Differential effect of the low-molecular-weight heparin, dalteparin, and unfractionated heparin on microvascular endothelial cell hemostatic properties

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Background and Objectives. Heparins, including unfractionated heparin (UFH) and low-molecular-weight heparins (LMWH), are glycosaminoglycans that are largely used as anti-thrombotic drugs. While the mechanisms of their anticoagulant actions in blood have been extensively studied, their effects on the hemostatic properties of the endothelium are still under investigation. The aim of this study was to compare the antithrombotic effects of a LMWH, i.e. dalteparin, with UFH on both microvascular (human microvascular endothelial cells [HMEC-1]) and macrovascular (human umbilical vein endothelial cells [HUVEC]) endothelial cells.

Design and Methods. Endothelial cells were incubated with dalteparin or UFH and exposed to an inflammatory stimulus (i.e. lipopolysaccharide [LPS]). The following parameters were evaluated: tissue factor (TF procoagulant activity, antigen and mRNA), tissue factor pathway inhibitor (TFPI), and thrombomodulin (TM).

Results. In HMEC-1 and HUVEC, both heparins inhibited LPS-induced endothelial cell TF expression. However, in HMEC-1, dalteparin was significantly more effective than UFH. Both heparins increased TFPI antigen release in HMEC-1 and HUVEC. Dalteparin also reversed LPS-induced reduction of TM in HMEC-1, while UFH did not.

Interpretation and Conclusions. These data show that both dalteparin and UFH suppress inflammatory-mediated TF expression and increase the anticoagulant properties of macro- and micro-vascular endothelial cells. However, dalteparin has significantly greater effects than UFH in the microvascular endothelium, a site that plays a central role in many processes involved in inflammation and thrombosis.

Key words: heparin, low-molecular-weight heparin, endothelial cells, tissue factor, thrombomodulin, tissue factor pathway inhibitor.

Haematologica 2006; 91:207-214

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Unfractionated heparin (UFH) and its low molecular weight (LMWH) derivatives are glycosaminoglycans used in clinical practice for the prevention and treatment of venous thromboembolism.[1] Heparins exert anticoagulant effects by accelerating the rate of factor Xa and thrombin inhibition through binding to antithrombin. In addition, heparins possess a broad variety of biological activities, including anti-inflammatory, antihypertensive, and antitumor properties.[2,3] It appears that heparins may have important actions on the vascular endothelium, which plays a key role in numerous physio-pathological processes, including hemostasis, inflammation, leukocyte trafficking, angiogenesis and tumor metastasis.[4] Under physiological conditions, endothelial cells generate an active antithrombotic surface that inhibits clotting activation and the adhesion of platelets and other circulating cells. However, when exposed to physical or biochemical stimuli, the endothelium undergoes a series of phenotypic changes that produce a shift to a prothrombotic condition. In particular, bacterial products (e.g. lipopolysaccharide, LPS), as well as inflammatory cytokines, stimulate endothelial cells to express procoagulant tissue factor (TF), downregulate anticoagulant thrombomodulin (TM), produce fibrinolytic inhibitors (e.g. plasminogen activator inhibitor-1 [PAI-1]), and express cell adhesion molecules. These modifications can lead to the activation of the blood clotting system, which culminates in fibrin generation, in addition to increasing cellular adhesion processes, which can further stimulate localized coagulation. These phenomena are also involved in the progression of chronic inflammatory diseases,[4,5] and are of particular relevance at the level of the microcirculation, where the interactions between the vascular wall and circulating cells are enhanced. In this context, it is of interest to determine the role of heparins at the microvascular level. Several *in vitro* studies have evaluated the impact of heparins on the hemostatic properties of the macrovascular endothelium, represented by human umbilical vein endothelial cells (HUVEC).[6-9] However, no data are currently available regarding the effect of heparins on microvascular endothelial cells, which have different properties to HUVEC.[10]

The aims of the present study were: (i) to evaluate whether LMWH can counteract the prothrombotic phenotype of microvascular

endothelial cells induced by a standard pro-inflammatory stimulus (i.e. LPS); (ii) to compare the responses of the microvascular endothelium with those of HUVEC; and (iii) to verify whether the effects of LMWH on the hemostatic properties of the two endothelial cell types differ from those of UFH. In particular, the effect of the LMWH, dalteparin, and UFH were compared in terms of the expression of endothelial TF procoagulant activity and anticoagulant properties (i.e. expression of TM and TF pathway inhibitor [TFPI]), in the presence and absence of LPS.

Design and Methods

Endothelial cells

The immortalized human microvascular endothelial cell line-1 (HMEC-1) was kindly supplied by Dr F.J.Candal (CDC, Centers for Disease Control and Prevention, Atlanta, GA, USA). To date, this is the best-characterized microvascular endothelial cell line.[11] HMEC-1 were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco, Gaithersburg, MD, USA), 100 μg/mL streptomycin, 100 U/mL penicillin, 5 μg/mL amphotericin B (Bristol-Myers Squibb, Princeton, NJ, USA), 2 mmol/L L-glutamine (Gibco), 10 ng/mL epidermal growth factor (ICN, Costa Mesa, CA, USA) and 1 μg/mL hydrocortisone (ICN). HMEC-1 were serially passaged in T25 flasks (Falcon, Becton Dickinson, Mountain View, CA, USA) and, at passages 8 to 16, seeded onto 24-well cell culture plates at the concentration of 30,000 cells/well, then grown to confluence in complete growth culture medium.[12] The cells reached confluence in 3 to 4 days.

