INITIATION OF COAGULATION BY TISSUE FACTOR

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I. INTRODUCTION

Tissue factor (TF) is a cell-surface protein which initiates coagulation; furthermore, it is probably the principal biological activator of this remarkable process. Four questions are addressed in this review:

1. What is it?
2. How does it work?
3. What is its biological significance?
4. What are the pathological consequences of aberrant TF expression?

The history of the subject is traced from the discovery of the thromboplastic activity of tissues by the 19th century physiologists[1-3] to the recent purification of the protein and cloning of the structural gene.[4-9]

Coagulation factor III, tissue thromboplastin, and TF are all used to describe this material. The first name, coagulation factor III, indicates that TF was the third component of the clotting system to be identified after fibrinogen (factor I) and prothrombin (factor II). As with the use of factor I for fibrinogen, the name is historically interesting but almost never employed. Tissue thromboplastin has been accepted as the term for the clot-promoting activity of tissues for most of this century. Its origin can be traced back to Nolf,[10] who described a "thromboplastic substance", and was later popularized in the seminal work of Howell.[11] From a review of the early work on thromboplastin by Milstone,[12] it is evident that the meaning of this term has undergone many changes over the years.

Although tissue thromboplastin was occasionally referred to as a tissue factor,[13,14] it was not until the work of Nemerson[15-19] that the term "tissue factor" was used for the protein. This transition was rooted in the concepts developed by previous investigators, particularly Howell and Chargaff. Implicit in the first efforts to purify TF by Chargaff and co-workers[20-22] is the assumption that a single protein is responsible for the thromboplastic activity. However, the failure to identify a bleeding disorder due to defective TF contributed to doubts about the single-protein hypothesis, particularly since genetic deficiencies of all the other clotting factors have been described. The view that this is a "nonspecific" property of tissues persisted until the molecule was purified. Final proof of the hypothesis was achieved in 1981 with the isolation of homogeneous bovine TF.[4] One consequence of the progress in purifying and characterizing the molecule has been a shift in terminology away from

thromboplastin to TF. This reflects the recognition that the clot-promoting activity of tissues is expressed by a unique protein.

II. STRUCTURE

A. Purification of the Protein

One of the first observations of coagulation research was the induction of clotting *in vivo* and *in vitro* by tissue homogenates.[1-3] These experiments were confirmed and extended in the early decades of this century in the first attempts to purify and characterize the procoagulant material. Most notable among this early work is a paper by W. H. Howell published in 1912.[11] He demonstrated that extraction of the active material with ether generated a lipid fraction which was partially active. On the other hand, an aqueous emulsion containing both lipid and protein was a relatively more potent initiator of clotting. Although it was not fully appreciated at the time, this simple fractionation experiment indicated that the activity was produced by a lipoprotein.

This set the stage for the next major advance in the study of TF, i.e., the proof that tissue thromboplastin requires both lipid and protein for full expression of biological activity. The papers by Chargaff and colleagues in the 1940s clearly demonstrated that tissue thromboplastin is a lipoprotein.[20-22] As already noted, they proposed that the "thromboplastic effect" was produced by a single protein and made a serious attempt to purify it, although the techniques of the time were not up to the task. Perhaps the most significant contribution of this work, at least to the subsequent efforts to purify the molecule, was the use of deoxycholate. Chargaff showed that the lipoprotein was sedimented at 31,000 g in the presence of 1 *M* NaCl or 5 *M* guanidine hydrochloride. However, when exposed to 0.012 *M* sodium deoxycholate, the active components remained in the supernatant. Furthermore, the activity was regenerated following removal of the bile salt by dialysis. This was the best evidence to date that the "thromboplastic protein" was complexed with lipid and that this association could be reversibly disrupted with deoxycholate. The ability of the detergent to solubilize TF eventually led to its successful purification. Although new detergents have been synthesized since Chargaff's initial work, largely replacing bile salts, this basic strategy has been exploited in virtually all subsequent efforts to purify TF as well as other membrane proteins.

In 1964 two important papers were published which relegated TF to the realm of obscure "artifacts" for nearly 2 decades. The proposals by Davie and Ratnoff[23] and MacFarlane[24] of their respective waterfall and cascade hypotheses brought together diverse elements of coagulation into a unified scheme. However, one component omitted from these formulations was TF. The theories dealt principally with the so-called intrinsic pathway, i.e., "contact activation" via Hageman factor and factor XI.

These postulates explained many aspects of this hemostatic process and thus quickly made the transition from hypothesis to dogma. TF was eventually added to the standard textbook diagram as the initiator of the extrinsic pathway, a second limb of the system converging with the intrinsic pathway at the activation of factor X. However, it was usually described as an auxiliary pathway without any obvious biological significance. Its inclusion was principally for the sake of describing the utility of the prothrombin time as a diagnostic tool.

In the 23 years since the publication of these seminal papers, additional information has accumulated regarding clotting factors and reactions. The original schemes of Davie and Ratnoff and of MacFarlane have undergone periodic reappraisal, sorting out the biologically significant aspects of the new data. For example, all the clotting proteins have now been purified and their structural genes cloned.[7-9,25-35] Likewise, several important reactions have been described which were not known in 1964. Also, TF, factor V, and factor VIII are now correctly identified as cofactors and not enzymes. Perhaps the most radical of these changes

is the reversal in roles for the intrinsic and extrinsic activation pathways. TF has been resurrected as the biological initiator of clotting while the significance of contact activation is now questioned.

There are at least four developments which account for the reemergence of TF. First, genetic deficiencies in the three proteins which comprise the intrinsic activation complex, i.e., Hageman factor, high molecular weight kininogen, and prekallekrein, are asymptomatic with respect to hemorrhage.[36-38] In other words, contact activation cannot be a significant biological mechanism for initiating clotting and may only operate when blood encounters an artificial surface such as the wall of a glass tube, artificial organs, heart valves, etc. The mild bleeding diathesis associated with factor XI deficiency indicates that it is involved in normal hemostasis, though the details of its contribution are as yet uncertain. Second, individuals who are severely deficient in factor VII do bleed abnormally.[39,40] Although TF deficiencies are as yet unknown, the hemorrhagic defects in factor VII deficiency constitute direct evidence for the biological significance of this mechanism for initiation. Third, in 1977 it was shown by Østerud and Rapaport[41] that, in addition to the previously described activation of factor X by TF-factor VIIa, factor IX could also be activated by this proteolytic complex. This discovery eliminated the most severe objection to the acceptance of TF as the principal biological initiator. As envisioned in the cascade and waterfall hypotheses, hemophilia A and B are exclusively diseases of the intrinsic pathway, thus giving great weight to this limb of the scheme. Showing that the extrinsic activation complex could cleave factor IX, an intrinsic factor, immediately suggested that the distinction between the pathways is arbitrary and provoked the obvious question: is hemophilia a disease of TF-initiated coagulation?[42] Finally, the purification of factor VII and TF to homogeneity[4-6,43-46] and the ensuing structure-function studies have muted the doubts regarding the existence of TF. Now that the molecules are accessible, the number of studies on the subject has risen rapidly over the last decade, converting TF from an esoteric topic to one of the more active areas of coagulation research.

During the decades of obscurity, several investigators persisted in their belief that TF is a biologically important clotting factor and continued their efforts to purify the molecule.[47-50] Following the lead of Chargaff, Nemerson[15-19] and Prydz[51-54] and their colleagues made significant progress in solubilizing the protein, separating it from lipid and other membrane proteins, and reconstituting the active lipoprotein complex with a variety of natural and synthetic phospholipids. A limited number of chromatographic techniques proved useful for isolating this membrane protein. One which was successfully employed, lectin-affinity chromatography, confirmed that the molecule is a glycoprotein,[55] as suggested by previous concanavalin A inhibition experiments.[56,57]

A major breakthrough came when it was shown by Bjørklid et al.[52] that TF could be denatured in sodium dodecyl sulfate (SDS) and recovered in an active form following renaturation and reconstitution into phospholipid vesicles. Exploiting this observation, the protein was extensively purified from human brain by polyacrylamide gel electrophoresis in SDS (SDS-PAGE). However, Bjørklid et al.[58] have recently shown that this procedure yields material which is heterogeneous. With two additional steps, they have further purified the protein ~15-fold to apparent homogeneity. The estimated 30,000- to 50,000-fold purification is in accord with the previous affinity purification data.[5,6]

The next significant advance in the purification process was the shift from bile salts to the nonionic detergent Triton X-100 for solubilizing the protein. Both the yield and stability of TF were significantly enhanced in this detergent. Differential Triton X-100 extraction was combined with lectin-affinity chromatography and preparative SDS-PAGE to purify bovine brain TF to homogeneity.[4] The protein was purified in excess of 100,000-fold, at least 50-fold greater than previous reports, yielding a polypeptide with an apparent molecular weight of 40,000 to 43,000 depending on the gel system employed.[4,59,60] Microgram quan-

