Perturbation of Human Endothelial Cells by Thrombin or PMA Changes the Reactivity of Their Extracellular Matrix Towards Platelets

Philip G. de Groot, Jan Hendrik Reinders,* and Jan J. Sixma

Department of Hematology, University Hospital Utrecht, Utrecht, The Netherlands; and *Department of Blood Coagulation, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands

Abstract. In this study we have examined the influence of perturbation of endothelial cells on the amounts of fibronectin and von Willebrand factor in their extracellular matrix and the consequences of a changed composition of the matrix on platelet adhesion. For this purpose, we have used an in vitro perfusion system with which we can investigate the interactions of platelets in flowing blood with cultured endothelial cells and their extracellular matrix (Sakariassen, K. S., P. A. M. M. Aarts, P. G. de Groot, W. P. M. Houdgk, and J. J. Sixma, 1983, *J. Lab. Clin Med.* 102:522–535). Treatment of endothelial cells with 0.1–1.0 U/ml thrombin for 2 h increased the reactivity of the extracellular matrix, isolated after the thrombin treatment, towards platelets by ~50%. The increased reactivity did not depend on de novo protein synthesis but was inhibited by 3-deazaadenosine, an inhibitor of phospholipid methylation, which also inhibits the stimulus-induced instantaneous release of von Willebrand factor from endothelial cells. However, no changes in the amounts of von Willebrand factor and fibronectin in the matrix were detected. Thrombin may change the organization of the matrix proteins, not the composition.

When endothelial cells were perturbed with the phorbol ester PMA or thrombin for 3 d, the adhesion of platelets to the extracellular matrix of treated cells was strongly impaired. This impairment coincided with a decrease in the amounts of von Willebrand factor and fibronectin present in the matrix. These results indicate that, after perturbation, endothelial cells regulate the composition of their matrix, and that this regulation has consequences for the adhesion of platelets.

In the normal situation, the endothelial cell lining of the vessel wall prevents platelet deposition. When endothelial cells are damaged, the subendothelium becomes exposed to the blood flow, resulting in adherence of platelets to the injured site (27, 35). Cultured endothelial cells have been shown to retain their in vivo polarity. The luminal cell surface, exposed to flowing blood, has kept its nonthrombogenic properties (33). In contrast, a highly thrombogenic extracellular matrix on the abluminal surface, which can support platelet adhesion almost independently of plasma factors, is synthesized (10, 34). The extracellular matrix produced by cultured endothelial cells has been shown to contain von Willebrand factor (14), fibronectin (13), thrombospondin (21), laminin (6), nidogen (4), collagen types III, IV, V and VIII, (20, 28-30), and proteoglycans (7). Both von Willebrand factor and fibronectin present in the subendothelium play an important role in the interaction of platelets with this extracellular matrix (10).

The process of vascular subendothelial biosynthesis, assembly, and the regulation of its formation is difficult to study in vivo, and, consequently, is poorly understood. The availability of cultured vessel wall cells that maintain their differentiated functions may be helpful in studying the formation of their extracellular matrix and the role of the different components of the matrix in the adhesion of platelets. The study of the interaction of platelets with cultured vessel wall cells requires the use of a well-defined flow system that mimicks the in vivo flow pattern (43). Recently we have constructed a perfusion chamber that enables us to investigate the adhesion of platelets in flowing blood with cultured vessel wall cells, and their extracellular matrices (33). In the present study we used this perfusion chamber to examine the effects of perturbation of endothelial cells on the reactivity of the extracellular matrix produced by these cells towards platelets. Upon stimulation of endothelial cells with thrombin or the phorbol ester 4β-phorbol 12-myristate 13-acetate (PMA), the extracellular matrix isolated 2 h after stimulation showed a markedly increased reactivity towards platelets. This enhanced deposition of platelets is not due to increased amounts of von Willebrand factor or fibronectin present in the extracellular matrix, nor to a direct proteolytic effect of thrombin on the matrix.

When endothelial cells were exposed to PMA or thrombin and the extracellular matrix was isolated 3 d later, the adhesion of platelets to this matrix was strongly impaired. This impairment coincided with a decrease in the amount of von Willebrand factor present in the matrix. These results indicate that endothelial cells can rapidly reorganize the struc-

tural organization of their matrix and their reactivity towards platelets.

Materials and Methods

Cell Culture

Human vascular endothelial cells were isolated from umbilical veins and cultured according to the method originally described by Jaffe et al. (12) with some minor modifications (44). The cells were identified by their typical characteristics (12). For the experiments described in this paper, endothelial cells of the second passage were subcultured on gelatin-coated 25-cm^2 culture flasks or on gelatin-coated glass coverslips. Before seeding the cells, the gelatin on the glass coverslips was fixated with 0.5% glutaraldehyde. To isolate the extracellular matrix, endothelial cells grown to confluence were exposed to 0.1 M NH_4OH for 30 min at room temperature with gentle shaking. The cell layer was completely removed by this procedure (33). This isolated extracellular matrix was washed three times with PBS (10 mM sodium phosphate, pH 7.4, and 150 mM NaCl) and either used the same day for perfusion experiments or extracted with 6 M urea and 1% Triton X-100 in PBS for analysis of the von Willebrand factor or fibronectin contents (22).

Assays

Von Willebrand factor antigen was determined by an enzyme-linked immunosorbent assay (ELISA). Pooled normal plasma containing 10 $\mu g \cdot ml^{-1}$ von Willebrand factor was used as standard. Rabbit polyclonal antibodies, monospecific for von Willebrand factor, served as solid phase, and a mixture of the murine monoclonal antibodies CLB-RAg 35 and CLB-RAg 50 (37) were used as an indicator, in combination with peroxidase-labeled sheep anti-mouse IgG (Institute Pasteur, Marnes-la-Garenne, France). The response of serial dilutions of extracellular matrix samples paralleled that of purified von Willebrand factor. Dissolving von Willebrand factor in extraction buffer followed by dialysis did not alter the results found in the ELISA.

Fibronectin was measured by an ELISA. Rabbit polyclonal antibodies against fibronectin were used as solid phase and monoclonal antibody against fibronectin CLB-HEC-FN-140 functioned as indicator antibody in conjugation with peroxidase-labeled sheep anti-mouse IgG (Institute Pasteur) (25). Fibronectin purified from human plasma, as described below, served as standard. The response of serial dilutions of extracellular matrix samples paralleled that of purified plasma fibronectin dissolved in extraction buffer.

