Hypoxia-Induced Mediators of Stem/Progenitor Cell Trafficking Are Increased in Children With Hemangioma


Objective—The mechanism of neovascularization during the proliferative phase of infantile hemangioma is poorly understood. It is known that circulating bone marrow–derived endothelial progenitor cells (EPCs) form new blood vessels in ischemic tissues using mediators regulated by the transcription factor, HIF-1α. Mobilization of EPCs is enhanced by VEGF-A, matrix metalloproteinase (MMP)-9, and estrogen, whereas homing is secondary to localized expression of stromal cell–derived factor-1α (SDF-1α). We examined whether these mediators of EPC trafficking are upregulated during the proliferation of infantile hemangioma.

Methods and Results—Surgical specimens and blood samples were obtained from children with proliferating hemangioma and age-matched controls (n=10, each group). VEGF-A and MMP-9 levels were measured in blood, and tissue sections were analyzed for SDF-1α, MMP-9, VEGF-A, and HIF-1α. The role of estrogen as a modulator of hemangioma endothelial cell growth was also investigated. We found that all these mediators of EPC trafficking are elevated in blood and specimens from children with proliferating infantile hemangioma. In vitro, the combination of hypoxia and estrogen demonstrated a synergistic effect on hemangioma endothelial cell proliferation.

Conclusions—These findings demonstrate that proliferating hemangiomas express known mediators of vasculogenesis and suggest that this process may play a role in the initiation or progression of this disease. (Arterioscler Thromb Vasc Biol. 2007;27:000-000.)

Key Words: hemangioma ■ hypoxia ■ vasculogenesis ■ endothelial progenitor cells ■ chemokines

Infantile hemangioma initially presents as a small red or white birthmark but grows into a rapidly expanding vascular tumor during the first year of life. This tumor is differentiated from other vascular anomalies by its unique natural history. It usually appears within weeks after birth and then proliferates for up to 12 months followed by a period of involution of 5 to 7 years.1,2 Although benign, infantile hemangiomas may range in severity from insignificant cutaneous discolorations to massive life threatening lesions occurring in the liver or central nervous system. These lesions are found more frequently in the head and neck regions with a 3-fold higher incidence in females.3–5 Infantile hemangioma is the most common tumor of infancy, yet few effective treatments are available due to a fundamental lack of knowledge in hemangioma biology.6

The majority of hemangioma research has focused on the mechanisms of angiogenesis as underlying the new blood vessel growth. However, a second pathway for neovascularization, that of adult vasculogenesis, is increasingly being invoked in a variety of states characterized by vascular growth. Postnatal vasculogenesis differs from angiogenesis in that new blood vessels arise from circulating bone marrow–derived endothelial progenitor cells (EPCs) rather than from the local endothelial cells.7 Although the initiating mechanism during the pathogenesis of infantile hemangioma has yet to be discovered, there is recent evidence that postnatal stem/progenitor cells may contribute to their explosive growth. Children with proliferating infantile hemangioma harbor increased levels of mobilized EPCs,8 and surgical specimens are positive for the coexpression of progenitor specific markers such as CD34, AC133, and vascular endothelial growth factor (VEGF) receptor-2.9,10 In this article, we address the possible mechanisms by which EPCs may be released into the circulation and recruited to sites of hemangioma growth.

The cellular pathways of EPC mobilization and trafficking are becoming clear. During tissue ischemia, the increased expression and stabilization of the transcription factor, hypoxia inducible factor-1α (HIF-1α), promotes the local production of stromal cell–derived factor-1α (SDF-1α) and vascular endothelial growth factor-A (VEGF-A) by hypoxic endothelial cells.11,12 These mediators are capable of enhancing the mobilization and recruitment of EPCs to ischemic tissue during postnatal vasculogenesis.12–16 In the bone mar-
MMP-9, which is also hypoxia responsive, may lead to the release of EPCs by cleavage of membrane bound kit ligand. Interestingly, children with proliferating hemangioma have increased urinary secretion of MMP-9, a finding which warrants further investigation of the role of this important mediator of stem/progenitor cell trafficking in hemangioma growth.

