Background and Purpose—Cerebral amyloid angiopathy (CAA) is a common cause of recurrent intracerebral hemorrhage in the elderly. Previous studies have shown that CAA induces inflammation and expression of matrix metalloproteinase-2 and matrix metalloproteinase-9 (gelatinases) in amyloid-laden vessels. Here, we inhibited both using minocycline in CAA mouse models to determine whether spontaneous intracerebral hemorrhage could be reduced.

Methods—Tg2576 (n=16) and 5xFAD/ApoE4 knockin mice (n=16), aged 17 and 12 months, respectively, were treated with minocycline (50 mg/kg, IP) or saline every other day for 2 months. Brains were extracted and stained with X-34 (to quantify amyloid), Perls’ blue (to quantify hemorrhage), and immunostained to examined β-amyloid peptide load, gliosis (glial fibrillary acidic protein [GFAP], Iba-1), and vascular markers of blood–brain barrier integrity (zonula occludins-1 [ZO-1] and collagen IV). Brain extracts were used to quantify mRNA for a variety of inflammatory genes.

Results—Minocycline treatment significantly reduced hemorrhage frequency in the brains of Tg2576 and 5xFAD/ApoE4 mice relative to the saline-treated mice, without affecting CAA load. Gliosis (GFAP and Iba-1 immunostaining), gelatinase activity, and expression of a variety of inflammatory genes (matrix metalloproteinase-9, NOX4, CD45, S-100b, and Iba-1) were also significantly reduced. Higher levels of microvascular tight junction and basal lamina proteins were found in the brains of minocycline-treated Tg2576 mice relative to saline-treated controls.

Conclusions—Minocycline reduced gliosis, inflammatory gene expression, gelatinase activity, and spontaneous hemorrhage in 2 different mouse models of CAA, supporting the importance of matrix metalloproteinase–related and inflammatory pathways in intracerebral hemorrhage pathogenesis. As a Food and Drug Administration–approved drug, minocycline might be considered for clinical trials to test efficacy in preventing CAA-related intracerebral hemorrhage. (Stroke. 2015;46:00-00. DOI: 10.1161/STROKEAHA.115.008582.)

Key Words: apolipoproteins E, cerebral amyloid angiopathy, cerebral hemorrhage, gliosis, matrix metalloproteinase-9, minocycline

Although ischemic stroke is the most common stroke subtype, intracerebral hemorrhage (ICH) results in greater morbidity and mortality. The most common cause of ICH in the elderly is cerebral amyloid angiopathy (CAA), a disorder caused by the deposition of β-amyloid peptide (Aβ) in small cerebral vessels—most prominently, penetrating arterioles of the cortex. Although CAA is commonly found in the brains of patients with Alzheimer disease (AD), it is also present in the elderly without AD. ICH is a recurrent complication of CAA; however, the pathogenesis of CAA-induced ICH has not been fully established. A better understanding of the underlying pathophysiology of CAA-induced ICH may result in the identification of potential targets for intervention. Furthermore, the frequent recurrence of ICH in patients with CAA provides opportunity for prevention in this high-risk population.

