Objective: To examine the location and degree of endothelial nitric oxide synthase (eNOS) protein expression in hemangioma growth, involution, and during propranolol therapy.

Design: Cross-sectional study.

Setting: University hospital.

Patients: Pediatric patients with hemangiomas.

Interventions: Fresh human hemangioma specimens at various stages of development were harvested. Effective propranolol therapy had been implemented in some patients. Quantitative assessment and localization of eNOS protein expression was performed on each specimen by Western blot analysis and immunohistochemical analysis, respectively.

Results: Hemangiomas in a proliferative phase (group 1: n=4; mean [SD] age, 4.25 [2.06] months), an early involuting phase (group 2: n=6; 12.00 [1.64] months), and a late involuting phase (group 3: n=6; 23.30 [1.97] months) were harvested. The mean (SD) eNOS protein expression was 0.88 (0.41) in group 1, 0.26 (0.26) in group 2, and 0.15 (0.08) in group 3, respectively. A statistically significant decrease in eNOS protein expression was observed between proliferating and involuting hemangiomas (group 1 vs group 2 and group 3; P<.01) but not between early and late phases of involution (P=.17). In a separate propranolol treatment group (n=7), the eNOS protein level was significantly lower than in age-matched controls (n=7; 0.08 [0.1] vs 0.45 [0.45]; P=.03). Immunohistochemical analysis demonstrated eNOS to be predominately in endothelial cells lining mature blood vessels.

Conclusion: Expression of eNOS protein decreases during the hemangioma lifecycle. Propranolol may suppress hemangioma growth by inhibiting expression of eNOS protein and subsequent production of nitric oxide.

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ejection of corticosteroids, chemotherapeutic agents (vincristine sulfate, interferon alfa), and laser therapy. Recently, Léauté-Labrèze et al, 3 for the first time, reported that propranolol can dramatically inhibit hemangioma growth. Much interest in propranolol therapy for hemangiomas has ensued since this original article. Similarly, our group demonstrated a 97% effective rate in reducing the size and growth of proliferating and involuting hemangiomas with low-dose propranolol. 4,5 However, minor surgical treatments were still necessary in nearly half of these patients.

Many related growth factors and receptors likely contribute to hemangioma proliferation and involution. In cultured hemangioma-derived endothelial cells and hemangioma tissues, fetal liver kinase-1 (Flk-1)/vascular endothelial growth factor receptor 2 (VEGFR-2), VEGFR-1, angiopoietin-2 are strongly expressed.6 VEGF expression is reduced in involuting hemangioma.7 In addition to VEGF, hemangioma cells express high levels of several cellular markers, including proliferating cell nuclear antigen, E-selectin, and basic fibroblast growth factors.8-10 Insulin-like growth factor-2 (IGF-2) expression is also upregulated in proliferating hemangiomas, indicating a role of IGF-2 in hemangioma growth.11 While apoptosis, known as programmed cell death, is thought to be critical during the involvment of hemangiomas.12

Evidence suggests that regulation of apoptosis and production of vascular associated growth factors is controlled by release of nitric oxide, a potent endothelial cell mitogen, vascular permeability factor, and vessel dilator.13 Its role in angiogenesis, vasculoegenesis, and vascular remodeling is well established. Three enzymes produce nitric oxide and include neuronal, inducible, and endothelial nitric oxide synthase. Primary production and release of nitric oxide is performed by endothelial nitric oxide synthase (eNOS). Because of nitric oxide’s role in vasodilation, we hypothesized that its constitutive production, by eNOS, may help regulate the hemangioma lifecycle and possibly the impact of propranolol therapy. Using fresh human hemangioma specimens from children of various ages, we explored this relationship through quantitative and qualitative measurement of eNOS during proliferation, involution, and propranolol therapy.

METHODS

PATIENT TISSUE COLLECTION AND PRESERVATION

This study was approved by the institutional review board of the University of Arkansas for Medical Sciences, Little Rock. Fresh tissue was harvested upon the surgical removal or sampling of hemangioma after written informed consent was obtained from the patient’s family. Following excision, the central core of each hemangioma was isolated and processed for experimental analysis. This provided some consistency in tissue sampling and removed any potential char debris from the specimen. Adjacent normal tissues in some patients with hemangiomas were harvested to provide control tissue specimens. Upon harvest, the tissue was immediately divided into 2 sections with 1 portion stored at −80°C and the remaining portion fixed in 10% formalin (pH, 7.0) and embedded in paraffin for further analysis. Tissue sections were stained with hematoxylin-eosin for routine histologic examination.

