Myeloperoxidase Is Increased in Human Cerebral Aneurysms and Increases Formation and Rupture of Cerebral Aneurysms in Mice

Yi Chu, PhD; Katina Wilson, BS; He Gu, MD, PhD; Lauren Wegman-Points, PhD; Sarah A. Dooley, BA; Gary L. Pierce, PhD; Guangjie Cheng, PhD; Ricardo A. Pena Silva, MD, PhD; Donald D. Heistad, MD; David Hasan, MD

Background and Purpose—Cerebral aneurysm (CA) affects 3% of the population and is associated with hemodynamic stress and inflammation. Myeloperoxidase, a major oxidative enzyme associated with inflammation, is increased in patients with CA, but whether myeloperoxidase contributes to CA is not known. We tested the hypotheses that myeloperoxidase is increased within human CA and is critical for formation and rupture of CA in mice.

Methods—Blood was drawn from the lumen of CAs and femoral arteries of 25 patients who underwent endovascular coiling of CA, and plasma myeloperoxidase concentrations were measured with ELISA. Effects of endogenous myeloperoxidase on CA formation and rupture were studied in myeloperoxidase knockout mice and wild-type (WT) mice using an angiotensin II–elastase induction model of CA. In addition, effects of myeloperoxidase on inflammatory gene expression in endothelial cells were analyzed.

Results—Plasma concentrations of myeloperoxidase were 2.7-fold higher within CA than in femoral arterial blood in patients with CA. myeloperoxidase-positive cells were increased in aneurysm tissue compared with superficial temporal artery of patients with CA. Incidence of aneurysms and subarachnoid hemorrhage was significantly lower in myeloperoxidase knockout than in WT mice. In cerebral arteries, proinflammatory molecules, including tumor necrosis factor-α, cyclooxygenase-2 (COX2), chemokine (C-X-C motif) ligand 1 (CXCL1), chemokine (C motif) ligand (XCL1), matrix metalloproteinase (MMP) 8, cluster differentiation 68 (CD68), and matrix metalloproteinase 13, and leukocytes were increased, and α-smooth muscle actin was decreased, in WT but not in myeloperoxidase knockout mice after induction of CA. Myeloperoxidase per se increased expression of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 in endothelial cells.

Conclusions—These findings suggest that myeloperoxidase may contribute importantly to formation and rupture of CA.

Key Words: inflammation ▪ intercellular adhesion molecule-1 ▪ intracranial aneurysm ▪ neutrophils ▪ peroxidase ▪ subarachnoid hemorrhage ▪ vascular cell adhesion molecule-1

Cerebral aneurysm (CA), typically with enlargement at an arterial bifurcation, affects 3% of the population. Subarachnoid hemorrhage (SAH), after rupture of CA, is a major cause of death or disability in these patients. The cause of CA involves hemodynamic stress and inflammation, with similarities and important differences to abdominal aortic aneurysms. Treatment for both unruptured and ruptured CA is surgical, with coiling and clipping to prevent rupture and rerupture; there is no pharmacological treatment.

Myeloperoxidase belongs to the heme peroxidase family, which includes vascular peroxidase 1, a plasma peroxidase produced by endothelial cells. Myeloperoxidase is a major oxidative enzyme produced by activated neutrophils, monocytes, and macrophages. Myeloperoxidase is increased in blood of patients with CA during SAH and vasospasm. It is not known, however, whether myeloperoxidase is increased locally within CA. It is also not known whether myeloperoxidase contributes to CA formation and rupture, although a study suggests that myeloperoxidase presence in human CA tissue is positively correlated with 5-year aneurysm rupture risk.

In this study, we hypothesized that myeloperoxidase may contribute to formation and rupture of aneurysms in a mouse model.
Methods

Plasma Levels of Myeloperoxidase in Patients

The study was performed using a protocol approved by the University of Iowa Institutional Review Board. Blood was drawn from the lumina of CA and femoral artery from 25 patients who underwent endovascular coiling of CA. Findings in a subcohort (13 with unruptured and 4 with ruptured CA) were described previously. This study added 1 patient with a ruptured aneurysm and 7 patients with unruptured aneurysms. Plasma myeloperoxidase was measured by ELISA (ab119605, Abcam), and values are reported as the average of 2 measurements using 2 ELISA kits. As a control for myeloperoxidase, plasma concentrations of vascular peroxidase 1, a homolog of myeloperoxidase that is a plasma peroxidase secreted by endothelial cells, were measured with immunoblotting against a panel of known standards.

