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Oxidative Stress-Induced Lipid per Oxidation Products Correlate with VEGF in Pterygia

Johnsen-Soriano S[1], Peris-Martínez C[1,2], Martinez-Belda R[1,3], Arnal E[1], Barcia JM[2], Forteza J[2], Francisco Javier Romero[2,4]

[1]Fundación Oftalmológica del Mediterráneo (FOM), Valencia, Spain

[2]Facultad de Medicina y Odontología, Universidad Católica de Valencia, Valencia,Spain

[3]Servicio de Oftalmología, Hospital Clínico Universitario, Valencia, Spain

[4]Facultad de Ciencias de la Salud, Universidad Europea de Valencia, Valencia, Spain

*Corresponding author: Francisco Javier Romero, Facultad de Ciencias de la Salud, Universidad Europea de Valencia, General Elio, 646010-Valencia, Spain, Tel: +34 91 740 72 72; E-mail: franciscojavier.romero@universidadeuropea.es

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Abstract

Purpose: To study the expression of angiogenic factors and oxidative stress markers in pterygium tissue.

Methods: Primary pterygia samples were harvested from 26 patients who underwent pterygium removal combining conjunctivalauto grafting technique with extensive excision of the hypertrophic subconjunctival tissue at Fundación Oftalmológica Del Mediterraneo (FOM), Valencia, and Spain. Primary pterygia and normal conjunctiva samples were fixed in 4% fresh formaldehyde for immunehistochemical staining.

Results: The pterygium tissue was found to have a significant increase in the number of VEGF positive cells. Moreover, the number of HNE (4-hydroxynonenal) and MDA (Malondialdehyde) positive cells were also increased in pterygium tissue, while that of GSH was found to be decreased, when compared with normal conjunctiva. Finally, the number of SOD positive cells was found to be increased in pterygium tissue compared to control tissue. Interestingly, a significant positive correlation between the numbers of HNE- and VEGF-positive cells was also reported.

Conclusion: Oxidative stress is undoubtedly involved in the pathophysiology of pterygium and a positive correlation between the lipid per oxidation marker HNE and the angiogenic regulator VEGF exists.

Keywords: HNE; Lipid per Oxidation; Oxidative Stress; Pterygium; VEGF

Introduction

Pterygium, a common ophthalmological disease, is a fibrovascularneoformation characterized by a triangular (wing-shaped) overgrowth of abnormal conjunctiva onto the cornea and is composed of epithelium and a highly vascular loose connective tissue[1]. It has been proposed that the typical location of the pterygium (nasal side)is due to corneal focusing of incident sunlight on the medial limbus [2-4]. In severe cases, a pterygium can grow into the central cornea and it frequently appears again after resection [5].

The pathogenesis of pterygium has not yet been clarified. A role of Ultraviolet (UV) radiation damage, irritation or inflammation is hypothesized in its etio-pathogenesis. The form, incidence, and distribution of pterygium support the role of UV radiation. Since UV radiation acts directly by phototoxicity or indirectly through free radicals, various studies have reported the implication of p53 in addition to 8-hydroxydeoxy-guanosine in pterygium tissue, suggesting a possible implication of oxidative stress in this disease [6-8].

Pterygium is a complex and intriguing pathology and shares many similar traits with tumors, such as cell proliferation, invasion of the cornea and recurrence after resection.

Recurrence of the pterygium is one of the most frequent problems faced by the ophthalmologist (as high as 40%) independent of the surgical procedures applied [9].

Moreover, the investigation of the vascular microdensity in pterygium has shown the existence of an intense angiogenic process in pterygium. Overexpression of the Vascular Endothelial Growth Factor (VEGF) and a decrease of the expression of Pigment Epithelium Derived Factor (PEDF) have earlier been reported [10,11]. These results indicate an involvement of pro- and anti-angiogenic factors in the development of pterygium. Interestingly, even though angiogenesis has been closely related to the development of pterygium, anti-VEGF drugs like bevacizumab fail to show a convincing effect in the treatment of pterygium [12-16]. In addition, apoptosis has been associated with pterygium, however there is no increase of caspase-3 expression in pterygium tissue [17], and indicating implication of other pathways.

