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Stromal Cell-Derived Factor-1 Is Associated with Angiogenesis and Inflammatory Cell Infiltration in Aneurysm Walls

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Abstract

Object—A small percentage of cerebral aneurysms rupture, but when they do, the effects are devastating. Current management of unruptured aneurysms consist of surgery, endovascular treatment, or watchful waiting. If the biology of how aneurysms grow and rupture were better known, a novel drug could be developed to prevent unruptured aneurysms from rupturing. Ruptured cerebral aneurysms are characterized by inflammation-mediated wall remodeling. We studied the role of stromal cell-derived factor-1 (SDF-1) in inflammation-mediated wall remodeling in cerebral aneurysms.

Methods—Human aneurysms; murine carotid aneurysms; and murine intracranial aneurysms were studied by immunohistochemistry. Flow cytometry analysis was performed on blood from mice developing carotid aneurysms or intracranial aneurysms. The effect of SDF-1 on endothelial cells and macrophages was studied by chemotaxis cell migration assay and capillary tube formation assay. Anti-SDF-1 blocking antibody was given to mice and compared to control (vehicle)-administered mice for its effects on the walls of carotid aneurysms and the development of intracranial aneurysms.

Results—Human aneurysms, murine carotid aneurysms, and murine intracranial aneurysms, all express SDF-1; and mice with developing carotid aneurysms or intracranial aneurysms have increased progenitor cells expressing CXCR4, the receptor for SDF-1 ($P<0.01$ and $P<0.001$, respectively). Human aneurysms and murine carotid aneurysms have endothelial cells, macrophages, and capillaries in the walls of the aneurysms; and the presence of capillaries in the walls of human aneurysms is associated with presence of macrophages ($P=0.01$). SDF-1 promotes endothelial cell and macrophage migration ($P<0.01$ for each), and promotes capillary tube formation ($P<0.001$). When mice are given anti-SDF-1 blocking antibody, there is a significant reduction in endothelial cells ($P<0.05$), capillaries ($P<0.05$), and cell proliferation ($P<0.05$) in the aneurysm wall. Mice given anti-SDF-1 blocking antibody develop significantly fewer intracranial aneurysms (33% versus 89% in mice given control IgG)($P<0.05$).

Conclusions—These data suggest SDF-1 associated with angiogenesis and inflammatory cell migration and proliferation in the walls of aneurysms, and may have a role in the development of intracranial aneurysms.

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CONFLICTS OF INTEREST/DISCLOSURES

None

Keywords

aneurysm; inflammation; stromal cell-derived factor-1; angiogenesis; wall remodeling

INTRODUCTION

Cerebral aneurysms are believed to occur in up to 5% of the population⁵⁴, however, the incidence of subarachnoid hemorrhage is only about 14.5 per 100,000 in the United States⁴⁵. This implies most unruptured aneurysms remain unruptured; but for the small percentage that do rupture, the effects are devastating, with up to 50% mortality and 30% dependency⁵³.

Current treatments for cerebral aneurysms are aimed at the surgical isolation of the aneurysm from the circulation (clipping) or intrasaccular packing to halt blood flow into the aneurysm (coiling); however, an alternative approach, that has yet to be identified, would be the development of potential drug therapies that could stabilize an unruptured cerebral aneurysm and prevent it from developing into one that is prone to rupture.

Ruptured cerebral aneurysms are characterized by inflammation-mediated wall remodeling^{9–11, 23, 24, 51}. It is believed that aneurysm wall remodeling consists of matrix degeneration by macrophages and other inflammatory cells, and regeneration by smooth muscle cells, but that a failure of regeneration leads to wall thinning resulting in aneurysm rupture^{9–11, 23, 24, 51}. Angiogenesis within the aneurysm wall is considered to regulate aneurysm wall remodeling and is suspected to have a critical role in aneurysm formation and rupture^{24, 26, 46}.

Stromal cell-derived factor-1 (SDF-1 or CXCL12) is a chemokine with a robust role in angiogenesis^{28, 29, 40, 47} and activation of the inflammatory cascade^{27, 41}. SDF-1 has been shown to be involved in the recruitment of lymphocytes within the wall of abdominal aortic aneurysms³⁵.

Our hypothesis is that SDF-1 has a key role in angiogenesis and inflammatory cell infiltration in the walls of aneurysms. We studied this in human cerebral aneurysm specimens, and in two murine models: an elastase carotid aneurysm model and a hypertensive elastase circle of Willis intracranial aneurysm model. We will show: 1) angiogenesis occurs in the walls of aneurysms; 2) angiogenesis is associated with inflammatory cell infiltration in the walls of aneurysms; 3) SDF-1 is expressed in aneurysms and aneurysm formation activates circulation of progenitor cells expressing CXCR4, the receptor for SDF-1; 4) SDF-1 promotes angiogenesis (by promoting endothelial cell migration and tube formation); 5) SDF-1 promotes inflammatory cell migration; 6) blocking SDF-1 inhibits angiogenesis in the walls of aneurysms; and 7) blocking SDF-1 inhibits cell proliferation in the walls of aneurysms.

METHODS

Human Aneurysm Specimens

All collection and studies of human aneurysm specimens and control arteries (superficial temporal arteries) were performed according to a research protocol approved by our Institutional Review Board (IRB). Patients signed informed IRB research consent before aneurysm and control superficial temporal artery specimens were harvested at the time of craniotomy and aneurysm clipping surgery. Tissue was collected from aneurysm domes and immediately fixed by 4% paraformaldehyde.

