Increased Risk of Hemorrhagic Transformation in Ischemic Stroke Occurring During Warfarin Anticoagulation
An Experimental Study in Mice

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Background and Purpose—The prevalence of long-term oral anticoagulant therapy is rising. Treatment options for patients who have an ischemic stroke under oral anticoagulant therapy are limited, and clinical data on the risk of hemorrhagic transformation (HT) in this condition are scarce. We therefore aimed to establish a mouse model of ischemic stroke occurring during oral anticoagulant therapy to assess the frequency and characteristics of HT.

Methods—C57BL/6 mice (n=59) were pretreated with warfarin. Untreated mice (n=32) served as controls. We performed a 3-hour transient filament occlusion of the right middle cerebral artery. In a first set of animals, ischemic lesion size and HT were evaluated macroscopically at 24 hours after middle cerebral artery occlusion. In a second set of mice, quantitative analysis of HT was performed at different time points after middle cerebral artery occlusion and in animals with different international normalized ratio levels using a photometric hemoglobin assay.

Results—Oral anticoagulant therapy at the onset of ischemia led to HT in all anticoagulated mice, whereas only 14% of the control mice showed HT. Mean HT blood volume 24 hours after middle cerebral artery occlusion was 0.3±0.4 μL in controls, 4.2±1.7 μL in mice anticoagulated to a mean international normalized ratio of 1.9±0.5 (P<0.05 versus controls), and 5.2±2.7 μL in mice with an international normalized ratio of 2.9±0.9 (P<0.001 versus controls). Anticoagulated mice euthanized at the time point of reperfusion had less HT than mice euthanized after 21 hours of reperfusion (1.6±0.5 μL versus 5.9±3.6 μL, P<0.05).

Conclusions—We present a mouse model of ischemic stroke occurring during oral anticoagulant therapy. Warfarin pretreatment dramatically increases the risk of HT 24 hours after middle cerebral artery occlusion. Reperfusion injury seems to be a critical component in this condition. (Stroke. 2011;42:1116-1121.)

Key Words: hemorrhage ■ MCAO ■ stroke ■ warfarin

Along with current demographic trends toward aging populations, the number of patients with atrial fibrillation is expected to double within the next decades.1,2 In consequence, strategies for the primary and secondary prevention of cardioembolic ischemic stroke will gain importance.3 Effective anticoagulation with vitamin K antagonists is currently considered to be the standard of care, but newer drugs such as direct thrombin inhibitors are in development.4

Despite having a largely reduced relative risk of experiencing cardioembolic ischemic events, many patients with atrial fibrillation under warfarin anticoagulation do have a stroke during their lifetime. Observational studies on the effect of anticoagulation on stroke severity in patients with atrial fibrillation have shown that approximately 30% of all patients with cardioembolic strokes are on warfarin anticoagulation at the time of stroke onset.5-8 Rates of effective anticoagulation (international normalized ratio [INR] >2) among warfarin-treated patients in these studies differ widely between 32% and 55%.

Less is known about the risk of hemorrhagic transformation (HT) in patients with stroke occurring under effective anticoagulation. In the largest of the previously named observational studies,7 the frequency of secondary intracranial hemorrhage was not increased in the warfarin group compared with the group not receiving anticoagulant treatment before admission. Nevertheless, thrombolysis is contraindicated in patients having INR values of >1.7 at hospital admission.9 Thus, therapeutic possibilities for those patients are very limited.

Recently, we developed an animal model of warfarin-associated (primary) intracerebral hemorrhage.10,11 Warfarin was applied via the drinking water leading to an effective anticoagulation with a drop of all vitamin K-dependent coagulation factors. The aim of the present study was to...
evaluate the frequency and characteristics of HT in a well-characterized model of focal cerebral ischemia performed in mice that were anticoagulated to different INR levels. Furthermore, we evaluated whether the rapid reversal of anticoagulation using prothrombin complex concentrates (PCC) reduces the risk of HT in this condition.

