doi: 10.17116/engoftalma20161326-2

SEM visualization of corneal epithelium through lanthanoid staining based on Ca/Nd isomorphous substitution in Ca-dependent molecular systems

S.E. AVETISOV, S.V. TRUFANOV, I.A. NOVIKOV, A.M. SUBBOT, A.A. FEDOROV

[1]Research Institute of Eye Diseases, 11 A, B Rossolimo St., Moscow, 119021, Russian Federation;

[2]The First Sechenov The Moscow State Medical University under Ministry of Health of the Russian Federation, 8-2 Trubetskaya St., Moscow, 119991, Russian Federation

Cumulative biomicroscopic evidence is usually sufficient for the diagnosis of recurrent corneal erosion or bullous keratopathy, however, exploration of the disease pathogenesis requires subcellular-level visualization of corneal structure. In the current study, lanthanoid staining and scanning electron microscopy were employed to visualize quite a number of structures responsible for epithelium organization. In particular, the study proves possible the use of Ca/Nd isomorphous substitution at Ca^{2+} sites of cytoadherence proteins for visualization of corresponding cellular structures. **Aim** — to assess the value of information provided by scanning electron microscopy of corneal epithelium that involves lanthanoid staining based on the Ca/Nd isomorphous substitution in Ca-dependent molecular systems. **Material and methods**. Anterior corneal epithelial scrapes were obtained from patients with recurrent corneal erosion or bullous keratopathy and cadaver eyes with no signs of any ophthalmic disease. Samples were then studied under a scanning electron microscope (Zeiss EVO LS10, BSE, EP — 79 Pa, 20—28 kV, Ln-staining with the BioREE assay kit). **Results**. In all cases, lanthanoid staining of biopsy material provided high-contrast SEM images with well-recognizable structural and ultrastructural elements associated with Ca^{2+} sites of cytoadherence proteins. **Conclusion**. Lanthanoid staining of biopsy material and subsequent SEM enabled detailed visualization of structural features of the corneal epithelium in various pathologies. Due to the Ca/Nd isomorphism we were able to evaluate structural position of the majority of protein molecules engaged in Ca-dependent processes and, consequently, in cytoadherence. Basing on the neodymium distribution within the basal membrane, we have described local effects of different substances on the lamina densa in the projection of basal layer cell borders that occur after unidirectional ultrafiltration. The results confirm the failure of the junctional adhesion complex in recurrent corneal erosion.

Keywords: *epithelium, cornea, bullous keratopathy, recurrent corneal erosion, scanning electron microscopy, SEM, scanning electron microscope, lanthanoid staining, cell adhesion molecules.*

Vestnik_Oftalmologii_2016-6_11EN

Eye surface epithelium is an example of the organism's first line of defense, particularly, a barrier between eye structures and the environment. The essential prerequisite for the barrier function of epithelium is its correct organization that is determined, to a considerable extent, by intercellular interaction and cellular interaction with the basal membrane. Normally, epithelial cells form multiple adhesive, tight, and gap junctions between each other that are created by microfilaments — cellular adhesion molecules — most of which (cadherins, laminins, integrins) are Ca-dependent transmembrane proteins.

The wide range of epithelial tissue building blocks that are responsible for its correct organization accounts for, among other reasons, the complex nature of most known corneal epithelium diseases and the versatility of factors that are simultaneously involved in pathogenetic chains.

In particular, recurrent corneal erosion (RCE) syndrome is a multi-factor disease caused by an unstable connection between the cellular epithelial layer and the basal membrane, and characterized by recurrent episodes of desquamation of considerable corneal epithelium regions [1, 2].

Modern optical intravital imaging techniques used in ophthalmology (biomicroscopy, confocal microscopy, optical coherence tomography) have allowed characterizing the overall picture of the damage caused by RCE. Currently, there have been described such RCE morphological features as irregularity, epithelial elevation, epithelial microcysts, bullae, as well as changes in the stroma (haziness and cellular infiltrates) [3, 4].

Bullous keratopathy (BK) is a condition that develops as a result of the functional decompensation of cornea and is characterized by persistent corneal edema with bullae in the epithelial layer.

Cumulative biomicroscopic evidence is usually sufficient for the diagnosis of RCE or BK, however, exploration of the disease pathogenesis requires a "finer", sub-

Corresponding author:
Anastasia Mikhailovna Subbot — Senior Research Worker in the Laboratory of Fundamental Research in Ophthalmology
e-mail: kletkagb@gmail.com

cellular level visualization of the corneal structure. This is essential for the assessment of the state and relationships of intercellular junctions, adhesion molecules, extracellular matrix elements, and proteolytic components, such as matrix metalloproteinases (MMPs).

Among the methods that enable marking of the processes at the biomolecular level, highly specific immunohistochemical staining techniques are the most frequently used ones in medical and biological research. Specificity is both an advantage and a disadvantage of such an approach: a researcher is limited by a set of suggested "study target objects", and by lack of possibility to simultaneously detect more than three of them. However, for complex pathological processes, which undoubtedly include abnormal corneal epithelium, the number of objects structurally involved in such processes may be fairly large **(see Table)**.

The described versatile factors which cause the disease and maintain the pathological process broadly range from genetic predisposition to an imbalance of MMPs and other regulating molecules. To reconstruct the links in pathogenetic chains, it is therefore required to simultaneously assess the interaction of cells and components of the extracellular matrix and the basal membrane — collagen of various types, laminin, fibronectin, etc., as well as to visualize the microrelief of basal layer cells, their anchoring structures, and other intercellular junction elements — integrins, cadherins [12].

Presumably, lanthanoid staining and subsequent scanning electron microscopy (SEM) may be used for simultaneous visualization of the three-dimensional sample structure, location of extracellular matrix components, and localization of a number of Ca^{2+} and ATP-dependent processes at the molecular level **(Fig. 1)** [13].

