Expression of leukocyte adhesion molecules by endothelial cells seeded on various polymer surfaces

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Received 28 May 2000; revised 19 January 2001; accepted 6 March 2001

Abstract: Although endothelial cell seeding in small-diameter vascular prostheses significantly improves graft survival, the detachment of adherent endothelial cells after the restoration of circulation remains one of the major obstacles. Because *in vivo* experiments indicate that leukocyte infiltration is involved in endothelial cell loss, we hypothesize that seeded endothelial cells become activated and express leukocyte adhesion molecules and cytokines because of an interaction with the underlying polymer surface. The aim of this study was to investigate the expression of the leukocyte adhesion molecules ICAM-1, VCAM-1, PECAM-1, and E-selectin by cultured human umbilical vein endothelial cells (HUVECs) and human adipose microvascular endothelial cells (HAMVECs). The cells were seeded on tissue culture poly(styrene) and the vascular graft materials Dacron and Teflon. The results of this study indicate that the expression of leukocyte adhesion molecules by cultured endothelial cells is mainly affected by the endothelial cell origin, that is, umbilical vein or adipose tissue. Expressions of both ICAM-1 and E-selectin by HUVECs and HAMVECs are characterized by the presence of two cell populations with distinct levels of expression. With respect to endothelial cell seeding in vascular prostheses, the increased expression of E-selectin by microvascular endothelial cells deserves further attention. © 2001 John Wiley & Sons, Inc. J Biomed Mater Res 56: 376–381, 2001

Key words: endothelial cells; leukocyte adhesion molecules; polymer surfaces; flow cytometry; immunofluorescence microscopy

INTRODUCTION

In reconstructive vascular surgery, there is an increasing need for small-diameter synthetic vascular grafts (<5 mm). Although the seeding of these prostheses with autologous endothelial cells before implantation would significantly improve graft survival,[1,2] major obstacles include the source of autologous endothelial cells, the efficiency of cell seeding, and the detachment of adherent endothelial cells from the graft surface after the restoration of circulation.[3] Histological studies in dogs showed severe endothelial cell detachment associated with massive leukocyte infiltration within 6 h after implantation of completely endothelialized vascular prostheses.[4] The observation that the implantation of such grafts in neutropenic dogs resulted in 80% endothelial cell retention after 6 h compared with 30% in untreated controls emphasizes the role of leukocytes.[5] Therefore, we hypothesize that seeded endothelial cells become activated and express leukocyte adhesion molecules and cytokines because of an interaction with the underlying polymer surface. This results in adhesion, extravasation, and activation of leukocytes similar to inflammatory processes. The subsequent release of cytokines, enzymes, and oxygen radicals causes additional recruitment of leukocytes and damage of the endothelial monolayer.[6]

The aim of this study was to investigate the effect of various polymer surfaces, including the vascular graft materials Dacron and Teflon, on the expression of the leukocyte adhesion molecules ICAM-1, VCAM-1, E-selectin, and PECAM-1 by seeded endothelial cells. Human umbilical vein endothelial cells (HUVECs) were used as a model for vascular endothelium. Moreover, because subcutaneous fat tissue is a candidate source of autologous endothelial cells, the expression of leukocyte adhesion molecules by human adipose microvascular endothelial cells (HAMVECs) was studied as well.

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MATERIALS AND METHODS

Endothelial cell cultures

HUVECs were isolated through the incubation of umbilical vein with 0.05% trypsin/0.02% ethylenediaminetetraace-

tic acid (EDTA) (Gibco, Paisley, United Kingdom) for 20 min at 37°C.[7] The endothelial cells were cultured in fibronectin-coated tissue culture poly(styrene) (TCPS) flasks (Costar, Cambridge, MA) containing a medium consisting of equal volumes of RPMI 1640 and M 199 supplemented with 2 m*M* glutamax-1, 100 U/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL fungizone (all from Gibco), and 20% human serum [pooled from 12 healthy volunteer donors; culture medium with serum (CMS) 20%]. HUVECs were subcultured via trypsinization and used after the third passage.

