Title
Intracranial aneurysms: links among inflammation, hemodynamics and vascular remodeling

Permalink
https://escholarship.org/uc/item/26w1t7bj

Journal
Neurological Research, 28(4)

ISSN
0161-6412

Authors
Yao, Jianhua S
Zhai, Wenwu
Fan, Yongfeng
et al.

Publication Date
2006-06-01

Peer reviewed

Interleukin-6 upregulates expression of KDR and stimulates proliferation of human cerebrovascular smooth muscle cells

Jianhua S Yao[1], Wenwu Zhai[2], Yongfeng Fan[1], Michael T Lawton[3], Nicholas M Barbaro[3], William L Young[1,3,4] and Guo-Yuan Yang[1,3]

[1]Department of Anesthesia and Perioperative Care, Center for Cerebrovascular Research, University of California, San Francisco, California, USA; [2]Comprehensive Cancer Center, University of California, San Francisco, California, USA; [3]Department of Neurological Surgery, University of California, San Francisco, California, USA; [4]Department of Neurology, University of California, San Francisco, California, USA

Interleukin-6 (IL-6) may play multiple roles in angiogenesis and vascular remodeling. Our previous study showed that a promoter polymorphism (174G > C) in IL-6 is associated with brain arteriovenous malformation hemorrhage; tissue expression is related to genotype. In this study, we investigated the effects of IL-6 on human cerebral smooth muscle cells (HCSMCs) and smooth muscle cells isolated from brain arteriovenous malformation surgical specimens (AVM SMCs) and surgical controls (control HCSMCs—from structurally normal temporal lobe taken during surgical treatment of epilepsy patients). We found that IL-6 (1.1 ± 0.27 versus 0.37 ± 0.04 pg/mL, $n = 5$, $P < 0.05$) and endogenous vascular endothelial growth factor (VEGF) receptor II (kinase domain-containing receptor (KDR), 15 ± 3 versus 1.5 ± 3 pg/mL, $n = 5$, $P < 0.05$) were increased in brain AVM SMCs compared with control HCSMCs. Further research revealed that IL-6 could stimulate SMC proliferation, VEGF release, and KDR activation in control HCSMCs. It could also stimulate KDR phosphorylation in control HCSMCs, further confirming a unique role of IL-6 in the triggering of KDR. Interleukin-6 could increase matrix metalloproteinase-9 (MMP-9) secretion through activating KDR in control HCSMCs ($P < 0.05$ versus control). Inhibiting IL-6-induced KDR could reduce MMP-9 activity at least 50% compared with the control group ($P < 0.05$). Increased MMP-9 activity was accompanied by increased control HCSMC proliferation, and blocking MMP-9 activity significantly reduced IL-6-induced control HCSMC proliferation ($P < 0.05$). Collectively, our results show that IL-6 could activate, amplify, and maintain the angiogenic cascade in HCSMCs. A novel role of IL-6 during HCSMC proliferation is upregulating KDR expression and phosphorylation. The results may contribute to the angiogenic phenotype of human brain vascular diseases, such as brain AVM.
Journal of Cerebral Blood Flow & Metabolism (2007) 27, 510–520. doi:10.1038/sj.jcbfm.9600365; published online 5 July 2006

Introduction

Brain arteriovenous malformations (AVMs) are an important cause of hemorrhagic stroke in young adults, but how brain AVMs develop in adults remains poorly understood. Human cerebral smooth muscle cells (HCSMCs), a major cell type in the brain vascular wall, are involved in vascular remodeling and angiogenesis. Proliferation and migration of HCSMCs are the important determinants of brain vascular diseases including AVMs. Many growth factors and cytokines play essential roles during vascular remodeling and angiogenesis, and among these interleukin-6 (IL-6) has received particular attention. Interleukin-6, a multifunctional cytokine, is a critical factor in inducing inflammatory, immunoregulatory, and hematopoietic responses (Akira *et al*, 1993;

Taga and Kishimoto, 1997). It plays an important role in both physiologic and pathologic angiogenesis (Wang and Newman, 2003). Our previous studies showed that IL-6 174C > G polymorphism is associated with brain AVM patients (Pawlikowska *et al*, 2004). Brain AVM patients with IL-6 174GG polymorphism have higher IL-6 protein in both blood samples and surgical tissue (Chen *et al*, 2006). Recent *in vitro* studies show that IL-6 can activate SMC, which has profound effects on the proliferation, migration, and secretion of cytokines (Wang and Newman, 2003). These processes have been most often related to the action of vascular endothelial growth factor (VEGF) (Wang and Newman, 2003).

Vascular endothelial growth factor is the most important growth factor in inducing vascular remodeling and promoting angiogenesis. Expression of VEGF is greatly increased during normal physiologic conditions (e.g., embryo development and wound healing) and abnormal physiologic conditions (e.g., tumor angiogenesis and brain AVMs) (Hashimoto *et al*, 2005). Vascular endothelial growth factor exerts its biologic function through specific membrane receptors: kinase domain-containing receptor (KDR) and the fin-like tyrosine kinase 1 (flt-1) (Plouet and Moukadiri, 1990). On ligand binding, VEGF receptors initiate autophosphorylation and induce tyrosine kinase activity, subsequently stimulating cellular responses (Miao *et al*, 1999). Although KDR and flt-1 are structurally highly similar, their mechanisms in vascular remodeling and angiogenic signal transduction are different: KDR is responsible for mediating the proliferation response to exogenous VEGF (Liu *et al*, 1995), whereas flt-1 is involved in SMC migration (Soker *et al*, 2001). Until now, there is no evidence that VEGF through KDR plays an angiogenic action in HCSMCs.

Smooth muscle cell activation is involved in increased production of matrix metalloproteinase-9 (MMP-9), which is a member of the family of zinc-dependent endopeptidase. It may promote angiogenesis through regulating cell attachment, proliferation, and migration. These actions of MMP-9 could be produced through direct or indirect growth factor release from the extracellular membrane (Jia *et al*, 2000). Growth factors and cytokines could stimulate MMP-9 expression during abnormal angiogenesis (Fabunmi *et al*, 1996). However, the relationship between growth factor release and MMP-9 activation, which may be involved in HCSMC proliferation, is unknown.

In the present study, we investigated VEGF and KDR changes in IL-6-stimulated HCSMCs, and evaluated functional activity of KDR by monitoring its phosphorylation. We further measured MMP-9 activity with IL-6 stimulation. Finally, we explored the role of IL-6 on brain AVM SMC and surgical control HCSMC proliferation.

Materials and methods

Smooth Muscle and Endothelial Cell Culture

The study was IRB-approved and patients gave informed consent. Brain AVM characteristics such as size, location, and venous drainage pattern were documented based on standardized guidelines (Joint Writing Group of the Technology Assessment Committee, 2001). Control cerebral cortex was obtained from patients undergoing surgical treatment of epilepsy as previously described (Table 1) (Hashimoto *et al*, 2004, 2005). Three types of brain cells were used: (1) AVM SMCs—cells isolated from brain AVM surgical specimens ($n = 5$); (2) surgical control SMCs—cells isolated from structurally normal temporal lobe taken during surgical treatment of epilepsy patients ($n = 6$), of which three were a gift from Dr Harry V Vinters of UCLA; these SMCs are also called control HCSMCs because no abnormal phenotype was detected; (3) normal HCSMCs and human cerebral endothelial cells (HCECs) purchased from Cell Systems (St Katharinen, Germany). The SMCs' homogenous nature was confirmed by immunostaining with anti-SM-α-actin, and no significant difference in morphology was found (Chemicon, Temecula, CA, USA; Figure 5A). All experiments were performed on control HCSMC two to five passages. In IL-6 treatment experiments, control HCSMCs were grown in 10% fetal bovine serum, whereas HCECs were grown in ECs growth medium (Clonetics, Walkersville, MD, USA) in 35 mm polystyrene plates to 90% confluent state, and rinsed three times with a serum-free medium before arresting with serum-free medium. The cells were then incubated with IL-6 and other antibodies in specified conditions.

