Expression of Endoglin (CD105) and Endothelial Nitric Oxide Synthase in Head and Neck Arteriovenous Malformations

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Importance: Endoglin (CD105) and endothelial nitric oxide synthase (eNOS) assist in regulating vascular development. Variation in expression of these factors is linked to errors in vascular growth and remodeling in invasive lesions.

Objective: To clarify the role of endoglin and eNOS in the growth of extracranial head and neck arteriovenous malformations (AVMs), an invasive and high-flow vascular anomaly.

Design and Setting: Immunohistochemistry and Western blot study at an academic research center.

Specimens: Frozen and formalin-fixed paraffin-processed human AVMs (n=14) were examined for expression of CD105 and eNOS. Expression in infantile hemangiomas (n=9) and in normal skin with subcutaneous tissue (n=9) was used for comparison.

Main Outcome Measures: Quantitative assessment and localization of CD105 and eNOS protein expression were performed on each specimen by immunohistochemistry and Western blot analysis. Protein expression levels were compared with β-actin level and were semiquantitatively assessed.

Results: Abundant CD105 protein was found in AVMs but was not present in infantile hemangiomas or normal skin with subcutaneous tissue. Expression of eNOS protein in AVMs and infantile hemangiomas was similar (P=.20) and was significantly greater than that in normal skin with subcutaneous tissue (P<.001 and P=.008, respectively). Immunohistochemistry demonstrated that CD105 and eNOS are predominantly located in AVM vascular endothelial cells.

Conclusions and Relevance: CD105 and eNOS are present and significantly expressed in head and neck AVMs. Expression of CD105 and eNOS may have an important role in the angiogenesis and vascular remodeling of AVMs. CD105 can be used as a specific marker for AVM endothelial cells.

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ARTERIOVENOUS MALFORMATIONS (AVMs) represent a rare form of high-flow vascular anomaly (VA) that most commonly occurs in the head and neck.1-2 They are present at birth but are usually clinically asymptomatic until later in life. The pathogenesis of AVMs remains unclear. They are known to arise from multiple aberrant arteriovenous shunts between arteries and veins and consist of numerous hypertrophic, poorly regulated, and tortuous arteries and veins. Unlike infantile hemangiomas (IHs), another form of high-flow VA that results from endothelial cell (EC) proliferation and excess angiogenesis, the development of AVMs is more likely associated with hemodynamic imbalance, vascular remodeling, and embryologic precursors.1-3

Arteriovenous malformations tend to progress slowly, but prior therapy, pregnancy, or trauma may cause their rapid enlargement.3-7 With time, AVMs will expand to excessive size and infiltrate local tissue. This relentless growth causes a mass effect on surrounding tissues, with functional deficits, aesthetic impairment, and life-threatening bleeding. Ultimately, most AVMs will need to be treated. Unfortunately, no current therapeutic modality is ideal for the control of AVMs. Exacerbating this issue is that AVMs are difficult to positively identify from other VAs by light microscopy and histopathology alone. Specific markers are unavailable to reliably diagnose AVMs. This diagnostic challenge is reflected and compounded by a poor understanding of the pathogenesis of AVMs, leading to invalid treatment protocols.8-11

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Angiogenesis and vascular development are closely regulated by molecular pathways generated by the transforming growth factor β receptor endoglin (CD105) and the nitric oxide (NO)–producing enzyme endothelial NO synthase (eNOS) in normal tissues and in tumors. Most importantly, their pathways intersect in the process of vascular remodeling and growth. Arteriovenous malformations are presumed to have an inherent disruption of this process, with CD105 recently discovered to be mutated in some forms of small AVMs and implicated in pathological development of brain AVMs. Similarly, eNOS has been found to be abnormally expressed in other forms of high-flow VAs.

Therefore, we hypothesized that CD105 and eNOS are involved in the growth and recurrence of extracranial AVMs. This study focused on the pathogenesis of AVMs through examination of CD105 and eNOS, factors known to be involved in hemodynamic control and vascular remodeling. Direct comparison of expression in IHs and in normal skin with subcutaneous tissue is made to help us better understand the role of these 2 factors.

METHODS

SPECIMENS

This study was approved by the institutional review board of the University of Arkansas for Medical Sciences. After obtaining informed consent, fresh surgical specimens of head and neck subcutaneous AVM tissues were obtained from 14 patients. Infantile hemangiomas and normal skin with subcutaneous tissue were obtained from 9 patients each. Histologic examination by a pathologist (C.-Y.F. or A.G.S.) experienced with VAs confirmed the diagnosis for each patient at the time of resection. GLUT-1 staining helped confirm the IH diagnosis (when positive) or the AVM diagnosis (when negative) in these high-flow vascular lesions. Specimens were then divided for storing at −80°C and formalin fixation (10% formalin) before deparaffinization and rehydration. The sections were heated for 3 minutes at room temperature. The sections were counterstained with hematoxylin for 30 seconds. Next, they were dehydrated through graded alcohol solutions and cleaned by xylene substitute. Then, they were mounted (with Permount; Thermo Fisher Scientific) and coverslipped.

