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α 2-Antiplasmin: New Insights and Opportunities for Ischemic Stroke

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Abstract

Thrombotic vascular occlusion is the leading cause of ischemic stroke. High blood levels of α 2-antiplasmin, an ultrafast, covalent inhibitor of plasmin, have been linked in humans to increased risk of ischemic stroke and failure of tissue plasminogen activator therapy. Consistent with these observations, α 2-antiplasmin neutralizes the therapeutic benefit of tissue plasminogen activator therapy in experimental stroke. In addition, α 2-antiplasmin has deleterious, dose-related effects on ischemic brain injury in the absence of therapy. Experimental therapeutic inactivation of α 2-antiplasmin markedly reduces microvascular thrombosis, ischemic brain injury, brain swelling, brain hemorrhage and death after thromboembolic stroke. These data provide new insights into the critical importance of α 2-antiplasmin in the pathogenesis of ischemic brain injury and suggest that transiently inactivating α 2-antiplasmin may have therapeutic value in ischemic stroke.

Keywords

Cerebral infarction; thromboembolism; fibrinolysis; blood brain barrier; survival

α 2-antiplasmin is a Potent Plasmin Inhibitor

Since its original description, ^{1–3} α 2-antiplasmin (a2AP) or α 2-plasmin inhibitor (serpinf2) has been known as the primary, fast inhibitor of plasmin. It is a member of the serine protease inhibitor family, whose members typically act as highly specific, suicide inhibitors. a2AP is such a potent inhibitor that the half-life of plasmin in vivo is thought to be less than 100 msec. ⁴ a2AP rapidly inactivates plasmin, forming covalent plasmin-a2AP (protease serpin) complexes, whose presence in the blood is a marker of plasmin generation in clinical states associated with plasminogen activation. ⁵ Aoki and colleagues showed that activated factor XIII crosslinks a2AP to fibrin during fibrin formation. ⁶ Crosslinking of a2AP to the fibrin significantly enhances the resistance of fibrin to degradation by plasmin. ^{6,7} The initial reactions between a2AP and plasmin appear to occur through kringle 1 and/or 3 of plasmin and one or more lysine residues in the C-terminus of a2AP. ⁸ When the kringles of plasmin are engaged with lysine residues of fibrin, or lysine analogs such as epsilon-aminocaproic acid, plasmin inactivation by a2AP is delayed. ⁹

Molecular Forms of a2AP

The primary site of a2AP synthesis in humans is the liver but low levels of transcripts for a2AP have been found in other tissues by gene profiling experiments. a2AP has been detected in neurons in the normal human brain and, for unknown reasons, its expression appears to be increased in A β plaques in Alzheimer's disease.¹⁰

The mean plasma level of a2AP is 1 micromolar (~70 micrograms/ml) and the plasma half-life is 2.6 days.¹¹ The rate of synthesis or catabolism was found to be 1.4 mg/kg/day.¹¹ During therapeutic fibrinolysis with plasminogen activators, a2AP disappears quickly (within 1 h) from the blood due to formation of plasmin-a2AP complexes.¹² Levels of a2AP levels rebound quickly (within 24 h) after streptokinase therapy.¹³ Higher levels are associated with myocardial infarction.¹⁴ Levels of a2AP increased with increasing total cholesterol, triglyceride, and plasminogen levels. Increased levels of a2AP are associated with an increased risk of stroke in univariate analysis.¹⁵ Atrial fibrillation, which is a risk factor for ischemic stroke, is associated with increased levels of plasmin-antiplasmin complex.¹⁶ Circulating levels of a2AP may be significantly reduced in patients with severe cirrhosis and severely diminished in patients with acute liver failure.^{17,18} In contrast, a2AP levels may be mildly elevated post-partum or post-surgery, or in patients with thrombosis, peripheral arterial disease, or coronary artery disease.^{17,19}