Analyses of Human Tissue Samples

Tissues were collected from CA and superficial temporal artery from 12 patients who underwent microsurgical clipping of CA. Samples were fixed in paraformaldehyde, and embedded in paraffin. After antigen retrieval (by microwaving in pH 6.0 citrate buffer), sections were incubated with anti-myeloperoxidase antibody (ab59777, Abcam), followed by horseradish peroxidase (HRP)-conjugated reagent (Boost, No. 8114, cell signaling) with 3,3'-diaminobenzidine (DAB) reaction (Vector Laboratories). Slides were stained with hematoxylin, dehydrated, and permanently mounted. Images were taken at x200 magnification with an Olympus BX61 microscope.

Mouse Model of Intracranial Aneurysm

The angiotensin II–elastase mouse model of CA was used as previously described. Two each of myeloperoxidase knockout (C57Bl/6J genetic background) and control C57Bl/6J mice (Jackson Laboratories), aged 17 to 18 weeks, were implanted with an osmotic mini-pump for continuous delivery of angiotensin II (1000 ng/kg per minute), and in-jected with 35 mU elastase (Sigma) into the right basal cistern. Systolic arterial pressure was monitored by tail cuff, and neurological function was assessed daily. Mice were considered symptomatic and euthanized if ≥1 of the following deficits were observed: decreased activity and hunched posture, leaning or circling to 1 side, or decreased food and water intake leading to weight loss of >20% of baseline. Mice without neurological signs were euthanized 19 to 19 days post CA induction, and the presence of CA and SAH was assessed.

Gene Expression in Mouse Cerebral Arteries

Cerebral arteries (anterior and posterior communicating arteries, middle cerebral arteries, and basilar arteries) were isolated and dissolved in TriZol (Life Technologies). Untreated mice (6 male WT and 6 male myeloperoxidase knockout) were used as baseline controls. Quantitative reverse transcription polymerase chain reaction was performed as described above. Two independent experiments were performed with n of 4 per group.

Mouse Brain Histology and Immunohistochemistry for Myeloperoxidase

Additional WT and myeloperoxidase knockout mice (n=3 and 4, respectively) underwent CA induction as described above. Brains were fixed in paraformaldehyde, paraffin-embedded, and 10-μm thick sections were cut. Sections were stained with hematoxylin and eosin. Leukocytes in cerebral arteries of comparable sizes were counted. Immunohistochemistry for myeloperoxidase was performed as described for human tissues except that a different anti-myeloperoxidase antibody was used for detecting mouse myeloperoxidase (ab9535, Abcam).

Effects of Myeloperoxidase on Gene Expression in Endothelial Cells

Primary human aortic endothelial cells were purchased from Lonza (CC-2535) and cultured according to instructions by Lonza (CC-3162). Passage 4 cells were incubated with human myeloperoxidase (30 nmol/L; Planta Natural Products) in the absence or presence of myeloperoxidase inhibitor 4-amino-benzoic acid hydrazide (ABAH, 50 μmol/L; Sigma) for 6 hours. Quantitative reverse transcription polymerase chain reaction was performed as described above. Two independent experiments were performed with n of 4 per group.

Statistical Analyses

All data are expressed as means±SE. Student t test was used for comparisons in the patient study. Fisher exact test was used for incidence of aneurysms and SAH in mice. ANOVA with Student–Newman–Keuls multiple comparisons post-test was used for gene expression. A P<0.05 was considered significantly different.

Results

Plasma Myeloperoxidase in Patients With CA

Myeloperoxidase concentrations were 2.7-fold higher in plasma from the CA sac (30±3 ng/mL) (mean±SE) than from femoral artery (11±1; P<0.001; Figure 1A). Among the 25 patients, 5 patients with ruptured aneurysms demonstrated similar values of femoral (11±3) versus aneurysm (35±10; P<0.05) concentrations of myeloperoxidase. No difference was found in 5 male (femoral, 15±5; aneurysm, 41±9; P<0.05) versus 20 female (femoral, 15±2; aneurysm, 33±4; P<0.05) patients. Myeloperoxidase concentrations of 7 healthy volunteers in blood drawn from an arm vein were 7.6±0.6 ng/mL. In contrast, concentrations of vascular peroxidase 1, a homolog of myeloperoxidase that is secreted to plasma by endothelial cells, were not different in blood from CA (2.3±0.3 ng/mL) versus femoral (2.4±0.4 ng/mL; Figure 1B).
hibmol/L) and femoral arteries of the patients (3.0±0.4; P=0.12; Figure 1B). These findings demonstrate for the first time that the local concentration of myeloperoxidase within the CA sac was elevated in comparison to circulating concentrations.

Tissue from ruptured CA appeared to have more myeloperoxidase-positive cells than unruptured CA, whereas superficial temporal arteries had no myeloperoxidase-positive cells except in the area containing blood (Figure 1C). These findings suggest that the presence of more myeloperoxidase-positive cells in human CAs could produce the local increase of myeloperoxidase in the CA sac.

