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Expression of endothelial leukocyte adhesion molecule 1 in the aqueous outflow pathway of porcine eyes with induced glaucoma

Tatiana Suárez, Elena Vecino

Departamento de Biología Celular e Histología, Facultad de Medicina, Universidad del País Vasco, E-48940, Leioa, Vizcaya, Spain

Purpose: To determine if the expression of endothelial leukocyte adhesion molecule 1 (ELAM-1) in the trabecular meshwork system of the porcine eye, when subjected to experimental glaucoma, is increased as it does in human glaucoma.
Methods: Immunohistochemistry using a specific mouse antihuman ELAM-1 antibody was performed on the trabecular meshwork system of five pigs. The episcleral veins of the left eyes were cauterized as reported elsewhere to induce glaucoma. Immunodetection of ELAM-1 was assayed in human trabecular meshwork samples obtained from trabeculectomy as a positive control.
Results: Pig eyes exhibiting elevated intraocular pressure (IOP) and damage to retinal ganglion cells (RGCs) due to experimental glaucoma as reported elsewhere, were found to exhibit ELAM-1 immunoreactivity in their trabecular meshwork.
Conclusions: ELAM-1 protein, the first molecular marker for human glaucoma, can also be considered a candidate molecular marker of induced glaucoma in the pig model of experimental glaucoma. The results of our study further validated the pig eye as an animal model of glaucoma, since increased expression of ELAM-1, which has been found in the trabecular meshwork of human eyes with glaucoma, is also found in pig eyes subjected to experimental glaucoma via episcleral vein cauterization.

Glaucoma is a chronic ocular disease characterized by increased pressure of the fluid within the eye. This is due to anatomical restriction of fluid drainage through the trabecular meshwork, the major outflow pathway of the eye [1,2]. This disease affects approximately 67 million people worldwide and is a leading cause of irreversible blindness [3].

The aqueous outflow pathway of the eye is composed of a series of channels lined with endothelial cells. It is located in the lateral edges of the anterior chamber and includes the trabecular meshwork, Schlemm's canal, the collector channels, and the episcleral venous system [4]. It has been hypothesized that trabecular meshwork cells modulate the outflow of aqueous humor from the anterior chamber by regulating the turnover of the extracellular matrix (ECM) [5]. Elevated intraocular pressure (IOP), which is commonly associated with glaucomatous conditions, is believed to arise as a consequence of impairment(s) in trabecular meshwork function [6] and is considered to be a principal risk factor for the development and progression of glaucomatous damage [7] to retinal ganglion cells (RGCs) and the optic nerve. Although important progress has been made in recent years in understanding glaucoma processes, the principal mechanisms that lead to glaucoma remain to be elucidated.

Endothelial leukocyte adhesion molecule 1 (ELAM-1) has been identified as the first molecular marker for glaucomatous trabecular meshwork cells in humans [8]. Wang et al. [8] ELAM-1 was expressed by trabecular meshwork cells in eyes with various types of glaucoma, but not in nonglaucomatous eyes. ELAM-1, also known as E-Selectin, is a 115 kDa cytokine endothelial cell surface glycoprotein that mediates the adhesion of neutrophils, monocytes, eosinophils, NK cells, and a subset of T cells to activated endothelium [9-11]. This molecule is known to be involved in several pathologies of different etiologies such as cutaneous inflammation [12] and arthritis [13], among others. It has been reported to be an inflammatory corneal disease-related molecule [14], and has been found to be expressed in induced uveitis [15], allergic eye disease [16], retinopathy in type 2 diabetic patients [17], and vernal keratoconjunctivitis [18], among others.

In many cases, glaucoma is asymptomatic for long periods of time. Consequently, by the time the illness is diagnosed, irreversible cellular and tissue damage may have already occurred. Therefore, several groups have employed different animal models to study its etiopathology. Our group has extensively documented the pig eye as an adequate model to study glaucoma [19-26]. By cauterizing episcleral veins in adult pig eyes to induce experimental glaucoma, we were able to observe IOP elevation, RGC loss, and the progression of glaucoma-like characteristics [19]. However, it is not known if the pig model of glaucoma also reproduces the phenomenon of ELAM-1 upregulation associated with human glaucoma.