A2AP is synthesized as a glycoprotein with a relative molecular mass of 70,000 Da. A partial X-ray crystal structure of a2AP shows unique structural elements.²⁰ In the blood, a2AP is secreted with an N-terminal methionine (Met-a2AP) and converted to another form, Asn-a2AP, when the amino terminal twelve residues are cleaved by a2AP cleaving enzyme, a soluble form of fibroblast activating protein.²¹ Crosslinking of a2AP to fibrin protects against fibrinolysis. Since Met-a2AP is crosslinked to fibrin at a slower rate by activated factor XIII, high levels of Met-a2AP may enhance fibrinolysis.²¹ This observation has prompted consideration of inhibitors of fibroblast activating protein as potential therapeutics.²¹ A tryptophan polymorphism in the N-terminus of a2AP (Arg6Trp) is associated with delayed N-terminal cleavage and the crosslinking of a2AP which may affect fibrinolysis.²² In the blood a2AP is also modified at the C-terminus which may slow its initial inhibition of plasmin.²³ While these modifications of a2AP may eventually be shown to affect fibrinolysis in vivo,⁸ recent studies failed to find a linkage between amino and carboxyl terminal modification of a2AP and, the risks of myocardial infarction.²⁴

Regulation of Fibrinolysis

Role of a2AP in fibrinolysis

Early studies using specific monoclonal antibody inhibitors showed that a2AP was a potent regulator of fibrinolysis. Inhibition of a2AP induced *spontaneous* dissolution of human plasma clots even without addition of exogenous plasminogen activators.^{25,26} The combination of a2AP-inhibition and plasminogen activators was synergistic, yet for the same degree of fibrinolysis, it did not enhance the degradation of fibrinogen.²⁵ In vivo, specific inhibition of fibrin-bound a2AP alone was sufficient to increase thrombus dissolution in experimental venous thrombosis.²⁷ In vivo studies of experimental pulmonary embolism

established that a2AP was a significant inhibitor of thrombus dissolution initiated by r-tPA.^{28,29}

Insights from a2AP Deficiency

Since a2AP is a less efficient inhibitor of fibrin-bound plasmin than of plasmin in solution, studies have suggested that the primary role of a2AP is not to regulate plasmin-mediated fibrinolysis, but to inhibit plasmin in the circulation in order to prevent degradation of clotting factors such as fibrinogen, factor V and factor VIII.³⁰ Similarly, illnesses associated with systemic activation of the fibrinolytic system may also cause depletion of a2AP and coagulation factors. Coagulation factor depletion has been associated with increased bleeding risk, particularly in patients undergoing invasive procedures. However, it is clear from studies of humans and mice, that primary genetic a2AP deficiency alone does not affect hemostatic parameters, coagulation or create a systemic fibrinolytic state (see a2AP-deficiency below).

Secondary or acquired a2AP deficiency

Pharmacologic activation of the fibrinolytic system by plasminogen activators, plasmin and microplasmin causes secondary or acquired a2AP-deficiency and α_2 -macroglobulin is also depleted.^{23,31} In fibrinolytic states, a2AP depletion induced by high plasmin levels leads to deficiencies of coagulation proteins such as fibrinogen and factor V, causing impaired coagulation with prolongation of prothrombin time (PT), activated partial thromboplastin time (aPTT), and reptilase time tests.^{31,32} Patients are at risk for bleeding complications, which are typically related to puncture wounds from cardiac catheterizations, arterial lines, etc.³¹

Patients with severe liver disease may have reduced synthesis of a2AP. Secondary depletion of a2AP may occur also with disseminated intravascular coagulation or systemic fibrinolysis such as in acute promyelocytic leukemia.³³ In these settings, patients usually have laboratory findings of hyperfibrinolysis or disseminated intravascular coagulation, which includes low platelet counts as well as decreased levels of fibrinogen, antithrombin and plasminogen. There is typically evidence of elevated fibrinogen degradation products.³³ Occasionally, these conditions are associated with serious bleeding, though treatment with antifibrinolytic agents such as epsilon-aminocaproic acid has been shown to stop hemorrhage.³³

