GYNECOLOGY

Immune activation enhances epithelial nerve growth in provoked vestibulodynia

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BACKGROUND: Provoked vestibulodynia manifests as allodynia of the vulvar vestibular mucosa. The exact mechanisms that result in altered pain sensation are unknown. Recently, we demonstrated the presence of secondary lymphoid tissue, which is the vestibule-associated lymphoid tissue in the vestibular mucosa, and showed that this tissue becomes activated in provoked vestibulodynia.

OBJECTIVE: The purpose of this study was to examine whether expression of intraepithelial nerve fibers and nerve growth factor are related to immune activation in provoked vestibulodynia.

STUDY DESIGN: Vestibular mucosal specimens were obtained from 27 patients with severe provoked vestibulodynia that was treated by vestibulectomy and from 15 control subjects. We used antibodies against the protein gene product 9.5, the neuron specific neurofilament, and nerve growth factor for immunohistochemistry to detect intraepithelial nerve fibers and nerve growth factor expressing immune cells in the vestibular mucosa. For intraepithelial nerve fibers, we determined their linear density (fiber counts per millimeter of the outer epithelial surface, protein gene product 9.5) or presence (neuron specific neurofilament). Nerve growth factor was analyzed by counting the staining-positive immune cells. Antibodies against CD20 (B lymphocytes) and CD3 (T lymphocytes) were used to identify and locate mucosal areas with increased density of lymphocytes and the presence of germinal centers (ie, signs of immune activation). B-cell activation index was used to describe the overall intensity of B-cell infiltration.

RESULTS: We found more protein gene product 9.5—positive intraepithelial fibers in vestibulodynia than in the control samples (6.3/mm [range, 0.0—15.8] vs 2.0/mm [range, 0.0—12.0]; P=.006). Neuron specific neurofilament —positive intraepithelial fibers were found in 17 of 27 vestibulodynia cases (63.0%) and in none of the control cases. Protein gene product 9.5—positive intraepithelial fibers were more common in samples with more pronounced immune activation. The density of these fibers was higher in samples with than without germinal centers (6.1/mm [range, 4.3—15.8] vs 3.0/mm [range, 0.0—13.4]; P=.020). A positive correlation between the fiber density and B-cell activation index score of the sample was found (Spearman's Rho, 0.400; P=.004; R^2=0.128). No significant difference, however, was found in the density or presence of nerve fibers between samples with high and low T-cell densities. We identified areas of minor and major vestibular glands in 16 of the patient samples and in 1 control sample. Protein gene product 9.5—positive nerve fibers were found more often in glandular epithelium surrounded by B-cell infiltration than in glands without B cells (P=.013). Also, the presence of neuron specific neurofilament—positive fibers in glandular epithelium was associated with B-cell infiltrates (P=.053). Nerve growth factor—positive immune cells were more common in mucosal areas with than without B-cell infiltration and intraepithelial nerve fibers.

CONCLUSION: Excessive epithelial nerve growth in provoked vestibulodynia is associated with increased B-cell infiltration and the presence of germinal centers. This supports the fundamental role of immune activation in provoked vestibulodynia.

Key words: germinal center, immune activation, immunohistochemistry, inflammation, nerve fibers, NGF, NF2F11, PGP9.5, vestibulodynia, vulvar pain, vulvar vestibulitis, vulvodynia

Provoked vestibulodynia (PVD), which also is referred to as localized provoked vulvodynia, manifests as allodynia (severe pain by touch) of the vulvar vestibular mucosa in the absence of any other disease or identifiable cause.[1]

Histopathologic investigation of PVD typically reveals increased lymphocytic infiltrates in the vestibular mucosa.[2,3] Recently, we demonstrated the presence of secondary lymphoid tissue, which is the vestibule-associated lymphoid tissue (VALT) in the vestibular mucosa, and showed that VALT becomes activated in PVD. We showed higher numbers of B cells in PVD than in control samples but found no difference in the density of T cells between the groups.[4] An exaggerated immunoinflammatory response and dysregulation of inflammation seem to be present in PVD.[5-7] The close relation between immune and neuronal systems can activate neuroinflammatory processes and lead to sensitization of nerve fibers. Immune cells produce nerve growth factor (NGF), which may induce nerve sprouting and enhanced signaling of the nociceptive nerve endings.[8] Thus, it is important to study the interrelation between immune activation and nerves in PVD. Previous studies have shown increased density of nerves in the vestibular mucosa in PVD and increased expression of transient receptor potential V1 (TRPV1) channels in these nerves,[9-12] but no specific data on the density of intraepithelial nerve fibers (IENF) or expression of NGF exist.

We wanted to find out whether the density and localization of IENFs and the expression of NGF are related to immune activation in the vestibular mucosal tissue in PVD. In addition to the standard neural marker, the protein gene product 9.5 (PGP9.5), we used a specific marker for neurofilaments.[13] To define the sites of immune activation, we used 2

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standard markers, CD20 (B cells) and CD3 (T cells). We explored the differences in the expression of IENFs and NGF between PVD and control samples and in relation to different B-cell and T-cell densities.

