Propranolol-Mediated Attenuation of MMP-9 Excretion in Infants With Hemangiomas

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IMPORTANCE Infantile hemangiomas (IHs) vary substantially in localization and extent of tissue involvement, but IH biological progression is remarkably unique and predictable. Propranolol is an effective treatment for symptomatic IH, but its mechanism of action remains unknown and understudied.

OBJECTIVE To compare excreted proteins in infants with IH being treated with propranolol vs prednisolone.

DESIGN, SETTING, AND PARTICIPANTS Exploratory urine proteomics profiling of patients with IH from July 2010 to September 2012 at a tertiary pediatric hospital. Participants were infants with IH treated at our institution who were participating in a blinded, randomized trial comparing prednisolone vs propranolol. They ranged in age from 14 days to 15 months at enrollment. Exclusion criteria included a history of diabetes mellitus, asthma, and/or cardiovascular disease including hypertension or hypotension. Urine samples were longitudinally collected from all participants. Specimens were desalted, concentrated, and gel fractionated, and the protein content was identified using liquid chromatography tandem mass spectrometry. Western blot analyses and enzyme-linked immunosorbent assays (ELISAs) were performed to validate mass spectrometry findings.

INTERVENTION Treatment with propranolol or prednisolone administered starting before the age of 6 months.

MAIN OUTCOMES AND MEASURES Proteins present in urine samples and change in urinary levels of proteins over time.

RESULTS Samples were obtained from 3 patients treated with prednisolone, 3 patients treated with propranolol, and 5 untreated controls with IH. More than 1000 urinary proteins were identified by proteomics. Patients treated with propranolol demonstrated attenuation of excreted matrix metalloproteinase 9 (MMP-9) in urine over the proliferative phase of the condition compared with prednisolone-treated patients. These findings were validated with Western blot analysis and quantified with ELISA, which confirmed mean urinary MMP-9 levels in the first year of life to be significantly lower in propranolol-treated patients with IH compared with prednisolone-treated patients with IH (0.118 vs 0.501 ng/mL; P = .03) or with nontreated patients with IH (0.118 vs 3.69 ng/mL; P = .02).

CONCLUSIONS AND RELEVANCE Propranolol treatment decreases urinary excretion of MMP-9 in patients with IH. Matrix metalloproteinase 9 may be a biomarker for IH propranolol responsiveness, and its signaling pathways may represent the molecular target of this drug.
Infantile hemangiomas (IHs) represent the most common tumor of infancy, affecting up to 10% of children. These benign vascular, endothelial cell tumors can vary substantially in localization and extent of soft tissue involvement, but IH biological progression is remarkably unique and predictable. Most lesions are not apparent at birth, undergo rapid growth during early infancy (first 6-8 months), and subsequently involute during the first several years of life. Historically, IHs are characterized primarily by the presence of endothelial cells. Although the exact mechanisms that govern the pathogenesis of IH lesions are not completely understood, it is thought to result from defects in angiogenesis because angiogenic growth factors have been shown to play an important developmental role. Peptides such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are known to be consistently expressed in IH tissues and in the serum and urine of patients with these lesions. Urine levels of bFGF and high molecular weight matrix metalloproteinases have been shown to correlate with the extent and aggressiveness of these tumors.

Although the majority of IHs require no therapy, clinical characteristics may mandate intervention in up to 20% of cases. Until recently, the mainstay of medical treatment included administration of glucocorticoids or interferon-α. Use of high-dose oral glucocorticoids is relative in halting the growth or even reducing the size of cutaneous hemangiomas, but use is limited by substantial and serious systemic adverse effects. Propranolol was serendipitously discovered to be effective in treating IHs in a recent small patient series. The use of propranolol has now been demonstrated to be effective for a majority of these lesions and has virtually become the standard of care. Importantly, however, the mechanism of action of propranolol in the treatment of IH is not completely known and remains understudied. We hypothesized that by using a global proteomics approach using mass spectrometry (MS), we could elucidate differential excretion of urine proteins in patients treated with propranolol vs those treated with prednisolone.