Primary or genetic a2AP deficiency

Congenital a2AP deficiency provides insights into the safety and tolerability of transient a2AP inactivation. Only a small number of individuals have been identified who are genetically deficient in a2AP and the true prevalence of complete a2AP deficiency is unknown.³⁴ Clinical assays for a2AP levels are still not widely available and most patients have been identified only during comprehensive hematologic evaluations for a cause of 'delayed re-bleeding' episodes after surgery or trauma.³⁵ Studies of humans with genetic a2AP deficiency have shown that their blood coagulation is normal, and they do not have evidence of a systemic lytic state or disseminated intravascular coagulation.³⁴ However, after trauma or surgery they are at risk of delayed oozing from the site of injury because the

process of blood clot dissolution or fibrinolysis is enhanced. Fourteen cases of homozygous a2AP deficiency have been reported worldwide according to the most comprehensive review.³⁴ In sharp contrast to coagulation factor deficiencies, none of these individuals were reported to have brain bleeding. Reports of bleeding in these individuals most commonly include hemarthroses and hematomas, some of which occur in muscle or bone. The ages of patients ranged up to 35 years of age at detection. More recently a patient was first identified at age 63, which indicates that severe a2AP deficiency can be associated with survival into older age.³⁶ The etiology of bleeding in 11 of 11 a2AP-deficient patients was thought to be related to premature dissolution of formed thrombi following trauma or surgery, but 'spontaneous hemorrhage' was also reported in 4 of 11 patients.^{34,37} Patients with a2AP deficiency suffering hemorrhage are treated with fibrinolytic inhibitors (tranexamic acid or epsilon-aminocaproic acid) or fresh frozen plasma. With antifibrinolytic therapy (epsilon-aminocaproic acid), patients with complete a2AP deficiency can be taken successfully through severe hemostatic challenges including major operations (open heart surgery, orthopedic procedures).³⁴

Mice with genetic deficiency of a2AP (a2AP^{-/-}) induced by gene targeting have normal fertility, viability and coagulation.³⁸ There was no increased bleeding or re-bleeding after surgical procedures, including tail bleeding.³⁸ In a similar fashion, selective a2AP inactivation in vivo by monoclonal antibodies has not been associated with bleeding or consumption of fibrinogen or plasminogen, in pre-clinical studies (see below). In considering the potential safety of transient, therapeutic a2AP inhibition, it is interesting to compare the effects of genetic deficiencies of pro-thrombin or factor X, which are targets of multiple current therapies. Genetic deficiency of pro-thrombin is lethal—no mice are born and no humans have been identified. Similarly, humans with complete deficiency of factor X do not appear to be viable and knockout of murine factor X is also lethal. In contrast, genetic deficiency of a2AP is tolerated in humans and in mice. Since the activity of both factor Xa and thrombin can be inhibited therapeutically with acceptable safety in patients, transient therapeutic a2AP inactivation may also prove to be safe.

When treated with anticoagulants, antiplatelet agents or r-tPA, a2AP-deficient mice do not have increased bleeding.^{29,39} In early studies of venous thromboembolism in ferrets, a2AP-inactivation did not cause bleeding.²⁸ a2AP-inactivation significantly reduced brain bleeding in ischemic stroke by comparison to controls (no treatment) and r-tPA; a2AP-inactivation had marked effects on reducing mortality by comparison to controls or r-tPA (see below).^{40,41}

a2AP in Ischemic Stroke

Epidemiologic and clinical linkage of a2AP to ischemic stroke

The Atherosclerosis Risk in Communities Study found that elevated a2AP levels were associated with an increased risk of subsequent stroke in univariate analysis.⁹ In patients treated with r-tPA for ischemic stroke, higher a2AP blood levels were associated with a decreased rate of successful reperfusion.⁴² a2AP-plasmin complexes are elevated in patients with atrial fibrillation and increased risk of ischemic stroke.¹⁶

Endogenous Activation of the fibrinolytic System in Ischemic stroke

There is significant activation of the fibrinolytic system after stroke in humans. Levels of a2AP-plasmin complex are higher, signifying increased endogenous plasminogen activation, dissolution of fibrin and inhibition of the process by a2AP.^{43–47} Levels of D-dimer and a2AP-plasmin complexes are increased in stroke patients versus controls, particularly in older patients.⁴⁸

