Effect of unfractionated heparin and a low molecular weight heparin (enoxaparin) on coagulant activity of cultured human endothelial cells

VICENTA MARTINEZ-SALES, VIRTUDES VILA, EDELMIRO REGANON, JAVIER GARCIA OMS, JUSTO AZNAR

Background and Objectives. Unfractionated heparin and low molecular weight heparins exert their anticoagulant effect by mobilizing tissue factor pathway inhibitor (TFPI) from the vascular endothelium into the blood circulation. We compared the influence of unfractionated heparin and enoxaparin on the anticoagulant function of cultured human endothelial cells.

Design and Methods. Monolayers of human umbilical vein endothelial cells were treated with 10 U/mL unfractionated heparin or enoxaparin for different periods of time (30 min-48h). Endothelial cell procoagulant activity was determined in the cell lysates by a chromogenic assay. Endothelial cell tissue factor (TF) and released TFPI and von Willebrand factor (vWF) were determined.

Results. In short periods of incubation (30 min-2h), both heparins reduced endothelial cell procoagulant activity, the inhibition produced by unfractionated heparin being greater than that induced by enoxaparin ($p<0.05$). However, no variations were observed in TFPI and vWF release. With long periods of incubation (24-48h), both unfractionated heparin and enoxaparin significantly increased TFPI release (control vs. unfractionated heparin, $p<0.05-0.001$; control vs. enoxaparin, $p<0.01-0.001$) and also reduced the release of vWF in the culture medium, though no variations in endothelial cell procoagulant activity or TF content were observed.

Interpretation and Conclusions. Our findings show that unfractionated heparin and enoxaparin exert different kinds of effects on endothelial cells. With short incubation periods, procoagulant endothelial cell capacity was reduced to a greater extent by unfractionated heparin, while after longer periods of incubation enoxaparin increased the anticoagulant activity of the endothelial cells to a greater degree than did unfractionated heparin.

Key words: heparin, enoxaparin, endothelial cells, tissue factor pathway inhibitor.

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Endothelial cells play a central role in the regulation of hemostasis by ensuring the cellular control of both procoagulant and anticoagulant mechanisms. The extrinsic coagulation cascade is initiated when tissue factor (TF) is exposed at a site of blood vessel injury. TF is a cell membrane integral protein receptor for circulating factor VII (FVII), and the rapid interaction of this factor and its receptor promotes the conversion of zymogen FVII to an active serine protease, activated factor VIIa (FVIIa). The TF-FVIIa complex in turn activates factors IX and X, which leads to thrombin generation. Thrombin induces procoagulant changes in endothelial cell function, including the release of von Willebrand factor (vWF). The control of the highly procoagulant activity of the TF-FVIIa complex occurs through feedback inhibition by tissue factor pathway inhibitor (TFPI), which is considered to be the principal physiologic inhibitor of the complex. TFPI is a serine protease produced chiefly by the endothelial cells. A major portion of intravascular TFPI is stored associated with endothelial cells under normal condition. TFPI is present in the plasma in free and lipoprotein-associated forms. The free form of TFPI is released from the endothelial surface into plasma as a result of the action of heparin, and exerts a much stronger anticoagulant effect than does the lipoprotein-associated form.

Heparin administration in vivo causes prompt mobilization of TFPI into the circulation, which is thus thought to contribute substantially to the anticoagulant action of heparin. Both unfractionated heparin and low molecular weight heparins exert their anticoagulant effect by accelerating the inhibitory action of antithrombin III against its target serine protease clotting factors (thrombin, factors IXa, Xa, Xla and Xlla), and by mobilizing TFPI from the vascular endothelium into the blood circulation. The exact profile of these effects depends on the molecular weight of the heparin. Enoxaparin is a low molecular weight heparin indicated for use in the treatment of coronary ischemic complications of unstable angina and non-Q wave myocardial infarction. Unfractionated heparin has for many years represented the standard in anticoagulation therapy for patients with acute coronary syndromes; however, recent studies suggest that enoxaparin is also a viable option for anticoagulant therapy in these patients. Data from the ESSENCE12 and TIMI 11B13 studies reported twice daily enoxaparin to be significantly more effective than unfractionated heparin in contin-
uous infusion in reducing death and serious cardiac ischemic events. Compared to unfractionated heparin, enoxaparin has 6-fold less anti-FIIa action and half the anti-FXa activity. Consequently, the ratio of anti-FXa to anti-FIIa activity is more than 3 for enoxaparin, whereas it is 1 for unfractionated heparin. In vitro studies have examined the ability of unfractionated heparin to modulate the procoagulant activity of stimulated endothelial cells, showing that unfractionated heparin reduces the procoagulant properties of stimulated endothelial cells. A recently reported study showed, in a spontaneously transformed immortal endothelial cell line (ECV304), that the procoagulant activity of the cells was downregulated by 36%, and the contribution of TFPI to the anticoagulant potency of ECV304 cells was moderately increased after 24 hours of heparin stimulation. It is suggested that TFPI release is of major importance for the anticoagulant function of heparins. The aim of the present study was to compare the influence of unfractionated heparin and enoxaparin on the anticoagulant function of cultured human endothelial cells.