Another putative modulator of EPC trafficking and vascular remodeling is estrogen. Endothelial cells possess estrogen receptors (ERs), and the addition of estradiol is known to be protective for hypoxia-induced apoptosis. A distinct mechanism for estrogen-mediated mobilization of EPCs was identified downstream of nitric oxide synthase. Additionally, SDF-1α expression is enhanced by estrogen which may lead to increased stem/progenitor cell recruitment and proliferation in peripheral tissues. The possibility that estrogen regulation is involved in the growth of infantile hemangioma is suggested by the increased incidence in females, evidence of ER positivity in proliferating tumors, and elevated levels of circulating 17β-estradiol in affected children. A possible link between estrogen and hypoxia mediated neovascularization in the pathogenesis of infantile hemangioma has not been previously examined.

This study aims to determine whether HIF-1α and the hypoxia-regulated mediators of EPC trafficking are increased during human hemangioma growth in vivo. A second arm of the investigation examines the effects of hypoxia and estrogen on human endothelial cell proliferation vitro. We demonstrate that HIF-1α and critical downstream targets are increased during hemangioma proliferation suggesting a new mechanism for the rapid progression of this tumor of infancy.

Materials and Methods

MMP-9 and VEGF-A ELISA

Peripheral blood samples were collected before surgery in children undergoing excision of proliferating hemangioma and age-matched controls undergoing strabismus correction (n=10). Plasma levels of total VEGF-A and MMP-9 (92 kDa pro- and 82 kDa active forms) were measured using commercially available ELISA kits (R&D Systems). All enrolled subjects signed an informed consent document approved by the institutional review board (IRB) of NYU School of Medicine.

Immunohistochemistry Analysis

Tissue was harvested either from freshly excised proliferating or involuting focal hemangioma, and confirmed to be infantile hemangioma from clinical examination, pathology consultation, and immunofluorescence for GLUT1 (n=10, each group). Samples were fixed within 5 minutes of excision in 10% neutral buffered formalin and embedded in paraffin blocks. After deparaffinization and rehydration, slides were treated with target retrieval solution (DAKO) at 95°C for 10 minutes following the manufacturer’s instructions. Sections were incubated with 4 primary antibodies: mouse anti-human SDF-1α (R&D Systems) at a dilution of 1:200, mouse anti-human MMP-9 (R&D Systems) at a dilution of 1:200, rabbit anti-human VEGF-A (Santa Cruz Biotechnology) at a dilution of 1:200, and mouse anti-human HIF-1α (Novus Biologicals) at a dilution of 1:1000 in PBS with 1% BSA. Primary antibodies were detected using biotinylated secondary antibodies (Vector Labs) and the ABC immunoperoxidase method (Vector Labs). Control slides included isotype-matched host-specific antibodies at a dilution of 1:100, 10% primary antibody host-serum, and single (no primary antibody) and double (no primary or secondary antibody) negative controls. For analysis of HIF-1α positivity, 2 blinded investigators evaluated slides and quantified HIF-1α nuclear staining from 5 nonconsecutive tissue sections at 40× magnification in 10 random fields. These counts were averaged, normalized to total nuclei, and expressed as a percentage of HIF-1α-positive nuclei per high-power field (HPF). ER staining of surgical specimens was completed during routine tissue processing in the NYU Department of Pathology.

Cell Culture

Normal human microvascular endothelial cells (HMECs) were isolated from foreskin, and hemangioma endothelial cells (HemECs) were isolated from proliferating surgical specimens using methods previously described (n=3). Breitly, under sterile conditions, 3 to 3-mm wedges from different areas of the tissue were minced and digested with Liberase 3 enzyme (Roche). CD31 labeled magnetic beads (Dynal Biotech) were used to isolate endothelial cells followed by culture in EGM-2MV (Cambrex) on gelatin coated plates at a density of 5000/cm².

[3H]Thymidine Incorporation Assay

Early passage (P2 or P3) HMEC and HMEC cultures (n=3) with similar population doubling times of 2 to 3 days, as calculated with a hemocytometer at cell seeding and harvest, were used to control for population doublings and biological age of the primary cell isolates. The cultures were subsequently incubated for 24 hours with endothelial basal medium-2 (Cambrex) and 1% fetal bovine serum to induce quiescence. Using methods previously described, DNA synthesis was reinitiated 6 hours before harvest by the addition of fully supplemented media. EGM-2MV, in the presence of 0.5 μCi/mL [3H]thymidine. Experimental cell cultures were treated with 100 nM estradiol (Sigma Aldrich) and exposed to normoxia (21% O2) or hypoxia (1% O2). Another set of HMEC cultures (n=3) was pretreated with 100 nM estradiol for 72 hours before initiation of the assay. After 24 hours, the cells were lysed for measurement of radioactivity in triplicate by liquid scintillation spectrometry (Beckman LS 1801).

