The effects of heparin on the adhesion of human peripheral blood mononuclear cells to human stimulated umbilical vein endothelial cells

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1 The effects of unfractionated heparin (UH) and a selectively O-desulphated derivative of heparin (ODSH), lacking anticoagulant activity, on the adhesion of human peripheral blood mononuclear cells (HPBMNC) to human stimulated umbilical vein endothelial cells (HUVECs), were investigated.

2 For comparison, the effects of poly-L-glutamic acid (PGA), a large polyanionic molecule without sulphate groups and two different molecular weight sulphated dextrans (DS 5 k and DS 10 k) were studied.

3 UH (50–1000 u ml^{-1}) significantly ($P < 0.05$) inhibited the adhesion of HPBMNC to HUVECs, stimulated with IL-1β (100 u ml^{-1}), TNF-α (1000 u ml^{-1}) or LPS (100 μg ml^{-1}), when the drugs were added together with stimuli to HUVECs and coincubated for 6 h. Such effects on adhesion occurred with limited influence on expression of relevant endothelial adhesion molecules (ICAM-1 and VCAM-1).

4 UH (100–1000 u ml^{-1}), when added to prestimulated HUVECs, significantly ($P < 0.05$) increased adhesion of mononuclear cells to endothelium at the higher concentrations tested, without any effect on adhesion molecule expression. In contrast, the opposite effect was observed when human polymorphonuclear leucocyte adhesion was examined, under the same experimental conditions, suggesting that the observed potentiation of HPBMNC adhesion is cell specific.

5 The effects of UH on HPBMNC adhesion were shared by the non-anticoagulant ODSH (600–6000 μg ml^{-1}) but not by sulphated dextrans or PGA (300–6000 μg ml^{-1}).

6 Heparin affects the adhesion of HPBMNC to stimulated endothelium, in both an inhibitory and potentiating manner, effects which are unrelated to its anticoagulant activity and not solely dependent on molecular charge characteristics.

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Abbreviations: DS, dextran sulphate; ELISA, enzyme linked immunosorbant assay; GAG, glycosaminoglycan; HPBMNC, human peripheral blood mononuclear cell; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule-1; IgG, immunoglobulin G; IL-1β, interleukin-1β; LPS, lipopolysaccharide; mac-1, macrophage-1 (CD11b/CD18); ODSH, O-desulphated heparin; PBS, phosphate buffered saline; PECAM-1, platelet endothelial cell adhesion molecule-1; PGA, poly-L-glutamic acid; PMN, polymorphonuclear leucocyte; TMB, 3,3′,5,5′-tetramethylbenzidine; TNF-α, tumour necrosis factor-α; UH, unfractionated heparin; VCAM-1, vascular cell adhesion molecule-1

Introduction

The role of endogenous heparin, synthesized exclusively by mast cells, is poorly understood but along with other glycosaminoglycans (GAGs) it is known to modulate a variety of biological functions. However, a possible immunomodulatory function is suggested, as increasing evidence, both experimental and clinical, indicates heparin to possess a range of anti-inflammatory properties (reviewed by Jaques, 1979; Tyrrell *et al.*, 1999). Many of these effects may be mediated through the ability of this large, negatively charged molecule to bind and inactivate an array of inflammatory proteins, including certain complement components (Matzner *et al.*, 1984; Strunk & Colten, 1976), chemokines (Miller &

Krangel, 1992) and products of activated granulocytes (Fredens *et al.*, 1991; Redini *et al.*, 1988; Walsh *et al.*, 1991b) as well as thrombin, a known proinflammatory mediator which acts through PAR-1 receptor activation. In addition, heparin is known to bind a number of adhesion molecules involved in leucocyte trafficking into tissues, including mac-1 (CD11b/CD18; Diamond *et al.*, 1995) and L-selectin (Koenig *et al.*, 1998) on inflammatory cells and the endothelial adhesion molecules P-selectin (Revelle *et al.*, 1996; Skinner *et al.*, 1991) and platelet endothelial adhesion molecule-1 (PECAM-1; Watt *et al.*, 1993). Accordingly, heparin and related molecules have been found to inhibit leucocyte-endothelial interactions both *in vitro* (Bazzoni *et al.*, 1992; Lever *et al.*, 2000; Silvestro *et al.*, 1994) and *in vivo* (Ley *et al.*, 1991; Salas *et al.*, 2000; Xie *et al.*, 1997), as well

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as inflammatory cell accumulation in tissue sites such as the lung (Sasaki *et al.*, 1993; Seeds *et al.*, 1993), skin (Teixeira & Hellewell, 1993) and peritoneal cavity (Nelson *et al.*, 1993).

The precise mechanisms by which heparin modulates leucocyte recruitment are not fully understood. The closely related molecule heparan sulphate is, however, found on vascular endothelial cells, where it is considered to play a role in preventing non-specific adhesion of blood elements since it is now known that certain inflammatory cells, most notably lymphocytes, release heparan-degrading enzymes in order to aid their extravasation (reviewed by Vlodavsky *et al.*, 1992) and that these enzymes are inhibited by heparin (Bar-Ner *et al.*, 1987). Indeed, the inhibitory effects of administered heparin on lymphocyte orchestrated processes such as graft *vs* host reactions (Gorski *et al.*, 1991; Lider *et al.*, 1989), delayed type hypersensitivity reactions (Sy *et al.*, 1983) and allergic encephalomyelitis (Lider *et al.*, 1990; Willenborg & Parish, 1988) are thought to involve heparanase inhibition. However, there is also evidence that heparin can affect the entry of lymphocytes to lymph nodes and Peyer's patches (Bellavia *et al.*, 1980), having potential consequences with respect to the recirculation of these cells. The aim of the present study was to examine whether heparin can influence the adhesion of mononuclear cells to vascular endothelial cells, a possible mechanism by which heparin might be active in reducing inflammatory responses *in vivo*.

Unfractionated heparin and several related molecules were investigated for their effects on the adhesion of human mixed mononuclear cell preparations to cytokine and LPS-stimulated endothelial cells. A structurally modified heparin, lacking in anticoagulant activity (Fryer *et al.*, 1997) due to the selective removal of O-sulphate groups, was used in order to assess the contribution of this property of the parent molecule to any observed effects. Poly-L-glutamic acid (PGA), a linear polyanionic molecule, was also tested, with a view to investigating the importance of charge characteristics to the actions of heparin. Given that heparin is a heavily sulphated molecule, whereas PGA does not possess these functional groups, we went on to consider the role of sulphate-derived negative charges through the use of dextran sulphate preparations of varying molecular weights.