Changeability of Main Structural Elements of Corneal Epithelium and Basal Membrane in BK and RCE Development

Morphological Structure	Structural Element	Changes	
		in RCE	in BK
Desmosome, a junction of neighboring cells	Cadherin, microfilaments	No changes in number [5]	—
Hemidesmosome, a junction between basal cells and underlying membrane	β4 integrin	Destruction after exposure to MMP-9 [6]	—
	Collagen XVII	Mutation in the coding gene, which contributes to disease development [7]	—
Lamina lucida	Laminin-5, fibronectin	Destruction after exposure to MMP-2, up to absolute disappearance [8]	Fragmentation [9]
Lamina densa	Collagen IV (main substance)	Destruction after exposure to MMP-2. Mutation in the coding gene, which contributes to disease development [10]	Deposits in subepithelial space [9]
	Collagen VII (anchoring fibrils)	Destruction after exposure to MMP-2, up to absolute disappearance [8]	—
Anti-adhesive glycoproteins	Fibrillin-1, tenascin-C	—	Diffuse accumulation [11]

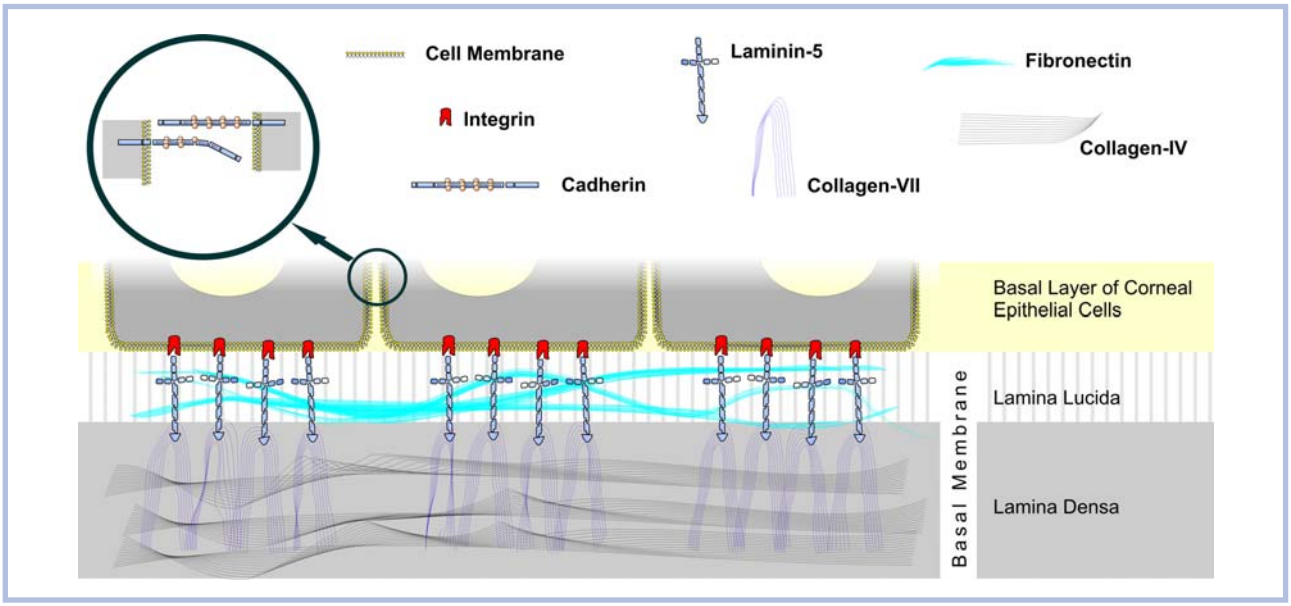

Fig. 1. **Basic Scheme of Relationships Between Structural Elements of Corneal Epithelial Cells and BM that are Imageable by Scanning Electron Microscopy with Lanthanoid Staining**

Basal epithelial cells are fixed to the lamina densa collagens through a system of anchoring proteins that pervade the lamina lucida.

The variety of Ca-dependent processes and Ca localizations in the protein structures of epithelial tissue justifies the use of Ca/Nd isomorphous substitution for staining the corneal epithelial structure.

The aim of the study is to assess the value of information provided by SEM of corneal epithelium that involves lanthanoid staining based on the Ca/Nd isomorphous substitution in Ca-dependent molecular systems.

Material and Methods

SEM was used to examine anterior corneal epithelial scrapes obtained in operating room conditions from patients with RCE (9 samples from male patients aged between 34 and 56) or BK (4 samples of male patients aged between 49 and 61), and from cadaver eyes with no signs of any ophthalmic disease (5 samples of male patients aged between 22 and 49).

The patients had undergone preliminary examination by conventional ophthalmic methods. Additionally, corneas (including donor ones) had been examined by biomicroscopy and cross-section imaging using the HRT II optical coherence tomograph with the Cornea Module.

Within the first minutes after biopsy material recovery, when cells maintain sufficient metabolic activity, the samples were stained using the BioREE assay kit (Glaukon LLC, Russia) following the protocol recommended by the manufacturer:

1) the first rinse to eliminate phosphates from the surface;

2) 45 min exposure to neodymium chloride solution for saturation with the staining agent;

3) the second rinse to remove excessive solutions.

On the SEM image, staining marked the zones where calcium was isomorphously substituted for neodymium, as well as localization sites of energy-dependent processes involving phosphate anion release and subsequent formation of insoluble neodymium phosphates [13, 14].

The prepared epithelial layers were spread over the specialized adhesive carbon tape for SEM. Each sample was positioned so that both the anterior epithelial surface and basal layer were observable at the same time (the epithelial layer was sliced in two and positioned with different surfaces outward, or partially turned under).

