Tissue factor (TF) is an integral membrane glycoprotein that serves as the receptor and essential cofactor for coagulation factors VII and VIIa (FVII and FVIIa, respectively), is the primary cellular activator of the coagulation protease cascade. Previous studies on the procoagulant activity of a variety of cell types (either lysed or in the intact state) have variously been interpreted as showing that TF is either stored intracellularly or is present in a cryptic form in the surface membrane. Using mAbs to TF, we have directly investigated the subcellular localization and functional activity of TF in lipopolysaccharide-stimulated blood monocytes and J82 bladder carcinoma cells. Blocking of surface TF of viable cells with inhibitory anti-TF mAbs abolished >90% of TF activity of the intact cells as well as of lysed cells. Furthermore, quantitative analysis of the binding of FVII and anti-TF mAb to J82 cells demonstrated that all surface-expressed TF molecules were capable of binding the ligand, FVII. By immunoelectron microscopy, TF was present only in the surface membrane of monocytes and J82 cells, although the latter also contained apparently inactive TF antigen in multivesicular bodies. On the intact cell surface the catalytic activity of the TF-FVIIa complex was investigated and found to be markedly less relative to cell lysates. Membrane alterations that affect the cofactor activity of TF may be a means of regulating the extent of initiation of the coagulation protease cascade in various cellular settings.

Abbreviations used in this paper: FVII, coagulation factor VII; FVIIa, activated coagulation factor VII; FX, coagulation factor X; FXa, activated coagulation factor X; LPS, lipopolysaccharide; PBM, peripheral blood mononuclear cell; TF, tissue factor.
to <5% of lysed cell activity (5-8, 10, 24, 27, 29, 39). This has been interpreted by some to indicate intracellular stores of TF, although other reports have suggested that TF is predominately surface expressed (23, 28, 39). In some cells, surface-expressed TF procoagulant activity has been shown to increase with exposure to proteolytic enzymes (28) or hydrogen peroxide (47), suggesting that a certain portion of TF activity is latent in the cell membrane or, alternatively, that TF is being mobilized from an intracellular compartment. Precedent for the latter exists in the monocyte membrane proteins MAC-1 and p150,95 for which up regulation of surface expression has been shown to be due to mobilization of proteins from intracellular vesicles and oxidase-negative granules to the cell surface (30).

We report here studies undertaken to determine the subcellular localization and functional expression of TF in endotoxin-stimulated human peripheral blood monocytes (PBMs) and a neoplastic human cell line (J82 bladder carcinoma cells). In initial studies, we have shown that these two cell types exhibit different localization of TF at the light microscopic level. While both express TF on the cell surface, permeabilized J82 cells, but not monocytes, contain immunoreactive TF intracellularly in a perinuclear granular distribution. Both cells, however, have similar increases in procoagulant activity after cell lysis. In the present study, we now present evidence that >90% of functional TF in cells is expressed on the surface membrane and is fully competent for binding its ligand, FVII, or FVIIa. However, the activity of TF on the intact cell surface is substantially lower than that in lysed cell preparations. These findings suggest that membrane alterations that modulate the catalytic activity of the TF–FVIIa complex on the cell surface might be an important means of regulating the extent of cellular initiation of the coagulation protease cascade.

Materials and Methods

Materials

Tissue culture media and supplements were obtained from Gibco Laboratories (Grand Island, NY), except for FBS which was from HyClone Laboratories (Logan, UT). All components were tested for endotoxin by Limulus amebocyte lysate assay (Sigma Chemical Co., St. Louis, MO), and only those with no detectable endotoxin (<10 pg/ml) were used. Tissue culture plastic ware was from Costar, Data Packaging Corp. (Cambridge, MA). TF was purified from human brain, and FVII and FX were from human plasma as described (15, 17, 33). FVIIa was prepared by activating FVII with FXa immobilized to Affigel 15 beads (2). Preparation and characterization of the mAbs to human brain TF has been described (32). Samples were added to 100 μl preserved CaCl2 (20 mM) in 15 × 75-mm borosilicate glass tubes. After 30 s, 100 μl human plasma was added and the time required for a visible fibrin gel to form was noted. The quantity of TF expressed was determined by comparison of clotting times with a standard curve established using purified reconstituted human brain TF in which 1,000 μl (equivalent to 1.2 ng protein) yielded a clotting time of 50 s. Mixing experiments were performed to control for possible carryover of unbound antibody that might not have been removed during the washing steps. In these, 50 μl of cell suspension that had been incubated with anti-TF mAb was combined with 50 μl of cell suspension that had been incubated with the control mAb. Both intact and lysed cell suspensions were tested.

