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# Immunohistochemical analysis of angiotensin converting enzyme in sardinian pterygium

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**Summary.** Pterygium is a common ocular surface disorder characterized by excessive cell proliferation, inflammation, fibrosis, angiogenesis and extracellular matrix remodeling. The Angiotensin converting enzyme (ACE or ACE I) is the major component of the Renin-angiotensin system (RAS) converting the inactive decapeptide Angiotensin I (Ang I) to the active octapeptide Angiotensin II (Ang II). Besides this 'classical role', it can act as transcriptional regulator in response to external stimuli that may lead to cell damage and tissue remodeling. Due to this role, it can be internalized into the nuclear compartment to act as transcriptional factor for proteins involved in the inflammatory response. The aim of the present study was to determine ACE expression and localization in pterygium and culture pterygium cells by immunohistochemistry. Our results are the first to demonstrate nuclear immunolocalization of ACE, more so in pterygium compared to conjunctiva epithelial cells in histological sections. ACE was not detected in the nuclei of subcultivated pterygium epithelial cells. The nuclear localization of ACE may be correlated with an anti-inflammatory path mediated by activation of its transcriptional role.

**Key words:** Pterygium, Renin-angiotensin system (RAS), Angiotensin converting enzyme (ACE), Tissue culture model, Immunohistochemistry

## Introduction

Pterygium is a common ocular disease that affects only humans (Chui et al., 2008). Anatomically, it appears as encroachment of a fleshy, wing-like shape that invades the cornea (Dushku and Reid, 1994). This invasion can lead to visual loss and diplopia (Lin and Stern, 1998). Pterygium is a degenerative and hyperplastic disease displaying inflammatory features, a rich vasculature, and extracellular matrix remodeling (Di Girolamo et al., 2004). Currently, ultraviolet radiation (UVR) is accepted as a major risk factor involved in the development of pterygium (Moran and Hollows, 1984; Mackenzie et al., 1992; Cullen, 2002; Kau et al., 2006; Perra et al., 2006). Moreover, anti-apoptotic mechanisms (Tan et al., 2000), cytokines (Di Girolamo et al., 2002), growth and angiogenic factors (Kria et al., 1998), and viral infections (Detorakis et al., 2000; Piras et al., 2003; Di Girolamo, 2011) have been proposed as causative agents in its pathogenesis.

The Renin-angiotensin system (RAS) is historically known as the most powerful system for regulating blood pressure and plasma volume (Peach, 1977). Its action is mediated mainly by angiotensin II (Ang II), a potent vasoactive peptide generated from angiotensin I (Ang I) by the action of angiotensin-converting enzyme (ACE, or ACE I, dipeptidyl carboxypeptidase I; E.C.3.4.15.1.) (Arregui et al., 1982; Wright and Harding, 1997; Gard, 2002; Sharma et al., 2010). Recent studies demonstrated that Ang II is not only a potent vasoconstrictor and a stimulant for the release of aldosterone from the adrenal gland, but also a growth factor and an immunomodulator that influences cell proliferation, apoptosis, tissue fibrosis, and participates in inflammatory responses in a nonhemodynamic manner (Nagai et al., 2005). Furthermore, it enhances the vascular endothelial growth factor (VEGF) and is implicated in recruiting inflammatory cells by inducing chemokines and adhesion molecules (Nagai et al., 2005). Several studies have indicated that, besides circulating RAS, local systems may exist. For example, RAS has been found in the vasculature (Sharma et al., 2010), adrenal gland, kidney, testis, (Naruse et al., 1985; Deschepper et al., 1986), brain (Ganten and Speck, 1978; Mizuno et al., 1987), and ovary (Glorioso et al., 1986; Schultze et al., 1989). Many components of RAS have also been

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identified in the eye. Human and rat retina (Wagner et al., 1996; Murata et al., 1997; Savaskan et al., 2004), dog and monkey choroid (Shiota et al., 1997), human ciliary body (Cullinane et al., 2002; Savaskan et al., 2004), and rabbit cornea (Sharma et al., 2010) showed protein and mRNA expression for ACE, Ang II, and its receptors, AT1 and AT2. There are two well known isoforms of the secreted ACE: somatic and testicular. Both enzymes are transmembrane proteins that include a N-terminal extracellular domain and a short C-terminal cytoplasmatic domain (Wei et al., 1991).

