

Induction of endothelial RAGE expression in pterygium

Samar Al-Swailem,¹ Zhenhua Xu,² Lijuan Wu,² Matthew J. Hartsock,² Samuel C. Yiu,² Elia J. Duh²

¹King Khaled Eye Specialist Hospital, Riyadh, Kingdom of Saudi Arabia; ²Wilmer Eye Institute, Johns Hopkins University School of Medicine, Baltimore, MD

Purpose: Chronic inflammation is a critical process in pterygium development and progression, including promotion of angiogenesis. Vascular endothelial cells (ECs) actively participate in and regulate inflammation. Pterygium research has uncovered multiple inflammatory cytokines that are upregulated, but there has been minimal focus on EC activation. The Receptor for Advanced Glycation Endproducts (RAGE), a major proinflammatory molecule expressed in the vascular endothelium and other cell types, is a major instigator of endothelial cell activation. In this study, we explored the hypothesis that RAGE is upregulated in ECs in pterygium. To this end, we examined RAGE expression and immunolocalization in human pterygium and normal conjunctival tissue, with a particular interest in assessing endothelial RAGE.

Methods: Pterygium specimens were obtained from 25 patients during surgery at the King Khaled Eye Specialist Hospital (KKESH). In the same patients, conjunctiva were obtained from the autograft during surgery. Tissue specimens were formalin-fixed and paraffin-embedded. Tissue sections were analyzed with immunohistochemistry with anti-RAGE antibody. Expression and localization of RAGE were evaluated in pterygium and corresponding conjunctiva.

Results: RAGE expression was detected in the vascular endothelium in all pterygium tissue specimens and most conjunctival specimens. Other cell types exhibited expression, notably epithelial cells, fibroblasts, and possibly macrophages. Strikingly, endothelial RAGE expression was increased in 19 of 25 pterygium tissue specimens, compared to the corresponding control conjunctiva.

Conclusions: Our data reveal that RAGE expression is upregulated in vascular endothelial cells in pterygium. RAGE upregulation is an important mechanism by which endothelial cells amplify the overall inflammatory response, and suppression of RAGE has been shown to prevent the progression of some systemic disease processes in experimental models. This suggests that pharmacologic targeting of RAGE, which is already being attempted in clinical trials for some diseases, could be useful in treating pterygium.

Pterygium is an ocular surface disease related to chronic ultraviolet light exposure. Pterygium is a proliferative, invasive process characterized by a fibrovascular conjunctival outgrowth that impinges on the corneal surface. Surgical excision can be a useful therapy for pterygia, but recurrences are common. There is a significant need to gain more insight into pterygium formation and recurrence, to enable the design of new therapeutic strategies, either for inhibiting pterygium growth, regressing pterygia, or preventing recurrent pterygia. The identification of new molecular pathogenic determinants of pterygia could lead to new therapeutic targets.

Chronic inflammation is a critical process involved in the development and progression of pterygium, including promotion of angiogenesis [1-3]. Inflammation has classically been conceptualized in leukocytes, but vascular endothelial cells (ECs) are now appreciated as active participants and regulators [4]. Pterygium research has uncovered multiple proinflammatory genes that are activated in pterygium tissue, including the proinflammatory transcription factor

nuclear factor- κ B (NF- κ B) [5], and several cytokines, including tumor necrosis factor α (TNF- α) [6]. However, there has been minimal focus on EC activation in pterygium, and more insights are needed into additional molecules that could lead to new strategies for pharmacologic treatment.

The Receptor for Advanced Glycation Endproducts (RAGE) is a member of the superfamily of immunoglobulin cell-surface receptors that plays an important role in promoting inflammation [7,8]. RAGE has multiple ligands, including advanced glycation endproducts (AGEs), HMGB1, and S100b. Interaction between RAGE and its ligands leads to induction of NF- κ B and proinflammatory gene activation. RAGE plays a particularly important role in vascular endothelial cells (ECs) and their activation [9]. Given the importance of endothelial cell activation (for inflammation and angiogenesis) in pterygium, we are interested in identifying molecular players that might regulate ECs in pterygia. In this study, we hypothesized that RAGE, a major instigator of endothelial activation, is upregulated in ECs in pterygium. We therefore investigated the localization of RAGE in pterygium tissue to determine whether RAGE is expressed in vascular endothelial cells. In addition, we compared endothelial RAGE expression in pterygium tissue with expression in normal conjunctiva,

Correspondence to: Elia J. Duh, Wilmer Eye Institute, Johns Hopkins University School of Medicine, 400 N. Broadway, Baltimore, MD 21287; Phone: (410) 614-3388; FAX: (410) 614-5471; email: eduh@jhmi.edu

TABLE 1. CLINICAL AND DEMOGRAPHIC INFORMATION FOR PATIENTS.

