Online First

Compatibility of Recombinant Tissue Plasminogen Activator and Bevacizumab Co-Applied for Neovascular Age-Related Macular Degeneration With Submacular Hemorrhage

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Objective: To investigate the compatibility of recombinant tissue plasminogen activator (rtPA) and bevacizumab in vitro because during surgery, rtPA or rtPA-induced plasmin may cleave and inactivate bevacizumab.

Methods: To simulate the intraoperative range of mixing ratios of rtPA, bevacizumab, and subretinal blood, we calculated the volumes of 12 submacular hemorrhages (SHs) with a spherical cap formula using measurements derived from fundus photographs and spectral-domain optical coherence tomographic images. Bevacizumab was incubated with rtPA or plasmin before gel electrophoresis with Coomassie blue and silver staining. The anti-angiogenetic activity of bevacizumab in the presence of rtPA with or without clotted human blood or of plasmin was quantified by vascular endothelial growth factor–enzyme-linked immunosorbent assay after incubation with the supernatant of porcine retinal pigment epithelium cell cultures.

Results: The mean (SD) volume of SH was 28.6 (24.7) mm³ (range, 6.2-94.6 mm³). In sodium dodecyl sulfate–polyacrylamid electrophoresis with Coomassie blue or silver staining, bevacizumab displayed characteristic patterns of protein bands. No additional fragments were detected in co-application of bevacizumab with either rtPA or plasmin. The anti-angiogenetic activity of bevacizumab remained unchanged in co-application with rtPA with or without blood or plasmin.

Conclusions: We demonstrated the absence of cleavage or functional inactivation of bevacizumab by rtPA in an in-vitro model of their intraoperative co-application as a treatment of SH.

Clinical relevance: In clinical practice, rtPA and bevacizumab can be co-applied as a treatment for neovascular age-related macular degeneration with SH to simultaneously clear SH and reduce choroidal new vessel activity.


Without treatment, the long-term prognosis of neovascular age-related macular degeneration (AMD) complicated by submacular hemorrhage (SH) is usually poor because the underlying choroidal neovascularization (CNV) lesion progresses and the resolution of the hemorrhage is associated with the formation of a macular scar. In addition to the progression of CNV, damage to sensory retinal tissue by SH has been attributed to a limitation of the passage of nutrients to the retina, shrinkage of the outer retinal layers owing to clot formation, and release of toxic substances such as fibrin, iron, and hemosiderin. Toxic effects of subretinal blood can be evidenced 24 hours after hemorrhage. Subretinal surgery with removal of a submacular blood clot has been abandoned because of poor functional outcome. To avoid surgical manipulation of the macular retina, the displacement of SH by intravitreal injection of recombinant tissue plasminogen activator (rtPA) and gas or by subretinal injection of rtPA during pars plana vitrectomy followed by an intravitreal gas tamponade have been proposed.

In the past, visual acuity often improved after successful displacement of SH by rtPA and gas but then frequently deteriorated because of progression of the underlying CNV. Since the advent of anti-vascular endothelial growth factor (VEGF) pharmacotherapy, new aspects have been added to the management of neovascular AMD with SH. Anti-VEGF agents administered by intravitreal injection are a safe and...
effective treatment for neovascular AMD for up to 2 years. In view of the intriguing clinical results, a combined intravitreal application of bevacizumab and rtPA could represent a significant improvement of current treatment concepts for neovascular AMD with SH.\textsuperscript{14,15,18,19} While the application of rtPA can help prevent a toxic effect from SH by effective displacement from the fovea,\textsuperscript{11-15,18,19} the simultaneous application of anti-VEGF agents could potentially prevent CNV progression.\textsuperscript{14,15,18,19}

We have previously reported the outcome of pars plana vitrectomy with subretinal co-application of rtPA and bevacizumab followed by intravitreal fluid-gas exchange. While the functional and anatomical results of our previous case series are indeed promising,\textsuperscript{14,15} it remains unclear whether co-applied rtPA and bevacizumab are compatible. Recombinant tissue plasminogen activator is a serine protease with its catalytic activity around the $\beta$ chain (light chain). The serine protease family possesses a triad characteristically formed of a serine, a histidine, and an aspartate. The hydroxyl group of the serine plays a role in the nucleophile and attacks the carbonyl peptide bond. Recombinant tissue plasminogen activator cleaves after the arginine, but other amino acids determine the specificity of rtPA. The main substrate of rtPA is plasminogen, which cleaves into active plasmin. Plasmin then degrades the fibrin matrix, which constitutes blood clots. However, other proteins such as hepatocyte growth factor or platelet-derived growth factor are also blood clots. However, other proteins such as hepatocyte growth factor or platelet-derived growth factor\textsuperscript{20,21} are also cleaved by rtPA. Hence, it is uncertain whether in co-application either rtPA itself, rtPA-generated plasmin, or other undetermined proteases activated during fibrinolysis of a subretinal blood clot may cleave, thereby functionally inactivating the antibody bevacizumab.

