

Human tissue-type plasminogen activator

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Summary

Tissue-type plasminogen activator (t-PA) plays an important role in the removal of intravascular fibrin deposits and has several physiological roles and pathological activities in the brain. Its production by many other cell types suggests that t-PA has additional functions outside the vascular and central nervous system. Activity of t-PA is regulated at the level of its gene transcription, its mRNA stability and translation, its storage and regulated release, its interaction with co-factors that enhance its activity, its inhibition by inhibitors such as plasminogen activator inhibitor type 1 or neuroserpin, and its removal

by clearance receptors. Gene transcription of t-PA is modulated by a large number of hormones, growth factors, cytokines or drugs and t-PA gene responses may be tissue-specific. The aim of this review is to summarise current knowledge on t-PA function and regulation of its pericellular activity, with an emphasis on regulation of its gene expression.

Keywords

Plasminogen activators, gene regulation, endothelial cells, nervous system

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Introduction

Tissue-type plasminogen activator (t-PA) is a serine protease that converts the proenzyme plasminogen into the proteinase plasmin. It is responsible for removal of intravascular fibrin deposits and exerts important functions in the brain and elsewhere. The ability of fibrin to specifically accelerate plasminogen activation by t-PA has led to its widespread use as a fibrin-specific thrombolytic agent for myocardial infarction and stroke (1, 2). The activity of t-PA in the pericellular space or in the blood circulation is controlled at six different levels, as presented in more detail below (► Table 1).

In this review we will summarise current knowledge on established functions for t-PA, the regulation of its pericellular activity, with an emphasis on regulation of human t-PA gene expression and the mechanisms influencing plasma t-PA concentrations and its relation to cardiovascular disease.

t-PA function

Depending on its site of expression, many distinct functions have been proposed for t-PA (► Table 2). Experimental evidence for the role of t-PA in the vascular system and in the central and peripheral nervous systems and for prevention of peritoneal adhesions appears to be conclusive. For other functions evidence is circumstantial and more work needs to be done to arrive at solid conclusions. Well-established functions proposed for t-PA are summarised below.

Fibrinolysis

The main function of t-PA within the vascular system is the removal of fibrin (3). Thus, in t-PA deficient mice, clot lysis was impaired, in particular when combined with urokinase deficiency (4, 5). In experimental primate models of acute disseminated intravascular coagulation, plasma concentrations of t-PA can acutely increase by more than two orders of magnitude, due to thrombin-mediated release from storage granules in endothelial cells (EC) (6, 7). The high local increase of t-PA precisely at the site of thrombin generation allows the efficient removal of fibrin deposits at the luminal side of an intact vascular endothelium and is important for reducing tissue damage.

Angiogenesis

Secretion of t-PA by EC is increased by vascular endothelial growth factor and by basic fibroblast growth factor and, in combination, these growth factors have a synergistic effect (8). As plasminogen activators contribute to angiogenesis (9) it is likely that t-PA contributes as well. Direct evidence for a role for t-PA in angiogenesis was obtained in a pancreatic tumor model, where antisense reduction of t-PA expression diminished tumour angiogenesis (10).

Central nervous system

t-PA is expressed by neurons, microglial cells, astrocytes and cerebral EC. Diverse physiological and pathological roles have been

Table 1: Mechanisms regulating t-PA activity in the pericellular space. Activity of t-PA is regulated by different mechanisms that act on at least six different levels.

1. t-PA gene transcription.
Agonists that affect t-PA gene transcription and/or mRNA turnover (for more information and references see Supplementary Table).
<ul style="list-style-type: none"> • Histone deacetylase inhibitors (butyrate, trichostatin, valproic acid, MS275). • Statins. • Thrombin. • Growth factors: VEGF, bFGF, TGFβ. • Hormones: dexamethasone, retinoic acid, estrogen. • Inflammatory agonists: TNF, IL-1, LPS. • cAMP inducers: vasopressin, adrenalin, epoxyeicosatrienoic acid.
2. mRNA stability, activation of t-PA mRNA translation and micro-RNA mediated translation inhibition.
3. Storage and regulated release of t-PA.
Storage of t-PA in Weibel Palade bodies in EC and in small regulated storage granules in EC, astrocytes and neuronal cells.
Secretagogues induce t-PA release via an increase in:
<ul style="list-style-type: none"> • intracellular calcium: thrombin, histamine, bradykinin, calcium ionophore. • intracellular cAMP: vasopressin, adrenalin, forskolin + IBMX.
4. Proteins that bind t-PA and plasminogen and enhance t-PA activity by >100 fold.
<ul style="list-style-type: none"> • Fibrin. • Cell surface proteins: annexin II, the voltage-dependent anion channel. • Extracellular matrix proteins. • Beta2-glycoprotein 1. • Denatured proteins, beta amyloid peptides.
5. Inhibition of t-PA by serine proteinase inhibitors.
<ul style="list-style-type: none"> • PAI-1, the principal inhibitor of t-PA. • Neuroserpin, a major t-PA inhibitor in the brain. • Other serpins: Protease nexin 1, PAI-2, a2-antiplasmin.
6. Clearance receptors.
Hepatic clearance, responsible for the short (~ 5 minutes) circulating half-life of t-PA.
<ul style="list-style-type: none"> • LRP1 on hepatocytes • Mannose receptor on liver EC
Clearance from the extracellular space
<ul style="list-style-type: none"> • LDL-receptor family members, LRP1, VLDL receptor • Mannose receptor
EC: endothelial cells; VEGF: vascular endothelial growth factor; bFGF: basic fibroblast growth factor; LDL: low-density lipoprotein; LRP1: low-density lipoprotein receptor-related protein 1; TNF: tumour necrosis factor alpha; IL-1: interleukin 1; LPS: lipopolysaccharide.

