Opposite Sorting of Tissue Factor in Human Umbilical Vein Endothelial Cells and Madin-Darby Canine Kidney Epithelial Cells

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Tissue factor (TF) is a 48-kD transmembrane glycoprotein that triggers the extrinsic pathway of blood coagulation by interacting with the plasma coagulation factor VII (FVII). TF is also a true receptor in that a cellular signal is generated when activated FVII (FVIIa) binds to TF. For both of these functions, the cellular surface distribution of TF is important, since FVII is primarily available on the apical side of vascular endothelial cells and on the basolateral side of epithelial cells lining the internal and external surfaces. We show that TFPI were kindly donated by Professor U. Hedner, Dr M. Ezban, Tissue factor (TF) is a 48-kD transmembrane glycoprotein that triggers the extrinsic pathway of blood coagulation by interacting with the plasma coagulation factor VII (FVII). TF is also a true receptor in that a cellular signal is generated when activated FVII (FVIIa) binds to TF. For both of these functions, the cellular surface distribution of TF is important, since FVII is primarily available on the apical side of vascular endothelial cells and on the basolateral side of epithelial cells lining the internal and external surfaces. We show that TFPI were kindly donated by Professor U. Hedner, Dr M. Ezban, since Wll is primarily available on the apical side of vascular endothelial cells, TF (both antigen and procoagulant activity) is sorted to the apical surface, whereas in wild-type and stably transfected Madin-Darby canine kidney epithelial cells (MDCK), which form tight junctions and express TF constitutively, TF antigen is on the basolateral surface. No significant clotting activity is detectable on this surface. Truncated TF (cytoplasmic tail residues 246 to 263 deleted) is sorted as wild-type in MDCK cells. © 1996 by The American Society of Hematology.
coding for the region up to cysteine 245 directly followed by a stop codon and the 3' untranslated region of the cDNA. This fragment was amplified with primers 1 + 4, digested with NcoI and HpaI, and subcloned into the NcoI and HpaI sites of wild-type human TF. The entire PCR-amplified sequence and the splice junctions were verified by restriction digests and sequencing.

This truncated TF construct (hTF$_{1-455}$) and wild-type human TF cDNA (hTF$_{1-265}$) were cloned into the mammalian expression vector pcDNA3. This vector contains the human cytomegalovirus promoter for high-level expression and a neomycin gene for selection of stable transfectants.

**Transfection**

All transfections were made using standard calcium phosphate coprecipitation procedures with glycerol shock. Clones with stably integrated constructs were selected with 500 µg/mL G418. Selection of transfectants for total TF expression was made by mixing cell homogenates (50 µL) with human plasma (50 µL) and 30-nanoliter Ca$^{2+}$ (50 µL) and recording clotting time as previously described.13

**Cell Preparation and Culture**

MDCK strain 1 and COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% inactivated fetal calf serum (FCS), 1-glutamine, and antibiotics. Stably transfected cells were maintained in the above medium supplemented with 100 µg/mL G418. For polarity studies, MDCK cells were plated either at low density (70,000 cells/cm$^2$ membrane) to obtain tight junctions. MDCK cells of high passage number were maintained in growth medium for 1 to 2 days after plating, and the experiments were performed 1 to 2 days after the formation of tight junctions. MDCK cells of high passage number were not used, due to their reduced ability to form tight junctions.

Tight junction formation was assessed by resistance measurements across the membrane using a Millipore Ohmmeter. A steep increase in electrical resistance accompanied the formation of junctional complexes across the membrane using a Millipore Ohmmeter. A steep increase every day, and the experiments were performed 1 to 2 days after plating or 24 hours and during the subsequent experiment. The medium was changed every day, and the experiments were performed 1 to 2 days after the formation of tight junctions. MDCK cells of high passage number were used, due to their reduced ability to form tight junctions.

Tight junction formation was assessed by resistance measurements across the membrane using a Millipore Ohmmeter. A steep increase in electrical resistance accompanied the formation of junctional complexes. Leakage controls were also made using radioactive insulin. There was no detectable leakage of FXa. Leakage of $^{125}$I-labeled antibody was less than 0.5% after 1.5 hours' incubation on ice. Where indicated, cells were incubated with 5 µg/mL BFA in growth medium for 4 or 24 hours and during the subsequent experiment.

HUVEC were isolated and cultured, and TF synthesis was induced with 100 U/mL IL-1β as previously described.16 The cells were grown on transparent permeable membranes to allow light-microscopic observation. Leakage of $^{125}$I-labeled antibody across the tight cell layers was, on average, 2% over a 1.5-hour incubation period on ice. Other tests to confirm the integrity of the tight monolayer were performed as recently described.17 DNA level was measured by Hoechst 33258 fluorometry. Immunofluorescence on cover slips performed using subconfluent cell layers.

