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.

TABLE
Immunohistochemistry procedures

Antibody	Clone; catalog number; manufacturer	Pretreatment buffer(pH); catalog number; manufacturer	Dilution; incubation time/°C	Detection system; catalog number; manufacturer	Staining instrument
Protein gene product 9.5 (PGP9.5)	Polyclonal	No pretreatment	1:1000	EnVision Detection SystemsPeroxidase/DAB	LabVision
	RA95101		30 Min/room temperature	K5007	
	Ultra Clone Ltd, Wellow, Isle of Wight, England			Agilent Technologies Inc, Santa Clara, CA	
Neuron specific neurofilament (NF2F11)	2F11	Tris-EDTA (pH 9,0)	1:200	EnVision Detection SystemsPeroxidase/DAB	LabVision
	M0762	S2367	30 Min/room temperature	K5007	
	Agilent Technologies Inc	Agilent Technologies Inc		Agilent Technologies Inc	
CD20	L26	CC1 (pH 8,0)	Ready to use	UltraView Universal DAB Detection Kit	Ventana XT
	760-2531		24 Min/room temperature	760-500	
	Roche Diagnostics Ltd, Rotkreuz, Switzerland	Roche Diagnostics Ltd		Roche Diagnostics Ltd	
CD3	2GV6	CC1 (pH 8,0)	Ready to use	UltraView Universal DAB Detection Kit	Ventana XT
	790-4341	950-124	32 Min/room temperature	760-500	
	Roche Diagnostics Ltd	Roche Diagnostics Ltd		Roche Diagnostics Ltd	
Nerve growth factor (NGF)	Polyclonal	EnVision FLEX Target Retrieval Solution (pH 6,1)	0.5 μg/mL	MACH 4 Universal AP Polymer Kit, BioCare Medical, Concord, CA	Manual
	sc-548	K8005	Overnight/4°C	M4U536	
	Santa Cruz Biotechnology Inc, Santa Cruz, CA	Agilent Technologies Inc		Biocare Medical Inc, Concord, CA	