For determination of rate of FXa generation, intact and lysed cells were resuspended in an identical volume of supplemented Hepes-buffered saline (10 mM Hepes, 137 mM NaCl, 4 mM KCl, 11 mM glucose, 0.5% BSA, pH 7.4), kept at 4°C, and added to the assay at various concentrations. A typical assay was performed by preincubating cells as a TF source with FVIIa for 10 min at 37°C in the presence of calcium ions. The reaction was started by the addition of FX. Fixed concentrations of FX (1,500 nM), FVIIa (0.2 nM), and CaCl2 (4 mM) were used. At varying times an aliquot of the reaction mixture was added to TBS containing 100 mM EDTA (final concentration) to stop the reaction. After removing the cells by centrifugation, FXa was quantitated in the supernatant with the chromogenic substrate S2222 at 0.2 mM final concentration using a kinetic 96 well multichannel ELISA reader (model Vmax; Molecular Devices Corp., Menlo Park, CA) for the determination of initial rates of peptide hydrolysis. Standard curves were prepared using purified FXa.

Quantitation of FVII and Anti-TF mAb Binding to Cells

FVII and mAb TP9-6B4 were labeled with 125I by the coupled lactoperoxidase/glucose oxidase method (Enzymobead radioiodination reagent; Bio-Rad Laboratories, Richmond, CA) to a specific activity of 1-1.7 × 106 cpm/μg, respectively. Binding characteristics to J82 cells were determined using the protocol described by Fair and MacDonald (16) with the modification that 5 mM CaCl2 was added to the washing buffer. Scatchard analysis was performed using the nonlinear least squares fitting procedure of the LIGAND program (34).

Immunelectron Microscopy

Suspensions of PBMs (1-3 × 107/ml) prepared as described above were incubated in plastic slide wells chambers at 37°C. After 2 h, the wells were washed five times with RPMI to remove nonadherent cells. Remaining adherent cells were incubated overnight in RPMI plus 10% FBS in 5% CO2 at 37°C. To stimulate TF expression, 100 ng/ml bacterial LPS in fresh medium was incubated with cells for 4 h. J82 cells were cultured in plastic slide well chambers as described above and studied when confluent, 2-3 d after passage. Cells were fixed by immersion in 0.2% glutaraldehyde plus 2% paraformaldehyde in PBS at 4°C for 60 min followed by washing in PBS.
with 0.1 M glycine for 15 min. Saponin (0.02%) was included in all wash and incubation solutions to maintain permeabilization of cells (51). Cells were then sequentially incubated for 60 min each at 37°C in blocking buffer (10 mM Tris-HCl, pH 7.4, with 2% dry milk solids and 0.02% saponin), primary antibody (TP9-9C3 and TP9-6B4 combined, as hybridoma culture supernatants diluted 1:5), biotinylated anti-mouse IgG (1:400), and streptavidin-peroxidase complex (1:200). These reagents were all diluted in a buffer of 0.053 M Tris-HCl, 0.15 M NaCl, 1% BSA, 0.02% saponin, pH 7.4. Except for the blocking solution step, slides were washed after each step for 15 min in 0.005 M Tris-HCl, 0.15 M NaCl, pH 7.4, with 1% BSA and 0.02% saponin. Before chromogen development, cells were fixed in 2% glutaraldehyde in PBS for 15 min at room temperature and then washed in PBS and distilled water before immersion in 2 mg/ml diaminobenzidine in 0.05 M Tris-HCl, pH 7.6, with 0.2% hydrogen peroxide for 15 min at room temperature. Slides were washed in distilled water and immersed in 0.1 M cacodylate buffer, pH 7.4, for 15-60 min before embedding.

Cells were postfixed in some cases in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4; in all cases, cells were dehydrated in graded ethanol solutions and then embedded in Epon 812. En face thin sections were cut on an Ultratome III (LKB Instruments, Inc., Gaithersburg, MD) and mounted on 200 mesh bare grids. Cells that had been postfixed in osmium tetroxide were stained with 1-2% uranyl acetate in 50% ethanol with or without light staining with lead citrate. Sections were examined with a 20× objective aperture. Some sections were examined without exposure to osmium, uranyl acetate, or lead citrate to enhance visibility of the diaminobenzidine reaction product.