**Determination of TF Activity**

**Total TF activity in cell homogenates.** Total TF activity in frozen, thawed, and homogenized cells was measured using a standard clotting assay with human plasma and 30 nM/ml CaCl$_2$.12 TF activity was determined from a standard curve made with a human brain TF preparation that clotted human plasma in 14 to 15 seconds. This was taken to represent 100 U TF (UTF)/ml. One unit TF corresponded to 1.5 ng TF as determined by the IMUBIND TF ELISA kit. The values are expressed as UTF/µg cell protein.12

Surface TF activity determined by the Xa chromogenic substrate assay was related to total TF activity determined by the clotting assay by reference to a common standard curve made with the human brain TF preparation.

**FX activating activity of TF/VIIa on the cell surface.** The method used was based on the activation of FX by the FX-VIIa-Ca$^{2+}$ membrane complex. The reaction between FXa and its chromogenic peptide substrate was monitored by the increase in OD$_{540}$ over 20 minutes. The cells were washed carefully thrice and incubated with BSA (5 mg/mL) in veronal-buffered saline (VBS), pH 7.4, for 30 minutes at 37°C with shaking at 100 rpm, in the presence or absence of a neutralizing monoclonal antibody (American Diagnostica anti-TF antibody no. 4507) as appropriate. This antibody neutralized human TF, but did not alter the endogenous canine TF activity in untransfected MDCK cells. CaCl$_2$ was then added to a final concentration of 4 nM/mL. The compartments to be tested for TF expression were supplied with FXIa (10 U/mL) and FX (0.5 U/mL), and the culture tray was again incubated with shaking at 100 rpm and 37°C. At 5, 10, 15, and 20 minutes, samples of 20 µL were taken from each compartment and added to a microtiter plate containing 150 µL VBS, pH 7.4, with 10 mM EDTA per well to stop the reaction. After thorough mixing, 20 µL (80 nM) FXa-I substrate was added to each sample. The OD$_{540}$ was monitored in a Titertek Multiscan at 10-minute intervals for 60 minutes, and ∆OD$_{540}$ was calculated. A linear increase in ∆OD$_{540}$ over this period confirmed sufficient amounts of substrate. FXIa and FX were also titrated to give linear curves within the time span of the assay. Controls with human brain TF demonstrated that the system was linear with increasing TF concentration under the established conditions. The first incubation (with cells, FXIa, and FX) was standardized to 10 minutes, and the second (with chromogenic substrate) to 20 minutes. The linearity of both reactions was controlled in each experiment. Under these conditions, a ∆OD$_{540}$ value of 1.0 corresponded to 0.71 UTF (1.1 nM TF) and the three standard curves (TF antigen, TF activity, and FX activating activity) were colinear.

**Amidolytic activity of TF/VIIa on the cell surface.** The cell surface activity of the TF/VIIa complex toward the peptide substrate S-2288 was determined essentially as described by Le et al.18 Cell monolayers were incubated with 20 nM VIIa in HEPES-buffered saline (HBS) 136 mM NaCl, 5 mM CaCl$_2$, 5 mM KCl, 1.2 mM MgCl$_2$, 11 mM BSA, pH 7.4) for 1 hour at 37°C to saturate TF specific binding. Following washing, the chromogenic substrate S-2288 was added to a final concentration of 0.5 mM/mL in HBS. After incubation at 37°C for 4 hours, 200-µL aliquots were transferred to a 96-well microtiter plate and absorbance at 405 nm was measured. Controls were cells not receiving VIIa and cells preincubated with a neutralizing anti-human TF monoclonal antibody (4504). The values are expressed as ∆OD$_{540}$hour·µg cell protein.12 Comparisons of this peptidolytic activity of TF/VIIa complexes on the cell surface to complexes formed using isolated brain TF/VIIa showed that with otherwise equal TF activities and antigen levels, isolated human brain TF demonstrated threefold to fourfold lower specific activity toward the chromogenic substrate. The cause remains unexplained.

**Immunofluorescence Microscopy**

For staining of TF on IL-1β-induced HUVEC, cells on cover slips were fixed in absolute methanol, incubated for 1 hour at 37°C with primary antibody (4 µg/mL, rabbit, in PBS with 1% BSA) and for 1 hour at 37°C with the rabbit antimonospecific FITC-conjugated antibody (25 µg/mL in PBS). Propidium iodide (100 ng/mL) was added during the last 10 minutes of the last incubation. Cells were rinsed four to five times in PBS between each step. A final rinse was made in PBS, pH 8. Controls of unperturbed HUVEC were included, with no evidence of nonspecific binding.

For detection of TFP-1 in wild-type MDCK cells, confluent cells were rinsed three times in serum-free medium at 37°C, fixed in 4% paraformaldehyde at 37°C for 30 minutes, and incubated with
polyclonal goat antiserum to human TFPI-1 at a 1:50 dilution in PBS with 0.1% BSA on either the apical or basolateral side for 1 hour. The cells were then rinsed in PBS at 37°C, and FITC-conjugated antigoat antiserum at a 1:50 dilution in PBS with 0.1% BSA on either the apical or basolateral side for 1 hour. The cells were then rinsed in PBS at 37°C. Staining with an irrelevant polyclonal antibody (anti-von Willebrand factor) was used as a control. Other controls were filters without cells incubated with medium and filters with cells stained with the second antibody only.