Effects of a2AP on Experimental Stroke

Experimental Studies of the fibrinolytic system in experimental stroke

The NINDS study demonstrated that r-tPA treatment reduced disability when given to stroke patients within three hours of the onset of ischemia.⁴⁹ However, in a series of surprising experiments, Tsirka and colleagues found that r-tPA had deleterious effects on the ischemic brain; r-tPA enhanced neurotoxicity and increased brain infarction following mechanical arterial occlusion.⁵⁰ Tsirka and colleagues induced brain ischemia by mechanical arterial occlusion and not by thrombosis. Their goal was to determine the effects of endogenous tPA and r-tPA on the ischemic brain, separate from their effects on thrombus dissolution and reperfusion. In studies of gene-deleted mice, Nagai and colleagues expanded these findings by examining the effect of endogenous tPA and other key components of the fibrinolytic system.⁵¹ In an open skull model of cerebral ischemia induced by surgical ligation of the middle cerebral artery (MCA), deletion of tPA or of a2AP (Table 1) significantly reduced ischemic brain injury.⁵¹ Nagai et al. also found that deficiency of PAI-1 or of plasminogen significantly increased stroke size, whereas deficiency of urinary-type PA mice had no effect.⁵¹ In subsequent studies, using the same model, Nagai et al demonstrated that depletion of a2AP by administration of microplasmin, plasmin or polyclonal antibodies significantly reduced ischemic brain injury.⁵² In related studies, Nagai and colleagues showed that in ischemia induced by MCA ligation, pharmacologic treatment with all plasminogen activators tested (r-tPA, streptokinase and staphylokinase) caused some degree of neurotoxic effects that enhanced ischemic brain damage.⁵³

Experimental models with translational relevance to human stroke

The deleterious effects of endogenous tPA and r-tPA in non-thrombotic, arterial occlusion studies led some to question the relevance of this non-thrombotic experimental model for human ischemic stroke, a disease largely caused by thrombotic occlusion.⁵⁴ Since almost all patients with ischemic stroke have evidence of an occlusive arterial thrombus,^{55,56} the Stroke Academic Industry Roundtable and other expert groups consider animal models of experimental thromboembolic stroke to have translational relevance to human disease,⁵⁷ particularly when evaluating the fibrinolytic system. In some models of thrombotic ischemic stroke, autologous clots are placed by catheter in the proximal MCA to induce hemispheric ischemia. Thrombotic obstruction of the proximal MCA is confirmed by greater than 80% reductions in relative hemispheric blood flow and by post-mortem inspection of the base of the brain. Thromboembolic stroke has been evaluated in a number of different animals including mice. The mouse fibrinolytic system is comparable to that of humans; mouse a2AP inhibits mouse plasmin with similar kinetics and a2AP is the dominant regulator of fibrinolysis.^{38,58,59} In these models, r-tPA has been shown to reduce stroke size in mice

when given within 15 minutes of thromboembolism⁶⁰ or 20 minutes of clot initiation,⁶¹ although its effectiveness diminishes significantly when given later.⁶²

Narrow therapeutic time window for r-tPA

When mice are treated after 15 min. after thromboembolism-induced ischemia, the standard r-tPA dose in mice (10 mg/kg) significantly increased dissolution of the MCA thrombus and markedly reduces brain infarction. However, treatment of mice one hour after stroke onset significantly increased dissolution of the MCA thrombus but did not decrease stroke size by comparison to controls. Treatment with r-tPA 2.5 hours after the onset of ischemic dissolved the MCA thrombus, but it markedly *increased* stroke infarction vs. controls.^{40,41} Treatment with r-tPA also significantly increased hemorrhage by comparison to controls.

