196 (159 - 237)</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LPS+neutrophils</td>
<td>191 (181 - 198)†</td>
<td>218 (195 - 240)*</td>
<td>153 (141 - 163)*</td>
<td>229 (198 - 261)*</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Neutrophils alone</td>
<td>137 (130 - 149)*</td>
<td>142 (127 - 154)*</td>
<td>126 (118 - 136)*</td>
<td>150 (129 - 173)</td>
<td>39 (34 - 43)†</td>
<td>37 (29 - 47)†</td>
<td>39 (31 - 49)†</td>
</tr>
</tbody>
</table>

Neutrophils were added to HUVEC prestimulated with IL1, TNF or LPS. GAGS were isolated from the cells and culture supernatants and quantified. After 4 hours, neutrophils caused a significant reduction in cell-associated GAGS in the presence of all of the mediators used. By 48 hours GAGS were undetectable (less than 20% that of unstimulated cultures). This was accompanied by a marked elevation in supernatant GAGS. The results show the GAG content, expressed as a percentage of unstimulated cultures. The mean and range of three experiments at 4 and 48 hours are shown. *P<0.05, †P<0.01, ‡P<0.001.

Table 4. The effect of cytokines and neutrophils on endothelial GAG synthesis

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Total GAG</th>
<th>HS</th>
<th>DS</th>
<th>CS</th>
<th>Total GAG</th>
<th>HS</th>
<th>DS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48 hours</td>
<td></td>
<td></td>
<td></td>
<td>48 hours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL1+neutrophils</td>
<td>207 (186 - 239)*</td>
<td>241 (207 - 271)*</td>
<td>163 (147 - 183)*</td>
<td>212 (173 - 214)*</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>TNF+neutrophils</td>
<td>351 (269 - 405)*</td>
<td>388 (330 - 441)*</td>
<td>309 (237 - 361)*</td>
<td>377 (261 - 473)*</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LPS+neutrophils</td>
<td>321 (277 - 348)*</td>
<td>196 (152 - 253)</td>
<td>413 (297 - 503)*</td>
<td>528 (329 - 560)</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Neutrophils alone</td>
<td>147 (142 - 153)†</td>
<td>151 (129 - 181)</td>
<td>134 (118 - 157)</td>
<td>163 (154 - 198)</td>
<td>49 (42 - 56)†</td>
<td>45 (38 - 53)†</td>
<td>53 (46 - 59)†</td>
</tr>
</tbody>
</table>

35S incorporation into supernatant and cell-associated GAGS was measured following the incubation of cytokine- or endotoxin-pretreated endothelial cultures with neutrophils for 48 hours. Supernatant GAGS isolated after this time had incorporated significantly more 35S than unstimulated cultures, indicating that the released GAGS were the result of de novo synthesis. The results are expressed as a percentage of 35S incorporation compared with that of unstimulated cultures. Mean and range of three experiments are shown. *P<0.05, †P<0.01, ‡P<0.001.
Fig. 3. Cationic gold staining of: (a) unstimulated cultures. An extensive fibrillar network can be seen overlying the monolayer with fine punctate staining of the cell surface. (b) HUVEC treated with heparinase II. Live cultures treated with 10 units/ml for 2 hours have almost completely lost their fibrillar network. Occasional fibrils are still present and moderate surface punctate staining remains. (c) HUVEC following incubation with TNF. Cultures exposed to TNF for 12 hours have lost much of their surface charge, with the remaining staining located at the intercellular junctions. (d) Cationic staining after IFNγ stimulation. 48 hours of IFNγ treatment induces cellular elongation and a redistribution of anionic sites to the intercellular and pericellular matrix. (e) Surface charge following the addition of neutrophils to LPS-stimulated endothelium. Neutrophils, added to HUVEC preincubated for 4 hours with LPS, cause a dramatic reduction in cationic gold.
shown). In order to elucidate the nature of the cationic binding, HUVEC were exposed to neuraminidase, chondroitinase ABC, heparinase II and nitrous acid. Although neuraminidase had a minimal effect on the distribution of charged sites, heparinase II and nitrous acid caused significant disruption of the fibrillar pattern and also reduced the intensity of punctate staining. These results indicate that the cationic gold binding observed in this study was predominantly directed against cell layer-associated heparan sulphate (Fig. 3b). Chondroitinase ABC had little effect on the fibrils, but inhibited cationic gold binding to the acellular zones of the culture and also reduced the membrane-associated punctate staining (data not shown).