HUVEC were freshly isolated from human umbilical cord veins, as previously described,[13] and grown in T25 flasks in RPMI 1640 medium supplemented with 20% fetal calf serum, 100 μg/mL streptomycin, 100 U/mL penicillin, 5 μg/mL amphotericin B and 2 mmol/L L-glutamine. Confluent primary cultures were harvested using 0.25% trypsin/0.02% EDTA solution (Sigma, St Louis, MO, USA), seeded onto 24-well cell culture plates pre-coated with 0.5% gelatin (Sigma) at the concentration of 30,000 cells/well and grown to confluence in complete growth culture medium. The cells reached confluence in 3 to 4 days. Both types of endothelial cells were grown in a humidified incubator in an atmosphere of 5% CO_2/95% air. At the end of each experiment, endothelial cells were counted by microscopy and the trypan blue exclusion test was used to determine cell viability. More than 95% of cells resulted viable in all experiments.

Experimental system

Confluent endothelial cell monolayers in 24-well plates were washed twice with RPMI 1640 medium and then incubated with RPMI 1640 medium (supplemented with 10% fetal calf serum and 2 mmol/L glutamine) containing either dalteparin (Fragmin®, Pharmacia legacy, Sweden) or UFH (Vister®, Parke-Davis, Lainate, Milan, Italy), at 0.01, 0.1, 1 and 10 IU/mL f.c., or saline (control cells) for 3 hours for TF mRNA measurement, 4-24 hours for TF activity and antigen assays, and up to 72 hours for TFPI and TM analysis. To evaluate the potential anti-inflammatory activity of both heparins, the study was also conducted in the presence of 10 μg/mL LPS (Lipopolysaccharide endotoxin from *Escherichia coli*, Sigma) or saline (control cells). Heparin doses were selected on the basis of the plasma concentrations achieved *in vivo* during therapy of venous thromboembolism. After incubation, endothelial cell treatment media were collected and centrifuged for 10 min at 13,000 rpm, the supernatants were then filtered through a 0.22 μm filter to eliminate cell debris, and stored at –40°C until assays. Endothelial cell monolayers were washed three times with phosphate-buffered saline (pH 7.4), to eliminate any heparin residues, and different endothelial cell samples were prepared as detailed below.

TF procoagulant activity

TF activity was evaluated in endothelial cell lysates (5×10^5 cells/mL in phosphate-buffered saline), obtained after three cycles of freezing/thawing, by the one-stage recalcification assay of normal human plasma, as previously described.[14] TF activity was identified and characterized as being associated with TF by the clotting assay of factor VII-, VIII- or X-deficient human plasmas (FVII-D, FVIII-D, FX-D, DADE Behring, Milan, Italy). In some experiments, TF activity was further characterized by incubating (15 min at 37°C) endothelial cell samples with a purified polyclonal rabbit IgG antibody (1 mg/mL) directed against human TF (#4502, American Diagnostica Inc., Greenwich, CT, USA) before the clotting assay. A normal non-immune rabbit IgG was the control in this assay. TF activity was referred to a calibration curve constructed with different dilutions (from 10^{-1} to 10^{-6}) of a standard rabbit brain thromboplastin (RBT; Sigma). Results are expressed as standard thromboplastin arbitrary units (RBT units): 1 unit = the activity of 1 mEquiv/mL of RBT in the coagulation assay.

TF antigen

TF antigen levels were measured by ELISA (Imubind Tissue Factor ELISA Kit; American Diagnostica) in endothelial cells extracted in TRIS buffer (5×10^5 cells/mL of 50 mmol/L Tris buffer, 100 mmol/L NaCl and 1% Triton X-100; pH 7.5) for 2 hours at 4°C, followed by three cycles of freezing/thawing. The ELISA was performed according to the manufacturer's instructions. Results are expressed as pg/10^5 cells.[14]

TF mRNA

Total cellular RNA was extracted using Trizol reagent (Life Technologies, Paisley, Scotland) according to the manufacturer's instructions. RNA was reverse-transcribed and used for parallel assays of TF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA by polymerase chain reaction (PCR) amplification. GAPDH acted as the internal control to normalize sample data to account for sample-sample differences.[15] The following oligonucleotides were used: F1 (sense: bp 178 to 198) and R1 (antisense: bp 495 to 515) from the coding sequence of the human TF, and GF1 (sense: bp 64 to 86) and GR1 (antisense: bp 581 to 603) from the coding sequence of the human GAPDH (Laboratoires Eurobio, Les Ulis, France). Quantitative PCR was performed with 5 μL of cDNA, as previously described.[15] The PCR products from TF (317 bp) and GAPDH (528 bp) were analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide. Band

intensities were quantified by densitometric analysis (ImageJ software, National Institutes of Health, Bethesda, MD, USA). The results are expressed as the ratio of TF band intensity/GAPDH band intensity.