**Methods**

**Sample Collection and Preparation**

Urine samples from children with IH being treated in a randomized trial of propranolol vs prednisolone were longitudinally collected over the first year of life under institutional review board (IRB) approval (Children’s National IRB, protocol 00000467) and parental consent. Infants with symptomatic hemangiomas ranging in age from 14 days to 15 months were recruited during the period from July 2010 through September 2012. Exclusion criteria included a history of diabetes mellitus, asthma, or cardiovascular disease including hypertension or hypotension. Samples were collected before the age of 6 months and between 6 and 15 months for each patient. All patients undergoing treatment started to receive either propranolol or prednisolone before the age of 6 months. For proteomics analysis, we compared the pretreatment samples (age <6 months) with the post-treatment samples (age 6-15 months). Only samples from the same collection dates were pooled; samples from different collection dates were never pooled. Urine was collected in a diaper sponge and immediately centrifuged at 13,000 rpm in a tabletop microcentrifuge after manual extraction from the sponge. The supernatants were then transferred, aliquoted, and frozen at −80°C.

**Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

Samples were thawed and desalted using a Millipore 3 kDa filter. Protein concentration was determined using bicinecinic acid protein assays (Pierce). Aliquots containing approximately 500 μg of total protein were dried by means of vacuum centrifugation on a Speed-Vac (Thermo Scientific). Samples were reconstituted in lithium dodecyl sulfate buffer containing 0.1 M dithiothreitol and subjected to 1-dimensional SDS gel electrophoresis using a 4% to 12% BIS-TRIS gel (Novex, Life Technologies) at 200 V for 50 minutes. The gel was fixed with a solution of 45:50:5 methanol:water:acetic acid for 30 minutes, rehydrated with water, and stained with Bio-Safe Coomassie (Bio-Rad) for 1 hour. Each gel lane was serially sliced into 32 segments, and each slice was digested with trypsin as previously described.

**Mass Spectrometry Protein Identification**

In-gel digested peptides were dissolved in 10 μL of 0.1% trifluoroacetic acid. Each sample (6 μL) was injected via a 10-μm silica tip (New Objective, Inc) adapted to a nano–high-performance liquid chromatography system. The mobile phases consisted of water with 0.1% formic acid and 90% acetonitrile with 0.1% formic acid aqueous. The sample was subsequently separated by a C18 reverse-phase column (3.5 μm, 75 μm inner diameter × 15 cm; LC Packings) at a flow rate of 250 nL/min using an Eksigent nano-high-performance liquid chromatography system. The mobile phases consisted of water with 0.1% formic acid (A) and 90% acetonitrile with 0.1% formic acid (B). A 65-minute linear gradient from 5% to 40% B was used. Eluted peptides were introduced into the mass spectrometer via a 10-μm silica tip (New Objective, Inc) adapted to a nano-electrospray source (Thermo Fisher Scientific). The spray voltage was set at 1.2 kV, and the heated capillary, at 200°C. The linear trap quadrupole mass spectrometer (Thermo Fisher Scientific) was operated in data-dependent mode with dynamic exclusion in which 1 cycle of experiments consisted of a full-MS (300-2000 m/z) survey scan and 5 subsequent tandem MS scans of the most intense peaks.

Each file was searched for protein identification using the Sequest algorithm in the Bioworks Browser software, version 3.3.1 (Thermo Fisher Scientific), against the Uniprot database indexed for human species and for partially tryptic peptides, 2 missed cleavages, and potential modification of oxidized methionine (15,9949 Da). The DTA generation parameters were peptide tolerance of 1.5 Da and fragment ion tolerance of 1 Da. Search result files were loaded into ProteiQ software (NuSep) and filtered on the basis of the following: XCorr greater than 1.9, 4 spectra per protein, 2
Western Blot Analysis
Briefly, samples containing 40 μg total proteins were solubilized in an equal volume of electrophoresis sample buffer (2.5% wt/vol SDS/4.5M urea/5% vol/vol β-mercaptoethanol/25% vol/vol glycerol/0.005% wt/vol bromophenol blue/0.08M TRIS hydrochloric acid, pH 7.5) and incubated for 15 minutes at 95°C. Electrophoresis was carried on 1.0% agarose gels (11 × 14 cm) prepared in electrophoresis buffer (0.1% wt/vol SDS/40M TRIS-acetate-ethylene diaminetetraacetic acid buffer) at room temperature for 1 hour at 35 V, then for 20 to 24 hours at 15 V. After electrophoresis, samples were transferred to polyvinylidene fluoride membranes (Millipore) by positive pressure transfer using a Posiblot apparatus (Stratagene) with saline-sodium-citrate buffer (0.6M NaCl/0.06M TRIS hydrochloric acid, pH 7.5) at a positive pressure of 75 mm Hg for 2.5 hours. Monoclonal MMP-9 antibodies (sc-21733) were purchased commercially (Santa Cruz Biotech, Inc). The membranes were then transferred to pouches containing 50 mL of 2.5% milk and antibodies (diluted 1:5000) and rocked overnight at 4°C. Following probing with the primary antibody, the membranes were washed 3 times for 10 minutes with PBST (phosphate-buffered saline containing 0.05% vol/vol Tween [Sigma]). Membranes were then incubated with horseradishperoxidase goat anti-rabbit antibody in 25 mL of 5% milk at a dilution of 1:5000 for 1 hour at room temperature and then washed 6 times for 10 minutes with PBST. After being washed in PBST, the blots were developed using the horseradish peroxidase chemiluminescence detection protocol using SuperSignal West Dura Extended Duration Substrate (Pierce) for 5 minutes. The developed blots were exposed and visualized with a charge-coupled device camera-equipped Gel Doc 2000 chemiluminescent imaging documentation station (BioRad). Exposure time was 1 second and equal for all blots. The respective intensities of the MMP-9-positive band relative to the positive control in the resulting images were densitometrically semiquantified with Quantity One image processing software, version 4.3.1 (Bio-Rad), using equal-sized box-shaped markers.

