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Pterygium: histological and immunohistochemical aspects

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Abstract

Introduction: Pterygium represents a triangular conjunctivo–epithelial overgrowth, proliferating from the bulbar conjunctiva and covering the cornea, causing severe vision loss. It is an abnormal growth and differentiation of the conjunctive epithelial structures of the corneal limbus. Chronic exposures to solar ultraviolet radiation, heat and dust, as well as viral agents, are the most common pathogenic entities involved in its evolution. Recent studies linked pterygium with neoplastic proliferation, as ocular limbic stem cells and p53-protein expression are altered. *Materials and Methods*: Our study was conducted on 84 fragments of pterygium, collected after surgery from patients admitted between 2008 and 2009 in the Departments of Ophthalmology of the Emergency County Hospital of Craiova and Hospital of Rovinari. Histological studies were performed by staining with Hematoxylin–Eosin, light green trichromic (Goldner–Szekely technique) and PAS–Hematoxylin. Immunohistochemistry highlighted the T-lymphocytes by using the CD3 antibody, B-lymphocytes by using the CD20 antibody and cells of the macrophage system using the CD68 antibody. The slides were analyzed under a 55I Nikon microscope, resulting pictures being captured with a 5 MP digital camera and digitally retrieved and enhanced using the dedicated NIS-Elements software. *Results*: Histology showed the presence of a conjunctivo–epithelial structure, significantly different from the structure from which it developed. In 20% of the cases, the covering epithelium was similar to that of the bulbar conjunctival mucosa. In some cases, when pathogens were more active in the environment, the appearance of the covering epithelium of the membrane appeared pleomorphic, with dysplastic aspects, suggesting significant alteration of cell proliferation and differentiation. In approximately 75% of patients, we identified goblet cells in the surface epithelium. They appeared either isolated or associated in variable numbers, structures resembling intraepithelial glands. These particular cells synthesize and accumulate PAS-positive mucines rich in glycosaminoglycans, and are usually found in conjunctival epithelium. We observed a number of invaginations in the connective tissue underlying the epithelium, mostly formed by goblet cells, this giving the aspect of mucous glands, similar to the "glands of Henle". A highly developed vascular neoformation network, consisting of arterioles, venules and a very large number of capillaries can also be found in the connective tissue. Immunohistochemistry suggested that B-lymphocytes marginally take part in the immune response in pterygium. T-lymphocytes formed the majority of the mass of immune system cell present in connective tissue of the pterygium. Macrophage-type cells were distributed unevenly in the pterygium tissue, as the intensity of the inflammatory process varies depending on antigen levels. *Conclusions*: Pterygium shows significant changes both in the epithelium and in the underlying connective tissue. Immune cell infiltrate was diffuse, more abundant in areas with erosion of the covering epithelium.

Keywords: pterygium, goblet cells, immunohistochemistry, inflammatory infiltrate.

Introduction

Pterygium represents a conjunctive epithelial overgrowth, triangular in most cases, which proliferates from the bulbar conjunctiva over the cornea [1], surpassing the anterior end, thus causing severe vision impairment. Regarded until recently as a chronic degenerative condition, pterygium is in reality a disorder of growth, development and differentiation of the conjunctivo-epithelial structures of the sclero-corneal limbus, characterized by the appearance of a new fleshy, translucent triangular formation on the surface of the cornea.

While most authors argue that chronic exposure to solar ultraviolet radiation plays a major role in the development and progression of lesions, the pathogenesis of pterygium is not fully understood. A number of factors have been involved in the pathogenesis of pterygium, among which the most frequently cited are: heat, dust, dry atmosphere [2] viral infections, immunological mechanisms, extracellular matrix remodeling, growth factors, some cytokines, antiapoptotic mechanisms and several angiogenic factors [3].

Histological and immunohistochemical studies in recent years associated pterygium with a neoplastic proliferation, as an alteration of the limbus stem cells [4] and altered p53-protein expression were found [5].

In the present study our goal was to highlight some aspects of histological and immunohistochemical changes that occur in pterygium and to evaluate the intensity of immune reaction in this disease of the anterior segment of the eye.

