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Angiogenesis in the pathogenesis of pterygium

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Abstract

Pterygium represents an epithelial hyperplasia associated with fibro-vascular growth. It is an active process, associate with cellular proliferation, remodeling of the connective tissue, angiogenesis and inflammation. The aim of this study consists of emphasizing angiogenesis involvement in the pterygium pathogeny. The material used for this study consisted of 21 pterygion fragments surgically removed in the Ophthalmology Clinic of the Emergency County Hospital, Craiova. Nine patients were men, 22 were women, and they were aged between 58 and 81 years. Ten fragments of epibulbar conjunctiva from the vicinity of the sclero-corneal limbus were used as control tissue. They were initially histological processed by paraffin inclusion. The immunohistochemical processing was made in the Histological, Histopathological and Immunohistochemical Techniques Laboratory of the University of Medicine and Pharmacy of Craiova. The working technique used was ABC/HRP (Avidin complexed with biotinylated peroxidase). Angiogenesis in the pterygion was investigated with CD31 marker that allows the identification of the vascular endothelium and the establishment of the vascular microdensity and with VEGF, which allowed the identification of the main source of proangiogenic factors in pterygium. Our study emphasized the existence of a much richer vascularization at the level of the pterygium, compared with the one of the normal conjunctiva. The respective blood vessels were best represented in the subepithelial conjunctive, due to the increased necessities of the proliferating pterygium epithelium. The morphology of the blood vessels is specific for the neoformation vessels, which have a small caliber, are branched and have a rarely visible lumen. The investigation of the vascular microdensity has shown the existence of an intense angiogenesis process at the level of the pterygium and the overexpression of the VEGF, mainly in the proliferating structures of the pterygium, plead for the pathogenic involvement of this growth factor in the development of the pterygium.

Keywords: pterygium, immunohistochemistry, angiogenesis, CD31, VEGF.

Introduction

Pterygium is a lesion of the ocular surface, involving only one or both eyes. It has the form of a triangular strap-like fibro-vascular tissue that lays over the epibulbar surface of the conjunctiva, with the bottom of the triangle on the nasal conjunctiva and pointing to the cornea. It can progress onto the center of cornea. Recently published data showed that the disease is an active process of cellular proliferation, ongoing connective tissue remodeling, angiogenesis and inflammation. Pterygium consists of epithelial hyperplasia accompanied by fibro-vascular proliferation, originating at the corneo-conjunctival junction. From this site, modified limbic stem cells migrate and surpass the cornea. It causes discomfort, lachrymation and photophobia. After surgical intervention, relapses are frequent. When pterygium takes a more aggressive course, it can cause blurred vision by inducing irregular astigmatism, injuries of the corneal stroma and obliteration of the visual axis, as well as ocular irritation through the inflammation of the ocular surface from its site [1]. Although considered a relatively benign process, pterygium can be locally invasive with various degrees of abnormalities, ranging from mild dysplasia to carcinoma *in situ*.

The subject of this paper is of great interest since the elucidation of the etiopathogenesis of this disease can have significant clinical consequences for the surgical treatment (preventing the frequent post-surgery relapses); also, it can help the development of new non-surgical treatments to reduce relapses, severity of inflammation, tissular invasion, proliferation and angiogenesis.

Materials and Methods

The material used in this study consisted of 24 pieces of surgical excision from human patients (fragments of pterygium), obtained from the Clinic of Ophthalmology of the Emergency County Hospital, Craiova. From a clinical perspective, nine of the patients were male and the rest were female; the patients' ages ranged between 58 and 81 years. Ten more fragments of epibulbar conjunctiva were obtained during surgical procedures for cataract; these were used as control tissue.

Pterygium fragments were initially treated with the regular method of paraffin inclusion and the resulting

sections were stained with Hematoxylin and Eosin (HE) and trichromic protocols: Goldner-Szekely and van Gieson.

The method employed for immunohistochemical tests was ABC/HRP technique (Avidin complexed with biotinylated peroxidase). During immunohistochemical study, we used two antibodies; their characteristics and working conditions are presented in Table 1.