Animals

All animal experimentation was performed in accordance with a protocol approved by our institution's Institutional Animal Care and Use Committee, and all studies were performed using C57BL6 female mice, 6–8 weeks old, 20–25 gm, (Charles River Laboratories, Wilmington, MA).

Murine Elastase Carotid Aneurysm Model

Carotid aneurysms were induced in C57BL6 mice by an elastase method we have previously described^{16, 17}.

Hypertensive Elastase Circle of Willis Intracranial Aneurysm Model

Murine circle of Willis intracranial aneurysms were created in C57BL6 mice using a modified version of a method previously described³⁴.

Briefly, in our modified version, the left common carotid artery and the right renal artery are ligated in mice fed a hypertensive diet (8% NaCl diet with 0.12% beta-aminopropionitril (BAPN), Harlan Laboratories, Indianapolis, IN). One week later, 20 uL of porcine pancreatic elastase solution (Worthington Biochemical Corp, Lakewood, NJ) 10 U diluted in 1 mL of 1 × phosphate buffered solution (PBS) (Invitrogen, Carlsbad, CA) is stereotactically injected into the right basal cistern at 1.2 mm rostral of bregma, 1.2 mm lateral of midline and 5.3 mm deep to the surface of the brain. Angiotensin II (Bachem, Torrance, CA) is continually infused via a subcutaneously-placed osmotic pump (Alzet, Cupertino, CA) at a dose of 1000ng/kg/min in PBS. Three weeks later, intracranial aneurysms are seen at the circle of Willis.

SDF-1 Blockade

SDF-1 blockade in mice was performed using anti-SDF-1 blocking antibody [monoclonal anti-human/mouse CXCL12/SDF-1 antibody, R and D systems, Minneapolis, MN, Cat# MAB-310] intravenously 24 hours pre-elastase (100µg/animal) and every 48 hours (50µg/animal) for three weeks post-elastase, for both the murine carotid aneurysm model and the murine intracranial aneurysm model. This antibody, Human/Mouse CXCL12/SDF-1 antibody (MAB310), detects human and mouse CXCL12/SDF1α and human CXCL12/SDF1β. The source of the antibody is Monoclonal Mouse IgG1 Clone # 79014. Regarding the neutralization, according to the R&D data sheet, 111µg/mL of this antibody will neutralize > 50% of the chemotactic effect due to 2 ng/mL Recombinant Human CXCL12/SDF1α in vitro. Control was performed using isotype matched IgG control (mouse IgG1 isotype control, R and D systems, Minneapolis, MN, cat # MAB002) using the same dose and schedule.

Mice were randomly selected from the cage and received either SDF-1 blockade or isotype matched Ig control by computer-generated random selection in syringes that were blindly labeled with numbers. The assigned agent for each numbered syringe was kept in a master data sheet to which the mouse surgeon and data collectors were blinded.

Immunohistochemistry

Immunohistochemistry was performed on human and mouse aneurysm specimens. Prior to harvesting murine aneurysms, mice underwent cardiac perfusion with 4% paraformaldehyde. After 24 hours of paraformaldehyde fixation, specimens were either frozen prepared or paraffin embedded. The tissues frozen prepared were transferred into 18% sucrose for 24 hours at 4 degrees Celsius, then embedded in a cassette with O.C.T. compound (optimal cutting temperature compound, Sakura/Tissue-Tek Company, Torrance,

CA) and frozen with dry ice and 2-methylbutane. Tissues were stored in -80°C before they were sectioned by a cryostat into $5\ \mu\text{m}$ sections. Tissues paraffin-embedded were transferred into 70% EtOH followed by paraffin embedding with a Rapid microwave histoprocessing unit (Milestone, Bergamo, Italy). The specimens were sectioned by a microtome into $5\ \mu\text{m}$ sections.

Immunohistochemistry was performed with the following antibodies: anti-human/mouse SDF-1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-human CD31 (DAKO Cytomation, Carpinteria, CA), anti-human CD45 (DAKO Cytomation, Carpinteria, CA) and anti-human CD68 (Abcam, Cambridge, MA), anti-MECA32 (BD Pharmingen, San Jose, CA), anti-mouse CXCR4 (Novus Biologicals, Littleton, CO), anti-mouse CD45 (Abcam, Cambridge, MA) and anti Ki67 (Leica, Buffalo, IL). Antigen retrieval with Citra buffer pH 6.0 was required for optimal staining with CXCR4. Anti-human CD31, anti-CD45, MECA 32, SDF-1 and Ki67 were heat retrieved in Target Retrieval Solution (DAKO Cytomation, Carpinteria, CA) following the manufacturer's instructions. Trilogy (Cell Marque Cooperation, Austin, TX) heat retrieval was used with CD68 staining. All slides were detected using 1:500 dilutions of species appropriate Alexa Fluor antibodies raised in donkey. Sections were mounted in VectaShield with DAPI (Vector Laboratories, Burlingame, CA) prior to imaging. Positive control tissues and concentration matched Ig controls were included with each immunoassay (data not shown).

Stereological counting rules were used for the cell and capillary counts. A total of five sections from each sample were created every $200\ \mu\text{m}$. The sections were then stained for MECA-32 and imaged with high-resolution. MECA-32-positive cells and capillaries were counted by two blinded observers. The area of the aneurysmal wall was measured by the blinded observers using ImagePro software.