Antibody Cytotoxicity was Determined using Colorimetric Thiazolyl Blue

Antibody cytotoxicity was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Lokeshwar *et al*, 2002). The HCSMCs and HCECs were grown in 96-well microtiter plates with 100 μL of culture medium for 24 h. The cells were treated with IL-6 at increasing concentration in 0.2% bovine serum albumin/Dulbecco's modified Eagle's medium. The plates were incubated for 36 h and the media were replaced with 100 μL fresh Dulbecco's modified Eagle's medium containing 0.2% bovine serum albumin. Next, 2 mg/mL MTT solution was added and the plates were incubated

Table 1 Characterization of patients

CCR no.	Age (years)	Gender	Diagnosis
145	37	M	AVM (left temporal)
237	43	M	AVM (frontal subcortical)
316	73	M	AVM (right parietal)
318	51	F	AVM (right occipital)
115	68	M	AVM (left parietal)
129	46	F	Epilepsy
205	36	F	Epilepsy
211	36	M	Epilepsy

again for 3 h. Finally, MTT-containing medium was aspirated off and 150 μL of dimethyl sulfoxide solution was added. Crystals were solvated by dimethyl sulfoxide and the absorbance was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader (E max, Molecular Devices, Sunnyvale, CA, USA).

HCSMC and HCEC Proliferation Assay

The HCSMCs and HCECs grew to 90% confluence in T-25 flash, and the cells were harvested using 0.025% trypsin. The cells (1×10^4) were seeded onto 96-well gelatin-coated plate overnight, treated with different antibodies for another 24 h, followed by incubation with 5-bromodeoxy-uridine for 2 h (BrdU cell proliferation ELISA kit, Roche, Penzberg, Germany) and measurement of absorbance at 450 nm (reference wavelength 690 nm) using an ELISA reader (E max).

Real-Time Polymerase Chain Reaction

Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using a RETROscript kit (Ambion, Austin, TX, USA). Oligonucleotide primers are as follows: MMP-9 forward: 5'-CCGCAGGGCCCCTTCCTTAT-3'; reverse: 5'-CCGCAGGGCCCCTTCCTTAT-3'; VEGF forward: 5'-TGTGAATGCAGACCAAAGAAAGA-3; reverse: 5'-GCTTTCTCCGCTCTGAGCAA-3'; probe: AGAGCAAGA CAAGAAAATCCCTGTGGGC; Glyseraldehyde-3-phosphate dehydrogenase (GAPDN) forward: 5'-GAAGGTGAAGGT CGGAGTC-3'; reverse: 5'-GAAGATGGTGATGGGATTTC-3'; probe: CAAGCTTCCCGTTCTCAGCC. Real-time polymerase chain reaction (RT-PCR) was determined using 2.5 μL (5 ng) cDNA, 12.5 μL SYBR Green PCR Master Mix ($2 \times$) for MMP-9, or Taqman PCR Master Mix ($2 \times$) for VEGF, 2.5 μL primer pair mix (5 pmol each primer), and water to a 25 μL final volume (ABI-prism 7000 sequence detector, Foster City, CA, USA). Thermocycler conditions consisted of an initial holding at 50°C for 2 mins and then 95°C for 3 mins. This was followed by 94°C for 15 secs, 56°C for 45 secs, and 72°C for 30 secs for 45 cycles for MMP-9. For VEGF, thermocycler conditions consisted of an initial holding at 50°C for 2 mins and then 95°C for 10 mins. This was followed by a two-step TaqMan PCR program consisting of 95°C for 15 secs and 60°C for 60 secs for 35 to 40 cycles. Results were analyzed using ABI sequence detection software, and MMP-9 and VEGF mRNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (18S rRNA endogenous control). The RT-PCR products were verified on agarose gels.

Western Blot Analysis

After harvesting, HCSMCs were homogenized in a lysis buffer. The protein concentration was determined using a Bio-Rad system. An equal amount of protein (70 μg) was loaded on 4% to 12% gradient gel (Invitrogen) for electrophoresis of protein. Subsequently, protein was electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad, Richmond, CA, USA) in a transfer buffer. The membrane was placed in 5% nonfat milk TBS with 0.1% Tween 20 for 1 h to block nonspecific binding, and was then immunoprobed overnight with anti-VEGF antibody (1:200 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-KDR antibody (1:200 dilution; Santa Cruz), or anti-flt-1 antibody (1:300 dilution; Santa Cruz). After washing, the membrane was incubated with horse-radish peroxidase (HSP)-conjugated anti-rabbit antibody or anti-mouse antibodies (Amersham). Finally, the membrane was incubated with an enhanced ECL (Amersham), and exposed to Kodak film. Protein bands were scanned and semiquantified by densitometry using NIH 1.63 image analysis software.

Phospho-KDR Expression

Phospho-KDR expression was semiquantified using Western blot analysis. The HCSMCs grew to approximately 80% confluent, and made quiescence with serum-free medium for 24 h. Then, IL-6 (100 ng/mL) was added and plates were incubated for 5 to 45 mins. The cells were washed with phosphate-buffered saline and scraped into a lysis buffer. Phospho-KDR was detected by incubating membranes overnight with rabbit polyclonal phospho-KDR antibody (1:100 dilution; Santa Cruz); secondary horseradish peroxidase-labeled anti-rabbit antibody (1:2,000 dilution; Amersham) was added for 1 h.

Gelatin Zymography

Zymography was performed as we have previously described (Hashimoto *et al*, 2003). A 70 μg sample protein was separated under nonreducing conditions in a 10% zymogram gel (Invitrogen) containing 0.1% gelatin as a substrate. After electrophoresis, gels were washed and incubated in developing buffer overnight at 37°C. The gels were then stained with 0.5% Coomassie blue R-250 and destained. The activity of MMP can be detected as white bands of lysis against the Coomassie blue-stained gel. Protein activity bands in zymography were quantitated through scanning densitometry.

Immunohistochemistry

The cell sections were fixed using 4% paraformaldehyde and quenched using 0.3% H_2O_2 in methanol for 30 mins. After washing, sections were incubated with primary antibodies: mouse anti-SM-α-actin (1:200 dilution; Chemicon, Temecula, CA, USA), mouse anti-human CD-31 (1:1,000 dilution; DAKO, Carpinteria, CA, USA), mouse anti-human-glial fibrillary acidic protein (1:500 dilution; Sigma, St. Louis, MO, USA), mouse anti-human flt-1 (1:500 dilution; Santa Cruz), and mouse anti-human-KDR (1:200 dilution; Santa Cruz). After incubating at 4°C overnight and washing in phosphate-buffered saline, the sections were incubated with biotinylated goat anti-mouse (Vector Laboratories, Burlingame, CA, USA) diluted

1:5,000 for 1 h at room temperature. The sections were treated with the ABC streptavidin detection system (Vector Laboratories) for 1 h. The resulting horseradish peroxidase signal was detected using 3,3′-diaminobenzidene. For fluorescent staining, after incubating overnight at 4°C with primary antibodies, sections were incubated with Alexa Fluor 594-conjugated goat anti-mouse or Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) at 1:500 dilution for 1 h at room temperature. The sections were then mounted and photographed with a fluorescent microscope. Negative controls were performed by omitting the primary antibodies.