a2AP contributes to the therapeutic failure of r-tPA

In the fibrinolytic pathway, a2AP is downstream of tPA and high blood levels have been associated with r-tPA failure to restore reperfusion in stroke patients.⁴² To examine this, blood levels of a2AP were experimentally increased (~doubled) by intravenous infusion prior to cerebral thromboembolism (Table 1). With r-tPA treatment, mice with high a2AP blood levels had 2.2-fold larger brain infarcts than mice with normal a2AP levels. During r-tPA treatment, high a2AP blood levels increased brain swelling (3.7-fold) and decreased dissolution of the MCA thrombus.

a2AP inactivation improves the effectiveness and therapeutic window of r-tPA

To confirm the effects of a2AP on r-tPA and stroke outcomes, mice were treated with the combination of r-tPA and a monoclonal antibody that inactivated a2AP (a2AP-I). This combination markedly enhanced the dissolution of the MCA thrombus vs. r-tPA alone (Table 1). Combination therapy also diminished brain infarction, swelling and hemorrhage. Combination therapy with a2AP-I and r-tPA extended the therapeutic time window for reduction in ischemic brain injury and improved survival by comparison to r-tPA therapy alone.

Effects of endogenous a2AP on ischemic stroke

The existence of a dose-response relationship between a2AP levels and ischemic brain injury was examined in mice with increased a2AP levels (following supplementation), normal a2AP levels and a2AP deficiency. Brain infarction was increased by more than 50% in mice with high a2AP vs. normal a2AP levels. Brain infarction was reduced by more than two-fold in a2AP-deficient (a2AP^{-/-}) vs. a2AP^{+/+} mice and, it was reduced about four-fold vs. mice with high a2AP levels. These data indicated a strong dose response relationship whereby high a2AP levels were deleterious and low a2AP levels were protective in ischemic stroke. The relative importance of circulating vs. thrombus bound a2AP was assessed in a2AP^{-/-} mice. There was faster MCA thrombus dissolution and brain reperfusion in a2AP^{-/-} mice with a2AP-deficient thrombi, than in a2AP^{-/-} mice with normal levels of a2AP in the thrombi. In these experiments, brain infarction was comparably reduced in both groups, suggesting that the presence or absence of a2AP in the MCA thrombus affected the ischemic brain injury less than circulating a2AP. Thus circulating levels of a2AP appeared to

be the most important determinant of outcomes, potentially through its effects on microvascular thrombosis (see below).

Comparative therapeutic time windows for r-tPA and a2AP-I

When blood flow is reduced due to arterial thrombosis, severely ischemic brain tissue begins to infarct. Brain tissue in the penumbra, the area between severe ischemia and normal blood flow, which may be rescued by therapy, remains marginally viable for a period of time. The duration of viability of the penumbra is much shorter in mice than in humans. The time-related effects of a2AP-I and r-tPA therapies alone were compared (Table 1, Fig. 1) in experimental ischemic stroke. Treatment 1 hour after stroke onset with r-tPA did not reduce brain injury. In contrast, treatment of ischemic stroke with a2AP-I 1 h after stroke onset significantly reduced brain infarction by comparison to control (un-treated) and r-tPA-treated mice. In addition, treatment of ischemic stroke with a2AP-I 2.5 hours after ischemia also significantly reduced infarction vs. control and vs. r-tPA. Treatment with r-tPA significantly increased brain hemorrhage vs. control or a2AP-I. However, a2AP-I treatment did not increase brain bleeding vs. controls.

Comparative effects of r-tPA and a2AP-I on longer term outcomes and survival

Since r-tPA treatment had a short therapeutic time window, mice were treated 30 min. following MCA thromboembolism. Treatment with a2AP-I decreased brain infarction by 4–5-fold by comparison to control or to r-tPA therapy. Treatment with a2AP-I also markedly reduced brain swelling (>10-fold). Importantly, a2AP-I treatment significantly reduced brain hemorrhage when compared with control or r-tPA treatment.

Proximal MCA thromboembolism was associated with large brain infarctions. There was significant mortality in mice treated with r-tPA and in control mice. In comparison to these two groups, mice treated with low dose a2AP-I showed a significant improvement in survival. All mice treated with high doses a2AP-I survived, which was a significant improvement over low dose a2AP-I, r-tPA therapy or controls. These experiments showed that a2AP inactivation treatment after stroke onset conferred a significant survival advantage.