In some experiments an alternative ELISA was used. The extracellular matrix of endothelial cells cultured in microtiter plates was used as solid phase, and the amount of von Willebrand factor and fibronectin was determined with monoclonal antibodies against von Willebrand factor and fibronectin, respectively, in conjugation with peroxidase-labeled sheep anti-mouse IgG.

Perfusion Studies

Perfusions with steady flow (32) were carried out with a rectangular perfusion chamber (33). This perfusion chamber can contain glass coverslips coated with extracellular matrix. Blood from normal human donors, anticoagulated with 1:10 vol 110 mM trisodiumcitrate, was directly used as whole blood in the perfusion system. Perfusions were also performed with washed platelets resuspended in a human albumin solution (HAS). In that case, platelets were first isolated and washed as described previously (31). The washed platelets were resuspended in HAS: 4% wt/vol human albumin (Behringwerke A.G., Marburg, Federal Republic of Germany) in Krebs-Ringer buffer (4 mM KCl, 107 mM NaCl, 20 mM $NaHCO_3$, 2 mM Na_2SO_4; pH 7.35) containing 19 mM citrate, 2.5 mM $CaCl_2$ and 5 mM glucose (31). To this suspension, washed and packed red cells were added to a hematocrit of 0.4.

After the coverslips with extracellular matrix had been inserted into the perfusion chamber, they were rinsed with 25 ml warmed 10-mM Hepes-buffered saline, pH 7.4. The perfusate was prewarmed for 5 min at 37°C. This perfusate was recirculated through the perfusion chamber for 5 min under nonpulsatile steady flow at a wall shear rate of 1,300/s. The coverslips were washed with Hepes-saline and fixed with 0.5% glutaraldehyde as previously described (31). After fixation, the coverslips covered with platelets were stained with May-Grünwald-Giemsa. Platelet adhesion was expressed as percentage of the surface covered with platelets. This was evaluated by en face light microscopy at 1,000× magnification. The light microscope was interfaced with an image analyzer (Quantimet 720; Imanco, Royston, United Kingdom). For every coverslip, 30 fields, each consisting of 500,000 image points (0.028 mm^2) were selected at random and evaluated. In recent studies we have found a good correlation between the percentage of adherent platelets covering the surface as determined by morphometry, and the number of deposited platelets as found by measuring the amount of radiolabeled platelets (33).

Purifications

Von Willebrand factor was isolated from fresh cryoprecipitates by agarose gel filtration on Sepharose CL-4B (Pharmacia, Uppsala, Sweden) as previously described (39). The von Willebrand factor in the void volume fraction was further purified by passing it over a gelatin-Sepharose column, equilibrated with 0.005 M Tris-HCl (pH 7.4). Von Willebrand factor was precipitated by dialysis against 1.6 M ammonium sulfate, pH 7.0, at 4°C for 18 h and stored as ammonium sulphate suspension at 4°C until use. The purified von Willebrand factor preparation (ristocetin cofactor activity 167 U/mg per protein) contained no detectable fibronectin and <1% fibrinogen.

Fibronectin was isolated from normal human plasma by affinity chromatography on gelatin-Sepharose (5). Plasma was passed over the gelatin-Sepharose column, which was equilibrated with 0.05 M Tris-HCl, pH 7.4. The column was washed with 1 M NaCl in 0.05 Tris-HCl, pH 7.4, followed by 1 M urea in the same buffer. Fibronectin was eluted with 6 M urea, 0.1 M citric acid, pH 4.7 (15). The buffers contained 0.1 M phenylmethylsulphonyl fluoride, 1 mM benzamidine, 5 mM ε-aminocaproic acid, 10 mM EDTA, and 0.02% wt/vol sodium azide. The pooled fractions were dialyzed at 4°C against Krebs-Ringer buffer, pH 7.4, supplemented with 19 mM trisodium citrate and 2.5 mM $CaCl_2$. The fibronectin was stored at 4°C and used for experiments within 2 wk.

Immunoprecipitation and Gel Electrophoresis

Endothelial cells were maintained in a medium with [^{35}S]methionine (Amersham International, Amersham, United Kingdom) for 2 d. The extracellular matrix was extracted as described above. The ureum/Triton X-100 extracts were extensively dialyzed against 15 mM imidazole-HCl, pH 7.0, 140 mM NaCl, 0.1% NP-40, 10 mM benzamidine, 5 mM EDTA. The dialyzed extract was precleared with Sepharose-CLB-CAg-117 (CAg-117 is a monoclonal antibody against factor VIII). Fibronectin was first absorbed with Sepharose-CLB-HEC-FN-140 (HEC-FN-140 is a monoclonal antibody against fibronectin) and then with Sepharose-gelatin. Finally, the extracts were incubated with Sepharose-CLB-RAg-20 (RAg-20 is a monoclonal antibody against von Willebrand factor) to absorb von Willebrand factor. Proteins absorbed to the antifibronectin and anti-von Willebrand factor Sepharoses were eluted with electrophoresis sample buffer (including 2% sodium dodecylsulfate and 0.016% β-mercaptoethanol), electrophoretically separated on 6% polyacrylamide gels and visualized by autoradiography.

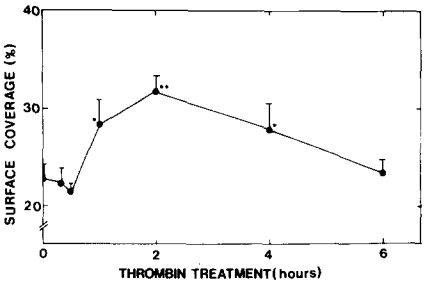

Figure 1. Effect of thrombin-treatment of endothelial cells on the ability of their matrix to support platelet adhesion. Endothelial cells were treated with 1 U/ml thrombin during the periods indicated. Then their matrix was isolated and perfused with whole blood for 5 min at a shear rate of 1,300/s. After fixation of the platelet-covered matrices, the percentage of surface coverage was determined morphologically. Results are expressed as mean ± SD (n = 4). ** $P < 0.005$, * $P < 0.01$ (Peritz' F test).

1. *Abbreviation used in this paper*: HAS, human albumin solution.

Statistical Analysis

Statistical analysis was performed according to the Peritz' *F* test.