Design and Methods

**Human umbilical vein endothelial cell culture**

Endothelial cells from human umbilical cord veins (HUVEC) were obtained by collagenase digestion and were grown to confluent monolayers according to a method previously described. HUVEC were grown in T-25 flasks pre-coated with endothelial cell attachment factor (Sigma), in Medium 199 with 15 mM HEPES supplemented with 20% fetal calf serum, 1% endothelial cell growth factor (Sigma), 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/mL penicillin, and 50 µg/mL streptomycin sulfate in an atmosphere of 95% air — 5% CO₂. The confluent endothelial cell monolayers were harvested from the culture flasks with 0.25% trypsin, 0.01% EDTA in 10 mM phosphate buffer, 150 mM NaCl, pH 7.4 (PBS), without Ca²⁺ and Mg²⁺. Cells were plated in 24-well polystyrene culture plates, at a density of approximately 30×10³ cells/well, precoated with endothelial cell attachment factor and grown to reach 80–100% confluence in the above-mentioned medium. Only cells from these first subcultures were exposed to the different experimental procedures.

**Cell treatment with heparins**

The endothelial cells were treated with unfractionated heparin (Rovi, 1000 U/mL) and low molecular weight heparin, enoxaparin (Decipar, Italfarmanco S.A. 10000 U/mL). Confluent monolayers of endothelial cells in 24-well plates were washed twice in Medium 199 with 15 mM HEPES (basal medium) and incubated with cultured medium (without antibiotics) supplemented with 2% fetal calf serum and unfractionated heparin or enoxaparin at 10 U/mL final concentration for 30 minutes, 2, 4, 8, 24 and 48 hours at 37°C. Controls without heparin were dosed in parallel dishes. At the end of the incubation periods with or without heparins, the supernatants were harvested, kept at −80°C until assay, and the cell monolayers were washed three times with PBS and processed.

**Determination of endothelial cell procoagulant activity with a chromogenic assay**

The procoagulant activity was measured in the cell lysates by a chromogenic assay using human plasma. In brief, cells were mechanically removed with rubber policemen and scraped off the plates with 0.5 mL of 0.9% NaCl, followed by transfer of the cell suspension to tubes. The cells were disrupted by 3 freeze-thaw cycles in liquid nitrogen. Endothelial cell procoagulant activity was determined by incubating 50 µL of HUVEC lysate with 50 µL of pooled normal plasma for 2 min at 37°C; then, 5 µL of 250 mM CaCl₂ were added and incubated for 20 min at 37°C. Thrombin generation was stopped by adding 10 µL of 200 mM EDTA. The amount of thrombin generated was determined using a thrombin chromogenic substrate (S-2238, Chromogenix-Instrumentation Laboratory Spa.). Absorbances at 405 nm were read and the corresponding amount of thrombin was determined using a standard curve with known dilutions of thrombin (Sigma). Procoagulant activity was defined as the amount of free thrombin generated by cells (mU thrombin/10⁴ cells).

**Tissue factor antigen assay**

Endothelial cell TF content was determined on cell lysates using a commercial ELISA kit (Immuno-Binding Tissue Factor, American Diagnostica Inc.). Cell lysates were obtained by lysis cells in Tris—buffer saline, pH 7.8 (TBS; 0.1 M Tris—HCl and 150 mM NaCl), containing 1% Triton X-100, 60 mM octyl-β-D-glucopyranoside and a cocktail of inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, and 10 mM EDTA) for 30 minutes at 37°C with vigorous vortexing.