Case number	Age	Sex	Case details	Area (mm ²)
1	76	M	Primary	11.3
2	71	M	Primary	24.8
3	39	M	Recurrent	14
4	72	M	Primary	33
5	27	F	Primary	25.6
6	68	M	Primary	12
7	17	M	Primary	15
8	57	M	Primary	17.5
9	53	M	Recurrent	15
10	70	F	Primary	17.5
11	69	F	Primary	24.5
12	53	M	Primary	14
13	85	M	Primary	37.8
14	75	M	Recurrent	24
15	41	M	Recurrent	7.5
16	57	M	Primary	13.5
17	70	F	Recurrent	22
18	41	M	Primary	13.8
19	53	F	Primary	27.5
20	63	M	Primary	13.5
21	64	F	Primary	30.3
22	50	M	Primary	13.5
23	50	M	Primary	11.3
24	76	M	Primary	26
25	46	F	Primary	13.8

to determine if there is induction of endothelial RAGE in pterygium.

METHODS

Patients and specimens: Pterygium specimens were obtained from 25 patients during pterygium surgery at the King Khaled Eye Specialist Hospital (KKESH). Patients were in good health and ranged from 17 to 85 years of age, with 18 males and 7 females (Table 1). Pterygium specimens were obtained during pterygium surgery at the King Khaled Eye Specialist Hospital (KKESH). All patients underwent pterygium excision, rotational conjunctival flap, application of (mitomycin C) MMC 0.2 mg/ml \times 1 min. Two patients required amniotic membrane transplantation (AMT) to cover bare sclera. In the same patients, 1.5 \times 1.5 mm conjunctiva specimens were obtained from the superior temporal quadrant of bulbar conjunctiva during the surgery. The tissues were fixed in 10% formalin and embedded in paraffin. Clinical details

pertaining to the study patients are described in Table 1. The study adhered to the tenets of the Declaration of Helsinki. The study protocol was approved by the Institutional Review Boards at the Johns Hopkins School of Medicine and the King Khaled Eye Specialist Hospital, and study participants gave informed consent to the treating surgeon.

Immunohistochemistry: Deparaffinized sections were boiled in 1 \times target retrieval solution (Dako, Carpinteria, CA) for 20 min. Endogenous peroxidase activity was quenched by treating samples with 3% hydrogen peroxide for 5 min. After blocking in 5% normal goat serum diluted in PBS (1X; 155 mM NaCl, 1.06 mM KH₂PO₄, 2.97 mM Na₂HPO₄-7H₂O, pH 7.4), the sections were incubated with anti-RAGE antibody (1:125, Abcam, Cambridge, MA), anti-CD31 antibody (1:100, Abcam), or the isotype- and concentration-matched normal immunoglobulin G (IgG) control (Abcam) overnight at 4 °C. After three washes with PBST (PBS plus 0.1% Tween-20), the sections were then incubated with the secondary biotinylated

goat anti-rabbit or anti-mouse IgG (1:2,000) for 1 h at room temperature. Sections were then detected with the alkaline phosphatase detection system (Vectastain ABC-AP kit, Vector Laboratories, Burlingame, CA), and a blue reaction product was produced by incubating sections with the alkaline phosphatase substrate (Vector Blue AP Substrate Kit III, Vector Laboratories). Sections were also counterstained with Nuclear Fast Red (Vector Laboratories).

Grading of immunohistochemistry: RAGE expression level in vascular endothelial cells of pterygium and normal conjunctiva tissues was scored based on the relative intensity of RAGE immunoreactivity. The intensity was graded by two independent observers in a masked fashion. The grading scores for intensity were as follows: 0, no staining (similar to non-immune IgG-incubated control staining); 1, mild staining; 2, moderate staining; and 3, strong staining.