To detect endogenous or binding of exogenous Annexin V, the following procedures were used at 4°C. Transfected or untransfected MDCK cells and HUVEC (with or without induction of TF synthesis) were first washed thrice in HEPES buffer with 5 mM CaCl₂ to conserve bound Annexin V or with HEPES buffer containing 1 mM EDTA to remove it. The HEPES buffer consists of 25 mM HEPES, 135 mM NaCl, 5 mM KCl, 0.01% wt/vol glucose, and 0.3% wt/vol BSA, pH 7.4. The cells were then incubated with Annexin V or FITC-conjugated Annexin V both at a final concentration of 142 mMol/L. NACL 5 mMol/L KCl 0.01% wt/vol glucose, and 0.5% wt/vol BSA, pH 7.4.) The cells were then incubated with Annexin V or FITC-conjugated Annexin V both at a final concentration of 142 mMol/L. NACL 5 mMol/L KCl 0.01% wt/vol glucose, and 0.5% wt/vol BSA, pH 7.4.) The cells were then incubated with Annexin V or FITC-conjugated Annexin V both at a final concentration of 142 mMol/L. NACL 5 mMol/L KCl 0.01% wt/vol glucose, and 0.5% wt/vol BSA, pH 7.4.) The cells were then incubated with Annexin V or FITC-conjugated Annexin V both at a final concentration of 142 mMol/L.

RESULTS

Using the Transwell culture system described, we have recently demonstrated that TF activity expressed in HUVEC induced with IL-1β is highly polarized to the apical (luminal) surface of the cells. To obtain this apical expression, the endothelial cells must be grown beyond confluence on a permeable support, to a state where junctional complexes are formed and reduced intercellular passage of macromolecules is evident. For comparison, we also used MDCK strain 1, an epithelial cell line that forms stable and well-characterized tight junctions. Transfected these cells with human TF constructs allowed studies of the sorting of both canine and human TF.

TF Activity in Transient Assays and Stable Clones

Constructs coding for wild-type (hTF1-245) and truncated (hTF1-245) human TF were transiently transfected into a monkey kidney fibroblast-like cell line (COS-1) that does not normally express TF. In total cell homogenates (Fig 1A), the truncated clone yielded about 40% higher activity than the wild-type clone, confirming that the cytoplasmic domain of TF is not needed for formation of an active complex with FVII, Ca²⁺, and phospholipids.

MDCK strain 1 cells were transfected with the same constructs and selected for stable integration with G418. TF activity was tested by coagulation of homogenized cells with human plasma and related to total cell protein (Fig 1B). MDCK cells expressed significant background levels of canine TF, which reacted with human FVII. The clone transfected with the hTF1-245 construct expressed more total TF activity than that transfected with the hTF1-243 construct. When these MDCK clones were grown to confluence in normal wells and tested for surface activity using a chromogenic factor Xa–dependent peptide assay, the ratios between the surface and the total activity of each clone (tested by the clotting assay) were similar (Fig 1B and C), indicating that the truncated TF reached the cell surface as efficiently as the wild-type.

In agreement with studies in other cell lines, we found that the activity of homogenized cells (Fig 1B) was 20- to 30-fold higher than that of intact cells (Fig 1C).
Ties were related to total cell protein and normalized to the activity of transfected COS-l cells determined by clotting assay of homogenized cells using human plasma. Clotting activity from transfected cells ranged from 0.5 to 6 UF/mg cell protein depending on the efficiency of the transfection. Data are the mean ± SD of 5 independent experiments, each in triplicate. An equal amount of DNA for each construct was transfected with an equal amount of DNA for each construct.

The total surface activity of the clone transfected with the wild-type human TF construct (hTF1-263) was set at 100%, and ranged from 0.5 to 6 UF/mg cell protein depending on the efficiency of the transfection. Data are the mean ± SD of 5 independent experiments, each in triplicate. An equal amount of cells were transfected with an equal amount of DNA for each construct. (B) Total TF activity in stably transfected MDCK clones determined by clotting assay of homogenized cells using human plasma. Data are the mean ± SD of 3 independent experiments with each clone, each in duplicate. (C) Surface activity of stable MDCK clones 1 day after plating. Surface activity of the high-density-plated layers of adherent cells was determined by chromogenic FXa substrate test and related to total cell protein. The total surface activity of the clone transfected with the wild-type human TF construct (hTF1-263) was set at 100%. The apical/basolateral distribution of TF antigen on the surface of polarized monolayers of HUVEC (after 6 hours of IL-1β stimulation) and MDCK was determined by binding of 125I-labeled anti-hTF antibodies or ligand FVIIa and by cell surface specific biotinylation followed by immunoprecipitation (MDCK cells only).