Enzyme-Linked Immunosorbent Assay
An enzyme-linked immunosorbent assay (ELISA) for MMP-9 levels was next performed on the samples from the same patients and from independent urine samples, including 5 additional patients who had not yet received any medical treatment for symptomatic cervicofacial IH. The ELISA was performed with Human MMP-9 Quantikine ELISA kits from R&D Systems, Inc, in accordance with the manufacturer's instructions, using 1 μg protein in 100 μL diluent to normalize protein content across all samples. Statistical analysis was performed using Mann-Whitney tests, with significance level set at .05.

Results

Patient Characteristics
For initial proteomics studies, samples were collected from 6 patients with symptomatic IH undergoing treatment at Children's National Medical Center in Washington, DC (3 prednisolone and 3 propranolol) (Table 1). All IHs were categorized as facial, and there were no clinically important differences in extent or location. All patients were white, and 5 of the 6 patients were female. Urine samples were collected before and after treatment over the first year of life and analyzed for proteomics in a paired fashion. In addition, 5 subsequent patients, all younger than 1 year and not receiving any medical treatment, participated as controls and had their urine analyzed by means of ELISA only (Table 2). There were also no clinically important differences in the extent or location of these tumors.

Proteomic Profiling
Proteins in urine samples were separated by means of SDS-PAGE and visualized using Bio-Safe Coomassie stain. The lanes from each sample were then identically cut into 32 gel segments and processed for proteomic analysis as described in the Methods.

Overall, more than 1000 proteins were identified in the urine of these children over time. The levels of most of the identified proteins did not differ over time between the 2 treatment arms (Figure 1). Given our aim of identifying potential mechanisms of action of propranolol, we initially focused on the excreted proteins that were most suppressed by propranolol treatment in patients with IH. Matrix metalloproteinase 9 was identified as 1 of the most decreased urinary proteins in the propranolol arm relative to the prednisolone arm (Table 3). As previously mentioned, MMP-9 is a key mediator of angiogenesis, and another group has reported that it is a key antiangiogenic molecule that propranolol targets in hemangiomas.16 A list of all of the proteins identified by proteomics can be found in the eTable in the Supplement. Note that this table lists the proteins most downregulated by propranolol at the top and the most upregulated at the bottom.

| Table 1. Patient Characteristics for Initial Proteomic Analysis |
|-------------------|-----------------|---------------|
| Patient No./Race/Sex | Drug       | Location     |
| 1/White/F          | Prednisolone  | Left Facial   |
| 2/White/F          | Prednisolone  | Left periorbital |
| 3/White/F          | Prednisolone  | Right Nasal   |
| 4/White/M          | Propranolol   | Left Nasal    |
| 5/White/F          | Propranolol   | Left Tragus   |
| 6/White/F          | Propranolol   | Right Periorbital |

Unique peptides per protein, peptide length of 6 amino acids, 0.98 peptide probability, and 0.95 protein probability. To achieve a semiquantitative analysis of the identified proteins, we first sorted the proteins by number of peptide spectral counts identified during MS. Next, we determined how these spectral counts changed over time in patients by determining the fold change in spectral count for each identified protein at older than 6 months compared with younger than 6 months. The difference in the mean fold change in each study arm (propranolol vs prednisolone) was then determined.