**Released tissue factor pathway inhibitor assay**

Antigenic concentrations of free TFPI in the supernatants of endothelial cells were determined using a commercial ELISA kit (Asserachrom free TFPI, Diagnostica Stago).
Statistical analysis

Results were expressed as the mean ± standard deviation (SD) of six experiments performed in duplicate. An unpaired t-test for normal distributions was used for the statistical comparison between controls and heparin-treated cells. Statistical comparisons between groups at different time points were performed using a paired-samples t-test. The SPSS version 10.0 statistical package was used throughout. A p value of ≤ 0.05 was considered statistically significant.

Results

Endothelial cell procoagulant activity

The endothelial cell procoagulant activity levels in HUVEC without heparin, with unfractionated heparin (10 U/mL) or with enoxaparin (10 U/mL) incubated at 37°C for different periods (30 min, 2, 4, 8, 24, and 48 h) were measured.

The results show that the interaction of unfractionated heparin and enoxaparin with HUVEC significantly decreased endothelial cell procoagulant activity by 30 minutes and 2 hours of incubation compared to the control (Table 1). Both unfractionated heparin and enoxaparin reduced endothelial cell procoagulant activity to the same extent at 30 min (70%, p<0.001). However, at 2 hours of incubation, unfractionated heparin (70%, p<0.001) produced greater endothelial cell procoagulant activity inhibition than did enoxaparin (45%, p<0.01): the difference between the effects of the two heparins was statistically significant (Table 1). After four hours of treatment with either unfractionated heparin or enoxaparin the observed procoagulant activity of the endothelial cells was slightly but not significantly less than that in the control group experiments (Table 1). Endothelial cell procoagulant activity was not modified by 8 to 48 hours of incubation (Table 1).

Table 1. Endothelial cell procoagulant activity (mU thrombin/10^3 cell). Effect of unfractionated heparin or enoxaparin.

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>30 min</th>
<th>2 hours</th>
<th>4 hours</th>
<th>8 hours</th>
<th>24 hours</th>
<th>48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>670±45</td>
<td>645±55</td>
<td>595±50</td>
<td>575±80</td>
<td>620±120</td>
<td>610±40</td>
</tr>
<tr>
<td>UFH</td>
<td>185±55*</td>
<td>185±50*</td>
<td>430±150</td>
<td>465±150</td>
<td>550±125</td>
<td>520±115</td>
</tr>
<tr>
<td>ENOX</td>
<td>215±50*</td>
<td>350±80†‡</td>
<td>470±125</td>
<td>525±50</td>
<td>580±100</td>
<td>540±80</td>
</tr>
</tbody>
</table>

Results are expressed in IU thrombin/mL as mean ± SD of 6 experiments done in duplicate. UFH, unfractionated heparin (10 U/mL); ENOX, enoxaparin (10 U/mL); Control, without heparin; *p<0.001, heparin vs. control; †p< 0.01, heparin vs. control; ‡p< 0.05, UFH vs. ENOX.

Tissue factor

We determined the endothelial cell TF content in cell extracts of HUVEC incubated at 37°C for 4, 8, 24 and 48 hours without heparin or with unfractionated heparin (10 U/mL) or with enoxaparin (10 U/mL). The endothelial cell TF content under basal conditions and in heparin-conditioned media at different time points are shown in Table 2. No significant differences in endothelial cell TF content were found between the control cells and HUVEC treated with UFH or with enoxaparin at any time point (Table 2). No significant differences were observed in endothelial cell TF content in relation to the two study treatments.

Tissue factor pathway inhibitor

We determined the free TFPI in the cultured media of HUVEC incubated at 37°C for 30 minutes and 2, 4, 8, 24, and 48 hours without heparin, with unfractionated heparin (10 U/mL) or with enoxaparin (10 U/mL). Results of free TFPI release by HUVEC without heparin and in heparin-conditioned media at different times are shown in Table 3. Both unfractionated heparin and enoxaparin induced a time-dependent and significant increase in free TFPI secretion (Table 3). After short periods (30 min– 4 h) of incubating HUVEC with unfractionated heparin or with enoxaparin, no significant variations were observed in endothelial cell-released free TFPI (Table 3). After 8 hours of incubation, only enoxaparin increased free TFPI release by the endothelial cells – this increase being significant compared with the release from both the control cells and the cells treated with unfractionated heparin. At 24 and 48 hours of incubation with heparins, unfractionated heparin and enoxaparin both induced a time-dependent and significant increase in free TFPI release (p<0.05 and p<0.001, respectively) (Table 3). Comparing the effects of the two heparins, it was seen that free
Heparins and coagulant activity of endothelial cells

Table 3. Free tissue factor pathway inhibitor released (ng/10⁶ cells) by HUVEC. Effect of unfractionated heparin and enoxaparin.