Human adipose microvascular endothelial cells (HAMVECs) were isolated by the digestion of human subcutaneous fat tissue with collagenase/dispase (Boehringer Mannheim, Mannheim, Germany; 3 mg/g of fat tissue) for 2 h at 37°C and the subsequent selection of endothelial cells via incubation for 10 min at 4°C with Ulex Europaeus Agglutinin 1 (UEA 1)-coated Dynabeads (Dynal, Oslo, Norway), as described elsewhere.[8] After the capturing and washing of the beads with a magnetic particle concentrator, the beads were incubated for 10 min at 22°C with 100 m*M* fucose to release the endothelial cells. HAMVECs were cultured just as HUVECs, except that the culture medium consisted of CMS 20% supplemented with human recombinant bFGF (Gibco; 0.3 ng/mL) and heparin (5 U/mL). To obtain homogeneous HAMVEC cultures, we repeated the selection with UEA 1-coated Dynabeads 2–3 times. HAMVECs were used after the fifth passage.

Polymer surfaces and cell seeding

TCPS 24-well plates were used as received (Costar; sessile drop water contact angle $\theta = 35°$). Poly(ethylene terephthalate) (PETP; Dacron; $\theta = 65°$) and fluoroethylenepropylene copolymer (FEP-Teflon; a translucent type of Teflon; $\theta = 102°$) surfaces were prepared as follows. Circular pieces 15 mm in diameter were punched from PETP and FEP-Teflon films (both from DuPont, Geneva, Switzerland), extracted in acetone (ultrapure-grade; 24 h), and rinsed with ultrapure water in an ultrasonic bath (three times for 5 min). Subsequently, the films were dried at 37°C and fixed in 24-well plates with silicon-rubber O-rings (Eriks, Alkmaar, The Netherlands). PETP and FEP-Teflon surfaces were incubated overnight at 37°C with penicillin (100 U/mL), streptomycin (100 μg/mL), and fungizone (2.5 μg/mL) in phosphate-buffered saline (PBS); afterward, they were washed four times with 1 mL of PBS.

Endothelial cells were seeded on surfaces coated for 1 h at 37°C with 2 mg/mL fibronectin [Central Laboratory of the Blood Transfusion Service (CLB), Amsterdam, The Netherlands] at a density of 50.000 cell/cm^2 and grown for 1–2 days until confluent monolayers were obtained. Subsequently, the basal expression of ICAM-1, VCAM-1, and E-selectin and the expression after stimulation with 10 U/mL recombinant human IL-1β (ICAM-1, 15 h; VCAM-1, 10 h; E-selectin, 4 h) were investigated. PECAM-1 expression was studied without stimulation of the cells.

Flow cytometry and immunofluorescence microscopy

Endothelial cell monolayers were incubated for 30 min at 37°C with 200 μL of monoclonal antibody (mab; 25 μg/mL) in PBS containing 1% (w/v) BSA. The following monoclonal antibody (mab) clones were used: Rec-1 [anti-ICAM-1 (CD 54)], 4B9 [anti-VCAM-1 (CD106)], and ENA-2 [anti-E-selectin (CD 62E)], provided by Dr. W. Buurman (University Hospital Maastricht, The Netherlands), and CLB-HEC 65 [anti-PECAM-1 (CD 31)], obtained from the CLB. Subsequently, the cells were washed three times with 500 μL of PBS/BSA to remove excess antibody. The second antibody was a polyclonal goat-antimouse IgG conjugated with fluorescein isothiocyanate (GAM-FITC; CLB). The cells were incubated for 30 min at 37°C with 200 μL of a 1/80 dilution of GAM-FITC in PBS/BSA, after which excess fluorescent antibody was removed by the cells being washed three times with 500 μL of PBS/BSA. Finally, the cells were detached with collagenase (1 mg/mL), centrifuged, and resuspended in PBS containing 1% (w/v) paraformaldehyde. For each cell type, the nonspecific binding of GAM-FITC was evaluated by the incubation of cells only with the second antibody (blanc).

Fluorescence intensities were measured by FACscan analysis (Becton Dickinson, Mountain View, CA). Within the endothelial cell area, as determined by forward and sideward scattering, 5000 events were collected, the mean fluorescence intensity of which was measured. Positive cells were distinguished from negative cells with the blanc sample. Mean fluorescence intensities were taken as a measure for the expression of adhesion molecules.