Materials

All culture plastics were obtained from Nunc (Roskilde, Denmark); the other tissue culture supplies (media, antibiotics, and trypsin) were purchased from Gibco Biocult (Paisley, Scotland). Thrombin (human, 3,000 U/mg protein) and PMA were from Sigma Chemical Co. (St. Louis, MO). Ionophore A23187 was obtained from Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany. Gelatin and glutaraldehyde were from E. Merck (Darmstadt, Federal Republic of Germany). All the other chemicals obtained from commercial sources were of the highest purity grade available. Immunoreagents were from the central laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands) unless otherwise stated.

Results

Short-term Effects of Thrombin on Platelet Adhesion to Endothelial Matrices

Endothelial cells grown to confluence on gelatin-coated glass coverslips were incubated with 1 U/ml thrombin for increasing time periods. The extracellular matrices of the cells was then isolated and the matrices were exposed to flowing blood to measure its reactivity towards platelets (Fig. 1). Thrombin treatment of the cells resulted in an increased platelet coverage of their extracellular matrix. The increase in platelet adherence to the matrix depended upon the time period for which the cells had been exposed to thrombin. After a 2-h incubation of the cells with thrombin a maximum in reactivity of the extracellular matrix was reached. Compared with the matrix of control cells, ~50% more platelets adhered. Prolonged incubation resulted in a steady decrease of platelet adherence and after 6 h of treatment, platelet coverage of the matrix had returned to values obtained with the matrices of untreated cells.

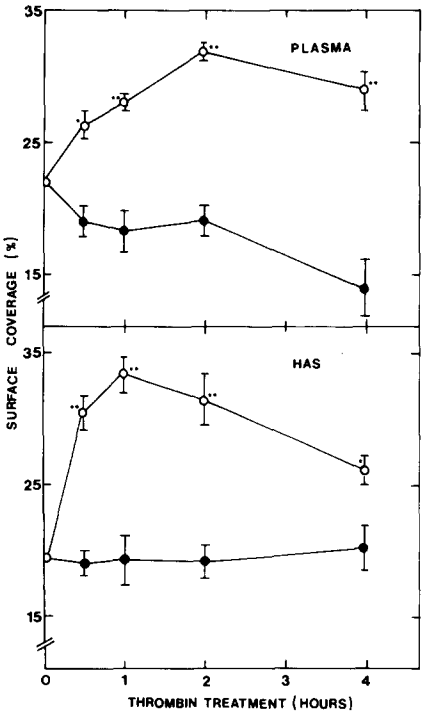

Figure 2. Comparison of the effects of thrombin on endothelial cells and endothelial cell matrix. Either endothelial cells were treated with thrombin (1 U/ml) after which their matrix was isolated (*open circle*) or extracellular matrices were isolated and subsequently incubated with thrombin (*solid circle*). After extensive washing with a buffer containing 100 μg/ml soybean trypsin inhibitor, the matrices were perfused for 5 min with whole blood (plasma) or platelets resuspended in HAS. After fixation the percentage of the surface covered with platelets was determined morphologically. Results are expressed as mean ± SD ($n = 4$). ** $P < 0.005$, * $P < 0.01$ (Peritz' *F* test).

To investigate if thrombin had a direct proteolytic effect on the matrix or if thrombin stimulated the endothelial cells to change the composition of their extracellular matrix, the influence of thrombin on endothelial cells and endothelial cell matrix was compared. For this purpose, part of the cultures were treated for indicated periods with thrombin and then the extracellular matrices were isolated. The extracellular matrices from other cultures were isolated, and then exposed to thrombin. Both types of matrices were exposed to whole blood or platelets resuspended in HAS at a shear rate of 1,300/s (Fig. 2). Pretreatment of endothelial cells with thrombin showed a significant increase in the number of platelets adhering to the matrix when the matrix were perfused with whole blood as well as with platelets resuspended in HAS. When the extracellular matrix was first isolated and then incubated with thrombin, no influence (HAS) or decrease (whole blood) in the number of platelets adhering to the matrix was found. When the matrix of thrombin-treated cells was perfused with platelets resuspended in HAS instead of plasma, the increase in platelet adherence started earlier but the maximal increase reached similar values. Fig. 3 shows the reactivity of the matrix of endothelial cells towards platelets when the endothelial cells were treated with different concentrations of thrombin. 0.1 U/ml thrombin already caused a significant increase in platelets adhering to the matrix. A maximum increase was reached with 0.5 U/ml thrombin.

Platelet adhesion to matrices of cultured endothelial cells in the absence of plasma factors has an absolute requirement for the presence of fibronectin and von Willebrand factor in

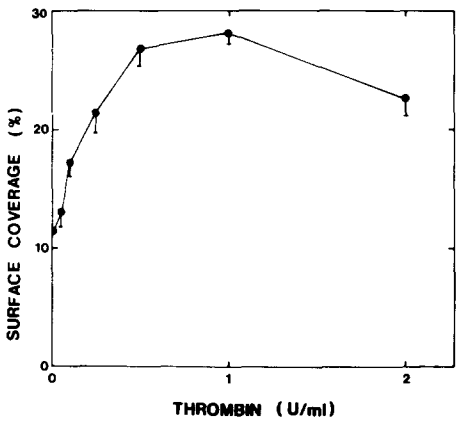

Figure 3. Dose-response curve of the effect of thrombin. Confluent cultures of endothelial cells were incubated with different concentrations of thrombin for 2 h. Then the matrix was isolated and exposed to platelets resuspended in HAS (5 min, 1,300/s). After fixation the amount of platelets adhered to the matrix was determined morphometrically. Results are expressed as mean ± SD of three to five different perfusion experiments.