Enzyme-Linked Immunosorbent Assays

Interleukin-6 expression from human brain AVM SMCs were measured using an ELISA kit according to the protocols provided by the manufacturer (R&D Systems Inc., Minneapolis, MN, USA). Cultured brain AVM SMCs were maintained at 37°C in 5% CO_2/95% ambient mixed air, and the culture media were changed every 2 days. The same amount of protein was examined for each sample. All experiments were performed on brain AVM SMC two to five passages.

Statistical Analysis

Brain AVM SMCs were compared with controls by analysis of variance. Results of the dose-dependent proliferation and inhibition experiments were analyzed using an analysis of variance with *post hoc* multiple comparison test. All data are represented as mean ± standard deviation (mean ± s.d.). A random probability value less than 0.05 is considered statistically significant for the comparisons.

Results

IL-6 Upregulation Stimulates VEGF Expression in Control HCSMCs

To investigate the effects of IL-6 on control HCSMCs, we exposed HCSMCs to IL-6 for 24 h. Interleukin-6 at 50 ng/mL (2 nmol/L) could stimulate the increase of VEGF protein in a dose-dependent manner ($P < 0.05$, IL-6 versus control; Figures 1A and 1B). Consistent with the protein expression, IL-6 could also stimulate VEGF mRNA expression in a dose-dependent manner ($P < 0.05$; Figure 1C). To
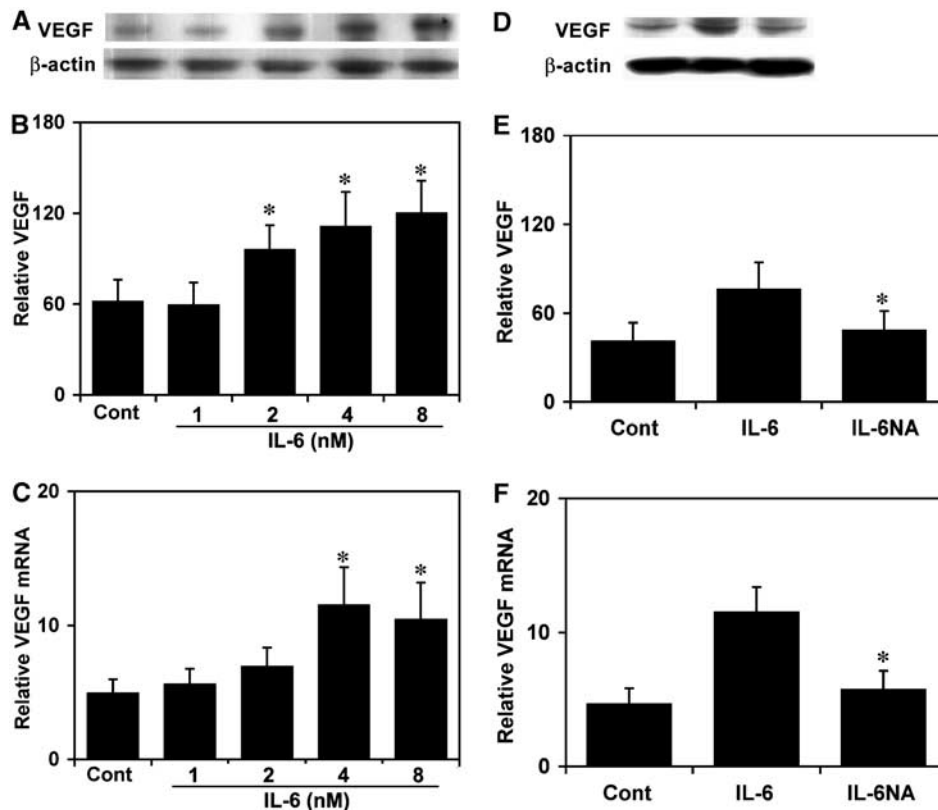

Figure 1 Interleukin-6 upregulates VEGF expression in control HCSMCs. (**A**) Photograph represents one experiment of Western blotting, which indicates VEGF expression after IL-6 (1 to 8 nmol/L) stimulation at the indicated dose for 24 h. (**B**) Bar graph shows semiquantified densitometry from Western blot analysis. (**C**) Bar graph shows IL-6-induced VEGF mRNA expression using RT-PCR. Data are mean ± s.d. ($n = 3$). *$P < 0.05$, versus control. The results show that IL-6 upregulates VEGF levels in a dose-dependent manner. (**D**) Photograph shows VEGF expression after being neutralized with IL-6 antibody in Western blot analysis. Bar graph shows semiquantitative VEGF protein (**E**) and VEGF mRNA (**F**) expression after being neutralized with anti-IL-6 antibody. Data are mean ± s.d. ($n = 3$). IL-6NA means IL-6 plus anti-IL-6 antibody. *$P < 0.05$, IL-6 versus IL-6 neutralizing. The results show that anti-IL-6 antibody completely abolishes IL-6 function.

determine if increased VEGF expression was because of IL-6 stimulation, we neutralized IL-6 with concentrations equivalent to 100 ng/mL of IL-6 in cultured HCSMCs. As anticipated, neutralizing IL-6 could abolish VEGF expression in both protein and mRNA levels (Figures 1D–1F), whereas the same concentration of nonimmune goat IgG could not reduce VEGF expression (data not shown). MTT assay shows no detectable cytotoxicity of tested antibodies in the experiment, indicating that the changes in cellular events were a result of neither nonspecific IgG nor cytotoxicity.

IL-6 Stimulates KDR Expression and Phosphorylation in HCSMCs

To explore possible signal transduction pathway for VEGF, we examined VEGF receptors flt-1 and KDR expression in control HCSMCs. There was no statistical significance in flt-1 expression between the control and the treated groups after IL-6 stimulation (Figure 2A). However, KDR expression was upregulated in a dose-dependent manner in control HCSMCs ($P < 0.05$; Figures 2A and 2B). The parallel result was obtained in immunofluorescent staining (Figure 2C). Prolonged exposure to IL-6 produced no further enhancement of KDR (data not shown). Treatment with human VEGF of 20 ng/mL for 24 h could upregulate KDR in normal HCECs but not in control HCSMCs; interestingly, IL-6 could upregulate KDR expression in both normal HCECs and control HCSMCs (Figure 2D). To ascertain that KDR expression was not because of an increase of endogenous VEGF induced by IL-6, we used anti-VEGF antibody to block endogenous VEGF. We found that blocking endogenous VEGF failed to abolish IL-6-induced KDR expression (Figures 2E
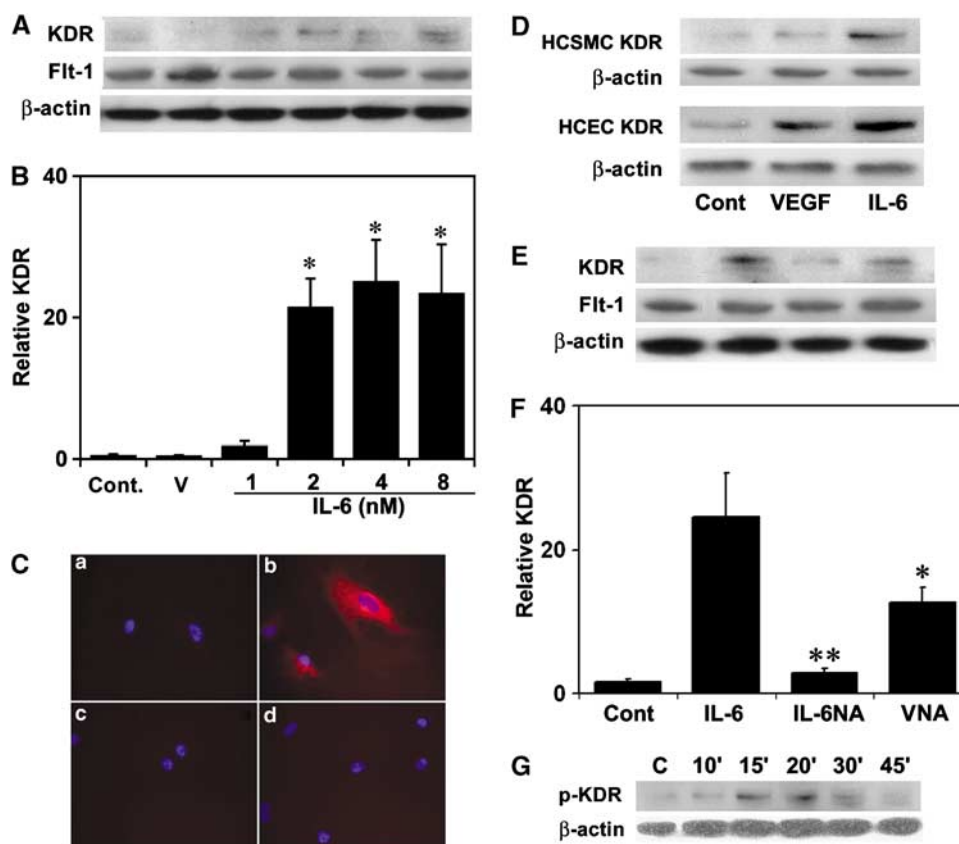