The recognition that several mouse models of AD, including Tg2576 and APP23, also develop CAA and microvascular hemorrhage has provided animal models to study CAA-induced ICH. These mice develop age-dependent accumulations of amyloid plaques and amyloid angiopathy, and at a later stage of pathogenesis spontaneously develop hemorrhage. In addition to providing a model to study the cellular and molecular pathogenesis of CAA-related ICH, these mice provide a preclinical model to test the efficacy of interventions to prevent recurrent ICH.
Earlier studies demonstrated that exogenous Aβ could induce expression and activity of matrix metalloproteinase-9 (MMP-9) and MMP-2, gelatinases implicated in vascular remodeling and hemorrhage, in cerebral endothelial cells and vascular smooth muscle cells, in vitro. Isolated rat microvessels exposed to exogenous Aβ demonstrated increased gelatinase expression and decreased endothelial tight junction proteins, claudin-1 and claudin-5, suggesting a mechanism for blood–brain barrier (BBB) breakdown. Moreover, gelatinase expression was found to be increased in amyloid-laden vessel and especially prominent in vessels with evidence of prior hemorrhage in Tg2576 mice. Pharmacological inhibition of MMP reduced not only CAA-associated gelatinase activity but microvascular oxidative stress in Tg2576 mice. In another microvascular CAA model associated gelatinase activity but microvascular oxidative stress in Tg2576 mice. In another microvascular CAA model associated gelatinase activity but microvascular oxidative stress in Tg2576 mice.16 In another microvascular CAA model lacking spontaneous ICH (Tg-SwDI mice), minocycline reduced microglial activation and improved behavioral deficits.17 These studies raised the possibility that enhanced gelatinase activity in CAA vessels might play a role in CAA-related ICH. In this study, we examined the efficacy of minocycline, a Food and Drug Administration–approved antibiotic known to inhibit gelatinase activity and inflammation, in reducing spontaneous hemorrhage in 2 different mouse models of CAA.

Methods

Animals

Tg2576 (a.k.a. APPsw)18 mice of both sexes, expressing human APP, with Swedish mutations at positions 670/671 under control of the prion promoter, were used in this study. 5xFAD/apoE4-knockin mice were produced by crossing 5xFAD transgenic mice (Tg7031 lime)19 containing 5 familial AD mutations (APP K670N/M671L+I716V+V717I and PS1 M146L+L286V) with apoE4-targeted replacement mice (endogenous murine APOE gene was replaced with the APOE4 gene). All experimental protocols were approved by the Animal Studies Committee at Washington University, and all studies were conducted in accordance with the US Public Health Service’s Policy on Humane Care and Use of Laboratory Animals.

Minocycline Treatment

Mice were treated with minocycline (50 mg/kg, IP, Sigma-Aldrich, St. Louis, MO) or vehicle (saline) every other day for 8 weeks. Age- and sex-matched Tg2576 mice (17±2-month old) were treated with minocycline. Eight mice were used in each group (5 females and 3 males). In addition, male 5xFAD/ApoE4 mice (12±1-month old) were randomly assigned to treatment with minocycline (n=8) or saline (n=8). A power analysis was performed based on hemorrhage frequency (6.2 hemorrhages per hemisphere) and SD (2.77) in 12 mTg2576 mice (and unpublished data). A sample of size of 8 mice per group was found to provide at least 85% statistical power at a significance level of 5% to detect a 50% reduction in the mean number of hemorrhages because of the treatment. Total body weight was measured weekly and injection volumes were modified accordingly. Mice were euthanized 6 h after the last injection.

Brain Extraction and Preparation

Mice were deeply anesthetized with isoflurane and transcardially perfused with 0.01 mol/L PBS. The brains were removed, and hemispheres were separated. Left hemispheres were immediately dissected, snap-frozen on dry ice, and stored at −80°C for biochemical analysis. Right hemispheres were fixed in 4% paraformaldehyde for 24 h and transferred to 30% sucrose in 0.1 mol/L PBS. Coronal sections, 50-μm thick, were made with a sliding microtome and stored in 0.1 mol/L PBS, 30% sucrose, and 30% ethylene glycol at −20°C until further analysis.

Histology

Right hemispheres were sectioned and stained for hemorrhage using Perls’ blue. Hemorrhages were counted by an assessor who was blinded to treatment assignments and graded based on size (grade 1, 1–3 blue puncta; grade 2, 4–10 puncta; and grade 3, >10 puncta) in 25 to 30 brain sections (50-μm thick) from each mouse, spaced 300 μm apart. To quantify CAA and amyloid plaque load, 4 regularly spaced brain sections from rostral anterior commissure to caudal hippocampus from each mouse was stained with Congo Red or X-34. Area stained with the amyloid dye was quantified and expressed as mean fraction of section area from each mouse.2 Groups were compared using Student t test or Mann–Whitney rank-sum test.

