Chapter 4

Immunohistochemical profile of VEGF, TGF-β and PGE2 in human pterygium and normal conjunctivæ: experimental study and review of the literature

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Abstract

Human pterygium is made up of chronic proliferative fibro-vascular tissue growing on the ocular surface. This disease exhibits both degenerative and hyperplastic properties. Some fibroangiogenic factors have recently been shown to play a potential role in fibrovascular diseases via the angiogenesis process. The aim of this study is to evaluate VEGF, TGF-β and PGE₂ expression in the epithelial, endothelial and stromal cells of human pterygium and normal conjunctiva in order to determine whether these factors participate in the development of pterygium. Ten specimens from patients with pterygium and two normal conjunctivas (cadavers) were analyzed by immunohistochemistry using specific antibodies against these growth factors. The technique used was ABC/HRP (Avidin complexed with biotinylated peroxidase). Immunoreactivity of VEGF was significantly increased in the epithelium, vascular endothelium and stromal cells in primary pterygium as compared with normal conjunctiva. A moderate expression of TGF-β in the pterygium was observed in the epithelial and stromal layers. On the contrary, immunolabeling of this growth factor in the human normal conjunctiva was weak. PGE₂ was strongly expressed in the epithelium of patients with pterygium, as in control conjunctival tissues and the immunolabeling was moderate in the stroma from the same patients. Our results suggest that these growth factors may contribute to the progression of primary pterygium by increasing angiogenesis, thus leading to the formation of new blood vessels from the pre-existing vasculature. We conclude that VEGF, TGF-β and PGE₂ may be potential therapeutic targets in the treatment of this disease although proof of this evidence requires further studies.
Introduction

Pterygium is a lesion of the ocular surface that begins to grow from limbal epithelium and invades the cornea by conjunctival epithelium, leading to visual impairment (1,2). Recently, published data have shown that this pathological condition is an active process of cellular proliferation, ongoing connective tissue remodeling, angiogenesis and inflammation. Histologically, actively growing pterygia exhibit both degenerative and hyperplastic changes as well as proliferative and inflammatory disorders (3,4). This disease consists of an overlying conjunctival epithelium, which may appear normal or mildly hyperplastic. The underlying fibrovascular tissue usually presents a chronic inflammatory cellular infiltrate and rich vasculature (4). It causes discomfort, lachrymation and photophobia. After surgical intervention, relapses are frequent. Many therapies, including antimitotics and corticosteroids, have been proposed for the prevention of recurrence, but no really effective therapy has been established (5). When pterygium takes a more aggressive course, although considered a relatively benign process, it may become locally invasive with various degrees of abnormalities, ranging from mild dysplasia to carcinoma in situ (6). The pathogenesis of pterygium is still controversial, although epidemiological studies have firmly established that ultraviolet radiation is an etiologic agent for this disease (7). In addition, many fibroangiogenic growth factors have been implicated in pterygium pathogenesis, such as tumor necrosis factor-α (TNF-α), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and transforming growth factor-β (TGF-β) (8). Angiogenesis is defined as the formation of new blood vessels from pre-existing vasculature and underlies a large number of physiological processes (9), such as growth and differentiation, wound healing, and abnormal conditions, such as neoplasia and eye diseases, which cause severe loss of vision (10). The process of vascularization involves the activation of cell-derived angiogenic factors as well as the appropriate synthesis of extracellular matrix components necessary for anchorage of migrating endothelium. One of the most potent and specific
angiogenic factors is vascular endothelial growth factor (VEGF), also known as vascular permeability factor and vasculotropin (11). VEGF is a heparin-binding glycoprotein that has several important effects on vascular endothelial cells. This growth factor may be produced in response to environmental stimuli, mainly hypoxia, certain cytokines and estradiol (12). VEGF is considered to be the most selective mitogen for endothelial cells (13): it increases vascular permeability (14), induces alterations in ion flow, cell proliferation (15), and migration and release of proteinases (16). Prostanoids are a group of lipid mediators forming in response to various stimuli, including prostaglandin and thromboxane A2. They are released extracellularly immediately after their synthesis and they act by binding to a G-protein-coupled rhodopsin-type receptor on the surface of target cells. There are 8 types of prostanoid receptors that are conserved in mammals: the PGD receptor (DP), a subtypes of the PGE receptor (EP1, EP2, EP3 and EP4), the PGF receptor (FP), the PGI receptor (IP), and the TXA receptor (TP). The ocular surface, especially the human conjunctival epithelium, showed EP2, EP3 and EP4 receptors, that may down-regulate ocular surface inflammation (17). In the current study we investigated the expression of VEGF, PGE2 and TGF-β by immunohistochemical techniques in some cases of primary pterygia and 2 normal conjunctiva specimens as controls, to elucidate the etiopathogenesis of this disease, that can have significant clinical consequences in terms of surgical treatment, preventing the frequent post-surgery relapses. Also, it may play a role in developing new non-surgical treatments to reduce relapses, severity of inflammation, tissue invasion, proliferation and angiogenesis.