Figure 2 Interleukin-6 upregulates KDR expression and phosphorylation in control HCSMCs. (**A**) Photomicrographs show Western blots of KDR and flt-1 expression after 24 h of IL-6 stimulation at indicated doses. (**B**) Bar graph shows semiquantified densitometry of KDR from Western blot analysis. Cont. = control, V = VEGF (20 ng/mL). Data are mean ± s.d. ($n = 3$). *$P < 0.05$ versus control. (**C**) Photomicrographs show KDR immunofluorescent staining. (a) Negative control shows no KDR staining, blue color is cell nuclear staining (PI); (b) red color shows KDR-positive staining in HCSMCs treated with IL-6 (100 ng/mL); (c) control HCSMCs without any treatment show no KDR staining; (d) HCSMCs treated with VEGF (20 ng/mL) show no KDR staining. (**D**) Photomicrographs show KDR expression in HCSMCs and HCECs treated with IL-6 and VEGF, indicating that IL-6 upregulates KDR in both HCSMCs and HCECs; however, VEGF upregulates KDR in only HCECs but not HCSMCs. (**E**) Photomicrographs show KDR expression in HCSMCs treated with control, IL-6 100 ng/mL, IL-6 plus IL-6 antibody-neutralized (IL-6NA), and IL-6 plus VEGF antibody-neutralized (VNA). (**F**) Bar graph shows semiquantified densitometry of KDR. Data are mean ± s.d. ($n = 3$). *$P < 0.05$ IL-6 versus IL-6 or VEGF antibody-neutralized groups. (**G**) Phospho-KDR was detected by using Western blot analysis after treating cells with IL-6 (100 ng/mL) for the time indicated.

and 2F); furthermore, nonimmune goat IgG (2 μg/mL) did not influence KDR expression (data not shown). Taken together, these data indicate that IL-6 upregulating KDR was independent of VEGF. To find out if IL-6 upregulating KDR expression was functional, phospho-KDR was examined in the cell extracts from non-IL-6-stimulated or IL-6-stimulated control HCSMCs. The results showed that IL-6 retained the ability to activate KDR, which peaked after 15 mins of IL-6 stimulation (Figure 2G).

IL-6 Enhances MMP-9 Expression

To examine whether IL-6 enhances MMPs expression, we measured MMP-2 and -9 mRNA level in control HCSMC culture. Interleukin-6 did not increase MMP-2 mRNA expression (data not shown), but it greatly increased MMP-9 mRNA in control HCSMCs ($P < 0.05$; Figure 3C). Because gelatin MMPs are secreted as inactive zymoyens, it has to be activated by cleavage of N-terminal sequences to achieve its proteolytic activity. We therefore performed zymographic analyses in the

serum-free HCSMC cultured supernatants. Our results indicate that IL-6 did not change MMP-2 activity; however, it increased MMP-9 activity in control HCSMCs ($P < 0.05$; Figures 3A and 3B).

To determine the relationship among IL-6, VEGF, KDR, and MMP-9 activity, neutralizing antibodies against IL-6, VEGF, and KDR were used in the IL-6-treated HCSMCs. The result clearly indicated that anti-IL-6 antibody effectively reversed MMP-9 overexpression; however, inhibiting KDR and VEGF only partially reduced ($\approx 50\%$) IL-6 effect in both protein and mRNA levels, suggesting a direct role of IL-6 on MMP-9 activity (Figures 3C, 3D, and 3F). No changes were detected in the control cells treated with goat or mouse IgG at the same concentration, and additional administration of these inactivated antibodies failed to induce MMP-9 inhibition (data not shown).

IL-6 Stimulation Causes HCSMC Proliferation Through Upregulation of KDR

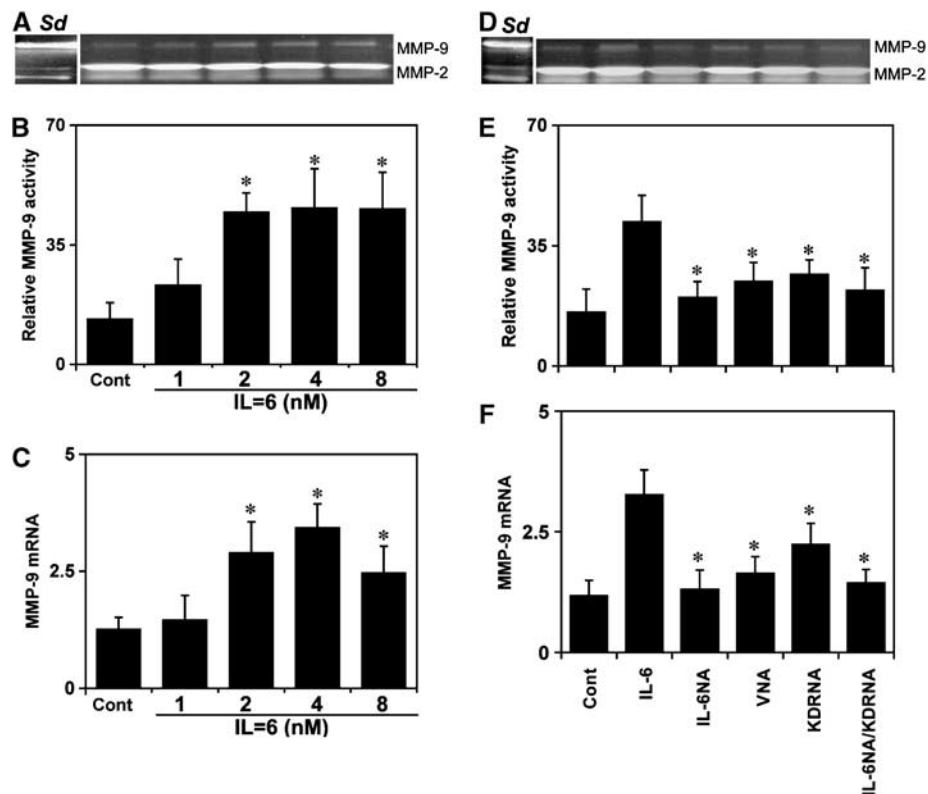The effect of IL-6 on HCSMC proliferation showed that IL-6 at 100 ng/mL could nearly double