Immunohistochemistry

For Aβ immunohistochemistry, brain sections were incubated with mouse anti-Aβ antibody (HJ3.4, 1:1000)23 overnight at 4°C. Vectastain ABC (1:400) followed by 0.025% 3,3-diaminobenzidine tetrachloride in 0.25% NiCl2, and 0.05% H2O2 was used to develop staining. Immunofluorescent double labeling was performed as follows: a mixture of primary antibodies was incubated with the brain sections overnight at 4°C. Fluorescently labeled secondary antibodies were then incubated at room temperature for 1 hour. Primary antibody pairs were mouse anti-GFAP monoclonal antibody (1:1000; Sigma, St. Louis, MO) and rabbit anti-Iba1 antibody (1:1000; Wako Pure Chemicals Industries, Tokyo, Japan); rabbit anti–ZO-1 antibody (1:250, Invitrogen, Camarillo, CA) and biotin-conjugated tomato lectin (1:400; Vector Laboratories, Inc, Burlingame, CA); mouse anti–collagen IV (Col IV) antibody (1:200, Sigma); and tomato lectin. Secondary antibodies were Cy3-conjugated donkey antirabbit IgG antibody (1:800; Jackson ImmunoResearch); AlexaFluor 488–conjugated donkey antimouse IgG antibody; AlexaFluor 488–conjugated streptavidin (1:100; Molecular Probes).

Aβ ELISA

To measure Aβ, dissected cortices were homogenized in PBS and then in 5 mol/L guanidine in TBS, pH 8.0. After each homogenization step, samples were centrifuged at 12,000 rpm for 20 minutes, and supernatants were collected for sandwich ELISA.23 Aβ1–40 and Aβ1–42 were assessed using mouse monoclonal capture antibodies HJ2 (anti-Aβ1–35–40) and HJ7.4 (anti-Aβ1–42),23 respectively, and a biotinylated central domain antibody, HJ5.1 (anti-Aβ1–39), was used as the detecting antibody followed by streptavidin-poly-HRP-40 ( Fitzgerald Industries). All ELISAs were developed using Super Slow ELISA 3,5,5′-tetramethylbenzidine (Sigma), and absorbance was read on a Bio-Tek Epoch plate reader (Winooski, VT) at 650 nm. Standard curves were generated from synthetic human Aβ1–40 or Aβ1–42 peptides (American Peptide, Sunnyvale, CA).

Quantitative Real-Time Polymerase Chain Reaction

Messenger RNA was extracted from the left hemisphere and reverse transcribed with the cDNA Reverse Transcription kit. Quantitative polymerase chain reaction was performed using the ABI 7500 in the default thermal cycling mode with Power SYBR. Mouse β-actin was used as a normalization reference. Relative mRNA levels were calculated using the comparative Ct method and expressed as a percentage of control. See Table I in the online-only Data Supplement for primer sequences used in reverse transcriptase polymerase chain reaction.

Gelatin Substrate Zymography

Some of the left cortices were homogenized in 500 μL of lysis buffer and supernatant was collected after centrifugation. The brain supernatant was incubated with 50 μL of gelatin-Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) for 1 hour with constant shaking. After centrifugation, the pellet was then incubated for 30 minutes with 50
µL of elution buffer consisting of lysis buffer plus 10% dimethylsulf oxide. Each 4 µL of eluted sample from the left cortex was mixed with 25 µL of 4x nonreducing sample buffer loaded onto 7.5% SDS-PAGE containing 0.1% gelatin and electrophoresed at 180 V. The gel was incubated for 20 hours at 37°C in buffer for 20 minutes and was stained for 1 hour with 0.1% Coomassie Blue.23

Statistical Analysis
All data were expressed as mean±SD. Differences between groups were determined using 2-tailed Student’s t test for 2-group comparisons, and 1-way ANOVA followed by post hoc Tukey test for comparison among multiple groups. Differences were deemed statistically significant at *P<0.05 and **P<0.01.