Cellular damage, caused by reactive oxygen species, occurs in biological systems because of an inadequate detoxification of free radicals that results in the accumulation of chemically altered macromolecules. Proteins, nucleic acids and particularly the polyunsaturated fatty acids, that constitute part of the biological membranes, are vulnerable to oxidation by free radicals. Lipid peroxides thus formed are degraded to lipid derived aldehydes (such as Malondialdehyde (MDA), and 4-Hydroxynonenal (HNE), among others), which are believed to contribute to the pathogenesis of several diseases such as diabetes, aging, retinopathy of prematurity, keratoconus, etc.[18-20]. Lipid per oxidation products contribute to toxicity by inducing pro-apoptotic signaling through multiple pathways and also by necrosis [21]. 4-Hydroxy-alkenals-modified proteins have been observed in pterygia [22], however, no mechanistic approach has been performed so far, as to what extent HNE could be responsible for other functional effects in pterygia. The intracellular concentration of 4-HNE has been described to be crucial for the nature of cell cycle signaling and may be a determinant for the signaling of differentiation, proliferation, transformation, or apoptosis [23]. In addition, HNE has earlier been reported to regulate the VEGF expression in retinal pigment epithelial cells [24]. Thus, the objective of this study is to investigate the possible increase of lipid per oxidation products and its relationship with various angiogenic markers in pterygium tissue.

Methods

Primary pterygia were harvested from 26 patients (14 males and 12 females), whose ages ranged from 39 to 75 years old (mean age 64, 8 years; standard deviation-SD 8, 51) (Table 1).

Demographic properties	Control (n=24)	Pterygium (n=26)
Age	69.30 ± 7.80	64.80 ± 8.51
sex		
Female	6	12
Male	18	14
Values are given as mean ± SD		

Table 1: Demographic characteristics of the study population.

All patients underwent pterygium removal, combining conjunctivalautografting technique with extensive excision of the hypertrophic subconjunctival tissue at Fundación Oftalmológica Del Mediterraneo (FOM), Valencia, Spain. All lesions were located on the nasal side and only the head of primary pterygium was used as pterygium sample. We choose to study only primary pterygia, in order to have a homogenous group. Normal conjunctiva samples as controls were collected from medial conjunctiva of24 patients (18 males and 6 females; mean age 69, 3 years with standard deviation-SD 7, 8) without pterygium and pinguecula or other conjunctival degenerations while undergoing cataract surgery. Patients did not receive any medication prior to surgery, except for topical anesthetic, and no drugs or chemical agents were used during intervention. The study protocol was approved by the local research Ethics Committee, and informed consent was obtained from all volunteers in this study, after explanation of the nature of the research; complete information on patients was available in all cases.

Primary pterygia and normal conjunctiva samples were fixed in 4% fresh formaldehyde and embedded in paraffin. Three-micrometer sections were cut and stained with Hematoxylin& Eosin (H&E). In addition to the Hematoxylin& Eosin staining, the sections were also stained with antibodies against HNE (1:200, Alpha Diagnostic International, Cambridge, UK), VEGF (1:100, Roche, Basel, Switzerland), Glutathione (1:50 Abcam, Cambridge, UK), Caspase-3 (1:50 Thermo, Waltham, USA), SOD (1:100, AbDserotec, Oxford, UK) and MDA (1:1000, Abcam, Cambridge, UK) overnight at 4ºC. The staining was done using the Autostainer Benchmark Classic (Ventana Medical Systems, Inc., Tucson, Arizona, USA) and visualized using a standard peroxides technique (Ultra View Universal DAB detection kit, Ventana Medical Systems, Inc., Tucson, Arizona, USA). The positive immunoreactions of the primary antibodies were detected by a secondary antibody conjugated with peroxides-labeled polymer with Diaminobenzidine (DAB) as chromogen, incubated for 1 h at room temperature. Immunopositive cells were counted in three different sections on each slide in a triple blind-test. Statistical analysis. Data are ex-

pressed as means ± SD. Comparisons between groups were done using t-test and Pearson correlation. Statistical differences were set at the $p < 0.05$ levels.

Results

The demographic characteristics of the groups are summarized in Table 1. The primary pterygia and normal conjunctiva were immune stained for various proteins related to angiogenesis and oxidative stress (Figure 1).