TFPI antigen

Antigenic levels of TFPI were measured in endothelial cell cultured media and cell extracts (prepared as for the TF antigen measurements) by ELISA using a commercially available kit (Imubind TFPI, American Diagnostica) according to the manufacturer's instructions. This ELISA measures both free and bound forms of TFPI. Results are expressed as ng/mL.

TM antigen

The levels of TM antigen were measured in endothelial cell extracts (prepared as for the TF antigen measurements) by a commercial ELISA kit (Asserachrom thrombomodulin, Roche Diagnostics), as previously described.[16] Results are expressed as ng/10^5 cells.

Statistical analysis

The results are reported as mean ± SD from at least five independent experiments performed in triplicate. Student's paired t-test was used for the determination of significance levels between treatments. Differences were considered statistically significant when p values were <0.05.

Results

Effect of heparins on TF expression in stimulated EC (with LPS)

In preliminary experiments, treatment of HMEC-1 and HUVEC with LPS induced a time-dependent increase in TF expression evaluated as activity, antigen, and mRNA, with a maximum effect at 4-6 hours and then a progressive decline towards basal values at 24-48 hours. In order to evaluate the activity of heparins on LPS-induced TF expression, we choose the 4- and 24- hour time points, i.e. when the effect of the LPS stimulus was maximum or when it was greatly reduced, respectively. Treatment of HMEC-1 with LPS for 4 hours induced a significant increase in TF activity (control: 14.5±1.3, LPS: 46.2±5.2 RBT units/10^5 cells; p=0.001). The incubation of endothelial cells with either dalteparin or UFH counteracted the LPS-induced TF activity (Figure 1). Particularly, dalteparin significantly inhibited TF activity, reaching a maximum inhibition of 96% at 1 IU/mL (15.8±1.8 RBT units/10^5 cells, p=0.031 vs LPS) (Figure 1A), whereas UFH showed a maximum inhibitory effect of 55% with the dose of 10 IU/ml (28.6±2.8 RBT units/10^5 cells, p=0.038 vs LPS). Remarkably, TF inhibition by dalteparin was significantly greater than that of UFH at the doses of 0.1 IU/mL (0.012), 1 IU/mL (p=0.002) and 10 IU/mL (p=0.031). As observed in HMEC-1, 4 hours of incubation with LPS induced a significant increase in TF activity also in HUVEC (control: 9.9±0.8, LPS: 89.2±9.5 RBT units/10^5 cells; p=0.001). However, differently from what we found in HMEC-1, in these cells, heparins reduced LPS-induced TF activity to a similar extent (Figure 1B). In particular, at a concentration of 10 IU/mL, dalteparin significantly inhibited TF activity by 37% (62.3±6.4 RBT units/10^5 cells, p=0.002) compared with a significant reduction of 40 % for UFH (59.6±6.1 RBT units/10^5 cells; p=0.004).

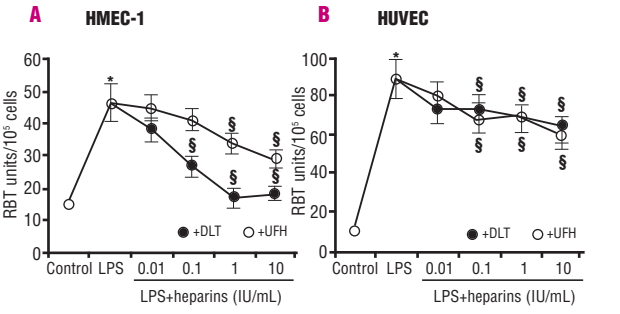

Figure 1. Effect of heparins on TF procoagulant activity expression by HMEC-1 (A) and HUVEC (B). TF activity was assayed in lysates of endothelial cells treated for 4 hours with LPS in the presence or absence of increasing doses of dalteparin (DLT) or UFH. The control was vehicle-treated endothelial cells. Data represent the mean±SD of five independent experiments performed in triplicate. *p<0.05 vs control; §p<0.05 vs LPS.

The measurement of TF antigen levels in the same experimental conditions showed that, similarly to TF activity, in HMEC-1, the antigenic TF expression induced by LPS was significantly (p=0.014) more inhibited by dalteparin (87±6% inhibition) than by UFH (67±8% inhibition) (Figure 2A). In HUVEC, both heparins reduced LPS-induced TF antigen levels to the same extent, confirming the results obtained with TF activity (dalteparin vs UFH: 45 ±13 % vs 43±5.7% inhibition; p=ns) (Figure 2B).

To evaluate whether the observed inhibition of TF activity and antigen expression was due to a possible effect of heparins on TF gene transcription, the levels of TF mRNA in HMEC-1 and HUVEC treated with heparins in the presence of LPS were evaluated by reverse transcriptase (RT)-PCR. The TF-mRNA analysis showed that control samples of both HMEC-1 and HUVEC expressed very low levels of TF-mRNA (Figure 3). Treatment with LPS markedly increased mRNA levels compared with levels in control cells, but not in the presence of heparins. In HMEC-1, dalteparin was more effective than UFH, with a 96% mean inhibition of LPS-induced TF-mRNA, compared to 72% inhibition with UFH (Figure 3A). In HUVEC, no significant differences were observed between the two heparins, both of which caused about 75 % inhibition of LPS-induced TF-mRNA (Figure 3B). TF expression was also measured after 24 hours of heparin treatments (Table 1). In both cell types, the LPS-induced increase in TF expression remained significantly higher than that in control cells, although it was inferior to that observed after the 4-hour incubation. The increase in TF activity in both HMEC-1 and HUVEC was significantly inhibited by both dalteparin and UFH. However, in contrast to the observations after 4 hours of incubation, the degree of TF inhibition in HMEC-1 after 24 hours of incubation was comparable for the two heparins. Similar levels of TF inhibition were obtained with the assay of TF antigen levels (Table 1).