Location of TF Surface Antigen in HUVEC and MDCK Cells

The apical/basolateral distribution of TF antigen on the surface of polarized monolayers of HUVEC (after 6 hours of IL-1β stimulation) and MDCK was determined by binding of 125I-labeled anti-hTF antibodies or ligand FVIIa and by cell surface specific biotinylation followed by immunoprecipitation (MDCK cells only). For binding of iodinated antibodies, intact monolayers were incubated on ice with 125I-labeled monoclonal murine anti-hTF antibody (htfl or 4504) to saturation of TF-specific binding. In preliminary experiments, this was determined to
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require about 1 hour. Both antibodies gave the same final result, although the htf1 antibody gave higher nonspecific binding to both HUVEC and MDCK cells. In HUVEC, 78% ± 5% of the surface available antigen was found on the apical surface (Fig 3A). In MDCK cells, the distribution of binding sites was the opposite of that in HUVEC: 94% ± 3% (htf1-263) or 92% ± 3% (htf1-245) of the surface available TF antigen was located on the basolateral surface of the MDCK cells (Fig 3B). Thus, in HUVEC, TF antigen and activity colocalized to the apical surface of the cell layer. However, in MDCK cells, the situation was different. More than 90% of the surface available TF antigen was detected on the basolateral side, whereas essentially all of the (very limited) TF surface activity was observed on the apical side. This is clearly illustrated in Fig 3C, where the apical and basolateral human TF activity of the MDCK htf1-263 clone (Fig 2B) was related to the number of apical and basolateral cell surface antibody binding sites on the same clone (Fig 3B). The specific activity of the basolateral TF was about 100-fold lower than that of the apical TF. All these binding experiments were performed at 4°C. Control experiments at 37°C gave similar results. Comparison of 125I-labeled antibody binding to intact and ethanol fixed (opened) cells on Transwells revealed that the surface available human TF antigen accounted for 70% ± 8% (n = 10) of the total human TF antigen in the transfected MDCK cells.

Binding of iodinated FVIIa was used as an independent method for determining surface available TF and also allowed determination of the distribution of canine TF. This was especially important in view of the discrepancy in MDCK cells between the very high amount of basolateral TF antigen (94% of total surface available antigen; Fig 3B) and the low basolateral procoagulant activity (less than 20% of total surface available activity; Fig 2B). Nontransfected cells and cells transfected with hTF1.263 were used. The binding assays were performed in the presence or absence of a 50-fold molar excess of cold FVIIa. The competitive binding (binding in the absence of cold FVIIa–binding in its presence) was taken to be the specific binding. These FVIIa binding data (Fig 3D) demonstrated a distribution of human TF that was similar to that obtained when antibody binding was studied, although the basolateral portion was slightly lower (88% rather than 94% of total surface available). A basolateral predominance was found also for the canine TF (84% of the total surface available).

The total number of antibody binding sites on the cell surface was essentially the same for wild-type and truncated human TF in MDCK cells (25.5 ± 3.1 and 25.9 ± 4.1 × 10^9 antibody binding sites/cm^2 filter, respectively, n = 21). The same number of total surface binding sites was found with FVIIa (24.4 ± 7.1 × 10^9 FVIIa binding sites/cm^2 filter, n = 9), suggesting that the number of binding sites corresponded to the number of cell surface TF molecules. The induced endothelial cells had much less TF antigen on their cell surface (3.4 ± 0.6 × 10^9 antibody binding sites/cm^2 filter, n = 10), partly due to the heterogeneity in response to IL-1β discussed below and to the lower number of cells per filter area (DNA content was determined by Hoechst 33258 to be 17 ± 6 μg/cm^2 filter for MDCK cells and 4.7 ± 0.8 μg/cm^2 filter for HUVEC).

For immunofluorescence studies of subconfluent HUVEC stimulated by IL-1β to synthesize TF, ethanol fixed on glass cover slips and costained with propidium iodide and an anti-hTF antibody showed marked cell-to-cell heterogeneity of
TF expression (Fig 4). Regularly, only one third of the cells responded to IL-1β induction of TF synthesis. Similar observations were made by Drake et al.22 and Kirchhofer et al.24 Stably transfected MDCK cells (hTF1.263) displayed TF positive staining in 100% of the cells. Immunofluorescence of paraformaldehyde-fixed tight layers of IL-1β stimulated HUVEC and stably transfected MDCK cells appeared to support the apical/basolateral expression pattern seen using iodinated antibodies, but staining of cells grown on Transwell filters gave too much background fluorescence.

