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Confocal Microscopy Study of Nerves and Blood Vessels in Untreated and Treated Port Wine Stains: Preliminary Observations

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BACKGROUND AND OBJECTIVE. Vascular ectasia in port wine stain birthmarks (PWS) might result from reduced innervation with loss of autonomic stimulation. We investigated this theory and evaluated nerve and blood vessel density, and mean blood vessel size in untreated and treated PWS skin.

METHODS. Skin biopsy specimens were obtained from uninvolved skin, untreated PWS, PWS with a good response to laser treatment and PWS with a poor response to laser treatment. Confocal microscopy was performed to determine nerve and blood vessel density, and mean blood vessel size.

RESULTS. Nerve density was significantly decreased in all PWS sites compared to uninvolved skin. Mean blood vessel diameter was larger in untreated compared to treated PWS. PWS with a good response to treatment had decreased nerve density but blood vessel density and mean diameter was relatively normal. PWS with a poor response to treatment had decreased nerve density but increased blood vessel density and mean blood vessel diameter compared to normal skin.

CONCLUSION. Nerve density was decreased in all evaluated PWS sites and this may be a factor in lesion pathogenesis. PWS blood vessel size correlated with pulsed dye laser response and may prove to be a useful prognostic indicator of therapeutic outcome.

PORT WINE stains are congenital vascular malformations characterized by multiple dilated vessels in the dermis that become larger or more ectatic with age.[1–3] Vascular ectasia occurs in the most superficial 800 μm of the skin and involves mature dermal vessels that can be identified using immunohistochemistry.[4]

Previous studies proposed that the pathogenesis of port wine stain is related to a reduction of neural innervation around the ectatic blood vessels.[5–9] The neural defect is likely to be autonomic in nature because there is no sensory loss within port wine stain. Blood flow in the absence of tonic modulation is thought to produce port wine stain vascular ectasia. Consistent with this theory, ectasia is progressive with age, resulting in lesion darkening from pink to purple.[2]

Development of the pulsed dye laser[10,11] and description of the concept of selective photothermolysis[12] significantly improved port wine stain treatment. Yellow light ($\lambda = 585-595$ nm) emitted by the pulsed dye laser is preferentially absorbed by hemoglobin (the major chromophore in blood) in the port wine stain vessels and, after being converted to heat, causes thermal damage and thrombosis. The pulsed dye laser produces reasonably good results in a select population of port wine stain patients owing to its ability to selectively destroy cutaneous blood vessels.[13–15] Nevertheless, very few patients achieve complete port wine stain blanching despite receiving multiple laser treatments.[16–18] Further, although there are some factors (including anatomic location, size, color, nodularity, and patient age), which appear to influence treatment response, to date, none of these has been accepted as a reliable prognostic indicator of therapeutic outcome.[14]

We compared mean nerve density, blood vessel density, and mean blood vessel size in uninvolved versus lesional skin from port wine stain patients to investigate the proposed role of neural innervation. We further compared these values among untreated versus treated port wine stain sites.

Methods

Subjects

Informed consent was obtained from all subjects, and the study was approved by our institutional human research review committee in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

Three adult male patients with port wine stain were recruited for this study (Table 1). Patient ages ranged from 28 to 58 years (mean 38.7 years). Patient 1 had not received any pulsed dye laser treatment at the time of his enrollment in the study.

Patients 1 and 2 had port wine stain lesions on the upper extremity and Patient 3 had a lesion on the face and neck. Patients 2 and 3 had received pulsed dye laser treatments (eight and three treatments, respectively) for their port wine stain using 585-nm wavelength, 0.45-ms pulse duration, and energy densities ranging from 6 to 8 J/cm^2.

Biopsies

Skin biopsy specimens (3-mm-diameter punches) were taken from the following sites on the port wine stain subjects (Table 1):

1. Uninvolved skin (all patients);
2. Untreated port wine stain (Patient 1);
3. Port wine stain skin with a history of good blanching in response to pulsed dye laser treatment (Patients 2 and 3); and
4. Port wine stain skin with a history of poor blanching in response to pulsed dye laser treatment (Patients 2 and 3).