Effects of a2AP on the Ischemic Brain

Microvascular thrombosis

Downstream from a thrombotic occlusion, ischemia may trigger the formation of microvascular thrombi, which further reduce blood flow and thereby enhance blood brain barrier breakdown and worsen brain tissue injury.^{63–66} Microvascular thrombosis may worsen ischemia by causing vascular obstruction.⁶⁷ In experimental stroke, r-tPA treatment significantly enhanced microvascular thrombus formation vs. control (untreated mice); this is consistent with the pro-thrombotic effects of r-tPA therapy in humans.^{41,68} High blood levels of a2AP also increased microvascular thrombosis. However, a2AP-I treatment or a2AP deficiency significantly decreased microvascular thrombosis, by comparison to control or to r-tPA treated mice.⁶⁹ Consistent with this observation, plasminogen deficiency

enhances, and high plasminogen levels reduce microvascular thrombosis in experimental ischemic stroke.⁷⁰

The extent of microvascular thrombosis was more strongly associated with the sequelae of severe ischemic brain injury than the magnitude of dissolution of the middle cerebral thrombus. While r-tPA therapy more efficiently dissolved the MCA thrombus than a2AP-I, the a2AP-I induced a greater reduction in microvascular thrombosis, which was associated with larger decrease in ischemic brain injury.

Matrix metalloproteinase-9 expression

Fibrin stimulates neutrophil adherence;⁷¹ in turn neutrophils adhering to fibrin(ogen) promote thrombin generation and fibrin deposition.⁷² In human stroke, matrix metalloproteinase-9 (MMP-9) expression is associated with neutrophil infiltration in infarcted and hemorrhagic areas of the brain.⁷³ However, MMP-9 is also expressed by endothelial cells and to a lesser extent by astrocytes and neurons in ischemic brain.⁷⁴ MMP-9 is a potential mediator of breakdown of the blood brain barrier, brain swelling and hemorrhage. There was a dose-response relationship between a2AP and acute MMP-9 expression. High a2AP levels or r-tPA treatment, significantly increased MMP-9 levels. Deficiency of a2AP deficiency or a2AP-I markedly diminished MMP-9 expression. The relationship of a2AP to expression of MMP-3 and other MMPs, which are linked to breakdown of the blood brain barrier, is unknown.⁷⁵

Blood brain barrier breakdown

MMP-9 has been implicated as a cause of breakdown of the blood brain barrier, hemorrhage, and brain edema in ischemic stroke.⁷⁵⁻⁷⁷ Mice treated with r-tPA alone showed leakage of albumin outside vascular spaces, indicated by collagen IV immunostaining, consistent with blood brain barrier breakdown; this was reduced in mice treated with the combination of r-tPA and a2AP-I. By comparison to normal mice, or r-tPA-treated mice there was also significantly less breakdown of the BBB in mice treated with an a2AP-I alone (Reed, unpublished data).

Apoptosis

In comparative studies, combination treatment with r-tPA and a2AP-I was significantly more effective than r-tPA alone at reducing brain apoptosis as indicated by reductions in cleaved caspase-3 and TUNEL staining.

Therapeutic Potential of a2AP Inactivation

The therapeutic potential of a2AP inactivation in human ischemic stroke has been suggested by epidemiologic, clinical and experimental studies. While there is as yet no approved therapy for modulating a2AP activity, several approaches hold promise.