Experiments were repeated at least four times, representative results of which are presented. Statistical analysis was performed via a two-way analysis of variance (ANOVA) of the mean fluorescence intensities versus the polymer substrate and cell type (Minitab Statistical Software, State College, PA). Mean fluorescence intensities were considered to be significantly different at $p < 0.05$.

Alternatively, FITC-stained endothelial cells adherent to the test surfaces (the procedure previously described without collagenase treatment) were examined with a fluorescence microscope.

RESULTS

ICAM-1 expression

Although there are differences between basal expressions of ICAM-1 by HUVECs and HAMVECs cultured on TCPS, PETP, and FEP-Teflon [Fig. 1(a)], a two-way ANOVA of the mean fluorescence intensities versus cell the type and polymer substrate shows that these are not significant. Except for the basal expressions of ICAM-1 by HUVECs on TCPS and HAMVECs on PETP and FEP-Teflon, two populations of positive cells are observed.

After activation with IL-1β, both HUVECs and HAMVECs show increased expressions of ICAM-1 [Fig. 1(b)]. A two-way ANOVA of the mean fluorescence intensities versus the cell type and polymer substrate shows that HUVECs express significantly more ICAM-1 than HAMVECs ($p = 0.001$). Moreover, after

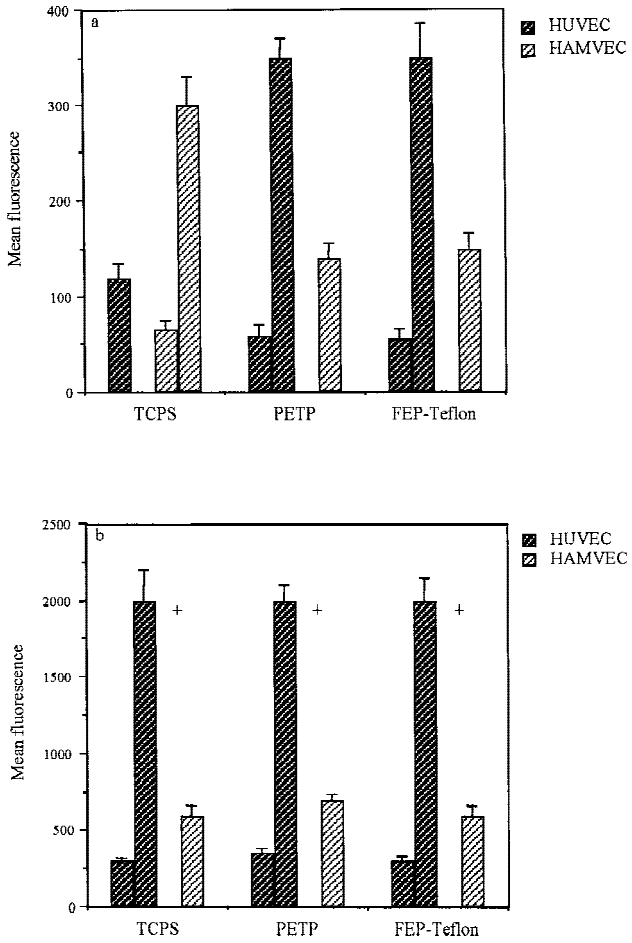

Figure 1. Expression of ICAM-1 by endothelial cells grown on various polymer surfaces as determined by FACscan analysis: (a) basal expression and (b) expression after 15 h of activation with 10 U/mL IL-1β. All data (means ± standard deviation, $n = 4$) refer to positive cells; mean fluorescence intensities of the blancs are subtracted. The presence of two bars per cell type cultured on a given surface indicates two positive cell populations with distinct fluorescence intensities. Two-way ANOVA of mean fluorescence intensities versus surface and cell type: (+) $p = 0.001$ for cell type.