Human and murine aneurysms were imaged using Optronics Magnafire digital microscopy (Meyer Instruments, Houston, TX) and Olympus IX71 inverted fluorescent scope (Olympus Inc., Center Valley, PA). Cell and capillary counting was performed by two independent blinded observers.

Human aneurysm data collectors were blinded to the clinical and aneurysm details of the human aneurysm specimens they were given. Murine aneurysm data collectors were blinded to any treatment mice were given.

Flow Cytometry

Peripheral blood samples were collected from mice 3 days post-elastase. Mono nuclear cells from peripheral blood were isolated using Ficoll-Paque (GE Healthcare Biosciences, Piscataway, NJ) and incubated with anti-mouse CXCR4 antibody conjugated with FITC (BD Pharmingen, San Jose, CA) and anti-mouse Sca-1 antibody conjugated with PE (BD Pharmingen, San Jose, CA).

Cell Culture

Mouse J774 macrophage (ATCC, Manassas, VA) was grown in DMEM (Mediatech, Manassas, VA) supplemented with 10% FBS, 1% Glutamax, and 1% Penicillin/Streptomycin. Human umbilical vein endothelial cells (HUVEC) were grown in Vasculife VEGF medium (Lifeline Cell Technology, Frederick, MD) prepared as per manufacturer's instructions.

Cell Migration Assay

Cell migration assays were performed with endothelial cells and macrophages using 6.5 mm 8µm Transwell Cell Inserts (Corning Life Sciences, Lowell, MA). Cells were grown to 80% confluence, serum-starved overnight, and harvested. Cells were then resuspended in serum-free medium and seeded into the upper chamber of cell inserts at a concentration of 30,000 cells/well for endothelial cells, and 200,000 cells/well for macrophages. The bottom chamber was filled with 0.6 mL of medium with or without SDF-1 (100 ng/mL) and with or without anti-SDF-1 blocking antibody (1600 ng/mL). Endothelial cells and macrophages were allowed to migrate for 24 hours at 37°C 5% CO₂. At the end of migration period the inserts were washed and fixed in 100% methanol and stained with hematoxylin. The top portion of the membrane was wiped twice with a cotton swab and migrated cells were counted by a blinded observer.

Bottom chambers of the transwell assays were randomly assigned to SDF-1, no SDF-1, anti-SDF-1 blocking antibody, or no anti-SDF-1 blocking antibody with syringes blindly labeled with numbers. The assigned agent for each numbered syringe was kept in a master data sheet to which the person performing the cell migration assay and data collectors were blinded.

Microscopic image of total of three fields of a membrane from each assay (n=5 each group), which were taken by ×20 objective lens using *Volocity 3D Image Analysis Software*, were tested. All images were not overlapped with other fields. Since the cotton swabs were not able to wipe off the cells around the edge of membranes, the image of those areas were not taken. All fields were imaged with high-resolution by a blinded observer, and all hematoxylin positive cells were counted by a blinded observer.

Endothelial Cell Proliferation Assay

Human umbilical vein endothelial cells (HUVEC) were grown to 80% confluence, harvested by 0.05% trypsin - EDTA, and resuspended in Vasculife VEGF basal medium with 2% FBS, 50 µg/mL ascorbic acid, 1.0 µg/mL hydrocortisone sulfate, and 10 mM L-glutamine. Approximately, 7,500 cells were seeded into wells in a tissue culture - treated 96-well plate (Techno Plastic Products, Trasadingen, Switzerland) with or without SDF-1 at 100 ng/mL concentration and incubated for 48 hrs. To confirm the effect of SDF-1 on endothelial cell proliferation, SDF-1's activity was blocked in some wells using anti-SDF-1 antibody in 1:1 molar concentration. Pictures were taken of each well with a 5× objective on an inverted phase-contrast microscope shortly after, 24, and 48 hrs after seeding. No medium was replaced or added during the experimental period.

Microscopic image of total of three fields of a culture dish from each assay (n=5 each group), which were taken by ×5 objective lens using *Volocity 3D Image Analysis Software*, were tested. All images were not overlapped with other fields. All fields were imaged with high-resolution by a blinded observer, and the numbers of cells were quantified by a blinded observer.

Tube Formation Assay

Briefly, human umbilical vein endothelial cells were grown to 80% confluence, harvested, and resuspended in Vasculife VEGF basal medium with 2% FBS, 50 µg/mL ascorbic acid, 1.0 µg/mL hydrocortisone sulfate, and 10 mM L-glutamine. Approximately, 10,000 cells were seeded into wells in a tissue culture - treated 96-well plate (Techno Plastic Products, Trasadingen, Switzerland) with or without SDF-1 (100 ng/mL) and incubated for 24 hrs. To confirm the effect of SDF-1 on endothelial cell tube formation, SDF-1's activity was blocked in some wells using anti-SDF-1 antibody in 1:1 molar concentration. The number of

complete loop networks formed by endothelial cells was quantified by a blinded observer. The number of cells were counted in each well to confirm that the effect of SDF-1 could not be attributed to an effect on cell proliferation.

Tissue culture wells were randomly assigned to SDF-1, no SDF-1, anti-SDF-1 blocking antibody, or no anti-SDF-1 blocking antibody with syringes blindly labeled with numbers. The assigned agent for each numbered syringe was kept in a master data sheet to which the person performing the tube formation assay and data collectors were blinded.

