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Concomitant Examination of Inflammation and Angiogenesis in the Pathogenesis of Primary Moderate Pterygium in a Well-designed Case-control Study

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Abstract: Pterygium is a common ocular lesion whose exact etiology is a point of contention. Chronic inflammation and angiogenesis are two major proposed mechanisms of the disease in the current literature. The objective of this study is to examine these two mechanisms in a very well-designed setting. In a case-control study, 24 tissue specimens from the patients with primary moderate pterygium (cases) and 15 specimens excised from the nasal bulbar region in healthy counterparts (controls) were compared in terms of the count of mast cells (inflammation), as well as the status of Cd31/vascular Endothelial Growth Factor (VEGF) expression (angiogenesis) in Tabriz Nikookari and Sina Teaching Hospitals. The case (mean age: 58.08±10.03 years, 84% males) and control (Mean age: 62.33±9.19 years, 80% males) groups were age-and sex-matched ($p = 0.19$, 0.75, respectively). The mean mast cell count was significantly higher in the case group (27.72±15.19 vs. 12.00±7.09 cells mm^{-2}, $p = 0.001$). The study of immunoreactivity revealed that the positive expression (moderate-severe) of CD31 was significantly more frequent in the case group (88 vs. 26.7%; $p<0.001$; Odds ratio = 20, 95% confidence interval 3.85-100). There was also higher rate of VEGF-positive (moderate-severe) cells in the group with pterygium (88 vs. 20%; $p<0.001$; Odds ratio = 33.3, 95% confidence interval 5.00-100). This study indicates that both inflammation and angiogenesis play pivotal role, in parallel, in pathogenesis of pterygium.

Key words: Pterygium, CD13, VEGF, mast cell infiltration

INTRODUCTION

An encroachment of a fibrovascular tissue onto the cornea is a neoformation which is called pterygium. The base of this umlateral or bilateral triangle is on the nasal conjunctiva and points toward the cornea (Lin *et al*., 2013; Bazzazi *et al*., 2010).

Although, benign in many cases, an aggressive pterygium may cause problems such as blurred vision, ocular irritation and in rare cases dysplasia to even carcinoma in situ. Furthermore, high prevalence and surgery recurrence (30-69%) have been always major concerns to physicians (Liang *et al*., 2010). Therefore, effective treatment of the disease is a monumental goal, which in turn, needs accurate knowledge of its underlying physiopathology (Mandour *et al*., 2011).

In spite of large amount of data available in the literature as to pterygium, its exact etiology is still an intriguing question. Various hypotheses have being put forward in this regard during the two last decades, such as limbal stem cell aberrations, apoptosis, metalloproteinases, infections and the immune system (Chui *et al*., 2008; Detorakis and Spandidos, 2009). According to the modern science, however, two wings have been hypothesized as the most important factors in the pathogenesis of pterygium including chronic inflammation and angiogenesis (Ribatti *et al*., 2007; Aspiotis *et al*., 2007; Marcovich *et al*., 2002). Mast Cells (MCs) play a key role in allergic inflammation and it has been proposed that they may be considered as a major contributor in pathogenesis of the disease through chronic inflammation (Nakagami *et al*., 1999).

On the other hand, angiogenesis, which is defined as growing new blood vessels from preceding ones, is modulated by a complex interaction between various regulating factors. One of these important factors is Vascular Endothelial Growth Factor (VEGF), a signal protein produced by cells that stimulates vasculogenesis and angiogenesis (Livezeanu *et al*., 2011). Platelet Endothelial Cell Adhesion Molecule (PECAM-1) or the Cluster of Differentiation 31 (CD31) is a structural protein in endothelial cell intercellular junctions. This protein involves in angiogenesis, integrin activation and leukocyte locomotion (Jackson, 2003). This study, for the

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first time in the literature, aimed to examine both wings of pathogenesis of pterygium in a simultaneous fashion. For this purpose the count of infiltrated MCs (the wing of inflammation) as well as the status of CD31/VEGF expression (the wing of angiogenesis) was considered as target goals. In addition, this is the first study of its type that tackled the confounding effect of the severity of pterygium by limiting the cases to stage II disease.

MATERIALS AND METHODS

Patients: In this prospective, case-control study, 24 tissue specimens were acquired from the patients with primary pterygia (the case group). For the control set 15 normal conjunctival tissues were examined, which were obtained from the nasal bulbar region next to the limbus during cataract surgery.

