GROWTH AND FIBRINOLYTIC PARAMETERS OF HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS SEEDED ONTO CARDIOVASCULAR GRAFTS

It was the aim of this study to investigate possible effects of biomaterials used to produce vascular grafts on the fibrinolytic system of endothelial cells. Therefore growth conditions for human umbilical vein endothelial cells on polytetrafluoroethylene and on polyurethane were optimized. Tissue culture polystyrene was used as a control material. We could demonstrate that precoating of the materials with fibronectin significantly increased the growth rate of human umbilical vein endothelial cells on these materials. Furthermore, we showed that human umbilical vein endothelial cells grown on polytetrafluoroethylene or polyurethane released more plasminogen activator inhibitor-1 and tissue type-plasminogen activator into the conditioned media than did human umbilical vein endothelial cells grown on tissue culture polystyrene. Human umbilical vein endothelial cells cultured on polytetrafluoroethylene also deposited more plasminogen activator inhibitor-1 into the extracellular matrix than did control cells grown on tissue culture polystyrene. Our results give evidence that human umbilical vein endothelial cells grown on two biomaterials used to construct vascular grafts, namely polytetrafluoroethylene and polyurethane, produce tissue-type plasminogen activator as well as plasminogen activator inhibitor-1, two major components of the fibrinolytic system also expressed by endothelial cells in vivo. In conclusion, our data suggest that endothelial cells grown on vascular grafts show functional integrity concerning their fibrinolytic system, which in turn might contribute to reduce the thrombogenic properties of the graft material. (J THORAC CARDIOVASC SURG 1995;109:1059-65)

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Although blood compatibility of biomaterials has improved over the years, a nonthrombogenic surface has not yet been found. The failure of many clinical implantation procedures is attributed to the early thrombogenicity of the graft surface and to the late development of initial hyperplasia and downstream atherosclerosis.[1, 2] Attempts to endothelialize synthetic graft surfaces of small-diameter vascular prostheses to overcome their thrombogenic properties have been made.[3-8] Endothelial cells play a key role in the development and dissolution of thrombi. They produce urokinase-type plasminogen activator, tissue-type plasminogen activator, and their major physiologic inhibitor, plasminogen activator inhibitor-1.[9] Tissue-type plasminogen activator, which activates the zymogen plasminogen to plasmin with much higher efficiency in the presence of fibrin, is thought to be mainly responsible for intravascular clot lysis, whereas urokinase-type plasminogen activator is involved in processes requiring extracellular proteolysis.[10]

The production of plasminogen activators and plasminogen activator inhibitor-1 by endothelial cells is regulated by a wide variety of stimuli,[11-15] whereby an increase in the production of tissue-type plasminogen activator would enhance the fibrinolytic potential of these cells and an increase in plasminogen activator inhibitor-1 production would decrease the fibrinolytic potential of the endothelium, rendering it antifibrinolytic. Impaired fibrinolysis, on the other hand, is thought to predispose to thrombus formation and atherosclerosis. Functional properties of endothelial cells in culture are strongly

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dependent on the extracellular matrix onto which cells had been seeded. Therefore functional properties of endothelial cells are strongly influenced by the biomaterials used for vascular grafting and are of major importance for maintaining a nonthrombogenic surface. It was the aim of this report to study the fibrinolytic system of human endothelial cells seeded onto different biomaterials used for the production of vascular grafts and to investigate possible effects of these materials on the fibrinolytic capacity of such endothelial cells.

Materials and methods

Cell culture. Human umbilical vein endothelial cells were isolated by mild collagenase treatment as described.[16] The endothelial cells were seeded into Petri dishes (Costar, Cambridge, Mass.) coated with 1% gelatin (Biorad, Richmond, Calif.) and grown to confluence in medium 199 (Sigma Chemical Co., St. Louis, Mo.) containing the following: 20% supplemented calf serum (HyClone, Logan, Utah), penicillin 50 IU/ml, streptomycin 50 μg/ml, amphotericin B 250 ng/ml (all JRH Biosciences, Lenexa, Kan.), ECGS 50 μg/ml (Technoclone, Vienna, Austria), and heparin 5 U/ml (Liquemin Roche, Hoffmann-La Roche, Basel, Switzerland) in a humidified atmosphere of 95% air and 5% carbon dioxide at 37° C. Cells were confirmed to be endothelial cells by their typical cobblestone shape,[17] by positive immunofluorescence with anti–von Willebrand factor antibodies (Cappel, Cochranville, Pa.),[18] and by uptake of acetylated low-density lipoprotein.[19] All human umbilical vein endothelial cells used in this study were between passages 2 and 3.

