Anniversary Issue Contribution

The changing faces of tissue factor biology

A personal tribute to the understanding of the "extrinsic coagulation activation"

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Up until the end of the 19th century the so-called "extrinsic coagulation system" was generally considered to consist of four components of which one frequently was derived from adjacent tissues. This tissue-derived component was called thromboplastin in Europe and tissue factor (TF) in the US. It was not until Prof. Owren's discovery (1944) and partial characterization of a patient with a hitherto unknown defect in haemostasis, not explainable by the activity of the previously known factors, that the need of a more complicated concept for the clotting system developed. Owren's discovery released a lot of scientific energy in the area of blood coagulation and haemostasis, which has been well described in H. Stormorken's book about Paul A. Owren and the "Golden Era of Haemostasis" (1).

Looking back upon the history of this "extrinsic" part of the clotting system, it appears reasonable to separate several stages of development where structure, function, cell biology and molecular genetics as well as clinical studies all have produced new knowledge and contributed to a closer understanding of functional mechanisms. Our intention has been to list the major steps forward from about 1950 to date to illustrate the progress as it becomes clear that TF is involved in a number of cellular reactions as outlined below. We emphasize that our intention is to give an overview of parts of the field and not to review all the excellent papers that have been published over all these years.

Biochemical characterization

In 1940 Chargaff stated that the thromboplastic protein could be considered as a lipoprotein. He described the fundamental finding that when separated into a lipid and a protein part the activity was lost and that both the protein component and the phospholipids were necessary for maximal activity (2). Sodium deoxycholate detached two-thirds of the protein in a non-sedimentable form with full thromboplastic activity (3).

Twenty years later the purification method was improved using deoxycholate extractions of the microsomal membrane fraction and by gel filtration the activity was detected in the protein peak (4). The thromboplastic activity in the presence of deoxycholate was connected with molecules or structures of a much smaller size than previously reported (4). Using acetone powder from lung tissue and a combination of salt-extractions and chromatography two major protein species with apparent molecular weights of 330 kDa and 220 kDa were detected (5). The difference between the two species was the phospholipid content. The use of sodium dodecyl sulphate (SDS) and preparative SDS-polyacrylamide gel electrophoresis (PAGE) after deoxycholate extractions and gelfiltration further improved the purification method (6). On analytical SDS-PAGE only one protein band with a molecular weight of around 51–53 kDa was detected. TF purified using this method induced monospecific antibodies in rabbits and goats that were inhibitory in clotting assays (7, 8).

The TF- gene and protein

The TF cDNA was cloned independently by four different groups in 1987 (9–12). The human TF gene contains six exons divided by five introns and spans 12.4 kb (13) on chromosome 1 (14). Transcription of the gene results in several transcripts, most abundant is the 2.1 – 2.3 kb mRNA but larger transcripts exist probably as a result of incomplete processing of introns (15, 16). The primary translation product contains 295 amino acids of which 32 residues are the leader peptide. The extracellular part of the mature protein contains 219 residues, the transmembrane domain contains 23 residues, and the cytoplasmic domain only 21 residues. The four cysteine residues in extracellular domain forms two disulfide-bonds and the cysteine residue in the cytoplasmic domain is potentially thioester-linked to palmitate or stearate (17) providing an extra anchor in the membrane. A family of cytokine receptors was defined using predictive structural analysis *in silico* and TF was classified as a member of the class-2 cytokine receptor family together with the interferon receptors (18). This structural family contains two domains with anti-parallel β-strands with topology similar to Ig-domains, a defined spatial clustering of conserved residues and characteristic cysteine pairs. The crystal structure of $TF^{1–219}$ (19) revealed that

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it indeed was made up of two immunoglobulin-like modules associated through a novel interface region.