In the case group the including criteria were as follow: primary moderate (grade II according to Awdeh *et al.* (2008) eye nasal pterygia with encroachment onto the cornea and an apex passing the limbus ≥1 mm, indication of surgery, no systemic immune diseases and/or previous use of immunosuppressants and no history of previous ocular surgery, injury or disease. The controls were age-and sex-matched counterparts without ocular diseases and/or immune compromise.

All the patients with pterygium and cataract were operated in Tabriz Nikookari Teaching Hospital from April 2012 through February 2013. The histopathological assessments were carried out in Tabriz Teaching Sina Hospital. This study is approved by the ethics committee of Tabriz University of medical Sciences and informed consents are obtained from the participants.

Procedures

Toluidine blue staining and MC counting: After sectioning the specimens along the longitudinal axis, both case and control tissues were fixed in a mixed solution (2.5% formalin plus 1% glutaraldehyde) for 24 h. Then the embedded specimens in glycol methacrylate were cut into 1 μm thick section and stained with diluted 1% toluidine blue for 10 min. Metachromatic cells were considered as MCs (Fig. 1a).

For MC counting, sections were photographed at 200X and the MCs were marked on the photographs while the entire tissue was skimming through at 400X. Finally, all the marked spots were counted by two observers and the mean count was reported as cells/mm^2.

Immunohistochemical staining: Paraffin-embedded specimens were fixed in 10% buffered formalin for a day.

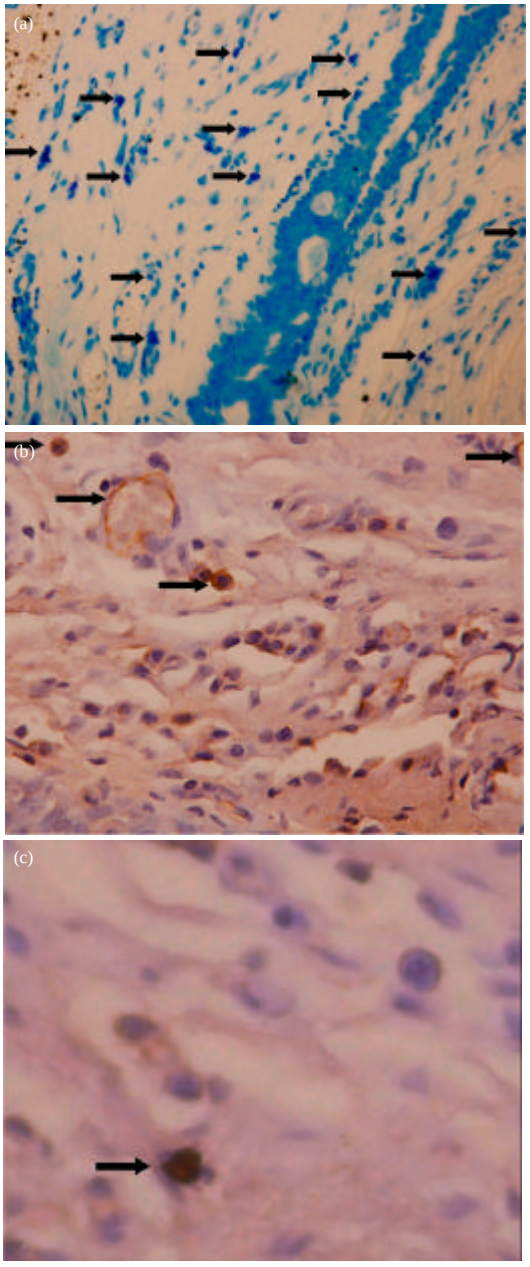

Fig. 1(a-c): (a) Infiltration of mast cells in a specimen of pterygium (Toluidine blue staining, original magnification 200X), (b) CD31-positivity in a specimen of pterygium (Hematoxylin eosin staining, original magnification 200X) and (c) Vascular endothelial growth factor (VEGF)-positivity in a specimen of pterygium (Hematoxylin eosin staining, original magnification 400X)

After dehydration, these specimens were prepared and examined for expression of CD31 and VEGF by a skilled dermatopathologist.

Then 4 μm sections were prepared and dewaxed in an oven for 30 min at 56°C. After being washed in Phosphate Buffered Saline (PBS), the specimens were pretreated with 0.5% H_2O_2 in 70% methanol for 30 min in order to ensure that the endogenous peroxidase was blocked. An avidin-biotin block provided by the manufacturer (DAKO) was used to block endogenous biotin.