The samples were put in the chamber of the scanning electron microscope (EVO LS10, Zeiss, Germany). They were observed in a low vacuum mode (EP, 70 Pa) at 20–28 kV accelerating voltage and 360–520 pA current on the sample. The LaB_6 cathode was used. The images were captured in the backscattered electron (BSE) detection mode.

Results and Discussion

In all cases, lanthanoid staining of biopsy material provided high-contrast SEM images with well-recognizable cellular structure.

In medical and biological research, electron diffraction patterns obtained with a SEM with a secondary electron detector are the most widely used ones. Lanthanoid staining presupposes the use of another detector — BSE detector. Therefore, the obtained images differ from the conventional ones, as the signal is detected from the greater sample depth and a considerable part of tissue volume remains transparent for an electron beam at high accelerating voltages. The generated images, to some extent, appear close to optical confocal images; and optical terms such as “opacity”, “brightness”, etc. can be used for their interpretation.

The contrast of the images obtained by a SEM in the BSE detection mode is determined by the average atomic weight of each sample substance lying under the surface of the local volume. Bright regions of an image correspond, thereby, to tissue structures saturated with heavy elements. An innovative *method of supravital lanthanoid staining* serves for selective saturation of individual structures with heavy lanthanoids, and for marking therewith several chains of cellular metabolism, which enables their subsequent SEM visualization [13, 14].

In terms of this study, lanthanoid staining of biopsy material and subsequent SEM allowed to obtain considerably different presentations of various corneal epithelium conditions (arbitrary norm, BK, and RCE) that characterized, directly and indirectly, the changes in the ultrastructure of epithelial tissue. It is also important to note the high repeatability of the observed changes. The patterns on the SEM images obviously reflect tissue elements involved in the similar pathological processes.

Lanthanoids are known for their ability to join calcium metabolic chains and block them [15]. Localizations associated with Ca/Nd isomorphous substitution give the possibility to visualize Ca-dependent processes, including cell adhesion ones, and the key types of protein molecules involved in this process. Here belong two types of heterovalent isomorphism: substitution of three calcium ions for two neodymium ions ($3Ca^{2+} \leftrightarrow 2Nd^{3+}$) — this is the case for cadherin structure; substitution of a couple of calcium ions for an ion of neodymium and alcali metal ($2Ca^{2+} \leftrightarrow (Nd^{3+} + Me^{+})$) — described for transmembrane Ca-pumps. Being Ca-dependent, proteins, integrins and laminins are also involved in such substitution processes. The most probable high local concentration of lanthanoids in the stained specimen is associated with the substitution of five triple Ca^{2+} sites in cadherins [16], ensuring the brightest staining at cell junctions, including desmosomes on the lateral surface of cells.

Another group of the structural positions of lanthanoid accumulation is a result of their binding with free phosphate anions. In this case, lanthanoids (neodymium) mark intracellular zones of active energy-dependent processes involving ATP consumption and phosphate anion release (e.g., tracks for cytoskeleton assembly, and flagellar and mitochondrial work) [17]. The suspension of simple neodymium phosphate ($NdPO_4$) is separated

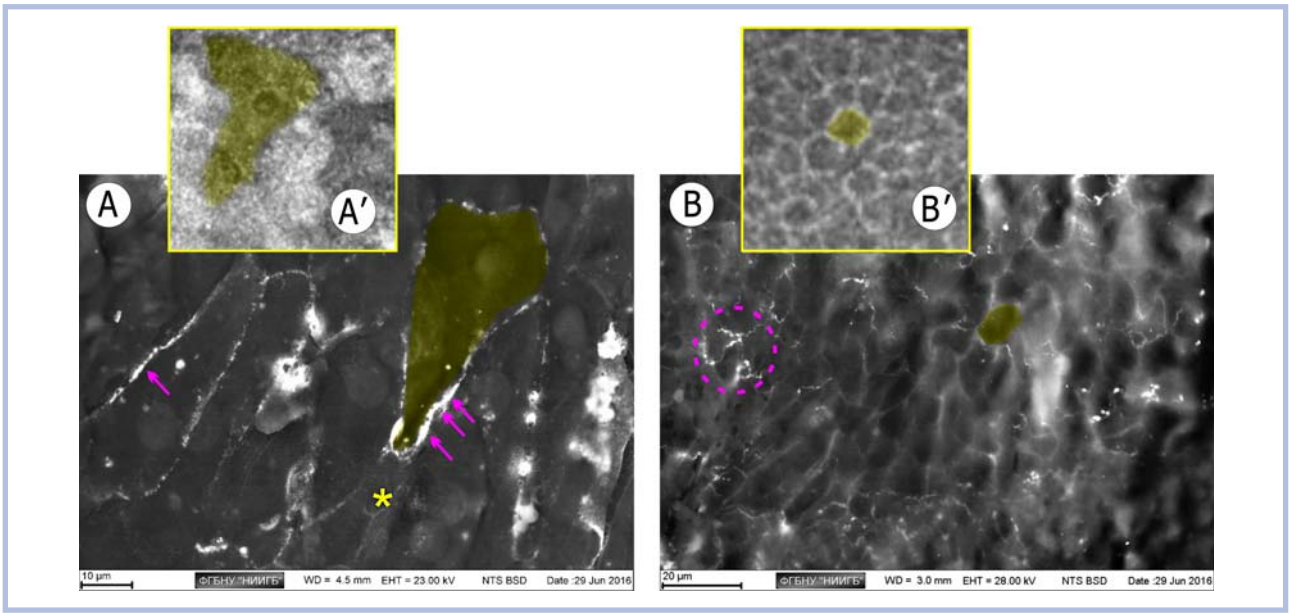

Fig. 2. **SEM images of corneal epithelium (biopsy material) unchanged by any pathological processes (BSE detector, lanthanoid staining).**