Methods

Preparation of HUVECs

HUVECs, at passage five or below, were cultured to confluency in endothelial basal medium (MCDB 131), supplemented with foetal bovine serum (2%), hydrocortisone (1 ng ml^{-1}), gentamicin (50 μg ml^{-1}), amphotericin-B (50 ng ml^{-1}) and human epidermal growth factor (10 ng ml^{-1}), in the central 60 wells of flat bottomed 96 well plates (200 μl culture medium per well; 5% CO_2; 37°C). Monolayers were stimulated by the addition of 22 μl per well of a solution of IL-1β, TNF-α or bacterial LPS, prepared in culture medium at 10 fold the final concentration required in the well, for 6 h.

Isolation of leucocytes

Peripheral venous blood was collected from healthy volunteers ($n = 6$) into citrated tubes (1 volume in 10 acid citrate dextrose), before being transferred to Accuspin tubes (containing Histopaque 1077) for centrifugation at 1000 $\times$ g for 10 min. The resultant plasma layer was carefully removed and HPBMNCs, contained within a distinct opaque layer, were carefully aspirated. Where necessary, contaminating red blood cells were lysed hypotonically (0.83% ammonium chloride, 10 min, ambient temperature). Cells were washed three times in modified Hank's Balanced Salts Solution (HBSS, without Ca^{2+} and Mg^{2+}) by centrifugation at 250 $\times$ g for 5 min. Samples of the cell suspension were removed and purity and viability assessed. Cell suspensions used were found to be more than 98% viable and to consist of not less than 95% mononuclear cells, with neutrophils being the main contaminating leucocyte. Within mononuclear cell populations, the mean ratio of lymphocytes to monocytes was approximately five. In some experiments, following removal of HPBMNCs, the remaining blood fraction was purified further in order to obtain autologous polymorphonuclear leucocyte (PMN) populations. In short, the cell mixture found below the Histopaque layer was mixed with an equal volume of dextran solution (Hespan, containing 18% PBS and 2% ACD) and centrifuged for 15 min at 20 $\times$ g to remove erythrocytes. The supernatant was further centrifuged (7 min, 175 $\times$ g) to collect the PMNs, remaining red blood cells were lysed as
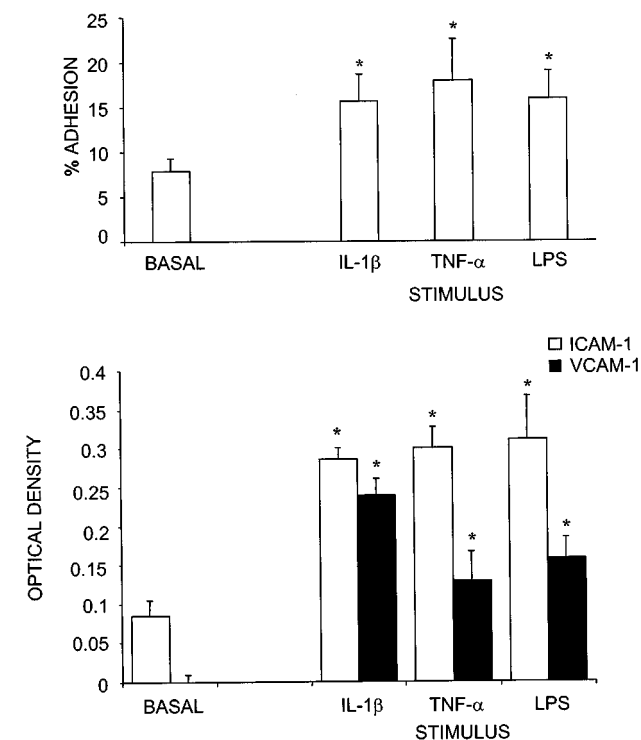

Figure 1 Effect of endothelial stimulation on HPBMNC adhesion and expression of adhesion molecules. HUVECs were stimulated with IL-1β (100 u ml^{-1}), TNF-α (1000 u ml^{-1}) or LPS (100 μg ml^{-1}) for 6 h. (a) ^{51}Cr-labelled HPBMNCs were added for 30 min and adhesion was quantified by γ-counting lysates of adherent cells and calculating the percentage of cells added which had adhered. (b) Endothelial ICAM-1 (open bars) and VCAM-1 (closed bars) were measured by ELISA. Data are expressed as the mean±s.e.mean of six separate experiments, each performed in triplicate. *Statistical significance ($P < 0.05$) is indicated.

before and cells were washed three times in modified HBSS. PMN preparations isolated in this manner were found to be more than 98% viable and to contain at least 95% neutrophils, contaminating cells typically being eosinophils.

^{51}Cr-labelling of leucocytes

HPBMNC or PMN pellets were incubated with aqueous sodium ^{51}chromate (37 kBq per 10^6 viable cells) for 60 min at room temperature. Excess radiolabel was removed by three washes in modified HBSS before cells were resuspended at a final concentration of 10^6 ml^{-1} in complete HBSS (with Ca^{2+} and Mg^{2+}). Cell viability was unaffected by this process.

Adhesion assay

Leucocyte-endothelial adhesion was examined using an assay described previously (Lever et al., 2000). Briefly, HUVEC monolayers, stimulated with cytokines or LPS in the absence or presence of the study drugs (see below), were washed with warmed HBSS prior to addition of 200 μl radiolabelled leucocyte suspension per well. Preliminary experiments were carried out to establish optimum concentrations of and exposure time to stimuli. A 6 h stimulation period, with IL-1β (100 u ml^{-1}), TNF-α (1000 u ml^{-1}) or LPS (100 μg ml^{-1}),

was selected for use in the study. Plates were returned to the incubator for 30 min, following which, non-adherent cells were removed by gentle aspiration and washing with warmed HBSS. Wells were examined microscopically and, upon confirmation of monolayer integrity, adherent cells were lysed by addition of 200 μl detergent solution (1% Igepal) per well. One hundred μl samples were transferred to scintillation vials and γ-counted alongside 100 μl samples of the original radiolabelled cell suspension (input). The number of adherent cells was calculated as the percentage of input counts present in sample counts, corrected for background radioactivity. Compounds under investigation (unfractionated heparin (UH, 0 – 1000 u ml^{-1}; specific anticoagulant activity $\approx$ 180 u mg^{-1}), O-desulphated heparin (ODSH, 0 – 6000 μg ml^{-1}), poly-L-glutamic acid (PGA, 0 – 6000 μg ml^{-1}) or dextran sulphates of molecular weight 5000 or 10 000 (DS 5 k or DS 10 k, respectively, 0 – 6000 μg ml^{-1})) were added to monolayers, either immediately prior to endothelial stimuli or, to stimulated HUVECs immediately prior to the addition of leucocytes.