Currently, the classical peptidyl-dipeptidase-dependent role of ACE protein, within the RAS system, has been expanded by way of cell surface activity and transcriptional regulation. In recent studies, exogenously added ACE resulted in transcriptional stimulation of bradikinin receptors B1 and B2 genes in smooth muscle cells (SMC) (Lucero et al., 2010). Moreover, it has been hypothesized that nuclear localization of ACE plays a key role in transcriptional regulation (Ignjacev-Lazich et al., 2005).

Camargo et al. (2006) detected ACE, Ang II, and Ang III within nuclei of mesangial cells from spontaneously hypertensive rats. ACE is already known as one of the most widespread enzymes. Besides its enzymatic effect at converting Ang I to Ang II in the regulation of blood pressure, it can modulate Ang II synthesis in cell proliferation, apoptosis, and tissue fibrosis control. This modulation is implicated in the inflammatory processes in response to external stimuli (Tamarat et al., 2002). Moreover, other studies described the ability of ACE to act on N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP), a novel peptide with antifibrotic and antiproliferative properties (Rhaleb et al., 2001). Taking all these studies into account and keeping in mind all pterygium features, we aimed to investigate the expression of ACE in pterygium and culture pterygium cells. In regard to the role of ACE in pterygium, we hypothesized that expression of ACE could result in local RAS activation.

## Materials and methods

### *Geographic location of the study. Outlines*

Situated to the west of mainland Italy, Sardinia is an island in the Mediterranean sea, located between 38° 51' and 41° 15' latitude north and 8° 8' and 9° 50' east longitude, with a high UVR exposure.

### *Primary and subculture of pterygium and conjunctiva cells*

Five pterygium specimens were obtained from Sardinian patients undergoing routine resection by bare sclera technique at the Department of Surgical Science, Eye Clinic, University of Cagliari, Italy. Four normal conjunctiva specimens were obtained from healthy donors undergoing strabismus surgery. One normal conjunctiva and two pterygium samples were not adequate to establish the cell culture model. Therefore, all participant demographic information is reported in Table 1. Human biospecimens were obtained in accordance with the tenets of the declaration of Helsinki and approved by the Human Study Committee of the Medical School, University of Cagliari, Italy and informed consent was obtained from all patients.

Three pterygium specimens and three normal conjunctiva specimens were used to establish cultured cell model. Fresh pterygium and conjunctiva fragments were placed in 8 $cm^2$ culture dishes (Corning-Incorporate; Corning, NY) containing 500 $\mu$l of Earle's MEM (Invitrogen Corporation; Carlsab, CA) serum free in 5% carbon dioxide in air atmosphere at 37°C. Explants were allowed to attach to the dish for at least 18-24 hours before adding complete media. After this time, 2 ml of EMEM supplemented with 10% fetal bovine serum (FBS; Invitrogen Corporation), 100 U/ml penicillin, 100 $\mu$g/ml streptomicyn and 2 mM L-glutamine (Invitrogen Corporation) was added. Explants were removed 10 days later, primary cultures grown to confluence, and passed at weekly intervals. Cells migrating from the explants were detached by incubating in trypsin/EDTA 0.5% (Invitrogen Corporation) for 10 minutes at 37°C, washed and then placed in 75 $cm^2$ flask (Corning) (Di Girolamo et al., 1999). For immunohistochemistry were used only cells from passage 6 and 12.

### *Characterization of pterygium and conjunctiva derived epithelial cells*

Cultured cells were examined by immunohistochemistry in chamber slides for markers of epithelial cell (cytokeratins), endothelial, and Langerhans cells. Monoclonal mouse anti-human cytokeratin 19 (clone RCK 108, Dako, Glostrup, Denmark), and monoclonal mouse anti-human cytokeratin (clones AE1/AE3, Dako) were used for cytokeratin expression. Von Willebrand factor (Dako) and CD1 (clone O10, Dako) were used for the detection of endothelial cells and Langerhans cells, respectively. Positive cells were counted in ten random fields at a final magnification of x400. The cut-off was set at 95% of positive cells per field. Cultured pterygium