Therefore, the aim of our study was to investigate the compatibility of rtPA and bevacizumab in an in-vitro model of their intraoperative co-application as a treatment for acute SH in neovascular AMD, assessing potential cleavage as well as the functionality of bevacizumab.

**METHODS**

**VOLUME OF SH**

For our study, 12 eyes were randomly chosen from a consecutive series of 40 eyes with neovascular AMD complicated by acute SH involving the fovea with a history of symptoms for a maximum of 2 weeks. Exclusion criteria were other etiologies of SH, massive hemorrhage beyond the equator, and preexisting macular scar. The area of SH was measured using digital fundus photographs and the maximal height using optical coherence tomographic images (Spectralis HRA + OCT; Heidelberg Engineering). Volume of SH was calculated with a spherical cap formula (volume = $\frac{1}{6} \times \pi \times $ height $\times 3 \times area^2 + $ height$^3$).

**CELL CULTURE**

Retinal pigment epithelium (RPE) cell culture was conducted as described elsewhere.\textsuperscript{22} In brief, fresh porcine eyes were obtained from a local abattoir and the preparation was initiated within 6 hours of death. Whole globes were briefly immersed in antiseptic solution. The anterior portion of the eye and the vitreous were removed by a circumferential incision at the pars plana. To each eye cup, trypsin was added and incubated for 5 minutes at $37^\circ$C. Trypsin solution was removed and replaced by trypsin-ethylenediaminetetraacetic acid (EDTA) for 45 minutes at $37^\circ$C. Retinal pigment epithelium cells were gently pipetted off the choroid, collected in media, and washed. The RPE of 3 eyes were collected and seeded in a 60-mm dish. Cells were cultivated in Dulbecco modified Eagle medium (PAA) and Ham F12 medium (PAA) (1:1) supplemented with 1% penicillin/streptomycin, 1-glutamine, 25 mM Hepes, 11 000 mg/dL of sodium pyruvate (to convert to micromoles per liter, multiply by 113.56), and 10% fetal call serum (Linares).

**INTERACTION STUDIES**

We tested the co-application of rtPA and bevacizumab and plasmin and bevacizumab.

In all experiments, we used bevacizumab (Roche), 25 mg/mL, dissolved in balanced salt solution (Alcon) and rtPA (Boehringer Ingelheim), 0.2 mg/mL, dissolved in balanced salt solution. Intraoperatively, as described in our previous patient study,\textsuperscript{14,15} 10 µg to 20 µg of rtPA dissolved in 0.05-0.1 mL of balanced salt solution, followed by bevacizumab, 1.25 mg, dissolved in 0.05 mL of balanced salt solution was injected subretinally. Accordingly, in our study, we used the same mixing ratio and incubated bevacizumab, 10 µL (250 µg), for each interaction experiment with 10 µL (2 µg) of rtPA for 2 hours at $37^\circ$C to a final volume of 20 µL. The mixture was collected and separated in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in dilutions of 1:10 and 1:100. The interaction of bevacizumab and plasmin was investigated using plasmin derived from human plasma with 2 or more units/mg of protein (Sigma). As the clinically relevant concentration of plasmin in SH treated with bevacizumab and rtPA is uncertain, 2 concentrations of plasmin were used: 5 µL (125 µg) of bevacizumab was incubated with 0.2 µg (mixture 1) or 2 µg (mixture 2) of plasmin, respectively, for 2 hours at $37^\circ$C at a final volume of 10 µL. The mixtures were collected and separated in SDS-PAGE in dilutions of 1:10 and 1:100.

**SDS-PAGE**

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was conducted under standard conditions using a discontinuous 12% SDS gel in a BioRad Mini-Protean II electrophoresis cell (BioRad).