Treatment of data

Results are expressed either as per cent adhesion or, when examining the effects of drugs on adhesion, as per cent of
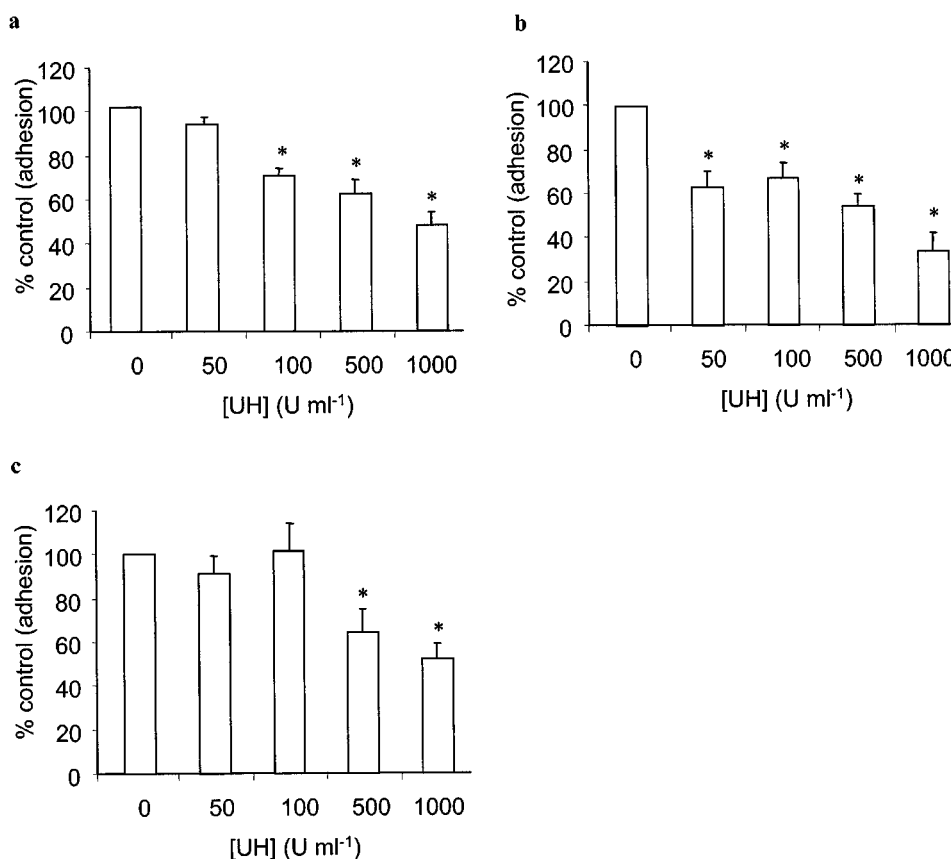

Figure 2 Effect of heparin on HPBMNC adhesion to HUVECs. HUVECs were stimulated with (a) IL-1β (100 u ml^{-1}), (b) TNF-α (1000 u ml^{-1}) or (c) LPS (100 μg ml^{-1}) for 6 h, in the absence and presence of different concentrations of unfractionated heparin (UH). ^{51}Cr-labelled HPBMNCs were added for 30 min. Adhesion was quantified by γ-counting lysates of adherent cells and was calculated as a percentage of that seen in control wells that had not been treated with heparin. Data are expressed as the mean $\pm$ s.e.mean of six separate experiments, each performed in triplicate. *Statistical significance ($P < 0.05$) is indicated.

control, that is, adhesion observed in control wells which had received stimuli but no test compounds. Data were analysed by ANOVA, followed by Dunnett's test.

Enzyme linked immunosorbant assay

In these experiments, HUVEC monolayers were treated identically to those used in adhesion assays, with respect to stimulation, addition of test compounds (in this case, UH only) and incubation times. Following the relevant treatments, plates were washed three times with a blocking solution of 5% w v^{-1} low fat powder milk in PBS, in order to eliminate non specific protein binding. Primary monoclonal antibodies (mouse anti-human ICAM-1 IgG, mouse anti-human VCAM-1 IgG or control mouse IgG$_1$; all at a concentration of 1 μg ml^{-1}) were added and plates incubated for 60 min at room temperature. Cells were again washed three times with blocking solution, before secondary antibody (goat anti-mouse IgG-horseradish peroxidase conjugate, 1:10 000 dilution) was added and plates incubated for a further 60 min at room temperature. Monolayers were washed thoroughly with PBS and 100 μl TMB substrate system (3,3'5.5'-tetramethylbenzidine) were added to each well. Plates were incubated for 10–15 min, until a blue colour developed and the reaction was quenched by addition of 2 M sulphuric acid. Plates were analysed colorimetrically at

450 nm in an Anthos HTIII plate reader. Readings from wells which received no primary antibody were subtracted from test values.

Treatment of results

Results are expressed as per cent of control values, that is, readings from stimulated wells which had received no UH. Data were again analysed by ANOVA, followed by Dunnett's test.