Biotinylation of transfected (hTF1.263 and hTF1.245) and untransfected MDCK cells (Fig 5) showed a distribution of biotinylated TF antigen to the basolateral (hTF1.263, 94% ± 1%; hTF1.245, 90% ± 4%) and apical (hTF1.263, 6% ± 1%; hTF1.245, 10% ± 4%) surface domains essentially identical to that obtained with binding of iodinated monoclonal antibody and FVIIa. Wild-type MDCK gave no recoverable biotinylated TF. 

Fig 3. Surface TF antigen on polar HUVEC and MDCK cell layers determined by binding of iodinated antibody and FVIIa (A) IL-1β-induced and control polar HUVEC layers labeled on ice for 1.5 hours with 125I-anti-TF antibodies on apical (■) or basolateral (□) surface. Binding to control HUVEC subtracted. The resulting total surface TF binding was set at 100% (3.42 × 10⁶ antibody binding sites/cm² filter). Data are the mean ± SD of 4 independent experiments, each of 2 to 3 parallels. (B) Polar cell layers of MDCK hTF1.263 and MDCK hTF1.245 labeled with 125I-anti-TF antibodies on apical (■) or basolateral (□) surface. Binding to nontransfected MDCK cells subtracted. Total surface binding to human TF was set at 100% for each clone (25.5 and 25.9 × 10⁶ antibody binding sites/cm² filter for MDCK hTF1.263 and MDCK hTF1.245, respectively). Data are the mean ± SE of 8 independent experiments, each of 2 to 3 parallels. (C) Specific activity toward macromolecular substrate of apical (■) and basolateral (□) surface anti-hTF antibody binding sites on MDCK hTF1.263 Transwells determined by relating results in Fig 2B (surface activity) and 3B (surface binding sites for anti-hTF monoclonal antibody). (D) Binding of 125I FVIIa to apical (■) or basolateral (□) surface of polar layers of stably transfected (hTF1.263) and nontransfected MDCK cells. Uncompetable binding (in the presence of 50-fold molar excess of cold FVIIa) was subtracted in both cases. Net binding to human TF caused by the stable transfection was obtained by further subtracting the binding to canine TF in wild-type MDCK cells. Total binding (the sum of specific binding on both sides after subtraction) was taken as 100% in each case (24.4 and 22.9 × 10⁶ FVIIa binding sites/cm² filter to human and canine TF, respectively). Data are the mean ± SD of 3 independent experiments, each in triplicate.

Fig 4. Heterogenous HUVEC response to IL-1β. HUVEC were grown on glass cover slips, induced by IL-1β to express TF, ethanol-fixed, and costained with mAb htf/FITC-rabbit-antimouse Ig (Ab'), and propidium iodide.
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immunoprecipitated with anti-hTF antibody and antimouse IgG biotin-HRP and magnetic beads, run on TFlFVIIa complex. hTF1.2.5 basolateral; lane streptavidin-biotin-HRP. lar-weight bands in lanes labeled with sulpho-NHS-biotin on apical hTF1.nu, MDCK hTF1.245, and nontransfected MDCK cells surface-(BFA). The concentration used polarized MDCK cell layers were treated with Brefeldin A to have an effect on the expression of proteins in MDCK cells under these conditions.* By attempting to induce a shift of the TF expression, we wanted to investigate the membrane domain that determines if the expressed TF is active. Treatment with BFA led to moderate cellular shape changes and reduced the electrical resistance by about 50% without causing detectable leakage through the layer, confirming previous observations.27 Redistriution of MDCK TF Activity and Antigen on Treatment With Brefeldin A

In an attempt to induce a shift of the polarity of TF antigen, polarized MDCK cell layers were treated with Brefeldin A (BFA). The concentration used (5 μg/mL) would be expected to have an effect on the expression of proteins in MDCK cells under these conditions.5,26 By attempting to induce a shift of the TF expression, we wanted to investigate the importance of the local environment for the activity of the TF/FVIIa complex. The cells were treated with BFA for 6 and 24 hours. After 6 hours, only a slight shift in antibody binding and TF activity toward the apical surface was seen. After 24 hours, total surface binding of the 125I-labeled monoclonal anti-hTF antibody increased by about 40%, caused by an approximately equal increase in binding sites on the apical and the basolateral side (Fig 6). This resulted in an apical shift from 6% to 8% to about 20% of the total surface binding. Cells transfected with the truncated construct (hTF1-245) showed a slightly higher increase on the apical side than cells transfected with the complete TF cDNA (hTF1-263). These observations were confirmed by direct surface biotinylation (Fig 7). Measurements of cell surface Xa generation showed a 2.3 ± 0.7-fold increase in apical activity compared with untreated cells, but hardly any (1.3 ± 0.2-fold) increase in basolateral activity (n = 12). The corresponding increase in apical antigen (4.0 ± 1.2-fold) was not significantly different from this activity increase. These results suggest that it is the local environment of the membrane domain that determines if the expressed TF is active. Treatment with BFA led to moderate cellular shape changes and reduced the electrical resistance by about 50% without causing detectable leakage through the layer, confirming previous observations.27 Activity of Basolateral TF in MDCK Cells