**COOMASSIE STAINING**

After SDS-PAGE, gels were briefly washed in TTBS and incubated overnight in Coomassie staining solution (Brilliant Blue R-250; BioRad). Gels were destained for approximately 1 hour (Destain Solution, Coomassie R-250; BioRad) until desired destaining was achieved.

**SILVER STAINING**

After SDS-PAGE, gels were washed twice for 5 minutes with ultrapure water and fixated in 30% ethanol and 20% acetic acid overnight. Gels were washed twice for 5 minutes with ultrapure water and incubated for 1 minute with a sensitizer solution (Thermo Scientific). After 2 brief washings, gels were incubated for 2 minutes in development solution (Thermo Scientific). The reaction was stopped by 5% acetic acid.

All gels were photographed using a Bio-Image System (ChemDoc; Biostep) and dried in a standard procedure using a 2% glycercine solution and cellophane (Roth). In all gels, the size of the bands was determined using TotalLab Software (Nonlinear Dynamics).
All experiments were independently repeated at least 3 times.

**ANTI-VEGF EFFECTIVENESS OF BEVACIZUMAB**

To determine the anti-VEGF effectiveness of bevacizumab in the presence of (1) rtPA, (2) plasmin, and (3) rtPA and clotted human blood, the ability of bevacizumab to inhibit VEGF detection by enzyme-linked immunosorbent assay (ELISA) was quantified as previously described.22

1. Bevacizumab and rtPA were incubated as just described. As control samples, rtPA (2 µg in 10 µL, plus 10 µL of sodium chloride) or bevacizumab (250 µg in 10 µL, plus 10 µL of sodium chloride) were separately incubated for 2 hours at 37°C. All mixtures were kept on ice until further use.

2. Bevacizumab, 250 µg, was incubated with 0.4 µg or 4 µg of plasmin to a final volume of 20 µL for 2 hours at 37°C. As control samples, plasmin (4 µg in 10 µL, plus 10 µg of sodium chloride) or bevacizumab (250 µg in 10 µg, plus 10 µg of sodium chloride) were separately incubated for 2 hours at 37°C. All mixtures were kept on ice until further use.

3. Intraoperative conditions were simulated to quantify the anti-VEGF effectiveness of bevacizumab in the presence of rtPA and human blood. Whole blood was obtained from a healthy volunteer (J.H.). Ten drops were collected in an uncoated Eppendorf tube and left for 15 minutes at room temperature, which resulted in complete clotting. Recombinant tissue plasminogen activator and bevacizumab were added at volumes and concentrations used in surgery (rtPA, 50 µL/10 µg; bevacizumab, 50 µL/1.25 mg). The mixture was incubated for 30 minutes at 37°C. Following incubation, the mixtures were centrifuged for 1 minute and the supernatant (approximately 160 µL) was collected and kept on ice until further use. As control samples, rtPA and bevacizumab were added to the conditioned medium as just described.

Table 1. Mean of Bands in Coomassie Stainings of Bevacizumab and rtPA

<table>
<thead>
<tr>
<th>Mean, kDa</th>
<th>rtPA</th>
<th>Bevacizumab</th>
<th>Mixture</th>
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<tbody>
<tr>
<td>230.1</td>
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<td>23.7</td>
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</tr>
<tr>
<td>18.8</td>
<td>20.5</td>
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</table>

Abbreviation: rtPA, recombinant tissue plasminogen activator.

**Figure 1.** Protein bands as seen in representative sodium dodecyl sulfate–polyacrylamide gel electrophoresis after co-incubation of bevacizumab and recombinant tissue plasminogen activator (rtPA) or bevacizumab and plasmin, respectively, in Coomassie and silver staining. Mixtures were applied in 2 dilutions for better visual analysis. For molecular weight (MW) analysis of bands, only the 1:10 dilution was considered (see tables for MW). Protein band patterns in mixtures correspond to untreated proteins; loss of single bands is caused by dilution. Bev indicates bevacizumab untreated; M, marker; and plas, plasmin untreated. Mix = 250 µg of bevacizumab and 2 µg of rtPA incubated for 2 hours at 37°C; mix1 = 125 µg of bevacizumab incubated with 0.2 µg of plasmin; and mix2 = 125 µg bevacizumab incubated with 2 µg of plasmin.
For the functionality assay, the medium of confluent primary porcine RPE cells was collected for 4 hours as primary porcine RPE cells have been shown to secrete a significant amount of VEGF into the supernatant. To a volume of 500 µL of conditioned medium, 20 µL of the mixtures 1, 2, or 3 was added, respectively, incubated for 15 minutes at 37°C, and centrifuged at 13,000 rpm for 5 minutes. The supernatant was collected and a VEGF ELISA (Quantikine; R&D Systems) was performed according to manufacturer’s instructions.