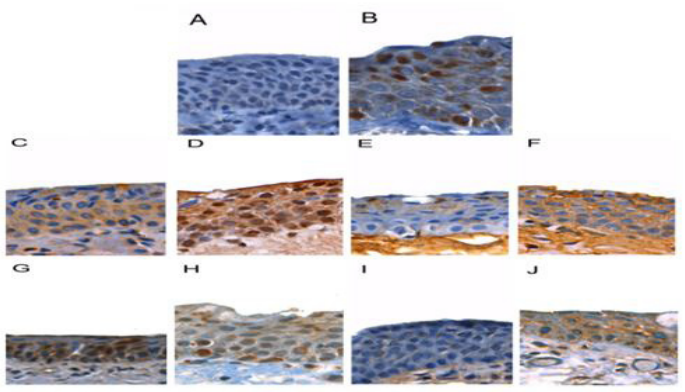

Figure 1: Immunostaining of different proteins and metabolites in conjunctiva tissue and pterygium.

VEGF in conjunctiva tissue (A) pterygium (B) HNE in conjunctiva tissue (C) pterygium (D) MDA in conjunctiva tissue (E) pterygium (F) GSH in conjunctiva tissue (G) pterygium (H) SOD in conjunctiva tissue (I) pterygium (J) Bar 50 µm. All slides were counterstained with Mayer's Hematoxylin. Original magnifications x 400.

No differences could be established for PEDF immunehistochemical (data not shown). VEGF nuclear and cytoplasmic immunostaining appeared in pterygium, whereas nearly no staining could be observed in conjunctiva (Figure 1A and B). Conjunctiva showed a reduced number of HNE positive cells (exclusively labeled in the cytoplasm) than pterygium, furthermore showing additional nuclear labeling (Figure 1C and D). Similarly, MDA staining appeared evenly distributed in the cytoplasm of epithelial cells of both conjunctiva and pterygium but more intense in the latter (Figure 1E and F). GSH appeared in the cytoplasm in epithelial as well as in stoma cells with more intense labeling in the conjunctiva than in pterygium (Figure 1G and H). SOD immune labeling appears very intense in pterygium when compared with the conjunctiva; it can also be observed that this staining is also present in the pervascular area (Figure 1I and J).

Angiogenesis

The result of the immunostaining for the angiogenic stimulating factor VEGF, showed that there were more VEGF immune positive cells (54.7 ± 9.6 %) in the pterygium samples than in the normal conjunctiva samples (43.0 ± 13.8 %, $p<0.05$, Figure 2).

VEGF+ cells / total cells

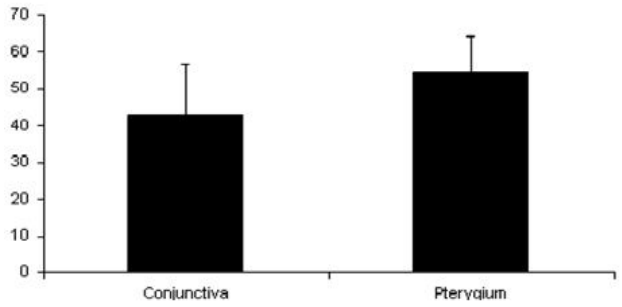

Figure 2: Quantification of angiogenesis markers in conjunctiva and pterygium tissues.

A. VEGF immune positive cells in conjunctiva and pterygium tissues in percentage B. PEDF immunopositive cells in conjunctiva and pterygium tissues in percentage*$p<0.05$.

Oxidative stress

Figure 3Quantification of oxidative stress markers in conjunctiva and pterygium tissues.The expression of oxidative stress markers was also studied. Immunostaining of the lipid per oxidation marker HNE revealed that there was a significant increase in HNE-positive immunostaining in pterygium tissue (77.4 ± 9.0 %) when compared to normal conjunctiva (53.7 ± 8.1 %, $p<0.05$, Figure 3A),

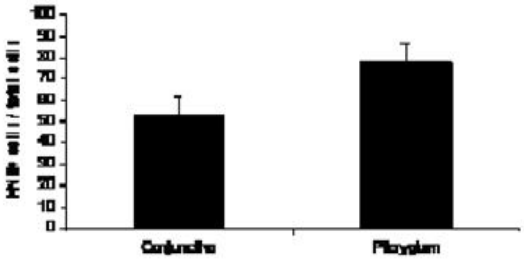

Figure 3A: HNE immune positive cells in conjunctiva and pterygium tissues.

As previously reported [22]. Furthermore, MDA, was found to be elevated in pterygium tissue (63.5 ± 8.0 %) when compared to normal conjunctivas (56.5 ± 5.1 %, $p<0.05$, Figure 3B).