RNA Extraction and Real-Time Quantitative RT-PCR

After 12 hours in hypoxia with or without estradiol, total RNA was extracted from HMEC cultures using the Totally RNA extraction protocol (Ambion). Complimentary DNA libraries were reverse transcribed using the RNA PCR Core Kit (Applied Biosystems) and quantitative real-time PCR (Roche Light Cycler 1.2) with SYBR Green (Applied Biosystems) was performed using the following primers:


All PCR products were analyzed by agarose gel electrophoresis and confirmed by DNA sequencing.

Statistical Analysis

Statistical analyses on data were performed by a Student t test or analysis of variance (ANOVA) with a post hoc Tukey test. All values are reported as mean±SEM. Statistical significance was determined by a probability value of P<0.05.

Results

Circulating Mediators of EPC Mobilization Are Increased in Children With Infantile Hemangioma

MMP-9 and VEGF-A play significant roles in the mobilization of EPCs from the bone marrow; therefore, we examined their levels in patients with proliferating hemangioma under-
going surgical resection and age-matched controls undergoing strabismus correction surgery with similar characteristics (supplemental Table I, available online at http://atvb.ahajournals.org). Age-matched controls were used instead of patients with involuting hemangioma to avoid the confounding effect of age on levels of specific plasma proteins. Circulating MMP-9 and VEGF-A in age-matched controls were in accordance with normal levels from other investigations (66.5±12.01 ng/mL and 114±14.93 pg/mL, respectively, Figure 1).32,33 In the peripheral blood of children with proliferating hemangioma, plasma levels of MMP-9 and VEGF-A were significantly increased by a factor of 3- and 5-fold, respectively (197±21.74 ng/mL, 530.8±70.44 ng/mL, \( P<0.001 \)).

Mediators of Progenitor Cell Trafficking Are Expressed in Proliferating Infantile Hemangioma

To further investigate hypoxia-induced mediators of progenitor cell trafficking in hemangiomagenesis, we analyzed tissue sections from involuting and proliferating specimens for SDF-1\( \alpha \), MMP-9, and VEGF-A. Involuting tissue sections demonstrated weak staining for all proteins in accordance with normal levels from other investigations (66.5±12.01 ng/mL and 114±14.93 pg/mL, respectively, Figure 1).34 In the peripheral blood of children with proliferating hemangioma, plasma levels of MMP-9 and VEGF-A were significantly increased by a factor of 3- and 5-fold, respectively (197±21.74 ng/mL, 530.8±70.44 ng/mL, \( P<0.001 \)).

HIF-1\( \alpha \) Stabilization Is Increased in Proliferating Infantile Hemangioma

Hypoxia directly leads to the stabilization of HIF-1\( \alpha \), a transcription factor that controls many genes involved in

Figure 1. A, Circulating MMP-9 levels (ng/mL) in children with hemangioma are increased nearly 3-fold compared with age-matched controls (\( *P<0.001 \)). B, Circulating VEGF-A levels (pg/mL) using the same plasma samples as above demonstrating an almost 5-fold increase (\( *P<0.001 \)).
vasculogenesis including VEGF-A and SDF-1. HIF-1α dysregulation has been implicated in the pathophysiology of other tumors, and we sought to determine whether it is involved in the proliferation of infantile hemangioma by evaluating tissues from involuting and proliferating hemangioma specimens for HIF-1α protein using methods in immunohistochemistry.

Tissue sections of involuting hemangioma demonstrated weak signal in the perinuclear cytoplasm (Figure 3A). In contrast, HIF-1α-positive nuclei in proliferating hemangioma sections were evident in numerous endothelial and interstitial cells (Figure 3B). Quantitative analyses demonstrated that the percentage of HIF-1α-positive nuclei was significantly increased in proliferating hemangioma compared with involuting tissues (95.6% ± 4.1 and 14.5% ± 6.8, respectively; *P < 0.001, Figure 3C).