Materials and Methods

Ethical considerations
The study group included 10 cases of surgically excised pterygium from patients (6 males and 4 females) aged 45-80 years, together with 2 autoptic specimens harvested as control cases (normal nasal epibulbar conjunctiva segments that showed the same features for immunohistochemical study; in fact, is it
impossible to use control specimens from normal conjunctiva of patients with pterygium due to potential abnormalities in the conjunctiva adjacent to the lesions). During excision, apart from topical anaesthesia, no other chemical or pharmaceutical product was administered. Experiments were performed in compliance with the Italian laws and guidelines concerning the patients informed consent. The ethical committee of the Hospital approved our study according to the European Community and Italian laws. Control morphological sections were stained with hematoxylin-eosin. The following molecules were investigated: vascular endothelial growth factor (VEGF), prostaglandin E2 (PGE$_2$) and transforming growth factor-$\beta$ (TGF-$\beta$).

**Immunohistochemical analysis**

Small fragments from pterygium and nasal epibulbar conjunctival tissues near the limbus (autoptic specimens) samples were washed in PBS, fixed in 10% formalin and embedded in paraffin according to a standard procedure. The method employed for immunohistochemical tests was ABC/HRP technique (avidin complexed with biotinylated peroxidase). Serial 3-μm thick sections were cut using a rotative microtome, mounted on gelatin-coated slides and processed for immunohistochemistry. These sections were deparaffinized in xylene and dehydrated. They were immersed in citrate buffer (PH 6) and subjected to microwave irradiation twice for 5 minutes. Subsequently, all sections were treated for 30 minutes with 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. To block non-specific binding, the slides were incubated in 3% normal goat serum in PBS for 30 minutes at room temperature. The slides were incubated overnight at 4°C with primary mouse monoclonal antibodies against human VEGF diluted 1/100 (abcam Cambridge Science Park, UK, ab1316), against human TGF-$\beta$ diluted 1/100 (abcam Cambridge Science Park, UK, ab49574) and with primary rabbit polyclonal antibody against human PGE2 diluted 1/500 (Abcam Cambridge Science Park, UK, ab2318). Optimal antisera dilutions and incubation times were assessed in a series of preliminary experiments. After exposure to the primary antibodies, slides were rinsed twice in phosphate buffer and incubated for 1 h at room
temperature with the appropriate secondary biotinylated goat anti-mouse or anti-rabbit IgG (vector laboratories Burlingame, CA, USA, BA9200 and BA1000) and with peroxidase-conjugated avidin (Vector laboratories, Burlingame, CA, USA, Vectastain Elite ABC Kit Standard* PK 6-100) for 30 minutes. After a further wash with phosphate buffer, slides were treated with 0.05% 3,3-diaminobenzidine (DAB) and 0.1% H$_2$O$_2$. Finally, sections were counterstained with Mayer’s hematoxylin and observed by using a light microscope. Negative control experiments were done: i) by omitting the primary antibody; ii) by substituting the primary antibody with an equivalent amount of non-specific immunoglobulins; iii) by pre-incubating the primary antibody with the specific blocking peptide (antigen/antibody = 5 according to supplier’s instructions). The staining assessment was made by two experienced observers in light microscopy. We assessed the immunoreactivity for VEGF, TGF-β and PGE$_2$ in epithelial, endothelial and stromal cells of these tissues. The intensity of the immune reaction was assessed microdensitometrically using an IAS 2000 image analyzer (Delta Sistemi, Rome, Italy) connected via a TV camera to the microscope. The system was calibrated taking the background obtained in sections exposed to non-immune serum as zero. Ten 100 $\mu$m$^2$ areas were delineated in each section by a measuring the diaphragm. Quantitative data of the intensity of the immune staining were analyzed statistically by analysis of the variance (ANOVA) followed by Duncan’s multiple range test as a post hoc test.