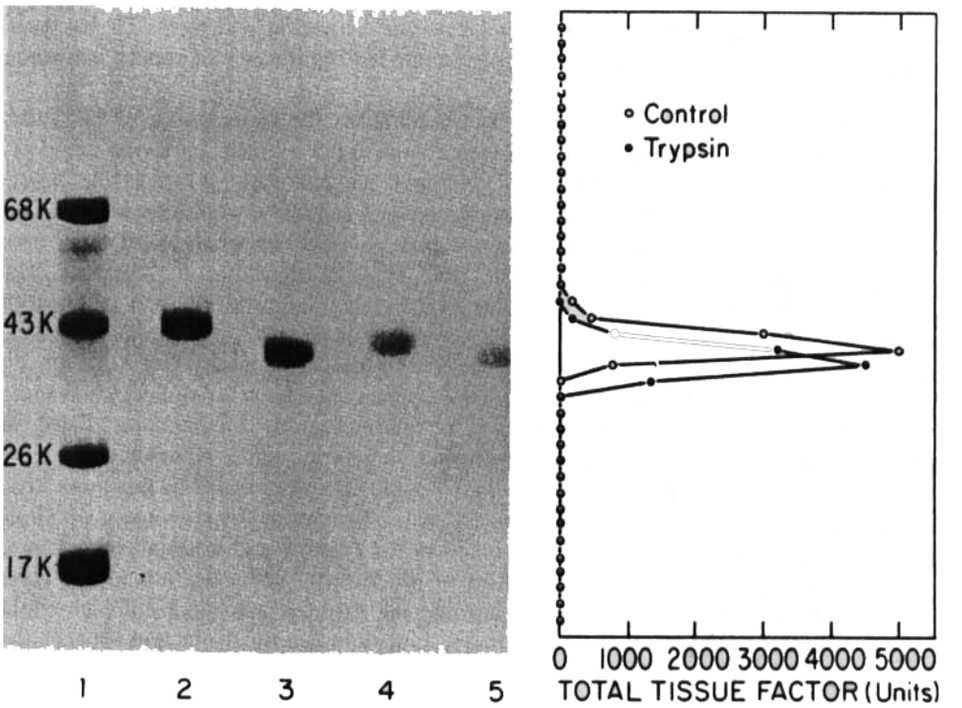

FIGURE 1. Assessment of the purity of bovine TF by digestion with trypsin and recovery of the activity from a stained SDS polyacrylamide gel. Bovine brain TF was isolated, digested with trypsin, and chromatographed by SDS-PAGE, as described by Bach et al.[4] The lanes include (1) molecular weight standards, (2) reduced bovine TF, (3) reduced bovine TF tryptic digest, (4) unreduced bovine TF, and (5) unreduced bovine TF tryptic digest. The protein was extracted from lanes 4 and 5, renatured, and reconstituted into phosphlipid vesicles. The TF activity of the material from each gel slice was determined in a two-state clotting assay. Tryptic digestion shifts the apparent molecular weight of TF from 43,000 to ~40,000. The discrete shift in the electrophorectic mobility of the protein after tryptic digestion indicates that the preparation contains a single polypeptide. The match of the stained bands and the activity profiles, both before and after digestion, demonstrates that the polypeptide is TF.

tities of this material were used to produce polyclonal antibodies. An immunoaffinity column was then constructed for purification of the protein, increasing both the speed and yield over the initial scheme. Subsequently, two monoclonal antibodies have been produced against this bovine TF by Carson et al.[60] Both of these antibodies compete with the enzyme for binding to the activator. Therefore, each presumably recognizes an epitope near the factor VII binding site. When utilized for immunoaffinity purification, an additional increase in efficiency over the polyclonal antibody columns was observed.[59,60]

The homogeneity of the resulting bovine brain TF was assessed by proteolytic digestion. As shown in Figure 1, tryptic digestion of Triton X-100-solubilized TF removes a small peptide from the protein. Release of this carboxyterminal tryptic peptide[189] did not alter the procoagulant activity of the molecule.[4] The protein and the procoagulant activity comigrated on the SDS gels before and after proteolysis. Thus, the homogeneity of the polypeptide and its identity as TF were established.

More recently, Broze et al.[5] and Guha et al.[6] have reported the isolation of homogeneous human brain TF by ligand-affinity chromatography using human factor VII coupled to agarose. The protein was extracted from brain powder with Triton X-100, essentially as previously described.[4] The procoagulant activity in the extract bound to the affinity columns in a calcium-dependent fashion and nearly homogeneous TF protein was eluted from the

matrix by chelation of the calcium. The material isolated in both reports was clearly the same protein as judged by electrophoretic mobility on SDS-PAGE, fold purification, and amino acid composition. The two papers reported different N-terminal sequences, both of which were in error and have been corrected in subsequent publications.[7,9]

A strong similarity between the bovine and human TF molecules is evident from the published data. Broze et al.[5] compared the amino acid compositions and estimated about 75% homology of the primary sequences. This has been confirmed by a direct matching of bovine TF peptide sequences (150 residues) with the entire derived human sequence. Greater than 70% homology is observed.[189] Carson et al.[61] have also produced a monoclonal antibody against human TF. This monoclonal antibody has been used to purify TF from human brain and placenta.[7,61] The molecules isolated from the two tissues are identical as judged by amino acid composition, N-terminal sequence, and specific procoagulant activity, and they also match the brain TF isolated previously.[5,6] As with the bovine purification, the antihuman TF monoclonal antibody column has greatly improved the efficiency of purification over the previous methods.

B. Isolation of the Structural Gene

The next logical step in the structural characterization of human TF has been the isolation of cDNA clones coding for the protein. Spicer et al.[7] have published the cDNA sequence of human TF, including the entire coding region as well as flanking 5′ and 3′ noncoding sequences. This coding sequence as well as portions of the flanking cDNA have been confirmed in reports by Morrissey et al.[8] and Scarpati et al.[9]

All three groups used partial-protein sequence data to verify the derived sequences. As shown in Figure 2, Spicer et al.[7] obtained 188 residues of amino acid sequence from placental TF with confirming sequence from the N terminus of brain TF. This covers 71.5% of the mature sequence; only one discrepancy between the derived and direct sequences was observed. The derived sequence predicts glu for residue 208 while the tryptic peptide obtained from placental TF yielded a gly at this position. Since the protein was isolated from the tissue of a single individual, this may be evidence of genetic polymorphism. Morrissey et al.[8] sequenced 67 residues of human brain TF, i.e., 25.4% of the total. Four discrepancies between the amino acid sequence data and the derived sequence were observed. Scarpati et al.[9] obtained 20 cycles of sequence from the N terminus of human brain TF. No conflicts with the derived sequence were observed in this 7.6% of the total protein sequence.

Two cDNAs were isolated and sequenced by Spicer et al.,[7] one of which is a full-length insert of 2147 base pairs (bp) (see Figure 2). It contains the entire coding region, 885 bp, as well as 5′ and 3′ untranslated sequences of 111 and 1145 bp, respectively, and a 6-nucleotide poly(A) tail. These clones were obtained from a human placental cDNA library and were identified by the use of synthetic oligonucleotide probes.

Morrissey et al.[8] have isolated six overlapping partial cDNA inserts from a human fibroblast (WI-38) cDNA library using both oligonucleotide and antibody probes. Analysis of these cDNAs was complicated by two apparent cloning artifacts. First, three of the inserts contained additional DNA sequences ranging from 142 to 997 bp which were not found in the other clones. This was interpreted as the product of "blunt-end concatemerization of unrelated cDNA molecules" generated during construction of the cDNA library. Second, one clone appeared to contain an unspecified 5% rearrangement. The length of the inserts used to deduce a portion of the TF cDNA sequence ranges from about 200 to 800 bp. Arrangement of these overlapping clones yielded a "consensus sequence" of 983 bp, including an open reading frame of 885 bp and 5′ and 3′ untranslated regions of 33 and 65 bp, respectively.

Scarpati et al.[9] have described the isolation and sequencing of four overlapping cDNA inserts from a human placental cDNA library. One of the clones (~1.1 kb) was obtained

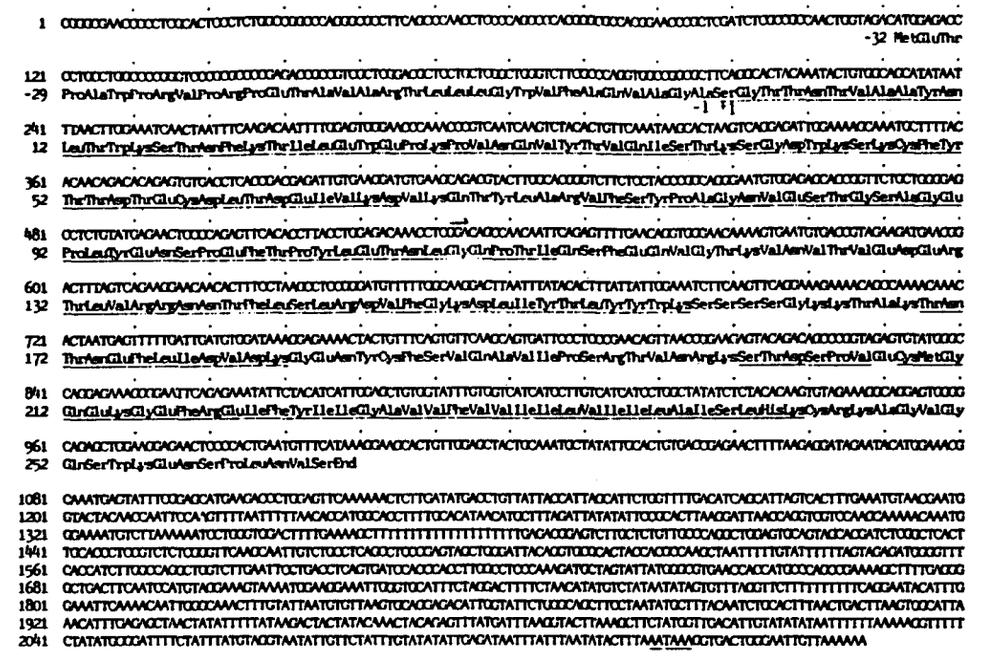

FIGURE 2. Nucleotide sequence of the cDNA containing the structural gene for human TF. The TF cDNA was sequenced by Spicer et al.[7] Nucleotides are numbered starting from the 5′ end of the full-length insert. The deduced amino acid sequence is numbered from the N terminus of the mature protein. The underlined amino acids correspond to regions of the protein sequence which were determined directly by gas-phase sequencing. The arrow indicates the boundary of the partial- and the full-length cDNA inserts which were characterized. The underlined sequence AATAAA near the 3′ end is the consensus polyadenylation signal.

using polyclonal anti-human TF antibodies as the probe. After confirming that this insert contained nucleotide sequences coding for TF protein, it was used to rescreen the library. From this secondary screening three additional inserts were characterized. These isolates range in size from about 1.1 to 1.8 kb. The cDNA sequence deduced from these clones is 2177 bp long with a coding region of 885 bp, 76 bp of 5′ untranslated sequence, 1141 bp of 3′ untranslated sequence, and a poly(A) tail of 73 nucleotides.

