Inflammation seems to play a key role in the formation and rupture of intracranial aneurysms.\textsuperscript{12} Various constituents of the inflammatory response seem to be increased in intracranial aneurysms, including cytokines, chemokines, growth factors, reactive oxygen species, leukocytes, matrix metalloproteinases, and vascular smooth muscle cells.\textsuperscript{2–4} Therapies targeting the inflammatory cascade have also shown promising results in humans and experimental animals.\textsuperscript{5–8}

Hepatocyte growth factor (HGF), initially discovered as a growth factor of hepatocytes, was shown to have mitogenic, motogenic, morphogenic, anti-fibrotic, and anti-apoptotic activities in several tissues.\textsuperscript{5–14} The biological responses to HGF are mediated through a tyrosine kinase receptor, the c-Met protooncogene.\textsuperscript{15} Emerging data suggest that HGF modulates the cytokine profile and protects various tissues, including arterial walls from inflammatory damage.\textsuperscript{10,13,16–20} A recent study found that HGF promotes an anti-inflammatory cytokine profile in abdominal aortic aneurysm tissue and concluded that pharmacological interventions enhancing endogenous HGF secretion could have efficacy in prevention and treatment of these aneurysms.\textsuperscript{13} There have been no reports regarding the role of HGF in intracranial aneurysms. The purpose of this study was to assess the role of endogenous HGF in the pathogenesis of intracranial aneurysms. Specifically, we sought to determine (1) whether HGF concentrations were higher in blood samples drawn from the lumen of human intracranial aneurysms as compared with femoral arteries of the same patients; (2) whether HGF and c-Met were expressed in the wall of human intracranial aneurysms; (3) whether a c-Met antagonist increases the risk of aneurysm rupture in a mouse model; and (4) whether HGF modulates the expression of inflammatory molecules in cultured endothelial, smooth muscle cells, and monocytes.
Methods

Human Studies
The human study protocol was approved by the University of Iowa Institutional Review Board. The nature, benefits, and risks of the study were explained to all patients before the study, and all participants read and signed the written Institutional Review Board–approved informed consent.

Measurement of HGF Concentrations in Plasma
All patients presented to the Department of Neurosurgery at the University of Iowa Hospitals and Clinics between November 2012 and December 2012. Consecutive patients harboring saccular intracranial aneurysms (ruptured or unruptured) who were candidates for coil embolization were enrolled in the study. Patients taking corticosteroids, aspirin, or immunosuppressant therapy were excluded. A total of 16 patients harboring 18 aneurysms were enrolled. Other findings in the cohort (12 with unruptured and 4 with ruptured CA) were described previously.16

In each patient, arterial access was obtained through femoral puncture by use of the Seldinger technique, and a 7-French arterial sheath was inserted. A blood sample was subsequently drawn from the femoral artery. The guiding catheter was navigated into the studied vessel and the aneurysm was identified. A microcatheter was subsequently advanced over a microwire and placed in the aneurysm lumen. A blood sample (5 mL) was taken from the aneurysm lumen before coil deployment. Blood was centrifuged, and the plasma was stored at −80°C until analysis. Serum concentrations of HGF in aneurysm and femoral samples were quantified with Luminesuence-based immunoassay.

Expression of HGF and c-Met in Human Intracranial Aneurysms
Samples of intracranial aneurysms (5 ruptured and 5 unruptured) were taken from patients undergoing microsurgical clipping. A segment of the aneurysm was resected, fixed in formalin, and embedded in paraffin. Superficial temporal arteries (STA) were used as controls. 4 μm sections were collected and immunostained with human HGF R/c-MET affinity purified polyclonal antibody (AF276), or human HGF affinity purified polyclonal Ab (AF-294-NA) from R&D systems (Minneapolis, MN). Antibodies were validated in sections of skin and liver at the Histology Research Laboratory of the University of Iowa (Figure S1 in the online-only Data Supplement). Images were collected using a 20× objective lens in an Olympus BX-61 motorized microscope.

Mouse Studies

Assessment of the Role of Endogenous HGF in a Mouse Model of Intracranial Aneurysms
Studies were performed in 41 adult (4.8±0.1 months old) c57/B16 mice obtained from the Jackson Laboratory (Bar Harbor, ME). All experimental protocols and procedures conform to the National Institute of Health guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Iowa.