Materials and Methods

Animals

Male C57BL/6 mice (8 weeks old, Strain J) from Charles River Laboratories, Sulzfeld, Germany, were used in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publications No. 80-23, revised 1996). The experiments were approved by the local governmental authorities (Regierungspraesidium Darmstadt, Germany, approval number F143/34). Following a recently established protocol, we administered warfarin by oral uptake through bottled drinking water. In brief, a 5-mg Coumadin tablet (warfarin sodium, crystalline; Bristol Myers Squibb, Munich, Germany) was dissolved in 375 mL tap water. Assuming a body weight of 20 g and a water consumption of 15 mL/100 g per 24 hours, this dosage corresponds to a warfarin uptake of 0.033 mg (0.83 mg/kg) per mouse for a 20-hour feeding period (leading to a mean INR of 1.9±0.5, n=3) and to a warfarin uptake of 0.040 mg (1.00 mg/kg) per mouse for a 24-hour feeding period (leading to a mean INR of 2.9±0.9, n=3). In nonanticoagulated animals, we measured a mean INR of 0.9±0.1 (n=3). Details of this mouse model of oral anticoagulation can be found elsewhere. After warfarin withdrawal, INR values remained stable for the next 6 hours and dropped to normal values within 24 hours. All 4 vitamin K-dependent coagulation factors were diminished in this mouse model, thereby mimicking full warfarin anticoagulation.

Experimental Procedures

The operator was blinded to the treatment status of the animals. Transient middle cerebral artery (MCA) occlusion was performed as described previously. Mice were anesthetized with 1.5% isoflurane (Forene; Abbott, Wiesbaden, Germany) and 0.1 mg/kg buprenorphine (Temgesic; Essex Pharma, Munich, Germany) under spontaneous respiration. Focal cerebral ischemia was induced by introducing a silicone-coated 7-0 monofilament until it occluded the ostium of the right MCA. Regional cerebral blood flow was monitored by laser Doppler flowmetry (PF5010; Perimed, Järfälla, Sweden) to confirm vessel occlusion. The filament was withdrawn after 3 hours to allow reperfusion of the ischemic hemisphere. After the operation, all animals received regular drinking water, withdrawing the warfarin supply of the previously anticoagulated animals. Animals were euthanized at the indicated time points after assessment of their neurological functions with a 6-point neuroscore (0=no deficit, 1=failure to extend left forepaw, 2=circling to the left, 3=falling to the left, 4=“barrel rolling,” 5=unable to move spontaneously, 6=dead).

For the first exploratory part of the study, 41 mice were randomized to receive 24 hours warfarin feeding (n=21, INR 2.9±0.9) or no anticoagulation (control group, n=20) to assess the feasibility of the MCA occlusion model in warfarin-pre-treated animals and to compare the frequency and characteristics of HT. The size of the ischemic lesion was measured with National Institutes of Health Image J software after staining freshly prepared brain slices of 1 mm thickness with 2% 2,3,5-triphenyltetrazolium chloride (Merck KGaA, Darmstadt, Germany). Lesion size was corrected for edema by multiplying the infarct volume by the ratio of the contralateral to the ipsilateral hemisphere volume. Exclusion criteria were (1) lesion size < 40 mm³ (6 control and 5 warfarin mice excluded) because we presumed a rather small risk of HT in those animals; and (2) death not related to intracranial hemorrhage (1 control and 2 warfarin mice excluded). Mice that died from intracranial hemorrhage during the experiment were included in the analysis but because their ischemic lesions were not yet fully defined, they were excluded from the evaluation of ischemic lesion size. In mice that died prematurely, a complete autopsy was performed to exclude competing extracranial sources of hemorrhage. HT was graded into hemorrhagic infarction (petechial infarct without space-occupying effect) or parenchymal hematoma (hemorrhage with mass effect) using adapted criteria.

For quantitative analysis of HT, the whole brains of 6 mice anticoagulated to an INR of 2.9±0.9, 6 mice anticoagulated to an INR of 1.9±0.5, and 6 control animals (INR 0.9±0.1) were homogenized 24 hours after the induction of ischemia. After a 3-hour MCA occlusion, additional animals were euthanized at the time point of reperfusion (n=4) and 2 hours (n=4) and 21 hours after reperfusion (n=6). Transcardial perfusion with phosphate-buffered saline was performed and intracerebral blood volume was measured using a photometric hemoglobin assay.

To assess whether a substitution of the vitamin K-dependent coagulation Factors II, VII, IX, and X can reduce the risk of HT in anticoagulated mice subjected to transient MCA occlusion, 6 versus 6 mice were injected with human PCC (Frothromplex 600 IE; Baxter, Vienna, Austria) 100 IU/kg or saline intravenously through the tail vein 1 hour after MCA occlusion. Brains were homogenized and analyzed by the hemoglobin assay after 24 hours as described before. We have shown previously that human PCC can reverse warfarin-induced anticoagulation in mice.

Statistics

Graph Pad Prism 4 (Graph Pad Software Inc, La Jolla, CA) was used for statistical analysis. Results are given as mean±SD and graphically presented as a box and whiskers plot depicting the median, extreme values, and the 25 to 75 interquartile range. Statistical significance was assessed with a 1-way analysis of variance with Bonferroni correction, a Kruskal-Wallis test with Bonferroni correction, and an unpaired, 2-tailed Student t test where indicated.