Microscopic image of total of three fields of a culture dish from each assay (n=5 each group), which were taken by $\times 5$ objective lens using *Velocity 3D Image Analysis Software*, were tested. All images were not overlapped with other fields. All fields were imaged with high-resolution by a blinded observer, and the numbers of complete loops networks formed by endothelial cells were quantified by a blinded observer.

Statistical Analysis

Data are given as the mean and 95% confidence intervals. Fisher's Exact Test was performed to test for association between presence of capillaries and monocytes, macrophages, and hematopoietic-derived inflammatory cells in the walls of aneurysms. Data are summarized with means and standard deviations as well as medians and ranges. Since sample sizes for the two-group comparisons were small and possibly from non-normal distributions, making t-tests inappropriate, two-sided permutation tests (R software, V. 2.13.1) were used to determine whether group differences existed. For multiple-group comparisons, analysis of variance (SAS PROC GLM, V 9.3) to evaluate overall group differences was used and Tukey's method was applied to main the Type I error rate at .05 when making post-hoc pairwise comparisons. ANOVA assumptions were verified by checking the normality of the residuals visually with a histogram and a Q-Q plot, and by plotting the residuals vs. predicted values to check for homogeneity of variance.

Power Calculations

For the comparison of cells expressing CXCR4, the receptor for SDF-1, by flow cytometry analysis between mice given anti-SDF-1 blocking antibody, mice given IgG control, and sham-operated mice (1) for the carotid aneurysm model; and 2) for the hypertensive circle of Willis intracranial aneurysm model), we powered the experiment to have 80% probability of detecting a true difference in percentage of 3 points between groups. We anticipated an overall mean response of approximately 5% and a standard deviation of approximately 2 percentage points in both groups. Although we later chose non-parametric tests for the final analysis because our data was not normally distributed, we initially assumed we would be able to use t-tests to determine whether observed differences were significant. These assumptions yielded a required sample size of 8 per group. To ensure adequate power in the event that some mice died, we inflated the sample size to 10 per group.

For the comparison of endothelial cells, capillaries, and cell proliferation in the aneurysm walls between mice given anti-SDF-1 blocking antibody and mice given IgG control, we arrived at a sample size of 10 per group after making similar assumptions and performing similar calculations for each test. At assumed standard deviations of 100 (for endothelial cells and cell proliferation), and 10 (for capillaries), this sample size gave us 80% power to detect true differences by t-test of 2.6, 1.3, and 0.13, respectively.

For the comparison of endothelial cell migration, capillary tube formation, and inflammatory cell migration between SDF-1, SDF-1 with anti-SDF-1 blocking antibody, or none, we powered the experiment to have an 80% probability of detecting a true difference in cells/

field of 30% between any two groups. We assumed we would observe an overall mean of approximately 225 cells/field with a standard deviation of approximately 50 in both groups on all tests. Although we later chose non-parametric tests for the final analysis because our data was not normally distributed, we initially assumed we would be able to use t-tests to determine whether observed differences were significant. These assumptions led us to set our sample size at 10 per group.

Animal Exclusion

The animal experiments conducted in Figure 4 and Figure 6 were performed with an initial sample size of n=10 per group. Only mice surviving to the full time point were included in the analysis. The number of excluded animals due to mortality were as follows: Figure 4A C57BL6 (n=1), Sham (n=2), anti-SDF1 (n=3) and IgG control (n=3); Figure 4C elastase (n=4) and sham (n=1); and Figure 6A, B & C anti-SDF1 (n=3) and IgG control (n=3).

RESULTS

Angiogenesis Occurs in the Walls of Aneurysms

Human aneurysm specimens were harvested at the time of aneurysm clipping surgery and immunohistochemical staining was performed demonstrating endothelial cells (CD31+) and angiogenesis (capillary formation) within the media of the aneurysm walls, whereas control superficial temporal arteries did not (Table 1) (Figure 1A).

Elastase-induced carotid aneurysms from C57BL6 mice were harvested three weeks post-elastase and immunohistochemical staining was performed demonstrating endothelial cells (MECA-32+) and angiogenesis within the media of the aneurysm walls, but not in normal control murine carotid arteries (Table 2) (Figure 1B).

Angiogenesis is Associated with Inflammatory Cell Invasion in the Walls of Aneurysms

Human aneurysm specimens contain abundant monocytes and macrophages (CD68+), and hematopoietic-derived inflammatory cells (CD45+) within the media of the walls of the aneurysms (Table 1) (Figure 2). The presence of capillaries in the walls of aneurysms is associated with presence of monocytes and macrophages ($P=0.01$ by Fisher's Exact Test), and hematopoietic-derived inflammatory cells in the walls of the aneurysm specimens ($P=0.01$ by Fisher's Exact Test).

SDF-1 is Expressed in Aneurysms and Aneurysm Formation Activates Circulation of Progenitor Cells Expressing CXCR4, the Receptor for SDF-1

Human aneurysm specimens demonstrate positive SDF-1 expression by immunohistochemical staining, whereas control superficial temporal arteries do not (Table 1) (Figure 3A).

Elastase-induced murine carotid aneurysms harvested 3 hours post-elastase express SDF-1 and its receptor, CXCR4; but normal control murine carotid arteries do not (Table 2) (Figure 3B).

