Interleukin-1 Inhibits the Synthesis of von Willebrand Factor in Endothelial Cells, Which Results in a Decreased Reactivity of Their Matrix toward Platelets

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We have studied the influence of recombinant human and murine interleukin-1 (IL-1) on the synthesis and secretion of von Willebrand factor by human endothelial cells. Treatment of endothelial cells with IL-1 caused a decline in the steady-state level of von Willebrand factor mRNA in endothelial cells. This decline resulted in a decreased secretion to the culture medium, a decreased storage of von Willebrand factor in the Weibel-Palade bodies, and a decreased incorporation into the extracellular matrix. As a consequence of the decreased amount of von Willebrand factor in the extracellular matrix we have found a strongly impaired platelet adhesion to these matrices. When the matrices of IL-1-treated cells were incubated with purified von Willebrand factor, their ability to support platelet adhesion was restored. These results suggest that perturbation of endothelial cells by inflammatory mediators like IL-1 results in a decreased adhesion of platelets to the subendothelium owing to a diminished synthesis of von Willebrand factor.

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Endothelial cells form the inner lining of the vessel wall and, owing to this unique and strategic position, participate in a wide range of normal and pathologic processes including inflammation and atherosclerosis. There is a strong interaction between circulating blood cells and the endothelium. Localized adhesion of peripheral blood leukocytes to the endothelial lining is one of the key events in the pathogenesis of certain vascular diseases, and the adhesion of blood platelets to locally damaged vessels is a crucial step in the development of thrombosis and atherosclerosis. There is now ample evidence that interaction of monocytes or their secretion products with endothelial cells strongly affects endothelial cell functions. Interleukin-1 (IL-1) induces the synthesis of tissue factor and platelet activating factor in endothelial cells and increases the adhesiveness for circulating blood leukocytes. In addition, IL-1 infusion in rabbits has been shown to downregulate the protein C thrombomodulin anticoagulant pathway on the endothelial cell surface. Also the synthesis of prostacyclin and plasminogen activator inhibitor is enhanced by IL-1. Prostacyclin, the major arachidonic metabolite in endothelial cells, is an important inhibitor of platelet aggregation. Thus, IL-1 forms a link between the interaction of leukocytes and platelets with the vessel wall.

To further investigate the influence of IL-1 on the interaction of platelets with endothelial cells and their extracellular matrix, we have studied the effect of human and murine IL-1 on the synthesis of von Willebrand factor by endothelial cells. IL-1 treatment of endothelial cells resulted not only in decreased secretion and intracellular storage of von Willebrand factor but also in decreased incorporation into the extracellular matrix of the cells. With the use of a well-defined flow system that mimics in vivo flow patterns, we have found that the decreased deposition of von Willebrand factor results in impaired platelet adhesion to these matrices.

Methods

Cell Culture

Human vascular endothelial cells were isolated from umbilical veins and cultured according to the method originally described by Jaffe et al. with some minor modifications. The cells were identified by their typical characteristics. For the experiments described in this article, endothelial cells of the second passage were subcultured on gelatin-coated 25 cm² culture flasks or on gelatin-coated glass coverslips. Before seeding the cells, the gelatin on the glass coverslips was fixed with 0.5% glutaraldehyde. To isolate the extracellular matrix, endothelial cells grown to confluence were exposed to 0.1 M NH₄OH for 30 minutes at room temperature with gentle shaking. The cell layer was completely removed by this procedure. The isolated extracellular matrix was washed three times with phosphate buffered saline (PBS) (10 mM phosphate and 150 mM NaCl, pH 7.4) and either used the same day for
perfusion experiments or scraped off the bottom of the flask in 6 M urea and 1% Triton X-100 in PBS to analyze the von Willebrand factor content. Experiments in which the extracellular matrix of endothelial cells grown in the presence of 3H-leucine was solubilized showed that about 80% of the matrix proteins were extracted with 6 M urea and 1% Triton X-100.

Assays
Von Willebrand factor antigen was determined by an enzyme-linked immunosorbent assay (ELISA).\textsuperscript{16} Pooled normal plasma containing 10 \mu g/ml von Willebrand factor was used as standard. The response of serial dilutions of extracellular matrix samples paralleled that of sample dilutions of normal plasma.