Results

High Prevalence of HT in Anticoagulated Mice After MCA Occlusion

Ischemic lesion size did not differ between the controls and the anticoagulated group (116.7±32.2 mm³ versus 112.4±32.6 mm³, P=0.75). However, in contrast to controls, mice with effective oral anticoagulation showed a very high prevalence of HT after 3 hours of MCA occlusion (Figure 1). Whereas in the nonanticoagulated control group, only 2 mice revealed a slight HT (hemorrhagic infarction), all mice in the anticoagulated group (INR 2.9±0.9) developed HT (approximately 30% hemorrhagic infarction and 70% parenchymal hemorrhage; Table). Three mice in the anticoagulated group died before the end of the experiment, when the ischemic lesion was not yet entirely defined. All of them showed intracerebral bleeding as the presumable cause of death, which was limited to the right hemisphere. We excluded subarachnoid hemorrhage due to the intraparenchymal location of the bleeding. Lethal extracranial bleeding was excluded by a complete autopsy. The maximum of the hematoma was most often situated in the striatum and seemed to involve the boundary between ischemic and nonischemic tissue.

The Risk of HT After MCA Occlusion Is Elevated Both at the Upper and Lower Ends of the Therapeutic INR Range

In brain homogenates of control animals (n=6), there was hardly any blood detectable by the hemoglobin assay (0.3±0.4 µL). In sham-operated mice (n=3) that were
anticoagulated to an INR of 2.9±0.9 and euthanized 24 hours after MCA occlusion with immediate withdrawal of the filament after the drop of the Doppler flow, we were not able to detect a relevant amount of intracerebral blood either (0.1±0.05 μL). Quantitative analysis of HT blood volume after MCA occlusion in brains of mice anticoagulated to an INR of 1.9±0.5 (n=6) revealed a mean value of 4.2±1.7 μL (P<0.05 when compared with controls), whereas mice anticoagulated to an INR of 2.9±0.9 (n=6) showed a HT blood volume of 5.2±2.7 μL (P<0.01 when compared with controls; Figure 2A). No significant difference was observed between mice anticoagulated to a mean INR of 1.9 and 2.9.

Control mice reached a median neuroscore of 3 (range, 2 to 4) 24 hours after induction of ischemia, whereas mice anticoagulated to an INR of 1.9±0.5 were graded with a median score of 4.5 (range, 3 to 5; P<0.01). Mice anticoagulated to an INR of 2.9±0.9 reached a median score of 5 (range, 4 to 5; P<0.001; Figure 2B).

Reperfusion Appears to Be a Crucial Factor in the Development of HT
To assess the impact of reperfusion on the development of HT, we euthanized animals at different time points after 3 hours of MCA occlusion, that is, at the time point of reperfusion, 2 hours after reperfusion and 21 hours after reperfusion. In mice that were euthanized at the time point of reperfusion, only traces of intracerebral blood could be detected in the hemoglobin assay (n=4; 1.6±0.5 μL). The amount of blood did not differ significantly from nonanticoagulated control animals (n=6; 0.3±0.4 μL; P>0.05). In contrast, warfarin mice that were euthanized 2 hours after reperfusion showed a significantly increased HT blood volume compared with control animals (n=4; 5.3±0.5 μL; P<0.05). Similar results were obtained for animals that were euthanized 21 hours after reperfusion (n=6; 5.9±3.6 μL, P>0.05 when compared with animals euthanized 2 hours after reperfusion; Figure 3).

Rapid Reversal of Anticoagulation 1 Hour After MCA Occlusion Ameliorates HT
Anticoagulated mice that were injected with 100 IU/kg PCC for reversal of anticoagulation 1 hour after MCA occlusion showed lower degrees of HT as compared with the group of effectively anticoagulated animals that were injected with saline (hematoma volume 1.1±0.6 μL versus 4.5±1.4 μL; P<0.001; Figure 4).

Discussion
This is the first study applying the standard filament occlusion model of the MCA to mice orally anticoagulated to different INR values within the therapeutic range. In our previous studies on warfarin-associated intracerebral hemorrhage, we have demonstrated that warfarin feeding through the drinking water leads to a significant drop of all vitamin K-dependent coagulation factors in mice blood and to elevated INR values of 2 to 4 that remained stable for the first hours after warfarin withdrawal.10,11 We demonstrated that it is possible to perform the surgical procedures associated with the filament model of focal cerebral ischemia in such anticoagulated mice without increasing the amount of complications resulting from the surgical intervention itself.

