Morphologic and Confocal Investigation on Salzmann
Nodular Degeneration of the Cornea

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Purpose. To investigate the ultrastructure of advanced Salzmann nodular degeneration (SND) and to correlate it to confocal in vivo findings.

Methods. The corneal degenerative nodules from four patients with SND were examined with confocal microscopy and then removed and processed for light microscopy (LM) and transmission electron microscopy (TEM).

Results. The confocal examination revealed elongated basal epithelial cells and activated keratocytes in the anterior stroma near the nodules. Occasional subbasal nerves and tortuous stromal nerve bundles were observed. With LM and TEM, five zones were described: one internodular and four pertaining the nodule, each characterized by peculiar aspects of the epithelium and stroma. As also confirmed by the morphometry, in the zones corresponding to the nodules, the epithelium was lower and with fewer cell layers than the peripheral zones. Over the nodules, the basement membrane was discontinuous or absent and the Bowman’s layer, when present, had a granular-filamentous appearance. The nodular stroma was formed by many activated keratocytes and irregular lamellae. Subbasal nerves were always isolated and had degenerative changes in the center of the nodule.

Conclusions. This work illustrates the confocal microscopic findings associated with LM and TEM observations in patients with advanced SND. Our data revealed milder changes of the epithelium together with more pronounced changes of the basement membrane and Bowman’s layer, which are aspects of increased keratocyte activity and an altered nerve pattern. All of these structures seem to contribute to the characteristic corneal disorganization of SND. (Invest Ophthalmol Vis Sci. 2011;52:5910–5919) DOI:10.1167/iovs.11-7789

Salzmann nodular degeneration (SND) of the cornea is a non-inflammatory, slowly progressive, generally bilateral disease that is characterized by single or multiple whitish-gray nodules raised above the corneal surface. It is considered a rare corneal affection. It might follow recurrent and chronic corneal disorders,1,2 even if several cases without any previous corneal disease have been reported.3 In some patients, it follows a surgical4 or a traumatic5 wound of the cornea or it is associated with chronic inflammatory, slowly progressive, generally bilateral disease that is characterized by single or multiple whitish-gray nodules raised above the corneal surface. It is considered a rare corneal affection. It might follow recurrent and chronic corneal disorders,1,2 even if several cases without any previous corneal disease have been reported.3 In some patients, it follows a surgical4 or a traumatic5 wound of the cornea or it is associated with chronic inflammatory diseases, such as Crohn’s disease.6 However, the pathogenesis of SND is still unknown, and the coexistence of multiple factors that lead to a nonspecific corneal reaction based on individual disposition has been proposed.7 More recently, an analysis of some epithelial markers revealed that the basal cells of the nodular epithelium were metabolically active, suggesting their major role in the pathogenesis of SND.8

The common histopathologic findings are noninflammatory superficial nodules formed by dense connective tissue with hyaline degeneration that are localized subepithelially and extend until one-third of the anterior stroma.7,9 Among the existing reports regarding the structural analysis of SND, only four include electron microscopic data,3,9–11 and other four7,12–14 show an in vivo examination with the confocal microscope.

In this study, we propose a comparison in the same patients between in vivo confocal findings of bilateral advanced SND and the structural and ultrastructural analysis of the removed corneal layers, with particular regard to the corneal epithelium and the underlying tissue of the Salzmann’s nodules.

Materials and Methods

Four patients (one male and three females; mean age, 53.5 ± 8.6 years) with advanced bilateral corneal SND were studied. The nodules, sometimes confluent, were present in all corneal quadrants of the examined eyes. Ethical approval was granted by the Ethics Committee of the University Hospital of Messina, and the study was conducted in concordance with the tenets of the Declaration of Helsinki. Informed consent was obtained from all patients after explanation of the nature and possible consequences of the study.

In Vivo Confocal Microscopy

In vivo confocal microscopy was performed with a confocal microscope (ConfoScan 4; Nidek Technologies, Vigozna PD, Italy) after topical instillation of unpreserved 0.4% oxybuprocaine (Novesina; Novartis Farma, Origgio VA, Italy). The examination was performed with the 40× contact objective with the additional Z ring probe to allow a precise positioning of the probe over the selected corneal areas. An ophthalmic gel medium (Viscotear; Novartis Farma) was used. Two areas for each eye were studied: the clear optical zone, corresponding to the central cornea, and the nodular area.