To serve as an additional control, biopsies were obtained from the right forearm of three normal healthy volunteers of the same approximate age as the port wine stain subjects.

Specimens were fixed and sectioned and immunohistochemical staining for panneuronal and panendothelial markers was performed according to the following protocol. Specimens were immediately fixed in cold (4°C) Zamboni's fixative[19] and held overnight. The next day, specimens were transferred to 20%

sucrose phosphate-buffered saline and refrigerated for 24 hr (or until processing). Thick (100-μm) sections were cut with a frozen, sliding microtome (American Optical Co., Buffalo, NY), placed in spot plates, and flooded with 0.05 mol/L phosphate-buffered saline (pH 7.4) containing 0.3% Triton X-100 (phosphate-buffered saline/Triton X-100) (Sigma Chemical Co., St. Louis, MO) and 5% normal donkey serum for 1 hr (as a blocking serum) to avoid nonspecific background staining. Subsequently, the floating sections were incubated for 16 hr at 4°C on a rotating table with the following primary antibodies:

- Panneuronal marker for nerves, protein gene product 9.5 (Ultraclone, Wellow, UK) at a working dilution of 1:1000; and
- Panendothelial marker CD31 (Dako Corporation, Carpinteria, CA) at a working dilution of 1:80.

Primary antibodies were visualized with cyanine 2 or cyanine 3.18 fluorophores conjugated to the appropriate mouse or rabbit secondary antibodies (Jackson ImmunoResearch, West Grove, PA). For secondary antibodies, floating sections were incubated overnight at 4°C on a rotating table and then washed with 1% normal donkey serum in phosphate-buffered saline/Triton X-100 for four successive incubations. Washed sections were mounted without drying on coverslips, dehydrated in alcohols, cleared with methyl salicylate (Fisher Scientific, Pittsburgh, PA), and mounted in DPX mountant (a mixture of distyrene, a plasticizer, and xylene) (Fluka, Ronkonkoma, NY) for microscopy.

Confocal Microscopy

Specimens were initially examined with a Nikon (Melville, NY) epifluorescence microscope. The sections were then imaged with a MRC-1000 scanning confocal microscope system equipped with a krypton/argon ion laser (Bio-Rad Life Science, Hercules, CA). A series of optical sections (20 × magnification) was acquired at 2-μm intervals through the entire thickness of the specimen (approximately 60 μm) and then projected into a single-focus image with the software supplied (Confocal Assistant 4.02, a free 2D and 3D

Table 1. Subject Characteristics

Patient	Port Wine Stain Location	Biopsy Sites	Number of Pulsed Dye Laser Treatments
1	Left arm	Uninvolved skin: left arm Port wine stain skin: left arm, left forearm	None
2	Left arm, forearm, wrist, hand	Uninvolved skin: forearm Good blanching: upper arm Poor blanching: hand	8
3	Right cheek, jaw, neck	Uninvolved skin: subauricular Good blanching: neck, lateral face Poor blanching: nose, perioral	3

animation software created and copyrighted by Todd Clark Brelje).

Quantification

The densities of nerves and blood vessels ($\mu m^3/\mu m^3$ tissue) were quantified for all samples using the three-dimensional software Image3 LLC Velocity[2] Pro (Salt Lake City, UT). This software uses the digital images acquired by the confocal microscope to create a three-dimensional model of the nerves and blood vessels, which can then be used to calculate density values. Measurements of blood vessel diameters were made for all samples using the public domain NIH Image program (U.S. National Institutes of Health, available on the Internet at http://rsb.info.nih.gov/nih-image/). Diameter measurements were made for dermal vessels (superficial or deep) in four random, $20 \times$ magnification fields per skin biopsy sample.

Statistical Analysis

Nerve density and blood vessel density of uninvolved skin of port wine stain subjects was compared to forearm skin of normal healthy volunteers. For comparison of uninvolved to port wine stain skin, subjects acted as their own internal controls. Statistical analysis was performed using a two-sample Student's t test assuming unequal variance using computer software (Stat View, version 5.0.1, SAS Institute Inc., Cary, NC).