**Table 1.** Demographic information for the normal conjunctiva and pterygium specimens for tissue culture model.

| | Gender | Age | Eye r/l | Primary/Recurrent |
|---|---|---|---|---|
| Normal conjunctiva | | | | |
| Patient n.1 | F | 51 | L | - |
| Patient n.2 | M | 50 | L | - |
| Patient n.3 | M | 57 | R | - |
| Pterygium | | | | |
| Patient n.1 | M | 38 | L | P |
| Patient n.2 | F | 45 | L | P |
| Patient n.3 | M | 57 | L | R |

and conjunctival epithelial cells stained positively for cytokeratin 19 and cytokeratin expression, while no immunoreactivity was noted for Von Willebrand factor VIII and CD1 (data not shown), suggesting that cultured cells were unlikely to be contaminated by endothelial cells and Langerhans cells.

### *Immunohistochemistry on paraffin embedded sections and subcultured cells*

Twenty pterygium specimens and ten conjunctiva specimens were processed for paraffin embedding. Demographic information on these patients is recorded in Table 2. After surgery, tissue fragments were fixed in zinc-based fixative (Wester et al., 2003) for at least 18 hours. After fixation, tissues were washed in phosphate-buffered saline and processed.

Paraffin-embedded serial sections (5 $\mu$m thick) were assessed for ACE expression by immunohistochemistry. Briefly, sections were deparaffinazed in xylene, rehydrated in a graded alcohol series and then equilibrated in phosphate-buffered saline (0.1M PBS; pH 7.4). Antigen retrieval was not necessary. Prior to incubation in the primary antibody, endogenous peroxidase activity was quenched with hydrogen peroxide for 20 minutes and then the sections were incubated for 45 minutes with 4% skim milk solution to block non-specific sites. Sections were incubated for 1 hour in mouse primary antiserum monoclonal antibody to ACE (1:50, clone 2E2; Santa Cruz Biotechnology Inc, CA), and 30 minutes in anti-mouse peroxidase conjugated antibody (1:200, Chemicon International, Billerica, MA) as secondary antiserum. 3,3'-diaminobenzidine (DAB) was used as final chromogen. Archival autoptic human kidney paraffin embedded specimen was used as positive control, while negative controls included omitting the primary antibody and isotype control.

Cells derived from fresh pterygium and conjunctiva were seeded at $10^3$ cells per well in an 8 chamber slide (Lab Tek; Nunc Rochester, NY). When confluent, they were fixed in 100% methanol at 4°C but otherwise treated just as tissue specimens for ACE. Experiments were conducted in triplicate. Positive cells from histological sections and tissue culture were obtained by counting manually in ten random fields at x 400. The cut-off was set at 60% of positive cells per field.

**Table 2.** Demographic information for the normal conjunctiva and pterygium paraffin embedded specimens.

| | Gender | Mean age±SD | Eye r/l | Primary/ Recurrent | Nasal/ Temporal |
|---|---|---|---|---|---|
| Normal conjunctiva | | | | | |
| 5 | F | 52±22.45 | 3/2 | - | - |
| 5 | M | 48±13.04 | 4/1 | - | - |
| Pterygium | | | | | |
| 7 | F | 48±17.59 | 3/4 | 5/2 | 5/2 |
| 13 | M | 45±18.33 | 9/4 | 10/3 | 11//2 |

### *Statistical analysis*

Statistical analyses were performed using the SPSS Statistical Package for Social Science, version 15.0 (SPSS Inc., Chicago, IL, USA). The difference of ACE expression between primary and recurrent or nasal and temporal specimens, and the correlation of this protein with the clinicopathological variables, such as sex or age, were assessed by Fisher's exact test. All tests were two-tailed and P values of less than 0.05 were considered to be statistically significant.