Prostacyclin production was measured with a radioimmunoassay for 6-keto-prostaglandin F\textsubscript{1α}, the stable hydrolysis product of prostacyclin.\textsuperscript{17}

Immunofluorescence Studies
Cells grown on glass coverslips were extensively washed with PBS and fixed for 5 minutes with methanol at room temperature. The cells were then incubated with monoclonal antibodies against von Willebrand factor (gift of Dr. van Mourik, Amsterdam) followed by incubation with FITC-conjugated goat antimouse IgG.\textsuperscript{16} After each incubation the preparations were extensively washed with PBS. Antisera were diluted in PBS to which 2% bovine serum albumin (Sigma, St. Louis, Missouri) was added. Finally, preparations were embedded in mounting fluid: 80% (vol/vol) glycerol, 1 mg/ml phenylenediamide (Sigma), and 1 mM sodium phosphate buffer, pH 8.6.

Perfusion Studies
Perfusions with steady flow\textsuperscript{18} were carried out with a rectangular perfusion chamber.\textsuperscript{14} This perfusion chamber can contain glass coverslips coated with an extracellular matrix. Perfusions were carried out with washed platelets suspended in human albumin solution (HAS). For this purpose, platelets were first isolated and washed as described previously.\textsuperscript{19} Washed platelets were then resuspended in HAS: 4% (wt/vol) human albumin (Behringwerke A.G., Marburg, W. Germany) in Krebs-Ringer buffer (4 mM KCl, 107 mM NaCl, 20 mM NaHCO\textsubscript{3}, 2 mM Na\textsubscript{2}SO\textsubscript{4}) pH 7.35 containing 19 mM citrate, 2.5 mM CaCl\textsubscript{2}, and 5 mM glucose.\textsuperscript{18} To this suspension, washed and packed red blood cells were added to a hematocrit of 0.4. After this, 400 \mu l of ice-cold 20 \times concentrated standard saline citrate (SSC) (0.15 M NaCl, 15 mM trisodium citrate) was added, and the RNA preparation was directly applied to the nitrocellulose by using a manifold filtration apparatus (Schleicher & Schull, Dassel, W. Germany). The filter was baked for 3 hours at 80°C and prehybridized for 4 hours at 65°C in 3 \times SSC, 5 \times Denhardt's Ficoll [(1 \mu g/ml), polyvinylpyrrolidone (1 \mu g/ml), bovine serum albumin (1 \mu g/ml)], to which was added 0.1% sodium pyrophosphate, 5 mM EDTA (pH 8.0), and 0.1% sodium dodecyl sulfate (SDS) containing 100 \mu g of sonicated heat denatured herring sperm DNA per ml. Hybridization was performed overnight at 65°C in the same solution. The probe used was a \textsuperscript{32}P-labelled 1100 base pairs von Willebrand factor cDNA PsiI fragment described by Verweij et al.\textsuperscript{22} A full length cDNA probe for plasminogen activator inhibitor, as described by Pannekoek et al.,\textsuperscript{23} and a cDNA probe for hamster actin (gift of R. Nusse, van Leeuwenhoek Kliniek, Amsterdam) were used for comparison. The filters were washed at a stringency of 0.1 \times SSC at 65°C (for hamster actin cDNA the filters were washed with 0.5 \times SSC). For the quantification of the relative amounts of mRNA, densitometry was used.\textsuperscript{24} In short, a scan was made of the dots and the areas under the peaks were integrated and plotted against the amount of mRNA on the filter. The concentration of the mRNA for von Willebrand factor, plasminogen activator inhibitor, and actin in the IL-1-treated cells was expressed as a percentage of control cells.

Reagents
Experiments were carried out with recombinant murine IL-1 (provided by Peter LoMedico, Hoffman La Roche, Nutley, New Jersey), which has been described,\textsuperscript{16} and with recombinant human β IL-1 (Genzyme Corporation, Boston, Massachusetts). Heat treatment of the IL-1 prepara-
tion (90°C, 70 minutes) resulted in a loss of IL-1 activity toward endothelial cells. Thrombin (human, 3000 U/mg protein) and PMA (4-β-phorbol 12-myristate 13-acetate) were from Sigma. All culture plastics were obtained from Nunc (Rashilde, Denmark). The other tissue culture supplies (media, antibiotics, trypsin) were purchased from GIBCO Biocult (Paisley, Scotland). All other chemicals obtained from commercial sources were of the highest purity grade available.