The three papers report an 885 bp open reading frame for the human TF structural gene. These coding sequences match precisely with one exception. The Scarpati sequence reports C instead of T at position 890 of the sequence in Figure 2. The nucleotide was observed in two independent isolates and may be another example of genetic polymorphism. It is a conservative substitution, converting Val to Ala in the hypothetical membrane-spanning domain.

The noncoding regions of the cDNA include a very G + C-rich (72%) 5′ end, nucleotides 1 to 111, and an A + T-rich (63%) 3′ untranslated domain, nucleotides 1000 to 2141. The consensus polyadenylation signal AATAAA is located near the 3′ end of the sequence and is followed 17 bases later by the poly(A) tail. As noted by Scarpati et al.[9], the 3′ untranslated region contains several additional features. Residues 1366 to about 1665 comprise an inverted Alu sequence.[62] It is flanked on either side by 11 bp palindromic direct repeats. The significance of this common repetitive sequence in the mRNA coding for human TF remains undetermined. Furthermore, the A + T-rich 3′ end of the cDNA sequence is a characteristic feature of the mRNAs coding for many inflammatory mediators and may be responsible for their rapid degradation.[63,64]

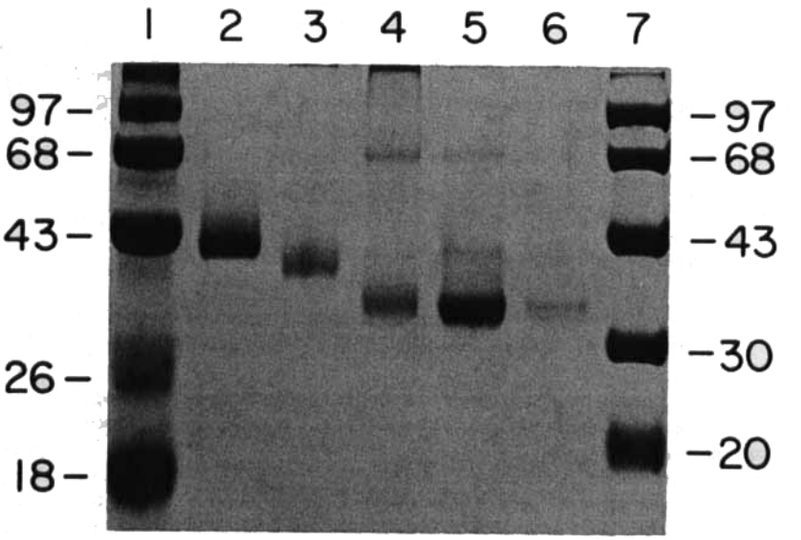

FIGURE 3. SDS-PAGE of human TF: intact, CNBr digestion, chemical deglycosylation, and enzymatic deglycosylation. Human placental TF was prepared and digested as described by Spicer et al.[7] SDS-PAGE was performed on a 7.5% polyacrylamide SDS-urea gel,[187] as indicated. The lane assignments are (1 and 7) molecular weight standards, (2) human TF {$M_r \sim 42{,}000$}, (3) human TF CNBr digest {$M_r \sim 39{,}000$}, (4) human TF trifluoromethanesulfonic acid digest {$M_r \sim 34{,}500$}, (5) human TF endoglycosidase F digest {$M_r \sim 33{,}500$}, and (6) endoglycosidase F alone.

The apparent molecular weight of human TF, as judged by SDS-PAGE, ranges from 42,000 to 46,000, depending on the gel system employed. This is considerably larger than the 29,593 Da calculated from the derived sequence. As shown in Figure 3, even after enzymatic and chemical deglycosylation, the apparent molecular weight of 33,500 to 34,500 still deviates significantly from the calculated value. Thus, either the protein binds SDS in an anomalous manner or there are as yet uncharacterized posttranslational modifications which alter its electrophoretic mobility on SDS PAGE.

The start of the coding region is predicted by the location of only one ATG codon upstream from the DNA coding for the N-terminal end of the mature protein. The open reading frame codes for a polypeptide of 295 residues including a leader sequence of 32 residues and a mature protein of 263 amino acids. Although the leader is somewhat longer than most eukaryotic signal peptides, it is otherwise typical of such domains, particularly in its high content of nonpolar residues. The N-terminal amino acid sequence of human brain and placental TF yielded two residues with each cycle.[7-9] These data can be ordered into two overlapping sequences out of phase by two residues which match the derived sequence. In other words, the purified protein is a mixture of two polypeptides, one of which begins at ser#1 and the other at thr#3. The origin of this ragged N-terminus is uncertain, but is most likely the result of alternate cleavage by the signal peptidase or perhaps a later proteolytic event.

The mature TF protein is presented schematically in Figure 4. The primary sequence suggests the following domain structure: an extracellular domain (residues 1 to 219), a membrane-spanning hydrophobic region (residues 220 to 242), and a cytoplasmic tail (residues 243 to 263). Four consensus N-linked carbohydrate attachment sites (Asn-Xaa-Ser/Thr) occur in the sequence. Three are contained in the proposed extracellular domain and two of these have been confirmed by protein sequencing as sites of posttranslational modification.[7-9] The fourth site exists within the cytoplasmic domain and as such would not be

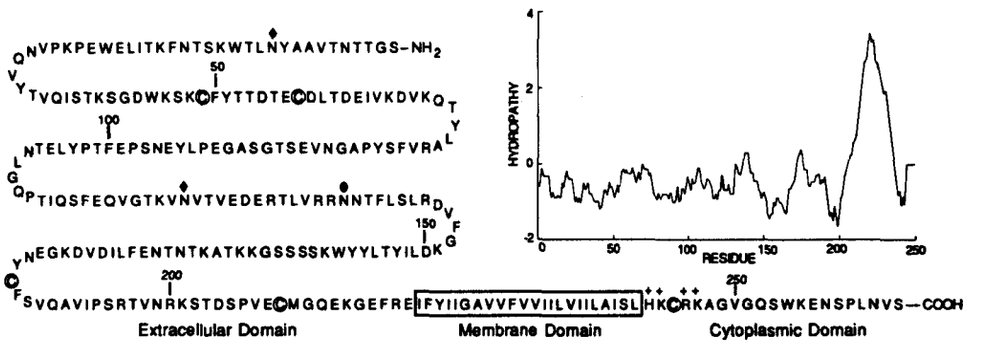

FIGURE 4. *Primary sequence, proposed domain structure, and hydropathy plot for human TF.* The amino acid sequence for the mature form of human TF is derived from the cDNA sequence shown in Figure 2. The hypothetical domain structure, as described in the text, includes an extracellular region (residues 1 to 219), a membrane-spanning domain (residues 220 to 242), and a cytoplasmic tail (residues 243 to 263). The potential N-linked glycosylation sites in the extracellular region are noted with black diamonds. The half-cystine residues are circled; the cluster of positively charged residues at the junction of the cytoplasmic domain and the membrane-spanning region is also indicated. The hydropathy plot was performed by the method of Kyte and Doolittle.[188]

conjugated. The attachment of carbohydrate to the putative extracellular domain is further evidence of the location of this portion of the molecule on the cell surface. As previously noted, the glycosylation of TF was first demonstrated by the binding of concanavalin A to the molecule.[55-57] The carbohydrate appears to be N-linked since both chemical and enzymatic deglycosylation reduced the apparent molecular weight of human TF by 7500 to 8500 (see Figure 3). Removal of the carbohydrate from human TF by endoglycosidase F has no measurable effect on its procoagulant properties as measured in a clotting assay, indicating that this posttranslational modification must be required for something other than expression of biological activity.[189]

Four half-cystine residues occur in the extracellular region, probably in the form of disulfides, while a single half-cystine is found in the intracellular domain. The evidence for disulfide bonds within the molecule comes from experiments showing irreversible loss of activity when the bovine protein was exposed to SDS in the presence of 2-mercaptoethanol. The denaturation was reversible if the reducing agent was omitted.[4] The cytoplasmic half-cystine, cys#245, is presumably sequestered on the inner side of the plasma membrane and thus is unavailable for the formation of an intramolecular disulfide bond. This has been confirmed by cyanogen bromide cleavage of the polypeptide at met#210 where the carboxyterminal peptide, residues 211 to 263, is released from the rest of the molecule without reduction.[7]

The status of cys#245 is yet to be determined. The reducing environment within the cell would not favor the formation of a disulfide bond. However, thioester linkages between intracellular half-cystines and palmitic acid have been reported on several integral membrane proteins.[65-70] Purified preparations of TF, both bovine and human, appear to contain a small amount of dimerized molecules, though most of the protein exists as monomers.[4-6,59-61] Some of this dimeric material can be reduced to monomers with 2-mercaptoethanol. Hence, it is probably a disulfide conjugate linked through cys#245. A portion of the dimers cannot be dissociated by reducing agents. They may be the product of peroxide-catalyzed cross-linking as a result of solubilizing the protein in a polyoxyethylene detergent such as Triton X-100.[71] Additional nonreducible cross-linking of the bovine molecule was observed when it was oxidized and then radiolabeled by reduction with tritiated sodium borohydride.[59]

Noncovalent TF dimerization was suggested by factor VII binding studies where positive cooperativity was observed in the interaction of enzyme and activator.[59] The appearance of

covalent dimers may be related to the apparent ability of TF to self-associate in a reversible fashion. These covalent complexes, in particular the proposed disulfide linkage through cys#245, are probably purification artifacts in that they are unlikely to occur in an intact cell where the intracellular glutathione would prevent oxidation of this residue to a disulfide. Following cellular disruption, the reduced half-cystine might then participate in disulfide-linked homodimer or heterodimer formation. In fact, in addition to the homodimers just described, an apparently unique heterodimer of TF and a 13-kDa polypeptide has been observed by others in some preparations of human TF.[8,72] It should be noted, however, that the heterodimer is elusive and has not been observed in most preparations of human TF.[4-7,59-61] This disulfide linkage appears to occur via cys#245.[190]

The proposed membrane-spanning region, residues 220 to 242, is a run of 23 nonpolar amino acids. The hydrophobic character of this region is graphically illustrated by the hydropathy plot in Figure 4. With respect to hydrophobicity and length, this is a classic membrane anchor and probably exists as an α-helix within the core of the plasma membrane.