MMP-9 Induction in Response to Estradiol and Hypoxia in Human Endothelial Cells In Vitro
A major goal of this study was to investigate whether hypoxia and estrogen could act as a switch for normal microvascular endothelial cells to start the hemangioma growth process. From studies which demonstrated strong binding of estradiol in proliferating hemangioma tissue, it was hypothesized that estrogen may be an important mediator in infantile hemangioma growth.28 In support of this data, we found increased ER staining in proliferating human hemangioma specimens (Figure 2).

In experiments to evaluate MMP-9 transcriptional regulation in endothelial cells in response to hypoxia and estrogen, we chose to use the well described HMECs rather than the less clearly understood HemECs. With estradiol in normoxia, MMP-9 expression in HMECs was downregulated compared with controls (61.6% ± 3.1, P < 0.01, Figure 4). Untreated HMEC cultures placed in hypoxia revealed an increase in MMP-9 mRNA copies (51.3% ± 5.8, P < 0.05), whereas supplementation with estradiol to hypoxic HMEC cultures markedly elevated MMP-9 expression (163.6% ± 3.8, P < 0.001).

These results suggest that estradiol and hypoxia may act in combination to enhance MMP-9 transcription in endothelial cells.

The Effects of Hypoxia and Estradiol on Hemangioma and Normal Endothelial Cell Proliferation
To further study the effects of hypoxia and estrogen on endothelial cell proliferation, [3H]thymidine uptake in stimulated HemEC and HMEC cultures was investigated. In these experiments, fully supplemented media, EGM-2MV, was used to expose the cultures to a microenvironment comparable to that found in infantile hemangioma. When measuring the proliferation of the cell cultures in hypoxia (1% O2), we found a significant increase over normoxia in both HemECs and HMECs (39.0% ± 8.6, P < 0.001 and 15.4% ± 7.2, P < 0.05, respectively; Figure 5).

In evaluating the potential for estrogen to further stimulate the proliferative phase of infantile hemangioma, we exposed normoxic and hypoxic HemEC and HMEC cultures to estradiol. In normoxic HemEC cultures, estradiol supplementation alone increased proliferation nearly as much as hypoxia (32.0% ± 4.5, P < 0.001; Figure 5) whereas HMECs did not significantly proliferate with estradiol treatment in normoxia or hypoxia compared with controls (0.4% ± 10.9, P > 0.48, 7.8% ± 5.2, P < 0.05, respectively). After estradiol was added to HemEC cultures in hypoxia, a dramatic increase in proliferation was observed when compared with unstimulated cultures in normoxia (99.6% ± 12.2, P < 0.001).

Based on analyses performed in this study and others,28,38 ERs are believed to be abundant on proliferating HemECs. Furthermore, preexposure of human endothelial cells to estradiol leads to significantly increased ER expression23,39; therefore, we preexposed HMECs to estradiol for 72 hours followed by treatment with a combination of estradiol and hypoxia. These cultures demonstrated enhanced proliferation compared with HMEC cultures that

Figure 2. Immuno histochemical detection of SDF-1α, MMP-9, VEGF-A, and ERs with intense brown capillary staining in proliferating hemangioma. There is minimal signal present in involuted hemangioma with evidence of fibrofatty deposition and hair follicles. Scale bars = 50 μm.
were not pretreated (42.9% ± 5.9, P < 0.001, Figure 5). It was also found that in hypoxia without further estradiol supplementation, preexposed HMECs exhibited increased proliferation compared with untreated HMECs (40.4% ± 9.3, P < 0.001). The data suggest that the mechanism of enhanced HemEC proliferation may be attributable to the convergence of ER signaling and hypoxia driven pathways.

Discussion

The unique growth pattern of infantile hemangioma has been under investigation for almost 40 years, yet little is understood regarding its pathogenesis. Studies of this disease are severely hindered by the absence of an animal model. We have been interested in the proliferation and regression of hemangioma because the natural history differs from classical genetic diseases, which have a slow, relentless progression. The initial clinical description of the promontory mark of hemangioma as “an anemic nevi” or “area of low blood flow” suggests that tissue ischemia, a powerful stimulus for neovascularization, may be involved. Additionally, a recent study showed that proliferating hemangiomas harbor several cell types that are known to preferentially migrate to zones of hypoxia. In our study, we observed that HIF-1α and its downstream effectors are upregulated in infantile hemangioma during the proliferative phase. Moreover, we reveal a synergistic interaction between estrogen stimulation and hypoxia signaling in endothelial cells that might explain the rapid growth of hemangioma in the perinatal period.

