

Targeted thrombolysis strategies for neuroprotective effect

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Abstract

Stroke is usually treated by systemic thrombolytic therapy if the patient presents within an appropriate time window. There is also widespread interest in the development of thrombolytic agents that can be used in cases of delayed presentation. Current agents that can be used in cases of delayed presentation of nerve damage by thrombus. Current systemic thrombolytic therapy is associated with adverse effects such as fibrinogenolysis and bleeding. In an attempt to increase the efficacy, safety, and specificity of thrombolytic therapy, a number of targeted thrombolytic agents have been studied in recent years. This review focuses on the concepts underlying targeted thrombolytic therapy and describes recent drug developments in this field.

Key Words: nerve regeneration; review; thrombolytic agent; fibrinolytic system; stroke; cerebrovascular disease; neuroprotective drug; fibrinolytic mechanism; fibrin targeting; platelet targeting; red blood cell targeting; cerebral hemorrhage; NSFC grant; neural regeneration

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Introduction

Cardiovascular diseases including pulmonary embolism, atherosclerosis, coronary heart disease, acute myocardial infarction, and stroke are major causes of morbidity and mortality worldwide (Capstick and Henry, 2005; Prasad et al., 2006; van der Worp and van Gijn, 2007; White and Chew, 2008; Jones et al., 2010; Collart et al., 2012; Siddiqui et al., 2013; Starmans et al., 2013). These diseases result from obstruction of blood flow by thrombus, and the most effective method of preventing morbidity and mortality associated with these diseases is to prevent thrombus formation. When thrombus has already formed, the best treatment strategy is to achieve rapid recanalization of the occluded vessel by angioplasty, surgery, or thrombolysis/fibrinolysis to remove the thrombus and prevent further propagation (Absar et al., 2013). Stroke is usually treated by thrombolytic therapy, which works by interacting with the body's intrinsic fibrinolytic system (Kowalski et al., 2009). Many thrombolytic agents have been found to effectively dissolve thrombus, including streptokinase, urokinase, and tissue-type plasminogen activator. These agents comprise proteolytic components of the blood clotting cascade, and as they are circulated throughout the cardiovascular system they do not selectively target specific organs or tissues. The systemic side effects of these agents such as fibrinogenolysis and bleeding (Kowalski et al., 2009; Absar et al., 2013) result in unavoidable clinical difficulties

(Oyama et al., 2013). Liu et al. (2006) reported that addition of a neuroprotective agent can increase the effectiveness of thrombolytic therapy, increase the therapeutic time window, and reduce cerebral ischemia-reperfusion injury. It is hoped that thrombolytic agents with neuroprotective effects can be developed for clinical use.

Mechanism of thrombolysis

Intravascular thrombus formation is a complex physiological process that involves interactions among many factors. When a blood vessel is injured, local accumulation of platelets and fibrin results in thrombus formation to prevent blood loss. Thrombus formation may also occur without vessel injury under some conditions. After thrombosis, plasminogen activator cleaves the sensitive Arg561-Val562 peptide bond of plasminogen (Lijnen and Collen, 2000; Lijnen, 2001; Oyama et al., 2013), thereby activating plasminogen to form plasmin (Gabriel et al., 1992; Castellino and Ploplis, 2005; Kunamneni et al., 2007; Baumer et al., 2013; Gomasrashi et al., 2013), which triggers the body's mechanisms for dissolving thrombus into soluble fibrin degradation products and restores the blood flow (Guyatt et al., 2012) (**Figure 1**).

Classes of thrombolytic drugs

In an attempt to increase the efficacy and safety of thrombolytic therapy, recent research has focused on the develop-