Table I. Effect of Stimuli on the Amount of von Willebrand Factor and Fibronectin Present in the Extracellular Matrix

Addition	Extracellular matrix	
	von Willebrand factor	Fibronectin
	ng/10⁶ cells	*μg/10⁶ cells*
No addition	108 ± 10	3.7 ± 0.8
+ Thrombin (1 U/ml)	97 ± 11	4.4 ± 0.4
+ A23187 (10 μM)	95 ± 18	5.7 ± 1.0
+ PMA (20 ng/ml)	111 ± 21	3.9 ± 0.2

Confluent cultures of endothelial cells were incubated for 2 h as indicated in serum-free medium. After removal of the cells, the matrix was scraped off the flasks in the presence of 6 M urea and 1% Triton X-100. After dialysis, the amounts of von Willebrand factor and fibronectin were determined. Results are expressed as mean ± SD (n = 3). Analysis of these results with the Peritz' F test showed no significant differences.

the matrix. Therefore, we measured the amounts of von Willebrand factor and fibronectin present in the matrix after treatment of the cells with thrombin for 2 h (Table I). After thrombin treatment, no significant alteration of the amounts of von Willebrand factor and fibronectin in the matrix were found. To confirm these results, the relative amount of von Willebrand factor and fibronectin in the extracellular matrix was measured in an alternative way. Endothelial cells were cultured in microtiter plates and after confluence the cells were stimulated for 2 h with PMA or thrombin. Then the cells were washed away with ammonia and the exposed matrix was used as a solid phase for an ELISA. The relative amount of von Willebrand factor and fibronectin, compared with control cells, was determined with monoclonal antibody CLB-RAg 35 directed against the platelet-binding site of von Willebrand factor (37) and a monoclonal antibody against fibronectin, respectively. No differences between stimulated cells and control cells could be detected (not shown).

To investigate the role of von Willebrand factor in the increase in platelet adherence, we preincubated the matrix with a murine monoclonal antibody, CLB-RAg 35, which inhibits platelet adhesion to subendothelium completely by blocking the platelet-binding domain on von Willebrand factor (37). Preincubation of thrombin-treated matrices with CLB-RAg 35 completely abolished platelet adhesion when perfusions were performed with platelets resuspended in HAS (Table II).

In endothelial cells, thrombin stimulates the methylation of phosphatidylethanolamine to form phosphatidylcholine (3). This process has been found to induce a Ca^{2+}-influx, and, due to this increase of cytosolic Ca^{2+}-concentration,

Table II. Effect of Preincubation of the Extracellular Matrix with Monoclonal Antibodies against von Willebrand Factor

Addition	Surface coverage	
	Control ascites	CLB.RAg 35
No addition	22.8 ± 2.4	1.1 ± 0.2
+ Thrombin	31.8 ± 3.5	1.9 ± 0.9

Confluent cultures of endothelial cells were incubated for 2 h as indicated. After removal of the cells, the matrices were incubated for 1 h at room temperature with the indicated monoclonal antibodies. Subsequent perfusions were performed with platelets resuspended in HAS for 5 min at a shear rate of 1300/s. Values are mean ± SD, n = 3.

Table III. Effect of Metabolic Inhibitors on Thrombin-stimulated Increase of the Reactivity of the Extracellular Matrix

Addition	Surface coverage Mean ± SD (n = 4)
	%
No addition	19.4 ± 1.7
+ Thrombin (1 U/ml)	29.7 ± 0.8*
+ 3-Deazaadenosine (10 μM)	21.4 ± 0.7
+ Thrombin + 3-deazaadenosine	20.2 ± 2.8
+ Thrombin + cycloheximide (10 μg/ml)	30.4 ± 4.5*
+ von Willebrand factor (2 μg/ml)	21.2 ± 4.2

Endothelial cells grown to confluence on coverslips were incubated for 2 h with serum-free medium supplemented as indicated. Then the matrix was isolated and exposed to platelets resuspended in HAS. After perfusion the matrices were fixed and stained, and the percentage surface coverage was determined by morphological evaluation. Results are expressed as mean ± SD (n = 4). * $P < 0.005$ compared with control incubation (Peritz' F test).

the release of a storage pool of von Willebrand factor is triggered (17, 41). Phospholipid methylation and von Willebrand factor release can be blocked by 3-deazaadenosine. 3-Deazaadenosine also blocked the increase of reactivity of the extracellular matrix towards platelets after treatment of the cells with thrombin (Table III). On the other hand, cycloheximide, an inhibitor of protein synthesis, was not able to inhibit the increase in reactivity of the matrix. When endothelial cells were incubated with purified von Willebrand factor in the culture medium for 2 h, no increase in reactivity of the extracellular matrix could be found (Table III).

Besides thrombin, the Ca^{2+}-ionophore A23187 and the phorbol ester PMA can instantaneously induce the release of von Willebrand factor from intracellular storage pools. 2-h treatment of endothelial cells with 10 μM A23187 or 20 ng/ml PMA also increased the reactivity of the extracellular matrix towards platelets (Table IV).

Long-term Effects of PMA and Thrombin on Endothelial Cell Matrices

Incubation of endothelial cells with PMA impairs the accumulation of fibronectin in the culture medium after 2 d, whereas the accumulation of von Willebrand factor increases under these conditions (25). On the contrary, prolonged incubation with PMA or thrombin resulted in a severe decrease in the amounts of von Willebrand factor and fibronectin present in the endothelial cell matrix after 3 d as quantified by ELISA (Table V). The decreases in extracellular matrix con-

Table IV. Effect of Different Stimuli on the Reactivity of the Extracellular Matrix towards Platelets

Addition	Surface coverage
	%
No addition	19.4 ± 1.7
+ Thrombin (1 U/ml)	29.7 ± 0.8*
+ A23187 (10 μM)	29.3 ± 1.1*
+ PMA (20 ng/ml)	26.7 ± 1.9*

Endothelial cells grown to confluence on glass coverslips were incubated for 2 h with serum-free medium as indicated. The matrices were isolated and exposed to platelets resuspended in HAS. After perfusion, the matrices were fixed and stained, and the percentage surface coverage was determined by morphological evaluation. Results are expressed as mean ± SD (n = 4). * $P < 0.0001$ compared with control incubation (Peritz' F test).

Table V. Effect of Prolonged Incubation with PMA on the Amount of von Willebrand Factor and Fibronectin in the Endothelial Cell Extracellular Matrix

Addition to cells	Incubation of matrix	von Willebrand factor	Fibronectin
		ng/10⁶ cells	*μg/10⁶ cells*
–	–	88 ± 17	11.5 ± 2.2
–	von Willebrand Factor	60 ± 23	NT
PMA	–	13 ± 10*	1.4 ± 0.7*
PMA	von Willebrand factor	56 ± 5	NT
Thrombin	–	24 ± 6*	NT

Endothelial cells were grown for 3 d in the presence of PMA (20 ng/ml). After removing the cells, the matrices were incubated for 1 h with Michaelis buffer with 1% BSA or Michaelis buffer with 1% BSA and 10 μg/ml von Willebrand factor. Then the matrices were scraped off the flasks. The amounts of von Willebrand factor and fibronectin were determined as described. Results are expressed as mean ± SD (n = 3).
* $P < 0.001$ (Peritz' F test).
NT, not tested.