proposed for t-PA in the brain: some related to its plasminogen activator activity, some plasmin-independent. In particular, t-PA contributes to synaptic plasticity, learning and long term potentiation and, during development, to neuronal cell migration. Pathological phenomena to which t-PA contributes include glutamate-induced cytotoxicity, disruption of the blood brain barrier or seizures. A full discussion of the many roles of t-PA in the central nervous system is outside the scope of this review and the reader is referred to several excellent recent reviews (11-18).

Peripheral nervous system

In a model of sciatic nerve crush injury, t-PA protected from axonal degeneration and demyelination, by removing fibrin deposits near the damaged nerves (19). Vascular sympathetic neurons store t-PA and release t-PA in response to bradykinin and phenylephrine (20, 21). In rats, chemical sympathectomy abolished nerve fiber associated t-PA, while maintaining endothelial t-PA; it strongly reduced plasma t-PA and t-PA release from isolated blood vessels (20, 21). Thus, vessel-associated sympathetic neurons contribute to t-PA release into the blood circulation. Conversely, t-PA plays a role in sympathetic nerve function because t-PA inhibition reduced sympathetic responses of the guinea pig vas deferens and t-PA null mice were deficient in norepinephrine release. This was independent of plasminogen and attenuated by plasminogen activator inhibitor type 1 (PAI-1) (22, 23).

t-PA and peritoneal adhesions

Intraperitoneal adhesions are a common complication of abdominal surgery. An imbalance between coagulation activation and fibrinolysis is responsible for excessive fibrin deposition followed by infiltration with fibroblasts and collagen formation. Inflammatory activation of peritoneal mesothelial cells increased tissue factor and PAI-1 and reduced t-PA expression; statin treatment increased t-PA and reduced PAI-1, resulting in reduced adhesion formation in a rat laparotomy model (24-26).

Agonists modifying t-PA expression in cultured cells or modifying plasma t-PA antigen concentrations

The regulation of t-PA gene transcription has been investigated in cultured primary human cells, in human foreskin and amnion explant culture and in human transformed cell lines. To date more than 30 different agonists have been identified that modify t-PA gene expression (► Table 3 and Suppl. Table 1, available online at www.thrombosis-online.com). It has to be stressed, however, that for most of these agonists, information on their *in vivo* effect in human plasma or human tissues is lacking. Some well-studied agonists will be treated in more detail below.

Table 2: Proposed functions for t-PA.

In the vascular system	
Cell Type	Proposed function of t-PA
Endothelial cells	Storage and regulated release of t-PA; binding of t-PA to fibrin, followed by fibrin degradation (3, 4, 6, 7)
	Angiogenesis (8–10) reviewed in: (11–13, 15–18)
In the central nervous system	
Cell Type	Beneficial effects of t-PA
Neurons (a,b,c,d,e,h,i)	a) Synaptic plasticity
Microglial cells (e,f,g,i)	b) Learning and long term potentiation
Astrocytes (g,i,j)	c) Neuronal cell migration
Cerebral EC (j)	d) Anti-apoptotic (activation of pro-neurotrophins)
	Pathological effects of t-PA
	e) Excitotoxicity and pro-apoptotic effects
	f) Demyelination
	g) Cerebral inflammation
	h) Alzheimer's disease
	i) Seizures
	j) Disruption of the blood brain barrier
In the peripheral nervous system	
Cell Type	Proposed function of t-PA
Peripheral nervous cells	Protection from axonal degeneration and demyelination (19)
	Regulated release of t-PA which may contribute to acute release of t-PA in the blood circulation (20, 21).
	Sympathetic responses (22, 23).
In the peritoneum	
Cell Type	Proposed function of t-PA
Peritoneal mesothelial cells	Prevention of intraperitoneal adhesion formation after injury (24–26).
NB. The strict division of the beneficial and pathological effects of t-PA in the central nervous system with respect to each cell type may be more complicated than presented here and requires further investigations. Expression of t-PA by many other cell types (monocytes, fibroblasts, keratinocytes, myocytes, gingival cells, dental pulp cells, retinal cells, hepatocytes and kidney cells, see Suppl. Table, available online at www.thrombosis-online.com) suggests that t-PA may have additional functions outside the vascular system, the central or peripheral nervous system or the peritoneum. The in vivo role, if any, of t-PA expressed by these cell types, still needs to be established.	