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Control</th>
<th>UFH</th>
<th>ENOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min</td>
<td>2.8±0.3</td>
<td>3.8±0.7</td>
<td>3.9±0.3</td>
</tr>
<tr>
<td>2 hours</td>
<td>3.3±0.4</td>
<td>3.8±0.7</td>
<td>4.3±0.7</td>
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<tr>
<td>4 hours</td>
<td>3.9±0.7</td>
<td>5.0±0.6</td>
<td>5.8±0.9</td>
</tr>
<tr>
<td>8 hours</td>
<td>6.0±3.5</td>
<td>15.7±7.5</td>
<td>30.0±3.6</td>
</tr>
<tr>
<td>24 hours</td>
<td>12.6±3.2</td>
<td>30.0±3.6</td>
<td>39.8±7.7</td>
</tr>
<tr>
<td>48 hours</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Results are expressed as mean ± SD of 6 experiments done in duplicate; UFH, unfractionated heparin (10 U/mL); ENOX, enoxaparin (10 U/mL); Control, without heparin; * p< 0.05, heparin vs. control; † p< 0.001, heparin vs. control; ‡ p< 0.01, heparin vs. control; § p< 0.01, UFH vs. ENOX; ° p< 0.001, UFH vs. ENOX

Table 4. Effect of unfractionated heparin or enoxaparin on HUVEC release of von Willebrand factor (% activity/10⁶ cells).

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Control</th>
<th>UFH</th>
<th>ENOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min</td>
<td>4.9±0.4</td>
<td>4.7±0.2</td>
<td>3.9±0.3</td>
</tr>
<tr>
<td>2 hours</td>
<td>4.7±0.2</td>
<td>3.9±0.3</td>
<td>6.1±0.1</td>
</tr>
<tr>
<td>4 hours</td>
<td>6.0±0.7</td>
<td>5.8±0.7</td>
<td>15.2±1.8</td>
</tr>
<tr>
<td>8 hours</td>
<td>15.2±1.8</td>
<td>15.2±1.8</td>
<td>15.2±1.8</td>
</tr>
<tr>
<td>24 hours</td>
<td>22.8±0.8</td>
<td>22.8±0.8</td>
<td>22.8±0.8</td>
</tr>
<tr>
<td>48 hours</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD of 6 experiments done in duplicate. UFH, unfractionated heparin (10 U/mL); ENOX, enoxaparin (10 U/mL); Control, without heparin; * p< 0.05, UFH vs. control; † p<0.01, ENOX vs. control.

TFPI release was greater with enoxaparin than with unfractionated heparin after both 24 (p<0.01) and 48 hours (p<0.001).

von Willebrand factor

HUVEC–released vWF was measured as the percentage activity of vWF in the cell culture media. We determined the vWF in the cultured media of HUVEC incubated at 37°C for 30 minutes and 2, 4, 8, 24, and 48 hours without heparin, with unfractionated heparin (10 U/mL) or with enoxaparin (10 U/mL). Results of vWF release by HUVEC without heparin and in heparin-conditioned media at different time points are shown in Table 4. The results show that during the first 24 hours of incubation with heparins, vWF release changed as a result of the action of heparin (Table 4). After 48 hours of incubation with heparin (UFH or enoxaparin) there was a significant decrease in vWF release by HUVEC (p<0.05 and p<0.01, respectively) (Table 4). However, when the effects of unfractionated heparin and enoxaparin were compared, no significant differences were recorded between the two heparins at any time.