Figure 3 Interleukin-6 enhances MMP-9 expression and activity. (**A**) Photograph shows MMP-9 activity in control HCSMCs after 24 h of IL-6 treatment at indicated doses. Sd = standard marker. (**B**) Bar graph shows semiquantified densitometry of MMP-9 activity from zymogram. Cont = control group. Data are mean ± s.d. ($n = 4$); *$P < 0.05$ versus control HCSMCs group. (**C**) Bar graph shows IL-6-induced MMP-9 mRNA expression in HCSMCs using RT-PCR. Data are mean ± s.d. ($n = 4$); *$P < 0.05$ versus control. (**D**) Photograph shows MMP-9 activity in control, IL-6-treated HCSMCs (100 ng/mL), IL-6 plus IL-6 antibody-neutralized (IL-6NA), IL-6 plus VEGF anti-VEGF antibody 10 μg/mL (VEGFNA), IL-6 plus anti-KDR antibody 5 μg/mL (KDRNA), and IL-6 plus IL-6 and KDR antibodies. Supernatants were used for gelatin zymographic analysis. (**E**) Bar graph shows semiquantified densitometry of MMP-9 activity from zymogram. Cont = control group. Data are mean ± s.d. ($n = 4$); *$P < 0.05$ IL-6-treated group versus antibody-neutralized groups. (**F**) Bar graph shows MMP-9 mRNA expression in control HCSMCs treated with IL-6, IL-6NA, VEGFNA, KDRNA, and IL-6NA plus KDRNA using RT-PCR. Data are mean ± s.d. ($n = 4$); *$P < 0.05$ IL-6 versus antibody-neutralized groups.
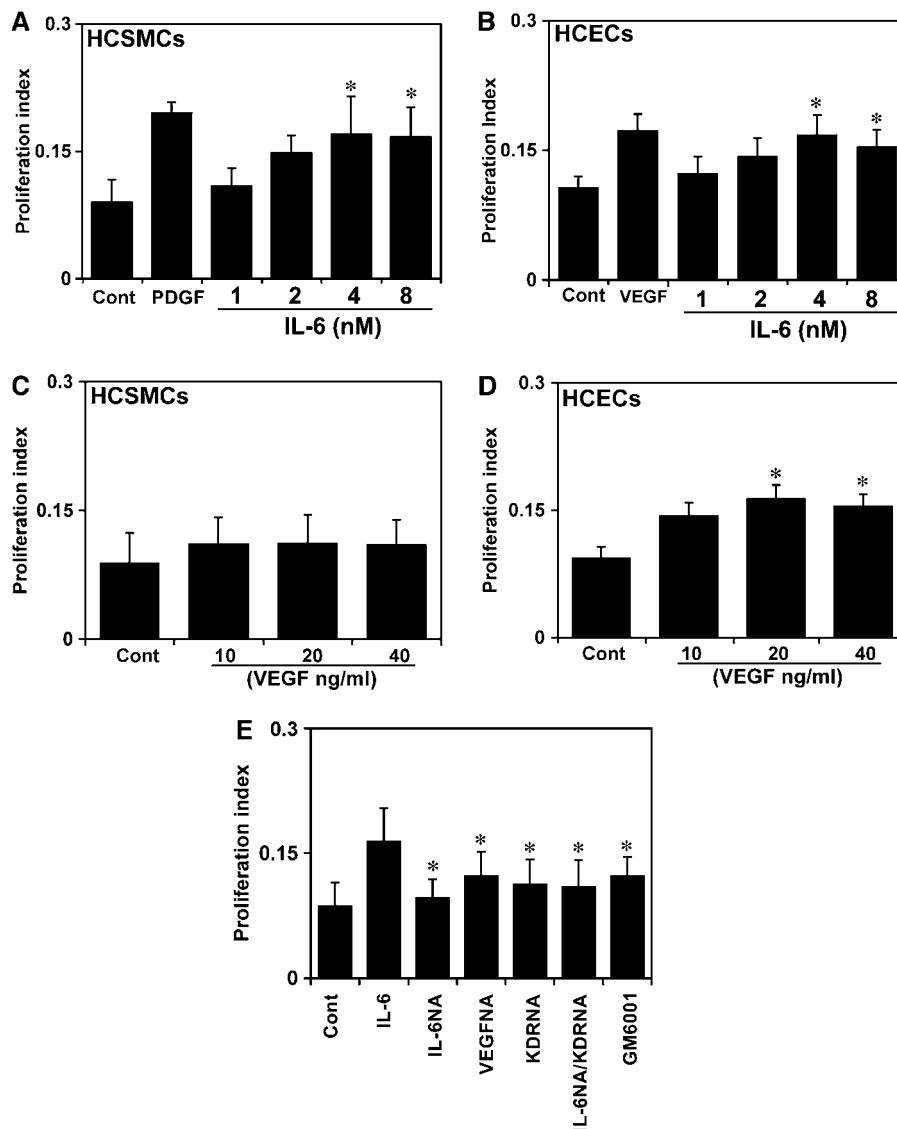
Figure 4 Interleukin-6 stimulation causes control HCSMC proliferation through upregulating KDR. Bar graphs show that IL-6 stimulates both HCSMC (**A**) and HCEC (**B**) proliferation in a dose-dependent manner. Vascular endothelial growth factor does not stimulate HCSMC proliferation at effective doses (**C**) whereas it stimulates HCEC proliferation in a dose-dependent manner (**D**). (**E**) Bar graph shows the HCSMC proliferation in HCSMCs treated with IL-6, IL-6NA, VEGFNA, KDRNA, IL-6NA plus KDRNA, and GM6001 (10 ng). Data are mean $\pm$ s.d. ($n = 4$); *$P < 0.05$, IL-6 versus antibody-neutralized and GM6001 groups.

proliferation rates of control HCSMCs. This effect is comparable with the effect of platelet-derived growth factor, which is a well-known cell proliferation stimulator (Figure 4A). A similar result was obtained when HCECs were stimulated by IL-6 (Figure 4B). Interestingly, VEGF failed to induce HCSMC proliferation but could stimulate HCEC proliferation (Figures 4C and 4D). Anti-VEGF and anti-KDR antibodies, in part, decreased IL-6-mediated HCSMC proliferation, which suggests that KDR activation is a downstream event in IL-6-induced proliferation. Furthermore, the increased proliferating cells were partially inhibited by MMP inhibitor GM6001, supporting a role of MMP-9 in the HCSMC proliferation process (Figure 4E) (McCawley and Matrisian, 2001).

Increase of IL-6 Expression in the Brain AVM Tissue

Here, we investigated if basic IL-6 level is higher in the AVM SMCs. We first confirmed that the isolated cells from human brain AVM tissue were SMCs (Figure 5A). An ELISA assay then showed that IL-6 level in AVM SMCs was three times higher than in control HCMSCs ($P < 0.05$; Figure 5B).