Results

All densities are presented in units of $\mu m^3/\mu m^3$ tissue. Papillary and reticular dermal blood vessels were included for calculation of mean vessel diameters (Table 2).

Uninvolved Skin Port Wine Stain Subjects

Uninvolved skin from port wine stain patients had mean nerve and blood vessel densities of 18.3×10^{-3} and 6.5×10^{-3}, respectively. These values were not statistically different compared to samples from normal forearm volunteer skin where mean nerve and blood vessel densities were 10.7×10^{-3} and 6.2×10^{-3}, respectively. The mean blood vessel diameter in uninvolved skin from port wine stain patients was $15.4 \mu m$ with a range of $6.2–28.9 \mu m$ (Table 2).

Port Wine Stain Skin

Nerve density was significantly decreased (range $0.3 \times 10^{-3}–1.0 \times 10^{-3}$) in all port wine stain sites compared to uninvolved skin ($p < 0.01$). Blood vessel density was variable as described below. Mean blood vessel diameter was larger in all port wine stain sites compared to uninvolved skin.

Untreated Port Wine Stain

Histologic examination of untreated port wine stain sites of Patient 1 revealed a decrease in nerve density compared to uninvolved skin from the same patient. Nerve density was slightly lower in the distal lesion (0.6×10^{-3}) compared to the proximal (0.8×10^{-3}) extremity site. Blood vessel density was increased in untreated port wine stain compared to uninvolved skin, slightly greater in the distal lesion (24.7×10^{-3}) compared to the proximal extremity site (22.3×10^{-3}).

Mean vessel diameter was increased in untreated port wine stain compared to uninvolved skin. The biopsy from a distal port wine stain site had a greater mean vessel diameter (mean $87.7 \mu m$) than the proximal port wine stain site (mean $57.8 \mu m$).

Table 2. Blood Vessel and Nerve Measurements, by Site, for All Patients

Biopsy Group	Nerve Density, 10^{-3} $\mu m^3/\mu m^3$ Tissue	Blood Vessel Density, 10^{-3} $\mu m^3/\mu m^3$ Tissue	Mean Blood Vessel Diameter (μm)	Blood Vessel Diameter Range (μm)
Normal healthy volunteer skin	10.7	6.2	Not measured	Not measured
Uninvolved skin	18.3	6.5	15.4	6.2–28.9
Untreated port wine stain				
Distal site	0.6*	24.7*	87.7	28.2–227.8
Proximal site	0.8*	22.3*	57.8	8.6–189.5
Port wine stain				
Good blanching response	1.0*	6.9	28.4	8.5–81.2
Poor blanching response	0.3*	31.9*	49.3	26.9–283.0

*$p < 0.01$ compared to uninvolved skin.

Port Wine Stain Skin with a History of Good Blanching in Response to Pulsed Dye Laser Treatments

For Patients 2 and 3, areas with a history of good blanching in response to pulsed dye laser treatment had a less than normal nerve density of 1.0×10^{-3} ($p<0.01$) but relatively normal blood vessel density of 6.9×10^{-3} (Table 2). Blood vessel diameter was relatively normal compared to uninvolved skin (Figures 1 and 2). The range of blood vessel diameters for both patients was 8.5 to 81.2 μm (mean 28.4 μm).

Port Wine Stain Skin with a History of Poor Blanching Response to Pulsed Dye Laser Treatments

For Patients 2 and 3, port wine stain with poor blanching in response to pulsed dye laser treatment had decreased nerve density (0.3×10^{-3}; $p<0.01$) and increased blood vessel density (31.9×10^{-3}; $p<0.01$) as compared to uninvolved skin (Table 2). Blood vessels were enlarged compared to uninvolved skin (Figures 1 and 2). The range of blood vessel diameters for both patients was 26.9 to 283.0 μm (mean 49.3 μm).