These data demonstrate that hypoxia-induced factors important for postnatal vasculogenesis (SDF-1α, MMP-9, VEGF-A, HIF-1α) are upregulated in children with proliferating hemangiomas. Other genes known to be expressed in hemangioma such as IGF-2 and GLUT-1 are also expressed in response to hypoxia and HIF-1α. Although HIF-1α protein is normally stabilized in the presence of decreased oxygen levels, overexpression in normoxic tissues can occur in some forms of cancer. Either mechanism of HIF-1α upregulation could potentially be responsible for the increased HIF-1α in proliferating hemangioma; however, the fact that later involuting specimens do not demonstrate increased HIF-1α suggests that physiological mechanisms are active and these lesions are truly hypoxic.

In the event that HIF-1α is stabilized by physiological mechanisms, infantile hemangioma may occur more frequently in ischemic tissues. There is data to suggest that hypoxia may be the initial stimulus for chorangioma, a tumor of the placenta that is histologically similar to hemangiomas. Infantile hemangioma also has a predilection for the head and neck region exhibiting a distribution that overlaps with embryonic fusion lines. It may be that these areas are more prone to ischemia because they are located in “watershed” vascular territories. Furthermore, environmental factors during fetal development may lead to insufficient vascularization of soft-tissues resulting in ischemia. Measurement of oxygen tension within the promontory mark would serve to confirm or refute whether these lesions are truly hypoxic, but these data are extremely difficult to obtain.

Previous studies have also suggested a role for estrogen in vasculogenesis; therefore, we examined the effects of hypoxia and estrogen on hemangioma endothelial cells. Circulating levels of estradiol are often significantly ele-
vated in the neonatal period when the tumor first begins to grow. At parturition, circulating levels of estradiol in the maternal plasma can reach as high as 50,000 pg/mL, over 500 times the normal value.\textsuperscript{48} This estrogen is free to cross the placenta into the fetal circulation,\textsuperscript{49} but, once transferred, its activity is suppressed by serum proteins including \( \alpha \)-fetal protein\textsuperscript{50,51} and sex-hormone binding globulin (SHBG).\textsuperscript{52} After transition to the postnatal environment, \( \alpha \)-fetal protein expression drops precipitously,\textsuperscript{53} and estradiol binding to SHBG is diminished.\textsuperscript{54} These physiological changes may result in an abrupt increase in free estrogen in the perinatal period which may stimulate areas of hypoxic endothelium to induce hemangioma formation.

In this article, we demonstrate the presence of hypoxia-induced mediators of progenitor/stem cell trafficking in proliferating infantile hemangiomma specimens and reveal that the combination of hypoxia and estradiol results in a synergistic effect on the upregulation of MMP-9 in endothelial cells in vitro, a key factor in EPC mobilization. The transcription factor HIF1-\( \alpha \) was also observed to be stabilized in proliferating hemangiomma specimens, providing evidence that these lesions may be hypoxic and that the vasculogenic response could contribute to their progression. Moreover, estrogen, which is ubiquitously present when these lesions arise, may potentiate these effects by acting synergistically with hypoxia on ER\( + \) endothelial cells to induce mitosis as shown by increased proliferation rates in HemEC- and ER-induced HMEC cultures in the presence of these 2 stimuli. Taken together, these data suggest a significant interplay between hypoxia and estrogen-mediated pathways of endothelial cell growth which may lead to new targets for the treatment of infantile hemangiomma.

Disclosures

None.

Figure 4. HMECs were treated with estradiol followed by hypoxia or normoxia for 12 hours. Representative total gene copy numbers (normalized to \( \beta \)-actin expression) demonstrated a 50\% increase in expression in the hypoxia group. With estradiol and hypoxia, MMP-9 mRNA expression was elevated to almost 3-fold the normoxic control (\( \ast P<0.001 \)).

Figure 5. HemEC and HMEC cultures were treated with estradiol (E) and exposed to hypoxia or normoxia for 24 hours. Pretreated HMEC cultures were exposed to estradiol for 72 hours before treatment. Supplementation with estradiol in hypoxia significantly increased mitosis in both the HemEC and pretreated HMEC cultures (\( \ast P<0.001 \)).
References


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**Supplemental Table 1.**

**Characteristics of Patients with Proliferating Hemangioma**

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**Characteristics of Patients with Involuting Hemangioma**

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**Characteristics of Control Patients Undergoing Strabismus Surgery**

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