Following the manufacturer's instructions the antigens were retrieved and the samples were incubated with monoclonal mouse anti-human CD31 antibody (DAKO A/S, Glostrup, Denmark; Clone JC70A; dilution 1:30) and monoclonal mouse anti-human VEGF antibody (DAKO A/S, Glostrup, Denmark; clone VG1; dilution 1:25) separately for 30 min at room temperature.

Tissues were then incubated with avidin-biotin-peroxidase complex (ABC, Vector Laboratories Inc, Burlington, USA). Accordingly prepared slides were treated with diaminobenzidene (DAB) chromagen substrate according to the manufacturer's instructions and counterstained with hematoxylin.

The slides were examined by a light microscope (Siemens, Mumich, Germany) at different magnifications (Fig. 1b, c).

According to previously established criteria (Jasani and Schmidt, 1993), the staining was considered positive when it was at least of focal or of moderate intensity, clearly visible only with medium magnification. This means that only moderate to severe staining was considered positive in this study.

Statistical analysis: Data were shown as Mean±standard deviation or number (%). The SPSS software for Windows (ver. 16) was used. Independent samples t test (for age and MC count) and the Chi-square test (for sex and the status of CD31/VEGF staining) were employed for analyzing. $p \leq 0.05$ was considered statistically significant.

RESULTS

In the case group there were 21 males (84%) and 4 females (16%) with a mean age of 58.08±10.03 (range: 41-72) years at the time of surgery. In the control group there were 12 males (80%) and 3 females (20%) with the mean age of 62.33±9.19 (range: 46-76) years. Both the case and control groups were matched for there subjects' sex ($p = 0.75$) and age ($p = 0.19$).

The mean MC count was 27.72±15.19 (range: 5-51) cells/mm^2 in the case group, which was significantly higher than the mean MC count in the controls (12.00±7.09, range: 4-30 cells mm^{-2}; $p = 0.001$).

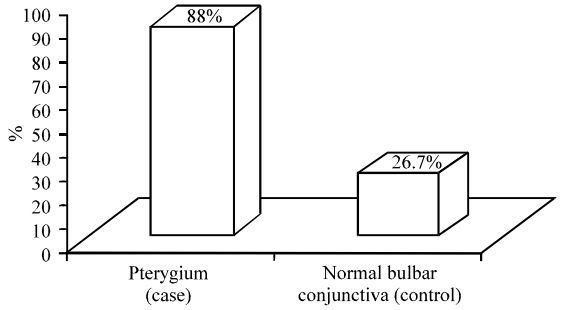

Fig. 2: Rate of CD31-positivity in the specimens of pterygium and normal bulbar conjunctiva

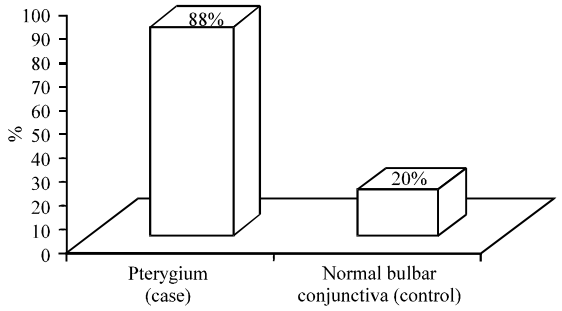

Fig. 3: Rate of VEGF-positivity in the specimens of pterygium and normal bulbar conjunctiva

The study of immunoreactivity at CD31 proved that this marker was significantly more frequent in the cases (22 patients) than in the controls (only 4 subjects) ($p<0.001$; Odds ratio = 20, 95% confidence interval 3.85-100). The percentage of the specimens with positive immunoreaction to CD31 in the two groups is shown in Fig. 2.

For the VEGF, there was again a significantly higher rate of positivity in the cases (22 patients) than in the controls (only 3 subjects) ($p<0.001$; Odds ratio = 33.3, 95% confidence interval 5.00-100). The percentage of the specimens with positive immunoreaction to VEGF in the two groups is shown in Fig. 3.

DISCUSSION

Although, there are many hypotheses available in the literature regarding the pathogenesis of pterygium, its exact underlying physiopathology is still unclear. However, modern approaches have proposed that two main processes may construct the basis of this condition; inflammation and angiogenesis (Chui *et al*., 2007; Livezeanu *et al*., 2011).