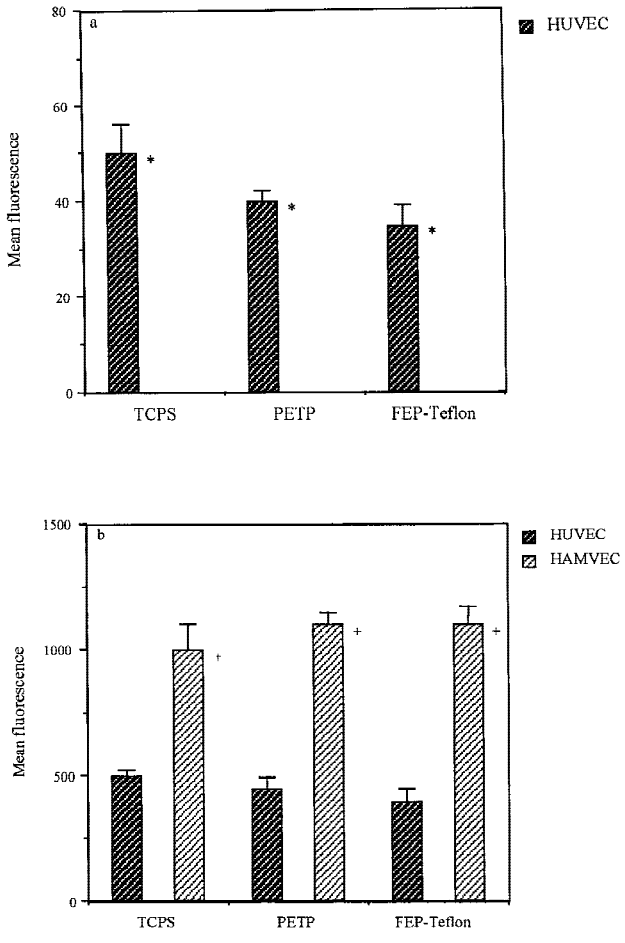

Figure 2. Expression of VCAM-1 by endothelial cells grown on various polymer surfaces as determined by FACscan analysis: (a) basal expression and (b) expression after 10 h of activation with 10 U/mL IL-1β. All data (means ± standard deviation, $n = 4$) refer to positive cells; mean fluorescence intensities of the blancs are subtracted. Two-way ANOVA of mean fluorescence intensities versus surface and cell type: (*) $p = 0.013$ for cell type and (+) $p = 0.009$ for cell type.

activation with IL-1β, two positive HUVEC populations are observed, whereas HAMVEC cultures show only one positive population.

VCAM-1 expression

In contrast to HAMVECs, which do not express VCAM-1 under basal culture conditions [Fig. 2(a)], HUVECs display a low but significant basal expression of VCAM-1 independent of the polymer substrate used for culturing (two-way ANOVA, $p = 0.013$ for cell type).

Activation with IL-1β results in upregulation of VCAM-1 expression on both HUVECs and HAMVECs [Fig. 2(b)]. A two-way ANOVA of the mean fluorescence intensities versus the cell type and polymer substrate shows that HAMVECs express significantly more VCAM-1 than HUVECs ($p = 0.009$).

PECAM-1 expression

Because PECAM-1 is constitutively expressed by endothelial cells, only basal expression of this adhesion molecule was investigated. Without activation, both HUVECs and HAMVECs display a similar expression of PECAM-1, which is independent of the polymer substrate used for culturing (Fig. 3).

E-selectin expression

In contrast to HUVECs, which do not express E-selectin under basal culture conditions [Fig. 4(a)], HAMVECs display a low but significant basal expression of E-selectin independent of the polymer sub-

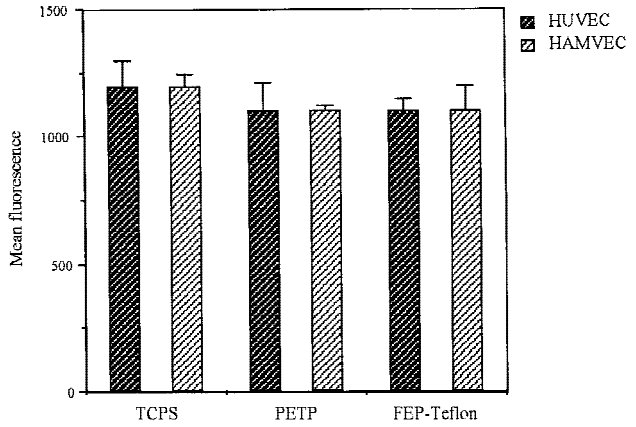

Figure 3. Basal expression of PECAM-1 by endothelial cells grown on various polymer surfaces as determined by FACscan analysis. All data (means ± standard deviation, n = 4) refer to positive cells; mean fluorescence intensities of the blancs are subtracted.

strate used for culturing (two-way ANOVA, $p = 0.002$ for cell type).

