Impaired Induction of Adhesion Molecule Expression in Immortalized Endothelial Cells Leads to Functional Defects in Dynamic Interactions With Lymphocytes

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Immortalization should overcome the problem of short lifespan and difficult culture of endothelial cells that limited their use in functional studies. We used four different immortalized endothelial cell lines to study dynamic interactions with lymphocytes. Surprisingly, tumor necrosis factor (TNF)α-stimulated human umbilical vein endothelial cells (HUVECs) or human dermal microvascular endothelial cells (HDMECs) readily supported rolling and binding of lymphocytes, whereas none of the immortalized cell lines did. As rolling interactions are primarily mediated by selectins and vascular cell adhesion molecule (VCAM)-1, the endothelial cells were analyzed regarding expression of selectins and other adhesion molecules. Interestingly, cell surface expression of E-selectin could only be detected on HUVEC and HDMEC. Immunocytochemistry showed that some immortalized endothelial cells expressed E-selectin intracellularly following TNFα stimulation, suggesting translation but defective post-translational processing or transport of the molecule. In contrast, other immortalized cell lines did not have detectable levels of E-selectin mRNA, suggesting impaired transcription. VCAM-1 could only be induced on normal and human placental microvascular endothelial cell-A2 endothelial cells, whereas all cell lines expressed intercellular adhesion molecule-1 following TNF stimulation. The immortalized endothelial cells tested here have lost functions that are required for dynamic interactions with immune cells and that are common to primary endothelial cells.

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INTRODUCTION

Selectins are interesting targets for immunomodulatory therapies (Boehncke and Schön, 2003; Ehrhardt *et al.*, 2004). The selectin family comprises E-selectin (CD62E, expressed on endothelial cells), P-selectin (CD62P, found on endothelial cells and platelets), and L-selectin (CD62L, expressed on leukocytes) (Bevilacqua and Nelson, 1993). L-selectin is constitutively expressed, whereas P-selectin is rapidly translocated from its storage vesicles to the cell membrane on activation (Bonfanti *et al.*, 1989; McEver *et al.*, 1989). The expression of E-selectin is transcriptionally regulated by inflammatory cytokines, such as tumor necrosis factor (TNF)α or IL-1, and peaks approximately 4 hours and declines within 24 hours after stimulation (Bevilacqua *et al.*, 1987). Research in interactions of lymphocytes with endothelial cells has been hampered by the difficulties in obtaining enough endothelial cells for functional studies and by their limited lifespan *in vitro*. Several immortalized endothelial cell lines have been described, which may facilitate disease-relevant functional studies (Ades *et al.*, 1992; Xu *et al.*, 1994; Schütz *et al.*, 1997; Greiffenberg *et al.*, 1998; Venetsanakos *et al.*, 2002). Immortalized endothelial cells are easy to maintain in culture and they can be passaged virtually indefinitely under the right culture conditions.

To study dynamic rolling interactions between lymphocytes and endothelial cells, we obtained four immortalized endothelial cell lines from different laboratories. When all of these cell lines failed to support selectin-dependent lymphocyte rolling, we further studied expression of E-selectin in these cells, as E-selectin plays a pivotal role in the transient adhesion of leukocytes to endothelial cells (Bevilacqua *et al.*, 1987). The intra- and extracellular expression of E-selectin was compared with that of non-transformed vascular endothelial cells. Moreover, intra- and extracellular vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 expression was determined to compare different TNFα-inducible adhesion molecules. VCAM-1 and ICAM-1 are both TNFα-inducible adhesion molecules, which

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Abbreviations: HBMEC, human brain microvascular endothelial cell; HDMEC, human dermal microvascular endothelial cell; HPEC-A2, human placental microvascular endothelial cell; HUVEC, human umbilical vein endothelial cell; ICAM, intercellular adhesion molecule; TIME, telomerase immortalized human microvascular endothelial; TNF, tumor necrosis factor; PBMC, peripheral blood mononuclear cell

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play an important role for firm adhesion of leukocytes to endothelia. Here, we report that immortalized endothelial cell lines lacked the inducibility of surface expression of E-selectin. In addition, most of the immortalized cell lines lacked cell surface expression of VCAM-1, but not ICAM-1. Thus, the usefulness of immortalized endothelial cells for studies of dynamic interactions with leukocytes appears to be limited by functional losses following transformation.

