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A failure of matrix metalloproteinase inhibition in the prevention of rat intracranial aneurysm formation

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Abstract We tested the hypothesis that nonspecific matrix metalloproteinase (MMP) inhibition with doxycycline would decrease the incidence of intracranial aneurysm formation in a rat aneurysm model. We performed common carotid artery ligation on 96 Long-Evans rats. A treatment group of 48 animals was chosen at random to receive oral doxycycline (3 mg/kg) in addition to standard rat chow, and the control group of 48 animals received standard rat chow only. The major circle of Willis arteries was dissected at 1 year following carotid ligation, and the proportions of animals with aneurysms were compared between groups using Fisher's exact test. Four animals given oral doxy-

cycline and ten control animals expired before 1 year. Of the examined animals, eight saccular intracranial aneurysms were found in 8 of 45 animals which had received doxycycline (17.8%) and seven saccular intracranial aneurysms were found in 7 of 37 control animals (18.9%). There was no significant difference in aneurysm formation between the doxycycline-treated and control groups ($P=0.894$). Nonspecific MMP inhibition with doxycycline is not effective in preventing intracranial aneurysm formation in a rat model.

Keywords Intracranial aneurysm · Animals · Matrix metalloproteinases (MMPs)

Introduction

Matrix metalloproteinases (MMPs) comprise a family of at least 23 enzymes that, through their collective ability to degrade most classes of extracellular matrix proteins, are instrumental in extracellular matrix remodeling. Their activity and regulation have been postulated to be a fundamental part of the biochemistry underlying many vascular pathological processes. Specifically, the proteolytic activity of certain MMPs has been associated with abdominal aortic aneurysm (AAA) formation and rupture in animals and humans [1, 8, 11, 17–19, 21, 23, 29]. Furthermore, some investigators have proposed an association between locally increased MMP-2 and MMP-9 activity and intracranial aneurysm formation in humans, despite some presumed differences in the etiologies of intracranial and abdominal aortic aneurysm formation, perhaps because of a shared involvement of extracellular matrix remodeling [6, 13, 14, 16, 22, 27].

Because of the apparent role of MMPs in AAA formation and rupture, pharmacological MMP inhibition has been proposed as a therapy for the inhibition of AAA growth [26]. Tetracyclines and other pharmaceuticals have been shown to inhibit MMP activity and aneurysm growth in animal models [3–5, 9, 15, 20, 24, 28], and experimental AAAs have been suppressed through the targeted gene disruption of MMP-9 in mice [25]. It has been shown that tetracyclines are taken up into the walls of human AAAs where they inhibit MMP activity [10, 12]. A human trial using doxycycline as a nonspecific inhibitor of MMPs for the therapy of small AAAs is currently underway [2].

We hypothesized that if MMPs are involved in the etiology of intracranial aneurysms, their pharmacological inhibition may decrease the incidence of intracranial aneurysm. Using a rat model of intracranial aneurysms, we compared the incidence of aneurysm formation between

control animals and animals treated with doxycycline, a systemic inhibitor of MMPs.

Methods

Aneurysm model

Our institution's Animal Care and Use Committee approved our protocol. Using aseptic technique, we performed unilateral (right) common carotid artery (CCA) ligation on 96 female 150 g Long-Evans rats, a strain susceptible to aneurysm formation with this technique [7]. Intraperitoneal ketamine (70 mg/kg; Phoenix Scientific, St. Joseph, Mo.) and xylazine (8 mg/kg; Phoenix Scientific) anesthesia was administered for the procedure according to weight. A longitudinal incision was made in the right lower neck and blunt dissection isolated the right CCA. The artery was divided by silk sutures at its mid-cervical portion. Absorbable suture was used for skin closure. Postoperative care included subcutaneous buprenorphine analgesia (0.4 mg/kg; Reckitt Benckiser Pharmaceuticals, Hull, UK).

As a secondary specific aim, we tested the hypothesis that bilateral CCA ligation would be more successful than unilateral CCA ligation in inducing intracranial aneurysm formation by performing bilateral CCA ligations in a subset of animals in two stages, 4 weeks apart.