The location of residues 243 to 263 on the cytoplasmic side of the membrane is hypothetical but highly probable. If residues 1 to 219 are on the cell surface as suggested by glycosylation and if residues 220 to 242 constitute the sole membrane-spanning region as suggested by the hydropathy plot, then the topology of the molecule is constrained and the carboxyterminus must form a cytoplasmic tail. This interpretation is supported by the cluster of four positively charged residues at the interface between the cytoplasmic region and the membrane domain, a common feature of many integral membrane proteins.[73] One proposed role of these charged amino acid side chains is association with acidic phospholipid head groups which are sequestered on the inner leaflet of the plasma membrane. The cytoplasmic tail of TF resembles the same regions of thrombomodulin[74,75] and low density lipoprotein (LDL) receptor[76] with respect to length, clustering of positive charges, and the presence of a single half-cystine residue. A function for this domain similar to the direct involvement of the LDL receptor cytoplasmic tail in receptor internalization[77] is purely speculative. Likewise, the role of cys#245 as a site of covalent modification *in vivo* or *in vitro* remains largely unexplored.

Since at least a portion of this carboxyterminal tail can be removed from the bovine molecule without any apparent effect on its biological activity,[4] the functional significance of this region is uncertain. Now that the structural gene has been cloned, site-specific mutagenesis of the cytoplasmic domain followed by expression in an appropriate eukaryotic cell is one obvious approach. This is analagous to the study of natural mutations in the cytoplasmic region of the LDL receptor which have a demonstrable effect on receptor function.[77] Modifying cys#245, for example, may directly answer questions concerning the role of this residue in covalent modifications of TF.

The location of the human gene was mapped by Carson et al.[78] This was achieved by a novel mouse-human somatic cell hybridization experiment which took advantage of species specificity; mouse TF does not clot human or bovine plasmas.[79-82] The gene was mapped to the short arm of human chromosome 1 (1pter-1p21). This was interpreted as evidence for a single TF gene in the human genome. The result has been confirmed by Scarpati et al.,[9] using a portion of the isolated cDNA as the probe in a chromosome-sorting experiment.

Morrissey et al.[8] have employed a cDNA probe in Southern blot analysis of human genomic DNA. From this restriction mapping, the size of the gene was estimated at about 9.4 kb and appears to contain at least two introns. By Northern blot analysis, the message for human TF was estimated at 2.2 to 2.3 kb.[8,9] This is consistent with the size of the full-length cDNA isolated by Spicer et al.[7]

The primary sequence of human TF was examined for homology against 4668 sequences in the protein sequence data base of the National Biomedical Research Foundation, as well as several procoagulant and anticoagulant proteins not yet in the data base.[7] No significant homologies were observed, suggesting an evolutionary origin for TF separate from that of

other coagulation factors. This is consistent with its unique role as the initiator of a proteolytic cascade.

III. FUNCTION

A. Unique Features of TF-Initiated Coagulation

The controlled expression of proteolytic activity is an essential feature of coagulation. Several mechanisms have evolved for modulating the enzymatic response to specific stimuli. The most universal of these is partial proteolysis, converting an inactive zymogen to an active enzyme. The proteolytic activations of trypsinogen to trypsin and chymotrypsinogen to chymotrypsin are classic examples of this process and are probably evolutionary precursors to the cascade of vitamin K-dependent serine proteases in clotting. A second level of regulation not employed by the digestive enzymes but essential for normal clotting involves the use of cofactors. The binding of TF, factor V, and factor VIII to their respective enzymes in 1:1 stoichiometric complexes is required for biological activity. In addition, factors V and VIII must be activated by partial proteolysis. On the other hand, the protein and cDNA sequence data in conjunction with structure-function studies of the pure protein suggest that TF is not activated or inactivated by hydrolysis of peptide bonds.

As already noted, the primary structure of TF is unrelated to that of factors V and VIII. Two additional features of TF which distinguish it from the other clotting cofactors include localization on cell surfaces that do not normally come in contact with the bloodstream and the expression of biological activity by the TF-factor VII complex without a prior proteolytic event. Thus, the physical barrier of the intact endothelium may be the first line of defense against uncontrolled TF-initiated coagulation. The activation of a serine protease zymogen by a protein cofactor without cleavage of either component is unusual but not unprecedented. For example, plasminogen and streptokinase form a complex with proteolytic activity capable of initiating clot lysis.[83]

Not only must the clotting system recognize the signals generated by bleeding, but the magnitude of the response must also be proportional to the extent of the injury. A quantitative description of how this coupling is achieved is one of the major goals of coagulation research. The obviousness of this tight regulation is obscured by the fact that the system works so well for the entire lifespan of most humans. We neither bleed to death as a consequence of shaving while still half asleep, nor do we clot solid from a pin prink. The contributions which TF makes to the normal operation of this exquisite process and the ways that it may be responsible for malfunctions of the system are explored in the following two sections. First, a picture of the reactions which constitute TF-initiated coagulation is constructed. The information required to resolve these events at the molecular level comes from *in vitro* experiments utilizing the purified components of the system. Finally, the evidence for the participation of TF in biological and pathological clotting is reviewed. In particular, the mechanisms which may regulate TF expression on cell surfaces are considered.

B. Proteolytic Activity of the Zymogen (Factor VII)

Factor VII, the plasma-derived component of the initiation complex, is a vitamin K-dependent serine protease. It has been purified from both bovine and human plasma.[43-46] Like TF, it is a rare protein and must be purified 100,000-fold or greater to achieve homogeneity. The circulating concentration of this protein, as judged by methods which are independent of the ambiguities inherent in functional assays, is ~13 n*M* in bovine plasma[84] and ~10 n*M* in human plasma.[85]

Conversion of the molecule from the one-chain factor VII (zymogen) to the two-chain factor VIIa (enzyme) may be catalyzed by thrombin, factor IXa, factor Xa, and factor XIIa[43,86-88] and it is generally accepted that factor Xa is the most potent activator. It has

also been shown that cleavage of the zymogen by factor Xa is accelerated by complexation with TF.[89] The activation of both human and bovine factor VII is achieved by hydrolysis of an Arg-Ile bond, residues 152 and 153 in the human protein.[31,86-88]

As previously noted, one-chain factor VII is an active protease when complexed with TF. The evidence for this conclusion is found principally in a paper by Zur et al.[90] One of the first suggestions that this zymogen might in fact be a protease came from experiments showing that the esterase activity of bovine factor VII and its rate of incorporation of diisopropyl fluorophosphate (DFP) differ from that of its two-chain counterpart by only two- and fourfold, respectively. This indicates that the zymogen has a functional catalytic center. However, these reactions with small molecules do not necessarily correlate with coagulant activity. It remains to be shown if factor VII, when complexed to TF, will cleave its natural protein susbtrates, factors IX and X.

Comparison of factor VII and factor VIIa in coagulation assays yielded a specific activity for the zymogen of 1 to 2% of the value measured for the two-chain molecule. The problem with these experiments is distinguishing between fractional activity inherent in a zymogen and trace contamination by its more active two-chain derivative. Zur et al.[90] established the activity of this zymogen by combining DFP inhibition with coagulation assays. The pseudo first-order rate constants for the incorporation of DFP into factors VII and VIIa were determined using ^{3}H-DFP. This analysis is insensitive to trace contamination of the zymogen by the enzyme. Thus, an accurate estimate of 0.032 and 0.130 min^{-1} for factor VII and VIIa, respectively, was obtained for 2 m*M* DFP. Next, the decay of coagulant activity for the one- and two-chain molecules was measured following DFP treatment. The activity of factor VIIa was inhibited by DFP at a rate of 0.127 min^{-1} in accord with the first determination. However, the disappearance of the coagulant activity in several preparations of factor VII was curvilinear on a first-order plot. This was interpreted as evidence for trace contamination of the zymogen by the enzyme with both forms of the molecule contributing to the observed activity. The degree of factor VIIa contamination was calculated by fitting the following double exponential equation to the DFP inhibition data:

$$y = C_1e^{-k_1t} + C_2e^{-k_2t} \quad (1)$$

where y is the measurable activity at time t, C_1 and C_2 are coefficients which describe the fractional contributions of zymogen and enzyme to the activity, and k_1 and k_2 are the previously estimated rate constants for DFP incorporation into factor VII and factor VIIa. A 1.5% contamination of factor VII by factor VIIa was estimated. From this analysis it was also calculated that the specific coagulant activity of the zymogen is 0.8% of that for the enzyme. In other words, conversion from the one-chain molecule to the two-chain form would produce a 123-fold increase in activity.

This conclusion was tested further by measuring the activation of factor VII, i.e., the increase in coagulant activity after conversion to factor VIIa as a function of time after addition of DFP. At each time point, DFP incorporation was stopped by dilution and the remaining zymogen was converted to factor VIIa by catalytic amounts of factor Xa in the presence of phospholipid and calcium. Coagulant activity was measured before and after activation and rose from an initial 37-fold to a plateau of about 110- to 130-fold after 40 min. The rate of decay of activity after 40 min was 0.034 min^{-1}, in excellent agreement with the previously determined rate of DFP incorporation into the zymogen. Likewise, after 40 min of exposure to DFP, the increase in coagulant activity following activation with factor Xa matched the previously calculated value. This experiment was technically possible because, even though both species are being inactivated, factor VIIa activity disappears more rapidly than factor VII activity. Thus, at 40 min only 0.55% of the initial factor VIIa will remain while 26.7% of the factor VII will still be available. Subsequently, it was shown