In the present study, we characterized the expression of ELAM-1 in pig eyes subjected to glaucoma and exhibiting RGC damage and elevated IOP. Our findings indicate that this

Correspondence to: Elena Vecino, Departamento de Biología Celular e Histología, Facultad de Medicina, Universidad del País Vasco, E-48940, Leioa, Vizcaya, Spain; Phone: +34-94-601-2820; FAX: +34-94-601-3266; email: elena.vecino@ehu.es

molecule is an appropriate marker for the glaucoma phenotype in the pig model of experimental glaucoma and validates the appropriateness and usefulness of this model for further studies of glaucoma.

METHODS

Experiments were carried out in accordance with European Union guidelines and the ARVO Statement for the use of Animals in Ophthalmic and Vision Research. The collection of human trabecular tissues was performed following the principles of the Declaration of Helsinki.

Induction of experimental glaucoma and intraocular pressure measurement: This study employed the same five adult pigs (*Sus scrofa*) used in a previous study by our group [19]. Increased IOP had been induced by cauterizing three episcleral veins of the left eye of each animal following the method described in rats by Shareef et al. [27]. Briefly, the pigs were deeply anesthetized with an intramuscular injection of ketamine hydrochloride (Ketolar) plus xylazine (Diazepan) at 20 mg/kg each. An intravenous cannula was applied to the ear to provide the animal with additional anesthesia (1 ml Propofol every 15 min), maintaining deep anesthesia throughout the operation. Three episcleral veins (nasal, dorsal, and temporal) were cauterized, and the animals were kept alive for 21 weeks after episcleral vein occlusion. The IOP was measured in both eyes 45 days before the episcleral vein operation to obtain the basal pressure values (15.2±2.2 mm Hg). One week after cauterization, both the nonoperated control and cauterized eyes were measured to evaluate changes in IOP. Although maximal increases in IOP in cauterized eyes were observed at weeks 16 (14.8±1.5 mm Hg in controls versus 21.0±2.9 mm Hg in cauterized; 1.41 fold increase) and 20 (15.1±2.3 mm Hg in controls versus 19.8±4.7 mm Hg in operated; 1.31 fold increase), animals were killed at week 21, when IOP values were 11.5±0.9 mm Hg in controls and 13.9±0.8 mm Hg in cauterized eyes (1.23 fold increase).

Anterior chamber tissue collection: At the end of this period, animals were sacrificed and their eyes enucleated. The anterior chambers were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS, pH 7.4) for 4 h at 4 °C and cryoprotected in 30% sucrose in PBS for 12 h at 4 °C. Sections corresponding to the angle of the anterior chamber containing the trabecular system were embedded in medium (Tissue Tek; Leica, Heidelberg, Germany), frozen in liquid nitrogen, cut at 14 μm using a cryostat, and stored at -20 °C.

Surgical sections containing the outflow pathway from human glaucoma patients were obtained at trabeculectomy. These sections were provided by the Department of Ophthalmology of the San Eloy Hospital (Barakaldo, Bilbao, Spain). These samples obtained at surgery were fixed and cryoprotected immediately upon receipt. The same experimental conditions employed for porcine eyes were used to analyze these human tissue samples.

Immunohistochemical assays: Mouse antihuman ELAM-1 antibody (E-selectin CD62E; Chemicon, Temecula, CA) was used as primary antibody for immunodetection of ELAM-1 protein. This antibody is known to present cross-reactivity with porcine ELAM-1 [28], and it was therefore used for both human and porcine immunodetection.

Sectioned anterior chambers and trabecular tissue were rinsed in PBS containing 0.25% Triton X-100 (PBST) and then incubated for 1 h at room temperature with blocking solution consisting of PBST and 1% bovine serum albumin (BSA fraction V; Sigma-Aldrich, St. Louis, Mo). Sections were incubated overnight at 4 °C with mouse anti-ELAM-1 antibody (CD62E, diluted 1:100; Chemicon). The next day, they were rinsed twice in 1X PBS containing 1% BSA for 10 min each, and incubated with goat antimouse IgG conjugated to BODIPY (diluted 1:200; BODIPY goat antimouse IgG (H+L) conjugate B-2572, Molecular Probes, Portland, OR) for 1 h. After incubation, the sections were rinsed in PBS, coverslipped with PBS/glycerol (1:1), and examined by epifluorescence microscopy. Labeling specificity was assessed by omission of the primary antibody, replacing it with PBST-BSA, and by omission of a secondary antibody.

