1. ABSTRACT

Tissue factor pathway inhibitor (TFPI) is a factor Xa dependent inhibitor of tissue factor initiated blood coagulation. In recent years, several alternatively spliced forms of TFPI have been identified. These alternatively spliced forms have different C-terminal regions and have different mechanisms for association with cell surfaces. They are differentially expressed in human and mouse tissues and may have distinct physiological functions.

2. INTRODUCTION

Tissue factor pathway inhibitor (TFPI) is an anticoagulant protein found primarily on the surface of endothelium, but also within platelets and circulating in plasma. TFPI directly inhibits the tissue factor-factor VIIa (TF-fVIIa) catalytic complex that initiates blood coagulation. The in vivo importance of TFPI as an inhibitor of TF-fVIIa activity has been demonstrated through studies of mice lacking TF, fVIIa and TFPI. TFPI null mice
Alternatively spliced forms of TFPI
die during embryogenesis from consumptive
cogulopathy. This embryonic lethal phenotype is rescued
by breeding TFPI null mice into mice with low amounts of
TFα or into mice lacking fVIIα, thereby demonstrating that
TFPI directly counterbalances TF-fVIIa activity. The initial
structural studies of TFPI described a protein containing an
acidic N-terminal region, three Kunitz-type serine protease
inhibitor domains and a highly basic C-terminal region.8
Functional studies identified the second Kunitz domain as a
direct inhibitor of factor Xa (fXa) and the first Kunitz
domain as a fXa dependent inhibitor of TF-fVIIa9. An
inhibitory function for the third Kunitz domain has not yet
been identified. Since its initial characterization,
alternatively spliced forms of TFPI have been identified
that are differentially expressed during mouse development
and may have distinct physiological functions10-13.

3. ALTERNATIVELY SPliced FORMS OF TFPI

TFPI is produced in four alternatively spliced
isoforms14 (Figures 1-4). Each isoform has the acidic N-
terminal region and the first two Kunitz domains that are
responsible for TFPI anticoagulant activity. However, they
differ in the domain structure of their C-terminal regions
and their mechanism of association with cell surfaces.

**TFPlalpha** is the full-length form of TFPI. It has
all three Kunitz domains and a basic amino acid rich C-
terminal region. The C-terminal region of human
TFPlalpha is more basic than mouse TFPlalpha, with
human having 14 basic amino acids, while mice have only
nine. The third Kunitz domain and/or the basic C-terminal
region may be important in mediating binding of TFPlalpha
to endothelium15. TFPlalpha associates with the

**endothelium surface in two ways. About 90% is indirectly
bound through an, as yet, unidentified GPI-anchored
protein.**16, 17 The association of TFPlalpha with
endothelium through a GPI-anchored protein localizes
TFPI to caveolae, where it may interact with caveolin-1
which increases its surface expression and anticoagulant
activity.18 The remaining 10% of TFPlalpha is non-
specifically bound to cell surface glycosaminoglycans17. In
humans, the glycosaminoglycan bound TFPI alpha is
released into plasma following heparin infusion causing the
plasma TFPI concentration to increase 2- to 4-fold19, 20.

**TFPibeta** is a C-terminally truncated form of
TFPI10. It has the first two Kunitz domains. Alternative
splicing occurs just prior to the third Kunitz domain and
produces a sequence encoding a GPI-anchor attachment
site and, consequently, TFPlbeta directly associates with
endothelium10, 11. After processing of the C-terminal region
to attach the GPI-anchor, human TFPlbeta protein has 12
amino acids not present in TFPlbeta, while mouse
TFPlbeta has eight. Thus far, attempts to make high affinity
antibodies that recognize the unique C-terminal region of
TFPlbeta have not been successful making it difficult to
directly identify TFPlbeta in tissues.

**TFPlgamma** is a C-terminally truncated form of
TFPI found only in mice12. It has the first two Kunitz
domains. Alternatively splicing occurs at the same 5’ splice
acceptor site as TFPlbeta, just prior to the third Kunitz
domain, but the 3’ splice acceptor site is 276 nucleotides
past the TFPlalpha stop codon within the TFPlbeta 3’
untranslated region12. This produces a protein sequence
with 18 amino acids not present in TFPlalpha or TFPlbeta.
As with TFPlbeta, attempts to make high affinity
Figure 2. The amino acid sequence and Kunitz domain structure of human TFPI beta. Red circles indicate acidic amino acids. Blue circles indicate basic amino acids. Yellow circles are cysteine residues.

Figure 3. The amino acid sequence and Kunitz domain structure of human TFPI gamma. Red circles indicate acidic amino acids. Blue circles indicate basic amino acids. Yellow circles are cysteine residues.
Alternatively spliced forms of TFPI

**Mouse TFPlgamma**

Figure 4. The amino acid sequence and Kunitz domain structure of mouse TFPI delta. Red circles indicate acidic amino acids. Blue circles indicate basic amino acids. Yellow circles are cysteine residues.

antibodies that recognize the unique C-terminal region of TFPlgamma have not been successful making it difficult to directly identify TFPlgamma in tissues. The C-terminal region of TFPlgamma does not encode a predicted GPI-anchor attachment sequence and following transfection into CHO cells, it is processed as a secreted protein.