Materials and Methods

The studied biological material was represented by 84 fragments of pterygium collected from individuals aged between 28 and 81 years, operated between 2008 and 2009 in the Departments of Ophthalmology, Emergency County Hospital of Craiova and Hospital of Rovinari. Immediately after harvesting, the biological material was placed in a fixative solution of 10% neutral formalin for 24–72 hours and then sent to the Research Center for Microscopic Morphology and Immunology of the University of Medicine and Pharmacy of Craiova, where they were worked in the classic histological technique of paraffin inclusion. Four millimeters thick histological cups were made using a HM350 microtome equipped with a special system for sections transfer (Section Transfer System, STS). For histological studies, we performed staining with Hematoxylin–Eosin, light green trichromic (Goldner–Szekely technique) and PAS–Hematoxylin. For immunohistochemistry studies, the histological sections were placed on slides coated with Poly-Lysine (Poly-L-Lysine) (Sigma), in order to increase adherence of sections on the slides. To highlight specific antigens, following standard procedures, histological sections were subjected to immunostaining techniques using the EnVision system (Dako), which we consider a superior method of detection to classical detection systems such as direct detection and detection mediated by Avidin and Biotin (ABC and LSAB). In our study, we wanted to highlight the T-lymphocytes by using the CD3 antibody, B-lymphocytes by using the CD20 antibody and cells of the macrophage system using the CD68 antibody. In the table below, we highlight the antibody clones we used, dilutions, specificity and antigen recovery method (Table 1).

Table 1 – ***Description of the antibodies utilized in this study***

Name	Specificity	Clonality	Clone	Working dilution	Antigene retrieval	Producer
CD3	T-lymphocytes	Polyclonal	–	1:100	Boiling in Citrate Buffer (CB), pH 6	Dako, Medicalkit Craiova
CD20cy	T-lymphocytes	IgG2a	L26	1: 200	CB, pH 6	Dako
CD68	Macrophages	IgG1	KP1	1:100	CB, pH 6	Dako

Examination of the histological sections was performed using a 55I Nikon research microscope equipped with a 5 MP digital camera and the automatic exposure and retrieval software for microscopic images, NIS-Elements (Nikon).

Results

Study of the histological cups evidentiated the presence of a conjunctivo–epithelial structure, with significant microscopic changes compared to the structural elements from which it developed.

Regarding the coverage epithelium, in approximately 20% of our patients the appearance of the covering epithelium was similar to that of the bulbar conjunctival mucosa, thus appearing as a stratified squamous type epithelium without keratinization. In its structure, we could observe several overlapping layers of cells. Relatively frequent, we found areas of squamous epithelium prone to pseudokeratinization, areas in which surface cells, called squamous cells, were loaded with cytoplasmic keratohialin granules or had intense eosinophilic due to the existence of granules with eleidin content. Also, in about 12% of cases, the covering epithelium presented areas of erosion on the surface, affecting virtually the entire epithelial thickness.

The most common changes of the covering epithelium were focal hyperplasia, where there was an excessive thickening of the intermediate layer, called polyhedral cell layer, microscopic aspect indicating a local disorder of the maturation and proliferation processes of cells.

In some cases, the appearance of the covering epithelium of the membrane appeared pleomorphic, with dysplastic aspects. We believe that in these areas the action of etiopathogenical factors was more intense, which caused a profound disruption of cell proliferation and differentiation.

In approximately 75% of patients we identified in the surface epithelium goblet cells, either isolated or associated in variable numbers, forming real "intra-epithelial glands" (Figures 1 and 2). Goblet cells are observed in classical stains, having the appearance of a cup or goblet, with a dilated apical pole, with foamy cytoplasm slightly stained due to the synthesis and accumulation of substances rich in glycosaminoglycans. PAS–Hematoxylin staining specifically revealed these cells, as the mucines are intensely PAS-positive. These cells are not normally found in the corneal epithelium, however they are found in conjunctival epithelium, where they produce mucines, substances that constitute the innermost layer of the tear film covering the outer surface epithelium of the conjunctiva and cornea. The emergence of an increased number of goblet cells in the epithelium of pterygium may be the consequence of the exposure of the anterior segment of the eye to irritant pollutants.