Activation with IL-1β results in upregulation of E-selectin expression on both HUVECs and HAMVECs [Fig. 4(b)]. A two-way ANOVA of the mean fluorescence intensities versus the cell type and polymer substrate shows no significant differences.

Immunofluorescence microscopy

In addition to FACscan analysis, the expression of leukocyte adhesion molecules was examined with immunofluorescence microscopy. The qualitative data obtained confirm the quantitative flow cytometry results. As representative images, the basal expression of ICAM-1 by HUVECs seeded on FEP-Teflon and the expression after activation with IL-1β are shown in Figure 5(a,b), respectively. Both IL-1β-activated and nonactivated HUVECs grown on FEP-Teflon show two positive ICAM-1 populations with distinct fluorescence intensities, as was observed with flow cytometry.

DISCUSSION

The efficiency of endothelial cell seeding in vascular prostheses would significantly improve with the reduction of endothelial cell detachment after the restoration of circulation. Because *in vivo* experiments indicate that leukocyte infiltration is involved in endothelial cell loss,[4,5] we hypothesize that seeded endothelial cells become activated and express leukocyte adhesion molecules and cytokines because of an interaction with the underlying polymer surface. This results in adhesion, extravasation, and activation of leukocytes similar to inflammatory processes.

The interaction of leukocytes with inflamed vascular endothelium can be divided into three separate processes that are mediated by different molecular mechanisms. Slow rolling of leukocytes along the endothelium is followed by firm adhesion, after which the leukocytes migrate across the endothelium. Upregulation of E- and P-selectin by activated endothelial cells in areas of inflammation initiates rolling of nonactivated leukocytes that constitutively express L-selectin.[9] Activation of leukocytes by chemokines or cytokines during rolling results in upregulation of leukocyte β1 and β2 integrins. Endothelial counter receptors for these integrins, VCAM-1 and ICAM-1, respectively,[10,11] are strongly upregulated on inflamed endothelium.[12] PECAM-1 is located at the endothelial cell border and on the membranes of monocytes and neutrophils. *In vitro* and *in vivo* studies suggest that the interaction of leukocyte PECAM-1 with endothelial PECAM-1 supports the transmigration of leukocytes across the endothelium.[13,14]

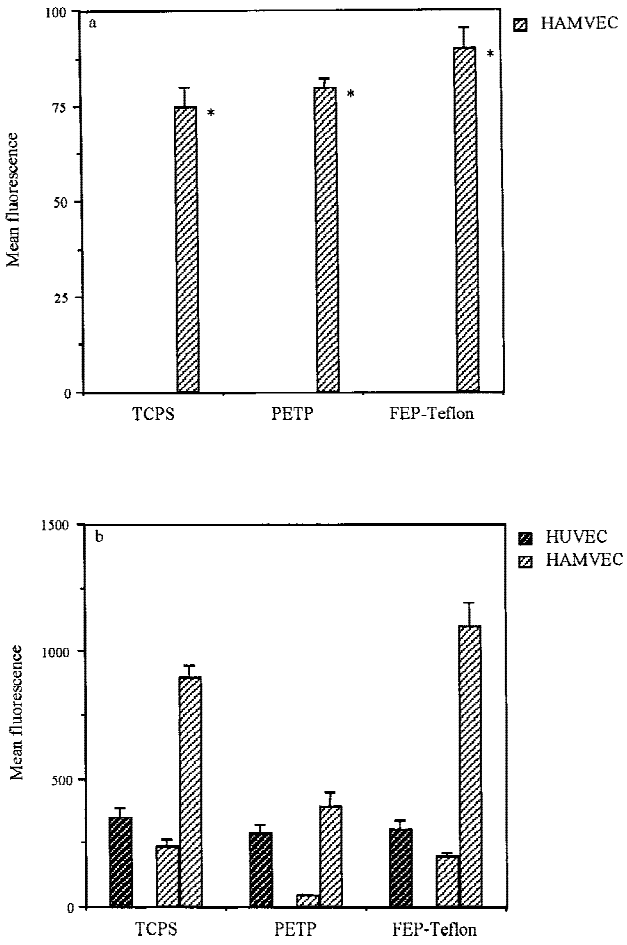

Figure 4. Expression of E-selectin by endothelial cells grown on various polymer surfaces as determined by FACscan analysis: (a) basal expression and (b) expression after 4 h of activation with 10 U/mL IL-1β. All data (means ± standard deviation, n = 4) refer to positive cells; mean fluorescence intensities of the blancs are subtracted. The presence of two bars per cell type cultured on a given surface indicates two positive cell populations with distinct fluorescence intensities. Two-way ANOVA of mean fluorescence intensities versus surface and cell type: (*) $p = 0.002$ for cell type.