Porcine trabecular meshwork tissue samples were prepared. They were examined by fluorescence microscopy and regular light microscopy using hematoxylin&eosin staining to determine the morphology of the trabecular system at the angle of the anterior chamber.

RESULTS & DISCUSSION

Morphological analysis of the pig anterior chamber (Figure 1A,B) revealed that the drainage outflow system of the pig eye is slightly different to that of humans (Figure 1C). Pigs have several small canals, collectively named the angular plexus (AP), instead of a single Schlemm's canal as observed in humans. These small canals are part of a scleral venous plexus, but are located closer to the trabecular meshwork. Indeed, some of them actually appear to enter just within the limits of the network. It has been reported that they do not carry blood and so are more like a true Schlemm's canal than the vessels of the more common scleral venous plexus [29]. This differential structure has previously been observed by our group in pigs subjected to experimental glaucoma using three different methods [30]. In that comparative study, only the cauterization of episcleral veins was able to produce a sustained IOP, and the lower effectiveness of the other two methods (i.e., the injection of a solution containing latex fluorospheres and a solution containing latex fluorospheres plus methylcellulose) could be explained on the basis of anatomic differences of the aqueous outflow system in primates and pigs [30]. Therefore, these differential morphological characteristics may underlie different results obtained using different methodologies in animal models of glaucoma.

We have previously validated the pig eye as a model of glaucoma based on chronic elevation of IOP and RGC damage at the level of the retina [19]. We next sought to evaluate if ELAM-1 expression in the pig anterior chamber also emulates that found in human glaucoma. To this end, we examined the immunohistochemical expression of ELAM-1 in the anterior chamber of four of the five pairs of pig eyes included in our earlier study i.e., those exhibiting the larger IOP increases. In those animals, both eyes of the same pig, the cau-

terized and the contralateral nonoperated eyes were analyzed under the same experimental conditions. The anterior chamber tissue sections were always cut at the same region, near the nasal region of the eye. A total of 10-12 glass coverslips for each analyzed eye were evaluated, each coverslip containing between five to six anterior chamber sections. ELAM-1 immunolabeling was observed in all tissue samples on the same coverslip corresponding to the four cauterized eyes; however, a slight variation of fluorescence intensity was observed among samples, possibly due to the level of cut inside the same tissue. The present results show that ELAM-1 is expressed in the canals that constitute the outflow pathway of all eyes that were subjected to episcleral vein occlusion (left pig eyes), but not in any of the nonoperated control right eyes (compare Figure 2A,C). It is important to note that the majority but not the total of the canals contained in a specific area of a sample were recognized by ELAM-1 antibody. We believe that the mesh-like tissue morphology as well as the angle of the court could be rational reasons for variations in the number of canals contained a particular area, and also for the differences of positive immunostaining of the canals.

Additionally, immunohistochemical assays to detect ELAM-1 protein in the human trabecular meshwork were also carried out as a positive control. Tissue from the trabeculectomy samples from glaucoma patients was found to be ELAM-1 immunoreactive (Figure 2E,F). These findings are consistent with the results previously published by Wang et al. [8], in which ELAM-1 was defined as a molecular marker for the glaucomatous trabecular meshwork in humans. In that work, immunohistochemical screening using a battery of cell-adhesion molecule (CAM) probes was used to determine whether CAM expression might be altered in glaucoma. Of the probes assayed, only ELAM-1 was able to differentiate the glaucomatous from the normal eye aqueous outflow pathway in the region of Schlemm's canal and the surrounding trabecular meshwork.