KDR Expression in AVM SMCs

We then examined whether VEGF receptors flt-1 and KDR expression exists in brain AVM SMCs. Immunostaining showed that all HCECs, control HCSMCs, and AVM SMCs express flt-1 in the
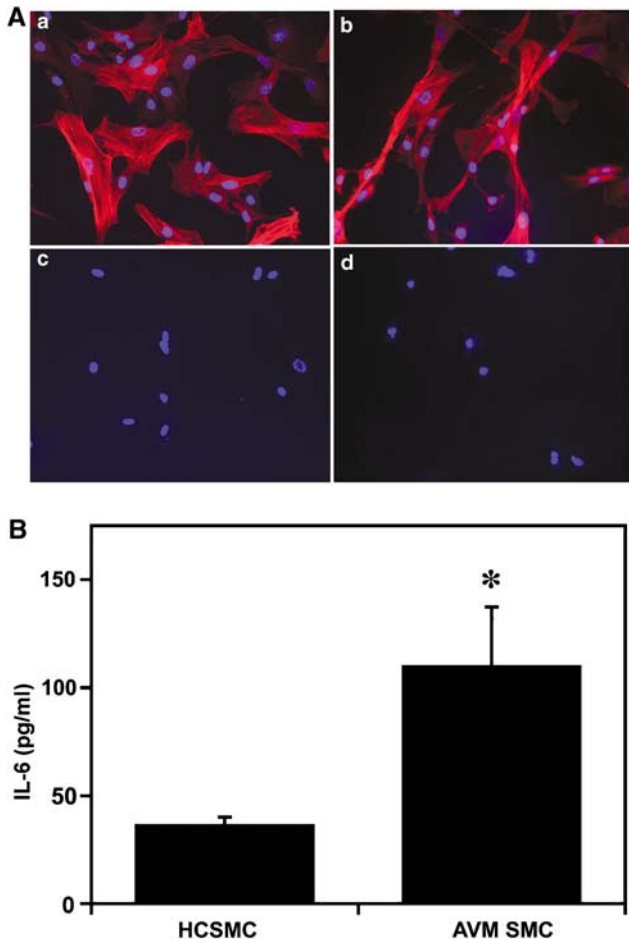
Figure 5 Increase of IL-6 in AVM SMC culture. (**A**) Photomicrographs show a representative of AVM SMC and HCSMC immunostaining. Both control HCSMCs (a) and AVM SMCs (b) show strong α-SM-actin-positive staining. The AVM SMCs show CD-31-negative staining (c), indicating that these cells are SMCs but not ECs. Negative control shows no staining (d). (**B**) Interleukin-6 protein levels in the AVM SMCs and control HCSMCs were determined by an ELISA assay. $N = 5$ per group. *$P < 0.05$, AVM SMCs versus control HCSMCs.

normal condition, and that there are no significant differences among cell sources. We showed that KDR-positive staining was detected in the HCEC and AVM SMCs; however, control HCSMCs showed negative KDR staining (Figure 6A). Semiquantitative Western blot result showed a similar result; KDR level in AVM SMCs was 10 times higher than that in control HCSMCs ($P < 0.05$; Figure 6B).

Discussion

In the present study, we revealed for the first time that IL-6 could trigger cultured HCSMC proliferation induced by increased endogenous VEGF and KDR in both mRNA and protein levels. This effect was completely abrogated by IL-6 antibody neutralizing

in HCSMCs, which verified the direct trigger effect of IL-6. Exogenous VEGF-treated HCSMC failed to activate KDR, suggesting a specific role of IL-6 in the upregulation of KDR. Interleukin-6 stimulating KDR phosphorylation further supported the role of IL-6 in upregulating KDR. In addition, the role of KDR was identified through IL-6 activating MMP-9. Activating MMP-9 was accompanied by increased HCSMC proliferation, which implies that MMP-9 plays a crucial function in HCSMC proliferation. Finally, we showed that AVM SMCs have higher IL-6 and KDR levels compared with control HCSMCs, indicating that increased IL-6 and KDR are involved in brain AVM development. It is unclear if increased IL-6 is the cause or the effect. However, our genomic study shows that GG genotype of the IL-6−174G > C promoter polymorphism is associated with clinical presentation of brain AVM patients (Chen *et al*, 2006), which suggests that IL-6 likely plays a role in the development of brain AVM. Our results provided strong evidence that IL-6 could trigger, amplify, and maintain HCSMC proliferation, which may be a major contribution to IL-6-regulated vascular remodeling and induced angiogenesis. Interleukin-6 is a potent preinflammatory cytokine in normal and neoplastic angiogenesis, and has been implicated in all stages of vascular diseases (Campbell *et al*, 1993). Its overexpression in the central nervous system is correlated to pronounced vasculization *in vivo* (Campbell *et al*, 1993). Interleukin-6 polymorphism is closely related to vascular diseases such as abdominal aortic aneurysms and brain AVMs (Jones *et al*, 2001; Pawlikowska *et al*, 2004). Increased IL-6 is beneficial to giant-cell arthritis patients, indicating that IL-6 activates a functional program related to angiogenesis (Hernandez-Rodriguez *et al*, 2003). It also increases in patients after stroke, which may reflect these patients' changing inflammatory-angiogenesis status (Heep *et al*, 2003). Monocyte-derived macrophage, T cells, ECs, and cardiac myocytes could synthesize IL-6 (Ikeda *et al*, 1992). We found that IL-6 expression is increased in AVM SMCs, an indication that both ECs and SMCs play a role in triggering pathologic angiogenesis in the brain. The IL-6 level we measured in the SMC culture medium is around 30 to 110 pg/10^5 SMCs in 1 mL culture medium. The result is similar to our previous study from AVM tissue samples (Chen *et al*, 2006). The concentration of IL-6 we chose for the stimulation study (25 to 200 ng/mL) is based on our previous research (Yao *et al*, 2006) and that of other groups (Huang *et al*, 2004; Loeffler *et al*, 2005). We did not observe VEGF and KDR expression in the low IL-6 level (less than 25 ng/mL). We believe that a higher dose of IL-6 appears necessary because only by maintaining a higher level of IL-6 within a short period of time can the therapeutic level of IL-6 be reached.

Interleukin-6 may activate signal transducers and activators of transcription 3 signal pathway by binding to gp130 subunit, which then transduces
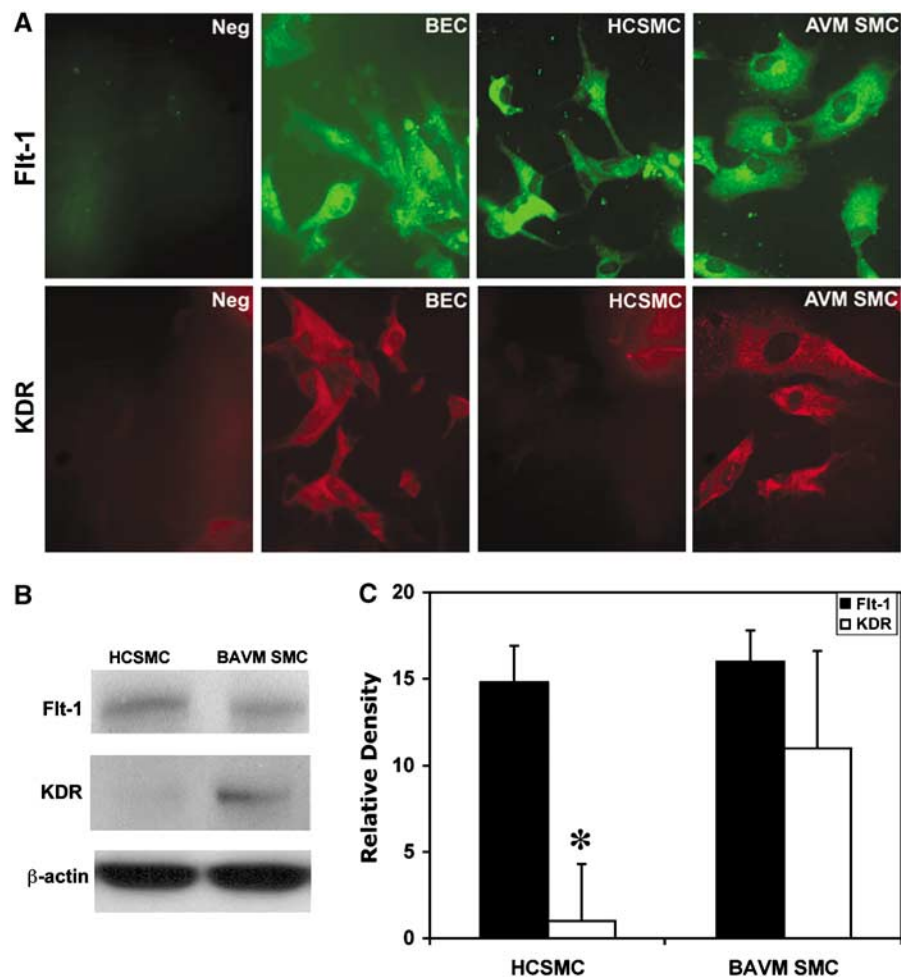
Figure 6 The expression of KDR in AVM SMCs. (**A**) Photomicrographs show VEGF receptors expression in the AVM SMCs and control HCSMCs. Green color shows flt-1 expression; brain ECs, control HCSMCs, and AVM SMCs have strong green fluorescent positive staining, indicating that flt-1 is expressed in these cell types. Red color shows KDR expression; brain endothelial cells (HCECs) and AVM SMCs have strong red fluorescent staining. However, control HCSMCs show negative staining, indicating that HCSMCs have undetectable KDR in the normal condition. (**B**) Image represents one experiment of Western blotting. The upper band shows flt-1 expressed in both AVM SMCs and control HCSMCs. The middle band shows KDR expressed in AVM SMCs but not in control HCSMCs. (**C**) Bar graph shows semiquantitative flt-1 and KDR expression in AVM SMCs and control HCSMCs. $N = 5$ per group; $P < 0.05$, AVM SMCs versus control HCSMCs.