Change of the cell phenotype, from a keratinous cell type to a mucus secretory cell is made progressively, by our observations, as in the epithelium we observed columnar cells which contained small quantities of PAS-positive mucin secretion at the apical pole. Increased mucin secretion without excretion will lead to expansion of the apical cell pole and the occurrence of such cells in form of a "cup of champagne" or caliciform (goblet) cell.

Another microscopic change highlighted in our study was the presence of intussusceptions in the connective tissue underlying the epithelium. These invaginations were formed mostly from goblet cells, giving the aspect of mucous glands, similar to the "glands of Henle",

present in the bulbar conjunctiva. Around these glandular structures, we revealed the presence of numerous cells belonging to the immune system, giving the appearance of chronic inflammatory infiltrate (Figure 3).

The general character of the connective tissue was similar to that of a subepitelial a loose connective tissue with a rich basic substance and a rich network of blood vessels, contrasting sharply with the underlying stromal connective tissue of the subjacent cornea. Fundamental substance was completely uneven, with areas of intensely positive reaction, rich in glycosaminoglycans, and areas with weak positive reaction.

Among the connective cells, we identified numerous fibroblasts, cells involved in the synthesis of non-fibrillary and fibrillary elements of the connective tissue. Besides fibroblasts, we found round mono-nuclear cells of lymphoplasmocytary macrophage type belonging to the immune system. The layout of the immune system cells was entirely uneven; however, we noticed very high densities in areas in which the covering epithelium showed erosion, which proves that at that level various antigens have penetrated.

Also, in the connective tissue we evidentiated a rich network of collagen fibers. Most often, these fibers had a loose arrangement with no particular orientation, and only rarely have tended to organize themselves and form collagen bundles.

Microscopic examination allowed us to note the presence of a highly developed vascular network, consisting of arterioles, venules and a very large number of capillaries (Figure 4). Blood vessels originate in the vessels of the sclera–corneal limbus, aspect macro-scopically visible through the transparent membrane of pterygium. Most appeared congested, with an enlarged lumen and multiple anastomoses. What was striking was the large number of vessels of angiogenesis. These vessels have come in the form of capillaries, with turgid endothelial cells with abundant cytoplasmic basophilia, containing a large, oval and hypochromic nucleus.

Another feature of the vascular network developed in the pterygium was the great fragility, demonstrated by numerous hematic extravasates, which probably occurred during surgery, with intact red blood cells and without macrophage reaction.

To assess immune response we used immunohisto-chemical techniques. B-cells, highlighted by using the CD20 antibody, were relatively few in the inflammatory infiltrate of the pterygium connective tissue compared with other cellular elements. Also, their distribution was uneven overall, being identified mainly around blood vessels or around congested vessels of angiogenesis. On some slides, we have highlighted the B-lymphocytes in the covering epithelium of pterygium. The presence of lymphocytes in the epithelium is caused by the fact that they are equipped with their own mobility, being able to cross non-keratinizated Malpighian-type squamous epithelia, in order to contact antigens, which penetrate the thickness, or on the surface of the cover epithelium. In areas where coverage epithelium showed erosion or discontinuities, the number of B-cells was much higher than in the rest of the pterygium tissue, which indicates that the antigens in those areas are more numerous in number and quantity, the epithelial barrier being severely affected (Figure 5).

These immunohistochemical aspects suggest that participation of B-lymphocytes is relatively low during the immune response in the pterygium, however also that the inflammatory reactions are minimal and chronic in pterygium.

T-lymphocytes were immunohistochemically high-lighted using CD3 antibody. T-lymphocytes were the best represented cells of the immune system in the inflammatory infiltrate of pterygium. They formed the bulk of the mass of immune system cell present in connective tissue of the pterygium. As B-cells, T-cells were arranged unevenly in the conjunctival structure of the pterygium. Most cells were identified as covering epithelium and around blood vessels (Figure 6).

Immunohistochemical study of the macrophage system cells was performed using CD68 antibody. Macrophage-type cells were distributed unevenly in the pterygium tissue. In some areas, we found clusters of macrophages, while in others the cells were highly dispersed. This allowed us to state that the intensity of the inflammatory process varies from one area to another in pterygium, probably depending on the amount of antigen present at this level.

