Thrombolysis in Experimental Cerebral Amyloid Angiopathy and the Risk of Secondary Intracerebral Hemorrhage

Björn Reuter, MD*; Saskia Grudzencki, PhD*; Eva Chatzikonstantinou, Dip; Stephen Mairs, MD; Anne Ebert, PhD; Patrick Heiler, PhD; Lothar R. Schad, PhD; Matthias Staufenbiel, PhD; Michael G. Hennerici, MD; Marc Fatar, MD

Background and Purpose—Intracerebral hemorrhage (ICH) is the most adverse event of thrombolysis in ischemic stroke. Cerebral amyloid angiopathy increases the risk for spontaneous lobar ICH. Although thrombolysis may be performed in cerebral amyloid angiopathy–affected patients, there is still little knowledge available on the risk for secondary ICH.

Methods—We investigated the effect of recombinant tissue-type plasminogen activator on experimental ischemic stroke in APP23 transgenic mice (n=18) and wild-type littermates (n=15). Focal ischemic stroke was induced in 26-month-old mice by temporal middle cerebral artery occlusion (filament model), followed by treatment with 10 mg/kg recombinant tissue-type plasminogen activator. Twenty-four hours later, a functional score was assessed and the mice were euthanized for histological analysis. ICH was classified as grades 1 to 3 depending on severity.

Results—The groups did not differ regarding mortality (P=0.67) and functional deficit (P=0.18). Compared with wild-type mice, the APP23 genotype was associated with a higher appearance for ICH in the infarct area (P=0.05). ICH severity grades 2 and 3 correlated significantly with infarct size (P=0.004 and 0.008, respectively).

Conclusions—The APP23 genotype was not associated with increased mortality or worse functional outcome. Our results suggest an increased risk for ICH in the cerebral amyloid angiopathy–affected brain; however, no ICH was observed outside the ischemic area. (Stroke. 2014;45:2411-2416.)

Key Words: cerebral amyloid angiopathy ■ cerebral hemorrhage ■ mice, transgenic ■ stroke ■ thrombolytic therapy

In cerebral amyloid angiopathy (CAA), the formation of oligomers and insoluble amyloid-β (Aβ) deposits in the leptomeningeal and cortical small arteries and capillaries results in vascular dysfunction.1,2 This consequently leads to various degrees of cognitive impairment, white matter lesions, cortical microinfarcts, superficial siderosis, and multiple cortical microbleeds (cMBs), with a higher incidence of spontaneous lobar hemorrhage.3,4 The spatial relationship between lobar cMBs and vascular Aβ deposition was demonstrated by histological studies and noninvasive T2*-weighted MRI and Pittsburgh compound B positron emission tomography.5-7 Population-based studies indicate a prevalence of severe CAA between 7% and 24% in nondemented and 37% and 43% in demented aged subjects.8 For sporadic CAA, which is the most common variant, the only established risk factors are age and abnormal apolipoprotein E genotyping.9-11 A high occurrence of silent cortical and subcortical microinfarcts, as well as cMBs, in patients with CAA is well documented, but the frequency of associated clinical symptoms is small.6,14 However, it seems likely that the lifetime risk for symptomatic ischemic stroke (IS) in patients with CAA is considerably high.6,15

Today, thrombolytic therapy with recombinant tissue-type plasminogen activator (t-PA) is the only approved treatment in patients experiencing acute IS. This overall benefit comes at the cost of an increased risk for severe side effects such as symptomatic intracranial hemorrhage (ICH), with an 1.8% to 4.5% of all thrombolysed patients being affected.16-18

The increasing availability of MRI, including T2* gradient echo imaging, facilitates rapid diagnosis of cMBs, which are a characteristic pattern of CAA. This raises questions about the individual benefit to risk ratio in patients with stroke hospitalized within the time window and suitable for thrombolysis. Nowadays, thrombolysis is not restricted in CAA-affected patients experiencing acute IS. However, inclusion of specific sequences in current MRI stroke protocols allows for an evaluation of the risk for thrombolysis-associated ICH in patients.
suspected to have CAA. The objective of our study was to determine the risk of ICH after thrombolytic therapy in an IS model under standardized experimental conditions using the APP23 transgenic (APP23-tg) mouse model of CAA.