Effect of heparins on TFPI in unstimulated endothelial cells (without LPS)

To evaluate the effect of heparins on endothelial cell anti-

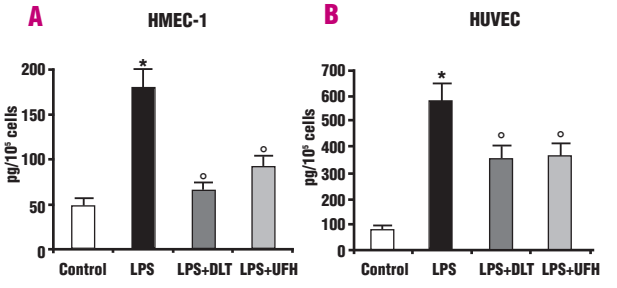

Figure 2. Effect of heparins on TF antigen levels in LPS-stimulated HMEC-1 (A) and HUVEC (B). TF antigen was measured in extracts of endothelial cells treated for 4 hours with LPS in the presence or absence of 10 IU/mL dalteparin or UFH. Data represent the mean±SD of five independent experiments performed in triplicate. $p<0.05$ vs control; °$p<0.05$ vs LPS.

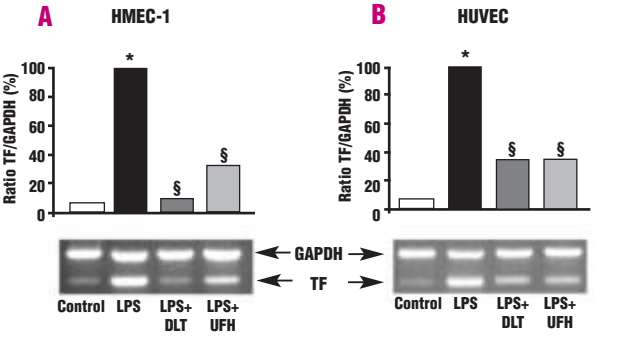

Figure 3. TF mRNA levels in HMEC-1 (A) and HUVEC (B) stimulated with LPS for 3 hours in the presence or absence of 10 IU/mL dalteparin or UFH. Quantification of the representative experiment is reported here, while statistical significances refer to the mean±SD of three independent experiments. *$p<0.05$ versus control; °$p<0.05$ versus LPS.

coagulant activity, the expression of TFPI by HMEC-1 and HUVEC was measured in basal condition (without LPS) and after stimulation with LPS. In unstimulated conditions, both heparins increased TFPI antigen levels in HMEC-1 cultured media at all the evaluated doses and all incubation times. The 1 IU/mL dose was the most effective for both heparins and produced a maximum effect on TFPI release after 72 hours of incubation (control: 34.7±2.9 ng/mL; dalteparin: 62.5±6.7 ng/mL, p=0.048; UFH: 70.5±8.3 ng/mL, p=0.021) (Figure 4A). In parallel to the induction of TFPI release in HMEC-1 cultured media, heparins significantly reduced TFPI levels in HMEC-1 extracts starting from 4 hours of incubation (Figure 4B). The most effective dose was 1 IU/mL for both heparins: at this dose, cell-associated TFPI levels were reduced 60% by dalteparin (control: 8.6±1.1 ng/mL, dalteparin: 3.4±0.4 ng/mL, p=0.032 vs control) and reduced 53.1% by UFH (4.08±0.4 ng/mL, p=0.038 vs control). This reduction was more marked after 24 hours of incubation, with mean reductions of 70 % for dalteparin (control: 5.51±0.7 ng/mL; dalteparin: 1.51±0.15 ng/mL, p=0.032) and 72.1% for UFH (1.2±0.11 ng/mL, p=0.019 vs control). After incubation for 48 and 72 hours, significant differences persisted between TFPI levels in heparin-treated cells and control cells, although these were less pronounced.

In HUVEC cultured media, an increase in TFPI antigen levels was observed for all incubation times with both dalteparin and UFH. As observed in HMEC-1, the 1 IU/mL was the most effective dose for both heparins, with a maximum increase in TFPI antigen levels observed after 48-hour incubation, i.e. 97% by dalteparin and 142% by UFH (control: 6.3±0.71 ng/ml; dalteparin: 12.4±1.4 ng/mL, p=0.005; UFH: 15.2±1.81 ng/mL, p=0.002) (Figure 4C). The increase in TFPI levels detected in the culture media of heparin-treated cells was much greater than the reduction observed in the levels of cell-associated TFPI. We could not perform the study in HUVEC extracts, because the levels of TFPI antigen in the extracts of these cells were below the detection limit of the ELISA (i.e.: 0.336 ng/mL).