Results

Influence of IL-1 on von Willebrand Factor Synthesis

Cultured human endothelial cells secrete von Willebrand factor toward the culture medium, they store it intracellularly in the Weibel-Palade bodies, and they incorporate it into extracellular matrix. When endothelial cells were incubated with 10 U/ml murine IL-1, a decreased secretion of von Willebrand factor was noted (Table 1). The decrease in secretion started after about 12 hours. Comparable results were found with human recombinant IL-1 (not shown). To investigate whether the intracellular amount of von Willebrand factor was also decreased, endothelial cells preincubated for 3 days with IL-1 were stimulated with thrombin and the phorbol ester PMA. Thrombin or PMA rapidly mobilizes the intracellular stores of von Willebrand factor. In contrast to control cells, cells pretreated with IL-1 did not show an increased secretion of von Willebrand factor to the culture medium (Figure 1), suggesting that the intracellular stores of von Willebrand factor were depleted.

Treatment of endothelial cells with IL-1 neither influenced the lactate dehydrogenase activity of the cells nor the number of cells (not shown). Endothelial cells preincubated with IL-1 were submitted to immunofluorescence studies (Figure 2). As has been shown previously in control cells, von Willebrand factor is located in rod-shaped structures, the Weibel-Palade bodies. When endothelial cells were cultured for 3 days in the presence of 10 U/ml IL-1, the rod-shaped structures with von Willebrand factor disappeared in most of the cells (Figure 2).

Prolonged incubation of endothelial cells with IL-1 resulted in a strong decrease in the amount of von Willebrand factor in the extracellular matrix (Table 1). When endothelial cells were incubated for 2 hours with 10 U/ml IL-1 and afterward cultured in the absence of IL-1, essentially the same inhibition of von Willebrand factor secretion was seen after 2 days when compared with cells cultured continuously in the presence of IL-1, although the amount of decrease was less (not shown). Thus, a brief perturbation of the endothelium has consequences after a longer period. The mechanism by which the endothelial cell is perturbed after such a long period is not yet clear.

Table 1. Effect of IL-1 on the Amount of von Willebrand Factor in the Culture Medium and Extracellular Matrix

<table>
<thead>
<tr>
<th>IL-1 stage (hours)</th>
<th>Medium (ng/10^6 cells/ml/24 hr)</th>
<th>Matrix (ng/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>76.4 ± 6.3</td>
<td>87.9 ± 9.2</td>
</tr>
<tr>
<td>8 hours</td>
<td>ND</td>
<td>69.2 ± 14.0</td>
</tr>
<tr>
<td>12 hours</td>
<td>62.8 ± 9.4*</td>
<td>68.5 ± 5.5*</td>
</tr>
<tr>
<td>24 hours</td>
<td>52.8 ± 8.0†</td>
<td>56.2 ± 3.5†</td>
</tr>
<tr>
<td>48 hours</td>
<td>40.3 ± 8.0†</td>
<td>35.2 ± 5.2†</td>
</tr>
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Endothelial cells were grown for the indicated time in the presence or absence of murine or human IL-1 (10 U/ml). To determine the amount of von Willebrand factor in the matrix, cells were removed with 0.1 M ammonia. Then the matrices were scraped off the flasks and after dialysis against PBS the von Willebrand factor content was measured with an ELISA. In a separate experiment, cells were also stimulated with IL-1 for the indicated time. During the last 12 hours of this period, the culture medium was replaced by a serum-free medium, and the amount of von Willebrand factor secreted in this serum-free medium was measured. Results are mean ± SD (n = 6); ND = not determined.

*p < 0.05; †p < 0.01.

To investigate if the decreased amounts of von Willebrand factor in all cellular compartments was from decreased synthesis after IL-1 treatment, the amount of endothelial cell mRNA for von Willebrand factor was determined. Serial dilutions of the total RNA preparations derived from the endothelial cells, cultured in the presence or absence of IL-1, were dot-blotted and hybridized with a 1100 base pair fragment of von Willebrand factor cDNA. As a control, the RNA was also hybridized with full length cDNA for plasminogen activator inhibitor and with cDNA for hamster actin (Figure 3). Incubation of cells with IL-1 decreased the amount of mRNA for von Willebrand factor by 35% as measured with densitometry. In contrast, IL-1 induces a twofold increase in the amount of mRNA for plasminogen activator inhibitor, which is in agreement with the increased secretion of plasminogen activator inhibitor after IL-1 treatment. IL-1 had no influence on steady-state levels of actin mRNA (with IL-1 94% of control value).
Figure 2. Von Willebrand factor in cultured endothelial cells. Endothelial cells were grown on coverslips; von Willebrand factor was visualized with monoclonal antibodies combined with an FITC-conjugated goat polyclonal antimurine IgG antibody. Control (A) and endothelial cells incubated for 3 days with human IL-1 (10 U/ml) (B). Comparable results were obtained with murine IL-1.