Graft materials. Polyurethane, which was kindly provided by The Institute of Chemistry (Chinese Academy of Sciences, China), was purified by precipitation with methanol from a dimethylformamide solution and then dissolved in dimethylformamide and cast into membranes on cover slips. Submerging the cover slips in water for 2 hours yielded polyurethane membranes. Reinforced thin-walled polytetrafluoroethylene patches, 0.1 mm, were purchased from W. L. Gore & Assoc. Ges.m.b.H. (Salzburg, Austria). The surface of tissue culture polystyrene flasks (Costar) was used as control material.

Precoating with fibronectin. Human fibronectin was isolated as described previously.[20] Patches of polytetrafluoroethylene and polyurethane, respectively, and tissue culture polystyrene 96 well plates (Costar) were coated with fibronectin in phosphate-buffered saline, pH 7.4, at the indicated concentrations for 30 minutes at 37° C. Thereafter the material was rinsed three times with phosphate-buffered saline and remaining binding sites were blocked by incubation at 4° C overnight with 1% bovine serum albumin in phosphate-buffered saline. After the material had been washed three times with phosphate-buffered saline containing 0.5% Tween 20 (Sigma), fibronectin binding was quantified by incubating the material for 2 hours at 37° C with a peroxidase-labeled monoclonal antifibronectin antibody (2FN, Technoclone), which was labeled with peroxidase according to the protocol of Nakane and Kawaoi.[21] The surfaces were washed again and a 1 gm/L solution of 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (Sigma) in sodium phosphate 0.11 mol/L, citrate buffer 0.05 mol/L, pH 5.85, made 0.03% (vol/vol) in hydrogen peroxide, was added to the fibronectin-coated tissue culture polystyrene 96 well plates or to pieces of polytetrafluoroethylene or polyurethane cut to size (0.64 cm diameter) and placed into untreated 96 well plates. Absorbances at 405 nm (492 nm reference wavelength) were read with an Anthos Reader 2001 (Anthos, Austria). As a control, the same sequence was performed with uncoated polytetrafluoroethylene, polyurethane, and tissue culture polystyrene.

For cell growth experiments, tissue culture polystyrene 96 well plates or patches of polyurethane and polytetrafluoroethylene, which were sterilized and cut to size (0.64 cm diameter) and placed into 96 well plates were coated with fibronectin 10 $\mu g/cm^2$ for 30 minutes at 37° C. The material was then rinsed three times with phosphate-buffered saline to remove nonbound fibronectin, and human umbilical vein endothelial cells were seeded onto the surfaces as described later.

Seeding procedure and determination of growth of human umbilical vein endothelial cells. Human umbilical vein endothelial cells, grown to confluence in Petri dishes under conditions described earlier, were seeded into tissue culture polystyrene-96 well plates (Costar) or onto patches of polytetrafluoroethylene or polyurethane placed into 96 well plates at a density of 9×10^3 cells/cm^2. Four days after the cells were seeded, cell numbers were determined with the EZ4U kit (Biomedica, Vienna, Austria).[22] In brief, a 25 μl dye solution that becomes incorporated into ribosomes of live cells was added to the wells containing human umbilical vein endothelial cells and 200 μl of regular growth medium. Thereafter the cells were incubated for 3 hours at 37° C and the absorbance at a wavelength of 450 nm (620 nm reference wave length) was determined with an Anthos Reader 2001 (Anthos). A calibration curve was constructed by means of wells containing different numbers of human umbilical vein endothelial cells. The cell number in these wells was determined by staining the nuclei with crystal violet.[23] A linear correlation existed between absorbance values and the number of human umbilical vein endothelial cells in the respective wells ($r = 0.98$).

Preparation of conditioned media and extracellular matrix. Confluent monolayers of human umbilical vein endothelial cells grown on polytetrafluoroethylene, polyurethane, or tissue culture polystyrene as described were rinsed twice with Hanks balanced salt solution (Sigma). Thereafter medium 199 containing insulin 5 μg/ml, transferrin 5 μg/ml, and sodium selenite 5 ng/ml (Sigma) was added to the cells. After the indicated time periods, conditioned medium of such cells was harvested, centrifuged at 1000 *g* for 5 minutes to remove cell debris, and stored at −70° C. So that extracellular matrix could be obtained from these cells, the monolayers were washed three times with cold phosphate-buffered saline, pH 7.4. Thereafter the cells were lysed with 0.1% Triton X-100 octyphenoxy polyethoxyethanol in phosphate-buffered saline, pH 7.4, and cellular components were removed by washing with distilled water.