Effect of heparins on TFPI in stimulated endothelial cells (with LPS)

The results of TFPI modulation by heparins in LPS- stimulated conditions showed that LPS incubation (Figure 5A) significantly increased TFPI levels in HMEC-1 cultured media, compared with vehicle-treated control cells, at all evaluated time points. The TFPI increase was maximum after 48 hours of incubation (control: 14.8±1.9 ng/mL; LPS: 23.5±2.1 ng/mL; p=0.001). Interestingly, in LPS-stimulated endothelial cells, heparins further increased TFPI release in culture media at all time points. As in unstimulated conditions, the heparin dose of 1 IU/mL was more effective than the 10 IU/mL for both heparins. After incubation for 72 hours, the following TFPI values were measured with the 1 IU/mL dose of heparin: LPS: 42.6±5.1 ng/mL; dalteparin+LPS: 78.5±9.5 ng/mL, p=0.031 vs LPS; UFH+LPS: 89.1±7.6 ng/mL, p=0.029 vs LPS.

Differently from what observed in cultured media of HMEC-1, LPS incubation did not significantly affect the levels of cell-associated TFPI (Figure 5B). However, the presence of dalteparin or UFH significantly reduced cell-associated TFPI compared to LPS alone, with no significant differences between the various doses of heparins utilized. After 4-hour incubation, maximum cell-associated TFPI reductions were observed. Mean reductions at the 1 IU/ml dose were the following: dalteparin: 73±5.9%, p=0.019 vs

Table 1. Levels of TF activity (RBT units/10^5 cells) and TF antigen (ng/10^5 cells) in HMEC-1 and HUVEC lysates after incubation for 24 hours with 10 μg/mL LPS in the presence or absence of 10U/mL of dalteparin (DLT) or UFH.

	HMEC-1	
	TF activity	*TF antigen*
Control	10.5±1.3	40.2±10
LPS	27.2±3.3*	96.8±16*
LPS+DLT	15.4±2.5°	65.7±12°
LPS+UFH	16.8±2.4°	69.4±11°
	HUVEC	
	TF activity	*TF antigen*
Control	4.8±0.7	81.1±15
LPS	23.7±2.6*	383±43*
LPS+DLT	11.3±1.9°	203±28°
LPS+UFH	12.4±1.4°	221±31°

**p<0.05 versus control; °p<0.05 versus LPS. Data are expressed as mean ± SD of three independent experiments.*

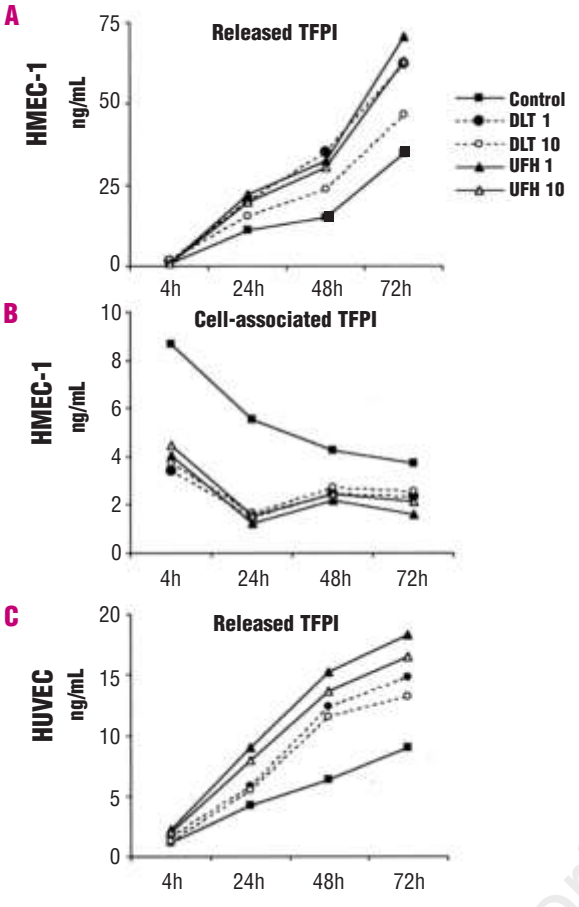

Figure 4. Effect of heparins on TFPI antigen expression by unstimulated endothelial cells (without LPS). TFPI was measured in cultured media (A) or cell extracts (B) of HMEC-1 or in cultured media of HUVEC (C), treated for 4, 24, 48 and 72 hours with 1 and 10 IU/mL of dalteparin or UFH. The control cells were vehicle-treated endothelial cells. Data represent the mean values from five experiments performed in triplicate.

