Angiotensin II: immunohistochemical study in Sardinian pterygium

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Abstract

The Angiotensin II (Ang II) is the principal effector peptide of the RAS system. It has a pleiotropic effect and, beside its physiological role, it has the property to stimulate angiogenesis and activate multiple signalling pathways related to cell proliferation. The purpose of the study was to determine the Ang II expression and localization in Sardinian pterygium and normal conjunctiva by immunohistochemistry, and its possible involvement in the development and progression of the disease. Twenty-three pterygia and eleven normal conjunctiva specimens obtained from Sardinian patients, were processed for paraffin embedding and assessed for the immunohistochemical revelation of Ang II. Significant Ang II expression was identified in pterygium and conjunctiva. Particularly, thirteen pterygium specimens (n=13) displayed exclusively moderate to strong nuclear staining; some specimens (n=5) showed exclusively a moderate cytoplasmic immunoreactivity, and few specimens (n=2) displayed moderate to strong immunoreactivity in both cytoplasm and nucleus. Only 3 specimens were negative. Statistical significance difference in respect of nuclear and cytoplasmatic localization was observed between normal conjunctiva and pterygium (P=0.026). The results showed a predominant intranuclear localization of Ang II in pterygium epithelial cells, in spite of conjunctiva that mainly showed cytoplasmatic localization. These findings suggest a possible role for Ang II in the development and/or progression of pterygium mediated by the activation of local RAS system.

Introduction

The renin angiotensin system (RAS) is one of the most studied physiological systems in humans. It is well known as major actor in the regulation of cardiovascular homeostasis, maintaining electrolyte balance, body fluid vol-
Twenty-three pterygium specimens and eleven conjunctiva specimens were processed for paraffin embedding. Demographic information on these patients is recorded in Table 1. After surgery, tissue fragments were fixed in formalin for at least 18 h. After fixation, tissues were washed in phosphate-buffered saline (0.1M PBS; pH 7.4) and processed. Paraffin-embedded serial sections (5 μm thick) were assessed for Ang II expression by immunohistochemistry. Briefly, sections were deparaffinized in xylene, rehydrated in a graded alcohol series and then equilibrated in PBS. Antigen retrieval was performed by citrate buffer (0.01M; pH 6) at 95°C. Prior to incubation in the primary antibody, endogenous peroxidase activity was quenched with a solution of hydrogen peroxide 3% in 100% methanol for 20 min. Then, the sections were incubated for 45 min with 5% skim milk solution to block non-specific sites. Sections were incubated overnight at 4°C in rabbit primary antiserum polyclonal antibody to Ang II (1:100; Novus Biological, Littleton, CO, USA), and 30 min in anti-mouse peroxidase conjugated antibody (1:200, Chemicon International, Billerica, MA, USA) 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. All sections were counterstained in haematoxylin and mounted in Entellan mounting medium (Merck KGaA, Darmstadt, Germany). Each section was viewed by two investigators, taking into account the location (cytoplasmatic and nuclear) and intensity of Ang II staining. Experiments were conducted in triplicate. Positive cells were obtained by counting manually in ten random fields at 400X. The cut-off was set at 60% of positive cells per field.

### Results

The results are summarized in Table 2. Significant Ang II expression was identified in pterygium and normal conjunctival epithelial cells (Figure 1). In conjunctiva, six specimens showed strong cytoplasmatic immunoreaction for Ang II (Figure 1A). Two samples showed moderate to strong immunoreaction in both cytoplasmatic and nuclear compartment (Figure 1B). Only one sample displayed strong nuclear immunoreactivity (data not shown). In regard to pterygium, a high number of pterygium specimens (n=13) displayed exclusively moderate to strong nuclear staining (Figure 1C). On the other hand, some specimens (n=5) showed exclusively a moderate cytoplasmatic immunoreactivity (Figure 1D). Furthermore, few specimens (n=2) displayed moderate to strong immunoreactivity in both cytoplasm and nucleus (Figure 1E). Occasionally, some sections showed immunopositive leukocytes-like cells (n=3) in the stroma and within the vessels (Figure 1F, arrows). No immunoreactivity developed in conjunctiva or in pterygium when incubated without a primary antibody and/or isotype control (Figure 1H). The kidney section showed strong immunoreactivity for Ang II in the cytoplasm and membrane of proximal renal tubules cells (Figure 1G).

### Statistical analysis

Statistical significance difference in respect of nuclear and cytoplasmatic localization was observed between normal conjunctiva and pterygium (P=0.038), while no significant association was obtained between Ang II expression and nasal or temporal eye localization, primary or recurrent pterygium specimens, and clinical features such as age and sex (P>0.05). Moreover, no correlation was found in temporal or nasal localization, clinical features, and between primary or recurrent pterygium and clinical features (P>0.05).