Assays for tissue-type plasminogen activator, urokinase-type plasminogen activator, and plasminogen activator inhibitor-1 antigen in conditioned medium. Tissue-type plasminogen activator, urokinase-type plasminogen activator, and plasminogen activator inhibitor-1 antigen in conditioned medium were determined by specific enzyme-linked immunosorbent assays (ELISAs) with monoclonal antibodies specific for tissue-type plasminogen activator, urokinase-type plasminogen activator, and plasminogen activator inhibitor-1 (Technoclone). The test ranges for these assays are 0.3 to 2.5 ng/ml for tissue-type plasminogen activator, 0.6 to 10 ng/ml for urokinase-type plasminogen activator, and 1.0 to 30.0 ng/ml for plasminogen activator inhibitor-1. The tissue-type plasminogen activator ELISA detects free tissue-type plasminogen activator and tissue-type plasminogen activator in complex with plasminogen activator inhibitor-1; the urokinase-type plasminogen activator ELISA detects free urokinase-type plasminogen activator and urokinase-type plasminogen activator in complex with plasminogen activator inhibitor-1; and the plasminogen activator inhibitor-1 ELISA measures active, complexed, and latent plasminogen activator inhibitor-1.

Determination of tissue-type plasminogen activator and plasminogen activator inhibitor-1 antigen in extracellular matrix. Extracellular matrix prepared as described earlier was incubated for 2 hours at 37° C with 100 μl/well peroxidase-labeled monoclonal antibodies against tissue-type plasminogen activator (MPW3VPA, Technoclone) or plasminogen activator inhibitor-1 (3PAI5, Technoclone). Thereafter, the extracellular matrix was washed three times with 300 μl/well of phosphate-buffered saline, pH 7.4, containing 0.5% Tween 20 (Sigma), and 100 μl/well of 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) in sodium phosphate 0.11 mol/L, citrate buffer 0.05 mol/L, pH 5.85, made 0.03% (vol/vol) in hydrogen peroxide, was added. As a control, the materials coated with gelatin were processed as described earlier. Absorbances were read at 405 nm (492 nm reference wavelength). Absorbances obtained with the gelatin-coated materials were deducted from the absorbance values obtained with the respective extracellular matrix to correct for nonspecific binding of the monoclonal antibody. Nonspecific binding was less than 10%.

Statistical analysis. Values are given as means ± standard deviation. Analysis of variance was performed to statistically compare cell proliferation and production of fibrinolytic components of the three experimental groups. When only two groups were compared, a Student's *t* test for unpaired observations was performed.

Results

When polytetrafluoroethylene was coated with increasing concentrations of fibronectin, a dose-dependent increase in the cell number of human umbilical vein endothelial cells determined 4 days after seeding was seen (Fig. 1). Maximal increase in cell proliferation was seen from a 5 μg/cm^2 concentration of fibronectin onward. As can be seen from the *inset* to Fig. 1, fibronectin coating efficiency was similar for all three materials tested.

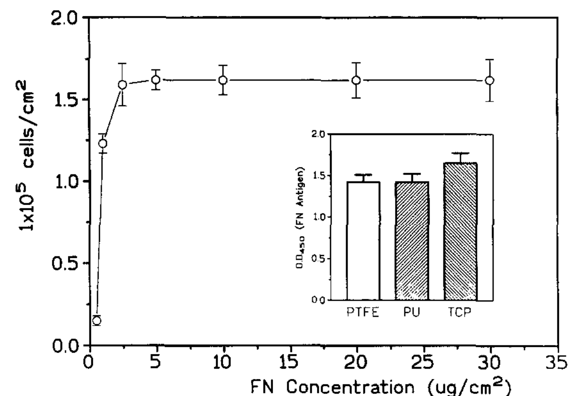

Fig. 1. The effect of fibronectin coating concentration on growth of human umbilical vein endothelial cells. Human umbilical vein endothelial cells were seeded on polytetrafluoroethylene coated with different concentrations of fibronectin (0.5 to 30.0 μg/cm^2) at a density of 9×10^3/cm^2 and counted 4 days after seeding, as described in the *Materials and methods* section. The data represent mean values ± standard deviations of three independent experiments. Nine pieces of the biomaterial were used. *Inset:* Fibronectin binding capacity of polytetrafluoroethylene, polyurethane, and tissue culture polystyrene. The material was coated with fibronectin (10 μg/cm^2). Fibronectin binding was determined with a peroxidase-labeled monoclonal antifibronectin antibody as described in the *Materials and methods* section. Values represent means ± standard deviations of three independent experiments. Nine pieces of the biomaterials were used. *FN,* Fibronectin; *PTFE,* polytetrafluoroethylene; *PU,* polyurethane; *TCP,* tissue culture polystyrene.

