EXTENDED REPORT

Recombinant tissue plasminogen activator injected into the vitreous cavity may penetrate the retinal veins of a porcine model of vascular occlusion

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Aim: To determine if recombinant tissue plasminogen activator (rtPA) injected into the vitreous cavity can penetrate the retinal vessels of porcine eyes with or without vascular occlusion.

Methods: Eight eyes (group I) of four pigs underwent clamping of the optic nerve flush with the globe for 90 minutes. One hour after reperfusion, one eye of each pig was injected with 75 µg of rtPA, and the fellow eye was injected with balanced salt solution (BSS). Eyes were processed for immunohistochemistry. Four additional eyes (group II) of two pigs were subjected to the same injections, but without optic nerve clamping.

Results: After reperfusion, the clinical picture was similar to that of a central retinal vein occlusion. Immunoperoxidase staining showed rtPA only in the retinal veins but not the retinal arteries in all eyes injected with rtPA in both groups I and II. Those eyes also showed intense rtPA staining at the level of the internal limiting membrane (ILM). No staining was seen at the level of the ILM or inside the retinal vessels in the BSS injected eyes. Immunofluorescence staining showed intense staining at the level of the ILM, but not inside the retinal vessels in the rtPA-injected eyes.

Conclusions: rtPA may penetrate the retinal veins, but not the arteries of porcine eyes with and without vascular occlusion. The ILM may play a part in preventing rtPA penetration.

Retinal vein occlusion is the second most common retinal vascular cause of visual loss after diabetic retinopathy.¹ Venous obstruction elevates the retinal venous pressure leading to scattered intra-retinal haemorrhage and macular oedema with decreased visual acuity. Associated capillary closure and subsequent retinal ischaemia can lead to intraocular neovascularisation in some eyes, which can cause further visual loss from vitreous haemorrhage or neovascular glaucoma.

Since histopathological studies have demonstrated a thrombus at the level of the lamina cribrosa in eyes with central retinal vein occlusion (CRVO),² thrombolytic therapy has been attempted. Systemic fibrinolytic therapy can improve experimental retinal vein occlusion in rabbits.³ Several studies using intravenous recombinant tissue plasminogen activator (rtPA) and intravenous heparin have shown an improvement in visual acuity.^{4–6} However, systemic fibrinolytic therapy can have significant risks, including stroke, bleeding that requires transfusion, and even death.^{4,7} More localised, targeted thrombolytic treatment can improve retinal venous outflow after injection in the ophthalmic artery.⁸ However, this technique is associated with systemic complications. Cannulation of the retinal veins and injection of rtPA is in progress but still not without complications.^{9–11}

Lahey and associates¹² have reported a pilot study of intravitreal rtPA for CRVO in which 70% of patients had stable or improved vision by 6 weeks. They hypothesised that intravitreal rtPA diffuses across the retinal internal limiting membrane (ILM) and into damaged retinal capillaries, but this has not been proved. Kamei and associates have shown that rtPA does not cross a normal rabbit retina.¹³ However, intravitreal injection of tPA has been shown to facilitate clot lysis in a rabbit model of subretinal haemorrhage.¹⁴ Whether intravitreal rtPA is able to enter a retinal vein, with or without ischaemic injury, is unknown.

The vascular supply of the porcine retina is very similar to the human and has four major retinal arteries and veins and a capillary network in multiple layers.¹⁵ A porcine model was

chosen for our study as this species has holangiotic retinal vascular pattern, whereas the rabbit eye is merangiotic, and much of the retina is avascular.

Our objective was to develop a porcine model of retinal vascular occlusion and to determine if rtPA injected into the vitreous cavity could be detected in the retinal vessels of the porcine eye with and without occlusion.

MATERIALS AND METHODS

All procedures were performed according to the Association of Research in Vision and Ophthalmology guidelines on use of animals in research, and the animal use protocol was approved by the institutional animal care and use committee at North Carolina State University.