Discussion

The present study shows that the interaction of heparin with endothelial cells modulates the anticoagulant potential of endothelial cells. First, a short period of incubation of heparins with endothelial cells resulted in potent inhibition of the procoagulant activity of the cells. Both unfractionated heparin and enoxaparin reduced endothelial cell procoagulant activity – though the reduction was comparatively greater with unfractionated heparin than with enoxaparin. Heparins stimulate the synthesis and the accumulation of heparan sulfate from endothelial cells, and this endothelial heparan sulfate shows anticoagulant activity.21 It seems reasonable that the reduced thrombin generation exhibited by endothelial cells when they are exposed to heparins could be due, at least in part, to increased production of heparan sulfate by the endothelial cells. However, no differences in endothelial cell antigenic TF were found between the control cells and cells treated with unfractionated heparin or enoxaparin at any point of incubation. These results are in accordance with those of Cadroy et al.21 who found that unfractionated heparin reduced coagulation activity, TF activity and prothrombinase activity, but did not modify TF antigen content in stimulated endothelial cells. Recently, other authors22 reported that shear stress reduces functional but not antigenic expression of TF by intact activated endothelial cell monolayers.

In confirmation of previous findings, cellular release of free TFPI increases during prolonged incubation of endothelial cells with heparin, probably through up–regulation of TFPI expression and synthesis.23 TFPI is produced predominantly by the endothelium, where it remains bound – presumably via glycosaminoglycan structures.24 TFPI associated with the cell surface of the endothelial cells is thought to act as a direct vessel–wall anticoagulant. During the two first hours of endothelial cell treatment with either unfractionated heparin or enoxaparin we found a decrease in endothelial cell procoagulant activity, unrelated to TFPI release. This fact suggests that there might be some mechanism of inhibiting endothelial cell procoagulant activity that is not related to TFPI release. Accordingly, both heparins showed a biphasic action on endothelial cells: first endothelial cell procoagulant activity decreased, then this was followed by an increase in TFPI release. Comparisons between the effects of unfractionated heparin and enoxaparin showed that the latter induced greater free TFPI release by endothelial cells. Thus, enoxaparin is clearly more efficient than unfractionated heparin in increasing the functional activity of TFPI in endothelial cells in vitro. Recently, Alban et al.25 performed a clinical study in healthy volunteers and found that unfractionated heparin mobilized
more free, but not total, TFPI than did enoxaparin. The apparent differences between in vivo and in vitro conditions could be related to the lower lipoprotein concentration in culture medium than in blood. In vivo unfractionated heparin efficiently prevents the binding of plasma lipoproteins to the released TFPI, whereas shorter heparin molecules with lower affinity to TFPI are partly displaced by lipoproteins.25

Our in vitro data could be interesting in relation to recent publications describing that the use of enoxaparin in patients receiving fibrinolytic therapy for acute myocardial infarction is associated with fewer acute cardiac events than is the use of unfractionated heparin,26 and that enoxaparin treatment reduces both cerebral lesions and functional defects induced by local ischemia.27 Our treatment reduces both cerebral lesions and functional defects induced by local ischemia.27

In conclusion, the present study shows that unfractionated heparin and enoxaparin exert two different kinds of effects on the procoagulant activity of endothelial cells. With short incubation times, both heparins reduce plasma thrombin generation, acting as a direct vessel-wall anticoagulant, with effect of unfractionated heparin being greater. After long periods of incubation, however, enoxaparin increases the anticoagulant activity of the endothelial cells to a greater extent than does unfractionated heparin.

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Peer Review

Contributions
VMS, VV, ER contributed to the conception and
design of the study, they carried out part of the ana-
lytical assay and contributed to the analysis and
interpretation of the results. JGO was involved in
obtaining the umbilical cords and contributed to data
analysis and interpretation. JA critically revised the
different versions of the manuscript. The order in
which the names appear is based on the time spent
by each contributor on this research. The authors
wish to thank Guadalupe Manzano, Aurelia Royo,
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the publication and for each Table and Figure: VMS.

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In the following paragraphs, Professor Cazzola
summarizes the peer-review process and its out-
comes.

What is already known on this topic
Unfractionated heparin and low molecular weight
heparins exert their anticoagulant effect by mobiliz-
ing tissue factor pathway inhibitor.

What this study adds
While unfractionated heparin is more efficient
than low molecular weight heparin (enoxaparin) in
reducing endothelial cell procoagulant activity,
enoxaparin is more efficient in increasing the release
of tissue factor pathway inhibitor, a crucial step in
the anticoagulant function of heparins.