Methods

Animals

All experiments were approved by the local ethical animal care and use committee. The procedures were in accordance with institutional animal protection guidelines. Heterozygote B6D2-TgN[Thy-APPsw]23-tg mice (B6D2F1), provided by Dr Matthias Staufenbiel (Novartis Institutes for BioMedical Research, Novartis Pharma AG, Basel, Switzerland), were backcrossed twice with C57BL/6 mice (12 weeks old; Janvier, Saint Berthevin Cedex, France). Eighteen APP23-tg (8 males and 10 females) and 15 wild-type (WT) littermates (2 males and 13 females) were kept 26 months under a 12/12 hour light/dark cycle with standard food and water ad libitum. Sample size calculation is described in the online-only Data Supplement.

MCAo and r-tPA Thrombolysis

Surgeries and the following animal handling including behavioral assessment, MRI, and brain harvesting were performed by an independent investigator blinded to the genetic status of the mice. Transient focal cerebral ischemia (Figure 1A) was induced via intraluminal filament technique by temporal occlusion of the left middle cerebral artery (MCA). The transient endovascular filament model technique is not the model of choice concerning the evaluation of tPA-induced clot lysis and reperfusion. But it has the advantage of easy performance, high success rates, and lower rates of complications compared with clot models of cerebral ischemia. Because reperfusion was obligatory in our model to investigate the bleeding risk within the area of infarction, we considered this model suitable for our experiments.

Mice at 26 months of age were anesthetized by face mask with 3.5% isoflurane followed by 1.5% isoflurane for maintenance (Abbott, Wiesbaden, Germany) in a mixture of oxygen/air (1:1). Body temperature was maintained by a heating pad regulated by a rectal temperature probe. A 0.19-mm silicone-coated monofilament suture (Doccol, Sharon, MA) was inserted into the left common cerebral artery and gently advanced through the internal carotid artery until the offset of the MCA to achieve occlusion (MCAo). After 30 minutes, the filament was removed for reperfusion. After MCAo, animals were treated intravenously with 10 mg/kg r-tPA (Actilyse, INN alteplase; Boehringer Ingelheim, Ingelheim, Germany). A higher dosage was chosen than used in humans (0.9 mg/kg) because the murine fibrinolytic system is known to show a weaker response to r-tPA. r-tPA was administered as a 10% bolus followed by continuous infusion for 39 minutes. Twenty-four hours after MCAo, a modified functional score was assessed based on that developed by Bederson et al: 0=no observable deficit; 1=forelimb flexion; 2=+decreased resistance to lateral push; 3=++circling; 4=falling down to paretic side. Afterward, the mice were euthanized under deep isoflurane anesthesia by transcardial perfusion with 4% acid-free formaldehyde and the brains were harvested for histological processing. Hematoxylin/eosin and thioflavin S staining protocols were provided in the online-only Data Supplement.

Magnetic Resonance Imaging

In vivo imaging of 2 randomly selected mice of each group was performed on a 9.4-T Biospec 94/20 USR small animal system equipped with 740 mT/m gradients and a 1H surface cryogenic probe (Bruker, Ettlingen, Germany). MRI was performed 20 to 22 hours after MCAo. T2-weighted images and T2*-weighted gradient echo images were used to demonstrate the ischemic area and possible signs of ICH. The MRI protocol is provided in detail in the online-only Data Supplement.