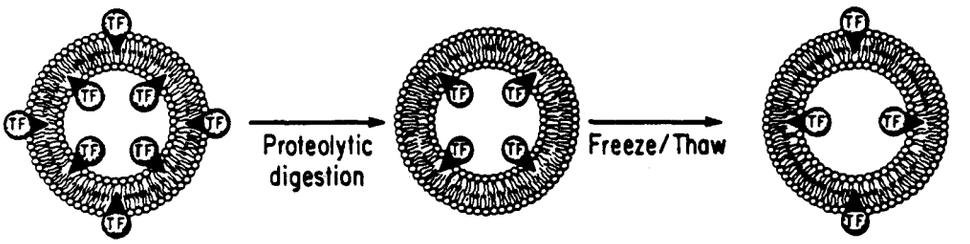

FIGURE 5. Schematic summary of protease digestion experiments used to discern the random orientation of TF in reconstituted phospholipid vesicles. Bovine TF was incorporated in phospholipid vesicles utilizing octyl glucoside dialysis, as described by Bach et al.[59] Digestion with subtilisin and freeze/thaw reorientation were combined, as described in the text, to demonstrate that the protein in these vesicles was randomly oriented. Following proteolysis, the undigested protein (50% of the initial activator) was sequestered on the inner face of the membrane. After freezing and thawing, half the remaining material again was available on the vesicle surface for interaction with subtilisin or factor VII.

that rapid immunoaffinity purification of bovine factor VII yielded zymogen preparations which could be activated 120-fold and thus appeared to be essentially free of the two-chain species.[84]

C. Activator-Enzyme Binding

The stoichiometric association of TF and factor VII/VIIa is an essential component of the model for the mechanism of cofactor action which we are constructing. The existence of this complex was suggested by the zymogen work just discussed in which it was shown that DFP-inactivated factor VII and VIIa are effective inhibitors of TF. In other words, they apparently compete with factor VIIa for binding to the activator and thereby block formation of the proteolytic complex. The binding of bovine TF and various forms of the enzyme has subsequently been studied directly.[59] The technique employed to measure the association of the activator and enzyme at equilibrium utilized ultracentrifugation to separate bound and free enzyme and liquid scintillation counting to quantify the concentrations of radiolabeled protein in these two pools.

One essential feature of this binding assay was the incorporation of TF in large phospholipid vesicles of defined structure and composition. Reconstitution of the pure activator was achieved by replacing the previously developed methods employing deoxycholate[4,91] with octylglucoside dialysis.[59] The advantage of this synthetic, dialyzable, nonionic detergent is that it generates large unilamellar phospholipid vesicles regardless of the ratio of phosphatidylcholine (PC) and phosphatidylserine (PS) in the membrane. The phospholipid and protein composition of these reconstituted vesicles was determined by the use of tracer amounts of ^{14}C-PC and ^{3}H-TF. The size of these TF-containing vesicles was estimated on a calibrated gel filtration column. The preparations were principally composed of vesicles with diameters greater than 100 nm for PS/PC ratios ranging from 0:100 to 40:60, and each vesicle contained on average only one molecule of TF. The requirement for large vesicles was imposed by the ultracentrifuge binding assay. Rapid and efficient separation of factor VII free in solution from the enzyme bound to TF on the vesicle surface could only be achieved with this material.

For use in direct binding studies, one additional feature of these vesicles had to be characterized, namely, the orientation of the protein in the lipid bilayer. A TF molecule with the factor VII binding site inside a vesicle is obviously unavailable to the enzyme. Thus, the inside-outside distribution of the cofactor must be known in order to calculate the stoichiometry of the activator-enzyme complex from binding isotherms. The orientation of reconstituted TF was determined by the protease digestion experiment summarized in Figure 5. Briefly, the protein on the vesicle surface was removed by digestion with subtilisin. The

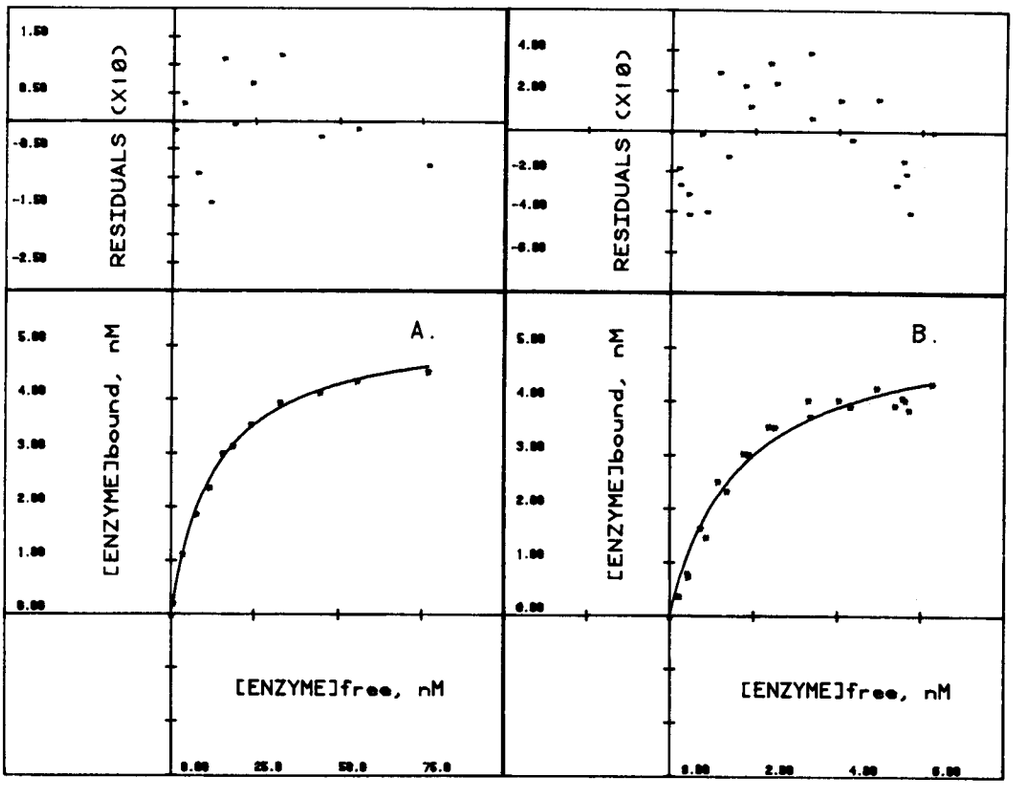

FIGURE 6. Binding of factor VII to TF in (A) uncharged PC vesicles and (B) charged PS/PC (30:70) vesicles. The association of activator and enzyme was measured as described by Bach et al.[59] The Langmuir single-site binding model was fit to the data. As indicated by the residuals, the binding in panel A is well described by this model, whereas the data in panel B show a systematic deviation. The sigmoidal shape of the second isotherm was well described by a two-site cooperative binding model.

progress of the reaction was monitored by both the release of trichloroacetic acid (TCA)-soluble tritium from radiolabeled TF and the disappearance of procoagulant activity. After one cycle of digestion, half the radioactivity was liberated while the biological activity was completely obliterated. The next step was to assess the status of the undigested protein. This was achieved by simply freezing and thawing the vesicles several times. The effect was a random reorientation of the sequestered activator; half the remaining material was again avialable for proteolytic degradation and interaction with factor VII. The exponential decay of intact protein (i.e., TCA-precipitable tritium) and sequestered procoagulant activity, through five cycles of proteolysis and reorientation, is fully consistent with a randomly oriented TF. Thus, in these vesicles the effective concentration of activator is 50% of the total.

The association of activator and enzyme was determined for TF in vesicles ranging from 100% PC to a PS/PC ratio of 40:60. Four forms of the enzyme were utilized: factor VII and factor VIIa, as well as their DFP-modified counterparts DIP-factor VII and DIP-factor VIIa. In all cases the binding isotherms yielded a 1:1 stoichiometry for the activator-enzyme complex. For TF in PC vesicles, the binding of all four forms of the enzyme was well described by a rectangular hyperbola. However, when acidic vesicles were employed, there was a systematic deviation of the data from the calculated best fit. The difference in the shape of the isotherms for enzyme binding to the activator in uncharged vs. acidic vesicles is illustrated in Figure 6. The sigmoidal shape evident in the second curve is a characteristic

Table 1
SYSTEMATIC DECREASE IN THE $K_{1/2}$ FOR ACTIVATOR-ENZYME BINDING WITH INCREASING VESICLE CHARGE[a]

	$K_{1/2}$ (n*M*)			
PS:PC ratio	Factor VII	Factor VIIa	DIP-factor VII	DIP-factor VIIa
0:100	12.2	4.27	5.68	3.25
5:95	6.19	4.84	4.05	2.75
10:90	6.67	4.34	5.06	2.06
20:80	2.12	3.44	3.66	1.77
30:70	1.10	2.23	2.16	1.45
40:60	0.66	1.69	0.91	1.76

[a] $K_{1/2}$ is the concentration of enzyme required to half-saturate the activator.

From Bach, R., Gentry, R., and Nemerson, Y., *Biochemistry*, 25, 4007, 1986. With permission.

of positive cooperativity. This complex binding was well described by a two-site cooperative-binding model in which occupancy of the first site increased the affinity of a second site for the ligand.

In addition to altering the mode of binding, PS apparently increases the affinity of the activator for the enzyme. This is best illustrated by calculating the $K_{1/2}$, the concentration of enzyme required to half saturate the TF sites. As shown in Table 1, the $K_{1/2}$ decreases with increasing PS for all forms of the enzyme. The binding of DIP-factor VII was cooperative only in the most acidic vesicles (PS/PC,40:60). Nevertheless, the $K_{1/2}$ decreased progressively with the addition of acidic phospholipid. Thus, the effect of the acidic lipid on the affinity is due only in part to the induced cooperativity. Also, a coordinate complex of enzyme, TF, and PS cannot be invoked to explain the increased affinity. The K_d for binding of the enzyme to acidic phospholipid vesicles is micromolar, while the K_d for activator-factor VII binding in PC vesicles is approximately 10^{-8} *M*. The standard Gibbs free energy change for these two events is about -8.2 and -10.9 kcal mol^{-1}, respectively. Hence, at a minimum, the free energy change resulting from ternary complex formation would be the sum of these two values, i.e., -19.1 kcal mol^{-1}. This translates to a K_d for the hypothetical ternary complex of about 10^{-14} *M*, while the observed K_d for activator-enzyme binding in the most acidic vesicles is only 10^{-9} *M*. Obviously, the free energy change predicted by the coordinate binding model does not fit the experimentally observed values.