The main finding of our study is that warfarin pretreatment significantly increases the risk for HT in brains subjected to focal cerebral ischemia. This is the case for INR values both at the lower and at the upper ends of the target INR range.
recommended for the prevention of stroke in atrial fibrillation (INR 2 to 3). Analyses of the pattern of HT on brain slices revealed that both hemorrhagic infarction and parenchymal hematoma occur more often in mice pretreated with warfarin. A quantitative analysis using photometric hemoglobin content measurement in mice anticoagulated to a mean INR of 1.9 and 2.9, respectively, demonstrated a 15- to 20-fold higher blood volume in the brains of warfarin-treated mice that investigated the effect of anticoagulation at the time point of stroke onset in patients with atrial fibrillation. Several authors reported that anticoagulation reduces initial stroke severity,7,8 and improves outcome6–8 without leading to an excess of HT.14 So far, there is no systematic clinical data on the impact of vessel recanalization on HT in patients who have cerebral ischemic events under oral anticoagulant therapy. The therapy. It has been shown that reperfusion injury comes along with a loss of blood–brain barrier integrity, which is associated with HT.14 So far, there is no systematic clinical data on the impact of vessel recanalization on HT in patients who have cerebral ischemic events under oral anticoagulant therapy.

Concerning the frequency of HT, our experimental results are not in line with clinical data from observational studies that investigated the effect of anticoagulation at the time point of stroke onset in patients with atrial fibrillation. Several authors reported that anticoagulation reduces initial stroke severity,7,8 and improves outcome6–8 without leading to an excess of HT.14 This discrepancy may at least partly be explained by the retrospective, observational nature of the latter studies. In addition, a systematic follow-up imaging to detect HT was not part of most of the study protocols, and there was no information available on whether patients had their anticoagulant treatment continued, stopped, or reversed after admission. Furthermore, the rather long period of MCA occlusion in our experimental study led to large territorial

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<th>Table. Descriptive Analysis of the Frequency, Extent, and Distribution of Secondary ICH in Controls (n=13) and Effectively Anticoagulated Mice (n=14) Who Were Subjected to 3 Hours MCA Occlusion and Euthanized After 24 Hours*</th>
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<td>Group</td>
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<td>Control, INR 0.9</td>
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<td>Warfarin, INR 2.9</td>
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ICH indicates intracerebral hemorrhage; HI, hemorrhagic infarction; PH, parenchymal hematoma.

*Striatal lesions were visualized by 2,3,5-triphenyltetrazolium chloride staining and lesion size was calculated correcting for edema. Hemorrhage was observed macroscopically. Mean lesion volume ±SD is given in cubed millimeters and statistical significance was evaluated with Student unpaired, 2-tailed t test.

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**Figure 2.** Quantitative analysis shows a significant increase in hemoglobin content in brains of mice anticoagulated to different INR values after transient MCA occlusion (MCAO). A, After 24 hours, mice were transcardially perfused with phosphate-buffered saline and the brains were homogenized and subjected to a photometric hemoglobin analysis. n=6 mice/group. Hematoma volume is represented in microliters in a box and whiskers plot with means and 25% to 75% interquartile range. Statistical significance of the difference between controls and treatment groups was tested by 1-way analysis of variance with Bonferroni correction, ***P<0.001 vs control; **P<0.01 vs control; *P<0.05 vs control. B, Mice were assayed on a 6-point neuroscore 24 hours after MCAO. Data are given as median and statistical significance between controls and treatment groups was tested with the Kruskal-Wallis test and Bonferroni correction. *P<0.05 vs control; **P<0.01 vs control; ***P<0.001 vs control.
lesions, which are comparable to total anterior circulation infarcts according to the Oxfordshire Community Stroke Project. Regarding those observational studies that provide information on ischemic lesion size, none or only 9% of the patients showed a total anterior circulation infarct. Thus, another reason of the much lower rate of HT in clinical series could be the absence of a relevant amount of large territorial infarctions.