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Results

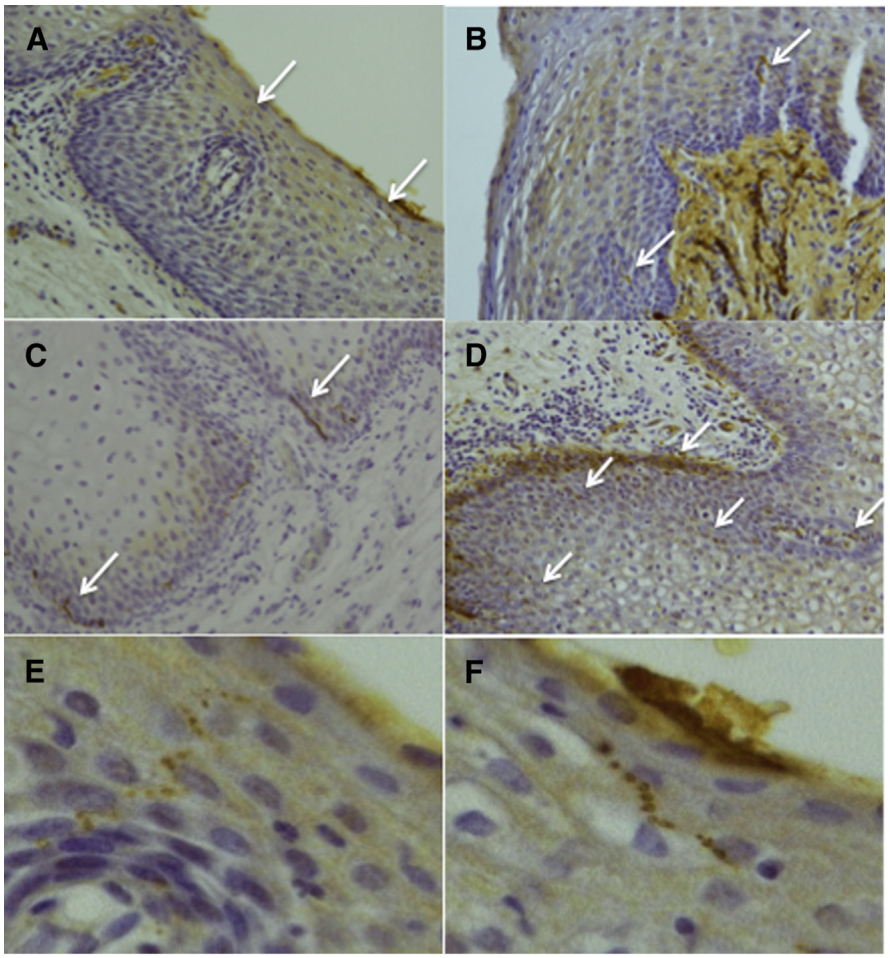
Intraepithelial nerve fibers

Because PVD may be related to increased pain sensitivity, we looked at individual nerve fibers in the vestibular epithelium. Both in PVD samples and control samples, PGP9.5-positive IENFs were expressed typically in clusters. Thus, such areas were studied for PGP9.5-positive IENFs. PGP9.5-positive IENFs were found in 24 of the 27 PVD samples (88.9%) and in 8 of the 15 control samples (56.2%). The density of PGP9.5-positive IENFs was significantly higher in PVD (6.3/mm [range, 0.0−15.8]; IQR, 4.4−9.2) than in control samples (2.0/mm [range, 0.0−12.0]; IQR, 0.0−4.3; P=.006; Figure 1, A and B). No significant difference was found in the density of IENFs between 8 primary PVD cases (7.5/mm [range, 3.3−15.8]; IQR, 5.3−8.3) and 15 secondary PVD cases (5.0/mm [range, 0.0−12.4]; IQR, 2.5−9.2; P=.332). NF2F11-positive IENFs typically occurred as solitary fibers or in clusters of 3−8 fibers (Figure 1, C) and were found in 17 of the 27 PVD cases (63%) and in none of the control cases (P<.001). NF2F11-positive IENFs were as common in primary and secondary PVD (P=.627).

IENFs in relation to B-cell infiltrates

Our previous study showed increased density of B lymphocytes in the vestibular

FIGURE 1
Intraepithelial nerve fibers in the vulvar vestibular mucosa



Intraepithelial nerve fibers (*white arrows*) **A,** in provoked vestibulodynia in a mucosal area without B-lymphocyte infiltration, **B,** in a control (stained by protein gene product 9.5), **C,** in provoked vestibulodynia (stained by neuron specific neurofilament), and **D,** in provoked vestibulodynia in a mucosal area with B-lymphocyte infiltration (protein gene product 9.5). **E** and **F** show the protein gene product 9.5—stained intraepithelial nerve fibers from panel **A** in a larger magnification. Histologic sections were counterstained with hematoxylin and photomicrographed with a ×20 objective (Nikon Eclipse E800; Nikon Instruments Inc., Melville, NY).

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mucosa of patients with PVD.[4] The density of PGP9.5-positive IENFs was greater in the areas with increased B-cell infiltration (5.3/mm; range, 0.0−23.3; IQR, 3.0−9.4) than in the areas with no B cells (4.0/mm [range, 0.0−11.3]; IQR, 1.0−8.0; *P*=.057; Figure 1, D). In control samples, no areas with increased B-cell infiltration were found.