Table 1 – ***Antibodies used in the study of pterygium***

Antibody	Clone	Manufacturer code	Antigen retrieval Microwave/enzyme digestion	Dilution	Technique used
Monoclonal Mouse Anti-Human CD31	JC70A	Dako/M0823	Seven 3-minute cycles using microwaves (750 W) in pH 6 citrate	1:30 in PBS, pH 7.2	ABC
Monoclonal Mouse Anti-Human VEGF	VG1	Dako/M7273	Seven 3-minute cycles using microwaves (750 W) in pH 9 Tris-EDTA	1:25 in PBS, pH 7.2	ABC

The criteria established in the scientific papers [2] were used for the interpretation of the immunohisto-chemically-studied cases and for reporting; according to these criteria, the intensity of staining is recorded as:

- (+++), if staining is intensely positive or specifically scattered, clearly visible with small magnification;
- (++), if staining is focal or of moderate intensity, clearly visible only with medium magnification;
- (+), if staining is weak or very localized, clearly visible only with a large magnification;
- (±), if staining is very poor;
- (–), if staining is negative.

For the assessment of vascular density, at least five microscopic fields were analyzed (so-called "hot-spot" – microscopic fields with the highest density of CD31+ vessels, with a ×400 magnification) for every pterygium case investigated.

For each case, we have determined a mean value and its standard deviation. Afterwards, we have compared these values.

The "hot-spot" fields were chosen after microscopic scanning of the sections with an objective with the magnification of ×200.

Results

We have examined under the microscope fragments of primary and relapsed pterygium from patients treated with surgical ablation and conjunctival autologous graft. These fragments are composed of epithelium and different kinds of stroma, in accordance with the evolutive stage.

The epithelium is stratified, columnar towards the sclera and squamous non-keratinized towards the cornea (Figures 1 and 2). This epithelium has a variable thickness, as it supplements the irregularities of the underlying stroma (Figure 3).

In some areas, the epithelium invaginates into the stroma and at this level there are increased numbers of goblet cells.

In the evolutive stage, the epithelium is thick and elevated by the proliferation of the underlying connective tissue.

The stroma of the pterygium's head is made of connective tissue, rich in fibrocytes and connective fibrils.

The stroma of the main body of pterygium contains numerous connective fibrils. Furthermore, there are many blood vessels, some of which having hyalinized walls (Figure 4).

An inflammatory infiltrate can be seen on some sections, especially in the perivascular and periglandular areas (Figure 5).

In the stationary phase, sclerosis is observed, and the connective fibrils are set in compact bundles; the blood vessels are prone to obliteration and the inflammatory process is reduced (Figure 6).

These findings are evident especially at the head of pterygium.

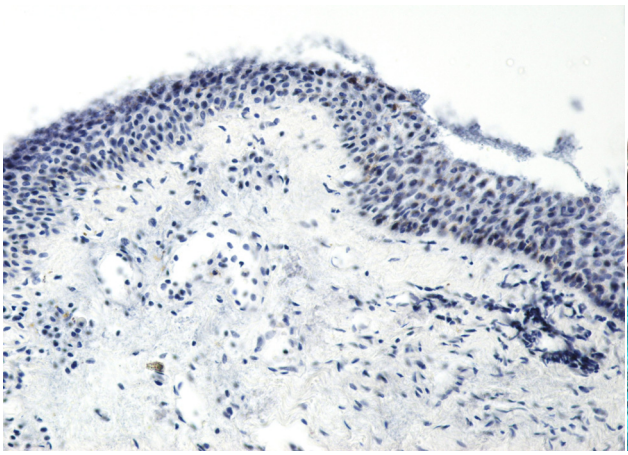

Figure 1 – ***Pterygium body. Non-keratinized stratified Malpighian epithelium (HE stain, ×200).***