RESULTS

Human PBMC roll on normal, but not on immortalized endothelial cells

Transient rolling interactions between human peripheral blood mononuclear cells (PBMC) and immortalized endothelial cells were assessed under conditions of shear flow and compared with the interactions between these PBMC and non-transformed human endothelial cells (Figure 1). As expected, PBMC attached to and rolled on TNFα-activated human umbilical vein endothelial cells (HUVEC) or human dermal microvascular endothelial cells (HDMEC) (four different populations), whereas no interactions were observed with unstimulated HUVEC or HDMEC indicating functional activation through TNFα (Video S1). The interaction was largely selectin-dependent, as rolling and adherence of PBMC on TNFα-stimulated endothelial cells could be inhibited by approximately 60% using an E-selectin-blocking antibody ($P<0.0001$ as compared with controls) and by approximately 80% when a cocktail of selectin-directed function-blocking antibodies was added ($P<0.0001$ as compared with controls, Figure 1 and Video S2). In contrast, PBMC did not interact with TNFα-stimulated telomerase immortalized human microvascular endothelial (TIME) cells, human brain microvascular endothelial cells (HBMEC), Center for Disease Control human microvascular endothelial cell line (CDC-HMEC)-1, or human placental microvascular endothelial cells (HPEC-A2) (Figure 1). These data suggested that functionally relevant molecules could not be induced by TNFα on immortalized human endothelial cells.

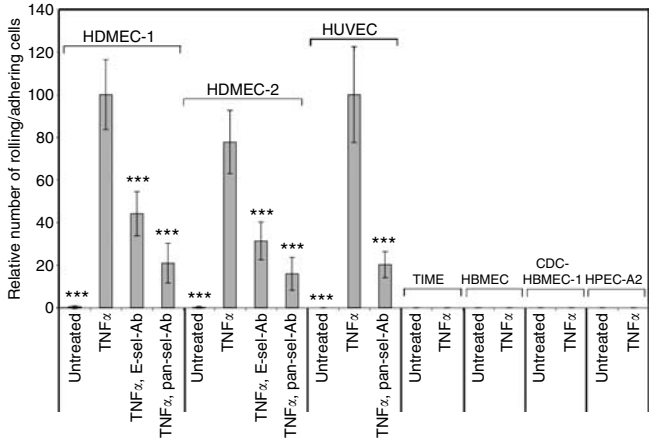

Figure 1. Selectin-dependent rolling interactions with lymphocytes are supported by stimulated primary, but not immortalized human endothelial cells. Adherence of PBMC to human endothelial cell lines was assessed in a dynamic flow chamber system in the absence or presence of selectin function-blocking mAbs as indicated (***$P<0.0001$ as compared with TNFα-stimulated controls). The values shown depict the number of rolling PBMC calculated relative to either the number of rolling PBMC on TNFα-stimulated HDMEC-1 when using HDMEC-1 and -2 or the number of rolling PBMC on TNFα-stimulated HUVEC when using HUVEC. These experiments were repeated twice with similar results.