As can be seen from Fig. 2, coating with fibronectin significantly increased the proliferation of human umbilical vein endothelial cells on polytetrafluoroethylene and polyurethane when compared with the untreated control. The proliferation rate of human umbilical vein endothelial cells was significantly higher on polyurethane than on polytetrafluoroethylene or tissue culture polystyrene ($p < 0.01$).

As can be seen from Fig. 3, a time-dependent increase in the number of human umbilical vein endothelial cells growing on polytetrafluoroethylene coated with fibronectin was paralleled by a time-dependent increase in tissue-type plasminogen activator antigen in the conditioned medium of these cells.

Plasminogen activator inhibitor-1 antigen and tissue-type plasminogen activator antigen were determined in the conditioned medium of human umbilical vein endothelial cells grown on polytetrafluoroeth-

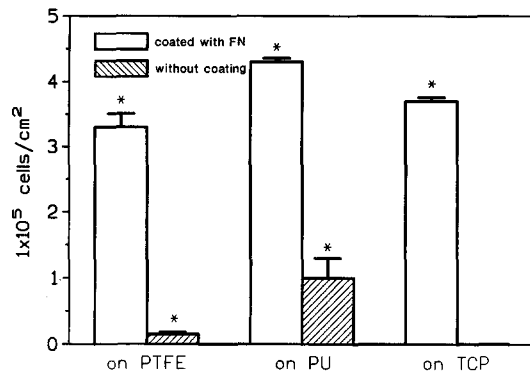

Fig. 2. Proliferation of human umbilical vein endothelial cells on polytetrafluoroethylene, polyurethane, and tissue culture polystyrene. Biomaterial surfaces were coated without or with fibronectin 10 $\mu g/cm^2$. Human umbilical vein endothelial cells were seeded at a density of $9 \times 10^3/cm^2$ and counted 4 days after seeding as described in the *Materials and methods* section. Cell numbers on each material were significantly different when compared with the two other materials (*$p < 0.01$). Values represent means ± standard deviations of three independent experiments. Twelve pieces of the biomaterials were used. *FN,* Fibronectin; *PTFE,* polytetrafluoroethylene; *PU,* polyurethane; *TCP,* tissue culture polystyrene.

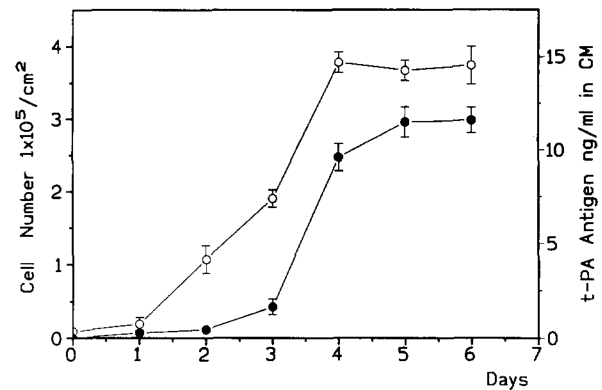

Fig. 3. Cell number and time course of tissue-type plasminogen activator antigen production by human umbilical vein endothelial cells grown on polytetrafluoroethylene. Human umbilical vein endothelial cells were seeded at a density of $9 \times 10^3/cm^2$ onto polytetrafluoroethylene coated with fibronectin (10 $\mu g/cm^2$). Cell numbers *(open circles)* at the days indicated and tissue-type plasminogen activator antigen *(closed circles)* in the conditioned medium harvested at the days indicated were determined as described in the *Materials and methods* section. Values represent means ± standard deviations of three independent experiments. Twelve pieces of the biomaterials were used. *t-PA,* Tissue-type plasminogen activator.