Statistical analysis: The two-sided Student *t* test was used to analyze the proportion of change in the levels of endothelial RAGE. Differences were considered statistically significant at $p < 0.05$. The statistical program used was SAS 9.2 version (SAS institute, Inc., Cary, NC).

RESULTS

RAGE expression and localization in pterygium and conjunctiva: We first investigated the localization of RAGE expression in pterygium, with specific interest in whether RAGE is expressed in vascular endothelial cells. RAGE expression in pterygium and conjunctival specimens was evaluated with immunohistochemical staining. In pterygium and conjunctiva, RAGE expression was detected in the epithelial layer in all specimens (Figure 1). In addition, RAGE was expressed in several cell types in the stroma, including fibroblasts, inflammatory cells, and vascular endothelial cells, as indicated by corresponding staining with the endothelial cell marker CD31/PECAM-1 (Figure 1). Of note, RAGE expression was consistently found in vascular endothelial cells in pterygium tissue, although to varying degrees (Figure 1; Table 2).

Induction of RAGE expression in vascular endothelial cells in pterygium: In the epithelium, there was no obvious difference in the level of RAGE expression between the conjunctiva and pterygium tissue. In light of the importance of RAGE in the activation of endothelium during inflammation [8], we were particularly interested in determining whether RAGE is induced in endothelial cells in pterygium specimens. For this purpose, we examined endothelial RAGE expression in 25 cases of pterygium and their corresponding normal conjunctiva. RAGE expression was graded by two independent observers (Table 2). As shown in Table 2, RAGE was expressed in vascular endothelial cells in all 25 pterygium

samples. RAGE was expressed in most, but not all, control conjunctiva samples. Most importantly, we found increased RAGE immunoreactivity in pterygium compared with their corresponding control conjunctiva in 19 cases (76%; $p = 0.0093$; Table 2); no difference was observed between pterygium and conjunctiva in the other six cases.

Among 20 primary pterygium cases, in 16 patients (80%), the RAGE levels were increased in the vasculature compared to the corresponding conjunctiva (Table 2; Figure 2). In five recurrent pterygium cases, three cases exhibited increased expression of endothelial RAGE (Table 2; Figure 3). Because of the small number of recurrent pterygium cases, the correlation between RAGE expression and disease stage (primary versus recurrent) could not be determined.

DISCUSSION

Inflammation is an important pathogenic process in pterygium, and multiple proinflammatory molecules have been identified in pterygium tissue. Vascular endothelial cells play an active role in the inflammatory process, interacting with leukocytes to promote inflammation. Interestingly, endothelial cell activation per se has received relatively little attention in pterygium progression. Our group is interested in identifying potential mechanisms of EC activation in pterygium. Given the known role of RAGE in ECs in multiple systemic diseases, we hypothesized that RAGE expression is induced in ECs in pterygium.

We first examined cellular localization of RAGE in pterygium and conjunctiva specimens. We detected RAGE expression in the epithelial layer and specific cell types in the stroma, including vascular endothelial cells, fibroblasts, and inflammatory cells. Although RAGE expression has not been hitherto studied in conjunctiva or pterygium, our finding for the epithelial expression of RAGE is consistent with previous reports of RAGE in other tissue, including epithelial cells in the colon [10], lung [11], and notably in skin [12].

Given the critical role for RAGE in endothelial cell activation during inflammation [8], we specifically scrutinized RAGE expression in the vascular endothelium. In our series, we found that pterygium tissue consistently exhibited expression of RAGE in endothelial cells and that endothelial RAGE was largely, albeit not uniformly, induced in ECs in pterygium compared to the control conjunctiva. This suggests that RAGE could be an important molecule that contributes to the inflammatory process and progression in pterygium.