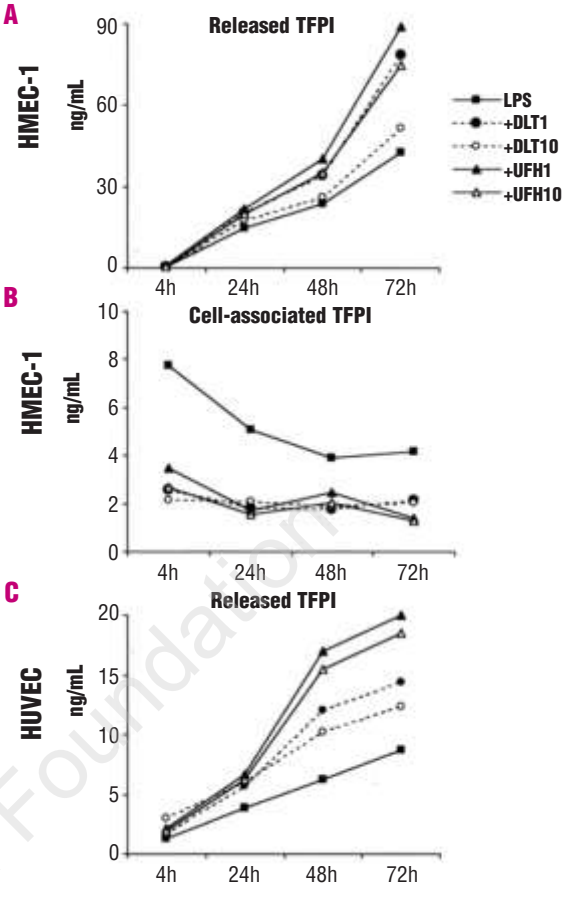

Figure 5. Effect of heparins on TFPI antigen expression by LPS-stimulated endothelial cells. TFPI levels were measured in cultured media (A) or cell extracts (B) of HMEC-1, and in cultured media of HUVEC (C), treated for 4, 24, 48 and 72 hours with 1 and 10 IU/mL of dalteparin or UFH in the presence of LPS. The control cells were vehicle-treated endothelial cells. Data represent the mean values from five experiments performed in triplicate.

LPS; UFH: 65±6.6 %, p=0.027 vs LPS. LPS did not significantly affect TFPI levels in cultured media of HUVEC, and no additive effects were observed when HUVEC were incubated with LPS in the presence of dalteparin or UFH (Figure 5C).

Effect of heparin on TM in unstimulated endothelial cells (without LPS)

In order to evaluate the effect of heparins on another anticoagulant property of endothelial cells, measurements of endothelial TM expression were conducted in both unstimulated and LPS stimulated conditions. In unstimulated conditions, TM antigen levels were significantly increased in HMEC-1 treated with 10 IU/mL of dalteparin for 24 hours (31±13% increase, p=0.04), 48 hours (28±12.1% increase, p=0.018) and 72 hours (20±8.8% increase, p=0.041), compared with control cells (Figure 6A). No increases in TM antigen levels were evident when the cells were treated with lower concentrations of dalteparin. In contrast, TM expression was not significantly affected by UFH treatment after incubation periods of up to 72 hours at all doses. In HUVEC, no significant effect of heparins was observed up to 24 hours of incubation (Figure 6C). However, after 48- and 72-hour incubation, both dalteparin and UFH increased the cell TM antigen levels. These results reached statistical significance at the dose of 10 IU/mL for both heparins.

Effect of heparins on TM in stimulated endothelial cells (with LPS)

In HMEC-1, 24-hour incubation with LPS induced a significant reduction in TM levels (11.8±1.0 ng/10^5 cells) compared with levels control cells (23±1.7 ng/10^5 cells; p<0.05) (Figure 6B). This effect of LPS was only observed after 24 hours of incubation, and not at other time points. This reduction in TM antigen levels was significantly counteracted by co-incubation of the cells with 10 IU/mL dalteparin, which prevented the LPS-induced reduction in TM antigen levels by 41% (16.4±1.31 ng/10^5 cells, p=0.01) (Figure 6B). As observed in unstimulated conditions, UFH did not show a significant effect on TM antigen levels in LPS-treated HMEC-1. LPS significantly reduced TM levels in HUVEC at all incubation times evaluated. Dalteparin and UFH prevented the reduction in TM levels, with no