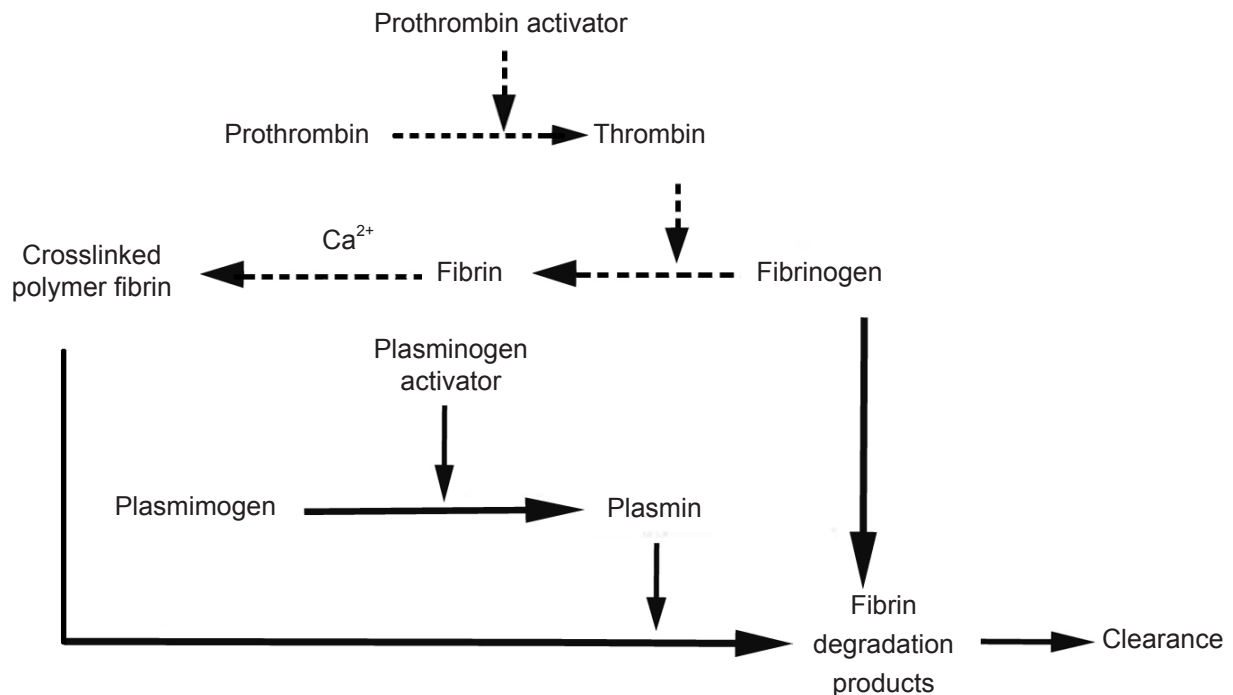


Figure 1 Thrombosis and thrombolysis pathways.

Thrombosis and thrombolysis are both important processes. Thrombosis is triggered by prothrombin activator, which causes formation of cross-linked polymer fibrin with the addition of Ca²⁺. Thrombolysis has a more complex feedback mechanism. Endogenous plasminogen is activated and converted to plasmin in the intravascular space by plasminogen activator, and the cross-linked polymer fibrin is degraded into soluble fibrin degradation products by plasmin and then cleared.

ment of targeted thrombolytic agents. Thrombolytic agents have been used with the aim of recanalizing the occluded vessel since streptokinase was first used in patients with acute myocardial infarction (Meyer et al., 1965). Four generations of thrombolytic agents have subsequently been used in clinical trials for the treatment of various clotting disorders (Kirmani et al., 2012). However, thrombolytic therapy is still associated with many problems. The first-generation fibrinolytic agents are effective for thrombolysis, but are not fibrin specific (Bentley and Sharma, 2005) and they may induce immunological responses resulting in drug resistance, fever, and allergic reactions (Verstraete, 2000). The second-generation fibrinolytic agents are more fibrin specific, do not induce adverse immunological responses (Balami et al., 2013), and have a shorter half-life (Collen and Lijnen, 1991; Eppler et al., 1998; Kim et al., 2009). The majority of third- and fourth-generation fibrinolytic agents have advantages over second-generation agents, but are currently only available in clinical trials (Longstaff et al., 2008). There is a need to improve the specificity, efficacy, and safety of the drugs available for clinical use (Toombs, 2001). Current research is focused on enhancing the ability to target the site of the thrombus and reduce adverse effects and complications (Table 1).

Targeted thrombolysis

Thrombus is mainly composed of a mesh of fibrin and plate-

lets (Cadroy and Hanson, 1990; Varin et al., 2013; Wadajkar et al., 2013) and may also include red blood cells (RBCs). Precise targeting of the thrombus site has been attempted by targeting these components.

Fibrin targeting

Fibrin (also called Factor Ia) is a fibrous, non-globular, insoluble protein that is produced in response to bleeding, and is the main protein component of thrombus (Ghasemi et al., 2012). There is a high concentration of fibrin in all types of thrombus, including acute and chronic, and arterial and venous thrombus (Sirol et al., 2005). Fibrin is a tough protein substance arranged in long fibrous chains that form from fibrinogen during blood coagulation (Mosesson, 2005). When tissue damage results in bleeding, fibrinogen in the wound is converted to fibrin monomers by the action of thrombin (Lord, 2007; Ariens, 2013). The fibrin monomers combine to form long fibrin threads that entangle platelets to build a spongy mass that gradually hardens and contracts, resulting in thrombus formation. Several proteins can bind to fibrinogen and/or fibrin and can thereby influence thrombus formation, structure, and degradation.