The potential importance of endothelial RAGE induction in pterygium is highlighted by present knowledge of the role of RAGE in systemic inflammation. Inflammation and

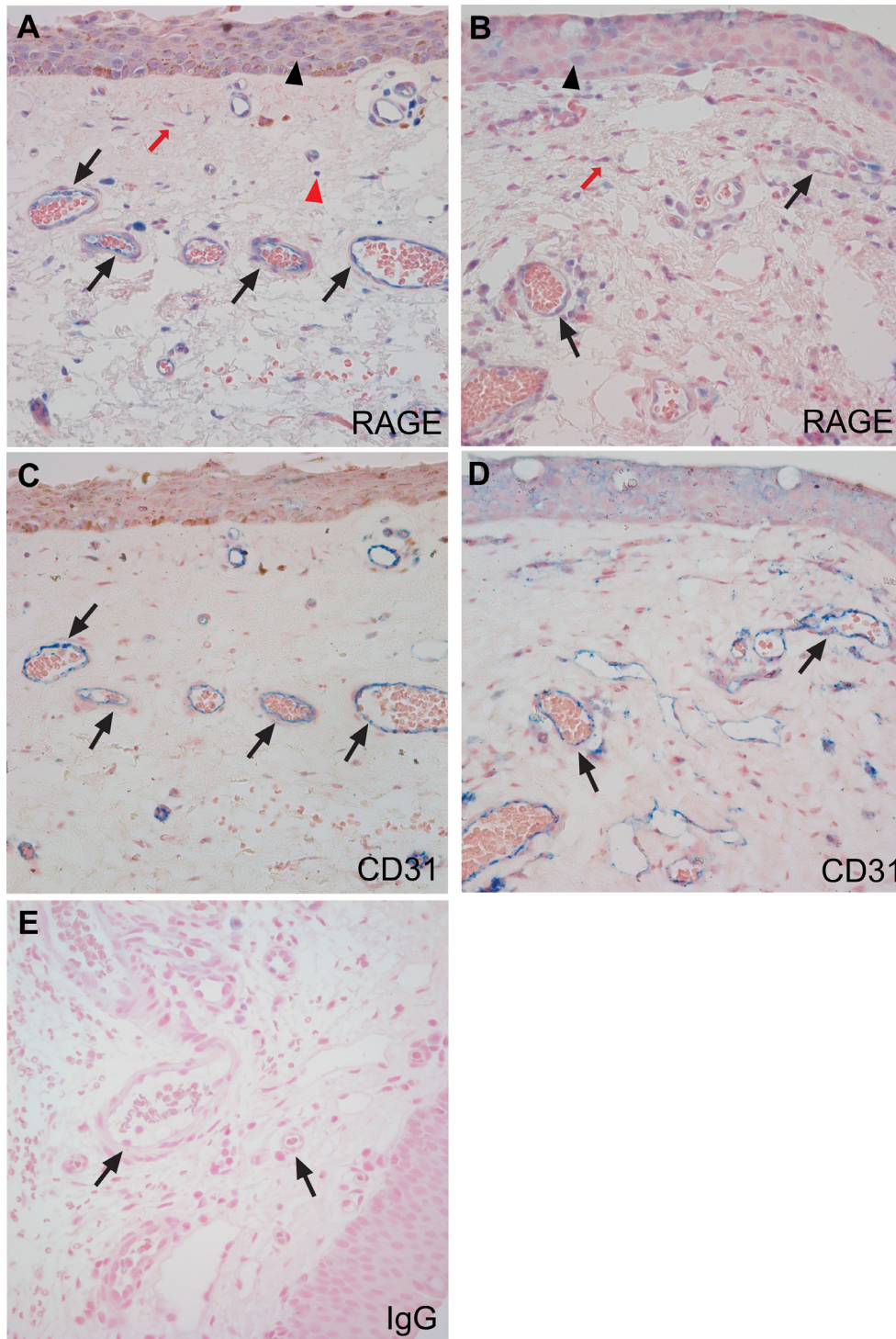


Figure 1. The expression of RAGE in human pterygium and normal conjunctiva tissues. **A, B:** Immunohistochemistry (IHC) staining of RAGE in conjunctiva and pterygium specimens. RAGE is expressed in endothelial cells lining the lumen of blood vessels (black arrows). In addition, other cell types also exhibit RAGE expression, notably epithelial cells (black arrow heads), fibroblasts (red arrows), and inflammatory cells (red arrow head). **C, D:** CD31 staining confirmed the distribution of microvascular endothelial cells in sections adjacent to the conjunctiva and pterygium samples used in **A** and **B**. **E:** Negative IHC staining in pterygium samples with control immunoglobulin G (IgG). The images were taken at 40X objective.