a — from the side of the anterior surface; b — from the side of the basal surface.
Contrasted borders between polygonal cells. Individual intercellular border zones of bright staining indicate the regions with the highest cadherin concentration, marking desmosomes, as well as tight and adhesive junctions (see arrows). Hazy cell cytoplasm with cell nuclei seen inside it; individual tree-like zones (see asterisk) associated with the most active energy-dependent processes (cytoskeleton assembly) are distinguished. From the side of the basal membrane, there are distinguished local regions with notably more contrasted intercellular borders within them, compared to the overall pattern (see dot lines). In respective insets — cross-section images obtained by HRT II with the Cornea Module within the desquamating (A') and basal (B') cell layers. Individual cells are illuminated with yellow light for comparison with SEM images.

and accumulated because of its low solubility and inability to be remobilized by cellular metabolism systems. Therefore, bright staining of individual structures in the depths of the cells is conditioned by the activity of metabolic processes located in such structures at the moment of sample lanthanoid staining [13].

Moreover, there is a possibility of non-specific binding between lanthanoids and structural proteins of the extracellular matrix, and therefore, of its illumination on SEM images [18].

Basing on the described theoretical premises, the images of corneal epithelium (biopsy material) that were obtained in this study can be interpreted as follows.

SEM of Normal Corneal Epithelium

The scans of the anterior surface of normal corneal epithelium reveal contrasted borders between cells; the polygonal shape of cells is clearly visible in the desquamating layer **(Fig. 2, a)**. Remarkably bright glowing of individual intercellular border zones shows the regions with the highest cadherin concentration, and therefore, marks the location of desmosomes, as well as tight and adhesive junctions (see arrow). Cell cytoplasm is moderately hazy, which indicates the activity of energy-dependent processes involving phosphate anion that proceeded in it. The scans demonstrate individual tree-like zones (see asterisk), possibly associated with the preferred directions of cytoskeleton assembly. Cell nuclei are seen through hazy cytoplasm. It can be noted that under such image capture conditions, electrons are backscattered not only in the first, superficial cell layer, but also at a greater depth, which is visually perceived as an overlay of semitransparent cells onto each other.

On the basal surface, cadherin-containing intercellular junctions glow all throughout the length of cellular borders, which suggests the greater regularity of their organization in respect to the anterior desquamating epithelial layer.

The features of the corneal epithelial structure visualized by lanthanoid staining and SEM techniques are well consistent with the images obtained by optical methods **(insets in Fig. 2)**.

The basal surface of the cellular layer is covered with the membrane that was removed together with the sample and is semitransparent for electrons. However, it cannot completely damp the glowing of intercellular borders, only making the image pattern blurred. The basal membrane structures (including the lamina densa) remain the same throughout the whole surface observed, which is evidenced by omnipresent cloudy glowing, presumably caused by non-specific binding of collagens to neodymium. **Fig. 3, a** provides the scheme of such separation of layers.

Fuzzy polygonal cellular borders are seen through the basal membrane (BM). In individual regions, the borders of the basal layer cells become clearly delineated and appear notably brighter **(see Fig. 2, b)**. This observation may have two principally different explanations: either the BM is thinned within these zones, or the reason is the binding of molecules that leaked from the anterior epithelial surface, to neodymium. Thereby, the geometry

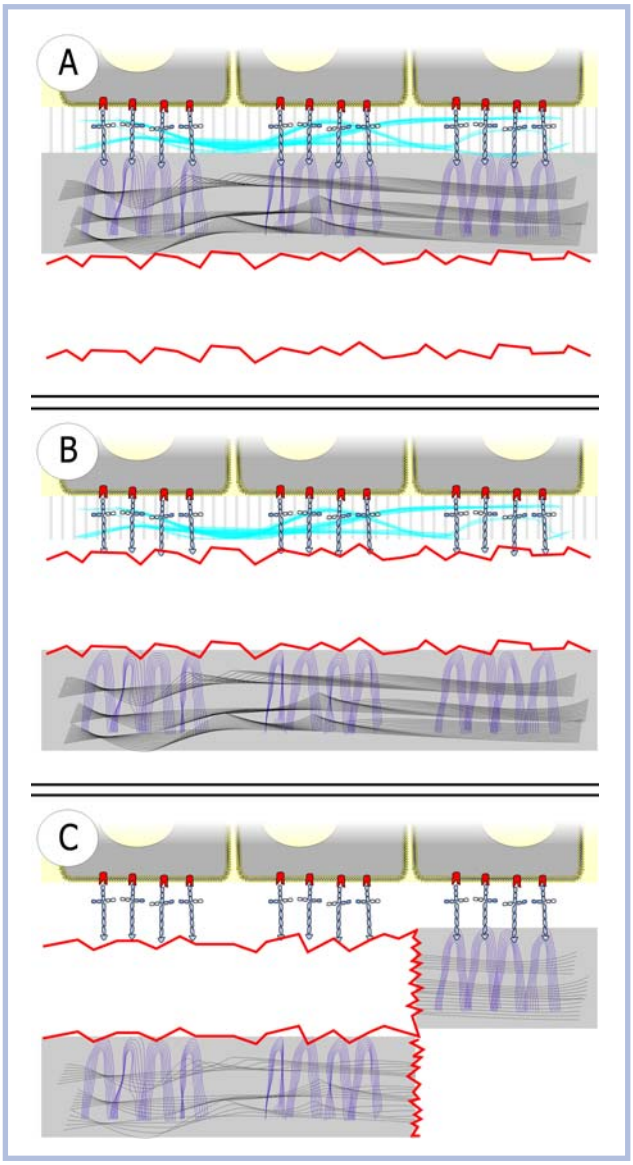

Fig. 3. **Scheme of Epithelial Layer Removal During the Obtainment of Biopsy Material Scrapes. Three options for the corneal epithelial layer to be removed from underlying structures.**

a — removal along with the BM structures (typical for unchanged epithelial tissue); b — removal along with the structures of the lamina lucida and anchoring filaments (typical for BK); c — removal along with anchoring filaments without other structures of the lamina lucida, and fragmentary integrity of the structures of the lamina densa (typical for RCE). See the legend in Fig. 1 and explanations in the text.