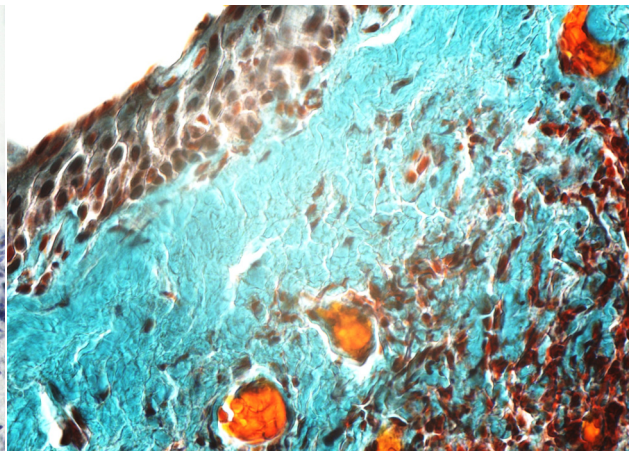

Figure 2 – ***Similar to prior image (trichromic Szekely stain, ×400).***

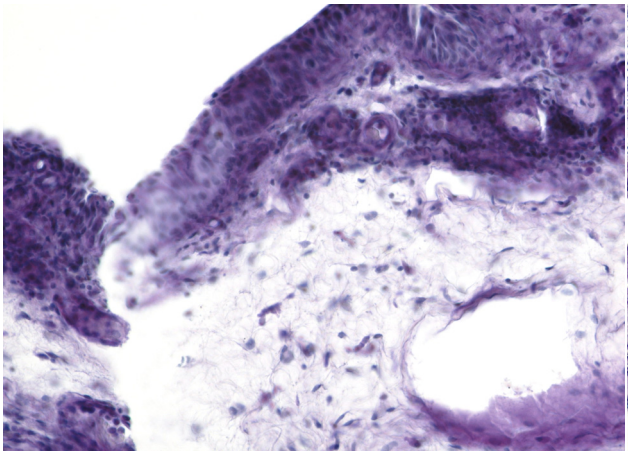

Figure 3 – ***The epithelium is likely to smoothen the stromal irregularities and in some areas becomes invaginated (HE stain, ×200).***

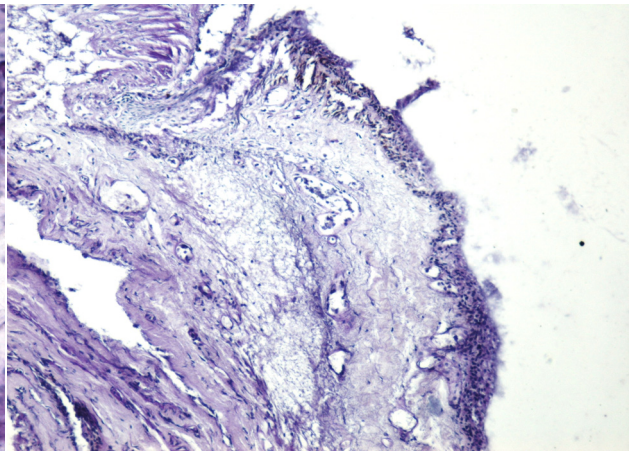

Figure 4 – ***Irregular epithelium and richly vascularized stroma (HE stain, ×20).***

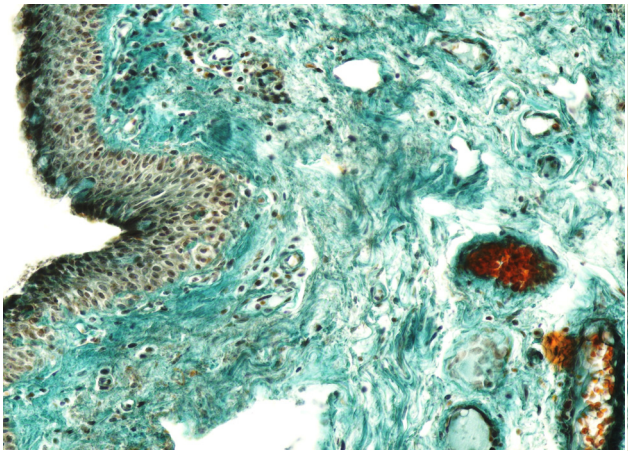

Figure 5 – ***Progressive pterygium. The stroma is a young connective tissue, containing a large number of fibrils and vessels. Perivascular inflammatory infiltrate (trichromic Szekely stain, ×400).***