Alternatively spliced transcripts have been detected that may give rise to TF variants. An example of this was recently reported in which exon 5 was spliced out and a different C-terminus formed (20) giving a protein, asHTF, where residues 1 to 166 are identical to full-length TF and residues 167 to 206 are unique. A novel exon IA (21) has been detected which can be included both in full-length TF and in the asHTF transcript. Such alternatively spliced TF transcripts have been detected in human tissues such as lung, placenta, and pancreas as well as in tumor cell lines (22). The recombinant asHTF protein produced in *E. coli* possessed procoagulant activity (20), but when produced in HEK cells no procoagulant activity was detected (23). To identify the biological function of asHTF further investigations clearly are needed.

Cell biology

During physiological conditions, cells in the blood or in contact with the blood stream do not express TF. A new era in the understanding of TF expression started with the possibilities that the methods for in-vitro culture of cells presented and revealed that TF synthesis could be induced in monocytes (24) and endothelial cells (25) by a number of agents (reviewed in [26]). A general feature is that increased TF synthesis is induced by agents elevating the intracellular calcium concentration and that TF synthesis is inhibited by agents that increase the cAMP concentration. Protein kinase C is part of the signal pathway when TF expression is induced by interleukin-1 (IL-1) and bacterial lipopolysaccharide (LPS) in endothelial cells (27), in monocytes (28), and in mesangial cells (29). TF is an immediate, early gene and mRNA is detected already 30 minutes (min) after adding stimulus to the cells. In endothelial cells the protein level reaches maximum after 6 to 8 hours (30) and declines with a half-life of 7 to 8 hours (31).

To initiate blood clotting TF must be in contact with the rest of the clotting machinery. Under normal physiological conditions TF is not expressed by or is inactive on the surface of monocytes and endothelial cells, but when induced, TF is located entirely in the plasma membrane in monocytes (32, 33). Both in the bladder carcinoma cell line J82 and in arterial smooth muscle cells surface and intracellular TF was observed (33, 34). In endothelial cells TF procoagulant activity (PCA) was detected on the apical or luminal side under growth conditions ensuring the formation of polarized cells with tight junctions (35). In Madine-Darby Canine Kidney (MDCK) cells the TF antigen was mainly observed on the basolateral surface (36). This TF protein was available for FVIIa binding, but did not display any PCA, opposite to the relatively small amount of apical TF antigen that displayed PCA. In endothelial cells TF antigen was detected on the apical side as expected (36). In endothelial cells, and smooth muscle cells TF was found in cholesterol-rich rafts or caveolae known to be devoid of anionic binding sites (37, 38). Taken together there is ample evidence that TF in cells of different origin is located in the cell surface as well as in intracellular pool(s). In fibroblasts, confocal microscopy studies revealed that the majority of TF was in the intracellular pool predominantly associated with the Golgi (39). When the TF-FVIIa complexes formed on the cell surface this intracellular pool moved to the surface resulting in increased expression of TF. In these cells, complex formation resulted in internalization of TF. In endothelial cells we observed decreased TF PCA due to formation of TF-FVIIa-TFPI complexes (31).

The complex of TF and FVII

TF is the cellular receptor and cofactor for the glycoprotein and serine-protease family member factor VII. This protein is mainly synthesized in the liver and secreted into the blood where it circulates as a single chain zymogen of 406 amino acids. Purification of human FVII from serum was obtained in 1970 using barium-sulphate and DEAE-sephadex chromatography followed by gel filtration and preparative PAGE (40). To obtain full enzymatic activity of FVII, cleavage of the Arg^{152}-Ile^{153} peptide bond and the subsequent insertion of the new N-terminal Ile into the hydrophobic core of the serine protease domain are required events. By associating with the transmembrane cofactor TF the fully active enzyme is formed. Different approaches have been used to map amino acids and regions important for formation and activation of the complex. Such studies include analysis of site-directed mutated TF variants (41–43) and regarding FVII analysis of both natural occurring variants (reviewed in [44]) and scanning of site-directed mutated variants (45). These studies combined with the data obtained from the crystal structure of the complex of TF and active-site inhibited FVIIa (46) identified extensive contacts including all sub-domains in both proteins. The extracellular TF domain and the light chain of FVIIa form a stalk-like structure with the heavy chain on top. Both the active site and the new N-terminus formed after zymogen activation are distant from TF. The energetically most important contact with TF is through the EGF-1 domain, which also has the largest contact area. The EGF-2 and SP domains interact with the amino-terminal module of TF but these interactions hardly contribute to the free energy of binding and serve to allosterically regulate the SP domain (47). In complex with TF and phospholipids the catalytic efficiency of FVIIa towards its substrates FX and FIX increases in the magnitude of 10^5 to 10^7 (48, 49 and references therein).