C57BL6 mice underwent right renal and left carotid ligation, infusion of angiotensin II (by subcutaneous osmotic pump), fed a hypertensive diet, and underwent stereotactic injection of elastase into the right basal cistern. The circle of Willis was harvested at 3 days post-elastase. Immunohistochemical staining demonstrated positive SDF-1 expression, but not in sham-operated mice (Table 2) (Figure 3C).

Flow cytometric analysis of blood collected 3 days post-elastase (n=9 mice) from mice induced to develop carotid aneurysms demonstrated increased circulation of progenitor cells (stem cell antigen-1 positive (SCA-1+)) expressing CXCR4, the receptor for SDF-1, compared to sham-operated mice (n=8) ($P<0.01$) (Figure 4A). When anti-SDF-1 blocking antibody was given to mice (n=7), there were significantly less progenitor cells (SCA-1+) expressing CXCR4, than mice given IgG control (n=7) ($P<0.01$) (Figure 4A).

Flow cytometric analysis of blood collected 3 days post-elastase (n=6 mice) from mice induced to develop intracranial circle of Willis aneurysms demonstrated increased circulation of progenitor cells (SCA-1+) expressing CXCR4, compared to sham-operated mice (n=9) ($P<0.001$) (Figure 4B). Additionally, immunohistochemical staining of the circle of Willis of mice that developed aneurysms demonstrate invasion of hematopoietic-derived inflammatory cells (CD45+), whereas none are found in normal circle of Willis (Figure 4C).

SDF-1 Promotes Angiogenesis (by Promoting Endothelial Cell Migration, Endothelial Cell Proliferation, and Tube Formation)

The effect of SDF-1 on endothelial cell migration was demonstrated by in vitro cell migration assays with human umbilical vein endothelial cells (HUVEC, C-003-5C, Invitrogen) cultured in the upper chamber and the bottom chamber containing either serum-free medium with SDF-1 (100 ng/mL); SDF-1 (100 ng/mL) with anti-SDF-1 blocking antibody (1600 ng/mL); or none. After 24 hours, there was significant migration of endothelial cells in the chambers containing SDF-1 compared to the chambers containing SDF-1 and anti-SDF-1 blocking antibody or none ($P<0.01$) (Figure 5A).

The effect of SDF-1 on endothelial cell proliferation was demonstrated in proliferation assays. Proliferation trajectory of cells exposed to 100 ng/mL SDF-1 was found to be significantly increased compared to control ($p=0.0004$) and anti-SDF-1 blocked ($p=0.0003$) groups. No difference in trajectories was found between control and anti-SDF-1 blocked groups ($p=0.919$). Next, endothelial cell growth at 24 and 48 hours were compared. No significant difference among the groups was found at 24 hrs ($p=0.18$) by ANOVA. However, at 48 hrs, cells exposed to 100 ng/mL SDF-1 showed 31% higher proliferation than both the control ($p=0.0012$) and the anti-SDF-1 blocked group ($p=0.0012$) by ANOVA with Tukey's pairwise comparison (Figure 5B). The control and the anti-SDF-1 blocked group were not found to be significantly different at 48 hrs by the same analysis.

The effect of SDF-1 on angiogenesis was demonstrated by in vitro endothelial cell tube formation assay in which HUVECs were cultured with SDF-1 (100 ng/mL); SDF-1 (100 ng/mL) with anti-SDF-1 blocking antibody (1600 ng/mL); or none. After 24 hours, there was significantly greater complete loop networks formed by endothelial cells cultured with SDF-1 compared to SDF-1 and anti-SDF-1 blocking antibody or none ($P<0.05$) (Figure 5C). There was no significant difference in cell numbers between each group after the assay, which indicates that the increased tube formation was not due to an effect of SDF-1 on increased cell proliferation.

SDF-1 Promotes Inflammatory Cell Migration

The effect of SDF-1 on inflammatory cell invasion was demonstrated by in vitro cell migration assays in which macrophages were cultured in the upper chamber of Transwell Cell Inserts (Corning Life Sciences, Lowell, MA) and the bottom chamber containing serum-free medium with SDF-1 (100 ng/mL); SDF-1 (100 ng/mL) with anti-SDF-1 blocking antibody (1600 ng/mL); or none. After 24 hours, there were significantly greater macrophage migration in the chambers containing SDF-1 compared to SDF-1 and anti-SDF-1 blocking antibody or none ($P<0.001$) (Figure 5D).

Blocking SDF-1 Inhibits Angiogenesis in the Walls of Aneurysms

Carotid aneurysms harvested three weeks post-elastase from C57BL6 mice given anti-SDF-1 blocking antibody (n=7) have significantly less endothelial cells ($P<0.05$) and number of formed capillaries ($P<0.05$) in the walls of the aneurysms, compared to mice given Ig control (n=7) (Table 2) (Figure 6A and 6B).

Blocking SDF-1 Inhibits Cell Proliferation in the Walls of Aneurysms

Ki-67 antibody staining performed on murine carotid aneurysms three weeks post-elastase from C57BL6 mice given anti-SDF-1 blocking antibody (n=7 mice) demonstrate significantly less cell proliferation, compared to mice given Ig control (n=7) ($P<0.05$) (Table 2) (Figure 6C).