Basolateral TF in MDCK cells had very low specific activity (Fig 3C). To study the possible causes, we have looked for TFPI-1 and Annexin V by immunofluorescence copy. With anti-TFPI-1 an almost uniform fluorescence was seen on the basolateral side; all the controls were negative. The fluorescence pattern was compatible with diffuse cellular or matrix staining. On the apical side all cells were unstained except for a few cells growing in dumps or piles. These cells expressed TF and were apparently not polarized. Thus, this antibody to human TFPI-1 recognized also the canine factor. TFPI-1 expression was essentially observed on the basolateral side. Treatment of the cells with heparin did not change the fluorescence pattern. Neither heparin (10...

Fig 5. Surface TF antigen on polar MDCK cell layers determined by biotinylation/immunoprecipitation. Polar cell layers of MDCK hTF1-263, MDCK hTF1-245, and nontransfected MDCK cells surface-labeled with sulpho-NHS-biotin on apical or basolateral surface, immunoprecipitated with an anti-hTF antibody and antimouse IgG magnetic beads, run on 12% PAGE, and visualized with streptavidin-biotin-HRP and ECL. One typical of 4 experiments. The high–molecular-weight bands in lanes 4 and 6 represent nonspecific binding of streptavidin-biotin-HRP. Lane 1, MDCK hTF1-263; lane 2, MDCK hTF1-245 basolateral; lane 3, MDCK hTF1-245 apical; lane 4, MDCK hTF1-263 basolateral; lane 5, MDCK apical; lane 6, MDCK basolateral.

Fig 6. Distribution of human TF antigen on the surface of polar MDCK cell layers treated with Brefeldin A for 24 hours determined by binding of iodinated antibody. Transfected MDCK hTF1-263 and MDCK hTF1-245 were labeled with 125I antibody on the apical (■) or basolateral (○) side after incubation for 24 hours in the presence or absence of Brefeldin A (5 μg/mL). Binding to nontransfected cells was subtracted. Total surface human TF specific antibody binding to cells not treated with Brefeldin A (normal) was taken as 100% (100% values are the same as in Fig 3B). Data are the mean of 3 independent experiments, each in triplicate.

Fig 7. Distribution of human TF antigen on the surface of polar MDCK cell layers treated with Brefeldin A for 24 hours determined by biotinylation/immunoprecipitation. Polar cell layers of MDCK hTF1-263, MDCK hTF1-245, and nontransfected MDCK cells were treated with 5 μg/mL Brefeldin A for 24 hours, surface-labeled with sulpho-NHS-biotin on apical or basolateral surface, lysed, and immunoprecipitated with anti-hTF antibody and antimouse IgG magnetic beads, run on 12% PAGE, and visualized with streptavidin-biotin-HRP and ECL. Lane 1, MDCK hTF1-263; lane 2, MDCK hTF1-245 basolateral; lane 3, MDCK hTF1-245 apical; lane 4, MDCK hTF1-263 basolateral; lane 5, MDCK apical; lane 6, MDCK basolateral.
U/mL for 6 hours) nor a neutralizing polyclonal antibody to human TFPI-1 significantly altered the activity of TF on the basolateral side.

Polyclonal antibodies to Annexin V did not disclose any endogenous Annexin V bound to the MDCK surface. However, purified human Annexin V bound to HUVEC and was easily detected using the same polyclonal antibodies. No binding was observed to MDCK cells, neither on the apical nor on the basolateral side, indicating a low content of negatively charged phospholipids in the outer leaflet of the plasma membrane.

**DISCUSSION**

The binding of FVII to the extracytoplasmic part of TF is an obligatory step in its triggering of blood coagulation. Recent studies in our laboratory have shown that TF has a dual function in that FVII binding also elicits an intracellular signal in the form of Ca^{2+} spikes. TF is constitutively expressed in many cell types that are not normally in contact with flowing blood, such as epithelial cells lining the body cavities. In two cell types, monocytes/macrophages and endothelial cells, which are both in contact with flowing blood, TF synthesis can be induced. In vivo, epithelial cells and endothelial cells form tight junctions or junctional complexes that seal off an apical surface area from the rest of the surface (basolateral) with regard to lateral diffusion in the cell membrane and thus allow the definition of these two surface domains. In vitro experiments to define characteristics of these domains must consequently be performed under conditions where the formation of such junctional complexes is possible, and only under such conditions can the polarity of TF expression be studied.

**TF Is Expressed Mainly on the Luminal Surface of IL-1β Induced Endothelial Cells**

By growing cells on permeable filters and carefully monitoring the formation of junctional complexes, we have recently shown that in endothelial cells induced by interleukin-1β, the fraction of total TF activity (~25%) that becomes available for triggering of coagulation on intact cells is mainly on the apical surface. In the present report, we confirm this distribution by measurement of binding of a labeled monoclonal antibody to the cell surface domains.