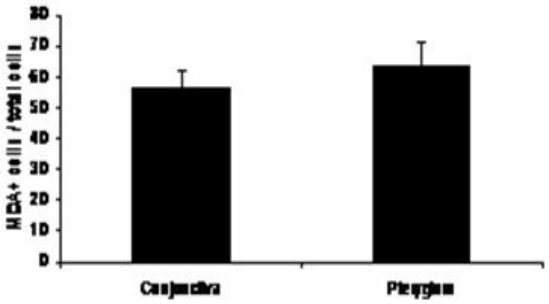

Figure 3B:MDA immunopositive cells in conjunctiva and pterygium tissues.

Some antioxidant defense mechanism markers were also analyzed: GSH-positive immunostaining was evaluated and the expression of GSH was found to be decreased in the epithelial layer of pterygia (54.2 ± 5.0 %) when compared to normal conjunctivas (60.9 ± 5.7 %, $p < 0.05$, Figure 3C).

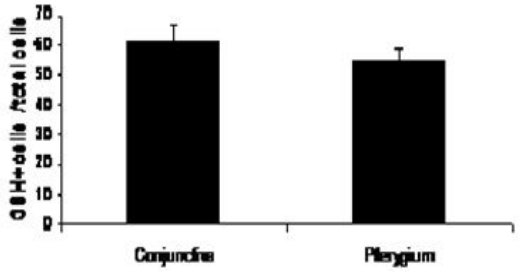

Figure 3C: GSH immuno positive cells in conjunctiva and pterygium tissues.

While the expression of SOD was found to be elevated in pterygium tissue (69.5 ± 6.1 %) when compared to normal conjunctivas (61.0 ± 7.7 %, $p < 0.05$, Figure 3D).

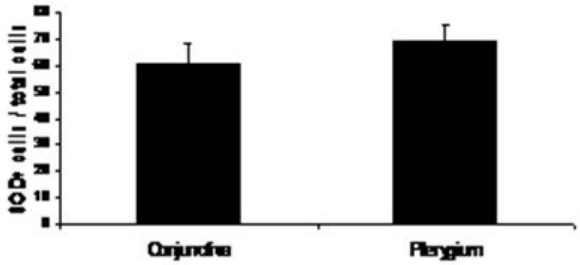

Figure 3C:SOD immuno positive cells in conjunctiva and pterygium tissues*$p<0.05$.All numbers are given in percentage.

A statistically significant positive correlation was found between HNE and VEGF in pterygium and conjunctiva tissues (Figure 4).

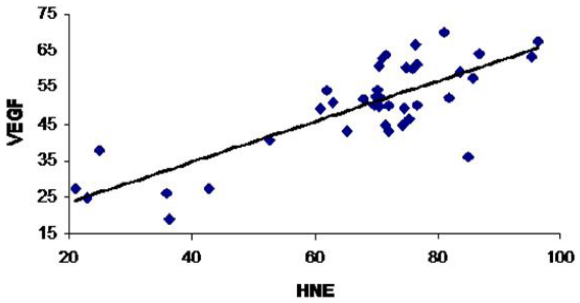

Figure 4: Correlation between the number of HNE and VEGF positive cells in conjunctiva and pterygium tissues. HNE was found to have a significant positive correlation with VEGF ($r = 0.794$; $p<0.05$).

Discussion

Pterygium is an epithelial hyperplasia characterized by the presence of abnormal fibro-vascular tissue formed by the advancement of altered conjunctival tissue onto the cornea [25]. The interest in this benign conjunctival degeneration is due to the fact that it is one of the most common ocular surface diseases, but its etiopathogenesis is still controversial [26]. Possibly, many pathways are involved and have a complex interaction between them.

Pterygium tissue was investigated for protein expression of various biomarkers for apoptosis, angiogenesis and oxidative stress. The caspase-3 staining in pterygium tissue was not found to be increased in it when compared to normal conjunctiva (data not shown). This result fits with earlier findings, where one study reports to find an increase of proliferative markers and at the same time an increase in the antiapoptotic marker BCL-2 and no increase in caspase-3 expression in pterygium tissue. Thus, it seems likely that cell apoptosis is downregulated and that the caspases are not involved in the development of pterygium, which suggests the involvement of other pathways [17].