## Results

### *Paraffin embedded sections*

ACE protein expression was detectable in all samples of conjunctiva and pterygium specimens (Fig. 1). For the conjunctiva, moderate to strong immunoreactivity was detected mainly in the cytoplasm of basal epithelial cells (Fig. 1A). Moderate nuclear immunoreactivity was occasionally observed. Weak ACE staining was noted in some blood vessels, while no immunoreactivity was observed in the stroma. In pterygium, immunoreactivity for ACE was observed in all epithelial layers (Fig. 1B,C). It was localized mainly in the nuclei (Fig. 1B-D). However, some cells showed immunoreactivity in both nuclei and cytoplasm (Fig. 1B). Strong to very strong nuclear immunoreactivity was noted, while mild to medium immunoreactivity was observed in the cytoplasm. Moreover, ACE positive spindle-shaped fibroblast-like cells, were noted in the stroma (Fig. 1B,C arrows). Moderate immunostaining in few subepithelial connective microvessels was also noted. No immunoreactivity developed in conjunctiva (Fig. 1E) or in pterygium (Fig. 1F) that were incubated without a primary antibody. The kidney section showed strong immunoreactivity for ACE in the cytoplasm and membrane of proximal renal tubules cells (Fig. 1G,H).

### *Tissue culture*

ACE staining was moderate to strong in the cytoplasm and weak in the nuclei of cultured conjunctival cells (Fig. 2A). Interestingly, ACE staining was faint in the cytoplasm and almost absent in nuclei of pterygium cells (Fig. 2B). No immunostaining was observable in conjunctiva (Fig. 2C) or pterygium (Fig. 2D) when the primary antibody was omitted.

### *Statistical analysis*

Statistical analysis showed no significant association between subcellular ACE expression and nasal or temporal eye localization, and primary or recurrent pterygium specimens, and clinical features such as age