In addition to establishing the 1:1 stoichiometry of the activator-enzyme complex, this binding study has proven several points which may be important for understanding how the biological activity is expressed. The binding of the zymogen is obviously required for its participation in clotting. Both factor VII and factor VIIa bind to the receptor with similar affinites: 13.2 and 4.54 n*M*, respectively, with TF in PC vesicles. This confirms and extends the work of Broze,[92] who demonstrated the binding of human factor VII and VIIa to human monocytes. Blocking of the enzyme active site by reaction with DFP did not interfere with the binding. On the contrary, the affinity was enhanced for both DIP-factor VII and DIP-factor VIIa (Table 1). This is consistent with the kinetic evidence to be examined next, that substrate binding to the activator-enzyme complex increases its stability. Finally, the addition of acidic phospholipid to the vesicles altered both the mode of binding and the strength of the association. Cooperativity in the binding of human factor VII to cell-surface TF in a human carcinoma cell line has been reported by Fair and MacDonald[93] and was suggested

in the binding study of Broze.[92] The positive cooperativity is evidence for dimeric TF in the PS/PC vesicles and on cell surfaces. The biological significance of the modulation of activator function by PS is undetermined, but it represents one potential cellular mechanism of regulation.

D. A Kinetic Model of TF

A kinetic model for TF-initiated coagulation has been developed by Nemerson and Gentry.[94] The radiometric assay used in these experiments was first developed for measuring the rate of hydrolysis of factor X[95] and has subsequently been applied to factor IX activation.[96] The assay requires that the sialic acid residues on the carbohydrate side chains of these glycoproteins be radiolabeled. This was done by oxidizing the carbohydrate with sodium periodate and then reducing it with tritiated sodium borohydride. The chemical modification does not alter the properties of the molecules either as substrates or as enzymes. Since a significant fraction of the carbohydrate on each protein is coupled to the small peptide released during proteolytic activation, the rate of hydrolysis can be monitored in a discontinuous fashion by simply measuring the appearance of TCA-soluble tritium. The assay is very sensitive, relatively simple to perform, and directly measures substrate cleavage. The radiometric assay has been successfully employed in several laboratories for measuring the kinetics of TF-mediated reactions with pure preparations of enzyme and activator, as well as crude material, e.g., plasma, cell extracts, etc.[95-98]

The experimental evidence and mathematical modeling which have gone into the construction of this kinetic model are complex and, to be fully appreciated, should be read in their original form. However, a brief overview is useful, if for no other reason than to stimulate the reader's interest sufficiently to examine the work in more detail. The experiments were performed with bovine activator, enzyme, and substrate since they had all been purified to homogeneity and well characterized. In addition to radiolabeling of the substrates, factor VII was activated to factor VIIa, and TF was reconstituted in PC vesicles. This choice of materials is important from the point of view of model building since it minimizes the number of species and reactions. In particular, with TF in uncharged vesicles, the cooperative activator-enzyme interaction, as well as binding of enzyme and substrate to the phospholipid, are excluded.

The first conclusion reached is that TF is an essential activator. The basis for this assertion is that in the activation of factors IX and X by TF-factor VIIa, no hydrolysis of the substrate is observed in the absence of either factor VIIa or TF. Furthermore, the catalytic activity in the system is limited by the concentration of both components of the proteolytic complex. This was demonstrated experimentally by fixing the concentration of either the activator or the enzyme and titrating the system with the other species. The reactions were performed with both 50 and 2500 n*M* factor X as the substrate and 0.075 n*M* activator or enzyme. The initial velocity of factor X hydrolysis in these titrations increased hyperbolically as a function of increasing activator/enzyme from zero in the absence of the reciprocal ligand to the same value at the plateau. The midpoint of the titration curves was significantly lower in the reaction performed at high substrate. This observation is consistent with the ordered assembly, where the increased substrate concentration would facilitate the formation of the catalytic complex.

This experiment suggests that the product-forming species in the reaction is a complex between the activator (A), the enzyme (E), and the substrate (S). One way that such a catalytic complex could form is by an ordered assembly:

$$A + E \underset{}{\overset{K1}{\leftrightarrow}} AE + S \overset{K2}{\leftrightarrow} AES \overset{kcat}{\rightarrow} AE + P \qquad (2)$$

where K1 and K2 are dissociation constants, kcat is the catalytic rate constant, and P is the product (factor IXa or factor Xa). AES could arise by other pathways involving ES or AS, but additional experiments indicated that the reaction is fully ordered with AE and AES as the only significant intermediates. Furthermore, the release of the product in the catalytic step could be accompanied by dissociation of the proteolytic complex into A + E. From mathematical modeling of these two alternatives, the dissociation of AE with each catalytic cycle did not agree with the experimental observations and was rejected.[99]

Experiments were designed to test this fully ordered essential-activation model of TF-initiated coagulation. The principal objective was to show that AE and AES are the only significant intermediates along the path to product formation. A monoclonal antibody against TF[60] was used to perturb the system. As previously noted, this antibody competes with the enzyme for activator binding and thereby inhibits catalysis. For the ordered-addition model, this antibody (Ab) inhibition may be diagramed as follows:

$$\begin{array}{lc} & \overset{K1}{\leftrightarrow}\ \overset{K2}{\leftrightarrow}\ \overset{kcat}{\rightarrow} \\ A + E \leftrightarrow AE + S \leftrightarrow AES \rightarrow AE + P \\ + & \\ Ab & \\ \alpha K1 \updownarrow & \\ AbA & \end{array} \qquad (3)$$

where αK1 is the dissociation constant describing the antibody-TF complexation. One intuitively obvious aspect of this type of inhibition is that it can be relieved by adding more substrate to the reaction. The "substrate tug" on the network would increase the concentration of the catalytic complex AES at the expense of the inhibitory complex AbA. The hypothesized kinetic effect of the inhibitor was demonstrated experimentally by substrate titrations performed in the presence and absence of the antibody. These reactions were performed with the activator as the limiting species. The inhibitor had no effect on the Vmax, but it increased the Km (app) for factor X hydrolysis more than 20-fold. These antibody-inhibition data are consistent with the ordered assembly of reactants.

A second inhibitor, DIP-factor VIIa, was also used to perturb the system. Inhibition with the chemically modified enzyme was more complex than that observed with the antibody. An order-of-addition effect was observed which produced either a decreased Vmax or an increased Km (app). When the inhibitor was added to the system prior to the substrate, the Vmax was decreased while the Km (app) remained unchanged. If the DIP-enzyme was added after initiating the reaction with substrate, then the Vmax was unaffected while the Km (app) increased. A model which accounts for these observations is as follows:

$$\begin{array}{lc} & \overset{K1}{\leftrightarrow}\ \overset{K2}{\leftrightarrow}\ \overset{kcat}{\rightarrow} \\ A + E \leftrightarrow AE + S \leftrightarrow AES \rightarrow AE + P \\ + & \\ I & \\ \alpha K1 \updownarrow & \\ AI + S \leftrightarrow AIS & \\ \beta K2 & \end{array} \qquad (4)$$

where I is DIP-factor VIIa and α and β are coefficients relating the additional dissociation constants to those described in the initial model. As with the antibody-inhibition experiments, the substrate titrations in the presence of DIP-enzyme were performed with TF limiting. The difference between DIP-factor VIIa and antibody inhibition is the hypothesized substrate

binding of AI. If this did not occur, the two inhibitors would be kinetically indistinguishable. Both would have an effect solely on the Km (app) which, as noted, is not the case for the DIP-enzyme.

This model predicts that if all components of the system are present initially, then the assembly of the various intermediates will proceed in accord with the kinetic and equilibrium constants describing the system. On the other hand, if TF is limiting and inhibitor is added after the activator has already formed AE and AES complexes, particularly at high substrate concentrations, then the free A available for the formation of AI and AIS will be significantly reduced. The first case could result in a decrease in the Vmax, while the second might produce an increase principally in the Km (app). Also, the observed kinetic effects of DIP-factor VIIa inhibition indicate that $K1K2 = \alpha K1\ \beta K2$. Generalizations regarding this model must be qualified, however, because the precise kinetic effects observed will depend on the relative concentrations of the components and on the constants which define the network. Mixed Km (app) and Vmax effects may be observed under the appropriate conditions.

In addition to these tests of the fully ordered essential-activation model, the quantitative aspects of the hypothesis were explored. The equations describing the model, using either rapid-equilibrium or steady-state assumptions, were derived. These equations were fit to a large set of initial-velocity data collected at varying enzyme, activator, and substrate concentrations. The adjustable parameters calculated by these two strategies were identical. However, the kinetically determined value for K1, the dissociation constant describing the binding of activator and enzyme, was substantially smaller than the direct estimate from the equilibrium-binding experiment,[59] 0.09 vs. 4.5 n*M*. This discrepancy forced a further evaluation of the characteristics of the kinetic model. Fully random, as well as partially random, models for AES formation were tested and summarily rejected as having properties inconsistent with the observed behavior of the system.

To resolve the difference between the calculated and measured values for K1, an alternative form of the ordered-addition essential-activation model was developed. It was noted that activator and enzyme do not appear to dissociate while residing in the ternary AES complex, i.e., the reaction is ordered. This suggests that the binding of substrate to AE may alter the structure of the complex and tighten the association of activator and enzyme. An additional form of the proteolytic complex, designated AE*, was incorporated into the following kinetic model:

$$\begin{array}{ccccccc} & & \overset{K1}{} & & \overset{K2}{} & & \\ A & + & E & \leftrightarrow & AE + S & \leftrightarrow & AES \\ & & \overset{K5}{\updownarrow} & & & & \overset{K3}{\updownarrow} \\ & & AE^* & + & S & \leftrightarrow & AE^*S \rightarrow AE^* + P \\ & & & & \underset{K4}{} & & \underset{kcat}{} \end{array} \qquad (5)$$

where K1, K2, and K4 are dissociation constants, K3 is the equilibrium-conformational constant for the transition from AES to AE*S, K5 is the equilibrium constant describing the transition from AE* to AE, and kcat is again the catalytic rate constant. In this model, it is the persistence of AE*, the essentially nondissociable form of the complex, which accounts for the disagreement between the kinetically determined value and equilibrium-binding estimate for the dissociation constant describing the complexation of TF and factor VIIa.[100]

The important features of this kinetic model include two conformational changes. In the first, the binding of enzyme and activator produces an enzymatic complex. The structural changes which occur as a result of this association are not known, but it is abundantly clear that TF-factor VIIa binds and hydrolyzes the substrates while the individual components do not. A plausible interpretation of this essential activation is that the cofactor induces a

conformational change in the enzyme creating the sites for binding and cleavage of factors IX and X. The second transition proposed in this model is induced as a consequence of substrate binding. The conformational change in the ternary complex from AES to AE*S tightens the association of activator and enzyme and traps the enzyme in the catalytic complex. This "conformational cage" was suggested by the kinetic and equilibrium analyses of this process. From a biological viewpoint, it is an efficient design which maintains the catalytic complex until the substrate is depleted.