Material and Methods

Study subjects

The study material consisted of 27 archival vestibulectomy specimens from posterior vestibulectomy operations. The patients were identified in the Helsinki University Hospital patient registry by matching the diagnosis (vulvar vestibulitis, vestibulodynia, and vulvodynia) and the surgical procedure (posterior vestibulectomy). Details of patient recruitment and data collection have been described previously.[4,14] A good quality paraffin block of the tissue specimen was required. All the included patients had a long disease history (4.0 years; range, 2−18 years) of PVD. The diagnoses for 8 patients were classified as primary (symptoms already at the first vaginal entry); the diagnoses for 15 patients were classified as secondary (symptoms appearing later after an interval of painless intercourses), and the classification for 4 patients was unknown. All patients had been refractory to conservative treatments. The time from the last attempted medical management was >6 months. As control subjects, we recruited 15 healthy volunteers with no vulvar complaints who underwent benign gynecologic surgery. All participants were premenopausal. The median age of the patients with PVD was 27 years (range, 18−48 years); the median age of the control subjects was 30 years (range, 24−44 years; P=.017). A 4-mm punch biopsy specimen from the posterior vestibule at 5 o'clock position was obtained from the control subjects. Both patients and control subjects had provided informed consent. The local Ethical Committee approved the study.

Tissues

All vestibular tissues were embedded routinely in paraffin after a maximum of 24 hours fixation in 10% buffered formalin. Five-micrometer sections were first stained with hematoxylin-eosin to exclude dermatologic diseases and to confirm the quality of the samples. Immunohistochemistry for nerve fibers (10-μm sections) and B and T lymphocytes (5-μm sections) was performed at the Helsinki and Uusimaa Hospital District Laboratory Services tissue laboratory. Routine staining procedures according to the manufactures' instructions were followed (Table). Immunostaining for NGF (5-μm sections) was performed at the Department of Clinical Chemistry, University of Helsinki (manual staining procedure[15]; Table).

Tissue analyses

Immunohistochemical scoring was performed under light microscopy (Nikon Eclipse E800; Nikon Instruments Inc, Melville, NY) at ×200 magnification. The scoring of each section was based on a consensus of 2 investigators (P.T., A.P., or S.M.) who were blinded to clinical data of the patients. The number of PGP9.5-positive IENFs was counted to calculate the linear density of IENFs (number of nerve fibers /millimeters of epithelial outer surface). For identification of individual fibers, we used the criteria that had been validated for the diagnostics of small fiber neuropathies.[16] Briefly, the fibers were considered to be separate if there were clearly 2 individual parallel fibers and if the distance between 2 different perpendicular sections of a stained axon exceeded 5 times the diameter of an axon. Only fibers clearly penetrating into the epithelium through the basal membrane were counted as IENFs. For neuron specific neurofilament (NF2F11)−positive IENFs only the presence or absence was documented. The overall density of neural fasciculi in the neural plexus region in the subepithelial stroma up to the depth of 1.25 mm (diameter of the ×20 high-power field) was scored semiquantitatively for both neural markers. A single number score from 1−3 (1=low density, 2=moderate density, 3=high density) was given.

Evaluation of the vestibular glands was also limited to the depth of 1.25 mm. The glands were identified on the basis of typical morphologic condition. All comparisons were made between PVD samples and control samples. In PVD samples, densities of epithelial nerve fibers were also compared between areas with or without increased B-cell infiltration. The representative areas of B-cell infiltration in each sample were located with the use of CD20 staining. To reflect the overall level of B-cell infiltration of each sample, we used the B-cell activation index (BAI). BAI is the calculated sum (0−12) of 3 different parameters that were analyzed from each sample: (1) overall density of B cells in the epithelium (score, 0−4), (2) overall density of B cells in the stroma (score, 0−4), and (3) absence (score, 0) or presence (score, 4) of germinal centers.[4] T-cell density was divided in the CD3-stained samples into 2 categories: "low density" (<50 cells/×20 high-power field) and "high density" (>50 cells/×20 high-power field). Germinal centers were visualized by CD20 and CD3 stainings.

For NGF quantification, 4 types of areas from the PVD samples were identified: (1) areas with increased B-cell infiltration without IENFs, (2) areas without increased B-cell infiltration with IENFs present, (3) areas with both increased B-cell infiltration and IENFs, and (4) areas lacking both B-cell infiltration and IENFs. The number of NGF-positive immune cells per visual field (×20 high-power field) in 3 of each type of areas in each sample was counted, and the mean number of positive cells was calculated. In the control samples, NGF-positive cells were evaluated only in the areas with IENFs present because no areas with increased B-cell infiltration were found.

The data were analyzed by Statistical Package for Social Sciences software (version 22; IBM Corporation, Armonk, NY). We report medians with minimum and maximum and interquartile range (IQR, 25−75%) when appropriate for continuous data. For comparisons, we used the Mann-Whitney U-test and Wilcoxon signed ranks test for continuous data and χ^2 analysis or Fisher's exact test for categoric data. For correlations, the Spearman's correlation test was used. A 2-tailed probability value of <.05 was considered significant.