Experimental groups

Animals were randomly assigned to one of four groups. Groups 1 and 2, considered the test animals, underwent unilateral and staged bilateral CCA ligation followed by long-term administration of doxycycline. Group 1, consisting of 38 animals, received doxycycline 3 mg/kg of standard rat chow (Dox Diet, Bio-Serv, Frenchtown, N.J.), mixed at the supply company. We had previously demonstrated serum levels of doxycycline (approximately 1.5–2.5 µg/ml) in the therapeutic range in rats receiving this dose (unpublished data). Group 2, a second treatment group of ten animals, also received doxycycline 3 mg/kg of standard rat chow, but additionally underwent surgical ligation of the contralateral (left) CCA at 4 weeks following right CCA ligation.

Groups 3 and 4, considered the control animals, underwent unilateral and staged bilateral CCA ligation without subsequent doxycycline administration. Group 3, consisting of 38 animals, received standard rat chow without doxycycline. Group 4, a second control group of ten animals, also received standard rat chow without doxycycline, but additionally underwent surgical ligation of the contralateral (left) CCA at 4 weeks following right CCA ligation.

Follow-up

Animals were housed for 1 year following carotid ligation and continued their respective diets. At the time they were killed, all surviving animals were given ketamine and xylazine anesthesia by weight, and perfusion-fixation was performed. Phosphate-buffered saline (PBS) was mechanically infused through a butterfly needle into the left ventricle for 10 min, followed by a 10-min infusion of 4% paraformaldehyde in PBS with 5% sucrose. A diluted solution of methylene blue in PBS was then infused to stain the intracranial vasculature. The animals' brains were removed for analysis, with the intracranial vasculature intact.

Gross aneurysm examination

The ventral surfaces of the animals' brains were examined under a dissecting microscope. A reader blinded to treatment group recorded the presence or absence of intracranial aneurysms in all animals.

Statistical analysis

The proportion of aneurysms found in the treatment group was compared to the proportion of aneurysms found in the control group using Pearson's chi-squared test without Yates' continuity correction. The proportion of aneurysms found in those animals which had undergone unilateral CCA ligation was compared to the proportion of aneurysms found in those which had received bilateral CCA ligation using Fisher's exact test. Mortality among control animals was compared with mortality among those treated with doxycycline using Pearson's chi-squared test without Yates' continuity correction. Power calculations were made using the binomial distribution. All statistical analyses were performed using S-Plus software (Insightful Corporation, Seattle, Wash.).

Results

A total of 14 animals died before 1 year: two from group 1, two from group 2, eight from group 3, and two from group 4. However, we were able to examine grossly one doxycycline-treated animal which died before 1 year; an intracranial aneurysm was found in this animal. We were not able to examine the remainder of the animals which expired before 1 year. Therefore, 45 of 48 total doxycycline-treated animals were able to be examined. One of the control animals, though surviving 1 year after carotid ligation, was rendered unexamined by its cage mates. Therefore, we examined 37 of 48 total control animals. The

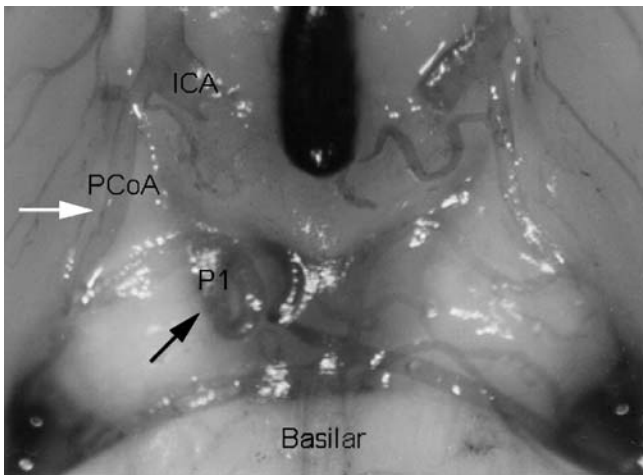


Fig. 1 Dolichoectasia of the P1 segment of the right posterior cerebral artery (*P1*, *black arrow*) and the right posterior communicating artery (*PCoA*, *white arrow*) is seen following right common carotid ligation (*ICA* internal cerebral artery, *Basilar* basilar artery)

majority of deaths were associated with the spread of a respiratory illness in the vivarium at approximately

10 months into the protocol, and a few deaths were of unknown cause. The difference in mortality between control animals and those treated with doxycycline did not reach statistical significance ($\chi^2=3.033$, $P=0.082$).