Anaesthesia and pupillary dilation

Six Yorkshire cross pigs (12 eyes), aged 10 weeks and weighing 15 kg were used in the study. The animals were premedicated with intramuscular ketamine (11 mg/kg) and xylazine (2 mg/kg), and maintained on isoflurane 1–5% administered through an endotracheal tube. Mydriasis was achieved with a combination of topical tropicamide 1% and phenylephrine 2.5%.

Monitored endotracheal anaesthesia

The heart rate ranged between 80 and 100 beats per minute, with a mean arterial blood pressure of 80 mm Hg. The mean respiratory rate was 30 breaths per minute, and the oxygen saturation was maintained above 96%. Lactated Ringer's solution was used intravenously at a rate of 180–270 ml/h with a maximum volume of 900 ml per pig to maintain mean arterial blood pressure. No intraoperative vasopressors were used.

Abbreviations: BSS, balanced salt solution; CRVO, central retinal vein occlusion; ILM, internal limiting membrane; IF, immunofluorescence; IP, immunoperoxidase; PBS, phosphate buffered saline; RPE, retinal pigment epithelial; rtPA, recombinant tissue plasminogen activator

Figure 1 The porcine eye and evolution of an experimental central retinal vein occlusion. (A) Normal porcine retina. Note the lack of disc oedema. (B) Ischaemic phase. Arrowhead points to attenuated vascular segment in the posterior pole, and in the periphery in (C). (D) Reperfusion phase. Dot-blot (arrow) and flame-shaped (arrowhead) haemorrhages. Note the oedematous optic nerve head. (E) Preretinal haemorrhage in the reperfusion phase. (F) Central retinal vein occlusion, full blown with four quadrant haemorrhage and disc oedema.

Vascular occlusion methods

Following a deep surgical plane of anaesthesia in four pigs, a temporal peritomy was performed and the lateral rectus insertion was excised. The posterior pole of the eye was exposed with blunt dissection through periocular fascia and a right angled long forceps was used to clamp the optic nerve flush with the globe. The clamping included the ciliary vessels from which originate the central retinal arcade at the optic nerve head.¹⁵ The clamp was released at 90 minutes.

Intravitreal rtPA administration

Group I

Eight eyes of four pigs underwent vascular occlusion according to the protocol described above. One hour after

release of the clamp and reperfusion, one eye of each pig ($n = 4$) was injected in a standard sterile fashion with 75 μg (0.075 ml) of human recombinant tPA (0.1 mg/0.1 ml; Genentech, San Francisco, CA, USA) using a 30 gauge needle, and the fellow eye ($n = 4$) was injected with the same volume of balanced salt solution (BSS).

Group II

Four additional eyes of two pigs received the same intravitreal injections as eyes in group I but without previous vascular occlusion by clamping. Two of these four eyes were injected with rtPA and the fellow eye was injected with an equal volume of BSS. It is to be noted that eyes in group II also underwent a temporal peritomy and the lateral rectus

insertion was excised. The posterior pole of the eye was exposed with blunt dissection through periocular fascia, but no vascular occlusion was attempted.

Injections in both groups were masked so that the physician injecting did not know about the contents of the syringes.

Enucleation

With the animals still under a deep plane of general anaesthesia, eyes were enucleated 2 hours after injection. Animals were euthanised using an intravenous injection of pentothal sodium after enucleation was complete.

Snap freeze technique

Preliminary studies performed in our laboratory suggested that processing of tissues with standard tissue fixatives including formaldehyde and glutaraldehyde interfered with immunohistochemical detection techniques for rtPA. We therefore devised a frozen section technique, in which the ocular tissues were snap frozen, that was associated with good immunohistochemical staining.

The globe was sectioned by making two peripheral posterior to anterior horizontal cuts and discarding the two calottes (side pieces). The remaining tissue included the central optic nerve vasculature and most of the posterior segment. The anterior segment was removed and discarded. The posterior eyecup was put in Tissue-Tek OCT Compound (Sakura Finetek, USA, Torrance, CA, USA) in a mould. The mould was placed on an aluminium tray cooled in the vapour phase of liquid nitrogen in a Styrofoam container. The frozen

specimens were wrapped in aluminium foil, transferred to dry ice, and subsequently transferred and stored in a -80°C freezer until tissue processing.