Reagents

Cryopreserved HUVECs and all culture media were obtained from BioWhittaker Ltd. Milton Keynes, Bucks, U.K.; Aqueous [51]sodium chromate from Amersham Life Science, U.K.; Accuspin Histopaque 1077, acid citrate dextrose (ACD), Percoll, Hank's balanced salts solutions, phosphate buffered saline, horseradish peroxidase linked goat anti-mouse IgG, TMB liquid substrate system, acid citrate dextrose, Igepal, poly-L-glutamic acid, dextran sulphates, bacterial (*E. Coli*) LPS (serotype 0111:B4) from Sigma-Aldrich Company Ltd. U.K.; human recombinant IL-1β; human recombinant TNF-α; mouse anti-human ICAM-1 IgG (clone BBIG-I1), mouse anti-human VCAM-1 IgG (clone BBIG-V1) and control mouse IgG$_1$ from
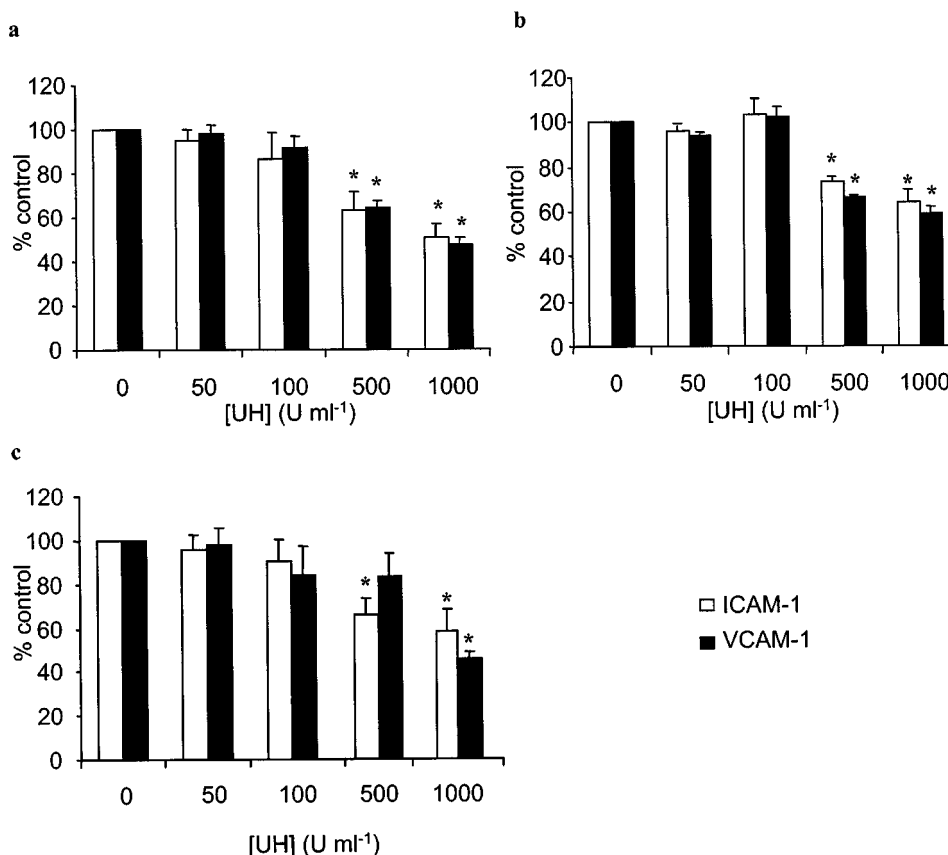

Figure 3 Effect of heparin on endothelial adhesion molecule expression. HUVECs were stimulated with (a) IL-1β (100 u ml^{-1}), (b) TNF-α (1000 u ml^{-1}) or (c) LPS (100 μg ml^{-1}) for 6 h, in the absence and presence of different concentrations of unfractionated heparin (UH). ICAM-1 (open bars) and VCAM-1 (closed bars) were measured by ELISA. Adhesion molecule expression was calculated as a percentage of that seen in control wells that had not been treated with heparin. Data are expressed as the mean±s.e.mean of six separate experiments, each performed in triplicate. *Statistical significance ($P<0.05$) is indicated.

R&D Systems Ltd. U.K.; Hespan from Du Pont Pharmaceuticals Ltd. Letchworth, Herts. U.K.; UH (Multiparin) from CP Pharmaceuticals Ltd. Wrexham, North Wales, U.K.; O-desulphated heparin was a kind gift from Dr T. Kennedy, Carolinas Medical Health Care Foundation, Charlotte, NC, U.S.A.

Results

A 6 h exposure of HUVEC monolayers to IL-1β (100 u ml^{-1}), TNF-α (1000 u ml^{-1}) or LPS (100 μg ml^{-1}) was found to significantly enhance the subsequent adhesion of HPBMNC to these cells (Figure 1a), commensurate with upregulation of the endothelial adhesion molecules ICAM-1 and VCAM-1 (Figure 1b).