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After deparaffinization and rehydration, the sections were heated to 97°C for 20 minutes in a water bath in the presence of antigen retrieval solution (CITRA, pH 6.0; Invitrogen) and cooled for 30 minutes. To block the endogenous peroxidase activity, all sections were incubated with hydrogen peroxide for 10 minutes and washed with a phosphate-buffered saline solution (pH 7.4; Sigma-Aldrich). The sections were preincubated with 2% nonfat milk for 30 minutes at room temperature. Then, the sections were incubated in primary eNOS (rabbit polyclonal antibody; Santa Cruz Biotechnology) at 1:500 dilution or CD105 antibody (mouse monoclonal antibody; Thermo Fisher Scientific) at 1:150 dilution for 30 minutes. To block the endogenous peroxidase activity, all sections were incubated with hydrogen peroxide for 10 minutes. To block the endogenous peroxidase activity, all sections were incubated with hydrogen peroxide for 10 minutes. To block the endogenous peroxidase activity, all sections were incubated with hydrogen peroxide for 10 minutes.

RESULTS

Western blot results were expressed as means (SDs). The differences between any 2 groups were calculated using the t test. P < .05 was considered statistically significant.

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All AVM specimens (n = 14) were positive for CD105 and eNOS expression by immunohistochemistry. Both CD105 and eNOS were located primarily in AVM ECs. All IH specimens (n = 9) were positive for eNOS but were negative for CD105. As in the case of AVMs, eNOS was located in the IH ECs. All samples of normal skin with subcutaneous tissue (n = 9) were negative for eNOS and CD105. Results from this staining are shown in Figure 1 and Figure 2.

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Semi-quantitative analysis of CD105 and eNOS protein expression was performed by Western blot. With β-actin as the loading control, the mean (SD) expression of CD105 protein level was 0.18 (0.10) in AVMs, 0.03 (0.02) in IHs, and 0.02 (0.02) in normal skin with subcutaneous tissue. CD105 protein expression was statistically significantly greater in AVM specimens vs IHs and normal skin with subcutaneous tissues (P < .001 for both). No statistically significant difference was noted between IHS and normal skin with subcutaneous tissue (P = .18). The mean (SD) expression of eNOS protein level was 0.20 (0.12) in AVMs, 0.27 (0.24) in IHs, and 0.03 (0.04) in human tonsil tissue was used as a positive control for CD105 antibody. Mouse brain tissue was used as a positive control for eNOS antibody. Slides with no primary antibody applied were used as the negative control. The staining results were validated by a blind review performed by a pathologist (C.-Y.F. or A.G.S.) with extensive experience examining VAs and immunohistochemistry. A strong staining in greater than 10% of the cells indicated a positive value.

STATISTICAL ANALYSIS

Total proteins were extracted from 10- to 30-μg sections of frozen specimens with 200 μL of tissue protein extraction reagent (Pierce) added with protease inhibitor (Mini Protease Inhibitor Cocktail; Roche). Thirty micrograms of the total protein was loaded on gels (NuPAGE 4%-12% Bis-Tris; Invitrogen) for electrophoresis, transferred to a nitrocellulose membrane, and probed with rabbit polyclonal antibody against eNOS (Santa Cruz Biotechnology) at 1:200 dilution, mouse monoclonal antibody against CD105 (Thermo Fisher Scientific) at 1:150 dilution, or mouse monoclonal antibody against β-actin (Santa Cruz Biotechnology) at 1:1000 dilution. The blot was incubated with a horseradish peroxidase–conjugated goat antimouse IgG (Invitrogen) or goat antimouse IgG (Invitrogen), and the protein was visualized using a kit (Novex ECL Chemiluminescent Substrate Reagent Kit; Invitrogen). CD105 and eNOS protein expression levels were semi-quantitatively assessed in comparison with β-actin level using available software (Image J; National Institutes of Health).
normal skin with subcutaneous tissue. Expression of eNOS protein in AVMs and IHs was statistically significantly greater than that in normal skin with subcutaneous tissue ($P < .001$ and $P = .008$, respectively). Expression of eNOS protein in AVMs and IHs was similar ($P = .20$). These results are shown in Figure 3 and Figure 4.

Endoglin (CD105) is a 180-kDa homodimeric transmembrane glycoprotein, acting as a component of the transforming growth factor β receptor complex.\textsuperscript{16,17} CD105 is important in angiogenesis, vascular homeostasis, and

Figure 1. Immunohistochemistry results for CD105. A and B, Endothelial cells of arteriovenous malformations stain strongly positive for CD105. C and D, CD105 expression is not found in infantile hemangiomas. E, Human tonsil tissue was used as a positive control for CD105 antibody. F, CD105 expression is not found in normal skin with subcutaneous tissue. Original magnification ×200 (A, C, and E) and ×400 (B and D).
It is expressed on activated vascular ECs and will mediate EC proliferation, migration, and tube formation when binding with transforming growth factor β. Although the exact mechanism of CD105 is unknown, there is no doubt that CD105 is a marker of proliferating ECs. CD105 is highly expressed in numerous solid tumors and is known to be involved in tumor angiogenesis and metastasis. CD105 is found on ECs and in mesenchymal stem cells, which are abundant in tumors. Many clinical studies have reported that CD105 is a useful tumor vasculature marker because it is more specific than traditional markers, such as CD31, CD34, and factor VIII. This is because inside the tumor CD105 is expressed predominantly in angiogenic ECs undergoing vascular remodeling but not in the stable ECs of normal vasculature.