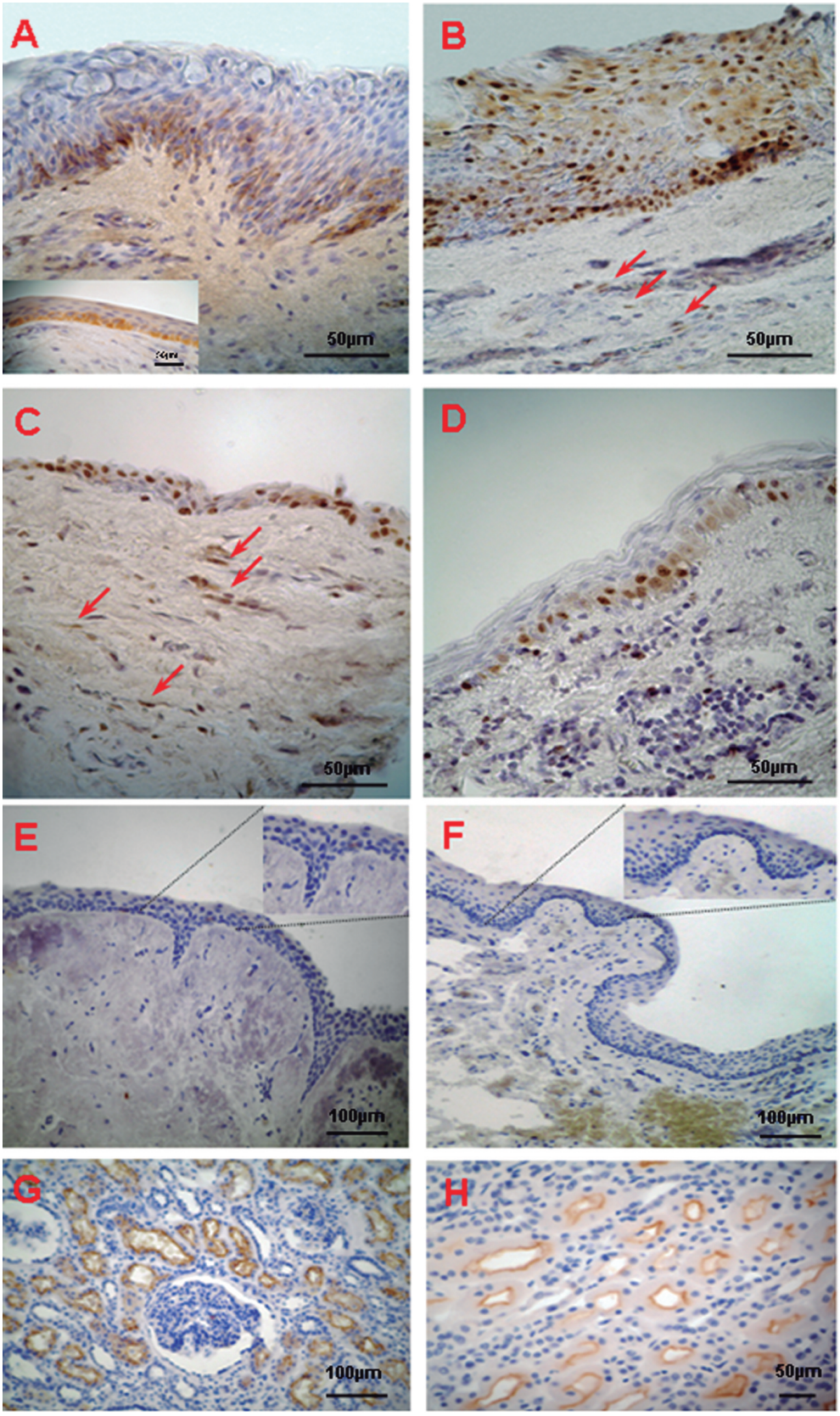


**Fig. 1.** Immunohistochemical expression of ACE in conjunctiva and pterygium. Positive cytoplasmic immunoreactive staining is notable mainly in epithelial basal layer cells of conjunctival sections **(A)**. Inset, higher magnification of ACE positive stain in basal epithelial cells. Images in panels **(B and C)** show predominant nuclear localization of ACE in all epithelial cell layers in two different pterygium samples. ACE positive spindle-shaped fibroblast-like cells are notable in the stroma (arrows). Nuclear localization is observable in the basal layer of epithelial cells in a further different pterygium sample **(D)**. Negative controls of normal conjunctiva **(E)** and pterygium **(F)** don't display any immunoreaction. Kidney **(G and H)** shows specific immunostaining to ACE. Each section was counterstained with haematoxylin. A-D: x 400; E-G: x 200; inset, H: x 630

and sex ($P>0.05$). Moreover, no correlation was found in temporal or nasal localization and clinical features; no differences between primary or recurrent pterygium and clinical features were observed ($P>0.05$).

## Discussion

The present study shows the expression and immunolocalization of ACE in pterygium. Different patterns of expression for ACE were observed between normal conjunctiva and pterygium in tissue sections and tissue culture. ACE is a crucial component in the RAS system. Besides its function of pressure regulation, it can promote vasoconstriction, inflammation, thrombosis, and vascular remodeling. Recently, the role of RAS and, in particular, of ACE was expanded. Currently, we know that ACE is not just viewed as an enzyme converting Ang I to Ang II, but even as a trigger that can lead to gene expression changes (Kohlstedt et al., 2005, 2009).

In rat isolated hepatocytes, Ang II was found to stabilize angiotensinogen mRNA and, more specifically, to stimulate mRNA synthesis, involving binding to putative nuclear Ang II receptors (Erdmann et al., 1996). Moreover, experiments on stimulation of gene transcription, in vascular smooth muscle (Takeuchi et al., 1990; Williams et al., 1995) and endothelial cells, (Erdmann et al., 1996) suggested an 'alternative' mechanism of Ang II mediated by ACE. In fact, it has been found that Ang II can induce the vascular permeability factor (VPF) mRNA expression, and thus it can directly influence the permeability, growth, and function of the vascular endothelium, independently of