vascular proliferation are major factors in pterygium [1-3]. From this perspective, it is clear that vascular endothelial cells are a significant cell type in pterygium pathogenesis. ECs play an active role in recruiting leukocytes to local tissue sites during inflammation, and activated ECs release cytokines

and chemokines involved in the inflammatory process [4,8]. In this light, endothelial RAGE activation could represent an important mechanism for pterygium progression. RAGE upregulation and activation are an important mechanism by which endothelial cells amplify the overall inflammatory

TABLE 2. THE GRADING SCORES OF RAGE IN VASCULAR ENDOTHELIAL CELLS IN HUMAN PTERYGIUM AND CONJUNCTIVA TISSUES.

Case number	Observer 1		Observer 2		Change*
	pterygium	conjunctiva	pterygium	conjunctiva	
1	2	1	1	0	↑
2	3	2	2	1	↑
3	1	0	1	0	↑
4	1	0	1	0	↑
5	3	2	3	1	↑
6	2	0	2	0	↑
7	2	1	2	1	↑
8	2	2	2	2	–
9	1	1	1	1	–
10	2	1	1	0	↑
11	2	1	2	1	↑
12	3	2	3	1	↑
13	2	2	2	2	–
14	3	1	3	0	↑
15	3	1	3	1	↑
16	3	3	3	3	–
17	3	3	3	3	–
18	3	3	2	2	–
19	3	2	3	2	↑
20	2	1	2	1	↑
21	2	1	2	1	↑
22	3	1	2	1	↑
23	3	2	3	2	↑
24	3	0	3	0	↑
25	3	1	2	0	↑

0 indicates negative staining; 1, mild staining; 2, moderate staining; 3, strong staining of RAGE in vascular endothelial cells in pterygium and normal conjunctiva tissue samples. *, ↑, means more RAGE expressed in pterygium specimen compared to respective conjunctiva tissue; –, means no change between pterygium and conjunctiva.

response [9]. RAGE is induced in vascular ECs during injury and inflammation, and migration of leukocytes across the endothelium is mediated by RAGE. The ligand-RAGE interaction triggers the activation of NF- κ B in endothelial cells, with subsequent cellular release of cytokines including TNF- α [7]. Interestingly, ligand-RAGE interaction generates reactive oxygen species [13], providing further relevance for RAGE given the role of oxidative stress in pterygium [2]. Finally, it is of interest that RAGE has been implicated in inflammation-associated angiogenesis, for instance, in a mouse model of laser-induced choroidal neovascularization, in which RAGE deficiency resulted in a significant impairment in pathologic angiogenesis [14].

To further elucidate the biology of RAGE in pterygium, it will be of interest to identify possible ligands involved in RAGE signaling in activated endothelium. Since RAGE binds multiple different ligands, it is considered a pattern recognition receptor (PRR), recognizing a structural motif [8]. RAGE has multiple ligands, including AGEs, members of the S100/calgranulin family, HMGB1, and Mac1 [15]. Multiple members of the S100 family are upregulated in pterygium tissue [16,17] and in the tear fluid of patients with pterygium [18]. To our knowledge, other potential ligands for RAGE have not yet been reported in pterygium tissue.

The current study relied on immunohistochemistry and a semiquantitative scoring system to evaluate the expression of endothelial RAGE in pterygium and conjunctiva.