Protocol for frozen section immunohistochemistry

Frozen sections were cut at $5\text{ }\mu\text{m}$ from all tissue samples and were then air dried for 30 minutes. The sections were then fixed in 4% paraformaldehyde for 30 minutes at room temperature. The sections were then washed in phosphate buffered saline (PBS) for 3 minutes, three times each. Background Buster (Innovex Biosciences, Richmond, CA, USA) was applied to sections for 30 minutes at room temperature. The tissues were again washed in PBS as above, incubated in 3% hydrogen peroxide in PBS for 30 minutes at room temperature, and again washed in PBS. The tissue sections were then incubated in a 1:100 dilution of primary antibody (mouse IgG₁ monoclonal antibody to recombinant human tissue plasminogen activator, American Diagnostica, Greenwich, CT, USA, product number ESP1) overnight for 12 hours at 4°C . The primary antibody that we employed is reported by the company to be specific for recombinant human tPA and not cross react with porcine tPA. The sections were then washed in PBS. Detection was with a stat-Q kit (Innovex Biosciences, Richmond, CA, USA) using a secondary linking antibody applied for 10 minutes at room temperature, washed in PBS, and peroxidase-streptavidin label applied for 10 minutes at room temperature. Sections were then washed in PBS and developed with Vector Red Substrate Kit (Vector Laboratories, Burlingame, CA, USA) for 3 minutes at room temperature. Levamisole solution (Vector

Figure 2 (A) Recombinant tissue plasminogen activator (reddish brown colour) is present inside a retinal vein (arrow) 2 hours after injection of $75\text{ }\mu\text{g}$ intravitreal rtPA in a group I porcine eye, after ischaemia reperfusion injury. Note the staining (reddish brown) of the internal limiting membrane (arrowhead). (B) Recombinant tissue plasminogen activator is present inside a retinal vein in a group II porcine eye without the ischaemic reperfusion injury, showing intravascular availability (arrow) after injection of $75\text{ }\mu\text{g}$ of intravitreal rtPA. Note the staining of the internal limiting membrane (arrowhead). (C) No rtPA is present inside a retinal artery in this group I porcine eye 2 hours after injection of $75\text{ }\mu\text{g}$ of intravitreal rtPA. Note the staining of the internal limiting membrane (arrow). (D) No staining inside the retinal vein after injection of intravitreal balanced salt solution. Note no staining of the internal limiting membrane (arrowhead). Scale = $50\text{ }\mu\text{m}$. Immunohistochemistry using a monoclonal anti-tPA antibody and avidin-biotin conjugate detection method. The primary antibody is a murine monoclonal antibody that binds with human rtPA (ESP-1). The secondary link-horse radish peroxidase is used with both mouse and rabbit antibodies. The tertiary antibody is a peroxidase streptavidin label.

Laboratories) was added to the Vector Red substrate solution to inhibit endogenous alkaline phosphatase. The slides were then washed in tap water for 10 minutes, counterstained with Harris haematoxylin (Fisher Scientific, Pittsburgh, PA, USA) for 15 seconds, and then washed again with tap water for 5 minutes. The sections were dehydrated through graded and absolute alcohol, followed by xylene, and then cover slipped with permanent mounting medium.

Negative controls, to detect non-specific binding of the primary antibody and/or non-specific peroxidase-streptavidin labelling, were run in parallel in the same fashion, except that Mouse Monoclonal IgG Isotype Control (Innovex Biosciences) was applied in place of the primary antibody. Immunofluorescent staining used an indirect procedure with similar steps except that fluorescein anti-mouse IgG (Vector Laboratories) was used for detecting primary antibody bound to injected rtPA.

RESULTS

Clinical findings

One minute after optic nerve clamping, the retinal arteries became attenuated, followed by segmentation of the blood column within the retinal veins (fig 1B, C). After reperfusion, immediate dilation and tortuosity of the retinal veins developed followed by disc oedema (fig 1D, E, F). Dot-blot (fig 1D), flame-shaped (fig 1D), and preretinal haemorrhages (fig 1E) in all four quadrants developed within 2 minutes. The ophthalmoscopic picture was characteristic of a human central retinal vein occlusion (fig 1F).