Protein kinase C

Ligand binding to Gq coupled receptors leads to an increase in intracellular Ca^{++} and to formation of diacylglycerol, an activator of protein kinase C which plays key regulatory roles in a multitude of cellular processes (27). The effect of protein kinase C activation on t-PA expression has been studied mostly by using phorbol ester (PMA), which mimics diacylglycerol. Treatment of EC, melanoma cells, non-small cell lung carcinoma cells, astrocytes or HeLa cells with (PMA), increased t-PA expression. The induction of t-PA by PMA was dependent on protein synthesis and preceded by a transient increase in c-fos (28, 29). Forskolin,

an inducer of intracellular cAMP potentiated the t-PA gene response to PMA (28, 30) in EC. In contrast, PMA downregulated t-PA gene transcription in HT1080 fibrosarcoma cells (31) and had no effect on t-PA in peritoneal mesothelial cells (24). The cell-type specific responses of the t-PA gene to PMA treatment may be due to differences in transcription factor binding to the proximal promoter (see below).

Inflammatory agonists

The profound effects of inflammation on haemostasis are well known. Inflammatory cytokines such as tumour necrosis factor

(TNF) or interleukin 1 (IL-1), IL-6 or the bacterial product lipopolysaccharide (LPS) exert strong procoagulant effects characterised by an increase in tissue factor and a decrease of thrombomodulin on endothelial cells. The effect of these inflammatory agonists on t-PA expression was found to be cell-type specific.

TNF reduced t-PA in human HT1080 cells (32) and in human umbilical vein EC (HUVEC), dependent on signalling via nuclear factor (NF)κB and p38 (33, 34). It increased t-PA expression in human peritoneal mesothelial cells (24) and gingival fibroblasts (35).

IL-1α suppressed t-PA in human peritoneal mesothelial cells (24) but increased t-PA in human gingival fibroblasts (35), in osteosarcoma cells (36) and in primary mesangial cells (37). The effect of IL-1α was dependent on MEK, p38 and PI3 kinase (36, 38).

IL-1β suppressed t-PA in HUVEC (39). This effect was dependent on NFκB, but not on p38 (39).

IL-6 induced a threefold increase in t-PA plasma concentrations in baboons (7, 40). It increased t-PA in dental pulp cells (41), but had no effect on t-PA production by HUVEC (39). As cultured HUVEC express no IL-6 receptor the effect of addition of soluble IL-6R was studied: addition of sIL-6R rather decreased t-PA (39).

LPS suppressed t-PA in human peritoneal mesothelial cells (24) and increased t-PA in gingival fibroblasts (42) and in microglial cells (43). Experimental endotoxaemia in human volunteers led to an increase in plasma TNF concentrations, which was followed by an increase in plasma IL-6 and t-PA (44). This most likely represents a hierarchy in which LPS first increases TNF, which stimulates IL-6 production leading to an increase in plasma t-PA, because TNF is capable of increasing IL-6 and t-PA (45) and IL-6 increases t-PA (see above).

Taken together, the effects of inflammatory agonists on t-PA expression are cell-type – and agonist – specific. In addition, inflammatory agonists may affect t-PA activity by increasing expression of PAI-1, the principal t-PA inhibitor (46).

Retinoids

Retinoids play an important role in cell differentiation and development. In most cell types studied, except keratinocytes and peritoneal mesothelial cells, retinoids increase t-PA expression. A requirement for de novo protein synthesis for RA-induction of t-PA gene transcription was shown for EC (47, 48), HT1080 fibrosarcoma cells (49) and human astrocytes (50). In EC, the increase in t-PA was dependent on a two-step mechanism: RA first increases production of RA receptor β (RARβ); thereafter RA increases t-PA via RARβ (47). RA interacts with PMA in a dose dependent manner. At moderate doses RA increased t-PA and synergistically potentiated the stimulatory effects of PMA in EC and HeLa cells, but at higher dosages RA reduced t-PA expression in HeLa cells, but not in EC (51). In rats, retinoic acid increased plasma t-PA activity and t-PA antigen in lung extracts by 1.5- and 1.3-fold, respectively. Conversely, a vitamin A deficient diet led to a reduction in plasma t-PA activity and t-PA antigen in lung extracts (48).

Statins

These cholesterol-lowering drugs are widely used in the prevention of cardiovascular disease. Their therapeutic effect is not only due to cholesterol lowering, but also to pleiotropic effects related to their inhibition of protein geranylgeranylation (52). Among the pleiotropic effects are a 10-fold increase of t-PA mRNA and a five-fold reduction in PAI-1 mRNA levels in human EC, smooth muscle cells, peritoneal mesothelial cells, and cardiac myocytes. The effect of statins is mediated by geranylgeranylated proteins of the Rho family of small GTPases (53), in particular of Cdc42 and Rac1 (54). Statins modify the structure of the cytoskeleton and disruption of actin filaments by latrunculin or cytochalasin D strongly increased t-PA (25, 53, 54). This suggests a direct relation between cytoskeletal function and t-PA expression. The statin-in-

Table 3: Agonists that modify t-PA gene expression in cultured human cells*.