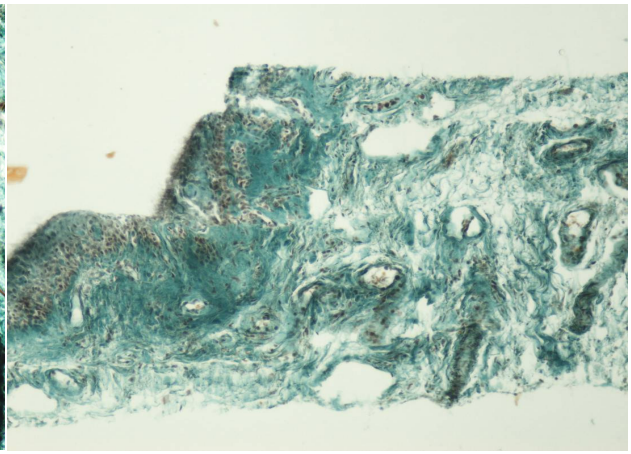

Figure 6 – ***Steady stage pterygium. The stroma contains a large number of fascicles of collagen. There is low inflammation (trichromic Szekely stain, ×100).***

Tissular morphology, distribution and microdensity of the blood vessels in the pterygium

The study of immunoreactivity at CD31 has proved the presence of a much richer vascularization in the connective tissue of pterygium, compared to normal conjunctive. The strongest reactivity to this marker was identified in the subepithelial region (Figure 7).

This particular distribution of the blood vessels can be explained by the need of the proliferating pterygium for an increased nutritive support.

Moreover, we have noted that the connective tissue contains especially vessels with small caliber, which tend to collabate (Figure 8). In addition, we have noted the presence of elongated, tortuous and branched blood vessels (Figure 9).

The morphology of these vessels is suggestive for the presence of an active angiogenesis in subepithelial connective tissue.

In the remaining connective tissue, the blood vessels had a regular diameter and morphology, very similar with normal conjunctive; their lumen is visible and sometimes distended, filled with blood.

In the areas of the connective tissue where an inflammatory infiltrate is observed, angiogenesis had the same intensity as in the subendothelial connective tissue.

Mean values for the microvascular density (MVD), determined with a ×40 objective were 19.58±6.7 and 7.6±4.5 for the pterygium and the normal conjunctive, respectively (Table 2).

Table 2 – ***Mean MVD in pterygium and normal conjunctiva***

Structure	MVD
Pterygium	19.58±6.7
Normal conjunctiva	7.6±4.5

The role of vascular endothelial growth factor (VEGF) in angiogenesis

In the epithelial part of normal conjunctiva, we have noted a weak reaction to VEGF; in the vascular endothelial cells and subepithelial stromal cells, no such reaction has been observed.

In all the cases studied, an immune reaction of the pterygian tissue to VEGF was present and this reaction was more intense than in normal conjunctival tissue.

From a qualitative perspective, the immunoreaction to

VEGF was heterogeneous (Figure 10), and its intensity was different for every case (Table 3).

Table 3 – ***Qualitative assessment of anti-VEGF antibody immunolabeling***

No. of pterygium cases	Qualitative measurement score of anti-VEGF immunolabeling				
	0	±	+	++	+++
21	0	0	4	11	6

A strong reaction to VEGF was observed in the pterygium for: epithelial cells, except goblet cells; vascular endothelial cells, fibroblasts and stromal inflammatory cells (Figures 11–13). The pattern of expression was granular, with diffuse cytoplasmic disposition. In the epithelium of pterygium, we have noted a gradual increase of immunoexpression towards the superficial layers (Figure 14).

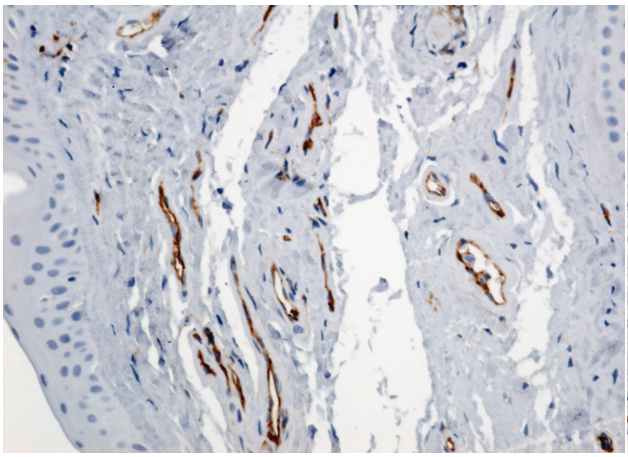

Figure 7 – ***Intense vascularization of the subepithelial connective tissue. CD31 positive, ×200.***