### Discussion

The present study shows the expression and immunolocalization of Ang II in pterygium and normal conjunctiva. Significant immunolocalization was noted in normal and pathological sections. It is widely known that Ang II is a multifunctional bioactive octapeptide of the renin-angiotensin system that plays a fundamental role in vasoconstriction, controlling cardiovascular function and renal homeostasis.²² In our study we were able to detect Ang II in different cellular compartments, with...
notable differences between normal conjunctiva and pterygium. Our findings show a predominant nuclear immunolocalization of Ang II in most of the pterygium samples. Solely few pterygium specimens show a moderate cytoplasmic immunostaining. Over the past two decades, has raised the hypothesis of local, tissue-specific, RAS system, that could act independently from circulating RAS. On this way, many authors, with different approaches, demonstrated local RAS systems.\textsuperscript{34-38} In 1971, Robertson and Khairallah\textsuperscript{39} demonstrated nuclear localization of Ang II in smooth and cardiac muscle cells. Later, other authors correlated the presence of intracellular/nuclear Ang II to different cellular mechanisms implicated in cell growth and proliferation\textsuperscript{2,40} and tumor growth, invasion, and angiogenesis.\textsuperscript{41-43}

In view of these studies, we found a similar pattern in pterygium. Thirteen samples showed nuclear localization versus five samples that showed positive cytoplasmic stain. This evidence led us to hypothesize a possible response by Ang II in the inflammatory/proliferative processes in pterygium mediated in the nuclear compartment. In fact, some studies reported the ability of Ang II to bind the chromatin,\textsuperscript{44,45} to modulate gene expression, and to mediate different mRNA transcripts.\textsuperscript{15} Recent discoveries showed Ang II inducing rapid synthesis of VEGF in proximal tubular epithelial cells (MCT), by stimulation of its mRNA translation.\textsuperscript{46} Moreover, it has been documented that Ang II regulates activity of several key mediators of injury, such as transforming growth factor-\(\beta\) (TGF-\(\beta\)).\textsuperscript{47-49} This intranuclear Ang II immunoexpression lead us to hypothesize a similar pathway in pterygium. Moreover, in the present study we were able to detect Ang II staining also in leukocytes-like cells: previous studies on human monocytes revealed that they contain high amount of Ang II, which may be potentially released under different stimuli.\textsuperscript{50} A well-known pterygium feature is the leukocytes infiltration consisting predominantly of lymphocyte T cells.\textsuperscript{51} In consideration of this statement, it is reasonable to think that a potential release of Ang II from leukocytes, in a chronic inflammation condition, could contribute to worsen the disease stage. All these evidences are reinforced by the results obtained from normal conjunctiva. Here we found a predominant epithelial cytoplasmatic staining. This leads to believe that in physiological conditions Ang II may basically play a role in the cytosol. Ang II microinjections on rat vascular smooth muscle cells have demonstrated an increase cytosolic Ca\textsuperscript{2+} levels inducing both intracellular stores and Ca\textsuperscript{2+} influx.\textsuperscript{52} This suggests a possible support function by Ang II to normal, physiological, cellular mechanism;\textsuperscript{1} postulated that actually intracellular RAS system, and particularly Ang II, may work in different ways under different conditions. In physiological condition the intracellular RAS may support the circulating RAS and, as proposed by Eggena et al., it may increase the local Ang II concentration by transcription rate of some RAS components when the Ang II concentration is low, or suppressing transcription when Ang II levels are high.\textsuperscript{53} To date we can not assert if the intracellular RAS is an independent regulation system, however our feeling is that it could represent a more accurate control mechanism to support local and circulating RAS. This hypothesis is supported by a study from Kalinyak and Perlman\textsuperscript{54} which stated that circulating Ang II concentration, in normal physiological conditions, is insufficient to induce the multiple responses attributed to Ang II.

Figure 1. Immunohistochemical expression of angiotensin II (Ang II) in normal conjunctiva and pterygium. A) Positive immunoreactive staining is visible in the epithelial cells of normal conjunctiva in cytoplasm. B) Positive immunoreactive staining is visible in both cytoplasm and nuclear compartment. C,D,E) Nuclear, cytoplasmatic and both nuclear and cytoplasmatic positive immunostain to Ang II in pterygium epithelial cells, respectively. F) Ang II positive leukocytes-like cells are notable in blood vessels and in the stroma (arrows). G) Pterygium negative control does not display immunoreaction. H) Kidney shows specific immunostaining to Ang II.
In conclusion, in this report we showed that pterygium epithelial cells exhibit Ang II in the nuclear compartment. Intranuclear localization could be attributable to the gene expression modulator role played by Ang II in pathological condition. Moreover, positive Ang II leukocytes-like cells in pterygium suggest a possible role due to the release of Ang II in pterygium. This study represents a tip of the iceberg and further studies will be needed to better clarify the intracellular role of Ang II in pterygium.

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