Endogenous Myeloperoxidase Contributes to Formation and Rupture of CA in Mice Induced by Angiotensin II and Elastase

The incidence of CA was lower in myeloperoxidase knockout mice (50%) than in WT control (90%, P<0.05; Figure 2A). The occurrence of SAH also was lower in myeloperoxidase knockout mice (8%) than in WT control (83%, P<0.001; Figure 2B). Systolic blood pressure increased to 140 to 150 mmHg from a baseline of 117 mmHg (WT) or 120 mmHg (knockout), with no difference between WT and myeloperoxidase knockout mice (Figure I in the online-only Data Supplement). Mortality during the course of study was not different between WT and myeloperoxidase knockout mice, with 1 death among 12 mice in each group. Consequently, comparisons in gene expression analyses were not subject to influences from different survival rates between the 2 groups. These findings indicate that the formation and rupture of CAs in this experimental model of CA were attenuated in the absence of myeloperoxidase.

Gene Expression in Cerebral Arteries of WT Versus Myeloperoxidase Knockout Mice

Induction of CA increased expression of the proinflammatory agents tumor necrosis factor-α and cyclooxygenase-2 (COX2) in WT mice but not in myeloperoxidase knockout mice (Figure 3A). Likewise, induction of CA increased expression of chemokine (C-X-C motif) ligand 1 (CXCL1) and matrix metalloproteinase (MMP) 8, mediators important for neutrophil recruitment and function, more significantly in WT, but not in myeloperoxidase knockout mice (Figure 3A). Furthermore, induction of CA increased expression of cluster of differentiation 68 (CD68), a macrophage marker, in WT but not in myeloperoxidase knockout mice (Figure 3A). In contrast, α-smooth muscle actin, a marker for vascular smooth muscle cells, was reduced in WT mice after induction of CA (Figure 3A). Myeloperoxidase deficiency attenuated reduction of α-smooth muscle actin in response to CA induction (Figure 3A).

Effects of Myeloperoxidase on Gene Expression in Endothelial Cells

To investigate whether myeloperoxidase exerts effects in the absence of myeloperoxidase-producing cells, human aortic endothelial cells were incubated with human myeloperoxidase (30 nmol/L) in the absence or presence of its inhibitor ABAH for 6 hours. Among >20 genes measured with quantitative reverse transcription polymerase chain reaction, adhesion molecules vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 and oxidative stress marker SOD2 (MnSOD) were increased significantly by myeloperoxidase (P<0.05; Figure 3B). The increase was eliminated by ABAH, suggesting that the catalytic activity of myeloperoxidase was required for the effects (Figure 3B). Interestingly, increased SOD2 and NOX4 (with a trend, P=0.05) may increase hydrogen peroxide, a substrate for myeloperoxidase enzyme.

Leukocytes in Cerebral Arteries

The number of leukocytes (based on morphology) in cerebral arteries of comparable sizes was decreased in myeloperoxidase knockout mice compared with that in WT mice, after induction of CA (Figure 4). Myeloperoxidase-positive cells appeared fewer in myeloperoxidase knockout than in WT mice (Figure 5). These findings are in concordance with gene expression data which suggested decreased recruitment of
neutrophils and perhaps macrophages in cerebral arteries of myeloperoxidase knockout mice.

**Discussion**

To our knowledge, this study is the first to demonstrate localized increase of myeloperoxidase concentration in the CA sac of patients, which may be produced by myeloperoxidase-positive cells (neutrophils and macrophages) in CA. The localized myeloperoxidase may contribute to formation, progression, and rupture of CA in at least 4 ways. First, myeloperoxidase may attract neutrophils, and perhaps other leukocytes, to endothelium of cerebral arteries, with negative charges on their surfaces, by physical forces because of strong positive charges on the surface of myeloperoxidase. Our finding of an increase in leukocytes in aneurysm tissue of patients as well as cerebral arteries of mice is consistent with this mechanism. Second, myeloperoxidase may enter into endothelial cells through direct cell-cell contact from neutrophils to cause impairment of endothelial function, which is a common characteristic in an early stage of CA as well as abdominal aortic aneurysms. Third, myeloperoxidase may transcytose through endothelium into the arterial media to catalyze oxidation reactions in the extracellular matrix, thereby reducing tensile strength of the wall of cerebral arteries. Fourth, myeloperoxidase per se may induce inflammation by increasing expression of adhesion molecules in endothelial cells, promoting leukocyte adherence and inflammation. Summary of our findings and interpretations are shown in Figure 6.