of such transmembrane ultrafiltration repeats the contours of cellular borders throughout the whole thickness of BM **(Fig. 4).**

Such explanation appears quite possible, given the paracellular liquid transport in epithelial tissue [19].

SEM of Corneal Epithelium in Bullous Keratopathy

In BK, SEM images of the epithelial layer from the side of the anterior surface demonstrate the preserved tissue architecture in the most of the sample area. The regular cellular borders in the unchanged surface regions are, to some extent, less contrasted than normal epithelium, although the reason is unclear.

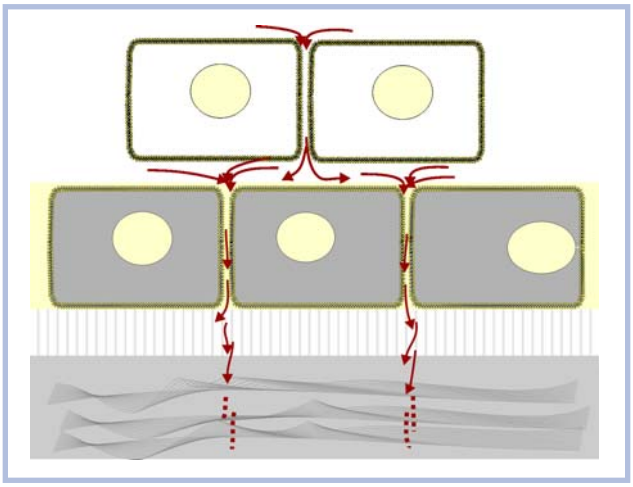

Fig. 4. **Scheme of the Suspected Mechanism of the Formation of the BM Tears along the Contours of Basal Epithelial Cells.**

Paracellular transport of proteinases leads to the formation of a zone with their increased concentration in the BM, where collagen destruction is more active. This results in a local decrease of the membrane's physical integrity.

In suspected location zones of bullae, where the increased pressure led to excessive stretching of the cellular layer, the cells appear considerably brighter delineated. The visible cellular borders in these regions are thickened; gaping intercellular spaces, serrated lines of junctions, and disconnection of cells can be seen **(Fig. 5, a)**. The dual structure of the desmosome zone, with slightly disconnected mating parts of one junction that belong to two adjacent cells, can be vividly seen in the image with large magnification **(see Fig. 5, b)**.

As a rule, the basal layer in BK is not overlapped by denser structures. Intercellular borders are crisp and clear **(see Fig. 5, c)**, which indirectly suggests that the mechanical interaction of cells with each other within the basal layer is not compromised. Large SEM magnifications allow to reveal the villous surface of the basal layer cells **(Fig. 5, d)**, which is possibly caused by molecular assemblies of laminin-5 (anchoring filaments) attached to the cell membrane. In respect to cytoplasm, these molecular groups of a typical structure appear sufficiently bright, which can be explained by Ca^{2+}-binding sites substituted for neodymium that are present in their structure. Apparently, during epithelium scraping, the separation line was at the border of the lumina lucida, with a tear of an intermolecular junction laminin-collagen-VII/collagen-IV, since molecular assemblies of laminin-5 remained adherent to the cell. **Fig. 3, b** illustrates the possibility of such separation. This assumption is consistent with the clinical and morphological data on the formation of bullae right under the cell layer, between it and the lamina densa [11]. It appears logical that during the scraping of epithelium in BK, it is removed in the zones of bullae, which also include anti-adhesive substances (tenascin-C, fibrillin-1).