Carboxypeptidase N (CPN) is an enzyme that cleaves C-terminal arginine residues from bradykinin, and belongs to the same family of zinc metallo-carboxypeptidases as thrombin-activatable fibrinolysis inhibitor (Walker et al., 2008). Talens et al. studied and identified CPN as a novel

Table 1 Classes of thrombolytic drugs

	Name	Origin	Advantages	Disadvantages	Status
First-generation fibrinolytic agents	Urokinase	Human urine and kidney tissue culture	Effective at thrombolysis; no immunogenicity	No fibrin specific; expensive	State Food and Drug Administration (SFDA) approved, 1994
	Streptokinase	β -Hemolytic streptococcus	Effective at thrombolysis	Immunogenic; inaction for organized embolus	SFDA approved, 1999
Second-generation fibrinolytic agents	Tissue plasminogen activator (t-PA)	Vascular endothelial cells	Fibrin selective; no immunogenicity	Short half-life; high doses	Listed in the U.S., 1987
	Single chain urokinase plasminogen activator (Scu-PA)	Renal cells	Fibrin selective	Short half-life	Not approved for clinical use
Third-generation fibrinolytic agents	Retenase	t-PA mutant	Fibrin selective <i>in vivo</i> ; long half-life	Low fibrin affinity <i>in vitro</i>	On the market in Germany, 1996
	Tenecteplase	t-PA mutant	High fibrin selectivity	Hemorrhagic side effects	Listed in the U.S., 2000
	Lanoteplase	t-PA mutant	Fibrin specificity	High rate of cerebral hemorrhage	Listed in the U.S., 2000
	Monteplase	t-PA mutant	Long half-life		On the market in Japan, 1998
Fourth-generation fibrinolytic agents	Plasminogen activator inhibitors (PAI)	Plasma	Low molecular weight; reduce plasma PAI-1 activity levels		Not approved for clinical use

thrombus component with possible antifibrinolytic properties (Talens et al., 2012a), but could not prove the presence of CPN in thrombus (Talens et al., 2012b). CPN should therefore be investigated further to determine whether it binds directly to fibrin or fibrinogen. The above-mentioned studies used surface plasmon resonance to determine that thrombus-bound CPN has the same molecular forms as CPN in the plasma, and that CPN may bind to fibrinogen and fibrin.

Fibrin has been considered as a molecular target for the selective delivery of thrombolytic agents to the thrombus. Recently, ultrasound-assisted drug delivery has been investigated as a method of targeting a specific area (Klibanov et al., 2010), and intrinsically echogenic liposomes have been used as a vehicle to achieve ultrasound-triggered controlled drug release (Huang, 2008; Greineder et al., 2013). Ultrasound was found to improve the effectiveness of tissue plasminogen activator (tPA), but was associated with hemorrhagic side effects (Datta et al., 2006; Holland et al., 2008; Meunier et al., 2009). Use of tPA-loaded intrinsically echogenic liposomes was found to be similarly effective to other treatment methods, while offering the advantages of ultrasound monitoring and enhanced thrombolysis with site-specific delivery (Shaw et al., 2009; Laing et al., 2012).

The fibrin-specificity of thrombolytic agents may be improved by conjugating them with fibrin-specific monoclonal antibodies (Vaidya et al., 2012). A hybrid molecule conjugated with tPA and the fibrin-specific monoclonal antibody 59D8 by a disulfide bond (Runge et al., 1987) has a 10-fold higher affinity for fibrin than urokinase and a 100-fold higher affinity for fibrin than tPA. Furthermore, experimental use of a hybrid recombinant plasminogen activator, anti-fibrin antibody 59D8–low-molecular-weight single-chain

urokinase-type plasminogen activator, for antibody targeting of fibrin increased the thrombolytic and antithrombotic potency with less impairment of hemostasis compared with recombinant tPA and recombinant single-chain urokinase-type plasminogen activator (Runge et al., 1996).