E. Acceleration of Clotting Reactions by PS

The coagulation reactions involving the cofactors (TF, factor V, and factor VIII) are accelerated by acidic phospholipids such as PS.[101] The vitamin K-dependent clotting factors, the enzymes and substrates for these cofactor-mediated reactions, bind to the charged vesicles. Likewise, factors V and VIII will bind to the acidic phospholipid surface. On the other hand, TF is always anchored in the membrane, regardless of the phospholipid composition. The role that substrate binding plays in the accelerating effect of charged surfaces in the TF system was recently examined by Forman and Nemerson.[102] One hypothesis is that this binding concentrates the substrate on the membrane surface in the vicinity of the bound activator-enzyme complexes and thereby increases the rate of catalysis.[103]

Since the concentration of the substrate is an essential parameter in any kinetic analysis, the partitioning of the substrate between the solution and the membrane surface is a serious complication for kinetic studies of clotting reactions. If charged vesicles are employed, then what is the true substrate concentration? Is it the concentration in the bulk phase or is it the concentration on the vesicle surface which drives the reaction? This obstacle was avoided in the kinetic model of TF by using activator incorporated in neutral PC vesicles. While PC supports TF-initiated coagulation, it does not work for the other cofactor. Thus, this strategy is not useful for the kinetic analysis of factor V- and factor VIII-mediated reactions.

The thesis tested by Forman and Nemerson is that the rate of hydrolysis of factor X by TF-factor VIIa in PS/PC vesicles is determined by the concentration of either bound or free substrate. Since the substrate is in equilibrium between these two pools, perturbing the concentration of factor X in one alters its concentration in the other. The equilibrium was successfully "uncoupled" in this work by the use of a competing ligand. Prothrombin fragment 1 was employed to displace the substrate from the vesicle surface, thereby decreasing the concentration of bound factor X and at the same time increasing the free factor X concentration. The ligand was shown to be kinetically neutral, i.e., it did not interfere with the hydrolysis of the substrate by the proteolytic complex formed on uncharged vesicles. However, when the TF was reconstituted in PS/PC vesicles, the addition of prothrombin fragment 1 had a dramatic effect on the rate of factor X activation. In all cases the displacement of bound factor X actually increased the rate of the reaction. Thus, it was concluded that the substrate hydrolyzed by TF-factor VIIa is the free factor X and the membrane-bound factor X does not contribute significantly to the formation of the catalytic complex. Obviously, this result contradicts the prevailing view of the role of charged phospholipids in the acceleration of clotting reactions. Whether this observation applies generally to all the clotting reactions occurring on lipid surfaces, or is restricted to TF-initiated coagulation, remains to be determined.

IV. BIOLOGICAL AND PATHOLOGICAL SIGNIFICANCE

A. Constitutive and Induced TF synthesis in Cultured Cells

In the remainder of this review, the experiments suggesting that TF participates in normal clotting, as well as disease-related thrombosis, are examined. Most of the work that is evaluated utilized cultured cells to study the synthesis and expression of the activator. The

basic observation is that when cells are grown in serum-containing media, most have measurable levels of TF. For example, fibroblasts and smooth muscle cells produce the procoagulant under virtually all conditions examined so far.[104,105] The notable exceptions to this rule are the cells which normally contact the blood directly. Of these, the most extensively studied are endothelial cells and monocytes/macrophages. In the absence of an exogenous stimulator of TF production, the latter cell types have little or no detectable activity.[105-112] Comparison of activity and antigen levels in confluent monolayers of bovine endothelial cells and fibroblasts illustrates this generalization. With a two-stage clotting assay and an ELISA calibrated with pure bovine TF, the endothelial cells had no detectable activity or antigen (i.e., <100 copies per cell), whereas the fibroblasts had ~100,000 copies per cell as judged in both assays.[189]

The hypothesized constitutive production of extravascular TF may create a large reservoir of procoagulant activity available for immediate response to vascular injury. Fibroblast, smooth muscle cells, and WISH amnion cells have all been examined in sufficient detail to permit some general conclusions.[104,105,113,114] There is considerable variability in the level of activity between cell types and even with different strains of the same cell. A portion of the variability may be attributed to the response of the cells to subculture. Following transfer, the TF activity per cell always increased to a maximum and then either declined in hours to a basal value or in some cases remained elevated for days. In addition, platelets stimulated TF production in fibroblasts and smooth muscle cells.[115] The variable kinetics of this response to replating makes any statement about the TF content of a particular cell type somewhat arbitrary since it is obviously very condition-dependent. Constitutive TF production in WISH amnion cells was suppressed by several metabolic and pharmacologic perturbations.[113,114] For example, serum starvation and conditioned media reduced the TF activity generated following subculture. Some steroids, as well as inhibitors of mRNA and protein synthesis, also effectively blocked production of the procoagulant activity. The transcriptional, translational, or posttranslational events which regulate synthesis and expression of TF remain largely undefined. However, with pure TF, monoclonal antibodies, and nucleotide probes for the message, these processes are currently being studied. It is reasonable to expect that understanding this modulation of TF gene expression in cultured cells may provide some insight into biological regulation.

For the monocyte/macrophage and endothelial cells with low levels of TF, a variety of agents have been found which induce the appearance of the clot-promoting activity, presumably by turning on the TF gene. Of particular interest are the naturally occurring substances which elicit this response. For example, endotoxin,[107-112,116] interleukin-1,[106,117-119] tumor necrosis factor,[120,121] an activated component of the complement system,[122] immune complexes,[123-125] some plasma lipoproteins,[126,127] a product of platelet arachidonate metabolism,[128] and exposure to some tumor cell lines[129] all generate procoagulant activity in one or more of the cells which are nonthrombogenic in the absence of an exogenous stimulus. For the endothelial cell, the action of the effectors is probably direct. For monocyte/macrophage stimulation, effector function often requires T cell collaboration,[126,130-132] which may be mediated by specific lymphokines.[133,134] However, in some cases the action on the mononuclear cell appears to be direct.[82] The potential involvement of many of these stimuli in inflammatory lesions is particularly intriguing.

B. Species Specificity and Characterization of Cellular Procoagulants

Simple clotting assays are employed in almost all the work on cell-derived procoagulants. The inherent ambiguities of these assays justifies a degree of skepticism. To evaluate this literature properly, the essential distinction between an unspecified procoagulant activity and TF activity must be understood. A procoagulant is simply any agent capable of accelerating clot formation in a one-stage recalcification time. A cell-derived procoagulant may be TF,

but other molecules such as specific proteases[135-137] and even phospholipid, as demonstrated in the earliest efforts to fractionate tissue thromboplastin, will also accelerate clot formation.[11] Obviously, attributing the activity to TF requires additional verification.

This is particularly important in light of the species specificity of TF. For example, it has been shown that human, rabbit, cow, sheep, horse, and pig tissue thromboplastins, i.e., crude brain extracts containing TF, accelerate the one-stage clotting time of the homologous and heterologous plasmas. On the other hand, tissue thromboplastins from mouse, rat, hamster, and guinea pig have very little or, in some cases, no clot-promoting activity in heterologous plasmas from the larger animals.[79-82] One procoagulant artifact relating to this problem of species specificity has been identified. Membrane vesicles shed from two guinea pig cell lines shortened the recalcification time of normal human platelet-poor plasma and were somewhat less active in factor VII-deficient human plasma. However, both these effects were obliterated if cephalin was added to the plasmas prior to assay.[138] Thus, the thromboplastic activity of the guinea pig material in human plasma was apparently generated by the phospholipid. Similarly, it has been shown that mouse monocyte procoagulant activity is undetectable in platelet-poor human plasma when the plasma is supplemented with exogenous lipid.[139] The potential for procoagulant artifacts is a serious complication which must be properly evaluated, particularly when species lines are crossed and when the plasmas employed have not been supplemented with saturating amounts of lipid.

Factor VII-deficient plasmas are most commonly used to distinguish TF from other procoagulants. However, as just noted, the human or bovine factor VII-deficient plasmas are probably not useful for the characterization of mouse, rat, hamster, or guinea pig TF. The destruction of the activity by phospholipase C has also been employed.[140] It is, however, an indirect proof and should not be accepted as definitive. Specific polyclonal or monoclonal antibodies may be used as inhibitors to determine if TF is responsible for the procoagulant activity of a crude sample.[107,138,141] Since pure protein has only recently become available, the specific antibodies produced so far have not yet been widely exploited for this purpose. Even with seemingly pure antigens, the resulting antibodies must be rigorously characterized to establish their immunochemical specificity.

Another approach to establishing the functional identity of a procoagulant as TF is direct evidence of its absolute requirement for factor VII. This analysis requires the use of pure enzyme (factor VIIa) and substrate (factor X or IX), and thus precludes the use of the one-stage recalcification time. Two-stage clotting assays,[4] radiometric assays measuring the release of a radioactive activation peptide,[95,96] or chromogenic assays measuring factor Xa amidolytic activity[72,142] are all serviceable for this purpose. The key is that the reactions can be performed with and without added enzyme, directly testing the factor VII-dependence of the signal. The possibility of factor VII contamination in the crude sample can be excluded by pretreating the material with DFP, which irreversibly inhibits both one- and two-chain forms of the enzyme without affecting TF, at least for bovine and human materials. It should be noted that human factor VII incorporates DFP much more slowly than bovine factor VII. Therefore, longer incubations are required to fully inactivate the human zymogen. A procoagulant which demonstrates an absolute requirement for the enzyme in these assays is, by definition, TF. Nothing has yet been identified which will substitute for the cofactor and yield a false positive, as long as the enzyme and substrate are pure and the phospholipid requirement of the assay is fully satisfied.