Cytokines	IL-1, IL-4, IL-6, TNF
Growth factors	TGFβ, EGF, VEGF, BDNF
Hormones	Estradiol, Dexamethasone, Retinoic acid, Vitamin D3
Toll-like receptor agonists	LPS, Poly I:C
cAMP inducers	8-bromo-cAMP and forskolin (synergy with PMA and EGF)
Ca ²⁺ inducers	Thrombin, Bradykinin
Protein kinase C activator	PMA
Product of phospholipase A2	Lysophosphatidylcholine
Epigenetic agonists	HDAC inhibitors: Butyrate, trichostatin, valproic acid, MS-275
	HAT inhibitors: garcinol, anacardic acid,
	Sirtuin agonists: Resveratrol, Quercetin
Drugs	Statins, Rapamycin, Paclitaxel
Actin polymerization inhibitors	Cytochalasin D, Latrunculin
Shear stress	Effect depends on type and intensity of shear stress
Epoxygenase product	EET
Radiation	UV, Ionizing radiation
Heat shock	
Various agonists	Fibrin, Ethanol, Glucose, HT-VLDL
	Protease peptone, Notoginsenoside R1
	Sonic Hedgehog

*For details and references see the Supplementary Table. BDNF: brain-derived neurotrophic factor (BDNF); EET: epoxyeicosatrienoic acid; EGF: epidermal growth factor; HDAC: histone deacetylase; HAT: histone acetyl transferase; HT-VLDL: hyper-triglyceridemic very low density lipoproteins; IL: interleukin; LPC: lysophosphatidylcholine; LPS: lipopolysaccharide; PMA: phorbol myristate acetate; Poly I:C : polyinosinic:polycytidylic acid; RA: retinoic acid; TGFβ: transforming growth factor beta; VEGF: vascular endothelial growth factor.

duced increase of t-PA in EC was dependent on activation of p38 (54). In contrast, prolonged statin treatment of hypercholesterolaemic patients or patients with diabetes type 2 or chronic kidney disease did not modify plasma t-PA antigen concentrations (55-58). At the present state it is not known whether these discrepancies are due to artefactual responses of cells in culture or are caused by the isolation of the cells from their natural stroma or from the paracrine and endocrine effects of their in vivo environment.

Epigenetic mechanisms regulating t-PA expression

All cells in an organism have the same DNA, whereas gene responses are cell-type specific. Whether a gene is inactive or active, and to what extent, depends on its epigenetic state, which is determined by histone modifications and CpG methylation. Epigenetic mechanisms may be important for regulating t-PA gene expression. Early studies observed that dibutyl cAMP increased t-PA secretion by EC, by liberating butyrate rather than via cAMP (59). Butyrate as well as other histone deacetylases (HDAC) inhibitors (trichostatin, MS-275, valproic acid) consistently increase t-PA production and storage in EC (60-63). t-PA is among the proteins most strongly induced by HDAC inhibition (63). Treatment with HDAC inhibitors increased the acetylation state of histones 3 and 4 associated with the t-PA promoter (62, 63) thereby modifying the t-PA associated chromatin into an active conformation. Expression of t-PA appears to require an unmethylated state of its proximal gene promoter. Indeed, in EC, which express t-PA, the proximal t-PA promoter is unmethylated, whereas in human pri-

mary hepatocytes, which express t-PA at low levels, it is partially methylated (62).

Promoter and enhancer elements in the t-PA gene

The t-PA gene has at least two distinct transcription initiation sites (TIS), leading to mRNA transcripts with 203 and 103 5'non-translated nucleotides, respectively (► Figure 1). The longer transcript was used in PMA stimulated HeLa cells and the shorter preferentially in a HUVEC derived cell line and in WI-38 human fetal lung fibroblasts (64-66). The position of promoter and enhancer elements described below is with respect to the TIS of the longer transcript (position 42'065'194 on chromosome 8 in GRCh37/hg19; <http://genome.ucsc.edu/>) (67).

Proximal promoter

Several regulatory elements have been identified in the proximal t-PA promoter (► Figure 1). The PMA responsive element, TRE), at position -115/-102, is important for basal and PMA-induced t-PA expression (68). A distinct profile of TRE binding proteins was observed for HT1080 cells and HeLa cells. In HT1080 cells, CREB and jun, but not CREM or ATF2, bound the TRE, whereas in HeLa cells TRE binding proteins were ATF2, junD, fosB and fra-2 but not CREB (66, 69). Treatment of HT1080 cells with PMA induced CREB phosphorylation and its binding to the TRE, while overexpression of CREB in HeLa cells reduced the magnitude of PMA mediated induction of t-PA (70). This suggests that down-regulation of t-PA transcription by PMA in HT-1080 cells requires CREB binding to the TRE.

Figure 1: Structure of the t-PA promoter. The figure shows the position of alternative transcription initiation sites (TIS) (doubly underlined) and transcription factor binding sites (underlined) in the t-PA gene promoter region. t1 represents the principal t-PA TIS in PMA stimulated HeLa cells (64), whereas t2 is the principal TIS in non-stimulated WI-38 human fetal lung fibroblasts major position at +110 (65) and in a HUVEC-derived cell line (66). t3 and t4 represent alternative, minor TIS. Sequences of the first and second exon are in bold type. Transcription factor binding sites and the TATA box are underlined. Position of CpG dinucleotides are shown in grey. In HUVEC all CpG's in the region -647 to -366 are fully methylated whereas the CpG's in the region -121 to + 159 are unmethylated (62). Note that most of the transcription factor binding sites contain or are closely flanked by a CpG dinucleotide. The TIS t1 is located at chromosome 8, position 42'065'194 (Assembly GRCh37/hg19; <http://genome.ucsc.edu/>).