Monoclonal antibodies

Because of their exquisite specificity and affinity monoclonal antibody therapy has emerged as a safe and effective treatment for cancer and inflammatory diseases, though none yet have

been developed for stroke. Potent and specific inhibitors of human a2AP have been described that markedly accelerate the dissolution of human clots in vitro.^{25,59,78–80} Some antibodies act through an elegant mechanism to convert a2AP from an inhibitor of plasmin to a plasmin substrate, which leads to permanent inactivation of the a2AP molecule.⁵⁹ Inhibition of a2AP in vivo significantly enhances dissolution of experimental venous as well as pulmonary and cerebral arterial thrombi.^{27,28,40,41,59,81} Recent studies in different laboratories have shown that monoclonal antibody inhibition of a2AP significantly reduces ischemic stroke injury.^{40,41,52}

Microplasmin

Microplasmin is a small fragment of plasmin that lacks the kringle domains, which modulate interactions with potential regulatory molecules and substrates. As a result, microplasmin is a relatively indiscriminant protease that is preferentially inactivated by α 2-macroglobulin and by a2AP. Infusion of microplasmin or plasmin, significantly depletes a2AP and reduces experimental brain infarction induced by surgical ligation of the cerebral artery.⁵²

Recombinant microplasmin also significantly reduced brain infarction and disability in a rat photochemical, thrombotic model of ischemic stroke.⁸² Intravenous microplasmin was investigated in a Phase 1, placebo-controlled, double-blind, ascending-dose study in 60 healthy volunteers.⁸³ Volunteers were given an intravenous bolus of microplasmin of 0.1 to 2 mg/kg over 15 min. Additional volunteers also received a bolus followed by an infusion of 1–4 mg/kg of microplasmin over 60 min. Subjects were monitored for 21 days after the infusion. There was a dose-dependent prolongation of the PT and activated PTT with a reduction in fibrinogen levels. The PT and aPTT levels normalized within 9 hours. There was no change in plasminogen levels.

Notable adverse events included: allergic-type (urticarial) events that were considered to be related to the study medication and were associated with transient decreases in total serum hemolytic complement; all resolved without treatment. Procedure-site reactions were reported in 6 subjects given microplasmin including pain at infusion site in leading to cessation of infusion (2), pain during (2) and after (1) infusion that didn't lead to cessation, bruising at the intravenous site 11 days post-infusion, prolonged bleeding after infusion (1) and a hematoma (1). No clinically significant changes were seen in the vital signs, urinalysis, hematology tests or clinical chemistry.

Microplasmin was subsequently tested in a Phase 2, randomized, placebo-controlled trial of patients with stroke who were not eligible for r-tPA and had ischemia of 3–12 h.⁸⁴ Three different bolus- infusion doses were tested. Microplasmin decreased a2AP levels up to 80% and reduced fibrinogen levels. The PT and aPTT prolonged and, D-dimer levels increased. Microplasmin decreased circulating MMP-2 but had no effect on other biomarkers of stroke or brain imaging tests. There were no allergic reactions, but one of thirty treated patients had a symptomatic intracerebral hemorrhage. Brain hemorrhage can be a complication of stroke itself and there were insufficient numbers of patients to conclude whether there was any statistical benefit or risk from therapy.⁸⁴ The company (ThromboGenics) producing microplasmin is not currently developing it for ischemic stroke.

Plasmin

While Nagai et al. had used infusions of plasmin to deplete systemic a2AP, Marder et al. conversely viewed a2AP as a molecular mechanism to control the systemic effects of catheter-infused plasmin.^{52,85} After promising studies shown experimental recanalization of thrombotically occluded middle cerebral arteries in rabbits, catheter delivery of plasmin into a thrombus was studied in a Phase 1 safety trial of 83 patients.^{86,87} Seven dose escalation cohorts of 25–175 mg of plasmin were given by 5 h catheter infusion.⁸⁷ There was major bleeding in 5% of patients and minor bleeding in 16%; the bleeding risk did not appear to be related to dose. At the highest doses, plasmin infusions lowered fibrinogen (20%), a2AP (39%) and α 2-macroglobulin (25%). Dissolution of the thrombus by 50% occurred in 79% of the patients receiving the highest doses vs. 50% receiving lower doses. Adverse events occurred in 57 subjects (69%) including hypertension (17%), γ -glutamyltransferase elevation (7%), constipation (7%), nausea (6%), and hypotension (6%), of which one event (hypertension and sinus tachycardia) was considered to be plasmin-related.⁸⁷ Serious adverse events occurred in 23% of patients: “postprocedural hematoma and hematoma, artery or graft complication or occlusion, pseudoaneurysm, peripheral ischemia, GI hemorrhage, MI, arterial rupture, hypertension, abdominal pain, wound infection, sepsis, compartment syndrome, joint effusion, convulsion, ischemic stroke, multiorgan failure, metastatic neoplasm, and psychotic disorder.”⁸⁷ However, some of these events were not attributed to plasmin, due to the timing of the event and the lack of a relationship between the dose of plasmin and the frequency of serious adverse events. Plasmin is now in a Phase 1/2a trial for treatment of ischemic stroke and a Phase 2 trial for peripheral arterial ischemia sponsored by Grifols.⁸⁵