ELAM-1 immunohistochemistry was carried out using a mouse antihuman ELAM-1 antibody (E-selectin CD62E; Chemicon), which cross-reacts with the human and porcine ELAM-1 protein, reacting strongly with activated and/or resting endothelial pig cells [28]. The intensity of the fluorescence signal observed in porcine canals (Figure 2A) was noticeably weaker than that observed in humans (Figure 2E), suggesting that the antibody may be more immunoreactive with human rather than porcine ELAM-1. Labeling specificity in the immunohistochemical assays was assessed by omission of the primary antibody, replacing it with PBST-BSA and by omission of the secondary antiserum (data not shown). Negative

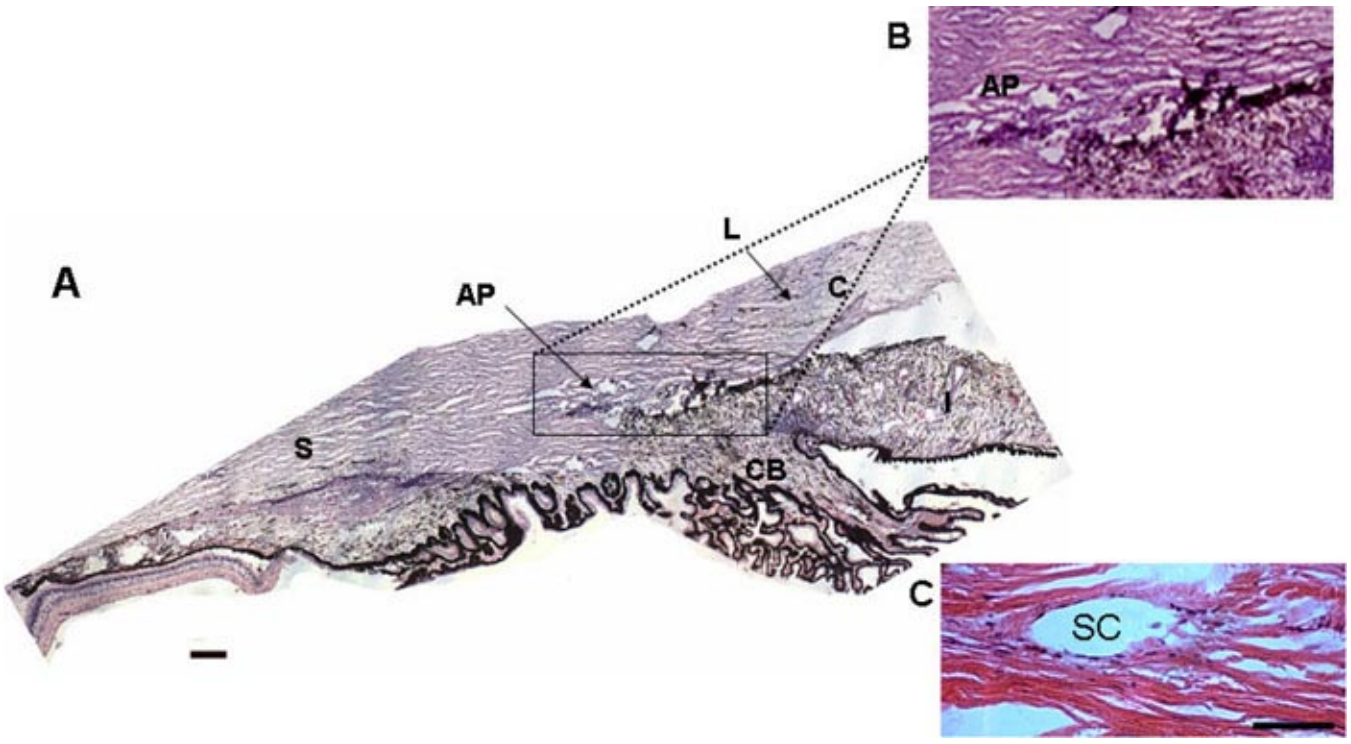

Figure 1. Morphological structure of the porcine and human anterior chamber using hematoxylin&eosin staining. **A**: Anatomy of the porcine aqueous outflow tract. The section corresponds to an iridocorneal angle. The boxed region marks the drainage outflow system, which is made up of several small canals forming the angular plexus (AP) instead of a single Schlemm's canal found in humans. The aqueous humor produced in the ciliary body (CB) proceeds through the trabecular meshwork into the AP. **B**: Higher magnification of the aqueous outflow section delimited by the box in **A**. **C**: Histological section from a human anterior segment included in the present study. A single Schlemm's canal is illustrated as well as its boundaries. AP, angular plexus; S, sclera; C, cornea; L, limbus; CB, cilliary body; SC, Schlemm's canal. Scale bar 25 μm