Previous reports have suggested different localizations of TF in endothelial cells at variance with our results and with those of others. As far as we can judge, neither of these groups have grown the endothelial cells on permeable supports (except for EM studies by Ryan et al), nor have they cultured the cells for the time we find necessary to establish junctional complexes. The differences are thus most likely due to difference in experimental designs. The inadequacy of such setups for the formation of junctional complexes in endothelial cells has also been suggested by Schleef et al.

If the polarized expression pattern described here also holds true for the in vivo situation, induced TF is uniquely positioned for interacting with FVIIa on the luminal surface of the endothelial cell. An important role is suggested for endothelial TF in the inflammatory response, contributing to an increased prothrombotic state of the endothelium without the requirement for endothelial disruption. This occurs in concert with novel synthesis of leukocyte adhesion molecules (E-selectin and vascular cell adhesion molecule-1) and with a decrease in the cell surface fibrinolytic activity (upregulation of luminally expressed type I plasminogen activator inhibitor, downregulation of cell surface thrombomodulin and tissue-type plasminogen activator production). All these factors would contribute to the initiation and stabilization of fibrin deposition on the perturbed endothelial surface, e.g., as part of a disseminated intravascular coagulation process. Whether these in vitro findings reflect what actually takes place in vivo is still in the dark, as expression of TF in perturbed vascular endothelium in vivo (except in spleen) has been hard to demonstrate. These difficulties may be related to the low level and heterogeneity of TF expression in perturbed endothelial cells, or possibly to masking of the antigen. Even very low levels of TF expression in vivo could still be of pathological significance, e.g., by disturbance of the plasma VIIa/VIII ratio, once the TF is presented directly to flowing blood as our findings suggest.

**TF Is Expressed Mainly on the Basolateral Surface of MDCK Cells**

In contrast to endothelial cells, epithelial cells would be expected to interact with FVII reaching the cell from the basolateral side. Studies of human TF antigen on the surface of transfected MDCK cells showed that 88% to 94% of surface TF indeed was localized to the basolateral side of the cells. The endogenous canine TF in the MDCK cells was distributed in the same way; i.e., both the human and canine TF in MDCK cells showed the opposite pattern of what was found for endothelial cells. The difference in localization suggests that the sorting machinery either looks at different signals in MDCK and endothelial cells or reads the same signals to a different conclusion. The strictly polar expression in both cell types suggests active sorting in both cases rather than active in one and default in the other, although the basolateral dominance in MDCK is reduced when the results are normalized per unit surface area constituting the two domains. Normalized for the ratio of apical to basolateral surface areas (~1:3.8), there is still a greater than two-fold basolateral preference for TF antigen expression. This is less compatible with passive delivery to the apical and basolateral membrane domains. Prolonged treatment of the MDCK cells with Brefeldin A caused an apparent redistribution of TF antigen as well as activity toward the apical surface. Whether this is caused by altered sorting of newly synthesized TF or (less likely) represents a true redistribution of basolateral or intracellular TF is not known. It is interesting to note that essentially all of the TF appearing on the apical surface becomes fully active. This local environment thus allows functional expression of an increased number of TF molecules.

**Discordant Expression of TF Antigen and Activity on the Surface of MDCK Cells**

Expression of TF antigen of the basolateral surface of MDCK was detected by antibody binding. FVIIa binding
and by biotinylation/immunoprecipitation but was not accompanied by detectable procoagulant activity. This was not a temperature-dependent phenomenon, as the same binding distribution was obtained at 37°C, nor was it due to limited access of activity assay components through the permeable filters, as the same activity distribution was observed with cells grown on large pore (3.0 μm) membranes. It was clear from our binding studies that FVIIa, a molecule similar to FX also used in the Xa chromogenic substrate assay, was able to reach and bind to the basolateral TF. The limited activity of the basolateral TF was not restricted to activation of macromolecular substrate, as a similar lack of activity was found when looking at direct hydrolysis of a peptide substrate (S-2288) by the TF/VIIa complex.

It thus appears that there was a large fraction of TF/VIIa complexes on the basolateral surface of the MDCK cells which was not active and a much smaller number of complexes on the apical surface which was highly active.

Several different not mutually exclusive explanations are possible. Restricted access of substrates to bind with TF is unlikely as discussed above. The presence of one or more inhibitors is possible. Candidates might be TFPI-1,2,42 TFPI-2,43 antithrombin III,44 Annexin V45 or sphingosine.46 The latter two are unlikely; Annexin V was not detected and may not be able to inhibit the TF/VIIa complex in intact surfaces.47,48 Sphingosine acts by inhibiting binding of VIIa to TF,49 which we have found to be unhampered in MDCK cells. Immunofluorescence studies of polar MDCK cell layers clearly showed basolateral localization of TFPI-1. This inhibitor is normally expected to require FXa for binding to the TF/VIIa complex, whereas we demonstrate lack of activity of the TF/VIIa complex also in the absence of FX.