**Results**

**Rate of FXa Generation by Intact and Lysed Cells**

The ability of lysed and intact LPS-stimulated PBMs to generate FXa was compared to confirm that the differences observed in the procoagulant activity of intact and lysed cells indeed represented true differences in functional TF expression (Table I). Incubation of PBMs with purified FVIIa and FX showed that the rate of FXa generation was substantially less with intact than with lysed cells, the intact cells averaging ~15-20% the rate of FXa generation of lysed cells over a range of cell concentrations.

Two-stage coagulation assays for TF were also performed with intact LPS-stimulated PBMs, comparing clotting times of cells that had been preincubated with FVIIa (1 nM final concentration) for 15 min at 37°C with cells that had equivalent amounts of FVIIa added immediately before (<15 s) assay with FVII-deficient plasma. No shortening of clotting times occurred with FVIIa preincubation (77.8 ± 1.7 s and 76.8 ± 1.3 s with and without FVIIa preincubation, respectively; lysed cells with or without FVIIa preincubation had clotting times of 40 ± 2 s). These experiments, in which the TF-FVIIa complex was allowed to come to equilibrium before adding plasma, demonstrated that the rate of FVIIa binding to TF on the cell surface was not a significant variable in the differences in activity between intact and lysed cells.

**Inhibition of Total Cellular TF by Surface-bound Anti-TF mAb**

To investigate directly if the majority of TF was present intracellularly or by other means not accessible to the extracellular environment, experiments were performed in which surface-expressed TF in intact cells was blocked with inhibitory antibody to TF, and cells were then washed to remove excess unbound antibody before lysis and assay of TF activity. It was observed that TF activity measured in lysed cell preparations was reduced by >90% if surface TF was blocked by an inhibitory mAb in this manner (Table II), with comparable results for both PBMs and J82 cells.

For these experiments, intact cell suspensions were incubated with either control or anti-TF mAb for 15 min at 37°C and then washed three times to remove unbound antibody before cells were lysed and the TF activity measured. Cells remained intact (>90%) during the preincubation and washing steps as determined by trypan blue dye exclusion, and 10 mM sodium azide was present throughout these steps to prevent energy-dependent internalization of membrane proteins or externalization of intracellular TF during the experiment. (Comparable results were also obtained when the experiments were conducted at 4°C in the absence of azide; data not shown.) Mixing experiments demonstrated that the washing steps effectively removed unbound antibody before cell lysis. Under these conditions, only surface-expressed TF should have bound antibody in solution, while TF potentially present intracellularly would remain unaffected. The results therefore support the conclusion that functional TF is nearly entirely surface expressed.

**Table I. Rate of FXa Generation by Intact and Lysed Cell Suspensions of LPS-stimulated PBMs**

| PBMs   | Lysed | Intact | Intact/lysed |
|--------|-------|--------|--------------|
| $n$    | nM/min| nM/min | %            |
| $4 \times 10^7$ | 39.24 | 8.46   | 21.6         |
| $8 \times 10^6$ | 15.59 | 2.67   | 17.1         |
| $1.6 \times 10^6$ | 4.95 | 0.69   | 14.0         |
| $0.3 \times 10^6$ | 1.14 | 0.25   | 21.7         |
|        |       |        | 18.6 ± 3.7   |

*Results are from one of three representative experiments.*
Table III. Binding of Anti-TF mAb TF9-6B4 and FVII to J82 Cells

|        | \( K_d \) (pM) | Molecules/cell |
|--------|----------------|----------------|
| FVII   | 1,200 ± 520    | 91,000 ± 4,100 |
| TF9-6B4| 193 ± 15       | 89,000 ± 4,200 |

Values are final estimates ± standard error given by the LIGAND program (\( n = 4 \)).

In these experiments cells that had been similarly incubated with control or anti-TF mAb, but not subjected to lysis after washing, were also assayed for TF activity. TF activity of intact cells that had been incubated with control mAb was <10% of that measured for cells that had been lysed (for PBMs and J82 cells, respectively, TF activity of intact cells was 4.2 ± 0.5% and 4.2 ± 3.6% that of lysed cells). Also, incubation with anti-TF mAb substantially inhibited TF activity measured on cells left intact (83.2 ± 9.5% and 92.8 ± 0.9% inhibition for PBMs and J82 cells, respectively).