Blocking SDF-1 is Associated with Reduced Intracranial Aneurysm Formation

C57BL6 mice given anti-SDF-1 blocking antibody (n=10 mice) developed significantly fewer intracranial aneurysms than C57BL6 mice given IgG control (n=10): 33% versus 89% respectively ($P<0.05$) (1 mouse from each group died perioperatively due to anesthetic issues) (Figure 7A–C). Mice given anti-SDF-1 blocking antibody also had fewer ruptured intracranial aneurysms (n=1 of 9) than mice given IgG control (n=3 of 9), but this was not statistically significant (Figure 7D).

DISCUSSION

The outcomes of cerebral aneurysm rupture can be devastating, with 30 to 50% mortality and 50% significant morbidity^{20, 30, 37, 50}. Only a small percentage of unruptured cerebral aneurysms go on to rupture, however, and their management consists of either watchful waiting, surgical clipping, or endovascular coiling. If the pathophysiology of how unruptured aneurysms go on to rupture were better understood, then a drug could potentially be developed that could stabilize and protect an unruptured aneurysm from becoming prone to rupture.

The same or similar type of drug could also stabilize aneurysms that have been coiled. Aneurysms that have been coiled are at risk for regrowth, which can occur in up to a quarter of coiled aneurysms³. Aneurysm regrowth after coiling is believed to represent growth of the aneurysm sac¹⁴. A drug that prevents aneurysm growth could be given to patients that have had aneurysm coiling to prevent aneurysm regrowth after coiling.

The pathophysiology of aneurysm growth is not well elucidated. Operative findings⁵⁶ and histopathologic analysis^{1, 2, 19, 33, 46} of human cerebral aneurysm specimens have identified angiogenesis or vaso vasorum in the walls of aneurysms. Additionally, angiogenic growth factors have been demonstrated in the walls of aneurysms^{9, 24, 46}, and in particular, ruptured aneurysms compared to unruptured aneurysms^{9, 24}.

Histopathologic analysis of human ruptured compared to unruptured cerebral aneurysms have demonstrated a significant association between the degree of inflammatory cell invasion and the fragility of the aneurysm wall²². Inflammatory cell, particularly macrophage, infiltration of the walls of aneurysm are associated with extracellular matrix degradation, loss of structural integrity of the wall, and these are associated with aneurysm rupture^{10, 22}. Complement activation has also been found in the wall of human cerebral aneurysm specimens, with a greater degree of complement activation in the walls of ruptured aneurysms compared to unruptured aneurysms. The presence of complement activation in the aneurysm wall further supports the hypothesis that inflammation is involved in aneurysm wall degeneration and rupture⁵².

Neovascularization of the vaso vasorum is believed to be the conduit by which inflammatory cells such as macrophages gain entryway to the walls of diseased arteries³². Aneurysm wall remodeling consists of matrix degeneration by macrophages and other inflammatory cells, and wall regeneration by smooth muscle cells. It is believed that a failure of regeneration is what leads to aneurysm wall thinning and eventual rupture. It has been suggested by other authors that a mechanism for cerebral aneurysm growth and rupture is: 1) the angiogenesis of vaso vasorum in the walls of aneurysms; 2) inflammatory cell infiltration of the aneurysm wall via the vaso vasorum; and 3) degeneration of the extra-cellular matrix of the aneurysm wall by inflammatory cell secretion of proteases resulting in fragility and loss of structural integrity^{19 26 33, 42 43 57}.

In this paper, we show that the presence of inflammatory cells in the media of human cerebral aneurysm walls is directly associated with the presence of capillaries (angiogenesis). We also show that SDF-1 is expressed in human cerebral aneurysms, murine carotid aneurysms, and murine circle of Willis intracranial aneurysms and that aneurysm formation triggers the circulation of progenitor cells expressing CXCR4, the receptor for SDF-1. We demonstrate in vitro that SDF-1 promotes endothelial cell and macrophage migration and promotes endothelial cell tube formation (angiogenesis). We show that blocking SDF-1 inhibits murine aneurysm wall angiogenesis and cell proliferation, and inhibits the formation of murine intracranial aneurysms.

The effect of SDF-1 in promoting angiogenesis has been demonstrated in animal models of myocardial infarction⁸, intracranial tumors²⁵, and Ewing's sarcoma³⁹. Additionally, blockade of SDF-1 has been shown to attenuate tumor growth by inhibiting angiogenesis¹³.

One theory is that SDF-1-mediated angiogenesis is a response to ischemia or arterial injury. Ischemia increases SDF-1 levels which then promote endothelial progenitor cell formation of new blood vessels in ischemic tissue⁶. In arterial injury and repair models, SDF-1/CXCR4 is a key mediator of vascular proliferation in response to injury³⁶. It has been shown that SDF-1 promotes proliferation, recruitment, and incorporation of endothelial-type progenitor cells into newly formed blood vessels^{7, 21, 44, 55}. In vitro studies have demonstrated SDF-1/CXCR4 signaling modifies the capillary-like organization of human embryonic stem cell-derived endothelium⁵. Additionally, SDF-1 induces endothelial cell migration and capillary tube formation³¹. The mechanism for endothelial cell migration is believed to be SDF-1-stimulated JNK3 activity via eNOS-dependent nitrosylation of MKP7 to enhance endothelial migration³⁸. Furthermore, in gene transfer studies of SDF-1, ischemic angiogenesis and angiogenesis is enhanced by SDF-1 expression via a vascular endothelial growth factor/endothelial nitric oxide synthase-related pathway¹⁵.