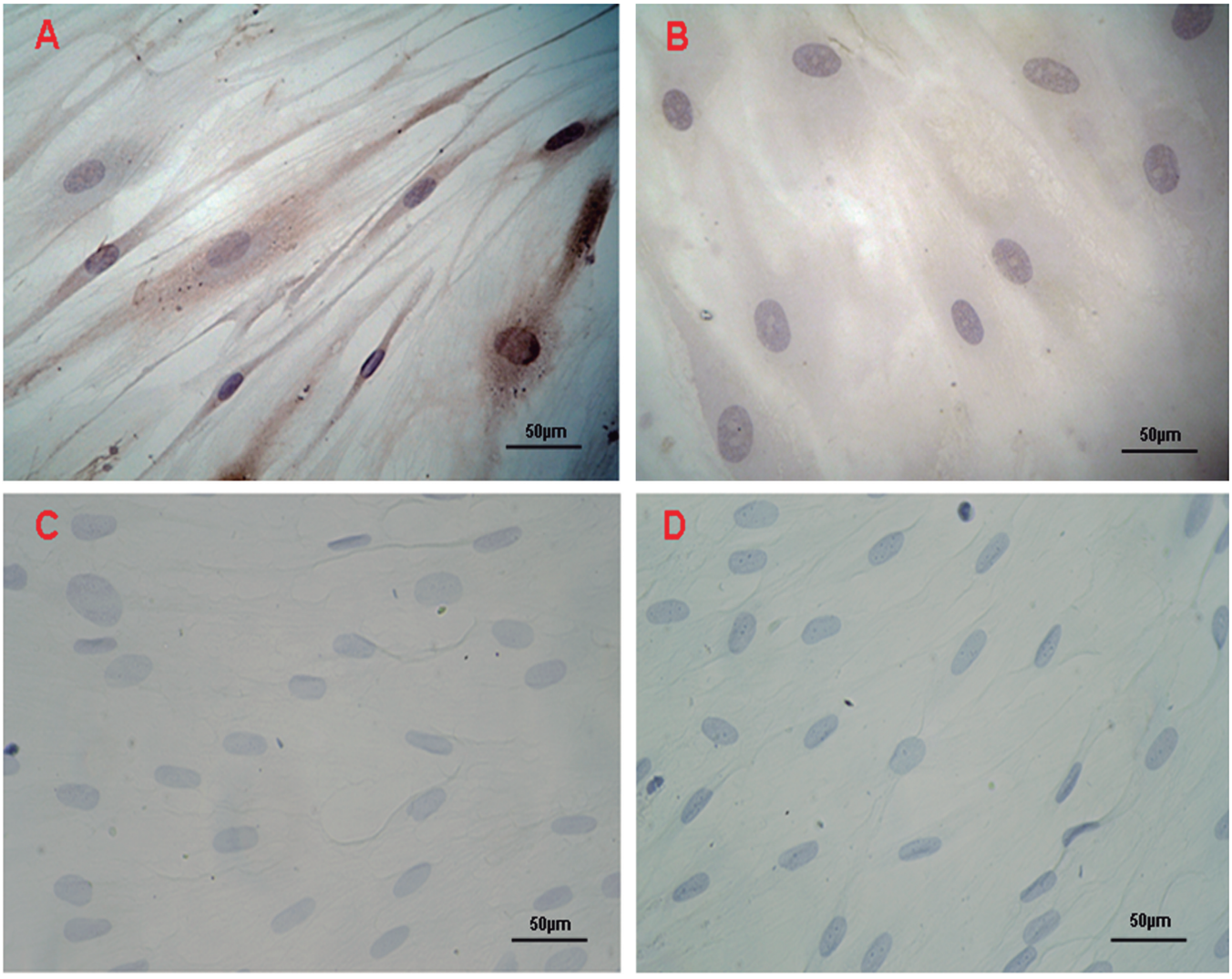


**Fig. 2.** ACE expression in tissue culture. ACE positive staining in conjunctiva **(A)** and pterygium **(B).** Cells in A show a strong and specific immunoreactivity in the cytoplasm. In **B**, very little reactivity is observable in pterygium epithelial cells. No immunoreactivity present when the primary antibody was omitted in conjunctiva **(C)** and pterygium **(D).** Each section was counterstained with haematoxylin. x 400

changes in hemodynamics (Williams et al., 1995).

In our study, paraffined sections showed nuclear localization of ACE in pterygium. This evidence could suggest an attempt by the RAS system, and more specifically by ACE, to promote an 'anti-inflammatory path' mediated by exogenous ACE transcriptional regulation, which may involve its nuclear localization (Lucero et al., 2010).