Determination of the Number of Cell Surface–binding Sites for FVII and Anti-TF mAb

The above experiments indicated that >90% of cellular TF was localized in the cell surface membrane where it was accessible to added antibody. One explanation for the difference in TF activity between intact and lysed cells could be that, although most of the TF is on the cell surface, many of the TF molecules on the surface of intact cells might exist in a state in which they were unable to bind FVII/FVIIa. Accordingly, the number of specific binding sites for both FVII and anti-TF mAb on J82 cells were quantitated and found to be the same (Table III). (A previous study showed that essentially all the specific binding of FVII to J82 cells is due to TF; 32.) J82 cells expressed ~90,000 sites per cell. Therefore essentially all of the surface-expressed TF molecules were competent to function as receptors for FVII/FVIIa.

Immunoelectron Microscopic Localization of TF

Supportive evidence for the surface localization of TF in cells was obtained by immunoelectron microscopic studies of both PBMs and J82 cells. Among adherent PBMs stimulated with bacterial LPS for 4 h, TF was detectable in
~10–20% of the cells and was only present on the surface membrane (Fig. 1). Endoplasmic reticulum and Golgi structures were negative in all cells examined, and no label was detected in internal membrane-bound vesicles of the cells.

When J82 cells were similarly examined, all cells were found to exhibit surface labeling and, in addition, most had label present in multivesicular bodies (Fig. 2). As in adherent PBMs, endoplasmic reticulum and Golgi structures were negative and there were only scattered small membrane-bound structures with label; these were always near the cell surface and could not be distinguished from invaginations of the surface membrane. The presence of label in the multivesicular bodies was useful to assure that antibody and reagents had adequately penetrated intracellular membrane-bound structures and, thus, that the absence of detectable TF elsewhere within the cells was not an artifact of the technique.

Discussion

Since TF expression on the cell surface in the presence of FVII or FVIIa is generally considered sufficient to initiate the coagulation protease cascade, regulation of surface expression of TF is a crucial process determining TF function. Previous studies of a variety of cell types have indicated that from 30 to >90% of the maximum observable TF activity is not functionally expressed at the cell surface, suggesting that TF might be present in a potentially mobilizable intracellular pool or, alternatively, expressed on the surface membrane in a state not accessible to FVII (5–8, 10, 24, 27, 29, 38). We have demonstrated in this study that essentially all the functional TF molecules in both LPS-stimulated monocytes and in a tumor cell line are fully expressed on the cell surface. This conclusion is supported by two independent observations: (a) >90% of lysed cell TF activity was inhibited by blocking surface TF with inhibitory anti-TF mAb before cell lysis; and (b) TF antigen is detectable on the cell surface membrane but not intracellularly by immuno-electron microscopy, except in multivesicular bodies of some cells.

We have also demonstrated that surface TF is fully accessible to FVII and that a decreased rate of association of FVIIa with TF on the cell surface cannot account for the reduced activity of TF observed in intact cells. The observation that apparently greater amounts of TF are detectable in lysed than in intact cells can be explained by reduced catalytic activity of the TF–FVIIa complex in the intact cell surface membrane relative to the environment of the lysed cell suspension.

Figure 2. Immunoperoxidase localization of TF in permeabilized J82 cells. (A, C, and D) Anti-TF mAb showing localization to plasma membrane (A) and multivesicular bodies (A, arrowhead, and C, MVB) but not the Golgi complex (D, G). Inset in A is a light micrograph of immunofluorescence localization of TF in a permeabilized J82 cell showing correspondence with electron microscopic localization. (B) Control mAb; inset shows multivesicular body at higher magnification. Bars: (A and B) 2 μm; (C, D, and B, inset) 0.25 μm.
Surface expression of TF indicates that the coagulation protease cascade will be activated when cells producing TF are accessible to FVIIa. However, the observation that the catalytic activity of the TF–FVIIa complex is submaximal in the intact cell surface membrane suggests that a significant degree of functional modulation may occur in response to changes in the cell environment. This was suggested by the earlier observations of Maynard et al. (28), that exposure of cells to proteolytic enzymes increased apparent TF activity, and by more recent studies of similar phenomena with sublethal peroxide injury to endothelial cells (47). Whether these or other physiologically relevant agents have similar effects on TF expressed by monocytes remain to be examined, as do the potential mechanisms by which this might occur. In vivo, the expression of maximal TF activity in lysed cells may also have protective hemostatic effects at sites of severe tissue injury.