Intracranial aneurysms were induced in mice according to previously published methods.15,16 Briefly, under anesthesia, a longitudinal craniectomy was made in the scalp and a 1 mm hole was drilled in the skull. A stereotactic injection of elastase (35 mU in 2.5 μL) was subsequently performed using the following coordinates: 2.7 mm posterior to the bregma, 1 mm to the right of the midline, depth of 6.3 mm from the skull. Then, an osmotic mini-pump that delivered a pressor dose of angiotensin II (1000 ng/kg/min) was implanted subcutaneously. Sham mice underwent surgery, but received physiological saline. Mice were allowed to recover and food and water were provided ad libitum.

The c-Met antagonist PF-0421790322 (10 mg/kg per day; Selleckchem, Houston, TX) or its vehicle (100% dimethyl sulfoxide) were given daily by gavage from day 0 (when elastase was injected in the basal cistern and infusion of angiotensin II is begun) until the end of experiment (21 days) or until the mice died or were euthanized. The dose of PF-04217903 was chosen according to previously published pharmacokinetic data.23 Sham controls were given 50 μL of 100% dimethyl sulfoxide daily, by gavage.

Mice were monitored daily and euthanized if signs of neurological deficit or weight loss (>20%) were observed. All asymptomatic mice were euthanized 3 weeks after aneurysm induction. Five mice were excluded from the study. One sham control died after complications of gavage. Four mice were euthanized because they did not recover completely after the surgical procedure (3 vehicle-treated mice and 1 mouse treated with PF04217903). Systolic blood pressure was recorded weekly using the tail-cuff method (Visitec Systems BP-2000).

After euthanasia, the chest and abdomen of the mouse were exposed and examined for major bleeding or aneurysms of the aorta. Then, mice were perfused transcardially with 10 to 15 mL of ice-cold physiological saline solution containing papaverine (100 μM) to cause systemic vasodilation followed by an infusion of a 8% gelatin-physiological saline mix containing 2 mg/mL of bromophenol blue to facilitate visualization of the cerebral circulation. The brain was dissected and inspected for the presence of intracranial aneurysms or subarachnoid hemorrhage. Aneurysms were defined as a localized outward bulging of the vascular wall with a diameter >1.5 times the parent artery. A survival curve was made according to the time of euthanasia or death.

Effect of HGF on the Expression of Inflammatory Molecules in Cultured Cells
Human aortic endothelial cells (HAECs; Lonza Inc.; 3–7 passages) were preincubated in normal growth media with human recombinant HGF (10 ng/mL), with or without 6 μg/mL of c-met antagonist (PF-04217903)22 for 1 hour. Cells were then treated with 100 ng/mL of lipopolysaccharides (LPS; E. coli 0111:B4 strain) for 3 hours. Real-time quantitative PCR was performed after reverse transcription using TaqMan primers for gene of interest and house-keeping gene (β-actin) in the same reaction. ΔΔCt method was used to quantify gene expression normalized by β-actin. Intercellular adhesion molecule-1, vascular cell adhesion molecule-1 (VCAM-1), E-selectin, tumor necrosis factor-α, interleukin (IL)-1β, monocyte chemoattractant protein-1 (MCP-1), cyclooxygenase (COX)-1, COX-2, or transforming growth factor-β were measured. All of the findings in tissue culture are based on measurements from n=3.

Similarly, human aortic smooth muscle cells (3–7 passages) and human acute monocytic leukemia cells were preincubated with recombinant human HGF (10 ng/mL) or HGF+PF-04217903. Human aortic smooth muscle cells and human acute monocytic leukemia cells were then treated with LPS for 3 hours, and the mRNA levels of inflammatory molecules were measured as described above.

Statistical Analysis
Data are presented as means±SEM for continuous variables and as frequency for categorical variables. Analysis was performed using unpaired t test, chi-square, and 1-way ANOVA as appropriate. Survival was analyzed with log rank (Mantel-Cox) test. P values <0.05 were considered statistically significant. Statistical analysis was performed with Prism 6 (Graphpad, La Jolla, CA) and Stata 10.0 (College Station, TX).

Results

HGF Levels in Aneurysm Lumens Versus Femoral Arteries
Of the 16 patients with intracranial aneurysms, 13 were women and 3 were men with a mean age of 55±13 years. Aneurysm size was 10±9 mm on average. Three of the 16 patients (18%) had ruptured aneurysms. The mean plasma concentration of HGF was significantly higher in samples taken from cerebral aneurysms (1076±656 pg/mL) versus samples taken from femoral arteries (196±436 pg/mL, 5-fold; P<0.001; Figure 1).
HGF and c-Met Are Expressed in Human Intracranial Aneurysms

Samples from human STA and ruptured or unruptured intracranial aneurysms were positively stained for HGF and its receptor c-Met. HGF and c-Met localized to the endothelium and smooth muscle layers (Figure 2).

c-Met Antagonist Increases the Risk of Aneurysm Rupture and Decreases Survival in Mice

After induction of aneurysms, systolic pressure increased significantly ($P<0.05$) and similarly in response to infusion of angiotensin II in mice that received PF-04217903 or vehicle compared with sham controls (Figure 3C). After induction of aneurysms, mice lost weight especially during the first 2 weeks of follow up ($P<0.05$). Weight loss was not observed in sham controls (Figure 3D). PF04217903 did not have an effect on blood pressure or weight loss compared with vehicle.