SDF-1 promotes inflammatory cell migration both directly, and by promoting angiogenesis which provides the capillary network by which inflammatory cells can infiltrate ischemic or injured tissue. Again, response to ischemia or injury seems to be critical. The recruitment of monocytes/macrophages is mediated by SDF-1, which is upregulated at the site of tissue injury. SDF-1 is key in recruitment of monocytes/macrophages and their perivascular retention around new blood vessels that arise from neovascularization¹². SDF-1 mediates proliferation, adhesion, migration, and homing of circulating progenitor cells that express the SDF-1 receptor CXCR4 and monocytes, thereby promoting tissue regeneration^{4, 18, 49}. Further, SDF-1 causes proliferation of CD34+ cells and differentiation of these cells into macrophages and foam cells⁴⁸.

SUMMARY/CONCLUSIONS

The data from this study combined with those discussed from previous studies leads us to believe that arterial injury or ischemia at the site of an aneurysm causes upregulation of SDF-1. SDF-1 is associated with angiogenesis by promoting proliferation, recruitment, migration, and incorporation of endothelial-type progenitor cells into newly formed blood vessels. SDF-1 is also associated with inflammatory cell infiltration of the aneurysm wall by promoting proliferation, recruitment, and migration of monocytes, macrophages, and progenitor cells. It has been suggested by other authors that newly-formed capillaries in the vessel wall are the conduit by which inflammatory cells can infiltrate the aneurysm wall. Future studies are needed to investigate the role of infiltrating inflammatory cells in creating conditions contributing to aneurysm growth and possible rupture.

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Figure 1.

Angiogenesis Occurs in the Walls of Aneurysms. A, Human aneurysms contain endothelial cells (CD31+) and angiogenesis (capillary formation) within the media of the aneurysm walls. Representative section is depicted here at 20×. Scale bar is 200μm. *Red: CD31+; blue: DAPI (4',6-diamidino-2-phenylindole)*. B, Elastase-induced murine carotid aneurysms contain endothelial cells (MECA-32+) and angiogenesis (capillary formation) within the media of the aneurysm walls, but not in normal control murine carotid arteries. Representative section is depicted here at 20×. Scale bar is 200μm. *Green: MECA-32+; blue: DAPI*.

Figure 2. Angiogenesis is Associated with Inflammatory Cell Invasion in the Walls of Aneurysms. Human aneurysms contain abundant monocytes and macrophages (CD68+), and hematopoietic-derived inflammatory cells (CD45+) within the media of the walls of the aneurysms. Representative sections are depicted here at 40 $\times$. Scale bar is 100 μ m. –Red: CD68+ left panel, CD45+ right panel; blue: DAPI.

Figure 3.

SDF-1 is Expressed in Aneurysms. A, Human aneurysms express SDF-1. Representative sections are depicted here at 60 $\times$. Scale bar is 50 μ m. *Red: SDF-1+; blue: DAPI*. B, Elastase-induced murine carotid aneurysms express SDF-1 and its receptor, CXCR4; but normal control murine carotid arteries do not. Representative sections are depicted here at 10 $\times$ and 60 $\times$. Scale bar is 50 μ m for left top and left bottom panels, and 200 μ m for right top and right bottom panels. *Green: SDF-1+ left panel, CXCR4+ right panel; blue: DAPI*. C, Murine intracranial aneurysms express SDF-1, but normal murine circle of Willis do not. Representative sections are depicted here at 40 $\times$ and 60 $\times$. Scale bar is 50 μ m for left panel, and 400 μ m for right panel. *Red: SDF-1+; blue: DAPI*.

Figure 4. Aneurysm Formation Activates Circulation of Progenitor Cells Expressing CXCR4, the Receptor for SDF-1. A, Mice developing carotid aneurysms have increased circulation of

progenitor cells (stem cell antigen-1 positive (SCA-1+)) expressing CXCR4, the receptor for SDF-1, compared to sham-operated mice. When anti-SDF-1 blocking antibody was given to mice, there were significantly less progenitor cells (SCA-1+) expressing CXCR4, than mice given IgG control. B, Mice developing intracranial aneurysms have increased circulation of progenitor cells (SCA-1+) expressing CXCR4, compared to sham-operated mice. C, Murine intracranial aneurysms demonstrate invasion of hematopoietic-derived inflammatory cells (CD45+), whereas none are found in normal circle of Willis. Representative sections are depicted here at 10× and 60×. Scale bar is 100µm for left panel and 400µm for right panel. *Red: CD45+; blue: DAPI.*

Figure 5.

SDF-1 Promotes Angiogenesis (by Promoting Endothelial Cell Migration, Endothelial Cell Proliferation, and Tube Formation) and Inflammatory Cell Migration. A, In transwell cell migration assays, there is significant migration of endothelial cells in chambers containing SDF-1 compared to chambers containing SDF-1 and anti-SDF-1 blocking antibody, or neither. B, In endothelial cell proliferation assays, there was no significant difference between cells exposed to 100 ng/mL SDF-1 compared to control and anti-SDF-1 blocked groups at 24 hrs ($p=0.18$) by ANOVA. However, at 48 hrs, cells exposed to 100 ng/mL SDF-1 showed 31% higher proliferation than both the control ($p=0.0012$) and the anti-SDF-1 blocked group ($p=0.0012$) by ANOVA with Tukey's pairwise comparison. The control and the anti-SDF-1 blocked group were not found to be significantly different at 48 hrs by the same analysis. C, In capillary tube formation assays, there is significantly greater complete loop networks formed by endothelial cells cultured with SDF-1 compared to SDF-1 and anti-SDF-1 blocking antibody, or neither. D, There is significantly greater macrophage migration in chambers containing SDF-1 compared to SDF-1 and anti-SDF-1 blocking antibody, or neither.