A2AP cleaving enzyme inhibition

Inhibitors of a2AP cleaving enzyme or a soluble form of fibroblast activating protein may be of value in accelerating fibrinolysis by delaying the production of Asn-a2AP which is more rapidly crosslinked to fibrin.²¹ While there appears to have been no published clinical investigations of a fibroblast activation protein-targeted therapy for thrombotic disease, there are ongoing clinical trials to investigate the clinical utility of modifying its activity on carcinoma-associated fibroblasts as a novel chemotherapeutic strategy.⁸⁸

Summary and Prospects

Evidence suggests that a2AP plays a critically important role in ischemic stroke. Epidemiologic and clinical studies indicate that a2AP levels are linked to the risk of developing stroke and to the therapeutic success of r-tPA. Experimental studies confirm that a2AP levels affect brain injury and outcomes after thromboembolic stroke, as well as stroke induced by surgical ligation without reperfusion. Therapeutic inactivation of a2AP by monoclonal antibodies or microplasmin-plasmin significantly reduces brain infarction (Fig. 1). Experimental a2AP-I had a longer therapeutic window for reducing ischemic brain injury than r-tPA. By comparison to r-tPA, a2AP inactivation markedly diminishes brain swelling, brain hemorrhage and mortality. Inactivation of a2AP markedly reduces microvascular thrombosis, MMP-9 expression and breakdown of the blood brain barrier. Taken together, these studies demonstrate enormous potential for a2AP-targeted therapies in ischemic

stroke. Early clinical studies are planned or underway to test whether modulating a2AP by microplasmin, plasmin or monoclonal antibodies are effective in reducing thrombosis, morbidity and disability in patients with ischemic stroke.

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Figure 1.

Effects of a2AP-inactivation on outcomes in experimental ischemic stroke. See text for details. *Reed GL, Houg AK, Singh S, unpublished data.

Table 1

Effects of a2AP on Experimental Ischemic Stroke

Outcome	Effect and Comparison				
	Normal a2AP Blood Levels		High a2AP Blood Levels		
	a2AP-I vs. r-tPA ⁴¹	a2AP-I (or deficiency) vs. control ^{b, 41,51,52}	a2AP-I + r-tPA vs. r-tPA ⁴⁰	vs. normal a2AP levels ⁴¹	Effect on r-tPA treatment ⁴⁰
Ischemic brain injury	⇓⇓ ^b	⇓⇓	⇓⇓	⇓⇓	⇓⇓
Brain Swelling	⇓⇓	⇓⇓	⇓⇓	⇓⇓	⇓⇓
Brain Hemorrhage	⇓⇓	⇓⇓	⇓⇓	⇔	⇔⇓
Microvascular thrombosis	⇓⇓	⇓⇓	-	⇓⇓	-
MMP-9 expression	⇓⇓	⇓⇓	-	⇓⇓	-
Blood brain barrier breakdown	⇓⇓	-	⇓⇓	-	-
MCA thrombus dissolution	⇓⇓ ^c	⇓⇓	⇓⇓	⇓⇓	⇓⇓
Survival	⇓⇓	⇓⇓	⇓⇓	-	-
Apoptosis	-	-	⇓⇓	-	-

^aControl, normal a2AP levels.

^b⇓⇓ markedly greater; ⇓ greater; ⇔ no significant difference; ⇓ less than or ⇓⇓ markedly less in comparison

^cDose-related