In our previous papers, we showed a significant correlation between UV-B exposure, alteration of normal cell-cycle protein expression, and cellular damage induced by oxidative stress in pterygium (Perra et al., 2006; Maxia et al., 2008, 2009). From recent studies, it would seem that ACE primary sequence contains 9-amino acid long trans-activating domains (TAD). Recently, it has been described as a common transactivation domain of many transcription factors, including Gal4, p53 and NF-κB (Sandholzer et al., 2007). Because of this, the nuclear localization of ACE may be correlated to an anti-inflammatory role, gene transcription-mediated. This hypothesis may explain the abundant expression of ACE in the nuclei of pterygium epithelial cells, in histological sections, with respect to normal conjunctiva. The predominant cytoplasmatic expression of ACE in normal conjunctiva epithelial cells actually could represent a non-activation mechanism for the transcriptional role of the ACE in normal tissue.

Moreover, regarding the expression of ACE in the subepithelial connective tissue, only in pterygium some fibroblast-like cells appear to be immunopositive. By this time, it is accepted that, behind a circulating RAS system, there is the presence of several tissue-specific local RAS systems. High levels of ACE enzyme are shown in rabbit corneal fibroblasts (Sharma et al., 2010), using a rabbit model of corneal neovascularization, but also in nonocular tissues, such as heart myofibroblasts in rat cardiac tissue repair model (Weber et al., 1997). It is well known that high levels of ACE can promote large amounts of Ang II (Sun and Weber, 1996). The elevated level of Ang II may modulate expression of collagen, fibronectin, and other extracellular matrix proteins, suggesting that the local RAS, whose probable existence is demonstrated by ACE expression in fibroblasts, could actively participate in fibrosis and wound healing (Lijnen and Petrov, 2003; Min et al., 2004; Santos et al., 2009). Taking into account these studies, it is reasonable to think that in pterygium a tissue local RAS system could also exist, and the ACE expression in fibroblasts could mean a local RAS activation in the inflammatory response and tissue remodelling. However, further studies will be useful to clarify the role of fibroblasts in ACE expression. Furthermore, we were able to see a strong immunoreactivity in cell cytoplasm of normal conjunctiva tissue culture too. Rare nuclei showed a moderate immunoreactivity. As reported from Lucero et al. (2010), this evidence could suggest a sort of steady-state level of nuclear ACE, which could be subjected to a dynamic turnover or could be cell-cycle dependent. Moreover, different ACE expression in pterygium between histological section and tissue culture model may be due to different or lacking expression of several enzymes in tissue culture. It is scientifically accepted the 'in vitro' model as a closed system in a controlled environment, outside of a living organism. In mammalian tissues, cells connect not only to each other, but also to a support structure called the extracellular matrix (ECM). This contains proteins, such as collagen, elastin and laminin, that give tissues their mechanical properties, and help to organize communication between cells embedded within the matrix. Receptors on the surface of the cells, in particular a family of proteins called the integrins, anchor their bearers to the ECM and also determine how the cells interpret biochemical cues from their immediate surroundings. Often, the lack of a network among all different cell types, normally present in a specific tissue, may lead in tissue culture to different pattern in terms of expression or cell signalling (Abbott, 2011).

With respect to blood vessels in the histological sections, we would have expected to see at least a moderate endothelial immunoreactivity in almost all of them; indeed, only few showed immunoreactivity. Previous papers have already shown an intense immunoreactivity in blood vessels in different ocular districts (Savaskan et al., 2004); this fact could signify that, in conjunctiva, the local RAS system assumes a more predominant role than circulating RAS. However, further studies will be necessary to elucidate this hypothesis. In conclusion, this is the first report about ACE in pterygium. This study showed the nuclear immunolocalization of ACE in pterygium epithelial cells and in spindle-shaped fibroblast-like cells. The different pattern of expression, between pterygium and normal conjunctiva in histological sections, emphasizes the role of ACE, in the RAS system, as signal transducer for the activation of an anti-inflammatory and anti-proliferative 'alternative' role of the Ang II. Moreover, the immunolocalization of ACE in pterygium fibroblast-like cells could mean a direct involvement of a local RAS system instead of circulating RAS. Finally, we hypothesize that Ang II mediated by ACE could have a key role in the development of pterygium. The recent discoveries about the multiple 'alternative' roles of ACE, in addition to its well known classical role of enzymatic action, are very interesting. New prospects and new points of view in regard to this role may be expanded in the future.

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