intracellular signals and produces various biologic functions (Kishimoto *et al*, 1995). This signal pathway widely exists in HCECs and HCSMCs, and mediates pathogenesis of vasculization (Seino *et al*, 1994).

Involvement of IL-6 in angiogenesis is associated with upregulation of VEGF in many cell types (Cohen *et al*, 1996). Vascular endothelial growth factor, an essential angiogenic growth factor, plays a crucial role in promoting angiogenesis in HCSMCs. We showed that IL-6 could greatly upregulate VEGF in both transcription and protein levels. Increased VEGF is related to the increase of HCSMC proliferation and migration. Our data provide additional evidence that IL-6 acts as an indirect regulator in HCSMC angiogenic changes. The most important finding in our study is that IL-6 not only induces VEGF release but also directly upregulates KDR. The

neutralizing antibody against endogenous VEGF only partially downregulates KDR, indicating that activating KDR is not because of endogenous VEGF. Furthermore, treating HCSMCs with VEGF alone cannot upregulate KDR, presumably because a very low level of KDR in HCSMCs may not interact with exogenous VEGF. We found that IL-6 treatment does not change flt-1 expression in HCSMCs, and neutralizing flt-1 does not influence IL-6-induced HCSMC proliferation. This result implies that IL-6 promoting HCSMC proliferation is through activation of KDR but not flt-1.

Matrix metalloproteinase-9 plays an important role in local microenvironments during embryonic development and growth, as well as in tissue remodeling processes (Mason *et al*, 1999). Our data showed the role of KDR in upregulating MMP-9 release. Many reports have established that MMP-9

influences HCSMC migration but few have shown that MMP-9 affects HCSMC proliferation. Inhibiting KDR with the specific KDR inhibitor SU5416 decreases HCEC secreting MMP-9 (Wagner et al, 2003). This result strongly supports our similar finding in HCSMCs. Activated KDR is associated with signaling intermediates such as phospholipase C-1γ, GTPase-activating protein, Nck-Grb2, and Shc-Grb2 (Thakker et al, 1999). These downstream events of KDR phosphorylation could contribute to MMP gene regulation. Endothelial cell proliferation is restricted when MMP-9 is absent, whereas EC proliferation is greatly increased when functional MMP-9 is added (Werb, 1997). These results match our finding that upregulating MMP-9 is accompanied by an increased HCSMC proliferation. The mechanism of this process is unknown. One potential explanation could be that MMP-9 stimulates matrix-bound cytokines and growth factors, which act in a paracrine manner to influence cell growth (Bergers et al, 2000; Imai et al, 1997). There are convincing data that these growth factors are necessary for cell proliferation. Another explanation may be that MMP-9 directly cleaves active growth factors. Matrix metalloproteinase-9 directly processes transforming growth factor-β and IL-1β into an active ligand (Schonbeck et al, 1998; Yu and Stamenkovic, 2000), and enables them to diffuse more widely, thereby inducing cell proliferation. The final explanation is that MMP-9 may disrupt cell-to-cell interaction in HCSMC culture. This process may relate to expression of cadherin and β-catenin (Novak and Dedhar, 1999). Neutralizing endogenous VEGF and KDR partially inhibits MMP-9 expression, suggesting that upregulating MMP-9 by IL-6 is not involved in KDR signal pathway. Interleukin-6 could induce angiogenic molecules that play a role in the release of MMP and in stimulating HCSMC proliferation. Therefore, this would explain why blockage of VEGF and KDR, in part, inhibits HCSMC proliferation. Current evidence is clear that IL-6, as one of the principal angiogenic molecules, acts both as a direct and an indirect stimulator of HCSMC proliferation.

The sample size in our study is relatively small, and the results might not be representative of the whole population of brain AVM patients. This would be a limitation if we found no effect, as there might be many confounding factors that could influence the range of values. A false negative would be highly suspect. However, unlike complicated clinical or experimental in vivo studies, our in vitro study was on one type of HCSMC only, and there was a rather robust and uniform difference from control, which suggests a general effect. Therefore, it seems unlikely that we are dealing with a type I false positive effect. Nevertheless, we found a significant difference in IL-6 expression between AVM and controls. In future studies, a large sample size will have to be considered.

In summary, our findings provide evidence that IL-6 stimulating HCSMC proliferation depends on upregulation of KDR. The activation of KDR promotes MMP-9 release from HCSMCs. Interleukin-6 triggering MMP-9 production may have broad implications for brain vascular generation. All our data suggest that IL-6 can activate, amplify, and maintain the angiogenic cascade in HCSMCs. We further showed that AVM SMCs have higher IL-6 and KDR compared with control HCSMCs. As AVM has been considered a continuously progressive angiogenic change and vascular remodeling, and SMCs are one of the main components of the vascular wall, our results provide novel evidence that IL-6 as a chemoattractant can stimulate HCSMC proliferation by upregulation of KDR. This process may be relevant to AVM development in adults.

References

Akira S, Taga T, Kishimoto T (1993) Interleukin-6 in biology and medicine. Adv Immunol 54:1–78

Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, Tanzawa K, Thorpe P, Itohara S, Werb Z, Hanahan D (2000) Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol 2:737–44

Campbell IL, Abraham CR, Masliah E, Kemper P, Inglis JD, Oldstone MB, Mucke L (1993) Neurologic disease induced in transgenic mice by cerebral overexpression of interleukin 6. Proc Natl Acad Sci USA 90:10061–5

Chen Y, Pawlikowska L, Yao JS, Shen F, Zhai W, Achrol AS, Lawton MT, Kwok P-Y, Yang G-Y, Young WL (2006) Interleukin-6 involvement in brain arteriovenous malformations. Ann Neurol 59:72–80

Cohen T, Nahari D, Cerem LW, Neufeld G, Levi BZ (1996) Interleukin 6 induces the expression of vascular endothelial growth factor. J Biol Chem 271:736–41

Fabunmi RP, Baker AH, Murray EJ, Booth RF, Newby AC (1996) Divergent regulation by growth factors and cytokines of 95 kDa and 72 kDa gelatinases and tissue inhibitors or metalloproteinases-1, -2, and -3 in rabbit aortic smooth muscle cells. Biochem J 315(Part 1): 335–42