**Statistical analysis**

The comparison of the expression levels of VEGF, TGF-β and PGE$_2$ in the pterygium and normal conjunctiva was carried out by t-test. Statistical analyses were performed using the SPSS statistical software package version 12.0. The results were considered as statistically significant when P-value<0.05.
Results

Under light microscope fragments of primary pterygium from 10 patients treated with surgical ablation were examined. These specimens are composed of epithelium, endothelium and different kinds of stroma, in accordance with the evolutive stage. Intense angiogenic activity was observed particularly in the subepithelial region. Furthermore, pterygium tissues were more vascularized than normal conjunctiva. The particular distribution of the blood vessels can be explained by the need of the proliferating pterygium for increased nutritive support. We noted the presence both of small vessels as well as elongated, tortuous and ramified blood vessels: the morphology of these vessels is suggestive for the presence of an active angiogenesis in subepithelial connective tissue. The epithelium of the conjunctival tissues is stratified, columnar towards the sclera and squamous non-keratinized towards the cornea. In some areas, the epithelium invaginates into the stroma and an increased number of goblet cells are present in this area (Fig 1A). The epithelium is thick and often elevated by the proliferation of the underlying connective tissue. In the pterygium, the stroma is made of connective tissue, rich in fibroblasts, numerous connective fibrils and many blood vessels. In the stationary phase, sclerosis is observed, the inflammatory process is reduced and the connective fibrils are set in compact bundles (Fig 1B). Our study showed relevant immunoreactivity of VEGF in epithelial cells, except goblet cells (Fig. 1C); the normal conjunctiva demonstrated a statistically significant difference in comparison with pterygium, that presented extremely high expression level for VEGF in the epithelial layer. A strong reaction to VEGF was observed in the vascular endothelium, and in fibroblastic and inflammatory stromal cells of the pterygium tissue (Fig. 1D). In contrast to this pathologic tissue, no VEGF immunoreactivity was observed in endothelial or stromal cells of normal conjunctival tissues (Fig. 1C). The staining reaction was diffuse, granular, cytoplasmic, with intensification at the superficial layers of the epithelium. In the epithelium of pterygium we observed an increase of reaction towards the superficial region (Fig. 1D). TGF-β immunolabeling was very weak
in fibroblasts from normal conjunctiva, in contrast to the primary pterygium, that appeared strong in some stromal cells (Fig. 2A-B). These findings demonstrated that this growth factor may interact directly or indirectly in the pathogenesis of pterygium. Finally, we detected prostaglandin E2 (PGE$_2$) in the conjunctival epithelium of pterygium patients as we did in the control autopic conjunctival epithelium. Our study suggests that PGE$_2$ is strongly expressed in the conjunctival epithelium of patients with pterygium, as in control conjunctival tissues. PGE$_2$ immunolabeling was moderate in the stroma from patients with pterygium, because vascular endothelium expressing the PGE2 protein increased in the presence of inflammatory infiltrating cells in sub-conjunctival tissues (Fig. 2C-D). The intensity of staining for VEGF, TGF-β and PGE$_2$ in human normal conjunctiva and pterygium is presented in Tables I and II (Tables I-II). The percentage values of growth factors-positive cells and P-values are shown in Table III (Table III).