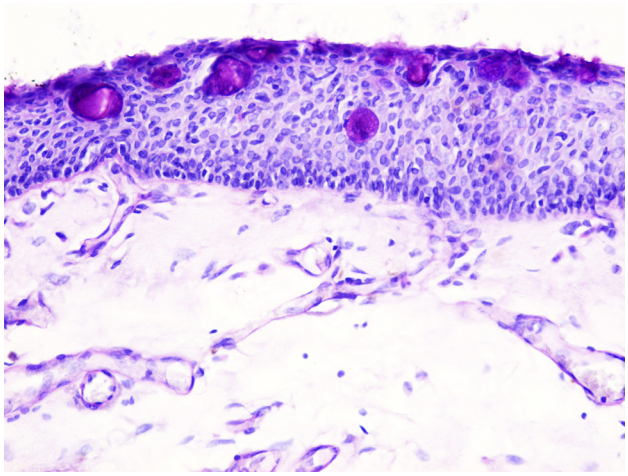

Figure 1 – ***Goblet cells randomly placed in pterygium epithelium (PAS–Hematoxylin staining, ×100).***

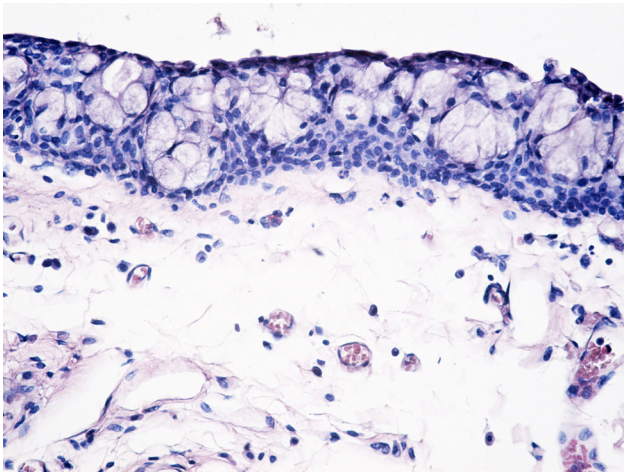

Figure 2 – ***Goblet cells grouped as intraepithelial glands (Hematoxylin–Eosin staining, ×200).***

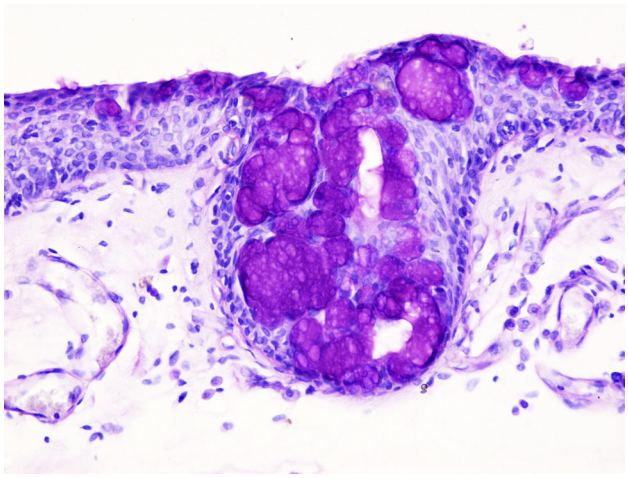

Figure 3 – ***Epithelial invaginations mostly formed by goblet cells with an aspect of a "Henle gland" (PAS–Hematoxylin staining, ×200).***

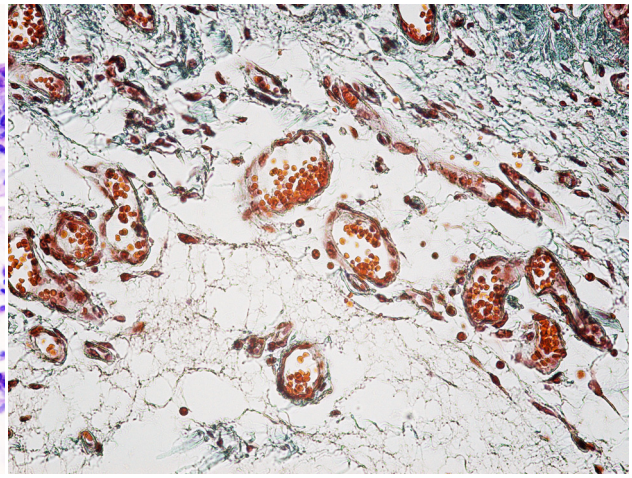

Figure 4 – ***Pterygium with rich vascular network (Trichromic Goldner–Szekely staining, ×200).***