Immunochemical analysis of TF antigen levels will be a useful complement to functional assays. For example, the identification of inactive forms of the molecule, as suggested by a recent study of the effect of cytotoxic drugs on HL-60 cell procoagulant activity,[143] may be achieved by comparing the concentrations of antigen and activity. At present, any work which does not provide independent verification of the functional or immunochemical identity of a cell-derived procoagulant must be regarded as provisional. The methods are now

available for a direct examination of the molecule. Therefore, it is reasonable to expect that subsequent work on this exciting topic will be subjected to more stringent standards.

C. Cellular Modulation of TF Expression

The experiments with cultured cells suggest several mechanisms for modulating the expression of TF procoagulant activity which may operate *in vivo*. Tests of constitutive production by extravascular cells and inducible production by the endothelial cell and the monocyte/macrophage indicate that mRNA and protein synthesis are required.[106,111,113,120] Thus, differential expression of the TF gene may sequester the activator on extravascular cell surfaces and prevent contact between the initiator and plasma-clotting factors in the absence of vascular injury. The relative contributions of transcriptional and translational controls to the hypothesized anatomical localization are as yet undefined.

In contrast to the TF synthesis just described, HL-60 cell procoagulant activity is stimulated by inhibitors of both protein and mRNA synthesis (puromycin, actinomycin D, etc.). This suggests that these cells contain a pool of inactive TF.[143] The response was not observed in normal monocytes and several other cell types, and was not simply the result of intracellular sequestering of the activator. Mobilization of the latent procoagulant activity might occur by a posttranslation modification which activates the TF protein or by the disappearance of an inhibitor. The observation may explain the high incidence of disseminated intravascular coagulation in patients with promyelocytic leukemia undergoing chemotherapy.

Finally, in the absence of mechanical or enzymatic perturbation, much of the TF activity in cultured cells is cryptic. Cellular disruption, or even brief tryptic digestion under conditions where cell viability is maintained, significantly enhances the activity.[105] The tryptic activation causes shedding of TF from the cell surface in plasma membrane vesicles, a process which occurs spontaneously in some tumor cells.[138] The difficulty in accurately estimating the activity of the unperturbed cell is analogous to measuring the activity of zymogen factor VII. Therefore, estimates for the magnitude of this stimulation vary widely, but values of tenfold or greater are commonly observed. The simple-minded interpretation of this observation is that at least a portion of the activator is unavailable for binding to the enzyme. It is also possible that the specific activity of TF is reduced in the unperturbed plasma membrane. The quantitative contributions of these two alternatives to the observed encryption are as yet undetermined. Inasmuch as synthesis of the activator and the HL-60 cell response to cytotoxic drugs require minutes to hours, while the unmasking of encrypted TF occurs in seconds to minutes, the latter is more likely to be a hemostatic response to vascular injury.

The binding of factor VII and/or factor VIIa to TF on unperturbed human monocytes[92] and other cells[93,144,145] has shown that at least some of the activator is available on the cell surface. In contrast, HeLa cell TF-factor VIIa activation of factor X measured in a chromogenic assay indicates that the intact monolayer has essentially no activity, while disrupted HeLa cells express the procoagulant.[142] On the other hand, unperturbed human fibroblasts in the same assay express procoagulant activity, although less than that of the perturbed monolayer.[190] From these seemingly contradictory results, it is evident that the mechanism of encryption remains largely undefined. Immunological analysis of the distribution of TF between cell surfaces and intracellular membranes, coupled with specific activity determinations, will clarify some of the ambiguities which remain regarding encryption. As already noted, acidic phospholipids facilitate the activator-enzyme association and increase the proteolytic activity of the complex. Since the outer leaflet of the unperturbed plasma membrane is composed primarily of uncharged phospholipids,[146,147] the appearance of PS on the cell surface in response to injury is one potential way of regulating TF cell-surface expression.

D. TF and Inflammation

As already noted, endotoxin, immune complexes, interleukin-1, tumor necrosis factor, etc. appear to induce TF gene expression in the endothelial cell and the monocyte/macro-

phage. This suggests that the appearance of these stimulators during the course of bacterial infection or immunologic disease may generate procoagulant activity on cell surfaces which are not normally thrombogenic. The pathological significance of this induction of TF synthesis is hypothetical. However, the clinically observed association between inflammation and thrombosis suggests that the process may occur *in vivo*. The stimulation of endothelial cells by interleukin-1, tumor necrosis factor, and endotoxin is particularly intriguing in light of recent observations that the increase of TF activity is accompanied by a decrease in thrombomodulin, a cell-surface cofactor for thrombin which alters its substrate specificity and converts it from a procoagulant to an anticoagulant enzyme.[116,118,120] This suggests that expression of these procoagulant and anticoagulant molecules might be coupled in an inverse relationship. The balance between the initiating and inhibitory pathways of clotting may be an important factor in determining the thrombotic potential of the endothelium. The mechanism of this coupling is obviously a question of considerable interest with respect to the role of the endothelium in thrombosis and hemostasis.

An increase in the procoagulant activity of circulating monocytes isolated from humans and animals with a variety of inflammation-related diseases has been reported. The list includes Crohn's disease,[148] Schwartzman reaction,[149] meningococcal infection,[150] renal allograft rejection,[151] lupus glomerulonephritis,[152] and rheumatic diseases.[153] The identity of the procoagulant as TF was not established unequivocally in all cases. Nevertheless, the implications are obvious and will no doubt stimulate basic studies into the cellular mechanism of the phenomenon, as well as clinical efforts to treat the thrombotic complications of inflammation by targeting the monocyte. Particularly interesting in this regard is the recent evidence that dipyridamole suppresses the generation of procoagulant activity in rabbit alveolar macrophages.[154]

E. Coagulation and Cancer

Thromboembolism is a serious complication of malignancy and may be the first manifestation of the disease in an otherwise healthy individual. One view holds this to be an epiphenomenon, a consequence of tumor growth and the associated destruction of normal tissues. However, there is evidence that the connection between coagulation and cancer is far more intimate. In fact, a tumor may utilize clotting and other processes involved in wound healing to its own benefit.[155] This view of tumors as "wounds that do not heal"[156] suggests that the growth and survival of a cancer is at least in part the result of successful mimicry. The details of this association between clotting and metastatic disease are described in a number of excellent reviews.[155-164]

One aspect of this model of malignancy is that TF may play an essential role in forming the stroma of solid tumors. Continuous fibrin deposition and turnover is observed in this extracellular matrix.[165] Since malignant cells in culture are often constitutive products of the cofactor, it follows that the clotting in and around tumors is to some degree TF-mediated, though other novel procoagulants have been identified and may also be involved.[135-137] Since the formation of this stroma seems to be required for the successful proliferation of a cancer, the possibility of blocking the spread of malignant cells with anticoagulants has received considerable attention. Impressive results have been achieved in some animal models.[166-168] However, the utility of this approach to the treatment of human tumors cannot be assessed at this time.

F. TF Inhibitors

High density lipoprotein,[169] apolipoprotein A-II,[170] a placental anticoagulant protein,[171] some lupus anticoagulants,[172] and serum amyloid P component[173] are inhibitors of TF-initiated coagulation assays. With the exception of the lupus anticoagulants, they have not been associated directly with hemostatic abnormalities, i.e., bleeding or thrombosis. There-

fore, an *in vivo* role for these proteins as inhibitors of coagulation is speculative. Kinetic analysis of apolipoprotein A-II inhibition indicates that the effect is principally on the Vmax for the hydrolysis of factor X.[172] The mechanisms of the other inhibitors have not been examined in detail. However, they appear to be nonspecific in that they also seem to inhibit other cofactor-mediated coagulation reactions.

An apparently specific inhibitor of human TF was identified in a patient with nephrotic syndrome of unknown etiology.[174] The individual had no bleeding abnormality, but exhibited a prolonged prothrombin time. The altered prothrombin time was enhanced in a time-dependent and dose-dependent fashion by incubation of the patient's plasma with thromboplastin, and Ca^{2+} was not required for this effect. The inhibition was observed only with human thromboplastin; thromboplastins from other species were unaffected. From the species specificity, the inhibition was judged to be a direct effect on the cofactor. The identity of the inhibitor and evidence for its complexation with human TF have not been established.

Another plasma inhibitor of TF was first identified by experiments showing that incubation of thromboplastin with serum blocked the procoagulant properties of the material.[175-178] The initial observations were subsequently confirmed and extended by Hjort.[14] It was concluded from these early studies that the inhibition is Ca^{2+}-dependent and the serum component(s) acts on the activator-enzyme complex. Furthermore, TF was not consumed in the process, since chelation of the Ca^{2+} reversed the inhibition. Recent work has shown that, in addition to factor VIIa, factor Xa must be present for the inhibitor to block the procoagulant activity of tissue factor.[179-181]

The inhibitor has been purified from HepG2 cell culture media[182] as well as from plasma.[183] The first procedure yields an apparently homogeneous protein of about 38,000 Da, while the plasma protocol isolated two discrete pools containing proteins of 34,000 and 43,000 Da, respectively. Although they all appear to inhibit TF in the same fashion, the chemical identity of these proteins has not been reported.

Catalytically active factor Xa is required for the inhibitor to manifest its activity.[180,181] Also, it directly interacts with factor Xa.[182] Together with the previous work, this suggests that the inhibition may be produced by formation of a stable quaternary complex of TF-factor VIIa-factor Xa-inhibitor, thus blocking further catalysis. The biological significance of this inhibition is uncertain. However, the activity appears to be slightly decreased in patients with disseminated intravascular coagulation.[184] Therefore, it is reasonable to surmise that this inhibition may act *in vivo* and suppress the generation of factors IXa and Xa by TF-factor VIIa.[185,186]

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