Defective intra- and extracellular expression of E-selectin and VCAM-1 on immortalized endothelial cell lines

The different cell lines were tested by immunofluorescence microscopy for intra- and extracellular expression of E-selectin before and after activation with TNFα. Following confirmation of the specificity of the used E-selectin-specific antibodies by immunoblotting (data not shown), immunofluorescence staining showed that no intra- or extracellular E-selectin could be detected before activation with TNFα in any of the cell lines (Figure 2a, first column). Following activation with TNFα, non-transformed endothelial cells expressed E-selectin on the surface, whereas none of the immortalized cell lines did (Figure 2a, second column). However, two distinct E-selectin-related staining patterns occurred in TNFα-activated immortalized endothelial cells when using intra- and extracellular staining methods. TIME and HPEC-A2 cells showed intracellular, but no surface expression, suggesting a defect in the post-translational processing or translocation pathway, whereas HBMEC and CDC-HMEC-1 cells showed neither intracellular nor surface expression of E-selectin, suggesting a transcription or translation defect. Importantly, the functionally relevant expression of E-selectin on the cell surface was lacking in all four immortalized lines. To test whether this effect was specific for E-selectin or a more general phenomenon, intra- and extracellular expression of VCAM-1 and ICAM-1 was determined. The results showed that only TNFα-activated normal endothelial cells and HPEC-A2 cells expressed VCAM-1, whereas all other cells were negative (Figure 2b). In contrast, all cell lines tested showed inducible surface expression of ICAM-1 (data not shown). Thus, the induction of both E-selectin and VCAM-1 was impaired in immortalized endothelial cells, whereas the induction of ICAM-1 was maintained, indicating that adhesion molecules can be influenced in different ways following immortalization.

Immunoprecipitation experiments were performed to define further the mechanisms behind the changed expression of E-selectin on immortalized endothelial cells, whereby the aim was to characterize the precipitated E-selectin from TIME cells by mass spectrometry. Unfortunately, these experiments were not successful, possibly owing to reduced antibody affinity to E-selectin in TIME cells, but more probably the quantity of E-selectin in these cells was too low to obtain sufficient amounts for mass spectrometry analysis.

Some immortalized endothelial cell lines show transcriptional defects of molecules involved in leukocyte adhesion

The distinct E-selectin and VCAM-1 expression patterns in TNFα-stimulated immortalized endothelial cell lines suggested that the defect in some of the cell lines might reside on the transcriptional level. Therefore, mRNA levels of various