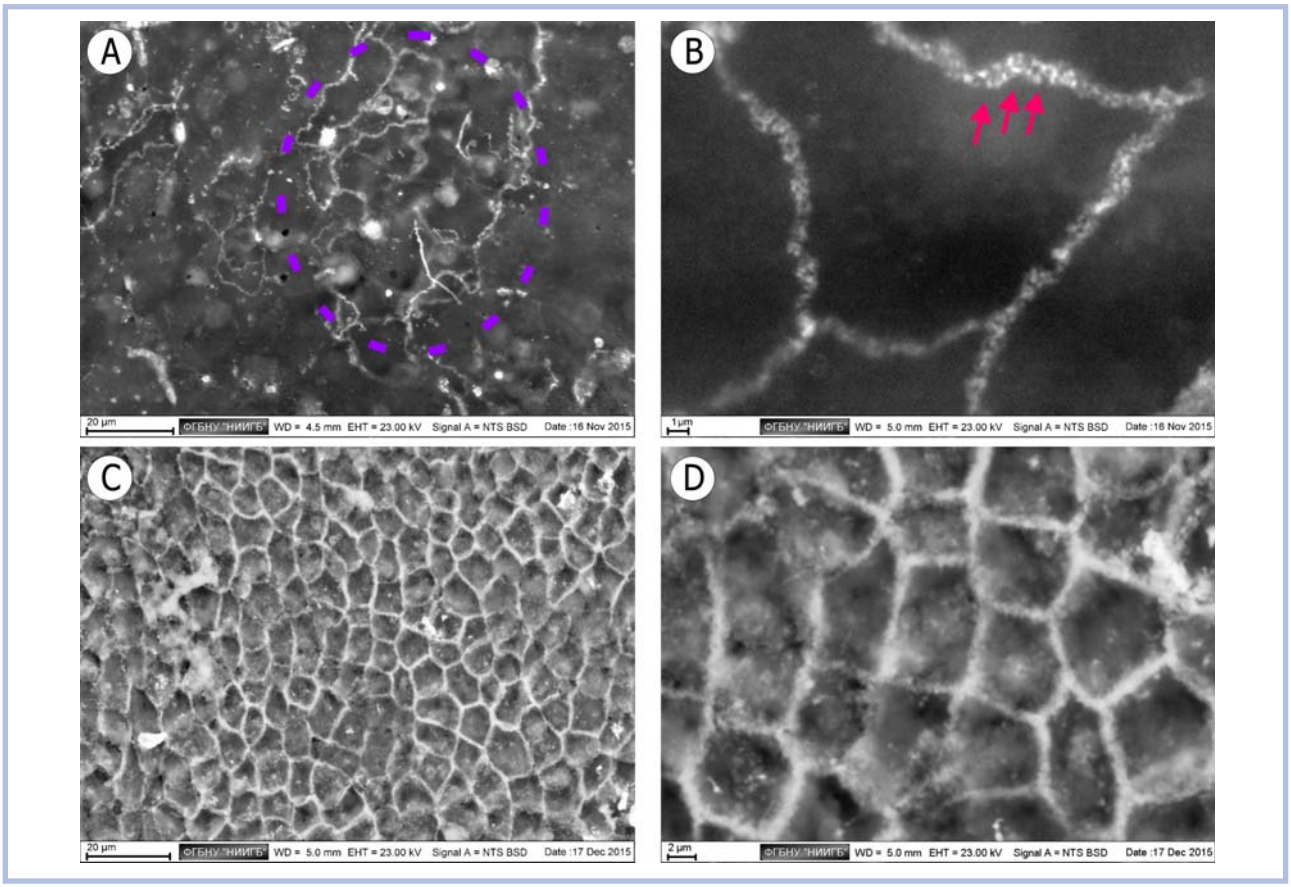

Fig. 5. **SEM images of corneal epithelium in BK (BSE detector, lanthanoid staining).**

a — from the side of the anterior surface (the bulla is shown with a dot line); b — from the side of the anterior surface with large magnification. Arrows point at the region where the zone of the intercellular junction is considerably tensile-stressed, revealing the dissociation of adhesive structural elements and their symmetrical location. Transverse cadherin bands can be seen in these zones; c — the view from the side of the BM. Intercellular borders are crisp and clear — the mechanical interaction of cells with each other is not compromised; d — from the side of the BM with large magnification. Villous cell surface due to anchoring filaments on it.

SEM of Corneal Epithelium in Recurrent Corneal Erosion

In RCE, the changes in the corneal epithelial structure were detected throughout the whole surface. The images show typical geographic (or map-like) and scalloped cellular borders **(Fig. 6, a, b)**. The images also reveal widespread dots on the anterior surface, which emphasize the irregularity of Nd accumulation in the tissue structure. This may be caused by the fact that the secretion covering the cellular surface is sparse because of a high concentration of proteolytic enzymes. Contrasted cell nuclei can be observed in sparse cytoplasm. In individual regions, the anterior surface structure becomes loose, no intercellular junctions can be visualized, and cell groups form desquamating layers. The desquamation of large cell groups reveals fibrotic motifs in the structure of the main substance of the epithelial tissue, which are not typical for normal epithelium **(see Fig. 6, c)**. The regions where a deeper layer is exposed under the superficial one demonstrate the fact that such changes virtually do not affect the middle layers — the mutual arrangement of the cells of the underlying layer is close to normal. Apparently, the viability of the surface layer cells in RCE is decreased to such a considerable extent that the internal cellular structure is passively contrasted through membrane pores. In the cases when membrane integrity is not impaired, one can observe cytoplasm that is virtually transparent for electrons. Its dark phototone without electron scattering on any objects in cells suggests the low level of the proceeded energy-dependent processes. The literature also provides descriptions of numerous abnormalities in the morphology of corneal epithelial cells in RCE (multinuclearity, strong size variability, cysts, vacuoles, desquamation) [20].

After neodymium staining, zones with no contrast of intercellular borders were noted from the side of the basal layer. The image **(see Fig. 6, d)** shows that the epithelial layer is mostly scraped off the BM without its dense part; although in some cases, the cellular layer was partially covered by the BM substance within the limits of its contours. In these regions, the cellular structure under the BM was poorly visualized because of a relatively bright layer of collagen fibers overlayed. It is obvious that in this zone the epithelial layer was removed together with the lamina densa and collagens that it contains.

RCE is known to be associated with excessive MMP-2 [8] that leads to the destruction of collagen IV — the