All experiments were independently repeated at least 3 times.

RESULTS

VOLUME OF SH

The area of SH ranged from 7.85 mm² to 51.61 mm², while the height ranged from 0.39 mm to 1.16 mm. Consequently, the mean (SD) volume of SH was calculated as 28.6 (24.7) mm³ (range, 6.2-94.6 mm³).

INTERACTION STUDIES

Bevacizumab and rtPA

With Coomassie staining, the mean value of the bevacizumab bands (applied 1:10 in the gel), rtPA bands, and the bands when both agents were co-incubated for 2 hours at 37°C are depicted in Table 1. No additional bands appeared in co-application, while the bands of high molecular weight (MW) were slightly moved to a higher molecular weight. A band corresponding to 37.8 kDa found in bevacizumab was missing in the mixtures. A representative gel is depicted in Figure 1.

By comparison to Coomassie staining, silver staining revealed additional bands. The mean value of the bevacizumab bands (applied 1:10 in the gel), rtPA bands, and the bands when both agents were co-incubated for 2 hours at 37°C are depicted in Table 2. No additional bands were seen in co-application. One bevacizumab band at 17 kDa did not appear in co-application (Table 2; Figure 1).

Plasmin and Bevacizumab

With Coomassie staining, the mean value of the bevacizumab bands (applied 1:10 in the gel), plasmin bands, and co-incubation bands of mixture 1 (0.2 µg plasmin) and mixture 2 (2 µg plasmin) are depicted in Table 3. No additional bands were seen in co-application. The bands of plasmin were lost in both mixtures (Table 3; Figure 1).

By comparison to Coomassie staining, silver staining revealed additional bands as depicted in Table 4. All bevacizumab bands appeared correspondingly in both mixtures.
tured. In the mixtures, 1 additional 27.5-kDa band appeared, which correlates with the 27.5-kDa plasmin band (Table 4; Figure 1).

Anti-VEGF Effectiveness of Bevacizumab

Significant amounts of VEGF (mean [SD], 290.2[101.8] pg/mL per hour) were detected in the medium of confluent cultured primary porcine RPE cells of second passage. In the control experiments, virtually no VEGF was detected by ELISA following the addition of bevacizumab, 250 µg in 20 µL, to 500 µL medium conditioned by RPE cells for 4 hours.

In mixture 1, when bevacizumab was co-incubated with rtPA (2 µg in a total volume of 20 µL) at 37°C for 2 hours, virtually no VEGF was detected in the supernatant. No loss of anti-VEGF effectivity was observed when bevacizumab and rtPA are co-incubated in the clinically used mixing ratio (Figure 2A).

In mixture 2, when bevacizumab was co-incubated with 0.4 µg of plasmin (data not shown) and 4 µg of plasmin, no VEGF was detected in the supernatant (Figure 2B).

In mixture 3, when bevacizumab was co-incubated with rtPA and clotted human blood, virtually no VEGF was detected in the supernatant (Figure 2C).

Our findings demonstrate that when rtPA and bevacizumab are co-applied, the presence of neither rtPA or plasmin, which is the main product of enzymatic rtPA activity, cleave bevacizumab, and the anti-angiogenic activity of bevacizumab remains intact in the presence of rtPA with or without human blood or in the presence of plasmin.

We used Coomassie staining and silver staining as a second method to confirm the findings and detect additional bands not stained by Coomassie. With both stains, the band patterns clearly show that no additional bands appeared when bevacizumab is co-incubated with either rtPA or plasmin, hence bevacizumab remains structurally intact (Figure 1). Some individual bands did not appear in co-incubation, which is most likely owing to a greater dilution of the proteins in the mixture. We used 12% gels optimized for the separation of proteins at a medium range of MW (30-70 kDa) because 12% gels provide an overall good separation across the entire MW spectrum and the main bands found in this study were within the optimum range. Twelve-percent gels are less reliable in the higher MW ranges (>150 kDa) and this may be the reason for the observed different high molecular weight band patterns of bevacizumab alone as compared to the mixtures (Figure 1).