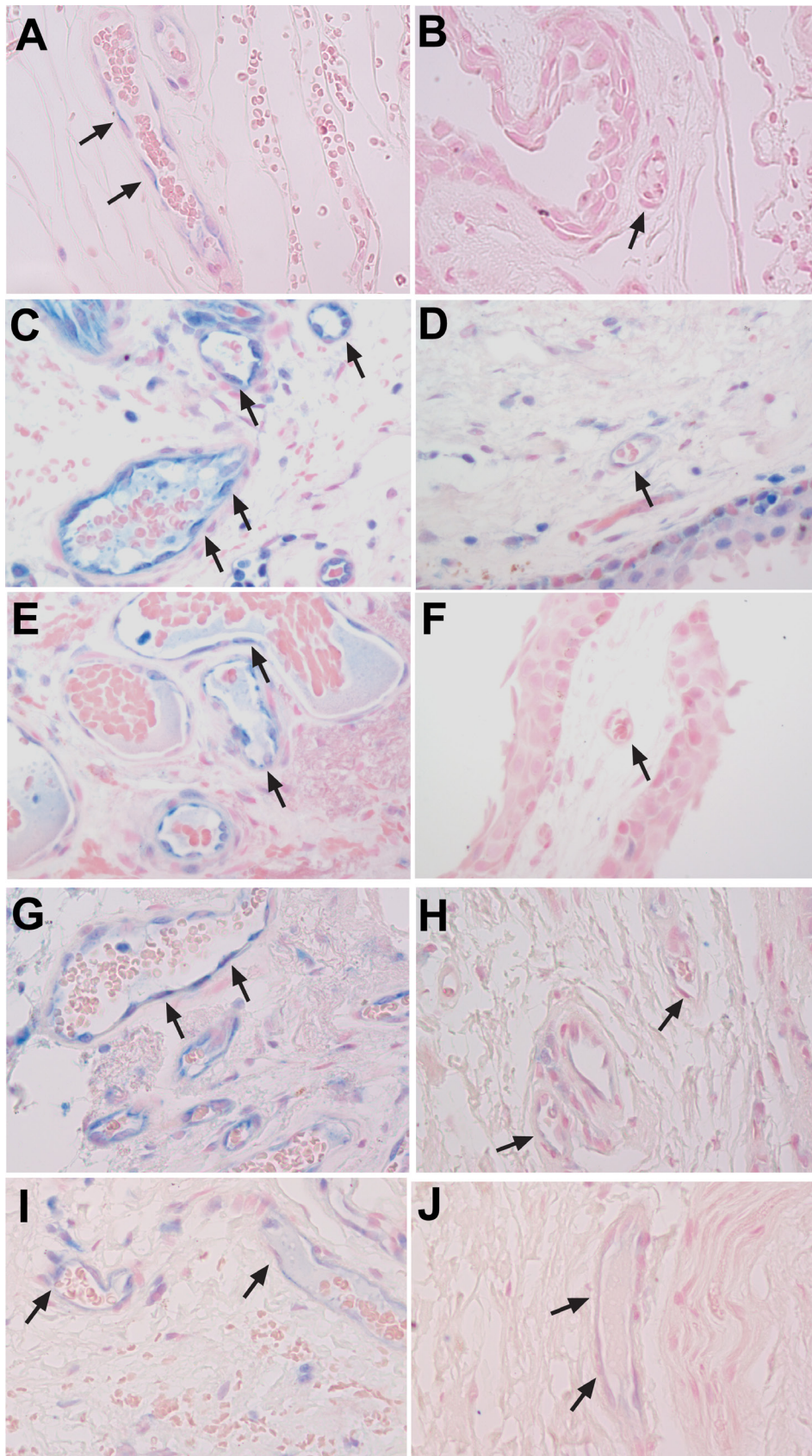


Figure 2. Increased RAGE expression in vascular endothelial cells in human primary pterygium compared to corresponding conjunctiva samples. RAGE staining in pterygium (A) and conjunctiva (B) samples from case 4. RAGE staining in pterygium (C) and conjunctiva (D) samples from case 5. RAGE staining in pterygium (E) and conjunctiva (F) samples from case 6. RAGE staining in pterygium (G) and conjunctiva (H) samples from case 24. RAGE staining in pterygium (I) and conjunctiva (J) samples from case 25. Black arrows indicate vascular endothelial cells. The images were taken at 40X objective.