In the present study, to the best of our knowledge, for the first time both these wings of pathophysiology have been examined at the same time.

For the wing of inflammation, the count of MCs was compared between the specimens of pterygium and those of normal bulbar conjunctiva. Accordingly, the mean count of MC was significantly higher in the first group (27.72±15.19 vs. 12.00±7.09 cells mm^{-2}; p = 0.001). This finding is in line with the outcome of a previous report by Butrus *et al.* (1995) who also showed that the count of MCs is higher in pterygium than normal tissue.

In another series by Nakagami *et al.* (1999), the mean count of MC was again higher in the specimens of pterygium comparing with that in normal conjunctival tissue (34.56±18.0 vs.15.56±9.0 cells mm^{-2}). As seen, these figures are very similar to the mean count of the MC in the present study.

Zhong *et al.* (2001) compared the count of MCs in 17 primary pterygia, 6 recurrent pterygia and 6 normal conjunctival specimens. The mean numbers of infiltrating MCs were 45.47±5.50 and 48.83±3.19 cells in primary and recurrent pterygium, respectively. These figures were significantly higher than that in normal connective tissue (4.24±2.36 cells mm^{-2}). Although the overall outcome is in concord with findings of the present study, the reported figures seem far different. This may be due to low number of samples (particularly in the control group), as well as the heterogeneity of the patients in term of primary and recurred pterygium. As mentioned before, only primary cases of pterygium were included in the present survey.

This heterogeneity also remains in the findings of another study by Beden *et al.* (2003). Based on this study, the difference in mast cell numbers between the pterygium and control groups was not statistically significant. However, they posited that MCs may participate in some stages of pterygium development. To obliterate this conflicting factor, only cases with grade II disease were recruited in the present study.

For the wing of angiogenesis, on the other hand, the expression of two its major contributors, i.e., CD31 and VEGF, were compared between the case and controls. Based on the findings, both the factors were expressed significantly higher in the specimens with pterygium than in the specimens with normal tissue.

Lee *et al.* (2001) compared the expression of VEGF in pterygium and in normal bulbar conjunctiva. They found a significantly higher rate if VEGF expression in the patients' group and asserted the major contribution of angiogenesis in the development of pterygium. This confirms the results of our study in this regard.

The role of VEGF in the pathogenesis of pterygium has been proposed in other studies, as well (Marcovich *et al.*, 2002; Jin *et al.*, 2003; Van Setten *et al.*, 2003). Aspiotis *et al.* (2007) compared the expression of both CD31 and VEGF in patients with pterygium and in normal bulbar conjunctive. They also found a higher rate of CD31/VEGF expression in the patients, confirming the findings of the present study.

Livezeanu *et al.* (2011) emphasized the presence of a much richer vascularization in pterygium, compared with normal conjunctiva. They also used the expression rate of CD31 and VEGF for this propose. Accordingly, the moderate-to-severe expression of VEGF was seen in 81% of the patients; a figure which is very close to that in our series (i.e., 88%).

Other studies also confirm that there might be a causative association between the expression of CD31 and emergence of pterygium (Ling *et al.*, 2012; Lin *et al.*, 2013; Fukuhara *et al.*, 2013).

Summing up the results of the present study and available data in the literature, it is apparent that the angiogenesis is a key factor in pathogenesis of pterygium. This is in conformity with a newly developed notion which surmises a causative role of ultraviolet exposure in pterygium, because this exposure has been connected with induction of angiogenetic factors such as VEGF (Ribatti *et al.*, 2009).

Likewise, it is suggested that both MCs and angiogenesis may be interconnected themselves (Ribatti *et al.*, 2007).

Although, this conjecture merits further well-controlled studies, the present work is the first one which examines both MCs and angiogenesis, simultaneously in patients with pterygium. In addition, the present survey carries two other preponderances comparing to available ones in the literature.

First, the patients in the present study were confined to those with primary, grade II disease. This is pivotal, as it is suggested that the severity of the disease may play an extra confounding role in pathogenesis of pterygium (Lin *et al.*, 2013).

Second, the positivity of CD31/VEGF expression was set to moderate-to-severe definition. It should be born in mind that as far as these two markers are associated with angiogenesis even in normal tissues, there expressions are expected even to mild degree. By omitting these "normal" expressions, the accuracy of the results seems to be higher.

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