Discussion

Port wine stains are progressive, vascular lesions composed of ectatic dermal capillaries.[2] As expected, our data found the mean blood vessel diameter to be larger in all port wine stain sites compared to uninvolved skin. The pathogenesis of this vascular dilatation is unknown but previous studies have proposed a reduction in neural innervation in areas of skin with port wine stain involvement.[5-7] Our confocal images confirm a significant decrease in nerve density from samples of both untreated and treated

Figure 1. Mean blood vessel diameters from port wine stain sites by blanching responses, after multiple pulsed dye laser treatments.
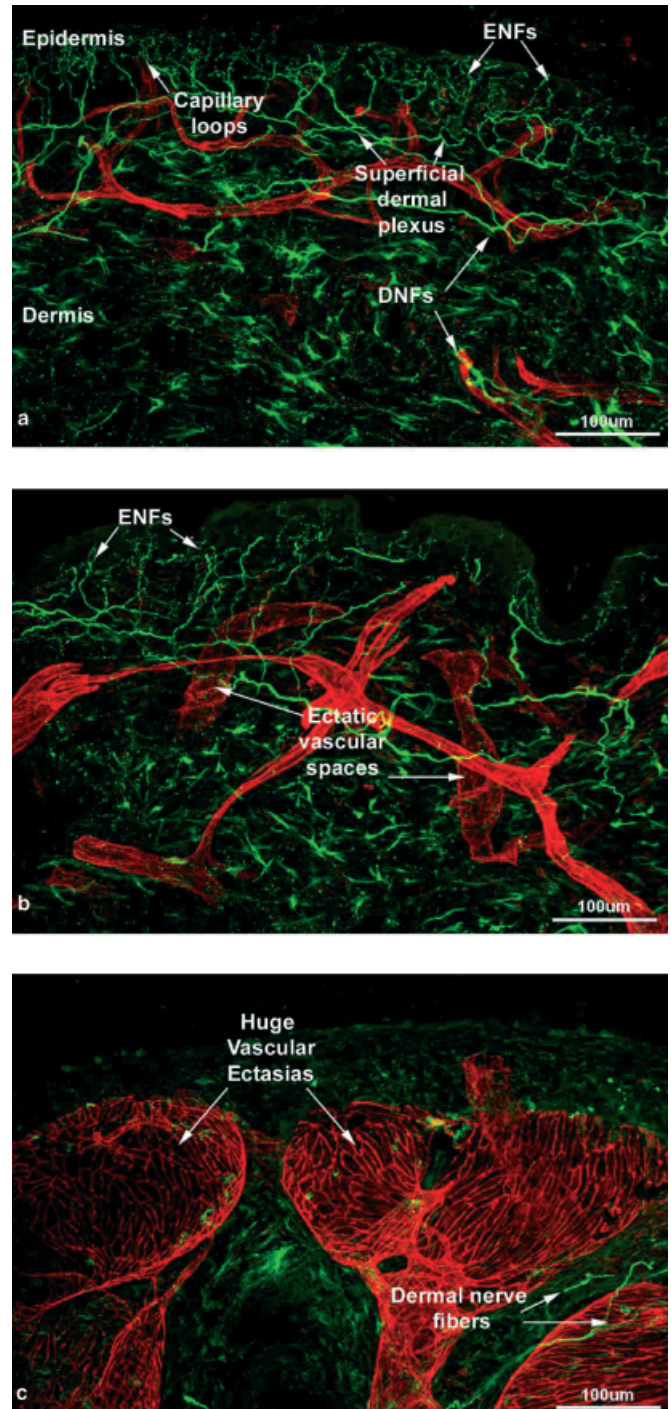