The complex and intracellular signalling

The cloning of TF and its apparent receptor configuration with an extracellular domain, a transmembrane domain and a short intracellular tail made us hypothesize that binding of the ligand FVII(a) to TF would start an intracellular signalling pathway resulting in altered gene expression. Calcium signalling is induced as a consequence of binding of many different ligands to their receptors and was the first signalling component to be investigated. Measuring the cytosolic free Ca^{2+} in human endothelial cells induced to express TF, a rise in Ca^{2+} was observed when adding 200 nM FVIIa to the cells (50). This effect was seen in several TF expressing cells; in the bladder carcinoma cell line J82, in COS-1 cells (monkey kidney fibroblasts) transfected with a TF cDNA, and in MDCK cells (50). The signalling events are specific in that they depend on the presence of the TF/FVIIa complex. Moreover, they depend on FVII being proteolytically ac-

tive, i.e. either added as the zymogen being activated upon binding to TF or added as the two-chain form. Addition of the thrombin inhibitor hirudin or the FXa inhibitor tick anticoagulant peptide (TAP) ensure that the signalling is independent of activation of coagulation proteases downstream of the TF/FVIIa complex.

This first report of intracellular signalling caused by complex formation opened up a new field. Until now studies on TF's biological function had been focused on extracellular events, mainly initiation of blood coagulation. To continue our studies of the intracellular signalling depended on finding a human cell line expressing TF and responding to FVIIa with increased intracellular calcium. Different cell lines were tested and the spontaneously transformed keratinocyte cell line HaCaT fulfilled all criteria (51). Rise in Ca^{2+} concentration is a classical event following activation of phosphatidyl inositol-specific phospholipase C (PI-PLC). The PI-PLC inhibitor U73122 inhibited the calcium response both in MDCK (52) and HaCaT cells, and the Egr-1 mRNA up-regulation in HaCaT cells (53). In BHK^{+TF} cells no detectable Ca^{2+} release was observed when stimulated with FVIIa (54).

MAP kinases are activated both in BHK^{+TF} and HaCaT cells. In the BHK^{+TF} cell line p44/$p42^{MAPK}$ were transiently phosphorylated with a maximum response after 10 min (54). In HaCaT cells increased phosphorylation of p44/$p42^{MAPK}$ was detected, but in these cells, opposite to the results obtained with BHK^{+TF} cells, phosphorylation of $p38^{MAPK}$ and c-Jun N-terminal kinase (JNK) were also induced by FVIIa (53). In both cell lines the induced phosphorylation of p44/$p42^{MAPK}$ was inhibited by PD98059, an inhibitor of the MAPK kinase MEK. Further studies revealed that the small G-protein Ras was activated in TF/FVIIa signalling and that p44/$p42^{MAPK}$ phosphorylation was dependent on functional p21Ras and the MAPKK kinase Raf (55). In HaCaT cells the p21Ras activation was mediated by a Src-family kinase, whereas in BHK^{+TF} protein kinase C was suggested to mediate this effect (55).

TF/FVIIa signalling induces phosphorylation of protein kinase B (PKB, also called Akt) in a fibroblast cell line (56), in BHK^{+TF}, and in HaCaT cells (57). This phosphorylation coincided with phosphorylation of the ribosomal S6 kinases p70/$p85^{S6K}$ and $p90^{RSK}$ as well as GSK3 and eIF-4E, both involved in the regulation of protein synthesis (57). Activation of Src-family kinases c-Src, Lyn and Yes, and subsequently the phosphatidylinositol-3-OH-kinase (PI3K) was upstream of the activation of p44/$p42^{MAPK}$ and PKB in a fibroblast cell line (56).