Results

Minocycline Reduced Hemorrhage Frequency in Tg2576 Mice
Mice tolerated minocycline treatment without adverse effects: average weights before, during, and after treatment did not differ between groups (Figure 1A in the online-only Data Supplement). One mouse died after 4 weeks of minocycline treatment and was subsequently replaced by another age- and sex-matched mouse.

After 2 months of treatment with minocycline (50 mg/kg, IP, QOD) or saline, brains sections were stained with Congo Red to assess amyloid load and Perl blue to quantify hemorrhage frequency. The Tg2576 mice treated with minocycline had almost half the number of hemorrhages compared with those treated with saline (Figure 1B). Hemorrhages were graded by size, based on the methods of Jucker (Figure 1A).7 Grade 1 hemorrhages were most frequent, regardless of treatment. Although minocycline treatment had fewer microhemorrhages for all grades, only grade 2 hemorrhages had statistically significantly fewer microhemorrhages in the minocycline-treated group compared with saline treatment (Figure 1C). Five of the 8 mice were females, and when compared separately, showed a trend toward fewer microhemorrhages (12 versus 7.4 hemorrhages per hemisphere; P<0.059).

CAA load was quantified by measuring the cross-sectional area of Congo Red–stained vessels and expressed as percentage area of the total section. There was no significant difference in CAA load between minocycline-treated versus saline-treated mice (Figure 1D). In addition, several other genes associated with vascular inflammation were reduced by chronic minocycline treatment, including NADPH oxidase 4 (NOX4) and MMP-9. NOX4 expression has been reported to be selectively increased in other amyloid precursor protein transgenic models.24 To confirm that decreased MMP mRNA resulted in a reduced MMP activity, we performed gelatin zymography on brain extracts from minocycline-treated mice. Minocycline significantly reduced both MMP-9 and MMP-2 activity compared with saline-treated controls (Figure 4B and 4C).

Minocycline Enhanced Expression of BBB Proteins
Previous studies have demonstrated that vascular MMP-9 expression can lead to degradation of basement membrane proteins (including Col IV) and endothelial tight junction proteins (ZO-1, occluding, and claudin 5), leading to compromise of the BBB.25 To determine whether minocycline reduces degradation of proteins involved in neurovascular integrity, we examined the expression of the tight junction protein, ZO-1, and the basement membrane protein Col IV. Focusing on vascular expression, we costained all sections

Figure 1. Chronic minocycline treatment reduced hemorrhage frequency without altering cerebral amyloid angiopathy (CAA) load in Tg2576 mice. A, Representative photomicrographs of Tg2576 brain sections stained with Perls' Blue and Congo Red, to demonstrate microhemorrhage size (grade 1, 1–3 puncta; grade 2, 3–10 puncta; and grade 3, >10 puncta). Bar=50 µm. B, The number of hemorrhages was significantly lower in the minocycline-treated mice compared with controls (n=8 mice per group; 30 brain slices per animal). C, Comparison of hemorrhage frequency by grade. D, CAA, stained with Congo Red, was quantified and expressed as percent area (4 equally spaced brain sections from each animal). No significant difference was found between treatment groups. Values are mean±SD. *P<0.05.
Minocycline treatment increased the expression of vascular ZO-1 and Col IV by >2-fold (Figure 5C and 5D). Staining of vessels with tomato lectin was unchanged by minocycline treatment (data not shown), indicating that minocycline increased expression of ZO-1 and Col IV without altering vessel density.

