Published in final edited form as:

Thromb Res. 2010 April ; 125S1: S26–S28. doi:10.1016/j.thromres.2010.01.028.

Posttranslational modifications and activity of natural and recombinant tissue factor

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Abstract

Tissue factor is a membrane protein, which in a complex with factor VIIa initiates *in vivo* blood coagulation. Due to the scarcity of natural tissue factor protein, most studies have relied upon recombinant tissue factor forms. However, there have been only cursory experimental comparisons of natural and recombinant tissue factor proteins. Our preliminary data suggested that placental tissue factor in a complex with factor VIIa was more efficient activator of factor X than the recombinant protein. After deglycosylation, both forms of tissue factor showed almost an identical activity in the extrinsic factor Xase. Analyses using tryptic digestion and mass-spectrometry revealed that the levels of glycosylation and the composition of carbohydrates present in natural placental tissue factor were different than those in its recombinant counterpart. These data indicate that natural and recombinant tissue factor proteins differ in their posttranslational modifications and that these differences translate into different cofactor activity. Thus the use of recombinant tissue factor proteins for the quantitation of natural tissue factor is misleading.

Keywords

Tissue factor; Posttranslational modifications; Extrinsic factor Xase; Mass-spectrometry; Carbohydrate composition

Structure of tissue factor

Tissue factor is an integral membrane protein, which in complex with factor VIIa, initiates the blood coagulation process. Tissue factor is expressed in the vascular adventitia, in astroglial cells, in organ capsules, and is found in the central nervous system, lungs and placenta at relatively high concentrations [1–3]. Many cells, including monocytes and macrophages, can express tissue factor when they are stimulated with various agonists, including inflammation-related cytokines [4–6].

Human tissue factor is a 263 amino acid lipoprotein containing three domains: 1) an extracellular domain, which represents the NH₂-terminal part of the protein (residues 1–219) and participates in binding factor VIIa [7]; 2) a transmembrane domain (residues 220–242), which anchors tissue factor to the membrane [8]; and 3) a cytoplasmic carboxyterminal domain

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Conflict of interest statement

None declared.

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(residues 243–263), which is related to signal transduction [9,10]. The extracellular domain is composed of two fibronectin type III domains with potential glycosylation sites at Asn¹¹, Asn¹²⁴ and Asn¹³⁷. Although it has been established that all three sites in the extracellular domain contain carbohydrates [11–13], the extent of glycosylation and detailed structure of carbohydrates at each glycosylation site was not established. There are also two potential disulfide bonds (Cys⁴⁹-Cys⁵⁷ and Cys¹⁸⁶-Cys²⁰⁹) located in this domain [12]. The carboxyterminal cytoplasmic domain of tissue factor contains a single Cys²⁴⁵ residue and three Ser residues. The Cys²⁴⁵ residue is linked to a palmitate or stearate fatty acyl chain [14,15] while Ser residues can be phosphorylated by a protein kinase C-dependent mechanism [16, 17].

Glycosylation and activity of tissue factor

Sufficient natural tissue factor was isolated over 20 years ago to identify, clone and express the recombinant protein in human kidney 293 cells and in *E. coli* [12,13,18]. Subsequently, various forms of recombinant tissue factor ranging from the full-length protein to the extracellular domain of tissue factor with different levels of posttranslational modifications have been expressed in a variety of vectors including yeast and insect cells [12]. Mutational studies [19] and an x-ray structure [20] have been derived using these recombinant tissue factor proteins. Although these recombinant tissue factor forms have been used extensively as surrogates for the natural protein, the limited availability of purified natural tissue factor has not allowed the certification of results obtained with recombinant proteins. The influence of posttranslational modifications on functions of tissue factor proteins, both natural and recombinant, is clearly underrepresented in the existing literature and is the subject of controversy. In a side-by-side activity comparison for glycosylated and non-glycosylated full-length recombinant tissue factor, it has been suggested that tissue factor glycosylation is not required for procoagulant activity [12]. Since no quantitative data were provided, it is impossible to establish whether glycosylated and non-glycosylated forms of tissue factor have identical activities. Similarly, a statement suggesting that the activity of recombinant tissue factor 1–263 is identical to that of natural tissue factor from brain is not supported by any data [21]. In contrast to these publications, several studies suggested that glycosylation of tissue factor plays an important role in tissue factor function. Thus Pitlick demonstrated that concanavalin A efficiently inhibits procoagulant activity of natural tissue factor by binding to the carbohydrates [22]. This inhibitory activity of concanavalin A can be reversed by the addition of α -methyl-D-glucoside. In another study, Shands demonstrated that an inhibitor of N-linked glycosylation tunicamycin inhibits the activity of endotoxin-induced cell surface tissue factor [23].