Myeloperoxidase deficiency may protect against CA through profound reduction in expression of tumor necrosis factor-α in cerebral arteries of myeloperoxidase knockout compared with WT mice, in response to induction of CA. In cerebral arteries, tumor necrosis factor-α may recruit leukocytes and increase inflammatory cytokines and chemokines, injure endothelial cells, produce phenotypic transformation of smooth muscle cells, and upregulate MMPs that deteriorate integrity of cerebral arteries.

CXCL1 is an important chemotaxant that recruits neutrophils to endothelium at the site of inflammation. A >10-fold reduction of CXCL1 in cerebral arteries of myeloperoxidase knockout mice compared with WT mice after induction of CA was associated with a 17-fold decrease of MMP8 (collagenase-2), an MMP expressed specifically in neutrophils. Thus, in addition to a reduction in recruited leukocytes, deficiency of myeloperoxidase may inhibit cellular production of proinflammatory molecules. Both leukocytes and vascular cells express MMPs. MMP9 has been linked to CA, using MMP9 knockout mice. In this...
In this study, we found not only a 4-fold decrease in gene expression of MMP9 but also a significant reduction of both MMP3 (3-fold) and MMP13 (18-fold; Figure II in the online-only Data Supplement), which are strong activators of MMP9.14,29 Thus, we speculate that there may be a significant decrease in MMP9 activity. The role of MMP3 and MMP13 has not been studied in CA. Thus, findings in this study provide a rationale for future studies of the role of MMP3 and MMP13 in CA.

A limitation in this study is that mechanistic findings to explain attenuation of CA by myeloperoxidase deficiency are based on quantification of mRNA levels, not by protein or activity levels. This limitation resulted from limited quantities of cerebral arteries in mice. However, quantitative reverse transcription polymerase chain reaction produces the most accurate quantification with limited material.

In summary, in patients with CAs, circulating myeloperoxidase concentrations are increased locally in the CA sac, which is associated with increased neutrophils and other myeloperoxidase-positive cells in aneurysm tissues. In a mouse model of CA, myeloperoxidase deficiency reduced expression in cerebral arteries of proinflammatory molecules, preserved expression of smooth muscle differentiation molecules, including α-smooth muscle actin, are decreased in cerebral smooth muscle cells in response to tumor necrosis factor-α in culture,29 in aorta of abdominal aortic aneurysms in mice,30 and after other vascular injuries.31 Thus, our finding of preserved expression of α-smooth muscle actin (also positive in myofibroblast) in myeloperoxidase knockout mice after induction of CA suggests that myeloperoxidase deficiency attenuates formation and rupture of CA via a mechanism that involves protection of smooth muscle cells from injury.

Figure 4. There are fewer leukocytes (hematoxylin–eosin staining) in cerebral arteries in myeloperoxidase (MPO) knockout (KO) mice than wild-type (WT) mice. Representative images of WT (A) and MPO KO (B) cerebral arteries stained with hematoxylin–eosin, with quantification of cell numbers per artery (C). n=3 to 4 mice in each group. *P<0.05 vs WT.

Figure 5. Immunohistochemistry for myeloperoxidase (MPO) in WT (A) and MPO knockout (KO; B) cerebral artery after cerebral aneurysm induction. An isolated aneurysm containing blood inside the sac was used for negative control (without anti-MPO antibody, C) and positive control (D) for MPO.

Figure 6. Summary of the findings and interpretations in this study. MPO indicates myeloperoxidase.
α-smooth muscle actin, decreased the number of leukocytes, and attenuated formation and rupture of CAs. Myeloperoxidase itself increased expression of adhesion molecules in endothelial cells. These findings suggest that myeloperoxidase, which is increased locally in the CA sac of patients, contributes importantly to the pathophysiology of CA in a mouse model. Because myeloperoxidase deficiency is common in human population,2,15 it would be of interest to examine the incidence of CAs in patients with myeloperoxidase deficiency.

Acknowledgments

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Disclosures

None.

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6. Shivashankar R, Tirumal R, Jagal D, Samard J, Aldrich EF. Myeloperoxidase deficiency is common in human population.2,15 It would be of interest to examine the incidence of CAs in patients with myeloperoxidase deficiency.

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Supplemental Material

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Supplemental Figure I. Systolic blood pressure was measured at baseline and weekly following induction of aneurysm and mini-pump implantation. Pressures were significantly elevated after mini-pump implantation. There was no significant difference in blood pressure between MPO KO and wild type controls at any time point.
Supplemental Figure II. Expression of MMP9, MMP3, and MMP13 in cerebral arteries of WT and MPO KO mice 17-19 days after CA induction. mRNA levels were quantified using TaqMan ΔΔCt method, with normalization to β-actin in the same reaction. * = p<0.05 vs. WT