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TACTGAAAGGCTTCGGTGGGCTTAAGGGCTGATGGCTTTGATCGAATTTACGGCATGTTG -601
GCCCCAAGGCCCTGTGTATATTCCCTGGGCCCACTCAAGGGGATGCTGGAGCCGGAAAGT -541
CCCCTGGAGGCCACCTACTGCAGCCCTGCACCTTTACAAAGAAGAGAAAGATTCTCCCTAAA -481
ATTACAGAACAGGGCCAAAGATGCCTACCGGAGCAAACCCCATGGGGGCACCTCCTACC -421
                                     GC Box
GCAGGTGAGCCCAAGGCTGGTCTGCCTTCTCAGTGGCTACCCCCCTGAGCTCCCGCCAC -361
CACACAAAGTGTTCGAATCCTTGTGCATCCTCCAGTCCTTTTAACTCTCATGTCTGAG -301
AGGCCAGAGCTACAGCCACAGATTCCAGAAGACACCCCACTCCAGCCCCAACCTGCTGC -241
CTTTAGAATTATAACACTTCTTGTGCATCACAGGGTCTGAAAGTCCCTTTTAAAGCCTGG -181
GACACTAGGACTCTAAAGGAAGATGATTCTTAAGGTCCCACTCCCAATTCTCTGC -121
TRE/CRE                                CTF/NF1
GATTCAATGACATCACGGCTGTGAATAATCAGCCTGGCCCGAAGCCAGGATGGGCTGTGC -61
                                     GC box
TGCTTCCACCGTGAACCTCCTCCCTGCTTTATAAAACAGGCCTGCCTCAGCTCCCTC -1
t1                                     GC box
ATGGCCCTGTCCACTGAGCATCTCCCGCCACACAGAAACCGCCAGCCGGGGCCACCG +60
                                     GCBox      ELK-1 t3                                t2
ACCCACCCCTGCCTGGAACTTAAAGGAGGCCGAGCTGTGGGAGCTCAGAGCTGAG +120
                                     t4
ATCCTACAGGAGTCCAGGGCTGGAGAGAAAACCTCTGCGAGGAAAGGAAGGAGCAAGCC +180
GTGGTAGG - - - 14 kb first intron - - - TCCAGAATTTAAGGACGCT
start codon
GTGAAGCAATCATGGATGCAATGAAGAGAGGGCTCTGCTGTGTGCTGCTGTGTGGAG
CAGTCTTCGTTTCGCCAGCCAGGTTGG - - - second intron

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GC boxes bind transcription factors of the Sp1 family. The GC boxes at +39 and +62 bind Sp1 (66, 69). Of these, the latter is important for basal and PMA induced t-PA expression in HeLa cells (68). In HT1080 cells, both Sp1 and Sp3 bind to the GC boxes at +39 and +62; both GC boxes were important for basal and RA-induced t-PA expression, whereas the TRE did not contribute to t-PA induction by RA (71). Induction of t-PA expression by quercetin was dependent on an Sp1 binding site located elsewhere at -365; inhibition of p38 reduced Sp1 binding to this site (72).

An NF1/CTF binding site, located 20 bp downstream of the TRE (69) exerts repressor activity in human EC and HeLa cells (73). An ELK1 site at +80 mediated the dexamethasone-induced downregulation of t-PA in breast cancer cells (74).

Enhancers located between -2.1 and -3.1 kbp

In Bowes melanoma cells enhancer elements were identified in the region between -2390 to -2129 and a repressor between -2129 and -2119 (75). The enhancer between -2288 to -2129 appeared to function in a cell type-specific fashion, because it was inactive in HeLa cells and HT1080 cells.

An NFκB binding motif was identified at position -3081. It contributed to PMA-induced upregulation of t-PA in neuroblastoma cells and to TNF- and IL-1β-mediated downregulation of t-PA in EC (33, 76).

Multihormone enhancer region at -7 kb

Induction of the t-PA gene by RA is mediated by a direct repeat of a GGTC A motif spaced by five nucleotides (DR5) at -7.3 kb (49). This motif is part of a multihormone responsive enhancer composed of four binding sites for the glucocorticoid receptor (77). RA treatment enhanced Sp1/Sp3 binding to a GC-box located at position -7351, which is associated with the release rate of t-PA *in vivo* (78). Formation of the complex of Sp1/Sp3 with this GC-box was inhibited by anti-RA receptor antibodies. This implies that Sp1/Sp3 and the RA receptor directly interact (79).

In WISH epithelial cells the RA-induced increase in t-PA occurred through interactions of heterodimers of the retinoid receptors, RARα or RARβ and RXRα, with the DR5 (80). At an early time period binding was preferentially to RARα/RXRα heterodimers and at later time points to RARβ/RXRα heterodimers (80).