tents of fibronectin and von Willebrand factor were also seen when [^{35}S]methionine-labeled proteins from extracellular matrix extracts were immunoprecipitated with monoclonal antibodies against fibronectin and von Willebrand factor (Fig. 4). Antifibronectin antibodies precipitated a 220,000-mol-wt protein that co-migrated with purified plasma fibronectin (not shown). Anti-von Willebrand factor antibodies precipitated a 225,000-mol-wt protein that co-migrated with purified plasma von Willebrand factor. A 55,000-mol-wt protein and two proteins of 45,000 and 47,000 mol wt were precipitated with both antibodies. These impurities were also found in control precipitations (not shown). The decrease in von Willebrand factor and fibronectin contents was accompanied by a decrease in the ability of the isolated extracellular matrix to support platelet adhesion when these matrices were perfused with platelets resuspended in HAS (Fig. 5). The addition of normal plasma to the perfusate or the addition of purified von Willebrand factor to the HAS overcame the PMA-induced inability to support platelet adherence to the matrix (Fig. 5, Table VI). Addition of purified plasma fibronectin had no influence on the number of platelets adhering to a PMA-treated matrix. Addition of purified von Willebrand factor dissolved in HAS to matrices of PMA-treated cells increased the amount of von Willebrand factor in this matrix (Table V).

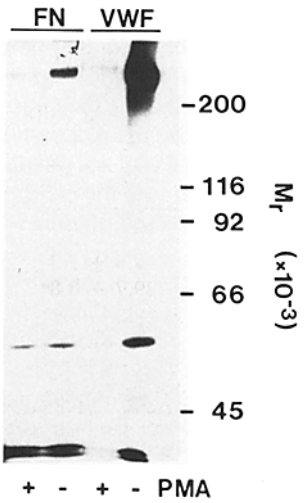

Figure 4. Influence of a 3-d culture in the presence of PMA on extracellular von Willebrand factor and fibronectin. Endothelial cells were maintained in serum-free medium with 0.2 MBq/ml [^{35}S]methionine for 48 h with or without 10 ng/ml PMA. Extracellular matrices were isolated and fibronectin and von Willebrand factor were immunoprecipitated and separated by SDS PAGE. Proteins were visualized by autoradiography as described in Materials and Methods. Lanes *1* and *2*, antifibronectin immunoprecipitate; lanes *3* and *4*, anti-von Willebrand factor immunoprecipitate; lanes *1* and *3*, cells incubated 48 h with PMA; lanes *2* and *4*, control cells.

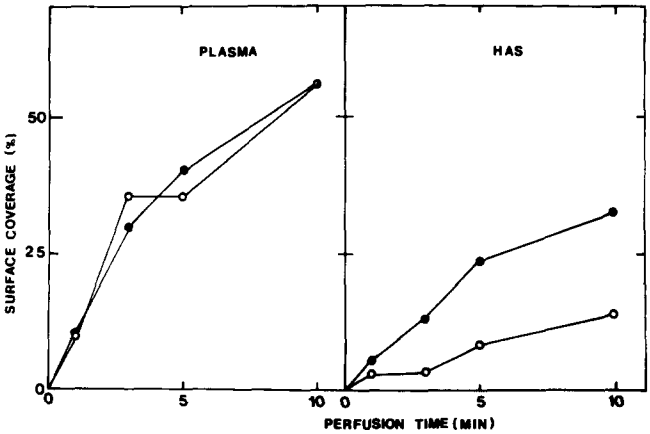

Figure 5. Time course of platelet deposition on extracellular matrices produced by endothelial cells (*solid circle*) and PMA-treated endothelial cells (*open circle*). Endothelial cells were treated for 3 d with 20 ng/ml PMA. After isolation of the matrix, the matrix was perfused with platelets resuspended in plasma or HAS. The amount of surface covered with platelets was determined morphometrically. Results are means of two determinations.

Besides PMA, thrombin treatment of endothelial cells also resulted in a strong decrease in the ability to support platelet adhesion (Table VI). This decrease could be abolished by the addition of von Willebrand factor to the perfusate, but not by the addition of purified plasma fibronectin.

Discussion

The extracellular matrix of cultured endothelial cells may be used as a model for subendothelium in studies of blood platelet–vessel wall interactions (1, 2, 33, 34, 40). We have previously reported on the interaction of platelets with the endothelial cell and their extracellular matrix in a perfusion chamber under well-defined flow conditions (33) and we have shown that the presence of von Willebrand factor and fibronectin in the extracellular matrix is an absolute requirement for normal platelet adhesion (10). Both von Willebrand factor and fibronectin are synthesized by endothelial cells

Table VI. Effect of (Purified) von Willebrand Factor and Fibronectin on Platelet Adhesion on Matrices of PMA-treated Cells

	Platelet coverage on matrix of		
Perfusate	Control cells percent coverage	PMA-treated cells percent coverage	Thrombin-treated cells percent coverage
HAS	26.7 ± 0.5	8.5 ± 5.3	9.1 ± 4.8
HAS + VWF	26.3 ± 3.1	22.0 ± 3.3	29.4 ± 1.4
HAS + FN	21.8 ± 1.7	10.8 ± 0.7	7.8 ± 4.0
Plasma	30.9 ± 2.5	22.2 ± 3.5	ND

Endothelial cells were grown for 4 d in the presence or absence of PMA (20 ng/ml) or thrombin (1 U/ml). The matrices were isolated and exposed to platelets resuspended in HAS, HAS supplemented with 10 μg/ml von Willebrand factor, HAS supplemented with 200 μg/ml fibronectin, or platelets resuspended in plasma. After perfusions, the matrices were fixed and stained, and the percentage surface coverage was determined by morphological evaluation. Results are expressed as mean ± SD (n = 4).
ND, not done.

(13, 14) and are incorporated into their extracellular matrix (9, 38). However, these major endothelial cell proteins are not localized in the same subcellular compartments, and, in spite of their co-distribution outside the cell, divergent routes of intracellular translocation of fibronectin and von Willebrand factor have been suggested (9, 25, 38).