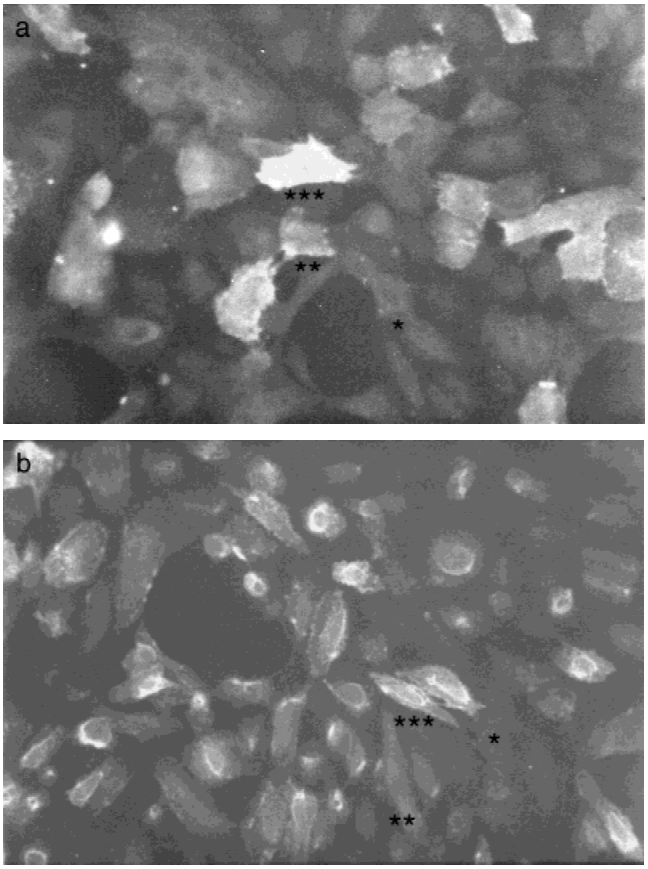

Figure 5. Expression of ICAM-1 by HUVECs grown on FEP-Teflon as determined by immunofluorescence microscopy: (a) basal expression and (b) expression after 15 h of activation with 10 U/mL IL-1β. In addition to (*) negative cells, two populations of (** and ***) positive cells can be distinguished with distinct fluorescence intensities.

In this study, we investigated the expression of ICAM-1, VCAM-1, PECAM-1, and E-selectin by endothelial cells grown on TCPS and the vascular graft materials Dacron (PETP) and Teflon (FEP-Teflon, a translucent type of Teflon). Human endothelial cells both from umbilical veins (HUVECs) and adipose microvessels (HAMVECs) were used. As a reference for the possible induction of inflammatory phenomena by the underlying polymer surface, the expression of leukocyte adhesion molecules by endothelial cells activated with IL-1β was compared to the basal expression of untreated cells. The results of this study indicate that, in addition to the activation of the cells with IL-1β, the expression of leukocyte adhesion molecules is mainly determined by the endothelial cell origin.

HUVECs cultured on PETP and FEP-Teflon show a higher basal expression of ICAM-1 than HUVECs cultured on TCPS. This agrees with data reported by Margiotta et al.,[15,16] who found an increased basal expression of ICAM-1 by human saphenous vein endothelial cells (HSVECs) grown on PETP and expanded Teflon with respect to HSVECs cultured on TCPS. Cenni et al.[17,18] reported that the culturing of HUVECs on pyrolytic carbon-coated PETP and woven Dacron does not upregulate the expression of ICAM-1 in comparison with culturing on TCPS. Furthermore, our results indicate that HAMVECs cultured on PETP and FEP-Teflon show a lower basal expression of ICAM-1 than HAMVECs grown on TCPS. Apparently, we cannot draw a general conclusion that the basal expression of ICAM-1 by endothelial cells is upregulated by hydrophobic polymers such as PETP and FEP-Teflon.