The final result of a signalling pathway conveniently used as an endpoint is altered gene expression. In HaCaT cells, the mRNA level for the transcription factor Egr-1 was elevated upon stimulation of the cells with FVIIa or FXa (53). The mRNA was rapidly up-regulated with maximum at 30 to 60 min after stimulation and returned to basal level within 2 hours. Using a human fibroblast cell line and differential display the poly (A)-polymerase was found to be up-regulated (58). Treating a human fibroblast cell line with FVIIa for 90 min five genes were found to be moderately up-regulated when tested in microarray analyses and, of these, increased mRNA for Cyr61 and CTGF (connective tissue growth factor) were detected on Northern blots (59). Using a similar approach with cDNA arrays and mRNA isolated from HaCaT cells stimulated for 30, 60, 90, 180 and 360 min with FVIIa, the mRNA for 24 genes including CTGF were found to be up-regulated (60). Accompanying increased protein synthesis and secretion of interleukin-8 was detected as well (61).

Although TF is a cytokine receptor and possesses several potential phosphorylation sites in the cytoplasmic tail there is no direct evidence for TF being the signal-transducing receptor. Transfecting COS-1 or BHK cells with constructs lacking the intracellular TF-tail and testing them for several TF/FVIIa stimulated end-points revealed that they responded equivalently to cells expressing full-length TF (53, 57, 62). Moreover, the requirement of FVII-proteolytic activity and the lack of proteolysis of TF following FVIIa-binding pointed to another receptor being the actual signal transducing receptor. A group of G-protein-coupled receptors called protease activated receptors (PARs) was an obvious choice when looking for this missing link in signal transduction. Hitherto four different PARs are cloned, whereby human PAR1, PAR3, and PAR4 are activated by thrombin (reviewed in [63]) leaving PAR2 as a likely candidate for TF/FVIIa activation, since desensitisation with thrombin did not inhibit the FVIIa induced Ca^{2+} response (64). Conflicting results have been reported on whether PAR2 is the signalling receptor in TF/FVIIa signalling. In *Xenopus* oocytes a FVIIa-signalling response was obtained only when expressing TF together with PAR1 or PAR2, and using a lung fibroblast cell line derived from PAR1 knockout mice a response to FVIIa was only seen when TF and PAR2 were co-expressed (65). The complex also activates downstream signalling via FXa and the PAR1 receptor. Activation of PAR2 signalling is responsible for the reported phosphorylation of TF cytoplasmic domain serine residues (66, 67). The initiation phase of TF/FVIIa signalling is even more complicated by yet another receptor activation. Increased phosphorylation of the epidermal growth factor receptor was detected (68) probably caused by release of membrane bound hb-epidermal growth factor (69). Transactivation of epidermal growth factor receptor is a key event preceding gene activation at least in HaCaT cells (69).

One question has been debated for many years; that is the phenomenon called cryptic TF. Cryptic TF is the part of the TF cellular pool that is non-coagulant. Recently, it was shown that TF-FVIIa signalling and coagulation involves distinct cellular TF pools and that disulfide isomerization may switch TF from coagulation to signalling (70).

TF – A target for cancer therapy

Last but not least, the advent of the siRNA technology (71, 72) has shown that it is possible to manipulate the cellular level of TF under tissue culture conditions (73), allowing new questions to be asked with high degree of precision. TF is involved in an increasing number of cell biological processes in systems like the endothelium and in cell-to-cell contact. Our findings suggest that there is at least a correlation between the level of TF and the degree of resistance to carcinogenesis in our cellular system (74).

In conclusion, we shall approach the end of our efforts to map and understand TF biology in terms of repair and rebuilding of 3D-structures in several uses of reconstructive surgery. The next 50 years will take us even further!

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