**Discussion**

Pterygium represents a vascular, potentially invasive surface ocular lesion, which originates from activated stem cells of the limbus (18). Immunopathogenic mechanisms and overexpression of extracellular matrix components seems to be implicated in its pathogenesis (19). Recent studies show that pterygium may be the result of a defective wound healing process, during which molecular events that lead to programmed cell death are modified (20). Normal conjunctiva contains a local surface immune system called conjunctiva-associated lymphoid tissue (CALT) that presents T cells, B cells, and plasma cells as well as local secretions of immunoglobulins (IgA, IgM, IgG, IgG, and IgE) and complement (21). Previous studies indicated that both immune cells and immunoglobulins are pathologically increased in pterygium tissue, suggesting that immunological process might be involved in its pathogenesis (21). Although T cells are the most frequently encountered type of inflammatory cell, CD68-positive macrophages are the most common cells encountered in pterygium and they are distributed both in the epithelial and
stromal layers, suggesting that they are related to the pathogenesis of the disease. Additionally, a previous study indicated that some of these macrophages strongly expressed COX-2 and VEGF in pterygium (22). COX-2 protein can upregulate VEGF production via the protein kinase C pathway in lung cancer cells (23). Prostaglandin E2 (PGE₂), the product of COX-2 activity, is also angiogenic by direct influence on endothelial cells or by inducing the release of angiogenic growth factors, such as VEGF (24). In fact, the present study revealed an intense expression of PGE₂ in the columnar stratified epithelium and moderate immunolabeling in endothelial and stromal cells, indicating the importance of this factor in the pathogenesis of pterygium. Although the pathogenicity 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 damage of DNA, RNA and extracellular matrix (25). Chronic ultraviolet-B (UV-B) exposure contributes to the pathogenesis of primary pterygium, which causes oxidative stress, leading to upregulation of many potential mediators of pterygium growth, such as cytokines and growth factors. Di Girolamo et al. (26) demonstrated that UV may induce the VEGF expression in the pterygium epithelial cells in a dose- and time-dependent manner (26). Therefore, the expression of VEGF in the epithelium of primary pterygium might be relevant due to UV-B exposure. The ingrowth of these vessels into the epithelium might be interpreted as a reaction to hypoxia or some cytokines. This process deserves further investigation and research. Angiogenesis is characterized by growing new blood vessels from pre-existing vessels. VEGF is one of the most potent and specific proangiogenic factors. It is also known to be a vascular permeability factor or vasotropin. VEGF is considered the most selective mitogen of endothelial cells (27); it increases vascular permeability (28), induces alterations of ionic fluxes, cellular proliferation (15), migration and release of proteases (16). Our study showed an increased expression of VEGF, mainly in the epithelium, endothelial cells and fibroblasts of the pterygium. Such reactivity is an argument for the pathogenic role played by this growth factor in the development of pterygium. The relationship between VEGF signal
transduction and the modifications in the behavior of epithelial cells from pterygium/conjunctiva has not been fully ascertained. Overexpression of VEGF in epithelial cells is, on the other hand, not capable of inducing angiogenesis and is seemingly consistent with allow level 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 cell can induce angiogenic activity, demostrated 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. demostrated a higher vascular microdensity in the fibrous subtype than in the vascular type, thus confirming the assumption that stroma plays a role in the pathogenesis of pterygium (29). Furthermore, the presence of pro-inflammatory cytokines secreted from the surface epithelium or from lacrimal inflammatory cells induces the fibroblastic production of proteins related to remodelling of extracellular matrix and angiogenesis. Growth factors with potent angiogenic activity, such as FGF, PDGF, TGF-β and TNF, have been found to be secreted from fibroblastic and inflammatory pterygium cells as well as in tissue cultures from pterygium fibroblasts (8). Our study showed a moderate expression of TGF-β in the epithelial and stromal cells of pterygium compared to normal conjunctiva, which presented weak staining in the endothelial, epithelial and stromal layers. From this and other studies, we can postulate that various cytokines and growth factors, including VEGF, might be involved in cellular proliferation, inflammatory reaction, remodelling of extracellular matrix and angiogenesis of pterygium. These data suggests that VEGF, PGE₂ and TGF-β can be therapeutic targets in the treatment of pterygium. Anti-angiogenesis therapy using anti-VEGF has recently been reported to be effective both for controlling primary pterygium and preventing recurrence after pterygium excision (30). We conclude that the overexpression of angiogenic factors and the concurrent decreased expression of angiogenesis inhibitors probably represents a possible pathogenic mechanism in the formation of pterygium. The development of synthetic inhibitors growth factors for therapeutic intervention
could bring about a reduction of relapse rate, inflammation intensity, tissue invasiveness, proliferation and angiogenesis in pterygium. Additional clinical and experimental investigations appear to be necessary to better clarify the biological role of these molecules in the development and progression of this type of disease.
References


Table I. Results of the immunohistochemical analysis for VEGF, TGF-β and PGE₂ in normal human conjunctiva.