Figure 2. Immunohistochemistry results for endothelial nitric oxide synthase. A-E, Endothelial nitric oxide synthase expression is found in the endothelial cells of arteriovenous malformations (A and B) and infantile hemangiomas (C and D) but not in normal skin with subcutaneous tissue (E). F, Mouse brain was used as a positive control for endothelial nitric oxide synthase antibody. Original magnification ×200 (A, C, and E) and ×400 (B and D).
Although AVMs are a type of VA, they have aggressive characteristics similar to those of locally invasive cancers. Qualities possessed by AVMs include the ability to undergo rapid expansion, achieve excessive size, infiltrate local tissue, and recur following extensive and ablative therapy. CD105 expression has been found to be abnormal in cerebral AVMs. Because of the constant remodeling presumed to occur in AVMs, the invasive quality of AVMs, and the presence of CD105 in intracranial disease, we hypothesized that CD105 was involved in the growth and recurrence of extracranial AVMs. In this study, we demonstrated that CD105 protein is predominantly located in AVM vascular ECs and that expression was significantly greater in AVM specimens than in IHs or normal skin with subcutaneous tissue. In IHs, another type of high-flow VA undergoing rapid vascular changes, CD105 protein could not be detected by immunohistochemistry or Western blot. This finding suggests that angiogenesis and vascular remodeling occur in AVMs and is consistent with recent clinical and experimental evidence.

Our study results also posit a role of CD105 in the invasive quality of extracranial AVMs. CD105 not only promotes angiogenesis by activating endothelial proliferation pathways but also affects NO production in ECs. Modulation of CD105 expression has been shown to influence NO-dependent vasodilation along with eNOS expression and activity in in vitro and in vivo models. These findings suggest that CD105 is an important coupler of eNOS activity and that eNOS has a major role in CD105-dependent angiogenesis.

Nitric oxide synthase comprises a family of enzymes that is responsible for production of NO from L-arginine. Three major isoforms of NOS have been found, including neuronal NOS, inducible NOS, and endothelial NOS (eNOS). Endothelial NOS is constitutively expressed in the ECs, hav-

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Figure 3. Western blot results for CD105. CD105 expression in arteriovenous malformations (AVMs) (A), normal skin with subcutaneous tissue (B), and infantile hemangiomas (IHs) (C) was detected by Western blot (D). The ratio of the intensity of the protein band of CD105 to β-actin was calculated. Arteriovenous malformations express higher levels of CD105 vs infantile hemangiomas and normal skin with subcutaneous tissue (P <.001 for both).

Figure 4. Western blot results for endothelial nitric oxide synthase (eNOS). Endothelial nitric oxide synthase expression in arteriovenous malformations (AVM) (A), normal skin with subcutaneous tissue (B), and infantile hemangiomas (IHs) (C) was detected by Western blot (D). The ratio of the intensity of the protein band of eNOS to β-actin was calculated. Arteriovenous malformations and infantile hemangiomas express higher levels of eNOS vs normal skin with subcutaneous tissue (P <.001 and P =.008, respectively). There is no statistically significant difference between AVM and IH eNOS expression (P =.20).
ing a key role in angiogenesis and vasculogenesis. Production of NO by eNOS regulates blood vessel tone and hemodynamics, inhibits vascular smooth-muscle cell proliferation, and modulates the interaction of endothelium with leukocytes.\textsuperscript{37} Ying and Hofseth\textsuperscript{38} demonstrated that the eNOS and NO pathways closely modulate events in tumors, including the promotion of angiogenesis and antiapoptosis in tumor epithelial cells, stimulating cancer cell cycle progression and proliferation, and enhancing tumor cell vascular invasion.

In this study, we demonstrated that eNOS protein is predominantly located in AVM vascular ECs. Its expression was greater in AVMs than in normal skin with subcutaneous tissue. Along with our CD105 results, this finding suggests that the angiogenesis and proliferation of AVM ECs may occur due to higher-than-normal levels of CD105 and eNOS expression. In this research, a notable phenomenon was observed: AVM ECs have high expression of CD105 and eNOS, while IH ECs have only high expression of eNOS. The deficiency of CD105 in IHs is unclear. A previous study\textsuperscript{39,40} demonstrated that eNOS protein level is decreased in involuting IHs. CD105 is perhaps involved in the constitutive expression of NO, is expressed at higher levels in the ECs of AVMs and IHs compared with normal skin. These results suggest that CD105 and eNOS expression may have an important role in vascular remodeling of AVMs and mark a collaborative and aberrant signaling pathway in the pathogenesis of extracranial AVMs. CD105 may also represent a histopathological marker for AVMs vs other VAs. Further investigation of CD105 in other malformations will help elucidate this possibility.

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