IENFs in relation to signs of immune activation

Germinal centers indicate immune activation and are the key component of the local lymphoid tissue, which we have termed as VALT.[4] Infiltrating lymphocytes were primarily B cells that were stained by the CD20 marker. T cells were distributed more evenly across the samples and did not form clusters. The density of PGP9.5-positive IENFs was significantly higher in samples with germinal centers (6.1/mm [range, 4.3−15.8]; IQR, 5.0−9.4) than in samples without germinal centers (3.0/mm [range, 0.0−13.4]; IQR, 0.0−8.4; *P*=.020). A positive correlation between the density of PGP9.5-positive IENFs and the BAI score was found (Spearman's Rho, 0.400; *P*=.004; R^2=0.128). NF2F11-positive IENFs were not associated with the presence or absence of germinal centers. However, the BAI scores of the samples with NF2F11-positive IENFs were higher (5.0 [range, 1.0−9.0]; IQR, 4.0−6.0) than the BAI scores of the samples without fibers (2.0 [range, 1.0−9.0]; IQR, 1.0−3.0; *P*=.005). No differences were found in the densities of PGP9.5-positive fibers between the low-density and high-density T-cell groups (6.8/mm [range, 0.0−15.8] and 4.6/mm [range, 0.0−12.4], respectively; *P*=.112) or in the presence of NF2F11-positive fibers (*P*=.080).

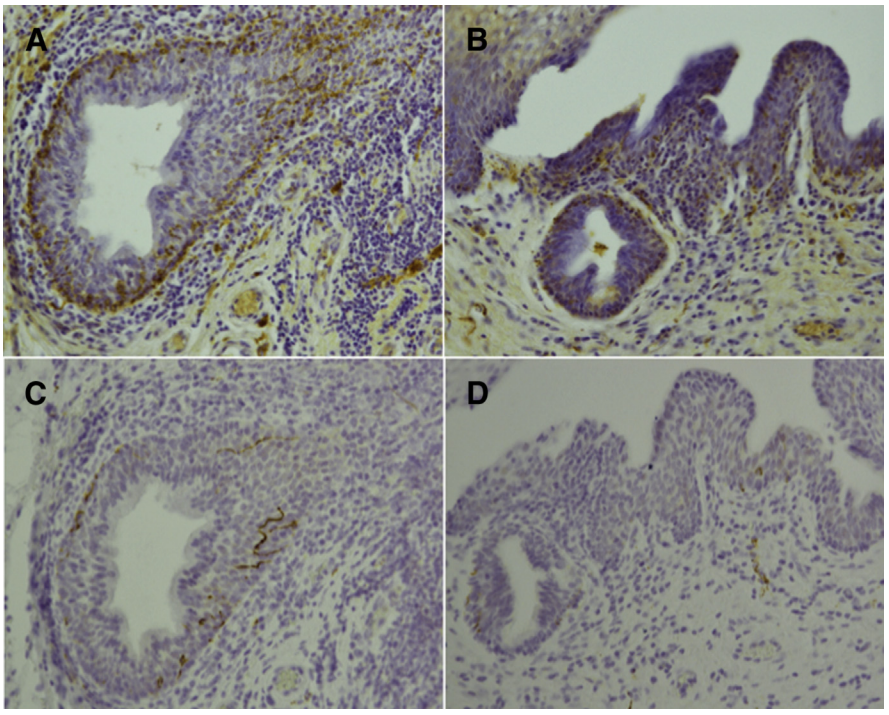
Glandular epithelium and nerve fibers in relation to B lymphocytes

Vestibular glands typically occur in the subepithelial layer of the vulvar vestibular mucosa.[17] In the PGP9.5-stained PVD samples, 14 regions with glandular epithelium were identified. Of these, 9 were in areas with increased lymphocytic infiltration that showed a glandular epithelial nerve fiber density of 25.0/mm (range, 3.1−48.0; IQR, 11.3−32.0; Figure 2, A). Five regions were in areas without increased numbers of lymphocytes. Here the glands showed significantly lower epithelial nerve fiber density of 2.0/mm (range, 0.0−17.0; IQR, 0.0−16.5; *P*=.013). In the control samples, only 1 area of glandular epithelium was identified with an epithelial nerve fiber density of 2.0/mm, which was located in an area with no lymphocytic infiltration. In the NF2F11-stained PVD samples, 16 glandular regions were identified, 12 in areas with lymphocytes and 4 in areas without. Again, epithelial nerve fibers were present more commonly in glands that were surrounded by lymphocytic infiltrates (11/12) than in glands not surrounded by lymphocytic infiltrates (2/4; *P*=.053, Fisher's exact test).