We have studied the influence of perturbation of endothelial cells on the amounts of fibronectin and von Willebrand factor in their extracellular matrix and the consequences of a changed composition of the matrix on their ability to support platelet adhesion. Platelet adhesion was evaluated by measuring the percentage coverage of the extracellular matrix with platelets. The percentage coverage correlates well with the number of platelets adhering to the matrix (33). A coverage of 10% means $\sim 10^6$ platelet/cm^2 (33). In the experiments described in this paper, treatment of endothelial cells with PMA or thrombin does not influence the spreading of the platelets on the matrix (data not shown), indicating that the same correlation between coverage and platelet number exists.

A 3-d culture of endothelial cells in the presence of the phorbol ester PMA (20 ng/ml) caused an 80% decrease in the von Willebrand factor contents of the endothelial cell extracellular matrix (Table V and Fig. 4). Comparable results were found with thrombin. This decrease in amount of von Willebrand factor coincided with a decrease in the number of platelets adhering to the matrix (Table VI and Fig. 5).

The decrease in platelet adhesion to matrices of PMA-treated endothelial cells was corrected when perfusions were performed with platelets resuspended in plasma or in the human albumin solution with the addition of purified von Willebrand factor. Addition of von Willebrand factor to a von Willebrand factor–depleted matrix increased the amount of von Willebrand factor in these matrices. Addition of plasma fibronectin to the perfusate had no influence on the number of platelets adhering to the matrix. These results indicate that upon perturbation with PMA or thrombin, endothelial cells alter the composition of their matrix, and that this alteration has consequences for the adhesion of platelets to this matrix. Evidently, the extracellular matrix of endothelial cells is subjected to a continuous process of construction and degradation. After PMA treatment, presumably by the induction of proteases (26), endothelial cells demolish their matrix more rapidly (16) and thus favor decreased deposition of not only structural extracellular matrix proteins, but also proteins, like von Willebrand factor, that are involved in platelet adhesion. PMA induces plasminogen activator secretion by endothelial cells (18). Guisasola et al. have showed that plasmin could destroy von Willebrand factor (8). The decrease in the amount of von Willebrand factor of the extracellular matrix might be due to the action of the induced plasmin activity. Prolonged incubation of endothelial cells with PMA results in an increased secretion of von Willebrand factor to the culture medium (25). Probably, in the presence of PMA, newly synthesized von Willebrand factor is predominantly secreted towards the culture medium and is no longer incorporated into the extracellular matrix.

In immunoprecipitation studies with monoclonal antibodies against von Willebrand factor and fibronectin from [^{35}S]methionine-labeled matrices we always found a strong 55-kD band and two weak bands of 45 and 47 kD. The nature of these bands is unknown, but they are also strongly influenced by PMA treatment. Evidently PMA changes the complete composition of the matrix, not only von Willebrand factor and fibronectin.

Treatment of endothelial cells for 2 h with thrombin or PMA had no influence on the total amounts of von Willebrand factor and fibronectin in the endothelial cell matrix. However, there was an increased ability of the matrix to support platelet adhesion. This increase was very distinct when the matrix was perfused in the absence of von Willebrand factor and fibronectin in the perfusate. Preincubation of the matrix of thrombin-treated cells with a monoclonal antibody against von Willebrand factor completely inhibited platelet adhesion to these matrices (Table II). Probably not only the total amounts of von Willebrand factor and fibronectin present in the matrix determine the number of platelets adhering, but also the way these proteins are organized in the matrix. Only relatively few von Willebrand factor molecules are present at sites in the subendothelium where they are reactive to platelets (37). Most other molecules are present at irrelevant sites, out of reach of passing platelets. Another possibility is that the multimeric composition of von Willebrand factor in the matrix has been changed, as a result of the perturbation. In all probability the support of platelet adhesion is best performed by multimers of high molecular mass. Perhaps the brief perturbation caused by PMA or thrombin results in an increase in multimers with high molecular mass. Our speculation is that thrombin treatment of endothelial cells results in a fast reorganization of their matrix, resulting in an enhanced amount of reactive von Willebrand factor or fibronectin.

Recently we have shown that treatment of endothelial cells with thrombin results in an instantaneous increase in the amount of von Willebrand factor secreted to the culture medium, and that this increased secretion depends on the methylation of phosphatidylethanolamine (3). Addition of 3-deazaadenosine, an inhibitor of the enzymic methylation of phosphatidylethanolamine, not only inhibited the release of von Willebrand factor into the culture medium (3), but also prevented an increase in the amount of platelets adhering to the matrix (Table II). Also, the increased secretion to the culture medium after thrombin treatment (17) and the increase in platelet adhesion could not be inhibited by cycloheximide, an inhibitor of protein synthesis. This suggests that the proposed alteration of the matrix organization is caused by the same cellular mechanism that releases receptor-mediated von Willebrand factor from cellular storage sites to the culture medium (3). On the other hand, addition of purified von Willebrand factor to the culture medium of the cells in a concentration at least 10 times higher than normally reached in the medium did not increase the reactivity. Thus, the increased reactivity of the matrix was not due to increased secretion of von Willebrand factor into the culture medium after perturbation followed by increased binding to the matrix.

The increased reactivity of the matrix after 2 h of thrombin treatment depends on the presence of cells during these 2 h. In the absence of cells, thrombin did not affect the ability of the matrix to support platelet adhesion (Fig. 2). Moreover, a 2-h incubation of the cells with the Ca^{2+}-ionophore A23187 or PMA also induced the increased reactivity of the

matrix (Table III). Thus, the increased reactivity is not likely to be due to a direct proteolytic effect of thrombin on matrix proteins.

The presence of von Willebrand factor and fibronectin in the endothelial cell matrix is required for normal platelet adhesion (10). However, this does not mean that they are the only matrix proteins involved in platelet adhesion. A co-distribution of von Willebrand factor in the extracellular matrix of endothelial cells with fibronectin and collagen types IV and V has been shown (42). ELISA studies showed an interaction of thrombospondin with collagen type V, von Willebrand factor, and fibronectin (23), and recently the participation of laminin and nidogen in platelet adhesion has been indicated (4, 11). It is possible that thrombin changes the amounts of other matrix proteins such as collagen types IV and V, laminin, or nidogen, and that these changes are responsible for the increased ability of the matrix to support platelet adhesion. Further studies are necessary to answer these questions.