Addition of IL1 (not shown) or TNF to the culture medium altered both the intensity and distribution of anionic sites. Fig. 3c shows that TNF induced a generalised reduction in the binding of cationic gold, and that in contrast to control cultures staining was mainly limited to the intercellular junctions.

The pattern of staining after the HUVEC were stimulated with IFN gamma differed significantly from that seen in the control and TNF-treated cells (Fig. 3d). Gold was distributed in clumps running between the longitudinal axes of the cells with minimal staining detected on the cell surface.

Neutrophils added to IL1-, TNF- (not shown) and LPS-treated cultures, caused a considerable reduction in cell surface staining by 4 hours, and by 12 hours there was only minimal cationic gold deposition (Fig. 3e). Trypan blue exclusion demonstrated that the neutrophil/cytokine combinations used in these experiments had not significantly altered HUVEC viability.

Cationic gold staining was very sensitive to fixation. The fibrillar structures seen with cold methanol were not observed if HUVEC were fixed in either warm methanol or an aldehyde fixative. Paraformaldehyde and glutaraldehyde fixation produced a more homogenous distribution of staining, predominantly located on the exposed surface of the endothelial cell, which was removed following treatment with heparinase II. Cells fixed with these aldehyde fixatives did however display significant alterations in response to the stimuli used in this study. In particular, neutrophils added to stimulated endothelium almost completely eradicated the cationic staining.

**Discussion**

Despite increasing recognition that GAGS are involved in many biological processes, elucidation of their functional significance has proved difficult to define accurately. The methods developed in this study to examine the metabolism of endothelial cell GAGS in vitro enabled both cell layer-associated and supernatant GAGS to be determined simultaneously. In addition, by labelling GAGS with $^{35}$S the contribution of newly synthesised molecules relative to the total extractable GAG could be assessed. Furthermore, poly-L-lysine/gold histochemistry has also enabled us to visualise the surface distribution of endothelial anionic sites in response to cytokines, endotoxin and neutrophils.

Unstimulated HUVEC in culture release heparan, dermatan and chondroitin sulphate into the culture supernatant with maximal release and synthesis occurring during growth to confluence. Only heparan and dermatan sulphate were detectable as cell layer-associated GAGS, and whilst these constituted less than 25% of supernatant levels, their rate of synthesis followed a similar time course. These observations are consistent with the reports of other investigators (Oohira et al. 1983; Gordon et al. 1985; Wight et al. 1986). Using a combination of cationic gold histochemistry, nitrous acid and enzymic treatment of endothelial cells, we have determined the surface location of these endothelial GAGS. Heparan sulphate was predominantly seen as an extensive network of fibrils extending above and between the cultured cells, whereas chondroitinase ABC-sensitive GAGS were located on the surface of the cell, and also constituted a significant component of subendothelial matrix.

Following the addition of TNF and IL1 to cultured endothelial cells, increased quantities of GAGS were continuously released into the HUVEC supernatants. However, using $^{35}$S incorporation as an indicator of de novo synthesis, it was apparent that the GAGS released in the first 12 hours were predominantly preformed and were not the result of increased biosynthesis. Indeed, GAG synthesis was actually depressed during the first 12 hours of cytokine incubation. Throughout the experiment, biochemical detection of cell layer-associated GAGS was diminished. Although this loss could be either from the intracellular compartment or from the cell surface, the dramatic changes seen in both the content and distribution of negatively charged sites suggests that at least a proportion of the released GAGS were derived from the endothelial cell surface. By 48 hours there was a significant increase in the synthesis of supernatant GAGS; however, this did not serve to replete the endothelial cell of its GAGS. This stimulation of GAG synthetic activity may be the result of IL1- and TNF-directed stimulation or, alternatively, it may represent recovery from earlier cytokine suppression.