Minocycline Reduces Microhemorrhage in 5xFAD ApoE4 Mice

Preclinical stroke studies during the past 3 decades have demonstrated the importance of replication using different animal models (Stroke Treatment Academic Industry Roundtable [STAIR] criteria).31,32 To confirm the efficacy of minocycline in reducing spontaneous hemorrhage, we repeated the minocycline study using a novel mouse model of CAA-related spontaneous hemorrhage. 5xFAD mice (line 7031)19 with targeted replacement of mouse apolipoprotein E with human apolipoprotein E ε4 (5xFAD/ApoE4) develop amyloid plaques and CAA by 5 to 6 months of age, and hemorrhages appear spontaneously beginning at 7 months of age (unpublished observation). Minocycline treatment (50 mg /kg, IP, QOD) was initiated at 12 months of age (when significant CAA and hemorrhages were already present) and treated for 2 months. Brain sections were stained with Perls’ blue to quantify hemorrhage frequency. Minocycline treatment reduced the number of hemorrhages by >50%. Both grade 2 and 3 hemorrhages were significantly reduced by minocycline (Figure 6A). Similar to the study in Tg2576 mice, 2 months of minocycline treatment did not alter CAA or plaque load (Figure 6B–6D) nor did it affect body weight during this period of time (Figure IB in the online-only Data Supplement).

Discussion

CAA is the leading cause of ICH in the elderly and recurrence is common; yet, there are no treatments for the prevention of CAA-related ICH. In the present study, we examined the efficacy of chronic minocycline treatment on hemorrhage frequency in 2 different mouse models of CAA. We found that...
Minocycline reduced hemorrhage frequency in both CAA models without affecting amyloid load. Minocycline also inhibited MMP-2 and MMP-9 activity, reduced gliosis and expression of select neuroinflammatory genes in brains of Tg2576 mice. These changes occurred in parallel with increases in vascular tight junction and basal lamina proteins, suggesting that minocycline preserved structural integrity of cerebral vessels, leading to reduction in hemorrhage frequency. In both models, treatment was initiated during advanced stages of disease, when microhemorrhage was already present. Therefore, the reduced frequency of microhemorrhage likely reflects a reduction in the accumulation of microhemorrhage during the 2-month treatment period.

Minocycline, a semisynthetic second-generation tetracycline with Food and Drug Administration approval for the treatment of acne vulgaris, has a large literature supporting its neuroprotective properties. The tetracyclines were first described to have anti-inflammatory, antiapoptotic, and anti-gelatinase activities limiting angiogenesis and tumor metastasis. Minocycline became the tetracycline of choice for central nervous system delivery, owing to its lipophilic nature allowing it to pass through the BBB. Indeed, minocycline has emerged as the most effective neuroprotectant among the tetracyclines, demonstrating activities in experimental models of cerebral ischemia, traumatic brain injury, and several neurodegenerative diseases such as Parkinson disease, Huntington disease, and amyotrophic lateral sclerosis. In AD mouse models, minocycline has been shown to improve behavioral performance, but have little effect on amyloid plaque load. Consistent with these earlier reports, our findings also demonstrate little effect on plaque accumulation.

Double-blind randomized clinical trials examining the efficacy of minocycline in a variety of neurodegenerative disease, including Parkinson disease, amyotrophic lateral sclerosis, and Huntington’s Disease, have not demonstrated definitive efficacy in attenuating disease progression. In these clinical trials, the precise target of minocycline’s mechanism of action was not identified, and therefore target engagement was not confirmed. In all trials except for one, safety after chronic administration of minocycline in these aged populations was clearly demonstrated. In the phase III trial in amyotrophic lateral sclerosis patients, neurological deterioration occurred more rapidly in the minocycline group than in the placebo group; however, a higher dose of minocycline was used in this trial compared with all others.