SND Removal

At least 7 days after confocal microscopy, the patients underwent manual removal of the nodules, as previously described.7,15 In brief, topical instillation of unpreserved 0.4% oxybuprocaine (Novesina) was used to anesthetize the ocular surface. A 9.5-mm diameter cone (J2909.2; Janach, Como, Italy) was placed on the cornea and was filled with a 20% ethanol solution in distilled water for 25 seconds. The cone was emptied with a sponge and the ocular surface was washed off with balanced salt solution. The tissue was lifted with a beaver blade and was peeled off with forceps.

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LM and TEM

The specimens were immediately fixed in 2.5% glutaraldehyde 0.2 M in phosphate buffer (pH 7.4) at 4°C for 4 hours, washed with 0.2 M phosphate buffer (pH 7.4), trimmed into small slices including the central cornea, and the entire nodular surface and postfixed in 1% OsO4 in 0.2 M phosphate buffer (pH 7.4) at 4°C for 1 hour. They were dehydrated in graded ethanol and acetone and flat-embedded in resin (Durcupan; Fluka, Buchs, Switzerland) for a better orientation of the specimens. Semithin sections (1-\(\mu\)m) were cut with an ultramicrotome (LKB Ultrotome V; Leica Microsystems, Wetzlar, Germany) stained with a freshly prepared aqueous solution of 1% toluidine blue in 1% borax and 1% pironine16 and viewed and photographed with a light microscope (Primo Star; Carl Zeiss Meditec, Inc., Dublin, CA). From the same specimens used for LM, ultrathin sections of gold–silver interference color were cut with a diamond knife on an ultramicrotome (LKB Ultrotome V; Leica Microsystems), collected on uncoated 200- to 300-mesh copper grids, and stained with methanolic uranyl acetate and lead citrate.17 Micrographs were taken with an electron microscope (CD-10; Philips, Eindhoven, the Netherlands) at 80 kV.

Morphometric Analysis

A morphometric analysis was carried out on the corneal epithelium with particular regard to its height and the number of the cell layers. The data were obtained from 15 semithin sections per subject, collecting one every 100. The sections were viewed with a microscope (Primo Star; Carl Zeiss Meditec, Inc.) with a \(40\times\) objective and the images were captured from different, nonoverlapping microscopic fields (three for each section) using a camera (A620 Powershot; Canon, Lake Success, NY) and saved as TIFFs with photo-editing software (Photoshop CS; Adobe, San Jose, CA). All micrographs were printed at the same final magnification of 800× and were assessed in a masked fashion by three independent observers. The mean height of the epithelium was calculated only where the epithelium was sectioned perpendicularly to the plane of the basement membrane, overlying on the single image two straight lines corresponding respectively to the epithelial surface and the basement membrane: then the distance between the lines at 10 equally spaced points along the epithelium was measured. All data were expressed in micrometers. The mean number of cell layers was calculated from the same images by counting all cellular layers where the nuclear profile was present and included between the lines. The mean and the SD of the results were recorded.

Statistical Analysis

Statistical analysis of the results was performed with statistical analysis software (SAS/Sta version 6.0.3; SAS, Inc., Cary, NC) using the Student’s \(t\)-test. \(P \leq 0.001\) was considered statistically significant.

RESULTS

Confocal Microscopy

In the central cornea, the epithelium had a normal aspect, exhibiting superficial squamous cells with prominent nuclei and bright cell borders and basal epithelial cells with dark bodies and bright borders (Figs. 1a and 1b). Rare subbasal nerve fibers were present and they exhibited an abnormal pattern with increased thickness.
and the absence of branching. Moreover, occasionally, reflective cellular elements were present near these fibers (Fig. 1c). The stromal corneal nerves had an extremely altered pattern: their branches were thick and tortuous, with both highly reflective segments along the bundles and tracts with granular aspect (Fig. 1d). Deep stroma and endothelium were normal.

In the peripheral zone, basal epithelial cells with an abnormal elongated shape were observed (Fig. 1e). In addition, activated, hyperreflective keratocytes were detected; more centrally, they were increased in number (Fig. 1e). Confocal images of the very central part of the nodules revealed highly reflective structures with strong light scatter (Fig. 1f).

LM and TEM
From the examination of the entire specimens, two morphologically different corneal zones, corresponding respectively to the central/internodular cornea and the nodular cornea, were selected.