Pterygium is a proliferative, invasive, and highly vascularized tissue [27]. The vascular endothelial growth factor VEGF plays a pivotal role in the regulation of vascular growth. In this study, we found an increased expression of VEGF in pterygium tissue when compared to control tissue. This finding agrees with other studies that investigated the vascular micro density and overexpression of VEGF in pterygium [28], suggesting the pathogenic involvement of this growth factor in the development of pterygium.

In the eye, PEDF expression is regulated by hypoxia in an opposite manner to VEGF [29]. PEDF was originally isolated from a human Retinal Pigment Epithelium(RPE) cell culture [30] but has subsequently been reported to be expressed in several other tissues

[31].In our study, we also investigated the expression of PEDF and found no differences in PEDF expression between pterygium and conjunctiva tissue. This is in contrast to an earlier report that found a decrease in PEDF expression when compared to control eyes [11]. However,in this latter report, the control eyes were obtained from donor eyes and it was not distinguished between primary or secondary pterygium.

In addition to angiogenesis, oxidative stress has also been related to the development of pterygium [32]. Herein we investigated the expression of lipid per oxidation markers MDA and HNE in pterygium tissue and both were found to be increased when compared to normal conjunctiva. The increased MDA immunostaining in pterygium tissue observed (Figure 1) agrees with an earlier report by Balci et al [32] and to a certain extent with a study by Uçakhan et al. where these authors reported an increase in MDA although not statistically significant [33].

The antioxidant defense GSH was found to be decreased in pterygium tissue. The detoxifying enzyme SOD was found to be increased in pterygium tissue (Figure 3D), which is in contrast to what was reported in an earlier study where SOD activity was found to be decreased [32]. The report by Uçakhan et al. also measured SOD expression in pterygium tissue where the SOD activity was similar to the control group [33]. However, this discrepancy might be due to the use of different techniques as we used fresh-fixed tissue with immunehistochemical staining and not frozen tissue on ELISA plates in our study. In addition, we used different study materials since we only used primary pterygium in our study.

Interestingly, when a correlation between the number of HNE and VEGF was done, a strong positive correlation was found. This result fits with an earlier result where HNE was found to regulate the VEGF expression in retinal pigment epithelial cells [24].

At low physiological levels, ROS are indispensable in numerous biochemical processes, functioning as red ox messengers and important molecules in intracellular signaling. In addition to having an important role in cellular differentiation, proliferation, and apoptosis, ROS are also key players in inflammatory processes and defense against microorganisms. However, at high levels, ROS may oxidize DNA, proteins, lipids, and carbohydrates, mediating numerous red ox-related pathological conditions. The guanine oxidative product, 8-oxo-2'-deoxyguanosine (8-oxodG), is one of the best studied oxidative modifications and is widely used as a biomarker for oxidative stress and carcinogenesis [6]. ROS have a dual role in tumor biology and has been reported to be involved in both tumor progression [34] and regression [35].

In this report, we did not find any correlation between the MDA and the VEGF, maybe because MDA is a more general LPO product that merges after a long chain reaction, thus it may have different origins and may mask

the specific effect of ROS on PolyUnsaturated Fatty Acids (PUFA). 4-HNE is far upstream of the chain reaction and provides a more direct correlation with the metabolic origin of the oxidative stress situation. Due to its nature, 4-HNE reacts with proteins once formed and has been proposed as a more accurate finger print for LPO than other smaller aldehydes, even in pterygia [22].

Pterygium has earlier been described to have tumor-like features and develop from limbal epithelial progenitors [36]. It can be speculated that development of pterygium is triggered by UV radiation that produces ROS and its derivatives (reactive aldehydes such as HNE and MDA) in limbal epithelial progenitor cells. Supporting this speculation, it has been reported that exposure to UV or blue light enhanced the labeling of 4-hydroxyalkenals-modified proteins in the nuclei of rat conjunctival epithelium [22]. The reactive aldehydes as HNE and MDA might thus act as second messengers which again could lead to regulation of apoptosis, proliferation and, as herein reported, increased VEGF. Interestingly, it has been reported that oxidative stress might be related with pterigyum recurrence [37], These findings might give these oxidative mechanisms further relevance also in recurrence. New mechanistic insights into the exact regulation and development of pterigyaare necessary in order to develop pharmacologically substances that could, by limiting oxidative stress- and/or lipid per oxidation-induced damage, offer new therapeutic approaches to this disease.

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