Histopathological findings

Histopathological examination of eyes in group I (occlusion) revealed intraretinal oedema and haemorrhages. Recombinant tPA was detected by immunoperoxidase in the retinal veins (fig 2A), but not arteries (fig 2C), in all eyes injected with tPA in both group I and group II. Staining within the lumen of the retinal veins was more marked in group I (occlusion) eyes (fig 2A) than group II (no occlusion) eyes (fig 2B). No labelling was detected inside the retinal vessels of eyes injected with BSS in group I, or group II (fig 2D). Diffuse labelling of the retinal internal limiting membrane (ILM) was detected in all eyes injected with rtPA (fig 2A,B, and C) but not in the eyes injected with BSS (fig 2D).

Indirect immunofluorescent technique did not show any staining within the retinal vessels of rtPA injected eyes in group I and II, but disclosed diffuse intense staining of the ILM in these eyes. No ILM staining was seen in the BSS injected eyes.

DISCUSSION

Recombinant tissue plasminogen activator injected into the vitreous cavity of porcine eyes was detected in the retinal veins using the immunoperoxidase technique. Our findings suggest that rtPA reached the lumen of the veins in greater quantity in eyes with the occlusion (group I) (fig 2A) compared to eyes without an occlusion (group II) (fig 2B). This may have been expected because of the breakdown of the blood-retinal barrier after vascular occlusion.

Owing to variable background staining in the immunoperoxidase stained negative control sections, we also performed indirect immunofluorescence, which did not reveal any staining inside the retinal veins of rtPA injected eyes but showed intense staining at the level of the internal limiting membrane (ILM). The cause for the different results using immunoperoxidase (IP) and immunofluorescence (IF) staining may reflect the possible lower avidity of the secondary antibody used in the IF method compared to the IP method. However, it is important to emphasise that with both

methods, there was an intense staining at the level of the ILM, only in the rtPA injected eyes. This highlights the important role of the ILM as a barrier to rtPA penetration.

The rtPA dose of 75 µg was chosen for this study after review of different doses used successfully in humans without side effects.^{12–16} Toxicity studies in cat¹⁷ and rabbit^{18–19} eyes suggested rtPA was toxic at intravitreal doses above 50 µg. However, the vitreous volume of the pig is much larger than in cats and rabbits and almost equal to the human volume, suggesting that an intravitreal dose above 50 µg would be safe in the pig. In fact, subretinal rtPA in a dose less than 200 mg/l was non-toxic in cats,²⁰ and our selected intravitreal dose of 75 µg is equivalent to 187.5 mg/l of human vitreous and is therefore considered by us to be non-toxic. The presence of retinal pigment epithelial (RPE) hyperplasia may indicate rtPA toxicity.¹⁷ We did not observe RPE hyperplasia in eyes injected with rtPA or BSS. However, our study was not designed to answer the toxicity profile of rtPA because of the very short duration of rtPA in the vitreous cavity before enucleation. It is to be noted that an intravitreal dose of 50 µg of rtPA with gas tamponade injected twice 3 days apart was found to be associated with RPE changes and reduced electroretinogram amplitudes in a case report.²¹

Given our study design, we cannot comment on the clinical efficacy of rtPA in eyes with retinal vein occlusion because the eyes were enucleated 2 hours after intravitreal injection. Recombinant tPA was not detected in the retinal arteries in our study, even in the occlusion eyes (group I). This may be due to the thicker arterial walls and perhaps less disruption of the blood-retinal barrier in the porcine model of vascular occlusion in arteries versus veins. This finding suggests that intravitreal rtPA may be less beneficial to eyes with retinal artery occlusion compared to eyes with CRVO. A recent study in rabbit eyes has demonstrated amelioration of the clinical picture of vein occlusion after 50 µg of intravitreal rtPA, but did not demonstrate any improvement in blood flow assessed by fluorescein angiography.²²