Microscopy, Infarct, and ICH Analysis

All analyses were performed blinded to the genetic status and functional outcome of the mice. For bright-field and fluorescence analysis, a Leica DM 4500 B fluorescence microscope and Leica IM50 Image Manager Software were used (Leica, Wetzlar, Germany). The infarct area was determined by coronal brain slides every 100 μm, with the total number of slides depending on the brain size using Image J (National Institutes of Health, MD). Infarct volume was calculated by multiplying the infarct area with the distance between the sections. Bleeding grades were determined and counted microscopically by classifying them into grades 1 (single erythrocytes), 2 (small aggregates of erythrocytes), and 3 (large aggregates of erythrocytes) as described before. Representative images of bleeding classification are shown in Figure 1 in the online-only Data Supplement.

Statistical Analysis

Statistical analysis was performed with a standard software package (SPSS for windows; SPPS Inc, Chicago, IL.). All parametric values are expressed as mean ± SD. Differences between the groups regarding mortality and ICH frequency were calculated using the Fisher exact test. The neurological deficit was assessed by Mann–Whitney U test. For comparison of infarct volumes, a parametric 1-tailed t test was used. Sex-dependent differences of infarct volumes were determined with a 2-tailed t test. The correlation between infarct volume and ICH grades was calculated using the Spearman p test. Correlation coefficient was specified R. A value of P≤0.05 was considered significant.

Results

Comparison of Mortality and Neurological Deficit

Four of 18 APP-tg mice and 2 of 15 WT mice undergoing MCAo died either under the procedure or within 24 hours.
after surgery. In the APP-tg group, 1 mouse died during reperfusion, 1 during lysis, and 2 within 24 hours after surgery. In the WT group, 1 mouse died during reperfusion and 1 within 24 hours after surgery. Mortality rates were comparable with 22% in the APP-tg group versus 13% in the control group ($P=0.665$). If the MCA was not reached with the nylon thread during surgery for any reason (eg, anatomic), this experiment was considered not successful. This criterion led to exclusion of 1 mouse of each group from further statistical analysis. Assessment of the neurological deficit showed no differences of functional scores ($P=0.18$). A detailed table including data about the functional deficit, ischemic volume, localization of the ischemic area (cortex and basal ganglia), distribution pattern of bleedings, and bleeding grades is provided in the online-only Data Supplement.

**MRI Demonstration of Infarcts and ICH**

Three representative T2-weighted images of MCAo-derived ischemic area involving cortex and striatum can be seen in the right hemisphere of an APP-tg mouse. Histology revealed an infarct volume of 50.2 mm$^3$. T2*-weighted images of the same slices reveal several bleedings located only within the area of infarction (Figure 1A). T2-weighted images in a WT mouse revealed an ischemic lesion of size 29.9 mm$^3$ as per histological assessment. In the corresponding T2*-weighted images, no hypointensities indicative of acute bleedings can be seen (Figure 1B).

**Aβ Deposits and Vessel-Associated Bleedings**

Histological analysis using hematoxylin/eosin and thioflavin S staining demonstrated the colocalization of acute intracerebral bleedings and vascular Aβ deposits (Figure 2). Bleedings were strictly located within the area of infarction.

**ICH and Infarct Volume**

With 9 of 13 (69%) APP23-tg mice compared with 3 of 11 (27.3%) WT mice, APP23-tg mice displayed a significantly higher risk to develop ICH after stroke and thrombolytic therapy ($P=0.05$). In APP23-tg mice, numbers of grade 1 bleedings ranged from 0 to 17 (median=0; interquartile range [IQR]=1), grade 2 bleedings ranged from 0 to 174 (median=8; IQR=29.5), and grade 3 bleedings ranged from 0 to 104 (median=2; IQR=5.5). Grade 2 and 3 bleedings correlated significantly with infarct size ($P=0.004$; $R^2=0.74$ and $P=0.008$; $R^2=0.699$, respectively). For WT mice, the correlation of bleedings and infarct volume was statistically not feasible because of low numbers of mice displaying bleedings (Figure 3).