Figure 6.

Blocking SDF-1 Inhibits Angiogenesis and Cell Proliferation in the Walls of Aneurysms. A, Carotid aneurysms from mice given anti-SDF-1 blocking antibody have significantly less endothelial cells in the walls of the aneurysms, compared to mice given Ig control. Representative sections are depicted here at 10× and 60×. Scale bar is 400μm for left top panel, and 50μm for right top panel. *Red: MECA-32+; blue: DAPI*. B, Carotid aneurysms from mice given anti-SDF-1 blocking antibody have significantly less number of formed capillaries in the walls of the aneurysms, compared to mice given Ig control. C, Carotid aneurysms from mice given anti-SDF-1 blocking antibody have significantly less cell proliferation, compared to mice given Ig control. Representative sections are depicted here at 20× and 60×. Scale bar is 400μm for left top panel, and 50μm for right top panel. *Red: Ki-67+; blue: DAPI*.

Figure 7. Blocking SDF-1 is Associated with Reduced Intracranial Aneurysm Formation A, C57BL/6 mice given anti-SDF-1 blocking antibody (n=10 mice) developed significantly fewer intracranial aneurysms than C57BL/6 mice given IgG control (n=10): 33% versus 89% respectively ($P<0.05$) (1 mouse from each group died perioperatively due to anesthetic issues). B, Representative example of intracranial aneurysm in mouse given IgG control. Scale bar is 5mm. C, Representative example of no intracranial aneurysm seen in mouse given anti-SDF-1 blocking antibody. Scale bar is 5mm. D, Representative example of ruptured intracranial aneurysm seen in mouse given IgG control. Scale bar is 5mm.

Table 1

Immunostaining of Human Aneurysms and Control Arteries

Location	Size (mm)	Ruptured?	CD31	Capillary	CD68	CD45	SDF1
PCOM	4	N	Y	Y	Y	Y	N
MCA	4	N	Y	N	Y	Y	Y
ICA terminus	5	N	Y	N	N	Y	Y
ACOM	5	Y	N	N	Y	Y	Y
ACOM	6	N	Y	Y	Y	Y	Y
ACOM	6	N	N	N	Y	Y	Y
MCA	7	N	N	N	N	N	N
MCA	7	N	Y	N	N	N	Y
ACOM	7	N	N	N	N	N	N
MCA	7	N	N	N	N	N	Y
ACOM	7	N	Y	Y	N	N	Y
ACOM	7	N	Y	N	Y	Y	Y
ICA terminus	7	N	N	N	N	N	Y
ACA	7	N	Y	Y	Y	Y	N
ACOM	8	N	Y	Y	Y	Y	Y
ICA terminus	8	N	Y	Y	Y	Y	Y
ACOM	8	N	Y	Y	N	N	Y
ACOM	8	N	N	N	N	N	Y
A2-A3	8	N	Y	Y	N	Y	N
MCA	8	N	Y	N	Y	Y	Y
MCA	9	N	Y	N	N	N	Y
MCA	9	N	Y	N	Y	Y	N
MCA	9	N	Y	Y	Y	Y	Y
PCOM	10	N	N	N	Y	Y	Y
ACOM	10	N	N	N	N	N	Y
ACOM	11	N	Y	Y	Y	Y	Y

Location	Size (mm)	Ruptured?	CD31	Capillary	CD68	CD45	SDF1
ACOM	11	N	Y	N	N	N	Y
MCA	11	N	Y	N	Y	Y	N
MCA	11	N	Y	Y	Y	Y	N
MCA	11	N	Y	Y	N	Y	Y
ICA terminus	12	N	N	N	N	N	N
ACOM	13	N	Y	Y	Y	Y	Y
MCA	13	N	Y	N	N	N	Y
MCA	14	N	N	N	N	N	N
MCA	14	Y	Y	Y	Y	Y	Y
MCA	15	Y	Y	N	Y	Y	N
control STA	-	N	N	N	Y	Y	N
control STA	-	N	N	N	Y	Y	N
control STA	-	N	N	N	N	N	N
control STA	-	N	N	N	N	N	N
control STA	-	Y	Y	N	N	N	N
control STA	-	N	N	N	N	N	N
control STA	-	N	Y	Y	N	Y	N
control STA	-	Y	N	N	Y	Y	Y
control STA	-	N	N	N	N	N	N

Table 2

Immunostaining of Murine Aneurysms

Model	Time stained	Treatment	N	Findings
Carotid	3h	none	6	SDF-1+, CXCR4+
Intracranial	3 days	none	5	SDF-1+
Carotid	1 week	none	7	CD45+, CD11b+, Ki67+
Carotid	3 weeks	IgG control	7	CD45++, Cd11b++, MECA-32++, Capillaries++, Ki67+
Carotid	3 weeks	Anti-SDF-1 blocking antibody	7	CD45+/-, CD11b+/-, MECA-32-, Capillaries-, Ki67+

+ = positive; ++ = robust positive expression; +/- = minimal positive expression; - = no expression