All animals were asymptomatic when they were killed except one, which was lethargic and stumbling. This particular animal was found to have acute subarachnoid hemorrhage and a large intracranial aneurysm at dissection. All animals which had received unilateral (right) CCA ligation had dolichoectasia of the first segment of the right posterior cerebral artery (P1) and of the right posterior communicating artery (Fig. 1). All animals which had received bilateral CCA ligation had dolichoectasia of the basilar, bilateral P1, and bilateral posterior communicating arteries (Fig. 2).

All aneurysms except three were quite large, and most or all of these large aneurysms would be considered giant. These large aneurysms generally did not have large patent lumens and only partially filled with methylene blue dye following fixation-perfusion (Fig. 3). Of the 12 large aneurysms, 11 were of the posterior circulation, although their origins were usually difficult to ascertain precisely. The one large anterior circulation aneurysm appeared to

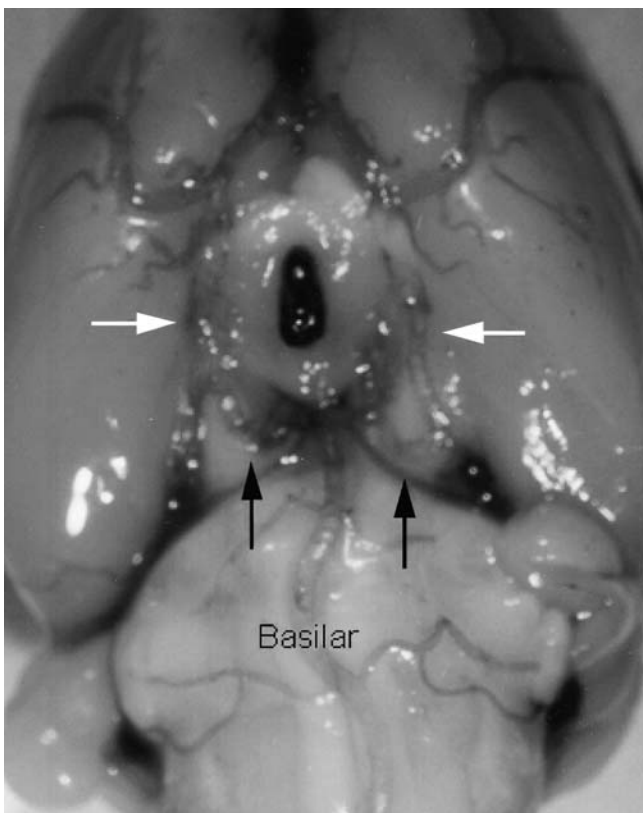


Fig. 2 Dolichoectasia of the basilar artery (*Basilar*), P1 segments of the bilateral posterior cerebral arteries (*black arrows*) and the bilateral posterior communicating arteries (*white arrows*) is seen following bilateral common carotid artery ligation