Neural fasciculi in the subepithelial stroma in relation to signs of immune activation

The densities of the neural fasciculi in the subepithelial stroma by PGP9.5 or

FIGURE 2
Nerve fibers in the vestibular glandular epithelium



Glandular epithelial nerve fibers in provoked vestibulodynia in vestibular mucosal areas with B-lymphocyte infiltration **A** and **B,** immunostaining for protein gene product 9.5, **C** and **D,** immunostaining for neuron specific neurofilament. Histologic sections were counterstained with hematoxylin and photomicrographed with a ×20 objective.

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NF2F11 staining did not differ between PVD and control samples (P=.110 and .498, respectively) or between primary and secondary PVD (P=.289 and .657, respectively). No association between neural tissue density and signs of immune activation was found (data not shown).

NGF-positive immune cells in relation to lymphocyte infiltrates and IENFs

NGF is essential in the development of peripheral nervous system by promoting growth and survival of neural cells.[18] In mature tissue, it has important roles both in acute nociception and chronic pain.[19] In the PGP9.5-stained PVD samples, the number of NGF-positive immune cells was higher in the areas with B-cell infiltrates and IENFs (20.0; range, 1.0−102.0) than in the areas lacking both B-cell infiltrates and IENFs (4.0 [range, 0.0−24.0]; P<.001). Because of wide variation, there was no significant difference between primary and secondary PVD in the density of NGF-positive cells (40.0 [range, 1.0−47.0] and 20.0 [range, 3.0−102.0], respectively; P=.256). Similarly, densities of these cells in areas with IENFs did not differ between PVD and control samples (20.0 [range, 1.0−102.0] vs 17.5 [range, 0.0− 68.0], respectively; P=.906; Figure 3). The numbers of NGF-positive immune cells did not differ in relation to T-cell density either (25.0 [range, 1.0−102.0] for the low-density samples and 17.0 [range, 0.0−60.0] for the high-density samples; P=.300).

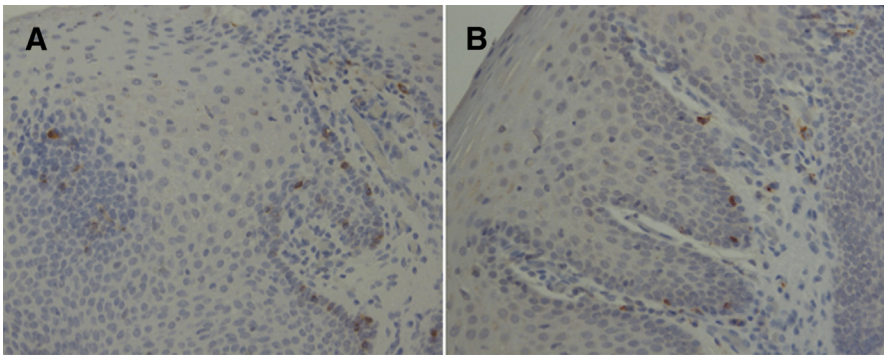
Comment

We conducted a thorough analysis of nerve fibers in the vestibular mucosa of patients with PVD and healthy control subjects by using 2 different neural markers, PGP9.5 and NF2F11. We showed that the density of IENFs in the vestibular mucosa was greater in PVD than in control subjects. Recently, we demonstrated the existence of the VALT[4] and showed evidence of immune activation in PVD by identifying germinal centers and higher densities of B cells in PVD than in control subjects. We now show that the epithelial neuroproliferation is associated with VALT and takes place around the areas with B-cell infiltration. Our results suggest that immune activation explains the neuroproliferation and may contribute to the altered pain sensation in PVD.

Germinal centers emerge in the mucosa as a sign of immune activation. Although germinal centers mostly consist of B cells, T cells are also present in the follicular area and have important functional roles as helper cells that stimulate the immune responses.[20] Density of T cells did not show effect on neuroproliferation. This is in line with our earlier study in which we, unlike others,[3,21] found no difference in T-cell density between patients and control subjects. Further studies are needed to dissect the different T-cell subgroups in relation to nerve fibers and development of germinal centers.