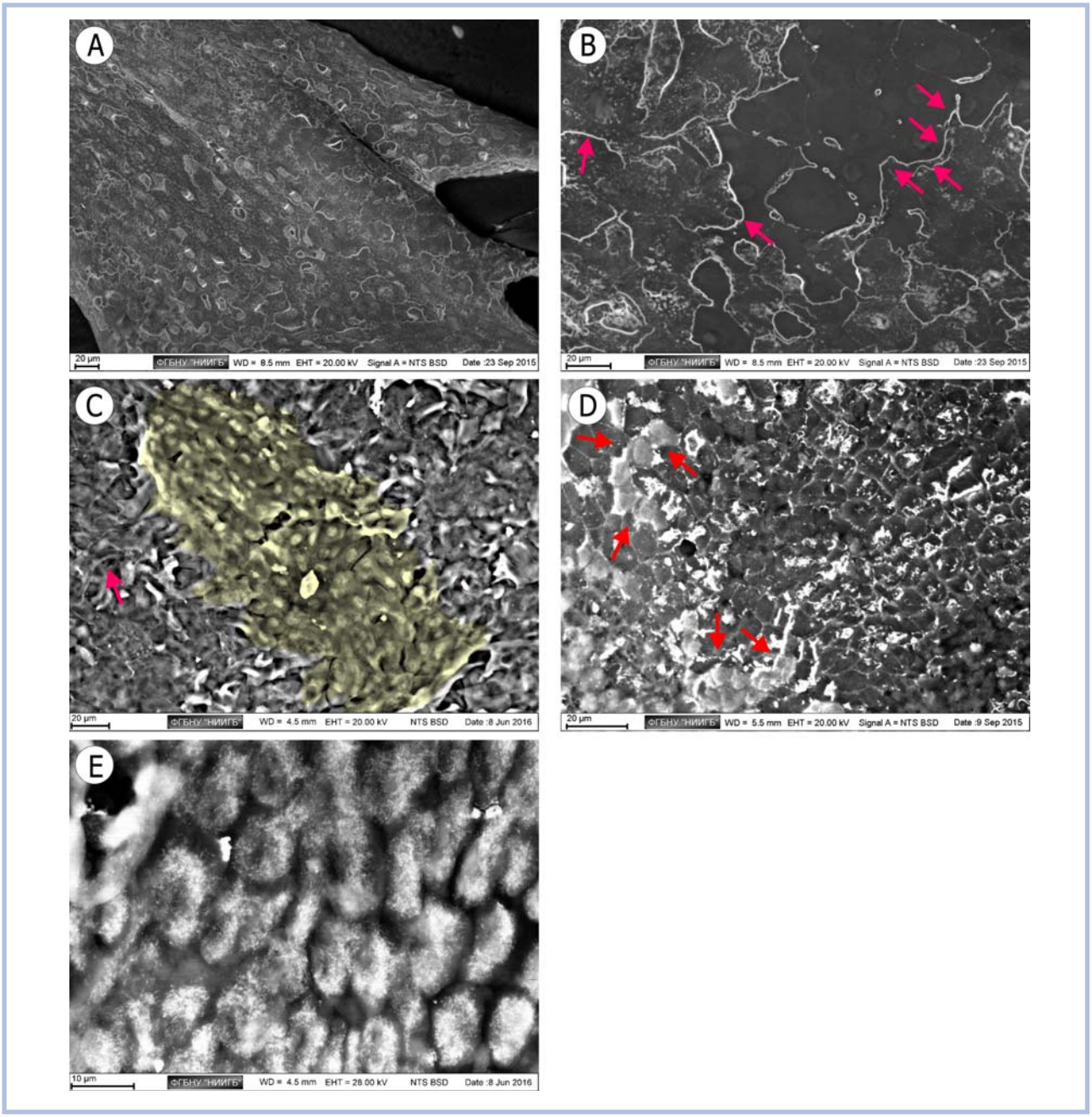

Fig. 6. **SEM image of corneal epithelium biopsy material in RCE (BSE detector, lanthanoid staining).**

a — from the side of the anterior surface, widespread dots on the anterior surface; this is presumably caused by sparse secretion covering the cells, as a result of a high concentration of proteolytic enzymes; b — the detailed region of the anterior surface with geographic (or map-like) motifs in the tissue structure (abnormal scalloped cellular borders showed with arrows), contrasted cell nuclei in sparse cytoplasm; c — the detailed region of the anterior surface with a desquamating layer (illuminated with yellow light) and fibers in the structure of the main substance of epithelium (see arrow); d — from the side of the BM. The cells covered with the dense substance of the BM (see arrow) have relatively homogeneous brightness, other regions were removed without the lamina densa; e — the region imaged with high resolution from the side of the BM. Villous cell surface due to anchoring filaments on it.

main substance of the BM, which may cause its softening.

For the basal layer of cells to be covered with the separated substance of the lamina densa in individual regions only, transverse tears of the lamina densa substance had to form during traction, which allowed to partially reject the lamina densa and partially leave it adherent to the epithelial structures. The most surprising observation is associated with the fact that in most cases, the collagen layer of the lamina densa was separated along the borders consistent with the ones of the above lying cells of the epithelial basal layer **(see Fig. 6, d)**. **Fig. 3, c** shows the scheme of the process. The mechanism of this phenomenon may be associated with the leakage along the intercellular space of epithelium of proteolytic enzymes, the increased concentration whereof is described above [8].

In this case, MMP concentration becomes focused, which results in weakening of the part of the BM directly below the cellular borders of the epithelial basal layer. This is well consistent with the supposed scheme of interstitial transport **(see Fig. 4)**.

The villous structure of epithelial cells **(see Fig. 6, e)** is observable in the places where the BM was separated along the lamina lucida **(see Fig. 3, c)**. The brightness of fibrillar protein structures (supposedly, laminin-5) on the image is determined not only by calcium isomorphous substitution, but also by the filamentous form itself. Given the integrity of the molecular assemblies of anchoring proteins, it can be supposed that weak adhesion to the lamina densa in this case is due to the failure of the anchoring fibrils of collagen VII [8].

Conclusions

1. Lanthanoid staining of biopsy material and subsequent SEM enabled detailed visualization of structural features of the corneal epithelium in various pathologies.

2. On the basis of the Ca/Nd isomorphism mechanism, we were able to evaluate the structural position of the majority of protein molecules involved in Ca-dependent processes and, consequently, in cytoadherence.

3. Basing on the neodymium distribution within the basal membrane, we have described local effects of different substances on the lamina densa in the projection of basal layer cell borders that occur after unidirectional ultrafiltration.