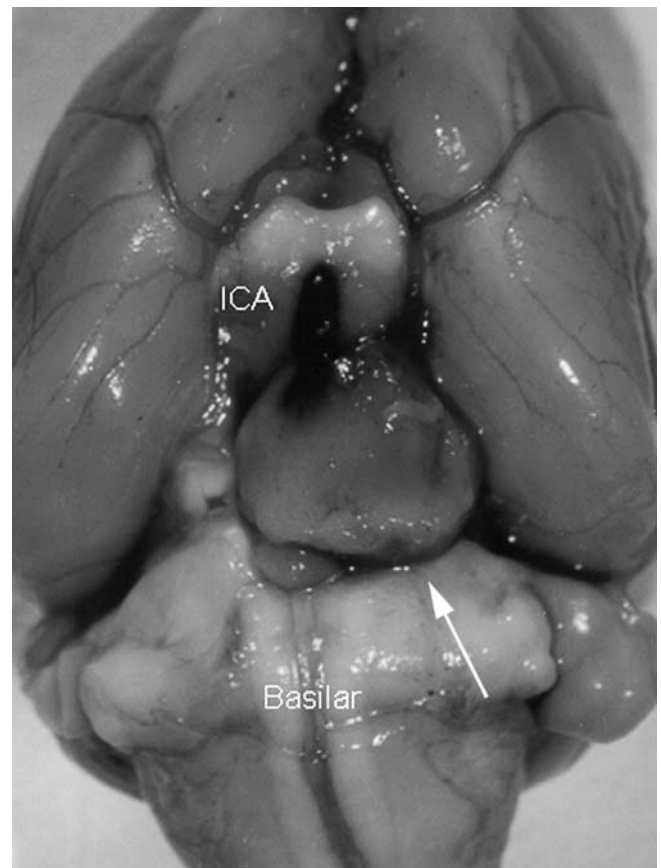


Fig. 3 A large aneurysm (*white arrow*) of the posterior circulation. (*ICA* internal cerebral artery, *Basilar* basilar artery)

arise from the first segment of the right anterior cerebral artery (A1).

A small aneurysm in one animal originated from the middle portion of P1, and the contralateral P1 and posterior communicating arteries were very small in caliber. Small aneurysms were seen arising from the proximal end of a left A1 fenestration in two animals. The three small aneurysms had completely patent lumens and filled completely with methylene blue dye (Fig. 4).

Of the 45 animals from groups 1 and 2 (doxycycline-treated), 8 (17.8%) had developed an aneurysm by 1 year following carotid ligation. Of the 37 animals from groups 3 and 4 (control), 7 (18.9%) had developed an aneurysm. The proportion of aneurysm formation from all four groups combined was 15 aneurysms per 82 animals for the 1-year interval or 18.3%. There was no statistically significant difference in the proportion of aneurysms between the doxycycline-treated and control groups ($\chi^2=0.0177$, $P=0.894$). Power to detect a 20% relative difference in aneurysm formation between the two groups was calculated at 77%.

Among those animals which had received unilateral CCA ligation, 7 (18.9%) of the 37 surviving doxycycline-treated (group 1) animals examined had developed one saccular intracranial aneurysm by 1 year following carotid ligation, and 5 (17.2%) of the 29 control (group 3) animals examined had developed one saccular intracranial aneurysm. Among those which had received bilateral CCA ligation, 1 (12.5%) of the 8 doxycycline-treated (group 2)

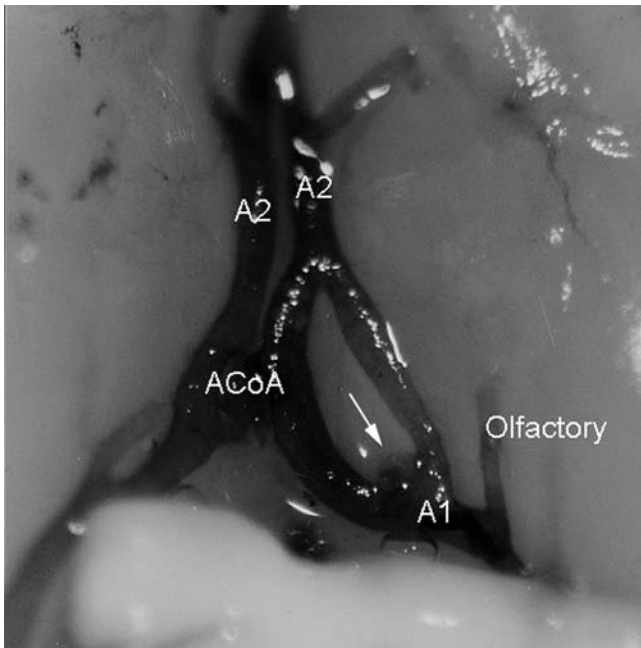


Fig. 4 A small anterior circulation aneurysm (*white arrow*) at the proximal end of an A1-A2 fenestration. (*A1* A1 segment of anterior cerebral artery, *Olfactory* olfactory artery, *ACoA* anterior communicating artery, *A2* A2 segment of anterior cerebral artery)