control preparations such as substitution of the primary ELAM-1 antibody by a matched isotype (such as mouse IgG1 antibody) or the pre-adsorption with an immunogenic peptide were not assayed. However, the fact that in the control group not staining was observed in any of the analyzed animals, provide an evidence of specificity. Nevertheless, the results should be interpreted under the differences of the staining pattern of the samples subjected to injury by episcleral veins occlusion, in comparison to the absence of a similar pattern in the control group.

The pathway represented by the trabecular meshwork/Schlemm's canal system is likely to be responsible for the regulation of most of the aqueous humor outflow. In humans, more than 75% of the resistance to aqueous humor is known to be exerted by this system [31]. Based on our previous results [19] and our present findings, we propose that in our pig eye model, induced glaucoma begins when cauterization of three of the four episcleral veins produces a decrease in outflow of aqueous humor, leading to altered drainage in the front of the eye [31] and subsequent elevation of IOP. The latter has a double effect: the activation of ELAM-1 expression in the outflow pathway system at the anterior chamber level as a response to the cellular stress generated by high pressure, and at the level of the retina, a progressive death of RGC, as well as an increase in the mean area of RGC somata, principally in the peripheral retina.

The finding that ELAM-1 is upregulated in the outflow pathway in pig and human experimental glaucoma models will undoubtedly motivate further studies in research areas related to the development of glaucoma treatments. As proposed previously [4], ELAM-1 could represent a new target for researchers trying to develop agents that act directly on the TM and SC to reduce outflow resistance. The pig model constitutes a valuable tool to determine the exact role of ELAM-1 in human glaucoma disease, and in other related glaucoma processes such as changes in cell morphology and cell-cell contact alterations. The availability of an animal model that reproduces human glaucoma can enable a more rapid evaluation of these hypotheses.