It has been postulated that rtPA injected within 21 days of acute CRVO may have a beneficial effect in some patients.^{12–23} If injected after more than 3 weeks of CRVO, tPA would not be effective as a result of fibrin cross linking occurring 21 days after thrombus formation.²⁴ Some studies showed that intravitreal rtPA was beneficial in non-ischaemic²⁵ and ischaemic²⁶ CRVO, if injected earlier in the course of the disease. Indeed, rtPA injected within 3 days of acute CRVO demonstrated a great effect on the course of the disease.²⁷ Early injection of rtPA in the course of CRVO may reach the retinal veins in greater amount when the blood-retinal barrier is maximally disrupted and exert an effective dissolution on the fresh thrombus. The effective time period for the maximum action of tPA from the beginning of the symptoms to the intravitreal injection is still to be determined. This would mainly depend on the speed of remodelling of the venous walls after an occlusion and the length of time the fresh thrombus is susceptible to rtPA. Intravenous tPA is an established treatment for acute thrombotic strokes, within 3 hours of presentation.²⁸ In one study, only 15% of the patients presented within the 3 hour time period when tPA would be of crucial importance and would lead to a better prognosis.²⁹ Although 15% may not be as high as one would desire, the effect of tPA on the better quality of life of these patients is of great significance. Some studies have also demonstrated that intravenous injection of tPA in patients with acute thrombotic stroke is useful within the first 6 hours, increasing the window of action of thrombolysis.^{28–30} A growing awareness in the ophthalmic and medical community of the possible effect that intravitreal tPA may have on prognosis, although not without complications, may

allow expedient referral, at least for the trial of early injection of tPA in the vitreous cavity in CRVO, to determine the effect on prognosis and compare it to the natural history of the disease.

In conclusion, our results suggest that rtPA injected into the vitreous cavity may penetrate into retinal veins but not arteries in this non-vitreotomized porcine model with and without vascular occlusion. The internal limiting membrane has a role as a barrier to rtPA diffusion into the retinal veins.