In contrast to IL1 and TNF, IFN gamma had no measurable effect on GAG metabolism until 48 hours of incubation, but by this time there was an increase in all three species of sulphated GAG in both cell layer-associated and supernatant fractions. For all three species, this was as a result of increased GAG biosynthesis. The microscopic pattern of cationic gold deposition was quite distinct from that seen with the other cytokines. Very little of the fibrillar network remained and the greatest proportion of negative sites were located at the periphery of the cells and in the extracellular matrix. The timing of these changes may be significant as many of the known effects of IFN gamma on HUVECs, including ICAM1 expression and neutrophil adhesion, are often observed later than those induced by IL1 and TNF (Dustin et al. 1986; Pober et al. 1986).
Previous studies in a variety of cell types in vitro, including lung and dermal fibroblasts (Elías et al. 1988; Postlewaite et al. 1989), chondrocytes (Ratcliffe et al. 1986) and human umbilical vein endothelial cells (Montesano et al. 1984), have shown that cytokines can influence GAG metabolism. In most of these reports hyaluronic acid was the GAG most affected. In endothelial cells, however, significant modulation of sulphated GAGs has been reported in response to cytokine stimulation. Montesano et al. investigated the influence of IL1, IFN gamma and IL2 on the ultrastructural organisation of alcianophilic material in HUVEC, and observed the induction of electron-dense pericellular deposits in response to IFN gamma alone or in combination with IL1. Enzymic analysis indicated that this material contained dermatan and chondroitin sulphate. In contrast to our results, Montesano et al. failed to detect alcianophilic material in unstimulated cultures. This difference may be explained by the different fixatives used in the two studies as we have shown that fixation is critical to the pattern of cationic staining obtained.

Whilst the molecular basis for the cytokine modulation of endothelial sulphated GAGs is not known, cytokine-directed regulation of proteoglycan metabolism has been investigated in other cell types. In common with the findings of this study, Tyler has demonstrated that IL1 induced both a depression in synthesis, and an increase in release, of cartilage-derived proteoglycans (Tyler, 1985a,b). It is suggested that the enhanced proteoglycan release from the cultured cartilage matrix may be caused by limited cleavage of the proteoglycan core, either by endogenous membrane-bound proteinases or by hydroxyl radicals. A similar mechanism has been postulated to explain the release of basic fibroblast growth factor/heparan sulphate complexes from bovine capillary endothelial cells (Saksela and Rifkin, 1990). Some proteoglycans contain protease-sensitive sites, and may be particularly vulnerable to membrane-bound protease modulation by inflammatory mediators (Hardingham and Fosang, 1992). Extracellular matrix proteoglycans may also be released when exogenous stimuli induce changes in other matrix proteins such as fibronectin, vitreorecin and laminin. Fibronectin, which contains at least two heparin binding sites (Ruoslahi, 1988) and is closely associated with heparan sulphate complexes from bovine aortic endothelial cells (Saksela and Rifkin, 1990), some proteoglycans contain protease-sensitive sites, and may be particularly vulnerable to membrane-bound protease modulation by inflammatory mediators (Hardingham and Fosang, 1992). Extracellular matrix proteoglycans may also be released when exogenous stimuli induce changes in other matrix proteins such as fibronectin, vitreorecin and laminin. Fibronectin, which contains at least two heparin binding sites (Ruoslahi, 1988) and is closely associated with heparan sulphate (Hayman et al. 1982), is lost from the pericellular matrix of HUVEC in response to TNF and IFN gamma (Stolpen et al. 1986). It would therefore seem likely that these cytokines would also influence the binding properties of matrix-associated proteoglycans.

In this study the most striking biochemical and histochemical results were seen when neutrophils were incubated with cytokine and endotoxin-treated endothelial cultures. After four hours of incubation with IL1, TNF or LPS, total cell-associated GAGs were reduced to less than 60% of that seen in unstimulated cultures with almost complete loss of negative charge from the cell surface. Although synthesis of all three classes of GAGs was significantly enhanced by 48 hours, cell-layer-associated GAGs continued to decline and were undetectable by this time. Neutrophils alone also caused a marked reduction in cell-associated GAGs, although not to the levels seen with cytokine and LPS stimulation. The correlation between histochemical cationic gold deposition and biochemical GAG isolation suggests that this reduction in endothelial surface GAG may be responsible for the loss of endothelial surface charge.