UH, when present during cytokine- or LPS-stimulation of HUVECs was found to inhibit ($P < 0.05$) the subsequent adhesion of HPBMNC to these cells, following washing of the monolayers to remove stimuli and any unbound drug (Figure 2). Treatment of HUVECs with heparin, in this manner, was found to have an effect on the expression of ICAM-1 and VCAM-1 on the endothelial cell surface, although inhibition of adhesion was achieved at concentrations lower than those required to elicit this effect on adhesion molecules (Figure 3). However, when UH was added to the assay system with the HPBMNCs, i.e. to HUVECs which had already been stimulated in the absence of any drug treatment, a potentiation of cellular adhesion was observed, with a maximum increase in adhesion of $68.9 \pm 21.01\%$ (IL-1β); $55.2 \pm 21.03\%$ (TNF-α) and $44.3 \pm 12.6\%$ (LPS). In previous studies which examined the
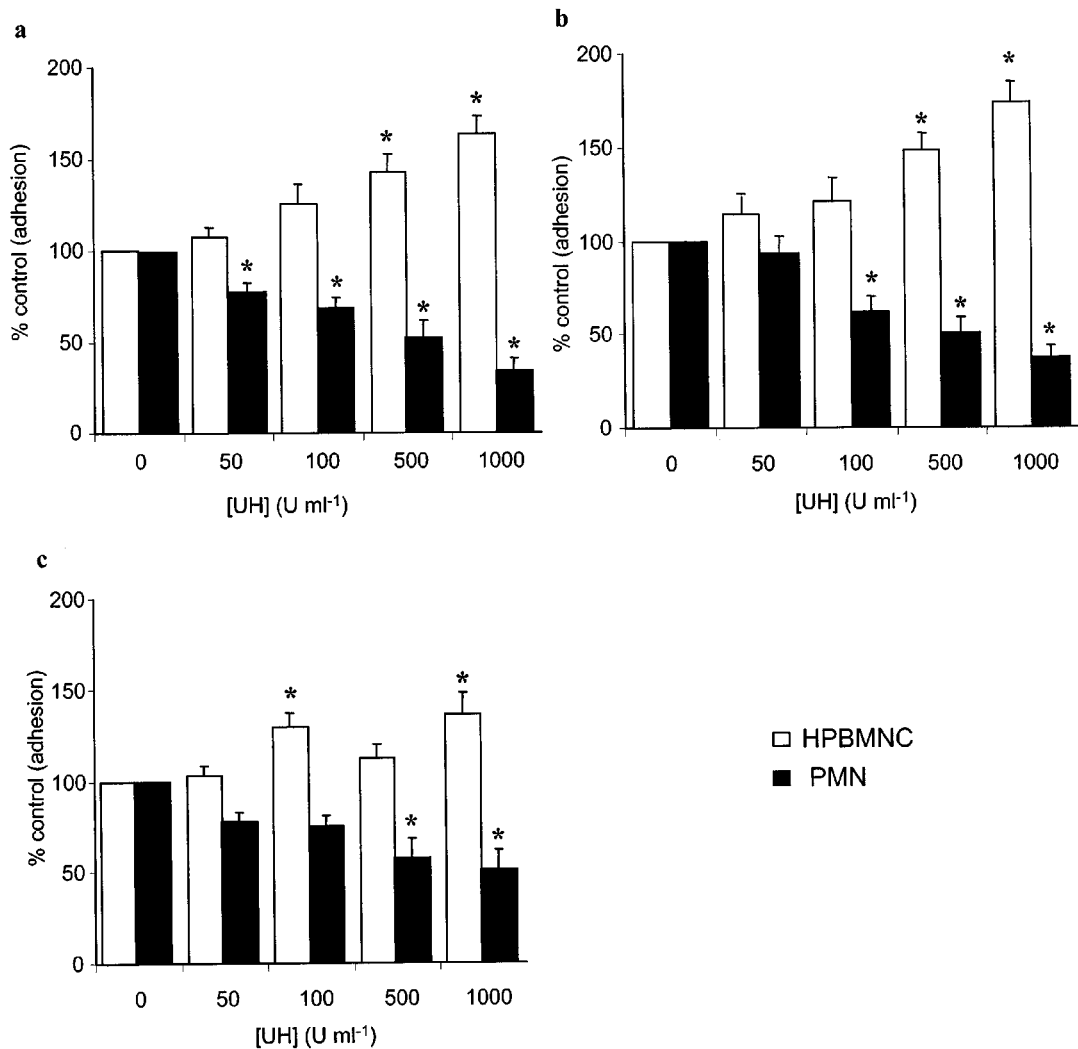

Figure 4 Effect of heparin on HPBMNC and PMN adhesion to pre-stimulated HUVECs. HUVECs were stimulated with IL-1β (100 u ml^{-1}), TNF-α (1000 u ml^{-1}) or LPS (100 μg ml^{-1}) for 6 h. ^{51}Cr-labelled HPBMNCs (open bars) or PMNs (closed bars) were added for 30 min, in the absence and presence of different concentrations of unfractionated heparin (UH). Adhesion was quantified by γ-counting lysates of adherent cells and was calculated as a percentage of that seen in control wells† that had not been treated with heparin. Data are expressed as the mean $\pm$ s.e.mean of six separate experiments, each performed in triplicate. *Statistical significance ($P < 0.05$) is indicated. †(Control PMN adhesion in wells stimulated with IL-1β, TNF-α or LPS was 19.16 ± 1.56, 17.5 ± 1.28 and $14.5 \pm 1.06\%$, respectively. Basal PMN adhesion was $7.74 \pm 1.62\%$).

Table 1   Effect of heparin on ICAM-1 and VCAM-1 adhesion molecule expression

[UH] U ml^{-1}	*ICAM-1 expression* (% of control)		
	IL-1β	*TNF-α*	*LPS*
0	100	100	100
50	115.2 ± 9.5	105.4 ± 6.3	114.7 ± 8.3
100	93.33 ± 6.4	102 ± 1.7	109.9 ± 12.4
500	98.22 ± 2.3	99.3 ± 2.7	117.6 ± 11.8
1000	101.22 ± 7.4	97.2 ± 6.9	105.6 ± 4.4
[UH] U ml^{-1}	*VCAM-1 expression* (% of control)		
	IL-1β	*TNF-α*	*LPS*
0	100	100	100
50	103.4 ± 2.4	108.2 ± 5.7	102.7 ± 7.3
100	99.5 ± 3.5	107.3 ± 4.1	106.3 ± 8.5
500	102.4 ± 2.2	111.3 ± 10.9	103.5 ± 6.9
1000	108.7 ± 4.9	100.5 ± 1	101.6 ± 6.1