The mechanism(s) responsible for the much greater catalytic activity of the TF–FVIIa complex in lysed cells remain to be determined. Studies of the catalytic activity of TF with FVIIa showing the dependence of phospholipid composition on TF activity may be relevant in this regard (35). The presence of phosphatidyl serine was associated with increased TF–FVIIa activity in vesicles. This phospholipid is unequally distributed in the plasma membrane, being mostly present on the cytoplasmic side of the phospholipid bilayer (44). Therefore, disruption of cells would be expected to increase its presence on the outer surfaces of resulting membrane vesicles. Additionally, the physicochemical effects of the dispersion of the TF–VIIa complex in small vesicles as a consequence of cell lysis could influence measurements of catalytic activity (I). The possibility of modulation by other membrane constituents must also be considered.

Functional activity of TF is dependent on its insertion in a phospholipid membrane; determination of the primary structure of TF by sequence analysis of cDNA clones has demonstrated features characteristic of a transmembrane protein (18, 31, 46, 48). Although TF may be released from certain cells in the process of membrane shedding (6, 13), a secreted form does not appear to exist. Results of this study are compatible, showing TF antigen to be present only in the surface membrane and, in J82 cells only, in multivesicular bodies. TF antigen detected in the latter site was very likely derived from internalized surface membrane and, based on the results of the functional studies, presumably represents inactive TF in the process of being degraded. Further studies will be needed to define the transit pathways of TF in cells, particularly internalization and degradation or possible recycling as described for some membrane proteins (49). However, this study has documented that relatively little (<10%) functional TF is present at sites other than the surface membrane. Although prior studies of J82 cells have shown negligible internalization of surface-bound FVII over 2 h (16), in limited experiments we were able to demonstrate some internalization over 4 h and subsequent localization to multivesicular bodies of an mAb that had previously bound surface TF (our unpublished observations). TF was not detectable by immuno-electron microscopy in endoplasmic reticulum, the Golgi apparatus, or in significant numbers of transport vesicles presumably because of the relatively small absolute amounts of TF present within cells and the lack of TF accumulation in any single intracellular compartment.

Visualization of TF antigen in multivesicular bodies of J82 cells showed that lack of detection in other intracellular sites was not due to inadequate reagent access to internal membrane-bound structures. Leoni and Dean (26) have described intracellular accumulation of TF in monocytes incubated with NH4Cl. This compound can raise intracellular pH, and the resulting interference with the activity of lysosomal proteases could slow the degradation of TF, leading to its intracellular accumulation in an active state as surface membrane is internalized.

At a physiologic level, TF initiation of coagulation is primarily controlled by selective expression of TF in cells at anatomic sites not normally in contact with blood (11). At the cellular level, the inducible intravascular cells, endothelium and monocytes, are limited in the types of stimuli capable of causing TF synthesis. In these cells primary regulation appears to be at the transcriptional level, with additional control at the level of mRNA degradation (2I). For monocytes and one tumor cell line, translocation of an intracellular pool or exposure of TF from a sequestered membrane site has been excluded as significant means of cellular modulation of functional TF expression. The current study suggests that a third means of controlling functional TF expression by cells may be modulation of the catalytic properties of TF–FVIIa at the cell surface.

The authors thank Cheng-Ming Chang, Ph.D., Electron Microscopy Laboratory, Research Institute of Scripps Clinic, La Jolla, CA, for advice and assistance in performing the immuno-electron microscopic studies.

Supported in part by National Institutes of Health grants POI HL-16411 and POI CA-41085 and by a grant-in-aid from the American Heart Association (in part by the Florida Affiliate). This work was done during the tenure of an Established Investigatorship from the American Heart Association to J. H. Morrissey. W. Ruf was supported by the Deutsche Forschungsgemeinschaft. This is publication 5686-IMM of the Research Institute of Scripps Clinic.

Received for publication 6 January 1989.

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