WESTERN BLOT ANALYSIS

Total proteins were extracted from 10 mg of frozen hemangioma tissue with 200 µL of T-PER tissue protein extraction reagent (Pierce, Rockford, Illinois) added with Mini Protease Inhibitor Cocktail (Roche, Indianapolis, Indiana). Thirty micrograms of the total protein was loaded onto NuPAGE 4–12% Bis-Tris gels (Invitrogen, Carlsbad, California) for electrophoresis, transferred to a nitrocellulose membrane, and probed with either polyclonal rabbit antibody against eNOS (Santa Cruz Biotechnology, Santa Cruz, California) at 1:200 dilution or monoclonal rabbit antibody against CD31 (Santa Cruz Biotechnology) at 1:500 dilution or monoclonal mouse antibody against β-actin (Santa Cruz Biotechnology) at 1:1000 dilution. The blot was incubated with a horseradish peroxidase (HRP)-conjugated either goat anti-rabbit IgG (Invitrogen) or goat antimouse IgG (Invitrogen), and the protein was visualized by using Novex ECL Chemiluminescent Substrate Reagent Kit (Invitrogen). The eNOS protein expression level was semiquantitatively assessed, compared to β-actin level, by using NIH software Image J (National Institutes of Health).

IMMUNOHISTOCHEMICAL ANALYSIS

To examine eNOS protein expression and its location, formalin-fixed and paraffin-processed sections (5 µm) of hemangioma tissues were deparaffinized and rehydrated. Epitope retrieval was performed by steaming sections for 20 minutes in citrate buffer (pH, 6.0). After quenching the endogenous peroxidase activity and blocking the nonspecific binding sites, sections were incubated overnight with polyclonal rabbit antibody against eNOS (Santa Cruz Biotechnology) at 1:500 dilution at 4°C. In the negative control slide, no primary antibody was applied. The mouse brain tissue was used as positive control as suggested by Santa Cruz Biotechnology. After washing with PBS, the antibody staining was visualized by using UltraVision Detection System (Fisher Scientific, Hampton, New Hampshire). The staining results were validated by a blind review performed by a pathologist (C.-Y.F. or A.S.) who has rich experience with vascular anomalies and immunohistochemical analysis.

STATISTICAL ANALYSIS

Western blotting results were expressed as means (SDs), and the difference between any 2 groups was calculated with t test. P < .05 was set as statistical significance.
2 groups were established. One was a control group (n=7; age, 10.9 [4.1] months), in which patients did not receive propranolol treatment, another 1-treatment group (n=7; age, 11.6 [3.6] months), in which patients were treated with propranolol. There is no statistical difference between these 2 groups in terms of the age.

Examination of the patients providing propranolol-treated specimens revealed that surgical removal was performed due to either incomplete response (4 patients) or intolerance of therapy or adverse effects (3 patients). Propranolol therapy was continued at, or near, the time of excision.

Semiquantitative analysis of eNOS protein expression was performed by Western blot analysis. With α-tubulin being the loading control, the mean (SD) eNOS protein level was 0.88 (0.41) in group 1, 0.26 (0.26) in group 2, and 0.15 (0.08) in group 3, respectively. A statistically significant decrease in eNOS protein expression was observed between proliferating and involuting hemangiomas (for group 1 vs group 2, P = .01; for group 3, P = .001) but not among early and late phases of involution (P = .17) (Figure 1A and Figure 2A). In the propranolol treatment group, eNOS protein level was significantly lower than in the control group (treatment group, 0.08 [0.1]; control group, 0.45 [0.45]; P = .03) (Figure 1B and Figure 2B).

Normal specimens were harvested adjacent to hemangioma tissue as a representation of normal skin and subcutaneous tissue. Western blot analysis revealed bands so weak that detection was too difficult for comparison with the hemangioma results. In essence, eNOS expression was represented by very weak bands indicating very low levels (Figure 1C). To determine if patterns in endothelial cell concentration affected eNOS results, CD31, a known endothelial cell marker, was examined by Western blot analysis. CD31 expression level demonstrated considerable variability among the various age groups of hemangiomas excised (Figure 1D). No consistent changes in CD31 expression were correlated with age or treatment.

To examine the location and distribution of eNOS protein, hemangioma tissues were stained using a specific eNOS antibody. Like the positive control that is positively stained for eNOS in the neuron cell cytoplasm, eNOS was expressed mainly in the cytoplasm of clumped endothelial cells and primitive hemangioma vasculature. In some vessels that appeared normal in development, a few endothelial cells also stained positively. Smooth muscle and stromal cells were nonreactive to this antibody (Figure 3).