Compared with sham controls, >85% of mice that received elastase and angiotensin II displayed evidence of intracranial aneurysms and subarachnoid hemorrhage after euthanasia (Figure 3A). Intracranial aneurysms were found in 12/14 (86%) mice treated with vehicle versus 15/16 (94%) mice treated with PF04217903 (Figure 3E). Prevalence of subarachnoid hemorrhage was significantly higher in mice treated with PF04217903 (15/16 [94%]) than in vehicle-treated mice (9/14 [64%]; $P<0.05$; Figure 3F). Survival was significantly lower in mice treated with PF04217903 than in vehicle treated mice (25 versus 57%, respectively; $P<0.05$; Figure 3B). Sham controls have 100% survival after surgery, and they did not have evidence of intracranial aneurysms or hemorrhage.

HGF Attenuates Inflammation in Cultured Human Aortic Endothelial Cells

The expression of VCAM-1 and E-selectin was lower in HAECs incubated with HGF+LPS versus LPS alone (Figure 4). Treatment with c-Met antagonist and HGF abolished the protective effect of HGF on inflammatory markers; specifically, levels of VCAM-1 and E-selectin were similar in HAECs treated with HGF+PF-665752+LPS versus LPS alone. There was no significant difference in levels of tumor necrosis factor-$\alpha$, IL-$1\beta$, MCP-1, COX-1, COX-2, transforming growth factor, or intercellular adhesion molecule-1 in HAEC cells treated with HGF+LPS, HGF+PF-04217903+LPS, or LPS alone (data not shown).

There was no significant difference in levels of tumor necrosis factor-$\alpha$, IL-$1\beta$, MCP-1, COX-1, COX-2, transforming growth factor, VCAM-1, E-Selectin, or intercellular adhesion molecule-1 in human aortic smooth muscle cells or human acute monocytic leukemia cells treated with HGF+LPS, HGF+PF-04217903+LPS, or LPS alone (data not shown).

Discussion

Inflammation is a critical pathway underlying the development, progression, and rupture of intracranial aneurysms. Evidence suggests that proinflammatory and proliferative pathways are activated in endothelial cells in response to local hemodynamic stress. This is followed by monocyte

Figure 2. Examples of expression of c-Met and hepatocyte growth factor (HGF) in human superficial temporal arteries and intracranial aneurysms (ruptured and unruptured). Positive immunostaining for C-Met and HGF was noted in the endothelium and smooth muscle layers of the examined samples. Negative control excluded the primary antibody for c-Met or HGF. Similar findings were observed in tissues from 10 other patients. Scale bar=100 $\mu$m.
infiltration, activation, and release of several proinflammatory molecules in arterial walls. A later final common pathway seems to involve the release of matrix metalloproteinases and apoptosis of cellular constituents of the vessel wall, leading to aneurysmal remodeling, progression, and rupture.

HGF may have a protective role in vascular disease and in the pathogenesis of intracranial aneurysms. HGF as a growth factor is regulated in a paracrine manner after tissue injury, and it promotes organ regeneration and wound healing. In the vascular system, HGF is involved in angiogenesis, and it also regulates function of endothelial progenitors cells. For example, HGF decreases angiotensin II–induced senescence and oxidative stress in endothelial progenitors. At the cellular level, HGF signaling through the receptor c-Met protects cells against DNA damage, promotes DNA repair, and decreases apoptosis. HGF also regulates other molecules involved in vascular biology and inflammation. HGF modulates EGFR degradation during LPS-induced inflammation by controlling translocation of phosphatases, such as SHIP2 (Src homology domain 2-containing phosphatase). Several studies have reported that HGF and c-Met are involved in arterial repair.