The dose of minocycline used in the current study (50 mg/kg, IP) produces peak plasma concentrations extrapolated to be equivalent to that after 1000 mg, PO in humans, according to published pharmacokinetic studies in rodents. Given
every other day, this is ≈2.5× the usual daily dose of minocycline for treatment of bacterial infections. Minocycline is a known sclerosing agent and has been reported to induce pleuritis in rodents when injected intraperitoneally. In our study, we administered minocycline via intraperitoneal injection every other day to minimize its sclerosing effects. Although 1 mouse in the minocycline-treated group died after 1 month of dosing, the remaining animals appeared healthy (maintaining constant body weight) without overt evidence of peritonitis.

Our studies demonstrate that minocycline inhibits gliosis, certain vascular inflammatory mediators, mediators of oxidative stress, and gelatinases, consistent with findings in other studies. All of these mediators have been implicated in CAA-related pathology. Chronic minocycline treatment of transgenic mice expressing the vasculotropic Dutch/Iowa (E693Q/D694N) mutant amyloid precursor protein did not alter CAA load but reduced microglialosis and inflammation in the brains of these mice. Moreover, cognitive deficits were improved with minocycline treatment. Of note, this model of microvascular CAA does not result in spontaneous hemorrhage. In another study, CAA-induced free radical formation was inhibited by chronic treatment with minocycline and other more specific MMP-9 inhibitors in Tg2576 mice. Although these inhibitors did not have a direct antioxidant effect, it is hypothesized that minocycline’s antioxidative effect was indirectly mediated via its MMP-9 inhibitory activity. Therefore, the effect of minocycline in attenuating CAA-related pathology has been confirmed in several animal models and by several independent laboratories.

Our findings are the first to demonstrate that chronic minocycline treatment reduced spontaneous hemorrhage in an established CAA model (Tg2576 mice). In addition, we have confirmed these findings in another newly developed CAA mouse model, the 5xFAD/ApoE4 mouse (Figure 6). This mouse model is highly relevant to human disease, because ApoE4 genotype has been independently associated with severe CAA, CAA-related vasculopathy, and ICH. Even in the absence of CAA pathology, recent studies suggest that the ApoE4 genotype alone may induce neurovascular injury with resultant BBB leak. A recent study using targeted replacement of human ApoE genes in mice demonstrated that ApoE4 specifically induced a proinflammatory pathway in pericytes resulting in the induction of MMP-9, degradation of BBB proteins (ZO-1 and Col IV), and BBB leak. In parallel with its MMP-9 and MMP-2 inhibitory activity, we have found that minocycline preserved elevated expression of the tight junction protein, ZO-1, and the basement membrane protein, Col IV, in brain microvessels (identified with tomato lectin; Figure 5). Both of these proteins are known substrates of the gelatinases. Inhibition of MMPs with nonspecific inhibitors (such as minocycline) reduced spontaneous hemorrhage (see above) and neurobehavioral outcomes. Furthermore, more specific MMP-9 inhibitors reduced other downstream consequences of CAA, including CAA-induced oxidative stress.

Conclusions
The current study demonstrates the efficacy of minocycline in reducing spontaneous hemorrhage in 2 different mouse models of CAA, during advanced stages of disease. Minocycline reduced gliosis, inhibited the expression of inflammatory mediators and gelatinase activity, resulting in the preserved expression of the BBB proteins, ZO-1, and Col IV. Because minocycline is already Food and Drug Administration approved and known to be safe in elderly populations, we propose that minocycline be considered as a candidate treatment in clinical trials for the prevention of ICH in CAA patients.

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Disclosures
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References


Minocycline Reduces Spontaneous Hemorrhage in Mouse Models of Cerebral Amyloid Angiopathy
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Supplemental Table I

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Supplemental Figure and Figure Legends

Figure IA

Supplemental Fig I. Mice tolerated minocycline treatment without overt adverse effects. A. Tg2576 mice were treated with saline or minocycline (50mg/kg, ip, qOD), and weighed every other week. Average weights did not change throughout the study period. B. 5xFAD/ApoE4 mice treated with saline or minocycline (same dosing schedule) also did not demonstrate change in weights during the study period.