Current strategies for site-specific delivery are focused primarily on the local release of therapeutic agents by drug-eluting stents. However, this technique is expensive and can only be used in limited situations (Muni and Gross, 2004; Huang et al., 2008). Nanoparticles have recently attracted attention as potential vehicles for targeted drug therapy, and have been shown to increase therapeutic effectiveness (Cyrus et al., 2008; Tsuruta et al., 2009; Gu et al., 2012; McCarthy et al., 2012). Yurko et al. (2009) used *in vitro* fibrinolysis assays to show that use of 40-nm polystyrene-latex nanoparticles covalently conjugated to tPA and anti-fibrin antibody could deliver tPA directly to the site of the thrombus, thereby lowering the risk of hemorrhage. Since then, a thrombolytic agent that conjugates an antifibrin monoclonal antibody and urokinase to a perfluorocarbon nanoparticle has been developed, and its effectiveness for targeted thrombolysis has been evaluated (Marsh et al., 2007, 2011). In animal studies, the maximum lytic effect was achieved with an enzyme load of 100–400 per nanoparticle.

Platelet targeting

Platelets (also called thrombocytes) are small, disk-shaped, clear, anuclear cell fragments, 2–3 μ m in diameter, which are derived from fragmentation of precursor megakaryocytes. Platelets circulate in the blood of mammals and play a vital role in the process of thrombus formation to maintain hemostasis (Langer and Gawaz, 2008). Under pathophysiological conditions, platelet activation can also play a critical role

in various thromboembolic diseases (Harrison, 2000). Platelet membrane glycoprotein IIb/IIIa (GPIIb/IIIa) receptor activation is the final common pathway of platelet aggregation (Kulkarni et al., 2000; ten Berg et al., 2001; Davi and Patrono, 2007; Badimon and Vilahur, 2008; Gladding et al., 2008). The GPIIb/IIIa receptor is the most abundant protein on the platelet membrane (Jennings, 2009; Vaidya et al., 2011), and is a potential target for novel thrombolytic agents. L-arginine-glycine-aspartic acid peptide (RGD) is a GPIIb/IIIa antagonist that binds to activated GPIIb/IIIa receptors specifically on aggregated platelets (Meyer et al., 2006), and has been used to develop targeted thrombolytic drugs. Under normal conditions, the spatial configuration of GPIIb/IIIa receptors is stable, and the platelet is inactive. When platelet agonists such as thrombin, collagen, adenosine diphosphate, and thromboxane A₂ bind with their receptors on the platelet membrane, GPIIb/IIIa forms a functional dimer complex that exposes the platelet membrane (Huang et al., 2008), resulting in binding with RGD. Conversely, the GPIIb/IIIa receptors are hidden on the unactivated platelet membrane, and cannot bind with RGD. RGD therefore binds only with activated platelets in the thrombus, and has no effect on circulating platelets. The hexapeptide H-Pro-Ser-Nva-Gly-Asp-Trp-OH also binds to the GPIIb/IIIa receptor on activated platelets, and development of thrombus-targeted microbubbles has been attempted by binding this hexapeptide to microbubbles (Zhou et al., 2011). Binding of the hexapeptide to the GPIIb/IIIa receptor delivers the microbubbles specifically to the thrombus (Wang et al., 2006). Platelet-targeted microbubbles have also been investigated for the prevention of thrombus recurrence. The microbubbles were found to be good carriers of thrombolytic drugs, and to be beneficial for preventing thrombus recurrence *in vivo*. GPIIb/IIIa receptors have been used as a target for the delivery of thrombolytic agents by conjugation with a monoclonal antibody modified with N-succinimidyl-3-(2-pyridyldithio) propionate at 7E3 Fab' (Bates et al., 1991) to selectively bind urokinase to GPIIb/IIIa on the platelet membrane. The conjugated urokinase was found to have higher thrombolytic activity than unconjugated urokinase (Bode et al., 1991). Platelet activation also exposes phosphatidylserine on the platelet membrane. Annexin V has a high affinity for phosphatidylserine, and binding of annexin V to the B chain of urokinase-type plasminogen activator by a disulfide bond was found to increase the thrombolytic activity of the plasminogen activator in *in vitro* tests (Okabayashi et al., 1996).