Central/Internodular Cornea
The epithelium was formed of 8 to 10 layers (Fig. 2a). Beneath the epithelium, the Bowman’s layer was 5 to 7 μm thick and had an undulated course and an irregular optical density. In the adjacent stroma, isolated keratocytes were evident. When the superficial and the wing cells were observed with the TEM (Fig. 2b), the former were flattened and had a variable electron density of their cytoplasm. The latter were closely interlocked by a large number of desmosomes and had elliptical nuclei with dispersed chromatin. The basal cells (Fig. 2c) rested on the Bowman’s layer, had well preserved cytoplasm with numerous tonofilaments, and small mitochondria and were connected to the adjacent cells by desmosomes. The intercellular spaces were wider and contained small isolated nerve fibers. The deep surface of the basal cells (Fig. 2d) had an irregular course and was connected to the Bowman’s layer by a large number of hemidesmosomes. In the adjacent stroma, isolated keratocytes were present. On occasion, intraepi-
epithelial lymphocytes were observed between the lowest parts of two basal cells (Fig. 2e).

**Nodular Cornea**

On the basis of its topographical arrangement, the nodular cornea was divided into four different zones: paranodular, perinodular, supranodular, and centernodular (Fig. 3a).

With LM in the paranodular zone, corresponding to the transitional area between the clear cornea and the nodules (Fig. 3b), the epithelium was formed by 7 to 10 apparently normal layers; the basal cells rested on an undulated Bowman’s layer separated from the stroma by a clearer space. In the perinodular zone, corresponding to the periphery of the nodule (Fig. 3c), the epithelium was formed by six to eight layers, and the deepest cells had an uneven basal pole placed on an irregular Bowman’s layer. In the supranodular zone (Fig. 3d), the epithelium became thinner (from six to three layers), and the Bowman’s layer had large blebs penetrating into the basal cell layer. In the centernodular zone (Fig. 3c), the epithelium was formed by two to three superficial hyperchromatic layers and by a single discontinuous layer of basal cells; the Bowman’s layer was absent.

With TEM in the paranodular zone (Fig. 4a), the superficial cells had dark cytoplasm and evident desmosomes, particularly toward the wing cells, which contained a large number of tonofilaments and desmosomes. The basal cells had uniformly distributed tonofilaments, few small mitochondria, and perinuclear rough endoplasmic reticulum (RER). With regard to their intercellular junctions, the number of desmosomes increased toward the surface, so that the deeper parts were joined only by corresponding infoldings of the cellular membranes. Along the lateral surface of the basal cells, isolated nerve fibers were observed, while on the basal surface hemidesmosomes were present (Fig. 4a, inset). The Bowman’s layer (Figs. 4b and 4c) was formed by an irregular electron-dense material, in whose

![Figure 3](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933462/)

**Figure 3.** (a) An overall view of a nodule and of the adjacent cornea indicating the four nodular zones (b, c, d, and e) examined in detail and the central/internodular zone (asterisk). (b) Paranodular zone. The epithelium is formed by six to nine layers and rests on an undulated Bowman’s layer separated from the stroma by a vacuolated space (asterisk). (c) Perinodular zone. The epithelium is formed by six to seven layers; the deepest cells show an irregular basal pole which rests on a wide Bowman’s layer (arrows). (d) Supranodular zone. The epithelium is progressively reduced to three layers. The Bowman’s layer is evident (arrows) and has large blebs penetrating into the basal cell layer (arrowheads). (e) Centernodular zone. The epithelium is formed by a single discontinuous layer of basal cells and by two to three hyperchromatic cell layers; no Bowman’s layer is evident (arrows). Scale bar: (a) 80 μm; (b–e) 20 μm.
meshes many fibrils, continuous with the stroma, and isolated or confluent vesicles filled with blebs of electron-dense material were present. In the subepithelial stroma (Fig. 4d), keratocytes surrounded by an extracellular matrix comprised of both fibrils and granular material of variable electron density were observed.

In the perinodular zone, the superficial and wing cells revealed morphologic characteristics superimposable to that of the paranodular zone. The basal cells showed a dense cytoplasm and wider, convoluted intercellular spaces, with evident desmosomes (Fig. 5a). Between two basal cells, isolated nerve fibers were present (Fig. 5a, inset). The basal surface had long and thin processes that penetrated the granular Bowman’s layer, so reaching the fibrillary meshwork continuous with the stroma. At higher magnification (Fig. 5b), many hemidesmosomes connecting the basal processes to the discontinuous basement membrane were seen. Keratocytes (Fig. 5c) with euchromatic nuclei, evident nucleoli, and numerous organelles, morphologic features typical of an activated cell, were placed between the Bowman’s layer and the stroma. Some stromal fibrils penetrated through linear clefts in the Bowman’s framework.