Indicating that the decrease in von Willebrand factor mRNA is not from loss of cells during the IL-1 incubation.

**Influence of IL-1 on Prostacyclin Synthesis**

Prostacyclin is the most potent inhibitor of platelet aggregation known. Incubation of endothelial cells with IL-1 increased the basal production of prostacyclin by endothelial cells by about five times (Table 2). Endothelial cells were incubated for 2 days in the presence of IL-1 and subsequently tested for their sensitivity to a second stimulus, such as thrombin. IL-1 enhanced the response of endothelial cells to thrombin by a fourfold increase in prostacyclin synthesis (Table 2).

**Platelet Adhesion to Endothelial Extracellular Matrices**

To investigate if the decreased amount of von Willebrand factor in the endothelial extracellular matrix was accompanied by a decrease in the ability of the isolated matrices to support platelet adhesion, perfusion studies were performed with platelets resuspended in an albumin solution. Pretreatment of endothelial cells for 3 days with human or murine IL-1 before their matrix was isolated resulted in a strong decrease in the number of platelets adhering to it (Table 3). When the matrices of IL-1 treated cells were preincubated with plasma or with purified von Willebrand factor, their ability to support platelet adhesion was re-
Table 2. Effect of IL-1 on the Synthesis of Prostacyclin by Endothelial Cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Basal release during 24 hours (ng)</th>
<th>Stimulated release (ng/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without thrombin</td>
<td>With thrombin</td>
</tr>
<tr>
<td>Control</td>
<td>2.3 ± 0.7</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>IL-1</td>
<td>10.0 ± 3.9</td>
<td>3.4 ± 1.1</td>
</tr>
</tbody>
</table>

Endothelial cells were grown to confluence and incubated for 2 days in the presence or absence of murine IL-1. A part of the cells were washed and incubated for 10 minutes in the presence or absence of thrombin (1 U/ml). The other part was incubated for another 24 hours in culture medium. The amount of prostacyclin was measured with a radioimmunoassay for 6-keto-PGF_1α. Results are in ng 6-keto-PGF_1α/ml culture medium x 10^6 cells (mean ± SD, n = 3).

Table 3. Platelet Adhesion to Matrices of IL-1-Treated Cells: Effect of Purified von Willebrand Factor

<table>
<thead>
<tr>
<th>Group</th>
<th>% Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28.8 ± 3.0</td>
</tr>
<tr>
<td>Control + VWF</td>
<td>30.8 ± 2.3</td>
</tr>
<tr>
<td>IL-1-treated cells</td>
<td>10.1 ± 3.4</td>
</tr>
<tr>
<td>IL-1-treated cells + VWF</td>
<td>31.0 ± 1.3</td>
</tr>
</tbody>
</table>

Endothelial cells were grown for 3 days in the presence or absence of IL-1 (10 U/ml, human or murine). The matrices were isolated and exposed to platelets at a shear rate of 1300 sec^-1. A part of the matrices were first incubated with purified von Willebrand factor (VWF) (10 μg/ml). After perfusion, the matrices were fixed, stained, and the percentage surface coverage was evaluated by morphometric evaluation. Results are in mean ± SD (n = 5).

stored, indicating that the decrease in reactivity of the matrix was from the lower amount of von Willebrand factor (Table 3). The residual 40% platelet coverage after IL-1 treatment found on the matrices could be blocked by the addition of a monoclonal antibody against von Willebrand factor, indicating that the residual platelet adherence is from the remaining von Willebrand factor in the matrices.