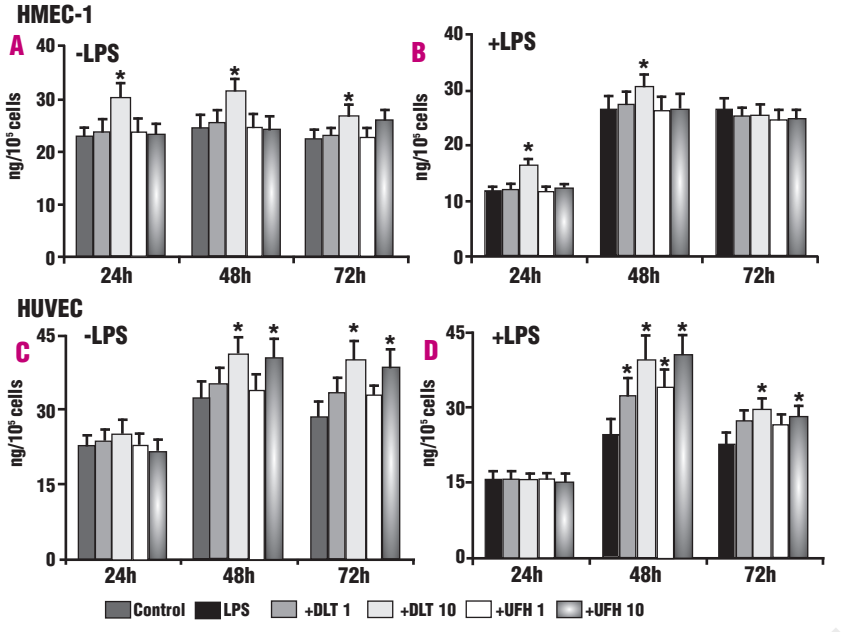

Figure 6. TM antigen expression according to incubation time in unstimulated or LPS-stimulated endothelial cells. TM was measured in extracts of HMEC-1 and HUVEC after treatment with 1 and 10 IU/mL dalteparin (DLT) or UFH, in the absence (A and C) or in the presence (B and D) of LPS. Data are the mean±SD of five independent experiments performed in triplicate. *p<0.05 vs control or LPS-treated cells.

significant differences between the two agents, starting from 48-hour incubation (Figure 6D). At this time, the maximum effect was achieved with 10 IU/mL of dalteparin (control: 32.7±3 ng/10^5 cells; LPS: 24.7±3.2 ng/10^5 cells, dalteparin: 39.8±4.6 ng/10^5 cells; p=0.01 vs LPS) or UFH (40.4±3.8 ng/10^5 cells; p=0.009 *vs* LPS).

Discussion

Heparins inhibit blood coagulation but also interfere with the physiological and pathological processes involved in inflammation and chronic diseases.[2,17] The underlying mechanisms are likely to include an action of these agents on the prothrombotic and pro-angiogenic properties of the vascular endothelium. The present study is the first to evaluate the effect of heparins (the LMWH, dalteparin, and UFH) on the hemostatic properties of microvascular endothelial cells as compared to HUVEC. Our study indicates that both dalteparin and UFH have an antithrombotic effect on endothelial cells by suppressing inflammatory stimulus-mediated procoagulant TF expression and by enhancing anticoagulant TM expression and TFPI release. However, we found significant differences in responses to these two heparins between the two types of endothelial cells. With regard to TF modulation, we found that: (i) dalteparin and UFH inhibited LPS-induced TF activity and antigen in HMEC-1 to a greater extent than in HUVEC, (ii) in HMEC-1 dalteparin was more active than UFH, and (iii) in HUVEC the two heparins had comparable inhibitory activity. The differences between the two heparins on microvascular endothelial cell TF expression were observed after 4 hours of incubation but they did not persist after longer incubation (i.e. 24 hours). However, it should be noted that LPS-stimulated TF expression by HUVEC has been shown to be maximal after 4 to 8 hours of incubation, and it declines thereafter.[22]

Our data on HMEC-1 are consistent with these findings. Our data show that dalteparin and UFH reduced TF activity and antigen levels, and also reduced TF mRNA levels in both endothelial cell types, suggesting that heparin-induced inhibition of TF expression occurs, at least in part, at the transcriptional level. Heparin has been shown to inhibit cell proliferation by preventing the activation of the mitogen-activated protein kinase kinase-1 (MAPKK-1).[23] Interestingly, MAPKK-1 activation in endothelial cells can be sensitive to LPS or cytokines, and is involved in the activation of the transcription factor complex NF-κB. This transcriptional complex is implicated in TF gene transcription,[24] and, as recently demonstrated, in TM repression by inflammatory cytokines.[25]

Previous studies have reported an inhibitory effect of heparins (both UFH and LMWH) on endothelial TF expression.[7-9] However, these studies were performed in different experimental conditions to those of the present study; different heparins were used and the experiments were conducted mainly in HUVEC. Cadroy *et al.*[9] showed that UFH reduces LPS-induced TF activity in HUVEC. Another study in unstimulated HUVEC showed a reduction in cellular TF activity by UFH and the LMWH enoxaparin[7] In that study, UFH produced significantly greater inhibition of TF activity than enoxaparin, although the heparins were only evaluated at one concentration (10 IU/mL). Interestingly, one study performed with the human endothelial cell line, EA.hy926, showed that dalteparin was more effective than UFH in reducing LPS-induced TF expression, and this is consistent with our observation in HMEC-1.[8]

Our study also examined the effect of heparins on the antithrombotic properties of endothelial cells, in particular, on the expression of the two main inhibitors of blood coagulation expressed by the endothelium, TFPI and TM. TFPI is a potent inhibitor of the extrinsic coagulation pathway and is constitutively synthesized by the vascular endothelium.[18] It acts by inhibiting the catalytic activity of factor Xa and of factor VIIa-TF complexes in the presence of factor Xa. TFPI is released from the endothelium by heparins, a

mechanism that contributes to the antithrombotic activity of this drug.[19] In particular, heparins release the pool of TFPI bound to the negatively charged glycosaminoglycans located on the luminal surface of the endothelial cells. This heparin-releasable TFPI is mainly constituted by the free form, which possesses a higher anti-factor Xa inhibitory activity compared to the truncated forms, i.e. without the C-terminal region, which normally circulate in the blood. Our results show that dalteparin and UFH increase TFPI release into endothelial cell cultured media, both in HMEC-1 and HUVEC. Similar effects have been described previously in HUVEC or other types of macrovascular endothelial cells,[6,8,20,21] but this is the first time they have been observed in an endothelium of microvascular origin. This heparin effect on TFPI release is maximum at the dose of 1 IU/mL and is associated with a reduction of the levels of this protein in cells, as we observed in HMEC-1 (in HUVEC, the study of cell-associated TFPI was not possible due to the low TFPI expression in cell extracts). In addition, we found that UFH was more effective than LMWH in this respect. Our study is the first to include an analysis of TFPI release in LPS-stimulated endothelial cells. In HMEC-1, both heparins were shown to further increase LPS-induced TFPI release. In contrast, LPS treatment did not affect TFPI release in HUVEC, a finding that is consistent with previous studies with this type of endothelial cell.[26]