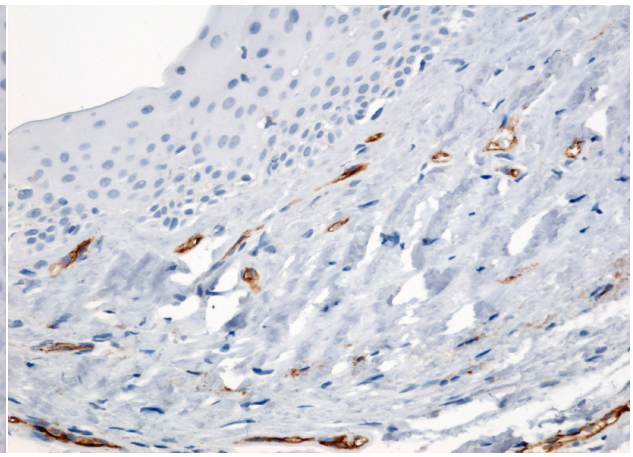

Figure 8 – ***Small sized flat CD31 positive blood vessels in the close proximity of the subepithelial connective tissue, ×200.***

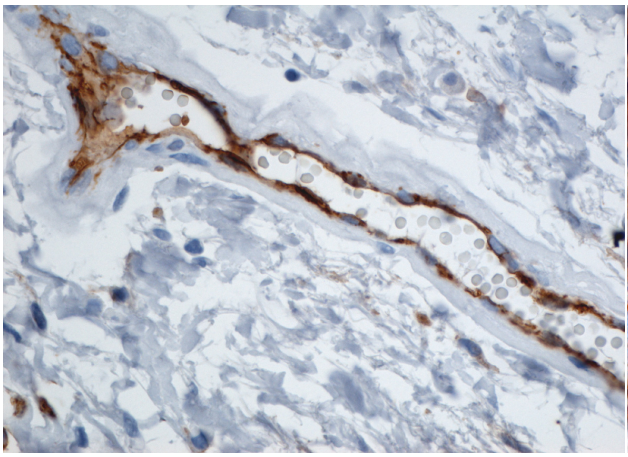

Figure 9 – ***Elongated, tortuous, ramified blood vessel CD31 positive, ×400.***

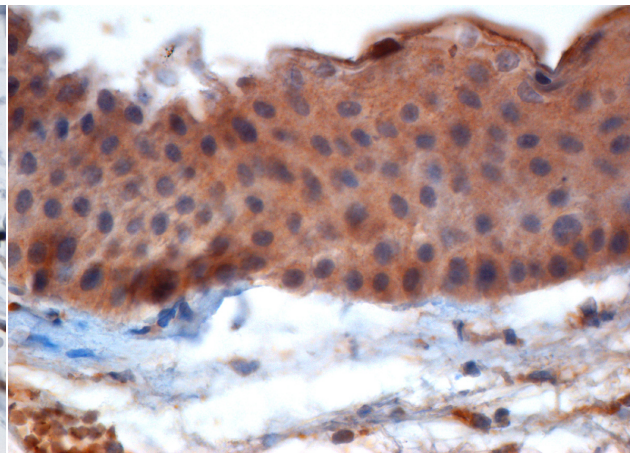

Figure 10 – ***VEGF positive through the epithelium thickness, ×400.***

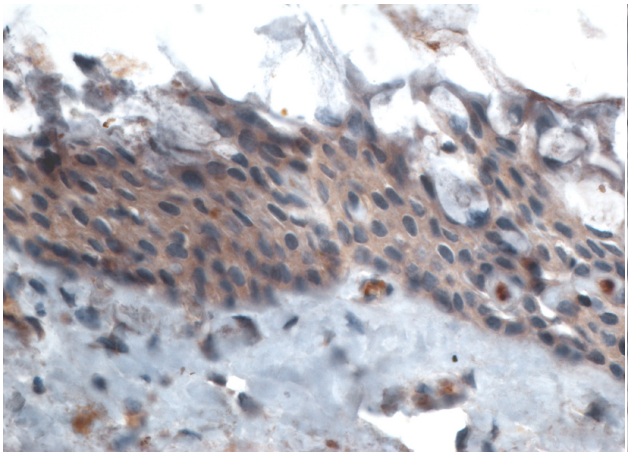

Figure 11 – ***VEGF positive in epithelial cells, except for mucous cells, ×400.***