Transient transfection of reporter genes, linked to the t-PA gene promoter and multihormone enhancer sequences in HT1080 cells revealed that Sp1 and Sp3 activate the t-PA promoter, but that the effect of retinoic acid required Sp1, but not Sp3 (71).

Potential regulatory elements elsewhere in the t-PA gene

In addition to the known regulatory elements described above, DNase I-hypersensitivity mapping and *in vivo* foot printing in human neuroblastoma and glioblastoma cells identified several

potential regulatory elements elsewhere within the t-PA gene (76). Analysis of the t-PA gene using the UCSC genome browser (<http://genome.ucsc.edu>) (67) illustrates the complexity of these potential regulatory regions. Suppl. Figure 1A (available online at www.thrombosis-online.com) shows the profile of histone modifications (H3K4me1, a marker for promoters and enhancers; H3K4me3, a marker for promoters, and H3K27Ac, a marker for active gene regions). In addition, it shows transcription factor binding sites, DNase hypersensitivity sites and RNA polymerase 2 (pol2) binding sites. The figure clearly identifies the proximal promoter, the enhancers at -2 to -3 kbp and the multihormone enhancer. In addition, a broad 5 kbp region in the first intron contains enhancer associated modifications. The pattern of histone modifications is cell-type specific, as illustrated by Suppl. Figure 1B (available online at www.thrombosis-online.com). To what extent this putative enhancer contributes to cell-type specific expression and agonist-mediated up- or down-regulation of the t-PA gene remains to be established. Other transcription factor binding regions are observed elsewhere in the t-PA gene.

Promoter/enhancer elements regulating t-PA expression *in vivo* in mice

Little is known about the promoter/enhancer elements that dictate tissue-specific expression of t-PA. Studies in transgenic mice expressing lacZ under control of the 5' flanking sequences of the t-PA gene revealed that elements between -9.5 kb and -3.0 kb are required to direct spatial and LPS-inducible expression to various regions of the mouse brain, while elements within the first 1.4 kb of the t-PA promoter direct expression exclusively to the medial habenula (81). None of the constructs directed lacZ staining to vascular tissue. We may, therefore, assume that elements required for EC-specific expression of t-PA are located elsewhere.

Post-transcriptional mechanisms regulating t-PA activity

Besides regulatory mechanisms based upon changes in t-PA gene transcription rate, t-PA activity in the pericellular environment is regulated by five additional post-transcriptional mechanisms (► Table 1), as summarised below.

mRNA turnover or translation

The t-PA mRNA contains in its 3' non translated region a highly conserved AU-rich element; removal of this region reduced t-PA mRNA turnover (82). There is also evidence for a mechanism of t-PA mRNA translation inhibition depending on a target site located in the same region (82). This suggests that t-PA mRNA translation is regulated by miRNAs, which inhibit mRNA translation through interactions with the 3' end of an mRNA. However, the effect of miRNAs on t-PA mRNA translation has not been reported yet.

The 3' untranslated region of t-PA contains furthermore a polyadenylation control element (ACE) (83). In mouse oocytes this element was responsible for deadenylation and translational silencing of the t-PA mRNA. During meiotic maturation, t-PA mRNA is re-polyadenylated and t-PA translation resumes. A mouse strain was created which expresses the enhanced green fluorescent protein (EGFP) coding sequence followed by the ACE element (84). High levels of EGFP mRNA was detected in all brain structures, but EGFP fluorescence was detected only in blood vessels, choroid plexus and Purkinje cells, suggesting cell-type specific translation inhibition. EGFP fluorescence increased after brain injury, presumably by release of translational inhibition. Similarly, glutamate induced a rapid increase in t-PA activity in hippocampal neurons resulting from translational activation of preexisting t-PA mRNA (85). These results imply that polyadenylation control of t-PA mRNA plays an important role in t-PA responses to brain insults.

Regulated release of t-PA from intracellular storage granules

An important aspect of t-PA function is its storage and regulated release. An early study in a rat hind leg model showed that platelet activating factor (PAF) and bradykinin were capable of inducing within a few minutes, an acute and transient simultaneous release of t-PA and von Willebrand factor, by increasing intracellular Ca^{++} (86, 87). Activation of guanylate cyclase reduced the PAF-induced release of t-PA and vWF (88). An alternative mechanism depends on cAMP. Indeed, isoproterenol, a beta-adrenergic agonist, or desmopressin, acting upon the V2 vasopressin receptor induced, via cAMP, an acute increase in plasma t-PA in vivo in man (89-92).

Acute t-PA release allows for a rapid increase in local t-PA concentrations. In chimpanzees and baboons injection of factor Xa and phospholipids led to wide-spread thrombin generation and a hundred-fold increase in plasma concentrations of t-PA (6, 7). This implies that blood vessels contain a large storage pool of t-PA. Two cell types may contribute: EC and vascular sympathetic neurons. In cultured EC, there is evidence for more than two types of t-PA storage granule: Weibel Palade bodies (61, 93, 94) and small storage granules (95-97). Both storage pools are released by agonists that increase intracellular Ca^{++} (thrombin, histamine, bradykinin, calcium ionophore) or cAMP (61, 86, 91, 93-98). Vascular sympathetic neurons may also contribute to acute increases in plasma t-PA. Indeed, these cells release t-PA in response to bradykinin or phenylephrine (20, 21). The acute release of t-PA by astrocytes suggests a similar mechanism of acute increases of extracellular t-PA in the central nervous system (50). In hippocampal neurons t-PA resides in dense-core granules (DCGs) that traffic to postsynaptic dendritic spines. Depolarisation by high potassium levels led to a Ca^{++} dependent release of t-PA (99).