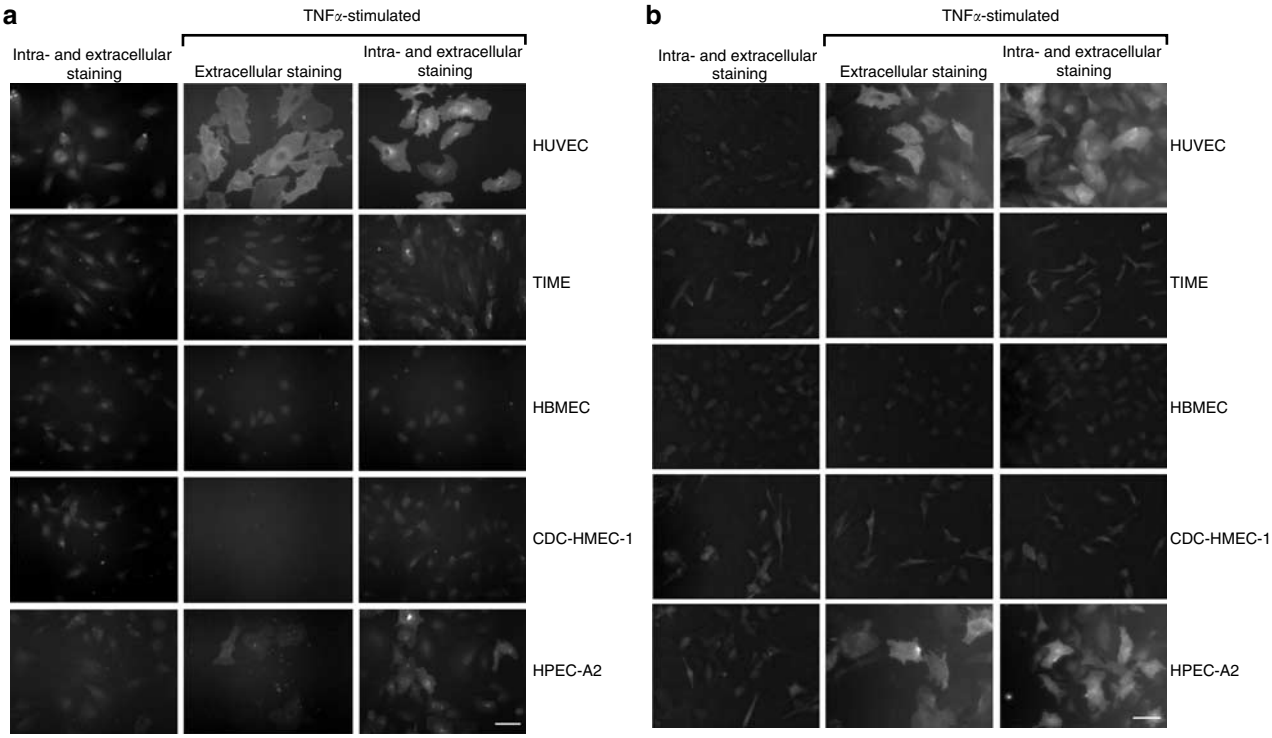

Figure 2. TNFα induces expression of E-selectin and VCAM-1 in normal, but not in all immortalized cells. (**a**) Unstimulated endothelial cells did not show any intra- or extracellular expression of E-selectin (first column). The expression of E-selectin on the cell membranes of TNFα-stimulated endothelial cells is shown in the second column and the third column depicts intra- and extracellular staining after stimulation with TNFα and permeabilization of the cells. HUVECs express intra- and extracellular E-selectin, whereas membrane expression of E-selectin was not found on immortalized ECs. Intracellular E-selectin expression can be visualized in TIME cells and HPEC-A2 cells. Bar = 20 μm. (**b**) As described in Figure 3**a**, the intra- and extracellular expression of VCAM-1 has been analyzed before and after stimulation with TNFα. HUVEC and HPEC-A2 express intra- and extracellular VCAM-1 after stimulation with TNFα, whereas all other immortalized ECs did not express this molecule. Bar = 20 μm.

molecules that are normally expressed on endothelial cells were analyzed. E-selectin (CD62E) mRNA was upregulated on TNFα stimulation in HUVEC, HDMEC, TIME cells, and HPEC-A2. In contrast, CDC-HMEC-1 showed weak inducibility of E-selectin and HBMEC could not be induced to express CD62E mRNA, suggesting a transcriptional defect. VCAM-1 (CD106) showed a very similar pattern compared with E-selectin, whereby the induction was most pronounced in HUVEC and HPEC-A2 (Figure 3a and b). CD54 (ICAM-1, a molecule involved in firm leukocyte adhesion to vascular endothelium) was not or only weakly expressed in unstimulated cells, but could be detected in all cell lines after TNFα stimulation, although the expression was low in HDMEC. Thus, CD54 could be induced in all endothelial lines, indicating that the E-selectin and VCAM-1 expression defect was not exclusively because of a failure of the transformed cells to respond to TNFα.

All cell lines, except HBMEC, expressed CD31 platelet/endothelial cell adhesion molecule (PECAM)-1 before and after stimulation. CD31 is constitutively expressed on endothelial cells and has been implicated in the transendothelial migration of leukocytes (Schön and Ludwig, 2005). CD34, a L-selectin ligand, was weakly detectable in HUVEC and strongly in CDC-HMEC-1 and HPEC-A2 cells, independent of stimulation. CD36, a platelet glycoprotein ligand, was weakly detectable in HBMEC and CDC-HMEC-1 cells and stronger in HDMEC. CD61 (a platelet/endothelial cell adhesion molecule-1 ligand) was present at low levels in all cells, independent of stimulation. CD62P (P-selectin) was detectable in non-transformed cells, independent of stimulation. CD144 (VE-cadherin) was clearly detectable in most cell lines, except HBMEC, and was weakly expressed in HDMEC (Figure 3a). These data suggested that E-selectin and VCAM-1 are not the only molecules affected by the immortalization procedure and that functional data obtained with these cells in other experimental systems should be interpreted with caution.