ylene, polyurethane, and tissue culture polystyrene. It was evident that human umbilical vein endothelial cells grown on polytetrafluoroethylene and polyurethane, respectively, released significantly more plasminogen activator inhibitor-1 and tissue-type plasminogen activator into the conditioned medium (plasminogen activator inhibitor-1: polytetrafluoroethylene, 2.51 ± 0.31 $\mu g/10^5$ cells per 24 hours; polyurethane, 2.24 ± 0.16 $\mu g/10^5$ cells per 24 hours; tissue culture polystyrene, 0.72 ± 0.10 $\mu g/10^5$ cells per 24 hours; $p < 0.001$ for polytetrafluoroethylene and polyurethane compared with tissue culture polystyrene; tissue-type plasminogen activator: polytetrafluoroethylene: 13.8 ± 1.5 $ng/10^5$ cells per 24 hours; polyurethane, 15.4 ± 1.2 $ng/10^5$ cells per 24 hours; tissue culture polystyrene, 7.7 ± $1.1/10^5$ cells per 24 hours; $p < 0.001$ for polytetrafluoroethylene and polyurethane as compared with tissue culture polystyrene). Urokinase-type plasminogen activator antigen was not detected in the conditioned medium under the conditions tested (Fig. 4).

As can be seen from Fig. 5, human umbilical vein endothelial cells grown on polytetrafluoroethylene deposited significantly more plasminogen activator inhibitor-1 antigen into the extracellular matrix than did human umbilical vein endothelial cells grown on tissue culture polystyrene ($p < 0.01$). No tissue-type plasminogen activator could be detected in the extracellular matrix of human umbilical vein endothelial cells grown under these conditions.

Discussion

Endothelium on vascular grafts has been shown to diminish platelet deposition and to improve the patency of prostheses.[8, 24, 25] However, adhesion and proliferation of endothelial cells on pure graft materials is minimal.[2, 26] Fibronectin and fibrin glue have been used to coat biomaterials to enhance adhesion and proliferation of endothelial cells. We could demonstrate that proliferation of human umbilical vein endothelial cells increased in a dose-dependent fashion when seeded onto polytetrafluoroethylene coated with increasing concentrations of fibronectin. The binding capacity for fibronectin of the two biomaterials used, namely polytetrafluoroethylene and polyurethane, was similar. Proliferation of human umbilical vein endothelial cells, however, was significantly higher on polyurethane than on polytetrafluoroethylene. This was also true when proliferation of human umbilical vein endothelial cells was measured on untreated polyure-

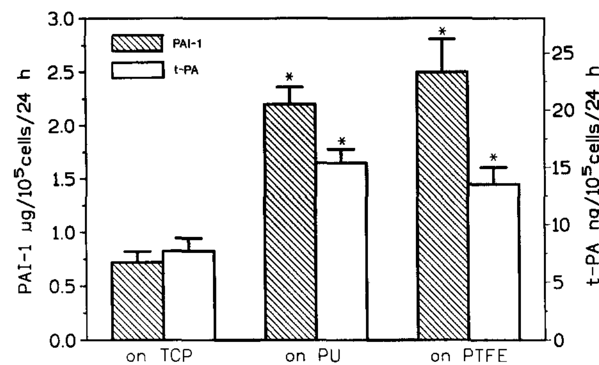

Fig. 4. Plasminogen activator inhibitor-1 and tissue-type plasminogen activator antigen secreted by confluent monolayers of human umbilical vein endothelial cells grown on polytetrafluoroethylene, polyurethane, and tissue culture polystyrene. Human umbilical vein endothelial cells were grown to confluence on polytetrafluoroethylene, polyurethane, or tissue culture polystyrene coated with fibronectin (10 $\mu g/cm^2$). Plasminogen activator inhibitor-1 and tissue-type plasminogen activator antigen in the conditioned medium of such cells incubated for 24 hours were determined by specific ELISAs, as described in the *Materials and methods* section. Significantly more plasminogen activator inhibitor-1 antigen and tissue-type plasminogen activator antigen were produced by human umbilical vein endothelial cells grown on polyurethane and polytetrafluoroethylene, respectively, as compared to human umbilical vein endothelial cells grown on tissue culture polystyrene (*$p < 0.001$). Values represent means ± standard deviations of three independent experiments. Twelve pieces of the biomaterials were used. *PAI-1,* Plasminogen activator inhibitor-1; *t-PA,* tissue-type plasminogen; *TCP,* tissue culture polystyrene; *PU,* polyurethane; *PTFE,* polytetrafluoroethylene.