Activation of t-PA by fibrin, extracellular matrix proteins, cell surface receptors or amyloid proteins

Most serine proteases need to be converted from a single chain pro-enzyme into a fully active two-chain form. In contrast, the

single chain and two chain forms of t-PA have a similar, low activity. The main activating event appears to be the binding of t-PA to cofactor proteins. For thrombolysis, the cofactor is fibrin itself, which increases t-PA activity by more than two orders of magnitude (100). This, in combination with the regulated release of t-PA from the vessel wall induced by thrombin assures that fibrinolysis is targeted towards forming fibrin deposits. Activation of t-PA by extracellular matrix proteins (101) may be of relevance for t-PA-mediated matrix degradation in the context of cell migration. Binding of t-PA to annexin 2 at the cell surface increases t-PA activity by two orders of magnitude (102). The activation of t-PA by beta-amyloid peptides, as well as the presence of t-PA in brain tissue, suggests that t-PA may play a role in Alzheimer's disease (103). Activation of t-PA by misfolded proteins in general, may facilitate removal of such proteins (104).

Inhibition by proteinase inhibitors

The principal inhibitor of t-PA is PAI-1 (105, 106). It inhibits single chain and two chain t-PA with high efficacy (107). PAI-1 is expressed by almost all cell types in culture and is, like t-PA, regulated by a large number of agonists. Among these are inflammatory agonists, transforming growth factor beta, hypoxia, insulin and statins (46, 108). Thus, t-PA activity can be regulated at the level of agonist-mediated changes in t-PA expression, in PAI-1 expression or both. PAI-1 plays a role in many clinical conditions, such as inflammation, sepsis, the metabolic syndrome, fibrosis and cancer. The physiological and pathological roles of PAI-1 are discussed in more detail elsewhere (109-112).

The brain contains a second t-PA inhibitor: neuroserpin (113-115). The relative importance of PAI-1 and neuroserpin in regulating brain t-PA activity is not known, but likely depends on the local cellular context and local physiological or pathological conditions. Neuroserpin and t-PA are co-expressed in neurons of many central nervous system regions (116). In patients with acute ischaemic stroke neuroserpin may have neuroprotective effects by inhibition of excitotoxicity, inflammation and blood brain barrier (117).

Other protein capable of t-PA inhibition are PAI-2, α_2 -antiplasmin and α_2 -macroglobulin. Based upon criteria of inhibitor concentration and inhibition rate constants, regulation of t-PA activity by these inhibitors appears to be unlikely of physiological relevance.

Clearance receptors

These play an important role in t-PA removal from the blood circulation, with a half-life of only a few minutes, and from the pericellular space. Two receptors may mediate t-PA clearance: the low-density receptor-related protein 1 (LRP1) (118, 119) and the mannose receptor (120). LRP1 not only functions as a clearance receptor for free t-PA and t-PA/PAI-1 complexes, it may also function as a signalling receptor (121, 122).

Mechanisms influencing plasma t-PA concentrations and relation to cardiovascular disease

Circadian variation of plasma t-PA

Acute myocardial infarction and stroke have a circadian pattern of occurrence with a peak in the morning (123). Considering the powerful capacity of t-PA to remove non-desired fibrin deposits, the circadian variation of plasma t-PA and PAI-1 antigen was investigated. Results of several studies showed a parallel circadian variation, with highest values in the morning (t-PA: 5 ng/ml and PAI-1: 21.9 ng/ml) and minima in the evening (t-PA: 3 ng/ml and PAI-1: 8.8 ng/ml) (124-126). As PAI-1 is in excess of t-PA, the effect of the circadian variation of t-PA and PAI-1 is that plasma t-PA activity is lowest in the morning (1 U/ml) and highest in the evening (1.8 U/ml) (125). It has to be stressed, however, that t-PA activity measured in these studies represented residual t-PA activity after reaction of t-PA with PAI-1 *in vivo* within the blood circulation and *ex vivo* during blood handling, centrifugation and, often, freezing and thawing. For a proper assessment of t-PA activity, blood samples should be collected on an acid anticoagulant, which prevents *ex vivo* inhibition of t-PA by PAI-1 (127). In mice, chronic time shifts reduced liver t-PA mRNA, but increased liver PAI-1 mRNA and plasma PAI-1 level (128). No information is presently available on the genes responsible for the circadian variation of t-PA, whereas the genes Clock and BMAL are known to contribute to circadian regulation of PAI-1 (129). It remains to be established to what extent the parallel circadian variation of t-PA and PAI-1 reflects common mechanisms or is fortuitous. One factor may be the effect of PAI-1 on hepatic t-PA clearance. In human subjects, clearance of injected free t-PA ($t_{1/2} = 2.4$ min) was faster than that of t-PA/PAI-1 complexes ($t_{1/2} = 5.0$ min). Also, clearance of free t-PA was faster in patients with low plasma PAI-1 activity than in patients with high PAI-1 activity ($t_{1/2} = 3.5$ and 5.3 min, respectively) (130).