TFPldelta is a C-terminally truncated form of TFPI. Sequences are present within the NCBI GenBank database, but no other information about this isoform has been published to date. Alternative splicing occurs immediately following the second Kunitz domain and a sequence encoding a new C-terminal region of 12 amino acids is present.

**4. EVOLUTION OF ALTERNATIVELY SPLICED ISOFORMS OF TFPI**

TFPlalpha is well conserved from man to zebra fish who last shared a common ancestor 430 million years ago. The third Kunitz domain has maintained a similar high degree of sequence identity as the first and second Kunitz domains suggesting it has an important biological function. In contrast, TFPlbeta-specific sequence has been identified in humans, other primates, mice, and rats. This is not surprising because TFPlbeta sequence conservation between man and mouse is fairly poor (43%) with the exception of the 7 amino acid region proximal to the predicted GPI-anchor modification site. Analysis of zebrafish genomic sequence between the exons encoding K2 and K3 revealed no potential alternative exon that might encode the TFPlbeta specific or the TFPlgamma specific sequence. This suggests that TFPlbeta represents a ‘recent’ evolutionary adaptation whereas TFPlalpha existed prior to the divergence of the bony fish over 430 million years ago.

**5. TISSUE EXPRESSION OF ALTERNATIVELY SPLICED FORMS OF TFPI**

Examination of the expression of TFPlalpha and TFPlbeta mRNA in human and mouse tissues and endothelial cell lines using real time PCR revealed that each tissue has more message for TFPlalpha than TFPlbeta. This ranges from 4- to 50-fold with message for TFPlalpha on average 10-fold more abundant than message for TFPlbeta. At the level of protein production, it appears that TFPlalpha is the major TFPI isoform produced in humans. TFPlalpha protein has been identified in human platelets and placenta. As mentioned, human plasma contains TFPlalpha and the heparin-releaseable form of TFPI is TFPlalpha. TFPI beta protein has not been identified in humans. The lack of a high affinity antibody that recognizes TFPlbeta but not TFPlalpha makes it difficult to differentiate TFPlbeta from partially degraded forms of TFPlalpha. Further studies are needed to definitively define the alternatively spliced isoforms of TFPI produced in different human vascular beds.

In mice, the placenta and embryo produce both TFPlalpha and TFPlbeta protein with placenta producing much more TFPlalpha than TFPlbeta and embryo producing approximately equal amounts of each. However, as mice mature, the production of TFPlalpha...
decreases and adult mice produce predominantly TFPIbeta protein in all major vascular beds. Mouse plasma contains essentially only TFPIbeta and heparin infusion has only minimal effect on the plasma TFPI concentration. This finding is consistent with the structure of TFPIbeta, which lacks the basic C-terminal region responsible for association of TFPIalpha with glycosaminoglycans in human vasculature. Interestingly, it appears that mouse platelets make only TFPIalpha (Maroney SA et al., ATVB 2011 In Press). Thus, adult mice produce TFPIbeta in all tissues except for platelets that make TFPIalpha. TFPIgamma and TFPIdelta protein have thus far not been identified in vivo. Since TFPIalpha message is more abundant than TFPIbeta message in adult mouse tissues, control of TFPI protein production in mice occurs during mRNA translation.

6. PERSPECTIVE: POTENTIAL FOR DIFFERENTIAL FUNCTION OF ALTERNATIVELY SPliced ISoFORMS OF TFPI

There is accumulating evidence that alternative splicing of TFPI produces physiologically relevant changes in TFPI activity. While all TFPI isoforms have the first two Kunitz domains and are capable of inhibiting TF-fVIIa and fXa catalytic activity, it is unclear whether they have relative equal inhibitory activity in vivo or whether they differentially inhibit TF procoagulant and pro-inflammatory activities. The evolutionary conservation of TFPIalpha and its conserved production by mouse embryo, placenta and platelets suggests that the third Kunitz domain and basic C-terminal region may have specific functions during vascular development and/or wound healing not performed by the other isoforms. The third Kunitz domain and C-terminal region of soluble TFPIalpha directly interact with fXa, perhaps partially explaining the enhanced anticoagulant activity of TFPIalpha compared to truncated forms of TFPI in solution phase assays. The anticoagulant activity of TFPIalpha is enhanced through interactions between the third Kunitz domain and protein S that produces enhanced inhibition of fXa by the second Kunitz domain, but not of TF-fVIIa by the first Kunitz domain in solution phase assays. Further studies are needed to determine how protein S may enhance the activity of surface associated TFPIalpha. This enhancing effect of protein S on inhibition of fXa was not observed in mouse plasma, a finding that is consistent with TFPIbeta as the major alternatively spliced form of TFPI in adult mice.

Several studies using in vitro and in vivo assays have reported physiological activities of peptides corresponding to the basic C-terminal region of TFPI that occur independent of TFPI anticoagulant activity further demonstrating that TFPIalpha may have unique functions not performed by the other isoforms. These include studies demonstrating that the TFPIalpha C-terminal peptide inhibits endothelial cell proliferation in vitro as well as primary and metastatic tumor growth in vivo. The C-terminal peptide has also been shown to have complement dependent antibacterial activity. It is important to note that these studies have been performed using soluble forms of TFPI. Yet, most TFPI within the body is associated with cell surfaces which can greatly alter its activity. Further studies of the biological activity of the different alternatively spliced isoforms associated with cell surfaces are needed to understand their physiological functions.

7. REFERENCES

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