The presence of E-selectin- and VCAM-1-encoding mRNA in stimulated TIME cells and HPEC-A2 was in agreement with immunofluorescence staining (Figure 2a), as was the absence (or very low presence) of the respective mRNA in HBMEC and CDC-HBMEC-1 (Figure 3a). Thus, it appears that inducible expression of some adhesion molecules in endothelial cells can be impaired at distinct steps following immortalization.

DISCUSSION

Research in the molecular basis of leukocyte recruitment has been hampered by the difficult generation of primary endothelial cell cultures and their limited lifespan. Immortalized endothelial cell lines were established to overcome this limitation.

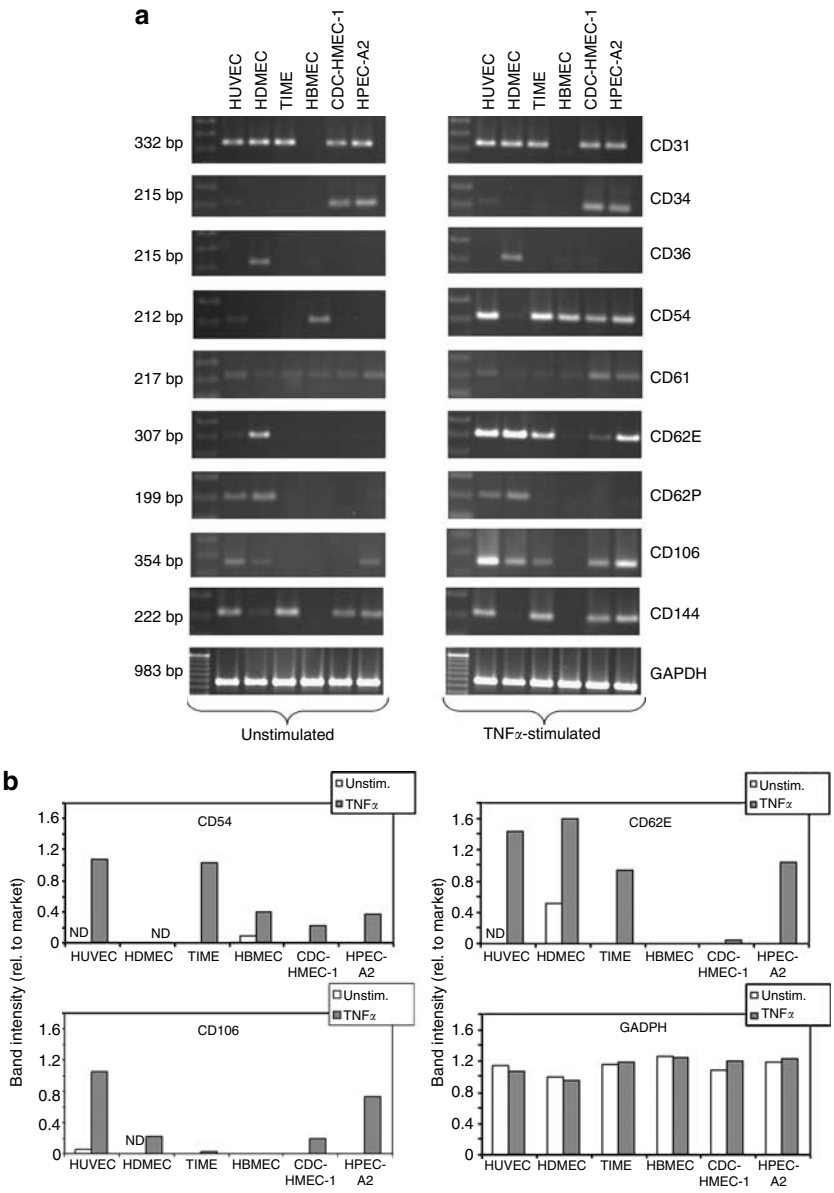

Figure 3. Differential inducibility of E-selectin and other adhesion molecules in normal and immortalized endothelial cells. (**a**) The mRNA was isolated from six different cell lines (stimulated or unstimulated) and expression of CD62E (E-selectin), CD54 (ICAM-1), and the other indicated adhesion molecules was determined. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as loading control. The sizes of PCR products are indicated on the left-hand side. (**b**) The intensity of the bands was analyzed using SigmaGel software and the results of CD54, CD62E, CD106, and GAPDH are shown before and after stimulation with TNFα. Bands that could be seen in the gel, but were too weak to analyze with this software are labeled ND (not detectable).

There are several ways to immortalize cells, whereby transfection of endothelial cells with a construct containing the simian virus 40 A gene product, the large T antigen, appears to be one of the most popular methods (Ades *et al.*, 1992; Hohenwarter *et al.*, 1992; Lassalle *et al.*, 1992; Vicart *et al.*, 1993). However, the expression of DNA tumor virus oncoproteins, such as the SV40 large T antigen, neutralizes the activity of pRb and p53, which are essential regulators of normal cell division, genomic integrity, and apoptosis (Venetsanakos *et al.*, 2002). Therefore, alternative methods have been employed, including immortalization of endothelial cells by expression of the catalytic subunit of telomerase (hTERT). This approach, when used on human fibroblasts, resulted in the generation of immortalized cells which had a largely normal phenotype (Jiang *et al.*, 1999; Morales *et al.*, 1999).