Another possibility might be the lack of phosphatidylserine (PS) in the outer leaflet of the plasma membrane. PS has previously been shown to be crucial for TF activity.49,50 In 1992, Le et al18 suggested the existence of two classes of TF/VIIa complexes on the surface of OC-2008 cells. Both fractions had the same binding affinity, both were active upon cell lysis, but only the minor fraction supported the activation of macromolecular substrate on the cell surface. To explain this, Le et al suggested that the recruitment of an additional surface component, essential for the catalytic activity of the TF/VIIa complex, was necessary. Cell lysis would supply more of this limiting compound, suggested to be PS. This would be consistent with the absence of binding of Annexin V, but the limited difference in gross composition of apical and basolateral plasma membrane domains51 does not support this hypothesis, although a very special distribution of PS between the outer and inner plasmamembrane leaflet52 is not excluded.

One should also consider the fact that the increase in apical TF antigen after treatment with BFA was coupled to an increase in activity of the same scale. This would be compatible with a limiting amount of an additional surface component required for activity only if the amount of this component was also affected by BFA treatment or by polarization of the cells.

Removal of the TF Cytoplasmic Tail Does Not Alter the Distribution of TF in MDCK Cells

Until recently, all of the proteins studied which localize to the basolateral domain of MDCK cells, have had active sorting signals within their cytoplasmic domains. Two types of sorting motifs have been revealed, one type dependent on a tyrosine residue and the other dependent on a double leucine (or other hydrophobic doublet).53 When these basolateral determinants are deleted, the proteins are distinctly mis-sorted to the apical surface of the cells.54-57 Cytoplasmic domain-independent sorting to the basolateral surface of polarized MDCK cells was recently demonstrated for the α2a-adrenergic receptor.58

The TF cytoplasmic domain does not contain any known signal for endocytosis or sorting. Amino acid sequence alignment of the human, murine, bovine and rabbit TF cytoplasmic domains shows a high degree of conservation comprising a serine/threonine phosphorylation site61 and several other amino acid residues (Fig 8). As was the case for the α2a-adrenergic receptor,59 deletion of the cytoplasmic domain of TF had no obvious effect on surface distribution of the protein; the truncated mutant showed the same basolateral phenotype as wild-type TF. We have not excluded the possibility that the three remaining membrane proximal amino acids of the cytoplasmic tail are of importance, as palmitoylation signal have in some cases been shown to have an effect on endocytosis.62,63 The cytoplasmic tail is not important for sorting of TF, but it may have other roles, such as in the modulation of TF half life on the cell surface or in mediating the intracellular signal generated by TF on binding of its ligand FVIIa.1 Why the activity of clones stably transfected with truncated TFcDNA1,245 was in fact greater than wild-type transfected clones is not known. Paborsky et al made the same observation20 and reported that there was no difference in specific activity between the two. In our studies, both constructs contained the 3' untranslated region of TF cDNA; therefore, it is not likely to be due to different mRNA stability.

Sorting of TF in MDCK cells is also unusual in the sense that although TF does not contain any known basolateral sorting or endocytosis signals, it still sorts to the basolateral domain of the cells. This, in combination with the lack of cytoplasmic tail involvement, raises the possibility of a new class of yet unidentified basolateral sorting signals (if the sorting is indeed active). Together with what was seen for the α2a-adrenergic receptor, this suggests that basolateral sorting in MDCK cells is not always regulated by a cytoplasmic determinant intimately linked to an endocytosis signal.

Prior to this study, there are few studies of sorting of the

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**Fig 8. Amino acid sequence alignment of the TF cytoplasmic domain in 4 species.** Conserved residues are boxed. Numbering as in human TF. Arrow indicates position of truncation in hTF,245.

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same protein in both endothelial and epithelial cells. GPI-anchors have been shown to direct proteins to the apical domain in both polarized endothelial cells and MDCK cells. The transferrin receptor is localized on the basolateral surface of MDCK cells, and on the apical surface of the high resistance endothelium of the blood-brain barrier, but not the normal low-resistance vascular endothelium. Sorting of TF to opposite domains in epithelium and normal low-resistance vascular endothelium remains without precedent.

ACKNOWLEDGMENT

We are grateful for the gifts of monoclonal TF antibody from Dr S. Carson (University of Nebraska Medical Center), TF cDNA from Dr J. Morrissey (Oklahoma Medical Research Foundation), polyclonal Annexin V antibody from Dr W. van Heerde (Hôpital de Bicêtre), and recombinant FVIIa, interleukin-1β, and TFPI antibodies from Prof. U. Hedner and colleagues (Novo-Nordisk), and to A. Simonsen for helpful advice.

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Opposite sorting of tissue factor in human umbilical vein endothelial cells and Madin-Darby canine kidney epithelial cells

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