Regarding the difference in the activity of heparins on TFPI release, in a study with HUVEC,[20] dalteparin was found to be more effective than UFH in increasing TFPI release in endothelial cell cultured media. In contrast, in the endothelial cell line, EA.hy926, the release of TFPI was greater in the presence of UFH than in the presence of dalteparin.[8] In addition, the TFPI increase in cultured media was accompanied by a concomitant reduction of cell-associated TFPI, and this is consistent with our findings. It has been suggested that the increase in TFPI release by heparins may also be due to enhanced transcriptional activity, as demonstrated by increased levels of TFPI-mRNA with heparin treatment.[8,27] In addition to its antithrombotic activity, as a physiological inhibitor of the extrinsic pathway of coagulation, TFPI has biological functions as an anti-angiogenic and anti-inflammatory agent. Furthermore, TFPI has been shown to possess anti-proliferative and anti-migratory effects on cultured smooth muscle cells.[28] Therefore, the release of TFPI by heparins likely mediates a role for these molecules in inflammation and atherosclerosis.

In the present study, we also demonstrated for the first time the effect of heparins on the other endothelial cell-associated coagulation inhibitor pathway, i.e. the expression of TM, a transmembrane protein expressed by vascular endothelial cells, which is a critical component of the anticoagulant protein C pathway. TM binds thrombin and alters its active site specificity to facilitate proteolytic activation of circulating protein C. In concert with its cofactor protein S, activated protein C enzymatically degrades factor Va and factor VIIIa of the clotting cascade, thereby suppressing further thrombin generation.[29] TM is downregulated in endothelial cells by various pro-inflammatory stimuli.[16,30,31] Our results show that in HMEC-1, dalteparin upregulates TM expression and counteracts the LPS-induced suppression of TM expression, while UFH does not significantly affect the expression of this protein. In HUVEC, both LMWH and UFH stimulated the expression of the anticoagulant TM, and attenuated the LPS-induced suppression of TM. These data, therefore, add a novel function to the wide range of activities of heparins.

Our findings indicate that, at the level of the microvascular endothelium, dalteparin appears to exert a greater effect than UFH in the expression of an antithrombotic phenotype. On the other hand, UFH was more effective than dalteparin in increasing TFPI release in the cultured media of both endothelial cell types. In line with our results, other studies have reported that endothelial cells show different responses to UFH and LMWH.[6-8,20] One explanation for this difference may reflect differences in the chemical composition of individual heparins. Heparins are heterogeneous preparations derived from mammalian tissues, and contain polysaccharide chains of different lengths and molecular weights. UFH has a mean molecular weight of 12-15 kDa, with molecules ranging from 3 to 37 kDa in weight. LMWH are derived from UFH by controlled enzymatic or chemical depolymerization, and have mean molecular weights ranging from 3 to 8 kDa. LMWH, therefore, have a much higher proportion of short chains than does UFH. This leads to an increased anti-Xa/anti-IIa activity ratio, a greater bioavailability, a lower effect on platelets, and a longer half life, compared with UFH.[1] It is possible that the different effects of heparins on endothelial cells observed in the present study might result from the different proportions of short and long polysaccharide chains within the two heparins.[3,31] Based on this assumption, we suggest that the mechanisms involved in heparin-induced TFPI release might be more responsive to long polysaccharide chains, which are prevalent in UFH, whereas TF and TM modulation might be optimized by the short polysaccharide chains, which characterize dalteparin. However, little is known about the molecular mechanisms by which heparins modulate cellular pathways and this hypothesis would need further investigation. In conclusion, both dalteparin and UFH ameliorate a prothrombotic state in endothelial cells by suppressing inflammatory-mediated TF expression in microvascular and macrovascular endothelial cells, confirming that the vascular endothelium is an important site of action of heparins. Interestingly, dalteparin inhibited the induction of TF by LPS more effectively than did UFH, and increased TM expression, irrespective of the presence of LPS.

In contrast, no significant differences were observed between the two heparins on TF and TM expression in HUVEC. The findings that heparins inhibit the effects of LPS and directly stimulate TM and TFPI expression in microvascular endothelial cells are consistent with their observed anti-inflammatory effects.

All authors directly participated in the conception, design, experimental work, data analysis, article drafting, and critical revision, and approved the final version of the manuscript. This study was partially supported by a grant from the 'Italian Society for the Study of Hemostasis and Thrombosis' (SISET), Italy. The authors declare that they have no potential conflict of interest.

Manuscript received June 14, 2005. Accepted November 25, 2005.

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