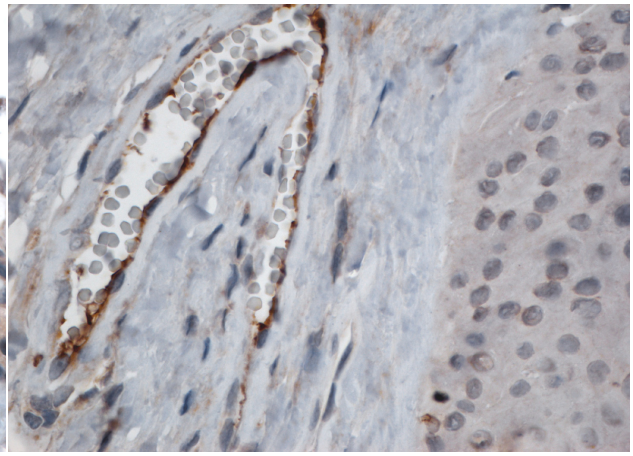

Figure 12 – ***VEGF positive in the endothelial cells of blood vessels, ×400.***

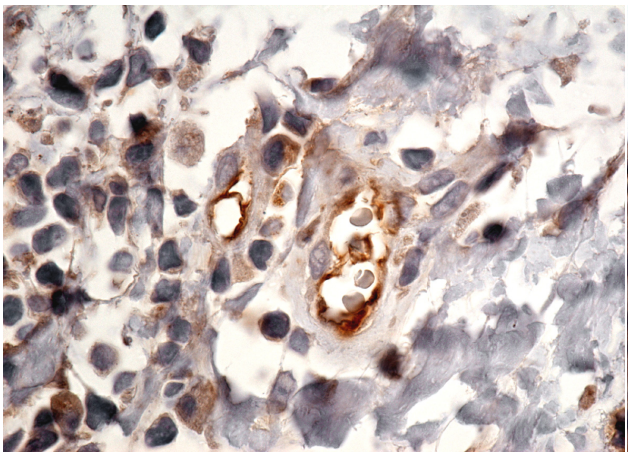

Figure 13 – ***VEGF positive in the endothelial cells of blood vessels and in the stromal inflammatory cells in the pterygium tissue, ×400.***

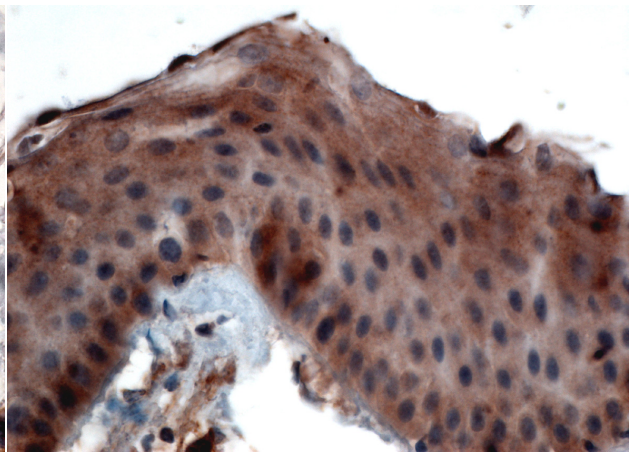

Figure 14 – ***Gradual increase of immunoreactivity to VEGF in the cells of superficial layers, ×400.***

Discussion

The incidence of pterygium was determined to be increased in tropical geographical areas, especially in equatorial regions between the northern and southern parallels of 30 degrees latitude [3]. This finding is explained by the excessive exposure to solar radiations. Although the pathogeny of pterygium is not fully understood, it is generally accepted that ultraviolet radiations are the most important etiological factor involved in its onset. Ultraviolet radiations trigger a series of events that ultimately lead to damages of DNA, RNA and extracellular matrix [4]. Actually, it has been proven that the polymorphism of Ku70 gene, responsible for repairing the damages of DNA, is linked to a genetic predisposition to pterygium [5]. More over, type B ultraviolet radiations induce the overexpression of cytokines and growth factors in the epithelial cells of the pterygium, which are considered the most important "driving force" in the development of pterygium [6].