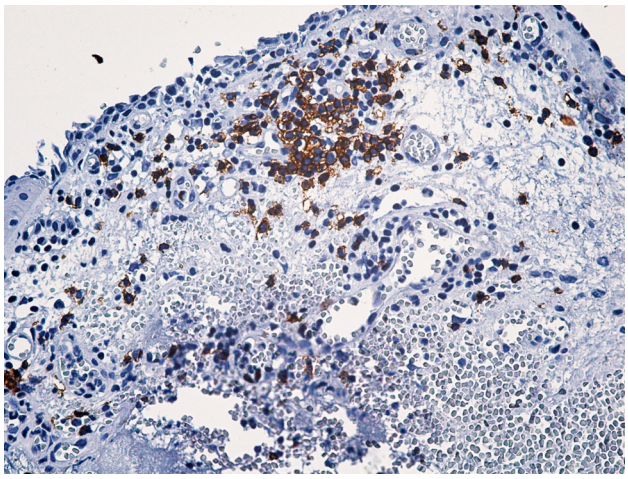

Figure 5 – ***Numerous B-lymphocytes with granular organization in a pterygium area, with the erosion of the covering epithelium (CD20 immunostaining, ×100).***

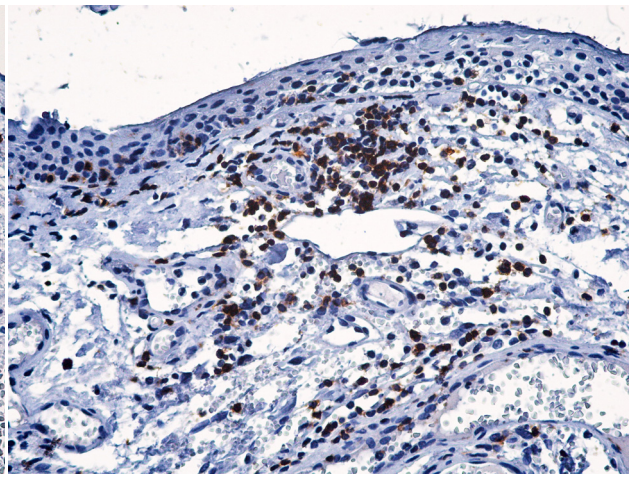

Figure 6 – ***Overview image of a pterygium area, characterized by a rich chronic inflammatory infiltrate mostly formed by T-lymphocytes (CD3 immuno-staining, ×100).***

Discussion

Pterygium represents an overgrown formation of fibrovascular tissue with a triangular layout, increasing progressively from the conjunctiva over the cornea [1, 3, 6]. Untreated, it can invade the cornea, occupying the optical axis of the eye, sealing the pupil, and eventually leading to loss of vision or the appearance of astigmatism [7, 8].

Although considered a benign condition, pterygium seems to be a condition with a very high prevalence, requiring repeated surgery. Currently, in Romania, there are no studies on the incidence and prevalence of the disease in the general population.

In a study published in 2009, Shiroma H *et al.* [9] have found a prevalence of 30.9% for pterygium in a single eye, compared with 13.3% for both eyes simultaneously. The highest prevalence reported was 33% in a population study conducted in China, which included subjects older than 50 years in the Doumen Region lying in the south of the country [10].

Microscopy and immunohistochemical studies on the structure and evolution of pterygium were imposed in recent years because the disease may recur several times after surgical removal, relapse being a new trauma for the patient.

According to some authors [11] recurrence would happen due to the fact that epithelial and connective cells with proliferative character remain on the edge of resection, hence the proliferative process can resume.

Other researchers have shown that although pterygium does not metastasize, it is a neoplastic disease presenting disordered cell proliferation. This cell proliferation is characterized by alterations in proteins involved in cell development and is enhanced by UV-light that causes specific mutations of the TP53 gene, known as a tumor suppressor gene. Therefore, most authors consider that ultraviolet radiation is a risk factor for pterygium [12–14].