Perturbation of endothelial cells with PMA induces a series of metabolic changes, resulting in an altered participation of endothelial cells in hemostatic processes. PMA induces the synthesis of tissue factor by endothelial cells (19), and enhances prostacyclin synthesis (24) and the secretion of von Willebrand factor and tissue-type plasminogen activator (17, 18). Here we show that long-term incubation of endothelial cells with PMA, a condition that may be relevant for endothelial cells in pathophysiological situations, resulted in decreased amounts of von Willebrand factor and fibronectin in their extracellular matrix, which had direct consequences for platelet adhesion. From these in vitro results, it seems likely that endothelial cells respond to perturbation by, among other things, increasing their prostacyclin synthesis and by decreasing the reactivity of their matrix, making it more difficult for platelets to adhere and aggregate on the disturbed surface of the vessel wall. This may be another good example of the potential of endothelial cells to control physiological processes such as hemostasis.

The findings described in this paper show that the endothelial cell matrix is not an inert protein layer, but that the composition and organization of the proteins in the matrix are closely regulated by the cells on top. After 3 d of PMA-treatment, 80% of the von Willebrand factor and fibronectin disappear, suggesting that these proteins are removed by an active process. Also, 2 h of thrombin treatment changes the reactivity of the matrix, suggesting that an active process reorganizes the already formed matrix. Perturbation of endothelial cells strongly influences their metabolism, resulting in a changed spectrum of secreted proteins and in the alteration of the insoluble matrix underneath the cells.

The authors thank Mrs. Mieke Ottenhof-Rovers, Mrs. Hetty de Boer and Mr. R. C. Vervoorn for their technical assistance, Mrs. Patricia Nievelstein for supplying purified fibronectin, and Miss Annemiek Beyer and Miss Maeyken Hoeneveld for their secretarial assistance.

J. H. Reinders was supported by grant 13-30-31 from the Netherlands Organization for the Advancement of Pure Research (ZWO).

Received for publication 31 December 1985, and in revised form 4 October 1986.

References

1. Booyse, F. M., A. J. Quarfoot, and S. Feder. 1982. Culture-produced subendothelium. I. Platelet interaction and properties. *Haemostasis.* 11:49–61.

2. Chazov, E. I., A. V. Alexeev, A. S. Antonov, V. E. Koteliansky, V. L. Leytin, E. V. Lyubimova, V.S. Repin, D. D. Sviridov, U. P. Torchilin, and V. N. Smirnov. 1981. Endothelial cell culture on fibrillar collagen: model to study platelet adhesion and liposome targeting to intracellular collagen matrix. *Proc. Natl. Acad. Sci. USA.* 78:5603–5607.

3. de Groot, P. G., M. D. Gonsalves, C. Loesberg, M. F. van Buul Wortelboer, W. G. van Aken, and J. A. van Mourik. 1984. Thrombin-induced release of von Willebrand factor from endothelial cells is mediated by phospholipid methylation. *J. Biol. Chem.* 259:13329–13333.

4. de Groot, P. G., M. Ottenhof-Rovers, M. Dziadek, R. Timpl, and J. J. Sixma. 1985. Nidogen is present in the extracellular matrix of human endothelial cells and is involved in platelet adhesion to this matrix. *Thromb. Haemostasis.* 54:231.

5. Engvall, E., E. Ruoslahti, and E. J. Miller. 1978. Affinity of fibronectin to collagens of different genetic types and to fibrinogen. *J. Exp. Med.* 147:1584–1595.

6. Foidart, J. M., E. W. Bere, M. Yaar, S. I. Rennard, M. Gullino, G. R. Martin, and S. I. Katz. 1980. Distribution and immuno-electron microscopic localization of laminin. A non-collageneous basement membrane glycoprotein. *Lab. Invest.* 42:336–342.

7. Gamse, G., H. G. Fromme, and H. Kresse. 1978. Metabolism of sulfated glycosaminoglycans in cultured endothelial cells and smooth muscle cells from bovine aorta. *Biochim. Biophys. Acta.* 554:514–528.

8. Guisasola, J. A., C. G. Cockburn, and R. M. Hardisty. 1978. Plasmin digestion of factor VIII: characterization of the breakdown products with respect to antigenicity and von Willebrand activity. *Thromb. Haemostasis.* 40: 302–315.

9. Hormia, M., V. P. Lehto, and I. Virtanen. 1984. Intracellular localization of factor VIII-related antigen and fibronectin in cultured human endothelial cells. Evidence of divergent routes of intracellular translocation. *Eur. J. Cell Biol.* 33:217–228.

10. Houdijk, W. P. M., P. G. de Groot, P. F. E. M. Nievelstein, K. S. Sakariassen, and J. J. Sixma. 1986. Von Willebrand factor and fibronectin but not thrombospondin are involved in platelet adhesion to the extracellular matrix of human vascular endothelial cells. *Arteriosclerosis.* 6:24–33.

11. Ill, C. R., E. Engvall, and E. Ruoslathi. 1984. Adhesion of platelets to laminin in the absence of activation. *J. Cell Biol.* 99:2140–2145.

12. Jaffe, E. A., R. L. Nachman, C. G. Becker, and C. R. Minick. 1973. Culture of human endothelial cells derived from umbilical cord veins. Identification by morphologic and immunologic criteria. *J. Clin. Invest.* 52:2745–2756.

13. Jaffe, E. A., and D. F. Mosher. 1978. Synthesis of fibronectin by cultured human endothelial cells. *J. Exp. Med.* 147:1779–1791.

14. Jaffe, E. A., L. W. Hoyer, and R. L. Nachman. 1974. Synthesis of von Willebrand factor by cultured human endothelial cells. *Proc. Natl. Acad. Sci.* 71:1906–1909.

15. Klebe, R. J., K. L. Bentley, P. J. Sasser, R. C. Schoen. 1980. Elution of fibronectin from collagen with chaotrophic agents. *Exp. Cell Res.* 130:111–117.

16. Laug, W. E., M. E. Weinblatt, and P. A. Jones. 1985. Endothelial cells degrade extracellular matrix proteins produced in vitro. *Thromb. Haemostasis.* 54:498–502.

17. Loesberg, C., M. D. Gonsalves, J. Zandbergen, C. Willems, W. G. van Aken, H. V. Stel, J. A. van Mourik, and P. G. de Groot. 1983. The effect of calcium on the secretion of factor VIII-related antigen by cultured human endothelial cells. *Biochim. Biophys. Acta.* 763:160–168.

18. Loskutoff, D. J., and T. S. Edgington. 1977. Synthesis of a fibrinolytic activator and inhibitor by endothelial cells. *Proc. Natl. Acad. Sci. USA.* 74: 3903–3907.