Overall, we can describe pterygium as a proliferative-invasive disease, complicated with localized insufficiency of the sclero-corneal limbus and associated with excessive exposure to ultraviolet radiations. It is characterized by chronic inflammation and angiogenesis, which ultimately lead to remodeling of the connective tissue [7]. This etiopathogenic hypothesis contradicts the traditional one, which considered the pterygium to be a simple degenerative and benign process.

Angiogenesis is the process of growing new blood vessels from pre-existing vessels; it is the foundation of many physiological processes [8], such as growth and differentiation, ovulation, wound healing, and pathological conditions, such as neoplasia and ocular diseases complicated with severe visual disturbances [9]. The vascularization process implies the activation of angiogenetic factors derived from cells, but also a proper synthesis of elements of the extracellular matrix, needed for anchoring endothelial cells during migration.

The paper published by Seifert P and Sekundo W [10] is one of the first studies on pterygium vascularization; the authors used electron microscopy to prove that capillary vessels are scarce in the epithelium, but not exceptional. At this level, their growth from the stroma can be considered a reaction to hypoxia or to the lack of another substance vehiculated by the circulation.

Our study revealed the existence of a much richer vascularization in the pterygium then in normal conjunctiva. In addition, these vessels were most evident in the subepithelial connective tissue. This finding was explained by the increased needs of the proliferating pterygoid epithelium. The morphology of these vessels is specific to neoformation: they have small caliber, are tortuous, branched and the lumen is rarely visible.

Another study shows that the vascular network developed in the pterygium presents a great fragility, proven by numerous hematic extravasates, which probably occurred during surgery, with intact red blood cells and without macrophage reaction [11].

There are relatively few studies about the microvascular density in pterygium. Lee PP *et al.* [12] have investigated the role of angiogenesis in the pathogeny of pterygium, by comparing the expression of von Willebrand factor and VEGF in pterygium and in normal bulbar conjunctiva. Their result, together with the overexpression of VEGF in pterygium, prompted some authors to assert the major contribution of angiogenesis in the development of pterygium.

Aspiotis M *et al.* [13] studied the relationship between vascular microdensity, measured by CD31, and the expression of angiogenetic factor VEGF and endogenous inhibitor endospondin-1 in patients with pterygium and in normal bulbar conjunctive. They hoped to clarify the role of specific angiogenetic factors in the pathogeny of pterygium. Thus, the authors found that the mean microvascular density was 17.97 and 5.72 per high-power microscopical field in pterygium and normal conjunctiva, respectively. The highest angiogenetic activity was observed subepithelially, and the mixed type of pterygium had higher microvascular densities than the fibrous type. Other authors [10, 14, 15] obtained similar results regarding the pathogenic significance of neoangiogenesis in pterygium.

Angiogenesis is governed by a complex balance between positive and negative regulating factors. VEGF is one of the most potent and specific proangiogenetic

factor; it is also known as vascular permeability factor or vasotropin (1996). VEGF is a glycoprotein that binds heparin, with significant effects over vascular endothelial cells. In response to surrounding stimuli, especially to hypoxia, this growth factor can induce the synthesis of certain cytokines and estradiol [16–18]. At present, VEGF is considered the most selective mitogen of endothelial cells [19]; it increases vascular permeability [20], induces alterations of ionic fluxes, cellular proliferation [21], migration and release of proteases [22]. On the contrary, trombospondin-1, a multifunctional protein that belongs to platelets and extracellular matrix [23], is involved in the regulation of cellular growth, cellular motility, inflammation and wound healing. It modulates the motility of endothelial cells, the growth, apoptosis and adherence of neoplastic cells, being considered an inhibitor of angiogenesis [24].

VEGF was identified in tissues of the eye that are normally vascularized: conjunctiva, iris and pigment epithelium of the choroid and retina [25]. The messenger RNA of the type 1 and 2 receptors for VEGF was detected in the normal iris of monkeys, in retina and pigment epithelium retino-choroidian [25]. VEGF and its receptors are overexpressed in the vascular and inflamed cornea of adults [26]. However, transcription of VEGF and its type 2 receptor was surprisingly detected in the eye during embryonary and fetal development, before the appearance of the retinal vascular system [27]. Moreover, increased expression of VEGF was found in various avascular tissues in healthy humans; normally, these tissues are not the site of angiogenesis: gastro-intestinal mucosa [28], thyroid cartilage [29] and cornea [30].