4. The received results confirm the data on the failure of the junctional adhesion complex in RCE development.

Authors' Contribution:
Study concept and design: I. N., A. S.
Material collection and processing: S. T., I. N., A. S.
Statistical analysis: I. N.
Writing: S. A., I. N., A. S.
Editing: S. A., A. F.
No conflict of interest is present.

REFERENCES

1. Mannis MJ, Holland EJ. *Ocular Surface Disease: Medical and Surgical Management*. Springer Science & Business Media; 2006:59-62.
2. Trufanov S.V., Malozhen S.A., Polunina E.G., Pivin E.A., Tekeeva L.Y. Recurrent corneal erosion syndrome (a review). *Oftal'mologija*. 2015;12(2): 4-12. (In Russ.).
3. Heyworth P, Morlet N, Rayner S, Hykin P, Dart J. Natural history of recurrent erosion syndrome — a 4 year review of 117 patients. *Br J Ophthalmol*. 1998;82:26-28.
4. Reidy JJ, Paulus MP, Gona S. Recurrent erosions of the cornea: epidemiology and treatment. *Cornea*. 2000;19:767-771.
   doi:10.1016/S0002-9394(01)00884-4
5. Mencucci R, Paladini I, Brahimi B, Menchini U, Dua HS, Romagnoli P. Alcohol delamination in the treatment of recurrent corneal erosion: an electron microscopic study. *Br J Ophthalmol*. 2010;94(7):933-939.
   doi:10.1136/bjo.2009.174409
6. Pal-Ghosh S, Blanco T, Tadvalkar G, Pajoohesh-Ganji A, Parthasarathy A, Zieske JD et al. MMP9 cleavage of the β4 integrin ectodomain leads to recurrent epithelial erosions in mice. *J Cell Sci*. 2011;124(Pt 15):2666-2675.
   doi:10.1242/jcs.085480
7. Oliver VF, van Bysterveldt KA, Cadzow M, Steger B, Romano V, Markie D et al. A COL17A1 Splice-Altering Mutation Is Prevalent in Inherited Recurrent Corneal Erosions. *Ophthalmology*. 2016;123(4):709-722.
   doi:10.1016/j.ophtha.2015.12.008
8. Garrana RM, Zieske JD, Assouline M, Gipson IK. Matrix metalloproteinases in epithelia from human recurrent corneal erosion. Invest Ophthalmol *Vis Sci*. 1999;40(6):1266-1270.
9. Ljubimov AV, Burgeson RE, Butkowski RJ, Couchman JR, Wu RR, Ninomiya Y et al. Extracellular matrix alterations in human corneas with bullous keratopathy. *Invest Ophthalmol Vis Sci*. 1996;37(6):997-1007.
10. Savige J, Sheth S, Leys A, Nicholson A, Mack HG, Colville D. Ocular features in Alport syndrome: pathogenesis and clinical significance. *Clin J Am Soc Nephrol*. 2015;10(4):703-799.
    doi:10.2215/CJN.10581014
11. Akhtar S, Bron A, Hawksworth N, Bonshek R, Meek K. Ultrastructural morphology and expression of proteoglycans, βig-h3, tenascin-C, fibrillin-1, and fibronectin in bullous keratopathy. *Br J Ophthalmol*. 2001;85(6):720-731.
12. Torricelli AAM, Singh V, Santhiago MR, Wilson SE. The Corneal Epithelial Basement Membrane: Structure, Function, and Disease. *Invest Opthalmol Vis Sci*. 2013;54(9):6390.
    doi:10.1167/iovs.13-12547
13. Novikov IA, Subbot AM, Fedorov AA, Griboedova IG, Antonov EN, Vakhrushev IV. Supravital lanthanoid staining for scanning electron microscopy of biological objects. *Geny i kletki*. 2015;10(2):90-96. (In Russ.).
14. Novikov IA, Subbot AM, Kiryushchenkova NP, Nesterova TV, Gabashvili AN, Sitnikov AV et al. Fast and easy method of lanthanoid staining for visualization of cellular ultrastructure and spatial arrangement. B: AIP Conference Proceedings. AIP Publishing; 2016 c. 20009. http://scitation.aip.org/content/aip/proceeding/aipcp/10.1063/1.4954343;jsessionid=kbwfzGpq96qMxcRo31dkHwy.x-aip-live-02
15. Ferreira-Gomes MS, González-Lebrero RM, de la Fuente MC, Strehler EE, Rossi RC, Rossi JPFC. Calcium occlusion in plasma membrane Ca2+-ATPase. *J Biol Chem*. 2011;286(37):32018-32025.
    doi:10.1074/jbc.M111.266650
16. Cailliez F, Lavery R. Cadherin Mechanics and Complexation: The Importance of Calcium Binding. *Biophys J*. 2005;89(6):3895-3903.
    doi:10.1529/biophysj.105.067322
17. Burton PR, Fernandez HL. Delineation by Lanthanum Staining of Filamentous Elements Associated with the Surfaces of Axonal Microtubules. *Journal of Cell Science*. 1973;12(2):567-583.
18. Evans CH. *Biochemistry of the Lanthanides*. Springer Science & Business Media; 2013.
19. Rubashkin A, Iserovich P, Hernández JA, Fischbarg J. Epithelial fluid transport: protruding macromolecules and space charges can bring about electro-osmotic coupling at the tight junctions. *J Membr Biol*. 2005;208(3):251-263.
    doi:10.1007/s00232-005-0831-y
20. Aitken DA, Beirouty ZA, Lee WR. Ultrastructural study of the corneal epithelium in the recurrent erosion syndrome. Br J Ophthalmol. 1995;79(3):282-289.