19. Lyberg, T., K. S. Galdal, S. A. Evensen, and H. Prydz. 1983. Cellular cooperation in endothelial cell thrombosplastin synthesis. *Br. J. Haematol.* 53:85–95.

20. Madri, J. A., B. Dreyer, F. A. Pitlick, and H. Furthmayr. 1980. The collagenous components of the subendothelium, correlation of structure and function. *Lab. Invest.* 43:303–315.

21. McPhearson, J., H. Sage, and P. Bornstein. 1981. Isolation and characterization of a glycoprotein secreted by aortic endothelial cells in culture: apparent identity with platelet thrombospondin. *J. Biol. Chem.* 256:11330–11336.

22. Mosher, D. F., and A. Vaheri. 1978. Thrombin stimulates the production and the release of a major surface-associated glycoprotein (fibronectin) in cultures of human fibroblasts. *Exp. Cell Res.* 112:323–334.

23. Mumby, S. M., G. J. Raugi, and P. Bornstein. 1984. Interactions of thrombospondin with extracellular matrix proteins: selective binding to type V collagen. *J. Cell Biol.* 98:646–652.

24. Nawroth, P. P., D. M. Stern, K. L. Kaplan, and H. L. Nossel. 1984. Prostacyclin synthesis by perturbed bovine aortic endothelial cells in culture. *Blood.* 64:801–806.

25. Reinders, J. H., P. G. de Groot, J. Dawes, N. R. Hunter, H. A. A. van Heugten, J. Zandbergen, M. D. Gonsalves, and J. A. van Mourik. 1985. Comparison of secretion and subcellular localization of von Willebrand protein with that of thrombospondin and fibronectin in cultured human vascular endothelial cells. *Biochim. Biophys. Acta.* 844:306–313.

26. Rifkin, D. B., J. L. Gross, D. Moscatelli, and E. A. Jaffe. 1982. Proteases and angiogenesis: production of plasminogen activator and collagenase by endothelial cells. *In* Pathobiology of the Endothelial Cell. H. L. Nossel, and

H. J. Vogel, editors. Academic Press, Inc., New York. 191–197.

27. Ross, R., and J. A. Glomset. 1976. The pathogenesis of atherosclerosis. *N. Engl. J. Med.* 295:369–377, 420–425.

28. Sage, H., E. Crouch, and P. Bornstein. 1979. Collagen synthesis by bovine aortic endothelial cells in culture. *Biochemistry.* 18:5433–5442.

29. Sage, H. 1982. Collagens of basement membranes. *J. Invest. Dermatol.* 79:515–595.

30. Sage, H., G. Balian, A. M. Vogel, and P. Bornstein. 1984. Type VIII collagen. Synthesis by normal and malignant cells in culture. *Lab. Invest.* 50: 219–231.

31. Sakariassen, K. S., P. A. Bolhuis, and J. J. Sixma. 1979. Human blood platelet adhesion to artery subendothelium is mediated by factor VIII- von Willebrand factor bound to the subendothelium. *Nature (Lond.).* 279:636–638.

32. Sakariassen, K. S., P. A. Bolhuis, and J. J. Sixma. 1980. Platelet adherence to subendothelium of human arteries in pulsatile and steady flow. *Thromb. Res.* 19:547–559.

33. Sakariassen, K. S., P. A. M. M. Aarts, P. G. de Groot, W. P. M. Houdijk, and J. J. Sixma. 1983. A perfusion chamber developed to investigate platelet interaction in flowing blood with human vessel wall cells, their extracellular matrix, and purified components. *J. Lab. Clin. Med.* 102:522–535.

34. Sakariassen, K. S., J. D. Banga, P. G. de Groot, and J. J. Sixma. 1984. Comparison of platelet interaction of subendothelium of human renal and umbilical arteries on the extracellular matrix produced by human venous endothelial cells. *Thromb. Haemostasis.* 52:60–65.

35. Sixma, J. J., and J. Wester. 1977. The hemostatic plug. *Semin. Hematol.* 14:265–299.

36. Sixma, J. J., K. S. Sakariassen, H. V. Stel, W. P. M. Houdijk, D. W. In der Maur, R. J. Hamer, P. G. de Groot, and J. A. van Mourik. 1984. Functional domains on von Willebrand factor: recognition of discrete tryptic fragments by monoclonal antibodies that inhibit interaction of von Willebrand factor with platelets and with collagen. *J. Clin. Invest.* 74:736–744.

37. Stel, H. V., K. S. Sakariassen, P. G. de Groot, J. A. van Mourik, and J. J. Sixma. 1985. Von Willebrand factor in the vessel wall mediates platelet adherence. *Blood.* 65:85–90.

38. Sussman, I. I., and J. H. Rand. 1982. Subendothelial deposition of von Willebrand factor requires the presence of endothelial cells. *J. Lab. Clin. Med.* 100:526–532.

39. van Mourik, J. A., and I. A. Mochtar. 1970. Purification of human antihemophilic factor (factor VIII) by gel-chromatography. *Biochim. Biophys. Acta.* 221:677–679.

40. Vlodawsky, I., A. Eldor, E. Hyam, R. Atzmon, and Z. Fuks. 1982. Platelet interaction with the extracellular matrix produced by cultured endothelial cells: a model to study the thrombogenicity of isolated subendothelial basal lamina. *Thromb. Res.* 28:179–191.

41. Wagner, D. D., J. B. Olmsted, and V. J. Marder. 1982. Immunolocalization of von Willebrand protein in Weibel-Palade bodies of human endothelial cells. *J. Cell Biol.* 95:355–360.

42. Wagner, D. D., M. Urban-Pickering, and V. J. Marder. 1984. Von Willebrand protein binds to extracellular matrices independently of collagen. *Proc. Natl. Acad. Sci. USA.* 81:471–475.

43. Weiss, H. J., V. T. Turitto, and H. R. Baumgartner. 1978. Effect of shear rate on platelet interaction with subendothelium in citrated and native blood. *J. Lab. Clin. Med.* 92:750–764.

44. Willems, C., G. C. B. Astaldi, P. G. de Groot, M. C. Janssen, M. D. Gonsalves, W. P. Zeijlemaker, J. A. van Mourik, and W. G. van Aken. 1982. Media conditioned by cultured human vascular endothelial cells inhibit the growth of vascular smooth muscle cells. *Exp. Cell Res.* 139:191–197.