COMMENT

Nitric oxide synthases (NOS) are a family of enzymes that are responsible for production of nitric oxide from...
The activated eNOS has variable complex functions in both physiologic and pathologic conditions. It produces nitric oxide that plays a critical role in regulating vascular permeability, dilation, and endothelial cell interactions with leukocyte. The role of eNOS is unknown, it is possible that eNOS is a major contributor to the rapid growth of hemangioma endothelial cells in proliferative phase. Although the exact role of eNOS is unknown, it is possible that eNOS is part of the VEGF pathway regulating hemangioma endothelial cell division. VEGF has been shown to upregulate eNOS messenger RNA and protein expression, while it is also detected at higher levels in proliferative than in involuting hemangiomas. Similarly, VEGF has been shown to promote proliferation of hemangioma vascular endothelial cells in vitro.

To examine the possibility that alterations in eNOS expression was related to a change in endothelial cell concentration, we checked expression of CD31, an endothelial cell marker, by Western blot analysis. The goal was to essentially normalize the denominator as the number of endothelial cells within the specimens tested. There was considerable variability of CD31 levels among the hemangioma specimens (Figure 1D) that was not consistent with age or stage of involution. This result suggests that despite changes in the endothelial cell density of the hemangioma architecture, changes are not predictable based on age. This further implies that changes in eNOS expression are not directly related to presumed changes in endothelial cell density at various stages of a hemangioma’s lifecycle.

Figure 2. The mean ratios of the intensity of the protein band of endothelial nitric oxide synthase to β-actin by Western blot analysis was calculated by Image J Software. The bars indicate standard deviations. A, P=.01 for group 1 vs group 2; P=.001 for group 1 vs group 3; P=.17 for group 2 vs group 3. B, P=.03 for the untreated group vs the treated group.
and exploring upstream and downstream regulators of hemangioma involution. Increasing our sample size the chemical cascade and cellular response that leads to biology and development undoubtedly contribute to mon birthmark. Numerous factors involved in vascular and the mechanism of propranolol in treating this com-

broader conclusions can be made. Inhibition of eNOS controls hemangioma endothelial division and differentiation is

due to the decreased expression and activity of eNOS. A effect of propranolol in hemangiomas may be partially

in the same phase of the hemangioma lifecycle (mean age, 10-11 months) so the effect of age and involution on eNOS expression could be considered negligible. Similarly, specimens examined in this study had a good response to propranolol but required definitive management owing to drug intolerance or tissue residuum. Thus, the eNOS expression determined in these tissues adequately represents levels following the successful impact of propranolol therapy on hemangioma.

Further, the results from this study are similar to those seen in a recent rat model of cirrhosis examining eNOS expression following propranolol therapy. In this model, propranolol decreased eNOS activity by 47% and eNOS expression by 75%.27 We thereby suspect that the effect of propranolol in hemangiomas may be partially due to the decreased expression and activity of eNOS. A low level of eNOS can be a direct correlate to the reduced NO production leading to vasoconstriction and reduced vascular tone. Physical evidence of this physiologic characteristic is present in treated lesions as they become softer and smaller within few weeks of administration.3,28 In addition, decreased eNOS can theoretically impair hemangioma endothelial cell proliferation and provide long-term control of hemangioma growth.

Despite these results, the study is limited in providing a clear picture on how reduced eNOS expression controls hemangioma proliferation and stability. Further investigation on the direct role of nitric oxide on hemangioma endothelial division and differentiation is necessary through in vitro and in vivo analysis before broader conclusions can be made. Inhibition of eNOS during such work may further elucidate the role of this ubiquitous enzyme in hemangiomas. Furthermore, we recognize this as pilot work in examining the complex array of cytokines involved in hemangioma regression and the mechanism of propranolol in treating this common birthmark. Numerous factors involved in vascular biology and development undoubtedly contribute to the chemical cascade and cellular response that leads to hemangioma involution. Increasing our sample size and exploring upstream and downstream regulators of eNOS expression is necessary to support the results from this study.

In conclusion, to our knowledge, this study is the first to suggest a potential role for eNOS expression, and subsequent nitric oxide production, in the life cycle of hemangioma development. Reduced levels of eNOS in involuting and propranolol-treated hemangiomas point to important hemodynamic and endothelial cell processes involved in hemangioma development. This study alludes to the mechanism by which propranolol has an impact on hemangioma dissolution.

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Figure 3. Endothelial nitric oxide synthase (eNOS) detection by immunohistochemical analysis (IHC) demonstrates specific expression in endothelial cells. Reductions in eNOS protein expression are incidentally evident by IHC in sequentially older patients: 7 months (A), 9 months (B), and 15 months (C). Samples were stained with polyclonal rabbit antihuman eNOS antibody (original magnification ×400). Quantification by IHC was not performed.