The expression of ICAM-1 after the activation of the cells with IL-1β is dependent on the endothelial cell origin; HUVECs display significantly higher mean fluorescence intensities than HAMVECs. In comparison with the basal expression of ICAM-1, HUVECs especially show a higher expression of ICAM-1 after activation with IL-1β, indicating that the upregulated basal expression of HUVECs cultured on PETP and FEP-Teflon is only limited. As determined by FACscan analysis, HUVECs and HAMVECs with a relatively high expression of ICAM-1, both basal and after stimulation with IL-1β, show two positive populations. This phenomenon, which was confirmed with immunofluorescence microscopy, may be due to differences in the cell cycles. The differential expression of adhesion molecule mRNA, depending on the phase of the cell cycle, may result in more than one positive population of cells. Alternatively, other mechanisms also depending on the cell cycle may be involved, such as differential translocation of adhesion molecules to the cell surface or differential glycosylation of adhesion molecules, thereby affecting binding efficiencies of the first antibody.

The expression of VCAM-1, both basal and after activation of the cells with IL-1β, was found to be dependent on the endothelial cell origin. In contrast to HUVECs, HAMVECs display no basal expression of VCAM-1. After stimulation with IL-1β, however, HAMVECs show significantly higher mean fluorescence intensities than HUVECs. Whatever the biological significance of these data, it can be concluded that the polymer surface used for culturing does not upregulate the expression of VCAM-1.

The basal expression of PECAM-1 is dependent neither on the endothelial cell origin nor on the underlying polymer surface. Both HUVECs and HAMVECs constitutively express PECAM-1 with similar mean fluorescence intensities. This is consistent with the *in vivo* situation, where PECAM-1 is found constitutively on the endothelium of all vessel types.[19] Recently, Cenni et al.[20] reported the downregulation of PECAM-1 expression by HUVECs cultured on knitted Dacron. This vascular graft material, however, was not coated with fibronectin, resulting in deficient cell spreading and many nonadherent cells, thus explaining PECAM-1 downregulation.

In contrast to HUVECs, a small but significant amount of HAMVECs displays basal expression of E-

selectin. In terms of mean fluorescence intensities, however, this expression is relatively low compared with the IL-1β-upregulated expression of E-selectin. Except for cells cultured on PETP, IL-1β-activated HAMVECs display higher mean fluorescence intensities than HUVECs. This is in agreement with data reported by Petzelbauer et al.[21] and Abbot et al.,[22] who observed higher mean fluorescence intensities with respect to IL-1α-upregulated E-selectin expression by dermal microvascular endothelial cells and synovial microvascular endothelial cells in comparison with HUVECs. This increased expression of E-selectin may reflect a general feature of microvascular endothelial cells exposed to cytokines.[21] Regarding E-selectin expression in the absence of cytokines, Cenni et al. reported upregulation of the expression of E-selectin by HUVECs cultured on knitted Dacron,[20,23] in contrast to HUVECs cultured on pyrolytic carbon-coated PETP and woven Dacron.[17,18] Apparently, surface morphology is an important parameter affecting E-selectin expression, as was also demonstrated for polytetrafluoroethylene (PTFE) and expanded PTFE surfaces.[24]

In conclusion, the results of this study indicate that the expression of leukocyte adhesion molecules by cultured endothelial cells is mainly affected by the endothelial cell origin, that is, umbilical vein or adipose tissue. Although the expression of ICAM-1 is especially affected by the polymer surface used for culturing, a general conclusion that the expression of leukocyte adhesion molecules is upregulated by hydrophobic polymers cannot be drawn. Both HUVECs and HAMVECs may display two positive populations of cells. With respect to endothelial cell seeding in vascular prostheses, the increased expression of E-selectin by microvascular endothelial cells deserves further attention.

The staffs of the Departments of Obstetrics and Surgery of the Medisch Spectrum Twente Hospital in Enschede are gratefully acknowledged for furnishing umbilical cords and subcutaneous fat tissue, respectively.

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