Mean infarct volumes for the WT group were calculated with and without exclusion of 1 mouse, which showed an enlarged ischemic lesion of 112 mm$^3$ and infarction within bihemispheric vascular territories. Affected were the left MCA territory and both anterior cerebral artery territories.
which can appear as a result of the variants of the intracranial vascular system. This ischemic lesion was significantly larger compared with the other WT mice, demonstrated by outlier analysis using an independent samples t test (P<0.001). Infarctions outside the vascular territory of the MCA were not a prespecified exclusion criterion of our study. However, in this case we regarded the exclusion feasible. The statistical analysis with inclusion of all WT mice is provided in the online-only Data Supplement. After exclusion of this WT mouse, the comparison of mean infarct volumes between the groups revealed significantly larger infarcts (±SD) of 32.5 (±24.9) mm³ in APP23-tg mice than in WT mice of 18.4 (±10.5) mm³ (P=0.04). Infarct volumes of APP-tg mice ranged from 4.5 to 76.9 mm³ (median=28.7; IQR=47.2). WT mice displayed infarct sizes from 7.22 to 34.4 mm³ (median=14; IQR=21.5; Figure 3). No sex-dependent differences in infarct volumes were determined between the groups, which we statistically verified to avoid any bias because of an imbalanced sex ratio.

Discussion

In the present study, we examined the effect of intravenous weight-adapted r-tPA treatment after MCAo in the aged APP23-tg mouse model of CAA and WT littermates. The APP23-tg mouse model was originally developed as a model of the Alzheimer's disease but demonstrated to deposit Aβ in the cerebrovascular system.28 Parenchymal Aβ deposition starts at the age of 6 months and vascular amyloid deposition at the age of 8 to 10 months.28,29 Subsequently, these mice develop characteristic morphological changes similar to those known from human CAA (ie, cMBs and rarely spontaneous lobar hemorrhage).29 Vascular Aβ deposits in 26-month-old mice are severe. In previous studies, CAA has shown to negatively affect cerebral vasoreactivity.30–32 In our study, the neurological deficit and mortality after MCAo did not statistically significantly differ compared with WT animals. However, the neurobehavioral testing might not have been sensitive enough to detect differences between the groups compared with more extensive test batteries.

Our results provide evidence that r-tPA treatment in the presence of Aβ causes an increased risk for ICH into the ischemic brain parenchyma and that ICH severity correlates with infarct volume. Aβ deposition induces proteolysis and anticoagulatory pathways, thus increasing the risk for ICH: Vascular Aβ deposition leads to matrix metalloproteinase activation and oxidative stress.33,34 In vitro data further suggest that Aβ promotes urokinase-type plasmin activator expression and plasminogen activation in human cerebrovascular smooth muscle cells.35,36

Although the APP23-tg mouse model of CAA is at greater risk for spontaneous vascular leakage with development of cMBs and lobar ICH, we observed no ICH after r-tPA treatment outside the infarct area. This difference from a previous study using the same mouse model may be related to the different interval between r-tPA treatment and analysis (24 hours in the present study versus 10 days in Winkler et al17). The occurrence of ICH strictly restricted to the area of ischemic injury might explain the missing impact on functional deficit and mortality in the APP23-tg group. APP23-tg mice demonstrated significantly larger ischemic lesions compared with WT mice. This observation is in line with a previous study reporting enlarged infarcts in the FVB/N transgenic APP mouse model, although this model does not develop vascular changes similar to CAA.38 Nevertheless, it has to be stated that differences in ischemic volumes after MCAo depending on genotype were not our primary outcome parameter. Prior sample size calculation was not performed to detect such differences. The inner-group variability of the ischemic lesions in our study is of relevance; therefore, the results have to be interpreted with caution and need to be confirmed.