HUVECs were stimulated with IL-1β (100 u ml^{-1}), TNF-α (1000 u ml^{-1}) or LPS (100 μg ml^{-1}) for 6 h. Different concentrations of unfractionated heparin (UH) were added for 30 min, following which, ICAM-1 and VCAM-1 were measured by ELISA. Adhesion molecule expression was calculated as a percentage of that seen in control wells that had not been treated with heparin. Data are expressed as the mean ± s.e.mean of six separate experiments.
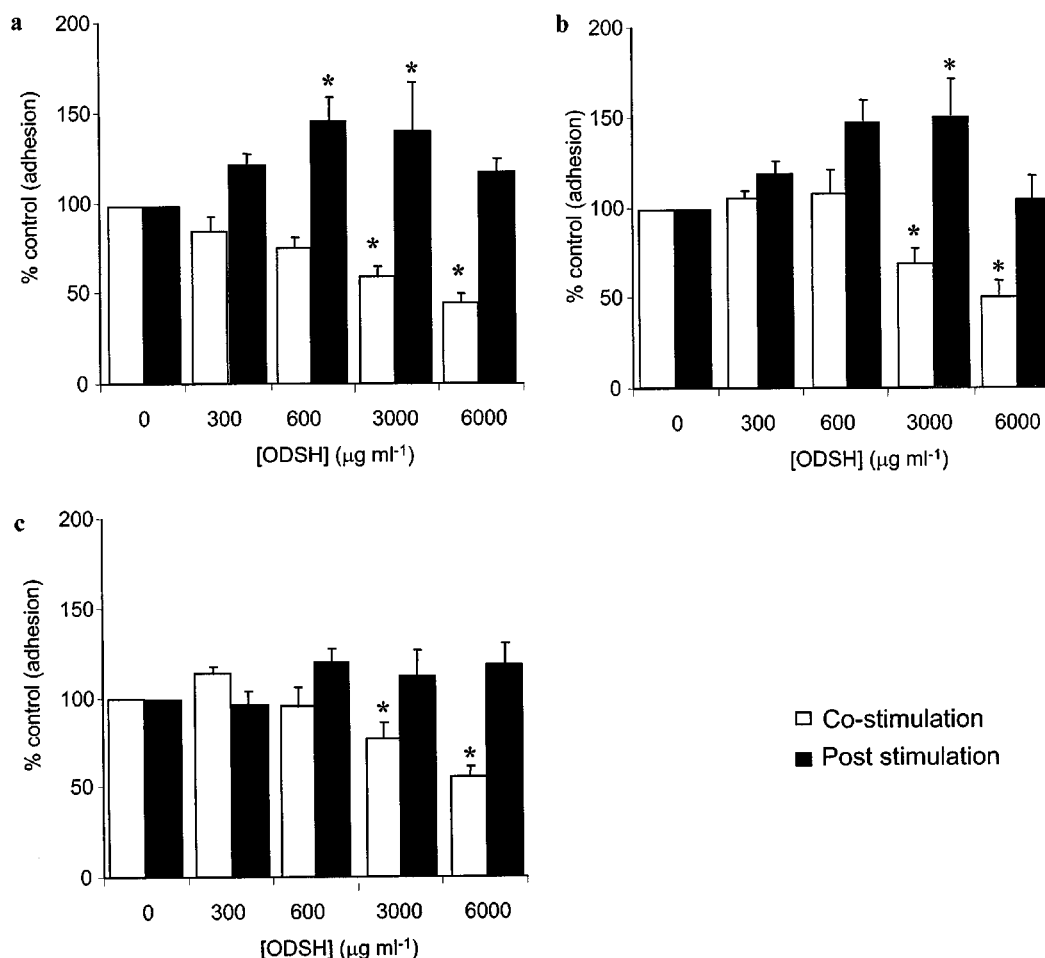

Figure 5   Effects of O-desulphated heparin on HPBMNC adhesion to HUVECs under different experimental conditions. HUVECs were stimulated with (a) IL-1β (100 u ml^{-1}), (b) TNF-α (1000 u ml^{-1}) or (c) LPS (100 μg ml^{-1}) for 6 h, in the absence or presence of O-desulphated heparin (ODSH). ^{51}Cr-labelled HPBMNCs were added for 30 min, either alone, to HUVECs stimulated in the presence of ODSH (open bars) or, in conjunction with different concentrations of ODSH, to HUVECs stimulated in the absence of the drug (closed bars). Adhesion was quantified by γ-counting lysates of adherent cells and was calculated as a percentage of that seen in control wells that had not been treated with ODSH. Data are expressed as the mean ± s.e.mean of six separate experiments, each performed in triplicate. *Statistical significance (P < 0.05) is indicated.
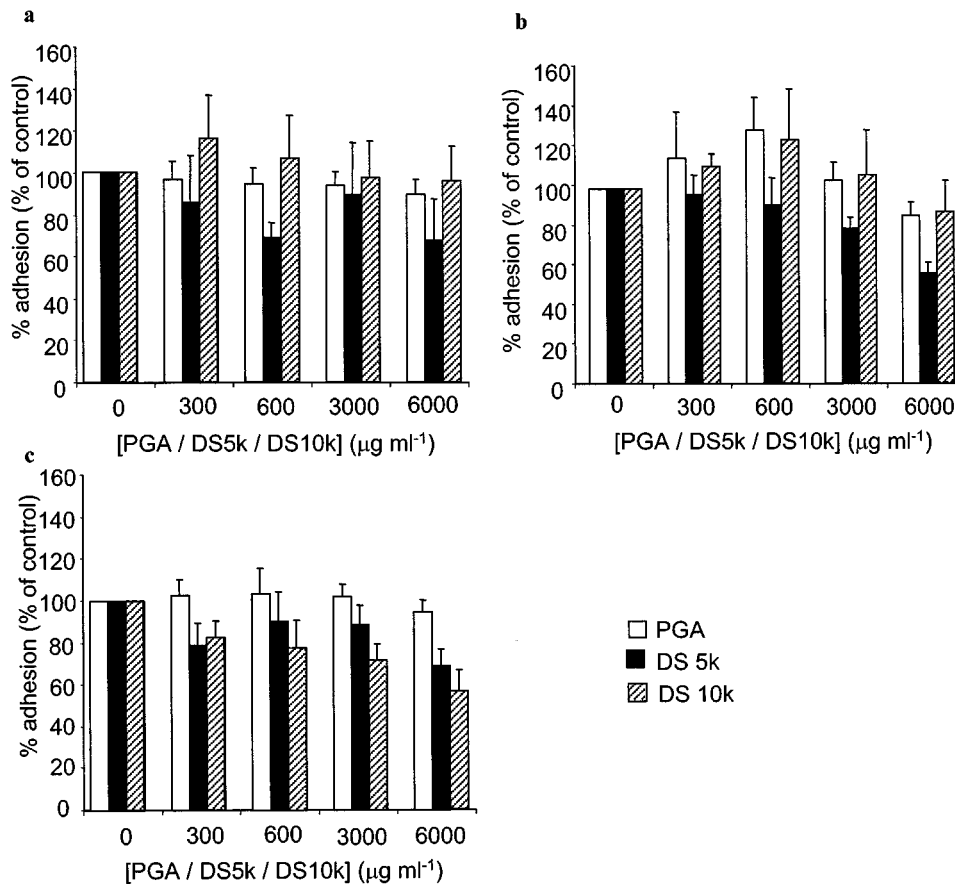
Figure 6 Effect of charged molecules on HPBMNC adhesion to HUVECs. HUVECs were stimulated with (a) IL-1β (100 u ml^{-1}), (b) TNF-α (1000 u ml^{-1}) or (c) LPS (100 μg ml^{-1}) for 6 h. ^{51}Cr-labelled HPBMNCs were added for 30 min, in the absence and presence of different concentrations of poly-L-glutamic acid (PGA, open bars) or dextran sulphates with molecular weights of 5000 (DS 5 k, closed bars) and 10,000 (DS 10 k, hatched bars), respectively. Adhesion was quantified by γ-counting lysates of adherent cells and was calculated as a percentage of that seen in control wells that had not been treated with PGA or dextrans. Data are expressed as the mean$\pm$s.e.mean of six separate experiments, each performed in triplicate. *Statistical significance ($P < 0.05$) is indicated.