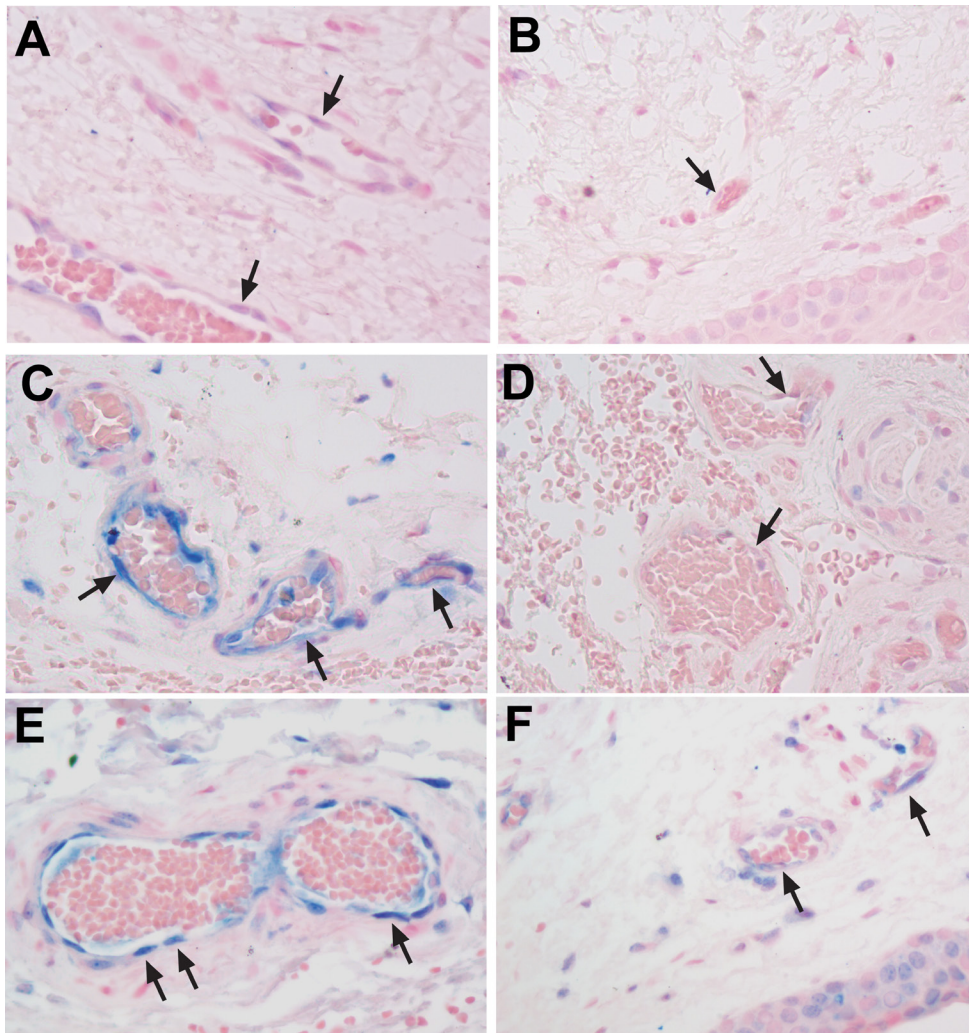


Figure 3. Increased RAGE expression in vascular endothelial cells in human recurrent pterygium compared to corresponding conjunctiva samples. RAGE staining in pterygium (A) and conjunctiva (B) samples from case 3. RAGE staining in pterygium (C) and conjunctiva (D) samples from case 14. RAGE staining in pterygium (E) and conjunctiva (F) samples from case 15. Black arrows indicate vascular endothelial cells. The images were taken at 40X objective.

This approach has been used by multiple investigators for studies in pterygium [19-22] as well as other ocular conditions including age-related macular degeneration [23] and diabetic retinopathy [24]. This semiquantitative approach has limitations, including reduced precision of results and a potential susceptibility to grader bias, compared to quantitative approaches such as western blotting and quantitative RT-PCR (qRT-PCR). Since the current study is focused on the expression of RAGE specifically in endothelial cells, western blotting was not a viable approach. In theory, qRT-PCR could be considered, but this would prohibitively require laser-capture microdissection of very large numbers of individual blood vessels in cryopreserved sections for each specimen. For the semiquantitative immunohistochemistry (IHC) approach, which was used in the current study, two helpful strategies have been used by many investigators, including two or more independent evaluators as well as a

masked grading approach [20,22], and we incorporated these approaches in the current study.

The presence and induction of endothelial RAGE in primary and recurrent pterygium indicate RAGE could potentially contribute to promoting pterygium progression and suggests that RAGE might represent a therapeutic target. Suppression of RAGE has been shown to prevent progression of some systemic disease processes in experimental models, and RAGE inhibition is actively being investigated for treating various systemic disease processes, with strategies including soluble RAGE species as well as small molecule inhibitors of RAGE [8]. The potential amenability of pterygium to local treatment approaches including topical delivery suggest RAGE targeting as an attractive pharmacologic strategy for this disease condition.

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