PGP9.5 is a well-established neuron-specific marker that detects the thinnest unmyelinated sensory C fibers, which are <1 μm in thickness.[22] PGP9.5 is the gold standard biomarker for IENFs. However, it is a pan-neuronal marker and, besides the small fibers, also stains subepithelial nerve bundles. Previous studies on PVD have shown an overall abundance of neural tissue in the vestibular mucosa of patients with PVD with no quantification of the IENF density.[3,11,23] Thus, by calculation of the linear density of IENFs, the validated method of evaluating IENFs, we were able to demonstrate an actual increase in the IENFs in PVD. Furthermore, we found that only the expressions of IENFs differed between PVD and control samples and between inflammatory and noninflammatory areas; the overall density of neural tissue in the stroma (detected by PGP9.5 and NF2F11) was comparable. This finding disagreed with a previous study that showed neural hyperplasia by PGP9.5 in PVD

FIGURE 3
Nerve growth factor positive immune cells in the vestibular mucosa



Immunostaining for nerve growth factor—positive immune cells (*red*) in **A,** provoked vestibulodynia and **B,** control. Histologic sections were counterstained with hematoxylin and photomicrographed with a ×20 objective.

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compared with control samples[9] and with studies that used S-100 staining.[3,23,24] However, the S-100 antigen is not specific to neural tissue but may also stain structures of dendritic cells.[25] Dendritic cells are numerous in the stroma and epithelium of vestibular mucosa, which may cause misinterpretation.[4] We did not find any difference in the IENF density or in the density of subepithelial neural tissue between primary and secondary PVD, contrary to previous reports that have suggested predominance of the overall neural tissue in primary PVD.[3,24]

NF2F11 detects neuronal axons by labeling the neurofilaments of the cytoskeleton. Neurofilament proteins are expressed exclusively in neurons and are essential for radial growth, axonal caliber maintenance, and myelination of the axons.[26] In mature neurons, the neurofilaments are composed of 3 subunits with different molecular weights. Phosphorylated forms of the subunits are expressed by axons; the nonphosphorylated forms represent somal and dendritic proteins.[27] Our study is the first to show NF2F11 expression in the vestibular mucosal tissue. We used the monoclonal 2F11 antibody, which detects the light molecular weight phosphorylated form of the subunit (the NFL subunit, 70 kDa), that is known to be crucial in the myelination process of the axons.[26] Thus, it is possible that these nerve fibers that are detected by the NF2F11 antibody might be myelinated A-delta fibers that signal a faster mode of sharp pain. It is important to note that no NF2F11-positive epithelial nerve fibers were found in the control samples.

The unmyelinated C-fibers and thinly myelinated A-delta fibers together constitute the small caliber nerve fibers. These fibers transmit cold, warm, and mechanical nociceptive stimuli.[28] Many neuropathic pain states (such as diabetic neuropathy, drug-induced neuropathies, and the burning mouth syndrome) are characterized by decreased density of these small nerve fibers.[29,30] Decreased density of these IENFs in a skin biopsy is the hallmark of the diagnosis of small fiber neuropathies.[31] Our current finding of a high density of intraepithelial small fibers in PVD suggests that PVD is not a small fiber neuropathy. To our knowledge, the only 2 other examples with increased pain sensitivity and increased density of IENFs that have been documented in the literature are rectal hypersensitivity disorder and dry eye disorder.[32,33] Little is known about the distribution of nerve fibers in the genital mucosal tissue of healthy women.

We, like others,[2,34] demonstrated the histopathologic characteristic of increased lymphocytic inflammation, especially around the vestibular glands in PVD. We also demonstrated nerve fibers in the glandular epithelium by both PGP9.5 and NF2F11 stainings. In agreement with our finding of IENFs, the density of nerve fibers was higher in the glandular epithelium of glands with surrounding B-cell infiltrates than in area without B cells. These are the first data on the nerve supply of the vestibular glands. The glandular innervation may also be a relevant factor in the sensitized pain perception in PVD.