effects of heparin on PMN adhesion, we found that adding UH with the PMNs led to a concentration related inhibition of adhesion (Lever *et al.*, 2000). Therefore, we repeated these experiments, comparing HPBMNC adhesion to that of PMNs, isolated from the same blood samples, in parallel. This difference in the effect of UH on adhesion of the two cell types, when included during the adhesion stage of the assay, was confirmed, in that HPBMNC adhesion increased whilst PMN adhesion decreased, in a manner dependent on the concentration of UH (Figure 4). ELISA experiments were carried out which demonstrated this short (30 min) exposure of stimulated HUVECs to UH to have no influence on levels of endothelial ICAM-1 or VCAM-1 expression (Table 1).

The selectively O-desulphated derivative of heparin (ODSH), devoid of anticoagulant activity, was found to mimic the effects of the parent molecule on HPBMNC adhesion. When present during cytokine- or LPS-stimulation of HUVECs, ODSH inhibited HPBMNC adhesion to these cells, following removal of unbound drug at the end of the stimulation period. Again, when added to stimulated HUVECs immediately prior to application of HPBMNC, adhesion was augmented (Figure 5).

The linear polyanion, PGA, had no effect on adhesion in our experiments, irrespective of the stage it was introduced to the assay. Of the two sulphated dextran preparations tested, DS 10 k had no effect on adhesion (Figure 6). DS 5 k was largely ineffective, although a small inhibitory effect was observed at the highest concentration, following treatment of HUVECs with this compound during stimulation with cytokines (Figure 7).

Discussion

The results of our study demonstrate that heparin is able to modulate the adhesion of mononuclear cells to vascular endothelial cells *in vitro*, in addition to its previously reported effects on granulocyte adhesion (Bazzoni *et al.*, 1992; Lever *et al.*, 2000; Silvestro *et al.*, 1994). However, we have found that depending upon the stage at which heparin is introduced to the adhesion assay, differential effects are observed, a phenomenon which would appear to be restricted to mononuclear cells, given that an inhibitory effect on neutrophil adhesion is seen under all the experimental conditions
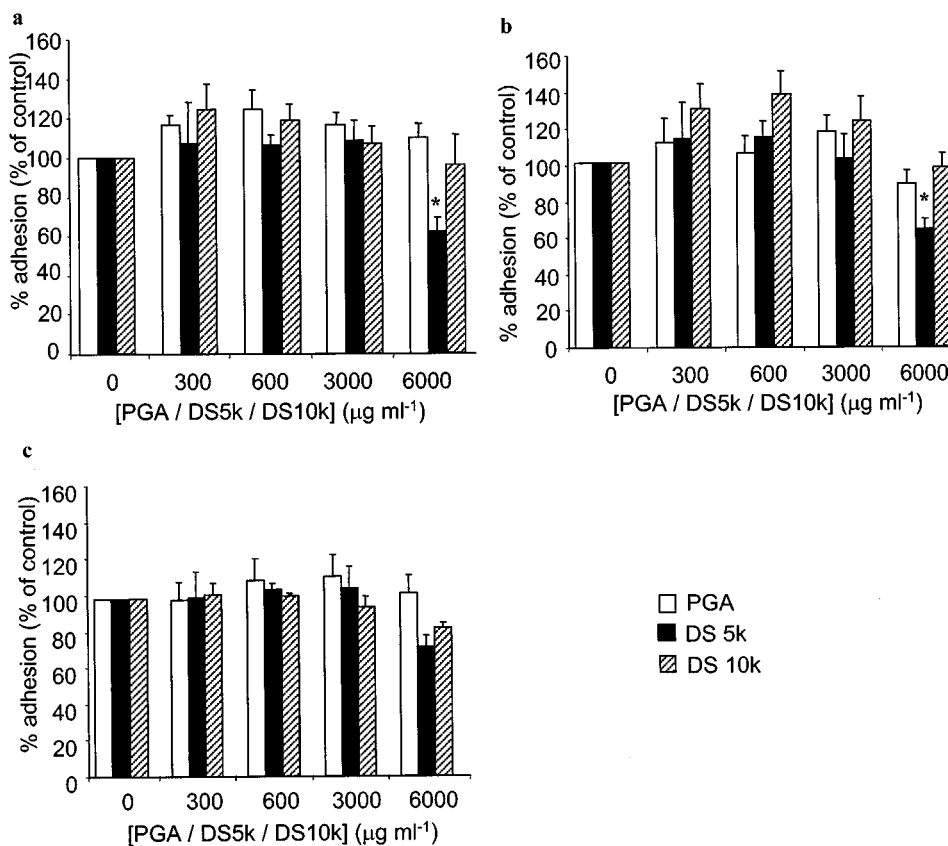
Figure 7 Effect of charged molecules on HPBMNC adhesion to HUVECs. HUVECs were stimulated with (a) IL-1β (100 u ml^{-1}), (b) TNF-α (1000 u ml^{-1}) or (c) LPS (100 μg ml^{-1}) for 6 h, in the absence and presence of different concentrations of poly-L-glutamic acid (PGA, open bars) or dextran sulphates with molecular weights of 5000 (DS 5 k, closed bars) and 10,000 (DS 10 k, hatched bars), respectively. ^{51}Cr-labelled HPBMNCs were added for 30 min and adhesion was quantified by γ-counting lysates of adherent cells and was calculated as a percentage of that seen in control wells that had not been treated with PGA or dextrans. Data are expressed as the mean $\pm$ s.e.mean of six separate experiments, each performed in triplicate. *Statistical significance ($P < 0.05$) is indicated.