In the supranodular zone, the epithelium (Fig. 6a) was formed by two to three layers of superficial cells, a single layer of wing cells, and flatter basal cells comprised of euchromatic nuclei, evident nucleoli, peripheral tonofilaments, small mitochondria, and perinuclear RER. The lateral membranes had few desmosomes and short infoldings. On occasion, large (≤4 μm) round structures, surrounded by a cellular membrane and filled with residual microtubules and granular material, were found between the deep poles of two adjacent basal cells, resembling a degenerated nerve fiber (Fig. 6a, inset). The basal surface was irregular because of the presence—instead of the Bowman’s layer—of either roundish cytoplasmic processes projecting into the stroma or slender evaginations of the granular material toward the cell. At higher magnification (Fig. 6b) of the basal

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**Figure 4.** Paranodular cornea. (a) In the basal cells (bc) tonofilaments (t), small mitochondria (m), and perinuclear rough endoplasmic reticulum (rer) can be seen. Along their lateral surface, occasional nerve fibers (n) are evident. The number of desmosomes (de) increases toward the surface and the basal cells are joined mainly by short infoldings of the cellular membranes (double arrows). On the basal surface, hemidesmosomes (asterisk) are present (inset). Wing cells (wc) contain a large number of filaments and desmosomes (de). Superficial cells (sc) have evident desmosomes (arrowheads), particularly toward the wing cells. (b) The Bowman’s layer is formed by an electron-dense material (M), in whose meshes fibers (t) penetrating from the stroma (arrow) and isolated vesicles (V) are present. (c) Confluent vesicles (V) are filled with blebs of electron-dense material. (d) Elongated keratocytes (K) with indented nuclei are placed in the subepithelial stroma: the extracellular matrix is composed of fibrils (t) generally parallel to the cell and of dense (G) and clearer (g) granular material. Scale bar: (a) 5 μm and (inset) 40 nm; (b, c) 2 μm; (d) 1 μm.
pole, many hemidesmosomes were placed only in correspondence to the residual basement membrane. Activated keratocytes were placed in close proximity to the basal material. The stroma (Fig. 6c) was formed by irregular lamellae of different electron density. In the less dense lamellae, activated keratocytes and microfibrils with occasional granular material were observed. The denser lamellae were formed only by granular material of different size and electron density.

In the centernodular zone (Fig. 7a), the epithelium was generally reduced to two to three layers of superficial cells, the inner layer of which had indented nuclei with evident nucleolus and few organelles. When present (Fig. 7b), basal cells were flatter, revealing an euchromatic nucleus, a large RER, small mitochondria, and peripheral tonofilaments, and were connected to the adjacent cells by desmosomes. Their basal surfaces rested on an electron-dense granular material and lacked hemidesmosomes. The basement membrane was absent. The centernodular stroma (Fig. 7c) was formed by irregular lamellae of activated keratocytes, surrounded by dense granular material and by fibrils parallel to the corneal surface. Activated keratocytes were often in pairs and showed nuclei with dispersed chromatin and evident RER (Fig. 7d).

**Morphometric Analysis**

The morphometric analysis carried out on the epithelial height revealed the greatest values in the central/internodular, paranodular, and perinodular zones (50.8 ± 6.6 μm, 55.2 ± 5.2 μm and 49.8 ± 2.9 μm, respectively), and the lowest in those of the supranodular and centernodular zones (26.2 ± 8.0 μm and
12.2 ± 2.4 μm, respectively). The statistical analysis revealed significant differences among all the considered groups (Fig. 8).

The mean number of the cell layers was greater in the central/internodular, paranodular, and perinodular zones (9.1 ± 0.7, 8.6 ± 1.2 and 7.4 ± 1.2, respectively) and particularly low in the supranodular and centernodular zones (4.3 ± 1.2 and 2.5 ± 0.5, respectively). The different zones of the cornea had a statistically significant difference for the number of the cell layers (Fig. 9).

**DISCUSSION**

The present work is the first investigation that describes the cornea of the same SND patients with both in vivo confocal microscopy and LM and TEM microscopy. Five zones were identified and indicated as central/internodular, paranodular, perinodular, supranodular, and centernodular. These areas were shown to be different in the organization of the epithelium, basement membrane, Bowman's layer, and stroma.