In this study, we found serum concentrations of HGF to be particularly high in the lumen of human intracranial aneurysms as compared with femoral arteries. Moreover, HGF and its receptor c-Met were expressed in the walls of both ruptured and unruptured human cerebral aneurysms. These findings provide the first evidence suggesting that HGF may be associated with human intracranial aneurysms (Figure 5). Thus, expression of HGF in the wall and lumen of human intracranial aneurysms may be a response to local cellular injury from hemodynamic stress and inflammation. Circulating HGF levels are changed in other cardiovascular diseases. Several

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**Figure 3.** Effect of a c-Met antagonist on intracranial aneurysm formation and rupture in mice. A, Cerebral arteries and intracranial aneurysm in situ (left) and after dissection (right). Note the hemorrhage surrounding the aneurysm. Survival (B), systolic blood pressure (C), weight loss (D), aneurysm formation (E), and prevalence of subarachnoid hemorrhage (F) in sham controls (n=5) and after induction of aneurysms in mice treated with PF-04217903 (n=16) or vehicle (n=14).

*P<0.05 vs sham; ψP<0.05 vs baseline; ΦP<0.05 vs vehicle.
studies have shown that HGF levels are increased in hypertensive patients compared with control subjects. On the other hand, HGF levels are decreased in diabetic patients and animal models of diabetes mellitus. HGF seems to have a protective role in vascular disease, and its levels may be modulated by mechanical, inflammatory, and metabolic stress.

HGF levels may increase locally in aneurysms to protect against vascular damage. Spin et al also showed that HGF expression is increased in aneurysms of the aorta. Our current studies suggest that HGF is upregulated in aneurysms and reduces inflammation and vascular damage because PF-04217903, an antagonist of c-Met, significantly increases aneurysm rupture and decreases survival in a mouse model of intracranial aneurysms. Importantly, protective effects of c-Met antagonism do not seem to modulate high blood pressure in this model of intracranial aneurysms.

We also explored, in cultured cells challenged with LPS, potential mechanisms through which HGF may prevent intracranial aneurysm rupture. We found that HGF mainly exerts its effects on endothelial cells (as opposed to smooth muscle cells or monocytes), where it attenuates the expression of VCAM-1 and E-selectin. The finding that HGF decreases the expression of adhesion molecules selectively in endothelial cells is particularly relevant because the infiltration of inflammatory cells in the intracranial aneurysm tissue is a hallmark of intracranial aneurysms. Aoki et al demonstrated that attenuation of macrophage migration through inhibition of MCP-1 halted intracranial aneurysm formation in mice. In addition, VCAM-1 and E-selectin were increased in experimental aortic aneurysm and human-ruptured cerebral aneurysm tissue, respectively. Collectively, these data suggest that attenuation of adhesion molecules (VCAM-1 and E-selectin) by HGF, and decreased infiltration of inflammatory cells in intracranial aneurysm tissue, may attenuate the inflammatory response associated with aneurysm formation, progression, and rupture.

Anti-inflammatory actions of HGF have also been reported in several other studies. Shintani et al demonstrated that attenuation of macrophage migration through inhibition of MCP-1 halted intracranial aneurysm formation in mice. In addition, VCAM-1 and E-selectin were increased in experimental aortic aneurysm and human-ruptured cerebral aneurysm tissue, respectively. Collectively, these data suggest that attenuation of adhesion molecules (VCAM-1 and E-selectin) by HGF, and decreased infiltration of inflammatory cells in intracranial aneurysm tissue, may attenuate the inflammatory response associated with aneurysm formation, progression, and rupture.

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**Novelty and Significance**

**What Is New?**

- This is the first report to suggest a protective role of endogenous hepatocyte growth factor (HGF) in cerebral aneurysms.
- We found increased levels of HGF in the lumen of intracranial aneurysms and found that HGF and its receptor c-Met are expressed in superficial temporal arteries and in ruptured or unruptured intracranial aneurysms.
- HGF decreased mortality and frequency of subarachnoid hemorrhage in a mouse model of intracranial aneurysms.
- HGF decreased the inflammatory response of cultured endothelial cells exposed to LPS.

**What Is Relevant?**

- HGF could have a therapeutic use by halting progression of cerebral aneurysms and preventing rupture of unstable aneurysms.

**Summary**

Plasma HGF concentrations are elevated in intracranial aneurysms. HGF and c-Met are expressed in superficial temporal arteries and in intracranial aneurysms. HGF signaling through c-Met may decrease inflammation in endothelial cells and protect against intracranial aneurysm rupture.
Novel Role for Endogenous Hepatocyte Growth Factor in the Pathogenesis of Intracranial Aneurysms

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A Novel Role for Endogenous HGF in the Pathogenesis of Intracranial Aneurysms

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Figure S1. Expression of c-Met and HGF in human liver and skin samples. Negative control excluded the primary antibody for c-Met or HGF. Scale bar = 100µm.