In specialized stroke centers, the percentage of IS patients treated with r-tPA nowadays exceeds 20%.39 This may be attributed to growing evidence that patients aged ≥80 years also benefit significantly from r-tPA treatment.16,18,40,41 Thus, it is likely that patients with stroke experiencing (undiagnosed) CAA are frequently undergoing thrombolytic therapy. Yet, the risk for ICH intracerebral bleeding complications in this subgroup is still unknown. For large observational studies evaluating this question, the most appropriate morphological parameter indicating possible CAA is cortical- and subcortical-located cMBs. However, even the largest prospective clinical study with 570 patients addressing the relationship between cMBs and ICH after IS and thrombolytic therapy was statistically not powerful enough to detect a significant increase.42 One prospective study revealed an association among r-tPA therapy, ICH, and amyloid burden by Pittsburgh compound B positron emission tomography but did not allow a quantitative risk assessment because of small numbers.43 Two recent meta-analyses tried to estimate the frequency of ICH after thrombolytic therapy in the presence of cMBs but failed to demonstrate a valid association, again most likely because of small numbers.15,20

Transgenic animal models are most helpful to investigate the impact of therapeutic strategies in the presence of a well-defined underlying pathology and under standardized conditions. However, their results only generate hypotheses and need to be confirmed in future animal studies and human clinical trials. Symptomatic ICH after thrombolysis occurs in 1.8% to 4.5% of all patients, and only a subgroup can be attributed to CAA.16,18

Our study suffers some specific limitations: (1) We are not able to disclose hemorrhagic transformation in our model after stroke but in the absence of r-tPA. This might lead to an overestimation of the hemorrhagic burden induced by r-tPA. Regarding the existing literature, this possible association was never systematically investigated in humans or rodents. However, several observational studies tried to evaluate the risk of CAA-associated ICH after stroke and thrombolytic therapy, because this is an ongoing debate in daily clinical routine. Therefore, we aimed to adjust our experimental design with these clinical studies to examine this specific and clinically important aspect from a rodent point of view. (2) Histological evaluation of the reason of death was not performed for the animals dying under the procedure or during the following 24 hours. We therefore might have missed lethal hyperacute brain ischemia and ICH in these mice, which could have biased our data.
In conclusion, our experimental findings indicate a higher risk for ICH in combination of CAA and stroke thrombolytic therapy. ICH severity in the CAA-affected brain increased with infarct volume. Intravenous thrombolysis of stroke in the presence of CAA did not negatively affect the clinical parameters mortality and neurological deficit, although larger ischemic volumes compared with WT mice were observed. Furthermore, although the APP23-tg mouse model of CAA is at greater risk for spontaneous vascular leakage with the development of cMBs and lobar ICH, we observed no ICH after r-tPA treatment outside the infarct area. The occurrence of ICH strictly restricted to the area of ischemic injury might explain the missing impact on functional deficit and mortality in the APP23-tg group.

Disclosures
Dr Staufenbiel has been an employee of Novartis and presently owns Novartis stocks. The other authors report no conflicts.

References


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Supplemental material

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* B.R. and S.G. contributed equally to this work
Supplemental Methods

Sample size calculation

Sample size calculation with the occurrence of intracerebral hemorrhage as the primary outcome parameter was performed using Fisher’s exact-test under a presumed rate of ICH of 0.2 in wildtype mice and 0.7 in APP23-tg mice. 15 mice per group were regarded necessary to detect a difference with a level of significance ($\alpha$) of 0.05 and a power (1-$\beta$) of 0.8. Due to an unexpected high mortality rate over the long keeping period, only 18 APP23-tg mice and 15 wt-mice were available for the experimental study, leaving 13 APP23tg-mice and 11 wt-mice suitable for statistical analysis according to either death during the experiment or the exclusion criteria.