The histological study conducted by us showed that in the structure of the covering epithelium of the pterygium appear abnormal cells, which alter the architecture of the conjunctival epithelium of which it has grown. Thus, the covering epithelium may appear pleomorph, with dysplastic aspects, features that indicate a profound disturbance of cell proliferation and differentiation process.

We consider a disturbance of cell proliferation and differentiation process the emergence of goblet cells, agglutinated in the form of intraepithelial glands, sometimes resembling hyperplastic and hypetrophic, as well as Henle's glands. We believe that these morphological factors are the result of direct action etiopathogenic factors, particularly dust particles in the air, because, as support and other authors [15], ocular mucosa is constantly exposed to particulate matter from the external environment and people living in areas with high concentrations of pollutants are often affected by eye symptoms [16]. Other authors [17] believe that goblet cell hyperplasia is a stereotypical response of conjunctival mucosa when there is significant air pollution. These arguments are supported by other authors [16, 18] who have detected abnormalities in the tear film and subclinical changes of the ocular surface in people who have lived in cities with a high level of air pollution. Moreover, Novaes P *et al.* [15] conducted a study involving 29 volunteers and found that the number of goblet cells increased proportionally to contaminant exposure, NO_2 in particular.

Concerning the appearance of erosions of the covering epithelium, we believe that their presence is due to the abrasive effect of dust microparticles and of microorganisms such as viral or bacterial agents that can temporarily penetrate the membrane of the pterygium epithelium.

Histological changes present in the connective tissue of the pterygium are extremely varied. According to some authors [19, 20] the first alteration of the connective tissue consists of a local hyalinisation, process through which degenerated collagen and a granular material probably resulted from degeneration of other components of the connective tissue appear.

Many papers have reported the presence of an inflammatory process in the connective tissue of the pterygium, and have involved inflammation and immune reactions in the pathogenesis and disease progression. Thus, in 1984, Pinkerton OD *et al.* [21] reported the infiltration of small lymphocytes and plasma cells in the pterygium tissue, and immunofluorescence techniques revealed the presence of IgG and IgE. On these considerations, the authors suggested that an immunological mechanism could also contribute to the pathogenesis of pterygium. They suggested that exogenous irritants such as pollen and dust particles could cause increased production of IgE to trigger a process leading to inflammation. This process induces the release of inflammatory cytokines and growth factors such as platelet-derived growth factor, which is responsible for the accelerated proliferation of the pterygium membrane, as well as angiogenesis. Other authors [22] have extended this hypothesis and suggested that ultraviolet light induces or causes damage to the ocular surface, which leads to an inflammatory response, which in turn induces fibrovascular proliferation and neovascularization of the cornea.

By the immunohistochemical techniques we used, we highlighted the presence of a quantitatively reduced inflammatory infiltrate composed of T-lymphocytes, B-lymphocytes, macrophages and plasma cells. In areas where coverage epithelium showed erosion or disruption, we have identified an increased number of cells belonging to the immune system. These facts make us believe that the inflammatory process is mild and is not responsible for cell proliferation, angiogenesis process or the recurrence of pterygium.

Conclusions

Pterygium, although considered an extension of the bulbar conjunctiva over the cornea, shows significant changes both in the epithelium and in the underlying connective tissue, compared with the structure of the cornea or conjunctiva.

In the epithelium, we found areas of hyperplasia, prone to pseudokeratinization, areas with erosion and even dysplastic areas. More than two thirds of patients with pterygium we identified numerous goblet cells, either diffusely scattered or clustered in the form of intraepithelial glands. Also, the epithelium presented, numerous intussusceptions in the underlying connective tissue forming "mucinous glands of Henle" composed primarily of the PAS-positive goblet cells.

Subepithelial connective tissue had the appearance of loose connective tissue, with a heterogeneous structure, rich basic substance, with numerous fibroblasts, round mononuclear cells of lymphocytary and macrophageal type, and a rich network of blood vessels.

Immune cell infiltrate (lymphocytes, macrophages, plasma cells) was diffuse, homogeneous, being more abundant subepithelial and in areas with erosion of the covering epithelium.

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