Recent research has revealed that neuropathic pain because of different diseases indicates different somatosensory profiles.[35] By quantitative sensory testing, it is possible to distinguish between disorders with irritable nociceptors that show positive (ie, sensory gain) sensory signs and disorders with nonirritable nociceptors that show negative (ie, sensory loss) sensory signs.[36] Many studies that have compared patients with PVD with pain-free control subjects have shown lower tactile, pain, and thermal thresholds and higher suprathreshold magnitude estimations for heat in the vulvar vestibule in PVD.[37,38] Only findings that have suggested sensory gain have been reported.[39] This might well be a result of increased density of irritable nociceptors.

Increased density and sensitivity of IENFs in PVD might be due to the upregulation of NGF because of a previous inflammatory process and immune activation. In addition to our finding of epithelial neuroproliferation and its association with immune activation, others have shown increased density of the TRPV1 in PVD.[12] NGF induces nerve sprouting and contributes to the generation of inflammatory hypersensitivity and allodynia.[40-42] Inflammatory and immune cells are major sources of NGF.[19] In a recent study on experimental chronic prostatitis in mice, Schwartz et al[43] showed elevated levels of NGF in the prostate tissue and upregulation of TRPV1 in the dorsal root ganglion neurons. We found an increased number of NGF-positive immune cells in the mucosal areas with B-cell infiltration and IENFs in PVD but could not show any difference in the number of NGF-positive cells between PVD and control samples. However, IENFs were present in only some of the

control samples, which may have caused bias. Furthermore, without analyzing the actual tissue level differences of NGF, no conclusions about the role of NGF in PVD pain generation can be drawn. It may be a question of the amount of NGF produced and not the number of cells producing it. Also, NGF might have been up-regulated already in an earlier phase of the disease and could no longer be verified in these samples that represent a late phase of the condition. NGF recently has been linked to many other syndromes that indicate chronic and neuropathic pain; research that targets blocking NGF is ongoing.[19] Encouraging results on the novel protein kinase inhibitor dilmapimod in reducing cytokine production of the immune cells have been reported.[44] Thus, as 1 potential drug candidate, the protein kinase inhibitor could inhibit neurogenic inflammation in PVD.

Our study is the first to report the linear density of IENFs in PVD and is the first to show the expressions of NF2F11-positive, possibly A-delta fibers, and innervation of the vestibular glands. A further strength is the higher number of proven cases and control cases than in most previous tissue studies on PVD.[3,11,45] The 3-year age difference between cases and control cases, all being premenopausal, is unlikely to play a role. In line with many previous studies on vestibular tissue, our challenge was the size difference between the PVD and control samples. To minimize the bias and to secure appropriate comparison, the control biopsy samples were taken exactly from the 5 o'clock position of the posterior vestibule. This is the most representative area of pain in PVD that has been indicated by studies on vulvar pain mapping and studies on the effect of surgical treatment of PVD.[46,47] In addition, the depth of all analyses was limited to 1.25 mm according to the depth of control biopsy samples to minimize the sample size bias. However, the size difference may have biased the evaluation of the focally distributed NF2F11 fibers. The lack of gland tissue in the control samples probably was due to the small size of the biopsy samples. Most importantly, however, the linear density evaluation of the C-fibers (numbers per length unit, millimeters) was not compromised because of the sample size difference. Dual staining to reveal the TRPV1 channels in the C-fibers would have been useful. The detection of myelin structures in the NF2F11-positive fibers could have confirmed the A-delta nature of the fibers.

We provide new information to the question of the key mechanisms of allodynia in PVD. NGF may be up-regulated because of the activation of VALT and in turn induces sprouting and sensitization of the nociceptive nerve fibers, which suggests a key role to the interplay between immune and neural systems. Future research that will focus on mechanisms in PVD might lead ultimately to the development of new therapeutic interventions. ■

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