As to the epithelium, the confocal examination of our patients with SND excluded any morphologic change of the basal cells and featured only an elongated shape of the superficial epithelial cells in the central/internodular cornea. This pattern has been previously described in the nodular epithelium of a single patient with SND. The abnormal cellular shape (up to 60 μm in length), which is not validated by the histopathologic analysis, could be referred to the in vivo mechanical stretching on the adjacent epithelial cells induced by the growth of the nodules. The structural and ultrastructural analysis of the corneal epithelium had a dramatic reduction of its height, particularly above the nodule. This pattern was also confirmed by the morphometric analysis of both the epithelial height and...
the number of the cellular layers. In fact, the epithelial thickness was approximately normal in the central/internodular, paranodular, and perinodular zones (50.8 ± 6.6 μm, 55.2 ± 5.2 μm, and 49.8 ± 2.9 μm, respectively) and highly reduced in the supranodular and centernodular zones (26.2 ± 8.0 μm and 12.2 ± 2.4 μm, respectively). The number of cellular layers (8-10 in the central/internodular zone) was also progressively reduced to three to four in the centernodular zone, where it consisted of a single discontinuous layer of basal cells and two to three hyperchromatic keratinized layers. A continuous epithelial covering over the nodule is confirmed.

In addition, in all the examined regions, the corneal epithelium was well preserved, thereby resembling the normal cornea as it was previously described. In particular, the basal cells had euchromatic nuclei, morphologically normal junctional complexes, and a large number of cytoplasmic organelles. These cells appeared to be functionally active, as was also shown by Eberwein et al., who revealed the expression of enolase-alpha, an enzyme with high proliferative capacities that is known to play a relevant role in the glycolytic pathway in cells.

In addition, neither degenerative changes nor focal degeneration nor single cell necrosis were observed in the corneal epithelium of the central/internodular and of the nodular zones.

The basement membrane of the SND corneal epithelium had evident changes in most of the examined zones. In fact, it was apparently well preserved only until the paranodular zone,
As to the morphologic behavior of the corneal nerves in the SND, they were described with the confocal microscope only by Ku et al., who showed in the deeper stroma very prominent nerves and an unusual pattern of grouped keratocytes, some of which were placed near the nerves. Our data revealed a close relationship between stromal cells and nerve fibers only in the subbasal plexus. In addition, altered stromal nerves were observed, with thick and tortuous branches similar to regenerating nerves after penetrating keratoplasty.

No studies are currently available on the ultrastructural organization of the nerve fibers in SND patients. Our data revealed the absence of nerve fibers in the nodular stroma and the existence of a peculiar subbasal plexus. In particular, the fibers were numerous in the central/internodular zone, fewer in the peripheral region of the nodule, rare, swollen, and degenerated in the supranodular zone, and absent in the centernodular zone. Their location, in all the examined zones, was restricted to the subbasal plexus, where they were placed between adjacent basal cells; therefore, no oblique fibers coursing into the more superficial cell layers were observed. This feature differed from the normal morphologic pattern observed by Müller et al., who described the presence of oblique fibers penetrating into the epithelium. In SND, the altered nerve organization could be related to both the degenerative changes observed in the corneal nerves and to the altered nodular stroma. In fact, the nerve fibers were always isolated, because no nerve bundles were observed in any of the examined zones.

Our data revealed aspects of increased keratocyte activity and altered nerve patterns in SND patients, including mild changes of the epithelium and more pronounced changes of the basement membrane and Bowman’s layer.

It was shown that the epithelium reacts in SND, producing proinflammatory substances. Matrix metalloproteinase-2 could induce disruption of the basement membrane and of the Bowman’s layer. TGFβ determined the activation of the stromal keratocytes toward myofibroblasts thereby promoting the deposition of an opaque stroma comprised of a rudimentary lamellar structure.

The nerve fibers appeared to be involved in such changes. In fact, their confocal appearance and the ultrastructural data suggest that corneal nerves could reach the subbasal layer of the SND epithelium.
the nodular cornea only through the periphery of the nodule itself, because we were not able to show nodular stromal nerves with both techniques. This hypothesis could be supported by the existence of only single axons, the absence of oblique fibers, the progressive reduction of their number toward the center of the nodule, and the occasional swollen and degenerated axon just near the center of the nodule.

In conclusion, SND poses an unsolved question about which, among the epithelium, the stroma and the nerves, might be the first to be involved in its pathogenesis. However, all these structure seem to contribute to maintain the corneal disorganization characteristic of SND.

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References