Table 2
| Patient Characteristics for Initial Proteomic Analysis |
|-------------------|-----------------|---------------|
| Patient No./Race/Sex | Drug       | Location     |
| 1/White/F          | Prednisolone  | Left Facial   |
| 2/White/F          | Prednisolone  | Left Periorbital |
| 3/White/F          | Prednisolone  | Right Nasal   |
| 4/White/M          | Propranolol   | Left Nasal    |
| 5/White/F          | Propranolol   | Left Tragus   |
| 6/White/F          | Propranolol   | Right Periorbital |
Of note, we did not identify bFGF and VEGF in the urine of any patients in the study.

**Protein Validation**

Given these preliminary findings from proteomics, we used Western blot analysis to validate the levels of MMP-9 in urine samples on 1% agarose gels. Western blot results revealed strong MMP-9 signal intensity in all samples (Figure 2). Semiquantitative densitometry results for each sample revealed a mean 3-fold increased signal in the prednisolone arm compared with the propranolol arm over the proliferative phase of the condition.

Next, we validated the semiquantitative Western blot densitometry findings by performing an ELISA for MMP-9 levels on the same samples and additional independent urine samples, including samples from 5 patients who had not yet started medical treatment for symptomatic cervicofacial IH. Mean levels of MMP-9 in the first year of life were found to be significantly lower in propranolol-treated patients with IH compared with prednisolone-treated patients with IH (0.118 vs 0.501 ng/mL; $P = .03$) or with nontreated patients with IH (0.118 vs 3.69 ng/mL; $P = .02$) (Figure 3). Prednisolone treatment also lowered the excreted MMP-9 levels compared with those of nontreated patients with IH, but the difference was not statistically significant (0.501 vs 3.69 ng/mL; $P = .07$).

**Discussion**

Propranolol has been demonstrated to be an effective treatment for a majority of IH lesions and has virtually become the standard of care because of its rate of success and its lower risk of adverse effects compared with existing IH treatments. The mechanism of action of propranolol in the treatment of IH is not completely known and remains understudied. Propranolol is a nonselective β-adrenergic receptor antagonist that has been shown to suppress angiogenesis via inhibition of migration, proliferation, and induction of apoptosis in primary endothelial cell cultures. The β-blockade action of propranolol is known to inhibit the adrenergic mediated regulation of cyclic adenosine monophosphate production, actin cytoskeletal dynamics, and proangiogenic factors such as VEGF and bFGF. Data from this study using a urine proteomics approach demonstrated that patients treated with propranolol had attenuated excreted MMP-9 in their urine in their first year of life compared with age-matched children treated with prednisolone or without any treatment. A recent study showed a selective role for propranolol in inhibiting MMP-9 gene expression and secretion from human brain microvascular endothelial cells. Matrix metalloproteinase 9 is a pericellular protease that has been linked to angiogenesis and tumor migration by activation, liberation, and modification of angiogenic growth factors (such as VEGF) and also by degradation of the endothelial and interstitial matrices. Given that urine MMPs have been shown to correlate well to IH extensiveness and aggressiveness, it is possible that our findings are merely a reflection of how propranolol is mitigating the clinical extent of the lesion. Whether the effect of propranolol on MMP-9 is direct is the subject of future investigations in our laboratory using an endothelial stem cell...
Our MS data did not identify other important angiogenic factors, such as bFGF and VEGF. It is possible that, given the small molecular weight of these cytokines and growth factors, they ran off the edge of the 4% SDS gel and thus were not detected during the proteomics analysis. In addition, our Western blots indicated a strong MMP-9 signal at approximately 65 kDa rather than the expected 92 kDa. Given that our samples reflect excreted protein, we believe this size to represent a processed, active form of MMP-9 as noted by others.19,20 Our work is further limited by the small number of patients recruited into our clinical protocol and by the lack of participation of infants without IH in the control arm.

In conclusion, in line with other studies that have demonstrated an important role of MMP-9 in angiogenesis regulation, we hypothesize that propranolol treatment alters IH proliferation through inhibition of MMP-9 activity. It is possible that excreted MMP-9 may be a biomarker for IH propranolol responsiveness, and its signaling pathways may represent the molecular target of this drug.

**REFERENCES**