animals examined had developed one saccular intracranial aneurysm and 2 (25.0%) of the 8 control (group 4) animals examined had developed one saccular intracranial aneurysm. The proportion of aneurysms was not significantly different between those which had received unilateral CCA ligation and those which had received bilateral CCA ligation ($P=0.631$).

Discussion

In this study we tested the hypothesis that systemic administration of an inhibitor of MMPs would protect against aneurysm formation in rats. We did not observe a protective effect of doxycycline, a nonspecific MMP inhibitor, against the formation of intracranial aneurysms in a rat carotid ligation model. The incidence of aneurysm formation was similar in both doxycycline-treated and control animals. This result suggests that intracranial aneurysm formation may not be directly dependent upon MMP-mediated weakening of the vessel wall. This may represent a general failure of pharmaceutical MMP inhibition to inhibit intracranial aneurysm formation; alternatively, our results may only apply to the aneurysm model and MMP inhibitor chosen.

We did observe predictable dolichoectatic remodeling of intracranial arteries with common carotid ligation, presumably in response to increased blood flow from the posterior circulation to the anterior circulation. Such remodeling was seen in both doxycycline-treated and control animals.

The incidence of intracranial saccular aneurysm formation in our Long-Evans rat model was approximately 18%, which is considerably less than what has previously been published with a similar model [7]. However, we performed gross analysis of the circle of Willis arteries under a dissecting microscope only and did not evaluate for microaneurysmal formations. We believe that an 18% incidence of aneurysm formation is a more reasonable expectation of this model, if one counts only true saccular aneurysms and not dolichoectasia or microaneurysms. We also did not observe a greater incidence of intracranial aneurysm formation in rats that had received bilateral CCA ligation compared to those that had received unilateral CCA ligation. We conclude that bilateral CCA ligation does not improve the efficiency of intracranial aneurysm induction over unilateral CCA ligation.

Pharmaceutical MMP inhibition has been shown to suppress AAA growth in animal models [4, 9, 20] and is now being evaluated in human clinical trials for AAA [2]. It remains possible that there are important differences in the role of MMPs in the formation and growth of AAAs as compared to that of intracranial aneurysms. Likewise, it is possible that there are important distinctions between MMP activity during aneurysm initiation and during various stages of growth and rupture; our model involved aneurysm initiation as well as growth.

It is also possible that doxycycline may have an effect size on aneurysm growth that was too small to detect with the power we had in our experiment. Larger studies or studies using aneurysm models with a higher incidence of aneurysm formation might be able to detect such a potentially small effect size. We do not know if arterial wall doxycycline concentrations in our model were sufficient for the degree of MMP inhibition that might be needed for aneurysm prevention, despite our achieving what are considered "therapeutic" serum levels. Finally, we do not know if another MMP inhibitor than doxycycline would be more successful.

We noted 14 premature deaths out of 96 animals; most deaths were secondary to respiratory illness. However, a greater number of premature deaths (ten) were seen in the control group than in the treatment group receiving doxycycline (four). Since we failed to determine whether aneurysms were present in 13 of the 14 animals that died prematurely, we cannot exclude the possibility that

aneurysms were, in fact, present. If there were aneurysms in these animals, and disproportionately in those not receiving doxycycline, the results may have been provocative.

As many treated or untreated human intracranial aneurysms are followed with imaging to ensure stability or to evaluate for growth, a safe and tolerable pharmaceutical product that could decrease growth and the chance of rupture would be highly desirable for these patients. As we learn more about the pathogenesis of intracranial aneurysm formation, growth and rupture, it may be possible to find such a pharmaceutical.

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