Discussion

The composition of the extracellular matrix of cultured endothelial cells resembles the composition of the subendothelium in vivo. Therefore, the extracellular matrix of cultured endothelial cells is a suitable model to study the interaction of blood platelets with the vessel wall. This interaction strongly depends on the shear rate of the blood. Recently, we constructed a perfusion chamber that enables us to investigate the adhesion of blood platelets in flowing blood with cultured vessel wall cells and their extracellular matrix. We showed that the composition of the extracellular matrix has a strong influence on the number of platelets adhering to the matrix, and that the presence of von Willebrand factor and fibronectin in the extracellular matrix is an absolute requirement for normal platelet adhesion. In the present study we have investigated the influence of IL-1, a potent mediator of inflammatory responses, on the interaction of platelets with endothelial cells and their extracellular matrix. IL-1 caused a decline in the steady-state level of the von Willebrand factor gene, which may have been from an inhibition of transcription of the von Willebrand factor mRNA or a fall in von Willebrand factor mRNA stability. The partial loss of the von Willebrand factor mRNA resulted in the decreased synthesis of the von Willebrand factor protein. The decreased synthesis resulted in a decreased secretion of von Willebrand factor to the culture medium, a decreased intracellular storage, and a decreased incorporation of von Willebrand factor into the extracellular matrix. This latter decrease will have direct consequences on the adhesion of platelets to the subendothelium. Because of the decreased amount of von Willebrand factor, fewer platelets will adhere to the subendothelium. In perfusion studies we and others have found that about half of the platelet adhesion to subendothelium is mediated by subendothelial von Willebrand factor. So, an altered amount of von Willebrand factor in the vessel wall might directly influence platelet adhesion. The Weibel-Palade bodies are storage vesicles for von Willebrand factor. After IL-1 treatment not all the von Willebrand factor disappeared from the Weibel-Palade bodies. We believe that at least part of the positive fluorescence still present after IL-1 treatment is coming from "old" Weibel-Palade bodies, which were synthesized by the cells before the IL-1 was added and are still present in the cell. Besides inhibiting platelet aggregation, prostacyclin also has a strong inhibitory effect on the adhesion of platelets. IL-1 stimulated the synthesis of prostacyclin by endothelial cells (Table 2). Not only was the direct synthesis of prostacyclin enhanced by IL-1, but the sensitivity to other stimuli was enlarged by preincubation with IL-1. So, only decreased amounts of von Willebrand factor in the extracellular matrix but also the enhanced production of prostacyclin by endothelial cells may contribute to a decreased adhesion of platelets to the vessel wall after perturbation of the endothelium by IL-1.

No differences have been found between the addition of human and murine IL-1. One of the first events in the pathogenesis of atherosclerosis is the influx of monocytes. This may result in IL-1 formation, leading to perturbation of endothelial cells. This perturbation will result in a changed metabolism in these cells, a decreased incorporation of von Willebrand factor in the subendothelium, and an increased prostacyclin synthesis causing an impaired platelet adhesion after injury of the endothelial layer.

From a number of studies it appears that IL-1 has a markedly prothrombotic effect on the endothelial surface. By inducing thromboplastin synthesis and reducing protein C activation it enables the formation of thrombin on the endothelial surface. (See 10) Also, enhanced secretion of plasminogen activator inhibitor decreases the activity of the fibrinolytic system. It is interesting to note that in contrast to the increased interaction of IL-1-perturbed endothelial cells with soluble plasma coagulation proteins, IL-1 induces a decreased adhesion of blood platelets to the vessel wall. The reason for these differences is not clear, and the in vivo relevance of this modulation of vessel wall cells remains to be established.

To evaluate the consequences of IL-1 perturbation of
the endothelial cells on their interaction with platelets, one must consider the important interactions between the clotting cascade and platelet function. Formation of thrombin on the endothelial cell surface undoubtedly influences platelet aggregation or, in other words, thrombus formation on an injured vessel wall. In the studies presented here, perfusions were carried out with platelets resuspended in an albumin solution, so thrombin formation is impossible owing to the absence of coagulation factors. In other studies, not reported here, we have used citrated whole blood, and because of the presence of citrate in the perfusate, there have been too few free Ca²⁺ ions to allow proper thrombin formation. The system as it is used here predominantly allows the study of platelet adhesion to the vessel wall, not the following aggregate formation. For the experiments described here, no conclusions can be drawn for the aggregate formation. So, the net result of the influence of IL-1 on the interaction of platelets with the vessel wall is not clear. The adhesion of platelets to the vessel wall is in all probability decreased. However, the consequences of IL-1 perturbation on the next step, aggregate formation, could not easily be predicted because of the contradictory effects of the presence of prostacyclin and thrombin on aggregate formation.

Acknowledgment
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