Functional studies of pathogenesis-relevant processes have been a primary goal when generating immortalized endothelial cell lines, but none of the TNFα-activated immortalized endothelial cells used in our study supported lymphocyte rolling under conditions of shear flow. In contrast, primary endothelial cells readily exhibited this trait. This functional impairment of immortalized endothelial cells was, at least in part, owing to a defect in the expression of E-selectin and

VCAM-1, adhesion molecules that mediate transient leukocyte–endothelial cell interactions (Bevilacqua *et al.*, 1987; Schön *et al.*, 2003).

Several not mutually exclusive explanations for the rolling deficiency of leukocytes on immortalized endothelial cells can be delineated. First, adhesion molecules might be expressed on the cell surface after activation, but post-translational modifications result in changed conformation of the molecule, which decreases binding interactions. Second, the post-translational processing or transport of the molecule to the cell membrane might be defective. TIME cells and HPEC-A2 probably have a defect in the transport of E-selectin to the cell membrane, as intracellular but not surface expression of E-selectin was detected following TNFα stimulation. Third, other molecules involved in lymphocyte tethering and rolling, such as L-selectin ligands, might have changed as a result of immortalization. Fourth, a phenotypic switch from vascular to lymphatic endothelial cells because of immortalization and prolonged periods of culture may have occurred (Nisato *et al*, 2004). As there was no difference in ICAM-1 expression between primary and immortalized endothelial cells, our data indicate that the defect in E-selectin and VCAM-1 upregulation in some cell lines is not because of a general defect of these cells to respond to inflammatory stimuli, but rather a more specific effect. The exact mechanisms behind these discrepancies need further study, because it might reveal potential pathways to specifically block the induction of relevant molecules in inflammatory diseases. Some immortalized endothelial cell lines have been described which express E-selectin. Immortalization does, therefore, not preclude E-selectin expression (Krump-Kovalinkova *et al.* 2001; Matsumura *et al.*, 2004).

In conclusion, our findings may have consequences for the use of immortalized endothelial cells in as much as such cell populations may not be suited to address some important questions in inflammation research. Therefore, results obtained with immortalized endothelial cells should be confirmed by primary endothelial cells.