Figure 2. Confocal images from Patient 3 showing the capillaries stained with CD31 (red) and nerves stained with protein gene product 9.5 (green) (ENF, epidermal nerve fibers; DNF, dermal nerve fibers). (A) Uninvolved skin; (B) port wine stain with a history of good blanching in response to pulsed dye laser treatments; (C) port wine stain with a history of poor blanching in response to pulsed dye laser treatment. (In C, note the very large vascular ectasia and the presence of only two nerves in the field.) Original magnification, ×20; bar, 100 μm.
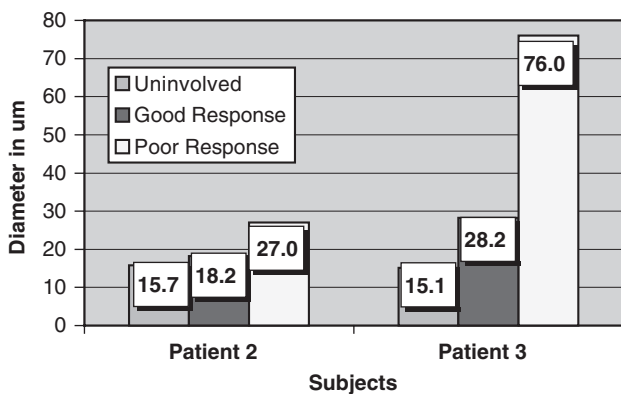
(p < 0.01) port wine stain skin compared to uninvolved skin. Our findings add support to the theory that port wine stain vascular ectasia may result from decreased neural innervation. Nevertheless, other possible explanations cannot be ruled out including the influence of a localized third factor (e.g., a cytokine), which could act during fetal development, promoting both vessel dilatation and inhibition of nerve development. It is interesting to note that the proposed role for decreased nerve density in lesion pathogenesis may be unique to port wine stain compared to other vascular lesions. Different mechanisms have been proposed for the development of other vascular entities such as hemangiomas.[20] Further study is required.

Although the port wine stain of the two laser-treated patients were from different anatomic locations (upper extremity vs. face) and received different numbers of pulsed dye laser treatments, the correlation between posttreatment blood vessel size and therapeutic outcome was consistent. Mean vessel diameter was larger in areas with a history of poor blanching in response to pulsed dye laser treatment compared to areas with a good blanching response. This relationship may be useful from a predictive standpoint as noninvasive imaging of port wine stain and determination of mean blood vessel diameter before treatment may be used as a prognostic indicator of therapeutic outcome. Patient age and a number of port wine stain characteristics such as size, color, and anatomic location have been considered as prognostic indicators of pulsed dye laser therapy. Nevertheless, to date, none of these has been accepted as a reliable indicator of therapeutic outcome.

Our data also revealed a trend toward larger vessels in untreated (Patient 1) versus treated (Patients 2 and 3) port wine stain sites. Because there were no pretreatment biopsies for Patients 2 and 3, we are unable to determine from this study if the decrease in mean vessel size in treated port wine stain was a result of laser therapy. Nevertheless, we believe this to be a likely explanation, which should be explored in future prospective studies.

In a single extremity port wine stain, mean vessel diameter varied depending on site (distal vs. proximal). Variance in vessel size within a port wine stain has important implications for laser treatment. At present, clinicians most often treat an entire port wine stain lesion with identical laser parameters such as wavelength, pulse duration, energy density, and cryogen spray cooling spurt duration.[21] Nevertheless, port wine stain blood vessel size and depth affect the optical absorption and scattering properties of the skin and, if these factors vary, laser parameter adjustments for different areas within a lesion is likely to be beneficial. Diagnostic imaging methods for port wine stain

characterization such as infrared tomography and optical Doppler tomography are being evaluated as means of rapidly and noninvasively determining blood vessel size and depth to optimize laser treatment parameters on a site-to-site basis.[22–24]

The sample size in this study is small and patients were not followed with serial biopsies during a course of treatment. Our conclusions will be confirmed in a larger study in which we will follow serial biopsies (beginning pretreatment) from multiple port wine stain sites obtained from the same and different individuals.

Conclusions

Nerve density was decreased in all evaluated port wine stain sites and this may be a factor in lesion pathogenesis. Port wine stain blood vessel size correlated with pulsed dye laser blanching response and may prove to be a useful prognostic indicator of therapeutic outcome.

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