MRI

The animals were anesthetised with 1.5 % Isofluran and positioned into the magnet with a laser-controlled system for the animal cradles. Respiratory frequency and body temperature (maintained with a water heating pad) were monitored throughout the measurement. For detection of cerebral ischemia, T2-weighted images were acquired with a coronal T2-weighted RARE sequence using the following parameters: TR/TE = 3300/60 ms, echo train length = 4, FOV=14 x 11 mm$^2$, matrix size = 256x200. 18 slices (four averages) were acquired in a total acquisition time of 4 min 24 s. Iron as a content of erythrocytes or hemosiderin leads to a decrease in the transverse relaxation time of water protons diffusing close to the cells, resulting in turn in signal loss in T2*-weighted gradient echo images $^1$. The visualization was realized with a 2D-FLASH sequence and the following sequence parameters: TR/TE = 600/8 ms, flip angle = 60°, FOV = 14 x 11 x mm$^2$, matrix size = 256 x 200, 18 slices, three averages. The total acquisition time was 6 min.
Histology

After incubation of the harvested brains in 4 % acid free formaldehyde at 4°C over night and dehydrating steps in ethanol and xylol (Roth, Karlsruhe, Germany), 2 mm blocs were embedded in paraffin and cut into 4 µm sections. Infarct size and bleeding score analyses were done using hematoxylin and eosin (H&E) stained sections (Hollborn&Soehne, Leipzig, Germany). After three dewaxing steps in xylene for 5 minutes each and rehydration in ethanol (100%, 96%, 80% and 70%) and aqua dest. for 2 minutes each, sections were incubated in hematoxylin for 5 minutes and washed in tap water for 15 minutes. After incubation in eosin for 3 minutes sections were dehydrated in ethanol (80%, 90%, 100%) and xylene (three times) for 2 minutes each. Sections were mounted in Eukitt For fluorescence analysis of Aβ and vessel status, after dewaxing and rehydration as described above, sections were incubated with 1 % Thioflavin S (Sigma-Aldrich, St. Louis, USA) followed by incubation in ethanol (70%) for 5 minutes. After two washing steps in aqua dest. for 2 minutes each and three steps in 1xPBS (Gibco/Invitrogen, Paisley, U.K.) (pH 7.4) for 5 minutes each sections were incubated with DAPI (KPL, Gaithersburg, USA) at a ratio of 1:500 for 1h at room temperature. After four last washing steps in 1xPBS sections were mounted in Mowiol (Roth, Karlsruhe, Germany) and stored at 4°C.

Calculation of infarct volume with inclusion of all wt mice

Without exclusion of one wt mouse with infarction in multiple vascular territories, a comparison of mean infarct volumes between the groups revealed similar infarcts (±SD) of 32.5 (±24.9) mm³ in APP23-tg mice and 26.2 ±28.9 mm³ in wt mice (p=0.57). Infarct volumes of APP-tg mice ranged from 4.5 to 76.9 mm³ (median=28.7; interquartile range (IQR) = 47.2). Wt mice displayed infarct sizes from 7.22 to 112.4 mm³ (median=15.65; IQR=22.2)
Supplemental Tables

Table I: Presented is the functional deficit measured as a neuroscore, infarct size, infarct and ICH localisation and numbers of ICH grade 1 to 3 for APP-tg and wt mice. Wt Mouse No. 25 was excluded from statistical analysis after histology revealed extended infarction in the vascular territories of the left middle cerebral artery and both anterior cerebral arteries.

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<tr>
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<td>f</td>
<td>2.5</td>
<td>29.9</td>
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<td>21 [65.6]</td>
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</tr>
<tr>
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<td>m</td>
<td>3</td>
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<td>51.85 [46.1]</td>
<td>60.52 [53.9]</td>
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Supplemental Figures

Figure I: Representative images of vessel associated bleedings grade 1, 2 and 3.

Vessel associated bleedings in a H&E stained section were classified in grade 1 (single erythrocytes) (A), grade 2 (small aggregates of erythrocytes) (B) and grade 3 (large aggregates of erythrocytes) (C).

Supplemental References