<table>
<thead>
<tr>
<th></th>
<th>EPITHELIAL CELLS</th>
<th>VASCULAR ENDOTHELIUM</th>
<th>STROMAL CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TGF-β</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>PGE₂</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
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+++ indicates strong immunoreactivity; ++ indicates relevant immunoreactivity; + indicates moderate immunoreactivity; +/- indicates weak immunoreactivity; - indicates absence of immunoreactivity.

Table II. Results of the immunohistochemical analysis for VEGF, TGF-β and PGE₂ in pterygium.

<table>
<thead>
<tr>
<th></th>
<th>EPITHELIAL CELLS</th>
<th>VASCULAR ENDOTHELIUM</th>
<th>STROMAL CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>TGF-β</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>PGE₂</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+++ indicates strong immunoreactivity; ++ indicates relevant immunoreactivity; + indicates moderate immunoreactivity; +/- indicates weak immunoreactivity; - indicates absence of immunoreactivity.
Table III. Levels of VEGF, TGF-β and PGE$_2$ examined in pterygium and control specimens, and respective levels of statistical significance (t-test)

<table>
<thead>
<tr>
<th></th>
<th>PTERYGIUM</th>
<th>CONTROLS</th>
<th>p-VALUE</th>
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<tbody>
<tr>
<td><strong>VEGF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>72.92%</td>
<td>42.03%</td>
<td>p=0.0035</td>
</tr>
<tr>
<td>Vascular endothelium</td>
<td>72.92%</td>
<td>0%</td>
<td>-</td>
</tr>
<tr>
<td>Stromal cells</td>
<td>87.80%</td>
<td>0%</td>
<td>-</td>
</tr>
<tr>
<td><strong>TGF-β</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>32.32%</td>
<td>22.30%</td>
<td>p=0.0294</td>
</tr>
<tr>
<td>Vascular endothelium</td>
<td>22.16%</td>
<td>19.10%</td>
<td>p=0.0380</td>
</tr>
<tr>
<td>Stromal cells</td>
<td>47.70%</td>
<td>19.36%</td>
<td>p=0.0209</td>
</tr>
<tr>
<td><strong>PGE$_2$</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>62.03%</td>
<td>36.26%</td>
<td>p=0.0334</td>
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<tr>
<td>Vascular endothelium</td>
<td>47.70%</td>
<td>0%</td>
<td>-</td>
</tr>
<tr>
<td>Stromal cells</td>
<td>49.90%</td>
<td>0%</td>
<td>-</td>
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**Figure legends: Fig. 1-A.** Hematoxylin and eosin stain of human normal conjunctival tissue. The epithelium of the conjunctival tissue is columnar stratified and elevated numbers of goblet cells are present in this area (40X).

**Fig. 1-B.** Hematoxylin and eosin stain of human pterygium. Irregular epithelium and richly vascularized stroma. The epithelium is adapted to smoothen the stromal irregularities and in some areas becomes invaginated. The stroma of the pterygium is made up of connective tissue, rich in new blood vessels, in fibroblasts and connective fibrils (40X).

**Fig. 1-C.** Immunohistochemical analysis of normal conjunctiva for VEGF. A moderate expression of VEGF is seen in the epithelial conjunctival cells while VEGF expression was not found in the vascular endothelial and stromal cells (40X).

**Fig. 1-D.** Immunohistochemical analysis of human pterygium for VEGF. VEGF immunoreactivity appears to be strongly positive in the endothelial cells of blood vessels, epithelial cells and in the stromal inflammatory tissue (40X).
**Figure legends:**

**Fig. 2-A.** Immunohistochemical analysis of normal conjunctiva for TGF-β. TGF-β immunolabeling was very weak in the epithelial, endothelial and stromal layers.

**Fig. 2-B.** Immunohistochemical analysis of human pterygium for TGF-β. Evident expression of TGF-β in the epithelial cells is visible. Immunoreactivity in the fibroblasts of the connectival tissue appeared strong in some cells. Weak immunolabeling in the endothelial cells of blood vessels is also visible (40X).

**Fig. 2-C.** Immunohistochemical analysis of normal conjunctiva for PGE₂. PGE₂ immunolabeling was moderate in the epithelial cells. PGE₂ expression was not found in the endothelial cells of the vessels (40X).

**Fig. 2-D.** Immunohistochemical analysis of human pterygium for PGE₂. Intense expression of PGE₂ in the columnar stratified epithelium and moderate immunolabeling in stromal cells are visible (100X).