Our results confirm the results of Faure et al,23 who also found an absence of cleavage of bevacizumab by rtPA in electrophoresis. However, our study adds important new findings because we show that besides rtPA, rtPA-generated active plasmin also does not cleave bevacizumab. We also used silver stain, a more sensitive staining method, to confirm the results. Furthermore, electrophoresis alone does not prove the therapeutic compatibility of the 2 agents. Electrophoresis itself fragments bevacizumab, which implies that bevacizumab fragments generated by rtPA would not be identified as such if they are of the same MW as the fragments generated by electrophoresis. Even cleaved bevacizumab could still be functional as long as the cleavage does not interfere with VEGF binding. Hence, to reliably determine the functionality of bevacizumab co-applied with rtPA, we quantified binding of VEGF by bevacizumab when co-incubated with rtPA or plasmin. Furthermore, other possible mechanisms of impairment of the antiangiogenic effect of bevacizumab when injected into the area of SH in patients include a possible interaction with blood as platelets have been shown to take up bevacizumab24 and bevacizumab could be cleaved and functionally inactivated by undetermined proteases activated during fibrinolysis of the subretinal blood clot. Therefore, we used an in-vitro model of the intraoperative situation with rtPA incubated with clotted human blood followed by a test of the effect of the resulting liq-
uid on the functionality of bevacizumab. The amount of human blood used in the ELISA experiments was greater than the calculated volumes of SH. However, an excess amount of blood ascertains that all bevacizumab molecules in the solution would be saturated with undetermined proteolytic enzymes potentially activated during fibrinolysis. The present ELISA experiments clearly show that neither rtPA, plasmin, or proteases activated during fibrinolysis of a subretinal blood clot functionally inactivate bevacizumab.

Our results are relevant to the clinical management of acute SH in neovascular AMD. Intravitreal18,19 and subretinal14,15 co-applications of rtPA and bevacizumab have been reported. Both rtPA and bevacizumab are relatively large molecules that exceed the experimentally determined molecular exclusion limit of human retina.25 Bevacizumab has been shown to traverse healthy retina,26 but it probably does so only at a slow rate because of its size.25 It is unclear whether intravitreally injected rtPA penetrates the retina because rtPA injected into the vitreous of rabbits failed to pass through healthy retina.27 On the other hand, molecules with similar MW (eg, albumin) have been shown to penetrate the diseased retina.28 A subretinal blood clot may hamper transretinal diffusion and following intravitreal injection, both agents may not reach the subretinal space and the underlying CNV at a sufficient concentration. However, while the intravitreal application of rtPA and bevacizumab may suffice to produce a therapeutic response,18,19 subretinal application14,15 ensures direct liquefaction of clotted subretinal blood by rtPA and the delivery of bevacizumab directly at the site of the CNV, which may enhance its effect. We found that bevacizumab is not functionally inactivated in the presence of rtPA and blood. This finding is clinically relevant because in intravitreal (and perhaps even more so in subretinal) co-application, not only the 2 drugs but also bevacizumab and proteolytic enzymes released from the liquefied blood clot could possibly interact.

Our findings cannot be directly translated from bevacizumab to ranibizumab. However, because ranibizumab is an antibody fragment derived from the same parent murine antibody as bevacizumab, it seems highly unlikely that when co-applied with rtPA, ranibizumab would behave differently from bevacizumab.

In conclusion, we demonstrate the absence of cleavage of bevacizumab by rtPA or plasmin and the absence of a functional inactivation of bevacizumab by rtPA with or without whole blood or by plasmin. In clinical practice, rtPA and bevacizumab can be co-applied to simultaneously clear subretinal hemorrhage and reduce choroidal new vessel activity.

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Author Contributions: All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Financial Disclosure: None reported.

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REFERENCES


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**Ophthalmic Images**

**Ciliary Body Medulloepithelioma in a 10-Year-Old Boy**

David A. Lewis, MD
Sarah Nehls, MD
Jocelyn Rowe, MD
Sherif Khedr, MD
Heather D. Potter, MD

**Figure 1.** A 10-year-old boy with a chronic red eye presents with a cystic, vascular, nonpigmented ciliary body mass extending into the anterior chamber.

**Figure 2.** Microscopic examination shows a medulloepithelioma consisting of cystic spaces of hyaluronic acid and primitive neuroepithelial cells arranged in sheets, cords, tubules, and rosette-like formations (hematoxylin-eosin, original magnification ×200).