Our comparison of recombinant tissue factor 1–243 produced in *E. coli*, recombinant tissue factor 1–263 produced in insect Sf9 cells and natural placental tissue factor in activity-based assays revealed that the highest specific activity in both membrane-independent (fluorogenic) and membrane-dependent (extrinsic factor Xase) reactions was exhibited by placental tissue factor, and that the lowest activity was observed for recombinant tissue factor 1–243 [24]. Based on mass-spectrometry data, it was determined that, consistent with the expression system, recombinant tissue factor 1–243 had no carbohydrates attached to the backbone of the protein and that placental tissue factor was modified more heavily than recombinant tissue factor 1–263. These data suggested that the cause of the different specific activities could be related to the differences in posttranslational modifications, primarily in glycosylation of the various tissue factor forms. To test this hypothesis, we compared glycosylated and fully deglycosylated forms of these three tissue factor proteins. As expected, “deglycosylation” did not affect recombinant tissue factor 1–243 activity and affinity for FVIIa and FX in the fluorogenic (membrane-independent) and the extrinsic factor Xase (membrane-dependent) assays, respectively. Somewhat surprisingly, deglycosylation had no effect on the activity of

recombinant and placental tissue factor in the fluorogenic assay, suggesting that differences in activity in this assay observed for different forms of tissue factor could be related to other posttranslational modifications. Similarly, Stone et al. showed that soluble form of tissue factor, which does not interact with the membrane, is not influenced by deglycosylation [25]. A possible cause for different activities of various tissue factor proteins in membrane-independent reactions could be related to differences in phosphorylation. It has been suggested in previous publications that the extent of phosphorylation directly correlates with the tissue factor activity [26–28]. Our preliminary data indicate that placental tissue factor is more heavily phosphorylated than its recombinant counterpart. These data are consistent with the observation that placental tissue factor, both glycosylated and deglycosylated, has higher activity than the recombinant protein. In contrast to the fluorogenic assay, deglycosylation of the full-length recombinant tissue factor 1–263 decreased the second order rate constant of the extrinsic factor Xase from $5.2 \mu\text{M}^{-1}\cdot\text{s}^{-1}$ to $3.8 \mu\text{M}^{-1}\cdot\text{s}^{-1}$. The most pronounced decrease in the activity of the extrinsic factor Xase was observed when natural placental tissue factor was deglycosylated. Deglycosylation caused a decrease in affinity of factor X for the factor VIIa/tissue factor complex, a decrease in the catalytic constant and, as a consequence of these changes, an almost 7-fold decrease in the second order rate constant (from $26.8 \mu\text{M}^{-1}\cdot\text{s}^{-1}$ to $4.0 \mu\text{M}^{-1}\cdot\text{s}^{-1}$). All of these parameters for deglycosylated placental tissue factor were almost identical to those determined for deglycosylated recombinant tissue factor 1–263, suggesting that carbohydrates have a pronounced effect on the activity of natural tissue factor. Similar decrease in activity was observed in a previously published study when tissue factor mutants with impaired glycosylation were generated [29], although authors assigned that decreased activity to the absence of the Cys¹⁸⁶-Cys²⁰⁹ disulfide bond in mutants.

Carbohydrate composition of tissue factor proteins

Tryptic digestion and mass-spectrometric analyses of recombinant tissue factor 1–263 and placental tissue factor fragments showed that three of the four potential glycosylation sites have N-linked carbohydrate chains attached [24]. All three sites are located in the extracellular domain of the tissue factor protein. Similar to previous publications [11,12], no carbohydrates were detected at Asn²⁶¹. The extent of glycosylation and carbohydrate composition was different between the two proteins as well as between each glycosylated site within the protein. Additionally, a quite high heterogeneity of carbohydrates was observed at each glycosylation site. Only 20% of Asn¹¹ in recombinant tissue factor 1–263 is glycosylated, predominantly with high mannose carbohydrates. In contrast, more than 75% of Asn¹¹ is glycosylated in placental tissue factor. This site in placental tissue factor is heavily fucosylated and no mannose-containing carbohydrates are detected. Asn¹²⁴ in both tissue factor proteins (natural and recombinant) is almost completely glycosylated, however the composition of carbohydrates is distinctly different. In recombinant tissue factor 1–263, more than 80% of this site is modified with high-mannose carbohydrates, whereas in placental tissue factor Asn¹²⁴ is glycosylated with hybrid and sialylated carbohydrates. Similarly, pronounced differences in carbohydrate structure are observed for Asn¹³⁷, although in both proteins this site is almost completely glycosylated. In recombinant tissue factor 1–263, the abundance of high-mannose carbohydrates is almost matched by that of fucosylated sugars, whereas in placental tissue factor Asn¹³⁷ is modified exceptionally with fucosylated-(sialylated) carbohydrates. Differences observed in carbohydrate composition between human placental tissue factor and the recombinant protein produced in Sf9 cells are consistent with general patterns of protein glycosylation in mammalian and insect cells [30,31].

Conclusion

It can be concluded that natural and recombinant tissue factor proteins are different with respect to their posttranslational modifications. These differences translate into different enzymatic

activities when the tissue factor complex with factor VIIa is formed. This observation is valid for low molecular weight synthetic substrates and, most importantly, for a natural substrate, factor X. Thus, caution should be used in data interpretation when recombinant protein is used as a surrogate for the natural, especially in diagnostic and biological experiments.

Acknowledgments

This work was supported by the P01 HL46703 grant from the National Institutes of Health.

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