IL1, TNF and LPS influence a wide range of neutrophil and endothelial functions including integrin expression, respiratory burst, arachidonic acid metabolism and cytokine release (Dahinden and Fehr, 1982; Dahinden et al. 1983; Smedley et al. 1986). Their role in relation to neutrophil/endothelial interactions and GAG metabolism remains to be determined, but it is interesting that a variety of inflammatory cells, including platelets, lymphocytes and neutrophils have the capacity to secrete GAG-degrading enzymes (Naparstek et al. 1984; Matzner et al. 1985). While little is known of the regulation of these endoglycosidases, Matzner et al. have shown that neutrophils, cooled to 4°C, released sufficient heparanase to degrade heparan sulphate from bovine aortic endothelial-derived extracellular matrix. This was not the result of non-specific protease release, as only small quantities of lactate dehydrogenase, lysozyme and globin-degrading enzymes were detected. The local release of GAG endoglycosidases may play an important role in enabling neutrophils and lymphocytes to migrate to inflammatory sites by facilitating their penetration through the vascular wall. Such a mechanism has been described in a rodent model of experimental autoimmune encephalomyelitis, in which only activated lymphocytes, reacting to a range of specific antigens, elaborated endoglycosidases capable of degrading heparan sulphate (Naparstek et al. 1984).

Platelets and neutrophils also synthesise a number of cationic proteins, which bind to surface GAGs and could effectively neutralise their negative charge (Camussi et al. 1986; Pereira et al. 1989). Cationic proteins have been shown to neutralise anionic sites in the glomeruli of rabbits with experimentally induced serum sickness, and in patients with systemic lupus erythematosus (Camussi et al. 1982; Camussi et al. 1986). In this study we found that while the addition of neutrophils to cytokine- and endotoxin-stimulated endothelium reduced the amount of chemically detected cell-associated GAGs to less than 50%, histochemical analysis revealed an almost complete loss of surface charge. This discrepancy between the loss of charged sites detected histochemically and the quantity of GAGs detected chemically may be explained by the intracellular or subendothelial location of remaining GAGs, which were therefore not detectable with the poly-L-lysine/gold, or by the neutralisation of surface anionic sites by neutrophil cationic proteins.

The dislocation of GAGs from the endothelial cell surface into the surrounding medium in response to inflammatory stimuli, could explain a number of the homeostatic and haemodynamic derangements seen in
inflammatory conditions. Vascular endothelium is known to exert a regulating influence on both procoagulant and anticoagulant mechanisms. ILI and TNF have been shown to enhance the thrombogenicity of vascular tissue by increasing the surface expression of tissue factor, decreasing endothelial surface thrombomodulin and increasing the secretion of tissue plasminogen activator inhibitor (Bevilacqua et al. 1986; Nachman et al. 1986; Nawroth et al. 1986; Nawroth et al. 1988). The roles of heparin, heparan sulphate and dermatan sulphate in preventing thrombosis through the stimulation of antithrombin III and heparin cofactor II activity have now also been well documented (Lindahl and Hook, 1978; Wright, 1980). The reduction in HUVEC surface GAG that we have observed in response to inflammatory stimuli would therefore be expected to result in a local reduction of endothelial cell surface anticoagulant activity. Paradoxically, GAGS released from the vascular wall could also behave as circulating anticoagulants as described in some metastatic and inflammatory diseases (Palmer et al. 1984; Tefferi et al. 1990).

In this study we have shown that cytokines, endotoxin and neutrophils can modulate endothelial sulphated GAG metabolism and thereby influence both the content and distribution of endothelial anionic sites. A number of recent studies have implicated host inflammatory mediators as important components in the pathophysiology of Gram-negative sepsis (Waage et al. 1987; Brandtzaeg et al. 1989). Our own results, considered in the context of these findings, may contribute to the further understanding of the vascular changes associated with endotoxic shock.

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References


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