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REFERENCES

- Clarkson J. Central retinal vein occlusion. In: Ryan S, eds. *Retina*. St Louis: Mosby, 1994:1379.
- Green WR, Chan CC, Hutchins GM, et al. Central retinal vein occlusion: a prospective histopathologic study of 29 eyes in 28 cases. *Trans Am Ophthalmol Soc* 1981;**79**:371-422.
- Oncel M, Peyman GA, Khoobehi B. Tissue plasminogen activator in the treatment of experimental retinal vein occlusion. *Retina* 1989;**9**:1-7.
- Elman MJ. Thrombolytic therapy for central retinal vein occlusion: results of a pilot study. *Trans Am Ophthalmol Soc* 1996;**94**:471-504.
- Hattenbach LO, Steinkamp G, Scharrer I, et al. Fibrinolytic therapy with low-dose recombinant tissue plasminogen activator in retinal vein occlusion. *Ophthalmologica* 1998;**212**:394-8.
- Hattenbach LO, Wellermann G, Steinkamp GW, et al. Visual outcome after treatment with low-dose recombinant tissue plasminogen activator or hemodilution in ischemic central retinal vein occlusion. *Ophthalmologica* 1999;**213**:360-6.
- The GUSTO investigators. An international randomized trial comparing four thrombolytic strategies for acute myocardial infarction. *N Engl J Med* 1993;**329**:673-82.
- Vallee JN, Aymard A, Paques M, et al. Value of local ophthalmic artery fibrinolysis in severe forms of central retinal vein occlusion. *J Radiol* 2001;**82**:137-44.
- Weiss JN. Treatment of central retinal vein occlusion by injection of tissue plasminogen activator into a retinal vein. *Am J Ophthalmol* 1998;**126**:142-4.
- Weiss JN. Retinal surgery for treatment of central retinal vein occlusion. *Ophthalmic Surg Lasers* 2000;**31**:162-5.
- Weiss JN, Bynoe LA. Injection of tissue plasminogen activator into a branch retinal vein in eyes with central retinal vein occlusion. *Ophthalmology* 2001;**108**:2249-57.
- Lahey JM, Fong DS, Kearney J. Intravitreal tissue plasminogen activator for acute central retinal vein occlusion. *Ophthalmic Surg Lasers* 1999;**30**:427-34.
- Kamei M, Misono K, Lewis H. A study of the ability of tissue plasminogen activator to diffuse into the subretinal space after intravitreal injection in rabbits. *Am J Ophthalmol* 1999;**128**:739-46.
- Coll GE, Sparrow JR, Marinovic A, et al. Effect of intravitreal tissue plasminogen activator on experimental subretinal hemorrhage. *Retina* 1995;**15**:319-26.
- Bloodworth JM Jr, Gutesell HP Jr, Engerman RL. Retinal vasculature of the pig. Light and electron microscope studies. *Exp Eye Res* 1965;**4**:174-8.
- Elman MJ, Raden RZ, Carrigan A. Intravitreal injection of tissue plasminogen activator for central retinal vein occlusion. *Trans Am Ophthalmol Soc* 2001;**99**: 219-21; discussion 22-3.
- Hrach CJ, Johnson MW, Hassan AS, et al. Retinal toxicity of commercial intravitreal tissue plasminogen activator solution in cat eyes. *Arch Ophthalmol* 2000;**118**:659-63.
- Irvine WD, Johnson MW, Hernandez E, et al. Retinal toxicity of human tissue plasminogen activator in vitrectomized rabbit eyes. *Arch Ophthalmol* 1991;**109**:718-22.
- Johnson MW, Olsen KR, Hernandez E, et al. Retinal toxicity of recombinant tissue plasminogen activator in the rabbit. *Arch Ophthalmol* 1990;**108**:259-63.
- Benner JD, Morse LS, Toth CA, et al. Evaluation of a commercial recombinant tissue-type plasminogen activator preparation in the subretinal space of the cat. *Arch Ophthalmol* 1991;**109**:1731-6.
- Chen SN, Yang TC, Ho CL, et al. Retinal toxicity of intravitreal tissue plasminogen activator: case report and literature review. *Ophthalmology* 2003;**110**:704.
- Nehemy MB, Passos E. Intravitreal recombinant tissue plasminogen activator (rt-PA) in experimental retinal vein occlusion in rabbits. *Invest Ophthalmol Vis Sci* ARVO E-Abstract, 2002:3524.
- Glacet-Bernard A, Kuhn D, Vine AK, et al. Treatment of recent onset central retinal vein occlusion with intravitreal tissue plasminogen activator: a pilot study. *Br J Ophthalmol* 2000;**84**:609-13.
- Folk JC, Hershey JM, Rivers MB. Lack of effectiveness of tissue plasminogen activator 20 or more days after vitrectomy. *Arch Ophthalmol* 1991;**109**:614.
- Weizer JS, Fekrat S. Intravitreal tissue plasminogen activator for the treatment of central retinal vein occlusion. *Ophthalmic Surg Lasers Imaging* 2003;**34**:350-2.
- Mahmoud T, Fekrat S. Ischemic central retinal vein occlusion in a patient with anticardiolipin antibodies on coumadin responds to intravitreal tPA [ARVO abstract]. *Invest Ophthalmol Vis Sci* 2001;**53**:S1270.
- Ghazi NG, Noureddine BN, Haddad RS, et al. Intravitreal tissue plasminogen activator in the management of central retinal vein occlusion. *Retina* 2003;**23**:780-84.
- Ringleb PA, Schellinger PD, Schranz C, et al. Thrombolytic therapy within 3 to 6 hours after onset of ischemic stroke: useful or harmful? *Stroke* 2002;**33**:1437-41.
- Katzan IL, Hammer MD, Hixson ED, et al. Utilization of intravenous tissue plasminogen activator for acute ischemic stroke. *Arch Neurol* 2004;**61**:346-50.
- Hacke W, Donnan G, Fieschi C, et al. Association of outcome with early stroke treatment: pooled analysis of ATLANTIS, ECASS, and NINDS rt-PA stroke trials. *Lancet* 2004;**363**:768-77.