Our study showed an increased expression of this factor, mainly in the proliferating elements of the pterygium: epithelium, endothelial cells and fibroblasts. Such reactivity is an argument for the pathogenic role played by this growth factor in the development of pterygium.

Many studies have shown that VEGF expression is greatly increased in pterygium [12, 31–33], suggesting its direct and indirect role in the pathogeny of this disease. The relationship between the VEGF signal transduction and the changing behavior of epithelial cells from pterygium/conjunctiva is not fully described.

Aspiotis M *et al.* [13] detected VEGF overexpression in the endothelial and stromal cells in the pterygium, while it was absent in the epithelial cells relative to normal conjunctiva. The results of this study contradict previous study results [31–34], which indicated high VEGF expression in the pterygium relative to normal conjunctiva. Unlike previous researchers, Aspiotis M *et al.* [13] noted that VEGF expression in the pterygium stroma was statistically higher than in the normal tissue. Overexpression of VEGF in epithelial cells is rather incapable to induce angiogenesis and it is seemingly consistent with low levels of vascular microdensity. This may be the result of VEGF lacking in endothelial and stromal cells. On the contrary, VEGF expression in the pterygium endothelial and stromal cells can induce angiogenic activity, demonstrated by increased vascular microdensity. Therefore, it seems that VEGF buildup in the epithelial cells is only a reflection of their secretory capacity, while buildup in the endothelial and stromal cells reflects its angiogenic activity. In this respect, Aspiotis M *et al.* [13] demonstrated higher vascular microdensity in the fibrous subtype than in the vascular type, thus confirming the assumption that stroma has a role in the pathogenesis of pterygium [4].

The study conducted by Gebhardt M *et al.* [34] demonstrated the presence of VEGF 121 and 165 isoforms both in the normal conjunctiva as well as in the pterygium. VEGF165 regularly induces angiogenesis and undergoes selective and potent inhibition by the connective tissue growth factor [35]. This growth factor prevents VEGF165 binding to the VEGFR2 receptor. However, it was proved that matrix metalloproteinases could release the VEGF165 isoform from the growth factor [36]. Since matrix metalloproteinase synthesis is common in pterygium [14, 37–40], this mechanism is also functional in the cornea and conjunctiva and therefore other antiangiogenic factors work through a similar mechanism *via* VEGF121, thus inhibiting neovascularization in these normal eye structures. The activation of matrix metalloproteinases in the pterygium may lead to the cleavage of the connective tissue growth factor and subsequent reactivation of the proangiogenic VEGF165 isoform, leading to endothelial cells growth and as a result to neovascularization in the pterygium.

Tsai YY *et al.* [41] highlighted that VEGF-460C gene polymorphism is associated with pterygium occurrence in women. One possible explanation is the impact of estrogens and progestins on VEGF expression and its receptors in the UV-exposed conjunctiva and cornea [42]. Therefore, young women with VEGF-460C gene polymorphism may develop pterygium.

Conclusions

Pterygium is a condition with a rather increased prevalence with multiple post-surgery relapses and involving a range of clinical issues; in severe cases, it reaches the corneal surface and induces vision impairment through irregular astigmatism, corneal stroma damage and obliteration of the visual axis.

The histological aspects described will contribute to the clarification of some issues relating to the etiopathogeny of the condition primarily regarding post-surgery relapses.

The study of vascular microdensity confirmed the existence of marked angiogenesis of pterygium, while VEGF overexpression particularly in the proliferative structures of the pterygium favors the causal involvement of this growth factor in pterygium pathogenesis.

This reactive response of the pterygium tissue proves on the one hand the proliferative-invasive, tumor-like nature of this condition. Moreover, this type of immunohistochemistry warrants the development of growth factors synthetic inhibitors, which may allow for the reduction of the relapse rate, inflammation intensity,

tissue invasiveness, proliferation and angiogenesis in pterygium.

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