Seasonal variation of plasma t-PA levels

In addition to its diurnal variations, plasma t-PA antigen concentrations also show a seasonal variation with lowest concentrations in late spring (3.5 ng/ml) and highest concentrations in autumn (5.6 ng/ml) (126).

Age and exercise

Release of t-PA is reduced with age in sedentary men. The age-related decline was not observed in endurance-trained men and exercise training normalised t-PA release in former sedentary men (131). This implies that exercise is a more important factor determining t-PA release than age.

Gene polymorphisms related to plasma t-PA concentrations

Initial studies on gene polymorphisms modifying the release rate of t-PA from the forearm vascular bed identified an Alu repeat insertion/deletion polymorphism located in intron 8 of the t-PA gene. Subjects homozygous for the insertion had a higher release rate (mean: $10.9 \text{ ng min}^{-1} \cdot \text{L}^{-1}$) than heterozygotes and homozygotes for the deletion (mean: 4.5 and $0.9 \text{ ng min}^{-1} \cdot \text{L}^{-1}$, respectively) (78). Later studies observed that the Alu polymorphism is in linkage disequilibrium with a C>T polymorphism located at position -7351 in the multihormone response enhancer (132). The C variant had a 10-fold greater affinity for the transcription factors Sp1 and Sp3 and, in reporter gene assays, a greater response to retinoic acid than the T variant (79). This polymorphism thus explains the relation between Alu polymorphisms and the release rate of t-PA. In human brain tissue more t-PA mRNA was generated from the C-allele than from the T allele (133).

The relation between t-PA gene polymorphisms and myocardial infarction or stroke is controversial with some studies finding a relation (134-136) and others not (137, 138). Plasma t-PA concentrations correlate with polymorphisms in the genes for several renin-angiotensin system proteins, suggesting that this system modulates t-PA expression *in vivo* (139, 140).

Mechanism of the effect of estradiol on plasma t-PA

The inverse relation between estradiol and plasma t-PA antigen concentrations can be explained by its effect on t-PA clearance. Indeed, clearance of t-PA was significantly faster in estradiol treated mice than in control mice; the difference in the two treatment groups was reduced by mannan, which inhibits mannose receptor mediated t-PA clearance, and not by RAP an inhibitor of LRP-1 mediated clearance (141). An estradiol-mediated increase in mannose receptor, and not in LRP-1 was observed.

Correlation of plasma t-PA with cardiovascular risk factors

In healthy controls, plasma t-PA antigen concentrations are correlated with cardiovascular risk factors, including serum lipids, body mass index and markers of systemic inflammation (142). Elevated t-PA antigen has been associated with an increased risk of coronary heart disease in some (142-144), but not all studies (145) and a recent meta-analysis suggested that elevated t-PA antigen was only modestly associated with coronary heart disease (146). One has to bear in mind, however, that several factors, relevant for cardiovascular disease, such as PAI-1 or the renin-angiotensin system (see below) may correlate with plasma t-PA levels. Another factor that may affect plasma t-PA antigen levels is the acute phase response, as shown by the correlation between t-PA antigen and C-reactive protein (142) and the three-fold increase in plasma t-PA antigen (but also the 20-fold increase in PAI-1 antigen) in IL-6-treated baboons (40). For a proper evaluation of correlations of t-PA antigen

or activity with cardiovascular disease, therefore, all parameters mentioned above, have to be taken into account.

Conclusions

In this review we addressed the functions of t-PA and the many mechanisms by which t-PA activity is regulated. Despite the huge amount of information gathered thus far, a number of important questions remain with respect to t-PA function and regulation of its gene expression. Among these: 1) what is the function of t-PA expressed by cells outside the vascular system or the central nervous system; 2) what are the intracellular signal transduction pathways mediating t-PA responses to the various agonists in the different cell types; 3) what are the regulatory elements that determine tissue-specific expression of t-PA; 4) is there a relation between the C>T polymorphism located at position -7351 and cardiovascular risk; 5) what is the function of the many thus far unknown putative regulatory regions that are identified in the t-PA gene; 6) Another important question is the *in vivo* relevance of the large number of hormones, growth factors and cytokines that modulate t-PA in cultured cells. Are these effects also relevant *in vivo* or artifacts due to the isolation of the target cells from their *in vivo* environment? Well-designed studies, targeted towards the specific cell type – agonist combination of interest, are required to provide an answer to these questions. Such studies may improve our understanding of the ways by which the many physiological and pathological functions of t-PA are regulated. This will aid in the design of therapeutic approaches to more specifically increase or decrease t-PA expression.

Taken together a large body of information is available on the many mechanisms that regulate t-PA expression in cultured cells and t-PA activity in the pericellular environment or in plasma. However, the translation of this information in clinically relevant understanding is still insufficient, in particular as concerns the role and regulation of t-PA *in vivo* under physiological and pathological conditions.

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Conflicts of interest

None declared.

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