Hashimoto T, Lawton MT, Wen G, Yang G-Y, Chaly Jr T, Stewart CL, Dressman HK, Barbaro NM, Marchuk DA, Young WL (2004) Gene microarray analysis of human brain arteriovenous malformations. Neurosurgery 54: 410–25

Hashimoto T, Wen G, Lawton MT, Boudreau NJ, Bollen AW, Yang GY, Barbaro NM, Higashida RT, Dowd CF, Halbach VV, Young WL (2003) Abnormal expression of matrix metalloproteinases and tissue inhibitors of

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metalloproteinases in brain arteriovenous malformations. *Stroke* 34:925–31

Hashimoto T, Wu Y, Lawton MT, Yang G-Y, Barbaro NM, Young WL (2005) Co-expression of angiogenic factors in brain arteriovenous malformations. *Neurosurgery* 56:1058–65; discussion 1058–1065

Heep A, Behrendt D, Nitsch P, Fimmers R, Bartmann P, Dembinski J (2003) Increased serum levels of interleukin 6 are associated with severe intraventricular haemorrhage in extremely premature infants. *Arch Dis Child Fetal Neonatal Ed* 88:F501–4

Hernandez-Rodriguez J, Segarra M, Vilardell C, Sanchez M, Garcia-Martinez A, Esteban MJ, Grau JM, Urbano-Marquez A, Colomer D, Kleinman HK, Cid MC (2003) Elevated production of interleukin-6 is associated with a lowerincidenceof disease-related ischemic events in patients with giant-cell arteritis: angiogenic activity of interleukin-6 asa potential protective mechanism. *Circulation* 107:2428–34

Huang SP, Wu MS, Shun CT, Wang HP, Lin MT, Kuo ML, Lin JT (2004) Interleukin-6 increases vascular endothelial growth factor and angiogenesis in gastric carcinoma. *J Biomed Sci* 11:517–27

Ikeda U, Ohkawa F, Seino Y, Yamamoto K, Hidaka Y, Kasahara T, Kawai T, Shimada K (1992) Serum interleukin 6 levels become elevated in acute myocardial infarction. *J Mol Cell Cardiol* 24:579–84

Imai K, Hiramatsu A, Fukushima D, Pierschbacher MD, Okada Y (1997) Degradation of decorin by matrix metalloproteinases: identification of the cleavage sites, kinetic analyses and transforming growth factor-beta1 release. *Biochem J* 322(Part 3):809–14

Jia MC, Schwartz MA, Sang QA (2000) Suppression of human microvascular endothelial cell invasion and morphogenesis with synthetic matrixin inhibitors. Targeting angiogenesis with MMP inhibitors. *Adv Exp Med Biol* 476:181–94

Joint Writing Group of the Technology Assessment Committee ASoIaTN, Joint Section on Cerebrovascular Neurosurgery, a section of American Association of Neurological Surgeons and Congress of Neurological Surgeons, and Section of Stroke and the Section of Interventional Neurology of the American Academy of Neurology (2001) Reporting terminology for brain arteriovenous malformation clinical and radiographic features for use in clinical trials. *Stroke* 32:1430–42

Jones KG, Brull DJ, Brown LC, Sian M, Greenhalgh RM, Humphries SE, Powell JT (2001) Interleukin-6 (IL-6) and the prognosis of abdominal aortic aneurysms. *Circulation* 103:2260–5

Kishimoto T, Akira S, Narazaki M, Taga T (1995) Interleukin-6 family of cytokines and gp130. *Blood* 86:1243–54

Liu B, Earl HM, Baban D, Shoaibi M, Fabra A, Kerr DJ, Seymour LW (1995) Melanoma cell lines express VEGF receptor KDR and respond to exogenously added VEGF. *Biochem Biophys Res Commun* 217:721–7

Loeffler S, Fayard B, Weis J, Weissenberger J (2005) Interleukin-6 induces transcriptional activation of vascular endothelial growth factor (VEGF) in astrocytes *in vivo* and regulates VEGF promoter activity in glioblastoma cells via direct interaction between STAT3 and Sp1. *Int J Cancer* 115:202–13

Lokeshwar BL, Selzer MG, Zhu BQ, Block NL, Golub LM (2002) Inhibition of cell proliferation, invasion, tumor growth and metastasis by an oral non-antimicrobial tetracycline analog (COL-3) in a metastatic prostate cancer model. *Int J Cancer* 98:297–309

Mason DP, Kenagy RD, Hasenstab D, Bowen-Pope DF, Seifert RA, Coats S, Hawkins SM, Clowes AW (1999) Matrix metalloproteinase-9 overexpression enhances vascular smooth muscle cell migration and alters remodeling in the injured rat carotid artery. *Circ Res* 85:1179–85

McCawley LJ, Matrisian LM (2001) Matrix metalloproteinases: they're not just for matrix anymore! *Curr Opin Cell Biol* 13:534–40

Miao HQ, Soker S, Feiner L, Alonso JL, Raper JA, Klagsbrun M (1999) Neuropilin-1 mediates collapsin-1/semaphorin III inhibition of endothelial cell motility: functional competition of collapsin-1 and vascular endothelial growth factor-165. *J Cell Biol* 146:233–42

Novak A, Dedhar S (1999) Signaling through beta-catenin and Lef/Tcf. *Cell Mol Life Sci* 56:523–37

Pawlikowska L, Tran MN, Achrol AS, McCulloch CE, Ha C, Lind DL, Hashimoto T, Zaroff J, Lawton MT, Marchuk DA, Kwok P-Y, Young WL (2004) Polymorphisms in genes involved in inflammatory and angiogenic pathways and the risk of hemorrhagic presentation of brain arteriovenous malformations. *Stroke* 35:2294–300

Plouet J, Moukadiri H (1990) Characterization of the receptor to vasculotropin on bovine adrenal cortex-derived capillary endothelial cells. *J Biol Chem* 265:22071–4

Schonbeck U, Mach F, Libby P (1998) Generation of biologically active IL-1 beta by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1 beta processing. *J Immunol* 161:3340–6

Seino Y, Ikeda U, Ikeda M, Yamamoto K, Misawa Y, Hasegawa T, Kano S, Shimada K (1994) Interleukin 6 gene transcripts are expressed in human atherosclerotic lesions. *Cytokine* 6:87–91

Soker S, Kaefer M, Johnson M, Klagsbrun M, Atala A, Freeman MR (2001) Vascular endothelial growth factor-mediated autocrine stimulation of prostate tumor cells coincides with progression to a malignant phenotype. *Am J Pathol* 159:651–9

Taga T, Kishimoto T (1997) Gp130 and the interleukin-6 family of cytokines. *Annu Rev Immunol* 15:797–819

Thakker GD, Hajjar DP, Muller WA, Rosengart TK (1999) The role of phosphatidylinositol 3-kinase in vascular endothelial growth factor signaling. *J Biol Chem* 274:10002–7

Wagner S, Fueller T, Hummel V, Rieckmann P, Tonn JC (2003) Influence of VEGF-R2 inhibition on MMP secretion and motility of microvascular human cerebral endothelial cells (HCEC). *J Neurooncol* 62:221–31

Wang Z, Newman WH (2003) Smooth muscle cell migration stimulated by interleukin 6 is associated with cytoskeletal reorganization. *J Surg Res* 111:261–6

Werb Z (1997) ECM and cell surface proteolysis: regulating cellular ecology. *Cell* 91:439–42

Yao JS, Zhai W, Young WL, Yang G-Y (2006) Interleukin-6 triggers human cerebral endothelial cells proliferation and migration: the role for KDR and MMP-9. *Biochem Biophysic Res Commun* 342:1396–404

Yu Q, Stamenkovic I (2000) Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev* 14:163–76