MATERIALS AND METHODS

Endothelial cell lines and cell culture

TIME cells (Venetsanakos *et al.*, 2002) were provided by M McMahon (Cancer Research Institute, UCSF, San Francisco, CA). HDMECs (CDC-HMEC-1), immortalized by transfection with SV40 T-antigens (Ades *et al.*, 1992) were a gift from FJ Candal (Centers for Disease Control, Atlanta, GA). HBMECs, immortalized by transfection with SV40 T-antigens (Greiffenberg *et al.*, 1998), were obtained from KS Kim (USC School of Medicine, Los Angeles, CA). SV40-transformed HPEC-A2s were generated as described (Schütz *et al.*, 1997). HUVECs were from Cambrex (Vervier, Belgium). HDMECs were isolated from four donors (Detmar *et al.*, 1990). The cells were cultured as described in the original articles or by the distributor. All cell lines have previously been tested to ensure their vascular characteristics before the immortalization procedure and the immortalized cell lines were exhaustively characterized following immortalization (Ades *et al.*, 1992; Schütz *et al.*, 1997; Greiffenberg *et al.*, 1998; Venetsanakos *et al.*, 2002).

Lymphocyte adhesion to endothelial cells under conditions of flow

Tissue culture-treated thermanox cover slips (22 × 60 mm; Plano, Wetzlar, Germany) were coated with endothelial cells (2×10^4) and incubated at 37°C/5% CO_2 overnight. Recombinant human TNFα (25 ng/ml) was added for 4 hours. For each cell line, unstimulated and stimulated conditions were used. PBMC were isolated from heparinized blood by density gradient centrifugation using Ficoll-Hypaque PLUS (Amersham, Freiburg, Germany) and resuspended at a density of 1×10^6 cells/ml in Hank's balanced salt solution containing 2 mM $CaCl_2$. Flow chamber experiments were performed as described using a wall shear rate of 150/s (1.04 dyn/cm^2) (Oostingh *et al.*, 2007). Function-blocking antibodies (10 μg/ml; ATCC, Manassas, VA) were added to some PBMC suspensions to establish the selectin dependence of lymphocyte rolling. Microscopic phase–contrast images were recorded in real time. Three different microscopic fields were recorded for each condition and experiments were repeated twice.

Intra- and extracellular detection of antigens

Intra- and extracellular expression of E-selectin, VCAM-1, or ICAM-1 was analyzed by immunofluorescence microscopy as described in the Supplementary Online Material.

Western blot analysis and SDS-PAGE

The specificity of the E-selectin-specific antibody was tested by Western blot analysis as described in the Supplementary Online Material.

Semiquantitative reverse transcriptase-PCR analysis

The presence of mRNA encoding for several different adhesion molecules was analyzed in TNFα-stimulated and unstimulated cells by semiquantitative reverse transcriptase-PCR (Kremlev and Palmer, 2005) using sequence-specific primers for these molecules (Supplementary Table S1), glyceraldehyde-3-phosphate dehydrogenase was amplified as control. The methods used are described in the Supplementary Online Material.

Statistical analysis

Statistical analyses were performed using Excel software (Microsoft, Munich, Germany). Data are displayed as mean (±SD); P values were determined using the two-tailed t-test, and P values <0.05 (confidence interval 95%) were considered statistically significant. All statistical tests were two-sided.

CONFLICT OF INTEREST

The authors state no conflict of interest

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SUPPLEMENTARY MATERIAL

Video S1. Human PBMC (1×10^6 cells/ml) rolling on and adhering to TNFα-stimulated normal human endothelial cells under conditions of shear flow, non-adhering cells are not visible owing to the high speed with which they pass through the chamber.

Video S2. Human PBMC (1×10^6 cells/ml) rolling on and adhering to the same human endothelial cells shown in video 1, but in the presence of

selectin-blocking antibodies (10 μg/ml). Non-adhering cells cannot be observed because of the high speed.

Materials and Methods.

Table S1. Oligonucleotide pairs used for reverse transcriptase-PCR.

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