RBC targeting

In most cases, labile and complex biotherapeutic agents such as enzymes, require precise delivery to the target site. The best way to achieve this goal is to use coupling drugs such as synthetic or natural polymers with various geometric configurations, phospholipid liposomes, albumin, antibodies, or other biological molecules as carriers (Simone et al., 2008). RBCs (also called erythrocytes) are anuclear, biconcave, disc-shaped cells with a diameter of 7–8 μm, thickness of 2–3 μm, and membrane surface area of about 160 μm², and are

the most common type of blood cell (Pierige et al., 2008). RBCs have many features that make them ideal carriers for drugs in the bloodstream (Magnani et al., 2002; Danchin et al., 2008), especially when sustained action is needed (Bax et al., 1999; Millan et al., 2004; Muzykantov, 2010; Greineder et al., 2013; Muzykantov, 2013). RBCs can transport many substances, and the RBC membrane is supported by a complex cytoskeleton comprising a hexagonal lattice of actin-spectrin filaments interconnected by anchoring integral plasma-membral proteins *via* numerous structural and connector proteins (Muzykantov, 2010; Luo et al., 2012). Ineffective delivery of plasminogen activators to the thrombus site limits their therapeutic effectiveness. This problem cannot be solved by increasing the dose because of the associated risk of adverse effects (Ganguly et al., 2006). However, the use of RBCs as drug carriers may help to solve this problem by enabling the use of plasminogen activators as thromboprophylactic agents (Ganguly et al., 2005). RBCs have recently been used as intravascular carriers for targeted drug delivery (Serafini et al., 2004; Rossi et al., 2005).

Once thrombus is established, it becomes progressively more impermeable to RBCs, and RBC carriers of plasminogen activators can therefore potentially prevent vascular occlusion in patients at imminent risk of thrombosis without lysing hemostatic clots. Murciano et al. (2003) hypothesized that tPA conjugated to RBCs would dissolve nascent clots while having minimal effects on preexisting hemostatic clots or extravascular tissues. This RBC-based drug delivery method alters the fibrinolytic profile of tPA, thereby permitting prophylactic fibrinolytic therapy. Conjugating of tPA to RBCs also reduces its central nervous system toxicity by spatially confining the drug to the vascular system. Administration of RBC-tPA before or after cerebral hypoxia/ischemia may preserve the responses to cerebral vasodilators and prevent neuronal injury mediated through the extracellular signal-related kinase (mitogen-activated protein kinase) pathway, indicating that use of RBC-tPA may increase the benefit/risk ratio of thrombolytic therapy (Armstead et al., 2009).

Plasminogen activators are currently not used for thromboprophylaxis because of their rapid clearance, associated risk of bleeding, and extravascular toxicity. Zaitsev et al. (2006) conjugated tPA to a monoclonal antibody against complement receptor type 1 expressed primarily on human RBCs, and found that tPA bound rapidly to RBCs in the bloodstream and circulated safely for many hours after injection in mice, providing prophylactic thrombolysis without hemorrhagic side effects, similar to use of preformed RBC-tPA. This approach provided rapid and tight binding of tPA to RBCs, which markedly prolonged the circulation of tPA, accelerated lysis of venous and occlusive arterial thrombus that formed subsequent to injection, and reduced bleeding from preexisting hemostatic clots. Subsequently, a single-chain antibody fragment-tissue type plasminogen activator fusion targeted to RBC glycophorin-A related antigen was developed that bound safely to circulating RBCs and had anti-thrombotic effects in a mouse model of thrombosis

that were qualitatively similar to RBC-tPA and superior to tPA (Zaitzev et al., 2010).

Conclusion

Currently, the ability to dissolve thrombus using conventional thrombolytic therapy is limited. Pharmacological agents have generally targeted the transformation of plasminogen and plasmin, thereby facilitating the natural process of fibrinolysis. However, these agents do not discriminate between healthy and at-risk vasculature, and are widely distributed in the circulation. Development of newer targeted thrombolytic agents should enable selective delivery to specific organs, tissues, or cells to enhance targeting of the thrombus and reduce adverse effects, thereby achieving superior efficacy and safety compared with existing therapeutic options.

Several approaches have been proposed for the targeted delivery of plasminogen activators, including conjugation with specific monoclonal antibodies, oligopeptides, and nanoparticles. Conjugation with fibrin-specific immunoconjugates seems to be a less promising approach because of the poor specificity of antibody binding to fibrin, and the inability to distinguish between hemostatic clots and occlusive thrombus. The effects of platelet-specific agents are obvious, but their safety and effectiveness need to be further studied. Strategies based on conjugating thrombolytic agents to RBCs are less practical. Use of a single-chain antibody fragment-tissue type plasminogen activator fusion targeted to RBC glycoprotein-A related antigen appears to be at least as promising as use of tPA-loaded nanoparticles for the prevention of both venous and arterial thrombus formation. Effective clearance of preexisting thrombus using methods such as ultrasound (Uesugi et al., 2010) and enhancement of the effectiveness of thrombolytic agents may provide a good approach to the treatment of stroke.

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