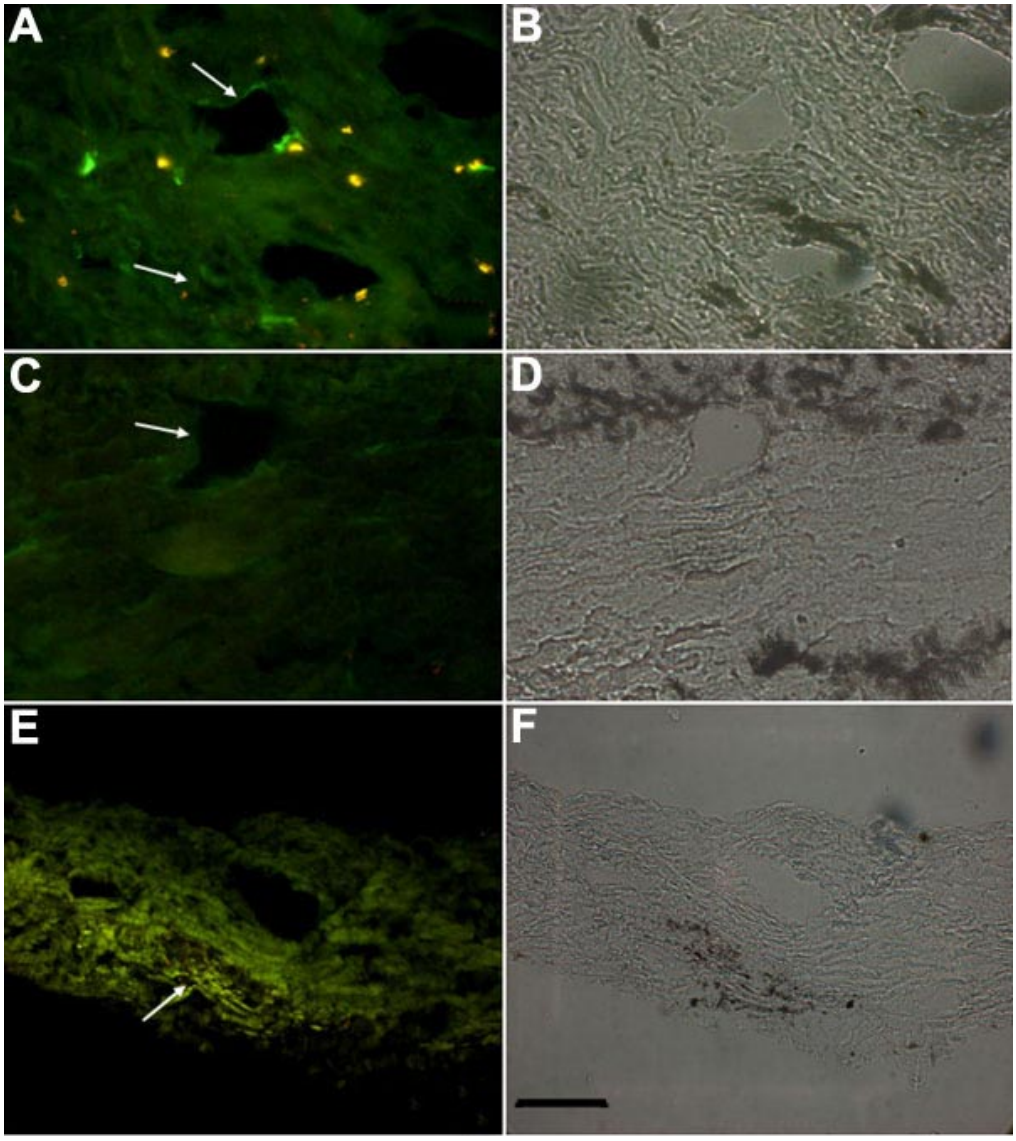

Figure 2. ELAM-1 immunoreactivity in the outflow pathways of porcine and human eyes. **A**: Immunohistochemical detection of ELAM-1 in the outflow pathway of the porcine eye subjected to experimental glaucoma. Note the fluorescent signal lining the canals and in the trabecular meshwork region (arrows). **B**: Phase contrast image of the same section presented in **A**. **C**: Immunohistochemistry of the outflow pathway of the control porcine eye in which glaucoma was not induced. ELAM-1 immunofluorescence was not observed. **D**: Phase contrast image of the same section presented in **C**. **E**: Detection of ELAM-1 in the human outflow pathway. The section corresponds to a surgical sample obtained from trabeculectomy of a glaucomatous eye. Note the fluorescent labeling at Schlemm's canal and the surrounding trabecular meshwork (arrow). **F**: Phase contrast image of the section presented in **E**. Scale bar 25 μm.

Additionally, given that ELAM-1 is a transmembrane protein that transduces signals into endothelial cells, leading to the activation of a variety of signaling pathways, such as the mitogen-activated protein kinase (MAPK) signaling cascade (reviewed in [4]), it will be of interest to use proteomic approaches to investigate the differential protein expression profiles of experimental glaucomatous vs. control tissues. This type of study could contribute to identifying other players involved in these signaling cascades and may help to identify novel molecules that play a role in glaucoma as well as new diagnostic markers and therapeutic targets.

Overall, our results indicate that ELAM-1 protein can be considered a potential marker for experimental glaucoma, and further validate our model of experimental glaucoma in terms of ELAM-1 expression. Using the model of pig experimental glaucoma induced by cauterization of the episcleral veins, we are able to induce an increase in IOP, as well as defects at the level of the retina and the outflow pathway, mimicking the cellular and physiological events that accompany the disease process in humans. This model is thus further validated as a suitable tool for the study of human glaucoma. Future characterization of the temporal expression profile of ELAM-1 during illness progression at several time points will further enhance our appreciation of the role played by ELAM-1 in this pathology.

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