In the last part of our study, we were able to demonstrate that the rapid reversal of warfarin anticoagulation using human PCC 1 hour after MCA occlusion prevents extensive HT. We have previously shown that the intravenous application of human PCC is able to rapidly normalize warfarin anticoagulation in mice. In an experimental model of collagenase-induced intracerebral hemorrhage, the rapid reversal of warfarin anticoagulation at 45 minutes after hemorrhage induction reduced intracerebral blood volume as compared with warfarin mice receiving saline by approximately 60%. Whether PCC, due to its prothrombotic properties, increases ischemic damage when administered in the context of an acute ischemic stroke has not yet been studied in animals or humans. In a report on 42 patients requiring immediate reversal of oral anticoagulant therapy, PCC did not induce an activation of coagulation as documented by plasma fibrinogen, D-dimer, and platelet count. One patient with sepsis and a beginning disseminated intravascular coagulation developed a lethal stroke. Among the studied patients, none had a stroke before PCC administration. Similar results are reported from a prospective study in 43 patients, among whom 1 patient with metastatic cancer had a fatal suspected pulmonary embolism, whereas the other patients, who did not experience adverse events, showed only a very brief rise in prothrombin activation fragments, which were judged to originate from the PCC medication itself rather than representing an activation of coagulation in the recipient. These 2 studies suggest that PCC, in the absence of severe predisposing conditions such as sepsis or tumor-associated hypercoagulability, may not induce a prolonged activation of coagulation. In our study, PCC treatment primarily served as a proof of concept to demonstrate the importance of the vitamin K-dependent coagulation factors in the development of HT after ischemic stroke occurring during oral anticoagulant therapy.

From a pathophysiological point of view, our study suggests that the plasmatic coagulation cascade and in particular the vitamin K-dependent coagulation factors are required to prevent HT in ischemic stroke. Considering our data, this may become crucial when it comes to reperfusion of the occluded vessel. In our study, we have demonstrated that the rapid reversal of anticoagulation reduces HT in anticoagulated ischemic mice. Apparently, a sufficient plasmatic coagulation system protects from an uncontrolled expansion of brain microhemorrhages. Thus, the application of PCC to patients with cerebral ischemia and high INR values may be a future strategy to prevent complicative secondary intracerebral hemorrhage. Again, it is important to mention that data on the application of PCC in the ischemic stroke paradigm are missing. Systematic studies are needed to clarify whether PCC treatment worsens the ischemic event per se, for example, by means of inducing additional thrombus formation.

Very recently, new anticoagulant drugs have been introduced to the market such as direct thrombin inhibitors. Apart from being more effective in preventing ischemic cerebrovascular events in patients with atrial fibrillation, the risk of complicative intracerebral hemorrhage is significantly reduced. However, until now, nothing is known on whether pretreatment with new anticoagulant agents also leads to a reduced risk of HT after ischemic stroke compared with warfarin anticoagulation. These and other aspects may be addressed in future studies on the basis of our experimental model. Our experimental model, however, does have several weaknesses. One cardinal point, like in many experimental stroke studies, is the fact that the study was performed in

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**Figure 3.** Reperfusion enhances hemorrhagic transformation after MCA occlusion (MCAO) in anticoagulated mice. Mice were anticoagulated to a mean INR of 2.9±0.8 by supplying warfarin through the drinking water for 24 hours. Transient MCAO was performed for 3 hours and mice were euthanized before reperfusion (second column, n=4 mice), 2 hours after reperfusion (third column, n=4 mice), and 21 hours after reperfusion (n=6 mice per group, first column control mice, fourth column anticoagulated mice). Hematoma volume is represented in microliters in a box and whiskers plot with means and 25% to 75% interquartile range. Statistical significance of the difference between groups was tested by 1-way analysis of variance with Bonferroni correction. The stars above the third and fourth columns represent the level of significance of the comparison to the control group.

**Figure 4.** PCC clearly reduces HT in anticoagulated mice after MCA occlusion (MCAO) when administered in a therapeutic time window. Transient MCAO was performed for 3 hours. One hour after the onset of ischemia, mice were injected intravenously with 100 µL of either 100 IU/kg PCC or saline through the tail vein. Brains were analyzed with the hemoglobin assay after 24 hours. n=6 vs 6 mice. Hematoma volume is represented in microliters in a box and whiskers plot with means and 25% to 75% interquartile range; statistical significance was evaluated with Student unpaired, 2-tailed t test. ***P<0.001 vs. control.
young mice that do not fully represent the clinical scenario of an (elderly) patient who is anticoagulated to prevent thromboembolism from atrial fibrillation. Because atrial fibrillation was most probably not present in these mice, this important factor could not be evaluated in our animal model. Besides that, our homogeneous group of large territorial infarctions only partly represents the heterogeneous degrees of stroke severity, which are observed in patients who have a stroke under oral anticoagulant therapy.

Taken together, our experimental data support the hypothesis that effective anticoagulation during ischemic stroke implies an increased risk of HT. Until more data are available, these findings may further justify withholding thrombolytic therapy to patients with elevated INR values at hospital admission.

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Disclosures

None.

References

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