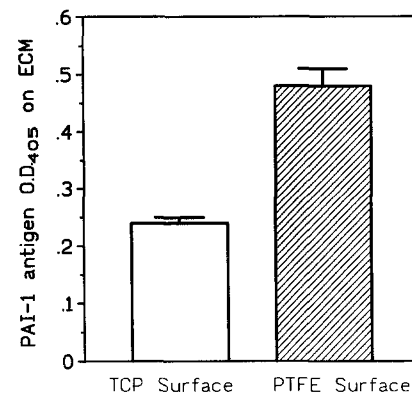

Fig. 5. Plasminogen activator inhibitor-1 antigen deposited by human umbilical vein endothelial cells grown on polytetrafluoroethylene or tissue culture polystyrene, respectively, into the extracellular matrix. Human umbilical vein endothelial cells were grown to confluence on polytetrafluoroethylene or tissue culture polystyrene coated with fibronectin (10 $\mu g/cm^2$). Plasminogen activator inhibitor-1 antigen deposited into the extracellular matrix by such cells incubated for 24 hours was determined by a specific ELISA as described in the *Materials and methods* section. Human umbilical vein endothelial cells grown on polytetrafluoroethylene deposited significantly more plasminogen activator inhibitor-1 antigen in the extracellular matrix than did human umbilical vein endothelial cells grown on tissue culture polystyrene ($p < 0.01$). Values represent means ± standard deviations of three independent experiments. Fifteen pieces of the biomaterials were used. *PAI-1,* Plasminogen activator inhibitor-1; *ECM,* extracellular matrix; *TCP,* tissue culture polystyrene; *PTFE,* polytetrafluoroethylene.

thane and polytetrafluoroethylene. The latter result could be explained by the fact that polyurethane provides a smooth surface for endothelial cell attachment whereas polytetrafluoroethylene shows a nodular structure.[1, 27]

The fibrinolytic system of human umbilical vein endothelial cells grown on artificial graft materials was chosen as a marker of functional integrity of these cells because it plays a major role in the development and dissolution of intravascular fibrin clots. Therefore functional integrity of the fibrinolytic system of endothelial cells seeded onto vascular grafts might contribute to the maintenance of a nonthrombogenic surface of the prostheses. We could demonstrate that human umbilical vein endothelial cells grown on polytetrafluoroethylene or polyurethane produce tissue-type plasminogen activator and plasminogen activator inhibitor-1, respectively, the two major components of the fibrinolytic system produced by human umbilical vein endothelial cells when cultured under in vitro conditions on tissue culture plastic. It is, however, noteworthy that human umbilical vein endothelial cells grown on polytetrafluoroethylene or polyurethane, respectively, produced significantly more tissue-type plasminogen activator and plasminogen activator inhibitor-1 than did human umbilical vein endothelial cells grown as a control on tissue culture polystyrene.

It has been suggested that activation of fibrinolysis by plasminogen activators produced by endothelial cells seeded onto vascular grafts would lead to the disintegration of fibrin layers coated onto the grafts to enhance endothelial cell attachment and subsequently to the dislodgment of these cells from such surfaces.[28, 29] In this study, however, we dem-

onstrate that human umbilical vein endothelial cells grown on polytetrafluoroethylene deposited significantly more plasminogen activator inhibitor-1 into the extracellular matrix than did human umbilical vein endothelial cells cultured on tissue culture polystyrene. Plasminogen activator inhibitor-1 deposited into the extracellular matrix by endothelial cells has been shown to remain active and to be capable of inactivating tissue-type plasminogen activator by forming a stable complex with the plasminogen activator.[30] Therefore one could speculate that plasminogen activator inhibitor-1 deposited into the extracellular matrix by human umbilical vein endothelial cells grown on artificial graft materials might act to prevent proteolytic damage to the endothelial layer on vascular prostheses.

In conclusion, our data emphasize the importance of precoating vascular graft materials with substances such as fibronectin, which enhance attachment and proliferation of endothelial cells on these materials. Furthermore, our results give evidence that endothelial cells grown on artificial surfaces used for the production of vascular grafts show functional integrity with respect to their fibrinolytic system. This fact might contribute to the decreased thrombogenicity of endothelialized grafts as compared with grafts without endothelial lining. Finally, the presence of plasminogen activator inhibitor-1 in the extracellular matrix of endothelial cells grown on artificial graft material suggests that the endothelial monolayer should be protected from proteolytic damage caused by plasminogen activators.

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