relevant to this study. The reason for this difference is not clear. When heparin is included in the assay during endothelial stimulation, then subjected to a washing stage prior to addition of mononuclear cells, adhesion is inhibited. Part of this effect may be due to binding and inactivation of the stimuli used to activate endothelial cells. However, in our previous studies, using the same endothelial stimuli but looking at neutrophil adhesion, this was not the predominant mechanism of action of heparin and related molecules, given that upregulation of the adhesion molecules ICAM-1 and E-selectin was affected only minimally by the drug and that inhibition of PMN adhesion to pre-stimulated HUVECs was achieved when heparin was added along with the leucocytes (Lever *et al*., 2000). Conversely, in the present study, heparin added with the mononuclear cells significantly increased adhesion, in a concentration dependent manner. It is possible that the inhibitory effects of heparin on mononuclear cell adhesion are related to inhibition of VCAM-1 expression on HUVECs, a molecule involved in mononuclear (Osborn *et al*., 1989) but not neutrophil (Hemler *et al*., 1987; Walsh *et al*., 1991a) adhesion. Indeed, in our studies, upregulation of VCAM-1 was found to be more sensitive to the presence of heparin than other endothelial adhesion molecules we have

measured. Similarly, the mechanisms by which heparin, in our alternative experimental protocol, increased mononuclear cell adhesion are equally indefinable at this stage. One theory is that heparin has an intercellular 'bridging' effect, binding structures on the mononuclear cell surface and on the endothelium, thus tethering one cell to the other. Heparin is known to bind readily to endothelium (Hiebert & Jacques, 1976; Bârzu *et al*., 1986) and possible candidates on the leucocyte surface include the adhesion molecule L-selectin, which is known, physiologically, to interact with heparan sulphate on the endothelial cell surface (Giuffrè *et al*., 1997) and to bind heparin (Koenig *et al*., 1998). However, if this were the case, one might expect the same effect to occur with neutrophils when, in fact, the opposite is seen. Moreover, binding of the integrin adhesion molecule mac-1 (Diamond *et al*., 1995) on neutrophils has been suggested to be at least partially responsible for the inhibitory effects of heparin on neutrophil adhesion (Salas *et al*., 2000; Silvestro *et al*., 1994). It is, however, plausible that such a 'bridging' effect may depend on the concentration of heparin available in the system and whether soluble or bound. In support of this suggestion, in the first part of our investigation, where inhibition of HPBMNC adhesion was observed, only heparin

which had bound to endothelial cells would have been present at the same time as the leucocytes. In our latter experiments, reasonably large amounts of, at least initially, soluble heparin were introduced at the same time as the HPBMNCs.

It is possible that heparin, a large and heterogenous polymer, when bound to endothelium, hinders specific leucocyte-endothelial adhesive interactions through steric interference, whereas molecules of the GAG in solution may be able to facilitate these interactions, as is now established to occur between heparin, basic fibroblast growth factor and its receptor (Schmidt *et al*., 1999). Given that neutrophils utilize different adhesion mechanisms to mononuclear cells, it does not necessarily follow that such an effect should be universal to all inflammatory cells.

The observed effects of unfractionated heparin on mononuclear cell adhesion, both inhibitory and augmentative, were shared by a selectively O-desulphated derivative of the GAG. As this molecule lacks the anticoagulant activity of the parent molecule, these data indicate that effects on mononuclear cell adhesion are separable from this property, as has been found to be the case with regard to many of the effects of heparin on inflammatory functions, including neutrophil adhesion (Lever *et al*., 2000), delayed type hypersensitivity reactions (Sy *et al*., 1983), and eosinophil trafficking (Seeds & Page, 2001). Moreover, unfractionated heparin has the ability to bind directly to serine proteases such as thrombin, in addition to its well established capacity to catalyze the inactivation of thrombin by antithrombin III (Damus *et al*., 1973). Given that thrombin is, in itself, a proinflammatory mediator, as well as the fact that haemostasis and inflammation are closely linked processes, this provides a further mechanism by which unfractionated heparin might be anti-inflammatory. However, since heparin molecules which lack the ability to bind thrombin and/or antithrombin III retain anti-inflammatory effects, both *in vitro* and *in vivo*, additional mechanisms must be involved (Ahmed *et al*., 1997; 2000; Campo *et al*., 1999).

In order to examine the contribution of anionic charge and, more specifically, sulphate-derived charge to the effects of heparin in our assay, we carried out the same experiments using the polyanionic molecule PGA and observed no effects on adhesion under any of the experimental conditions, suggesting that negative charge alone is not responsible for the effects of heparin. Again, this observation is in accordance with previous reports, which have found PGA to lack the effects of heparin on neutrophil adhesion (Lever *et al*., 2000) and accumulation of inflammatory cells *in vivo* (Sasaki *et al*., 1993; Seeds *et al*., 1993). As sulphation is deemed to be essential for many of the biological properties of this drug, we examined the effects of two dextran sulphate preparations, of different molecular weights and found, on the whole, these molecules to lack effect in our experiments. These results suggest that specific patterns of sulphation are involved in the effects of heparin on mononuclear cell adhesion, or the relative flexibility of the heparin chain, which exists as an α-helix at physiological pH (Mulloy *et al*., 1996), possibly rendering sulphate charges more accessible to relevant binding sites on leucocytes, endothelial cells or inflammatory mediators. Whereas dextran sulphates have been reported previously to inhibit neutrophil rolling in rabbit mesenteric venules (Ley *et al*., 1991), it is likely that such results, obtained *in vivo*, are a consequence of interference with carbohydrate interactions involved in leucocyte rolling, whereas our results reflect a lack of effect on firm adhesion mechanisms relevant to mononuclear cells. Since O-desulphated heparin behaves in a similar way to unmodified heparin in our assays, it will be interesting to see the effects of a selectively N-desulphated derivative of unfractionated heparin in such experiments, in order to investigate further the significance of sulphate groups and their molecular location with respect to mononuclear cell adhesion.

In summary, we have found the GAG heparin to possess mixed effects on the adhesion of HPBMNCs to stimulated endothelial cells *in vitro*, which are unrelated to anticoagulant mechanisms and which cannot be explained entirely by the physicochemical nature of the molecule.

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