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Endothelin-1 and endothelin-converting enzyme-1 in human granulomatous pathology of eyelid: an immunohistochemical and *in situ* hybridization study in chalazia

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Summary. Endothelin-1 (ET-1), a potent vasoconstrictor peptide, is involved in several functions of eye pathophysiology, such as regulation of intraocular tension and retinal reactive vasoconstriction. As ET-1 pro-inflammatory and fibrosing activity is emerging in different fields of pathology, we investigated the expression of ET-1 and endothelin-converting enzyme-1 (ECE-1) in chalazia, granulomatous lesions of the eyelid.

ET-1 and ECE-1 were analyzed by immunohistochemistry (IHC) in twenty surgically removed chalazia, with regard to expression in eyelid structures and inflammatory infiltrate. Phenotype of ET-1 expressing inflammatory cells was established by double immunofluorescence. The cellular localization of prepro-ET-1 (pp-ET-1) mRNA and ECE-1 mRNA was studied by nonisotopic in situ hybridization (ISH).

Neutrophils (PMNs), macrophages and T-lymphocytes were scattered in stroma, around alveoli and grouped in lipogranulomas. PMNs, macrophages, basal epithelium of meibomian adenomers and central ducts immunostained for ET-1. ECE-1 protein was found in meibomian adenomers, conjunctival epithelium, tarsal mucous glands and in inflammatory cells. Hybridization signals for pp-ET-1 mRNA and ECE-1 mRNA were recognized in healthy and degenerating meibomian ducts, adenomers, inflammatory cells, as well as in vessel walls. ECE-1 mRNA was also present in conjunctival epithelium and Henle's crypts.

Our findings suggest that the multifunctional peptide ET-1 may have a role in molecular genesis of tissue damage in chalazia. ET-1 cytokine activity is likely to support the migration of inflammatory cells and the setting of lipogranulomas; ET-1 stimulation might contribute to proliferation of fibroblasts and collagen synthesis. ET-1 upregulation on meibomian adenomers and ducts may further enhance granulomas formation by stimulating lipid release.

Key words: Endothelin-1, Endothelin-converting enzyme-1, Granuloma, Meibomian gland

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Introduction

Chalazion is a chronic granulomatous inflammatory lesion, involving the upper or lower eyelid. It originates from meibomian glands, producers of meibum, a lipidic component of the surface layer of the tear film. It may evolve from an internal hordeolum or may directly follow ducts engorgement and rupture from deposition of meibum and release of lipids on the neighboring tarsal plate (Lederman and Miller, 1999). An inflammatory process takes place as a foreign body reaction to the discharged lipids. Infiltration by PMNs, macrophages fusing into multinucleate giant cells (MGCs), lymphocytes and plasma cells then occurs with cell aggregation into lipogranulomas. Chalazia are often associated with other meibomian gland dysfunctions, such as chronic blepharitis, or systemic pathology of sebaceous glands, e.g. seborrhoea and acne rosacea (Nazir et al., 2004). Current treatment includes intralesional corticosteroid injection and surgical excision.

ET-1 is a potent vasoconstrictor (Yanagisawa et al., 1988) and a widely expressed growth-promoting mediator (Malendowicz et al., 1998). Besides physiological maintenance of vascular tone, the peptide

is also involved in the pathogenesis of vascular, inflammatory and fibrotic diseases (Clozel and Salloukh, 2005). ET-1 is chemotactic for monocytes and PMNs (Elferink and de Koster, 1994) and activates macrophages (Haller et al., 1991). The peptide also enhances expression of adhesion molecules (Ishizuka et al., 1999) as well as release of cytokines (Matsushima et al., 2004), chemokines (Mangahas et al., 2005) and prostaglandins (Schramek et al., 1995). Networks of reciprocal induction feedback, relating the endothelin system with other cytokines, such as interferon γ (IFN-γ) (Lamas et al., 1992) and tumor necrosis factor α (TNF-α) (Ruetten and Thiemermann, 1997; Lees et al., 2000), and with inflammatory effectors metalloproteases (Fernandez-Patron et al., 2001) are described in different biological systems.

ET-1 distribution in the human eye has been widely investigated in normal conditions and in pathology. ET-1 is detected in optic nerve neurons and astrocytes, in vascular, neural and epithelial retinal cells (Ripodas et al., 2001; Narayan et al., 2003, 2004), as well as in the iris, non-pigmented epithelial ciliary cells and ciliary muscle (Fernandez-Durango et al., 2003). A role of ET-1 in retinal hyperoxia-induced vasoconstriction (Dallinger et al., 2000) and in glaucoma pathophysiology (Nicolela et al., 2003; Prasanna et al., 2005) is experimentally recognized. Upregulation of ET-1 is detected in revascularized corneal epithelium with possible angiogenic activity (Kuhlmann et al., 2005).

Besides vascular endothelium, many distinct cell types produce ET-1. Active synthesis by macrophages (Ehrenreich et al., 1990), dendritic cells (Guruli et al., 2004), mast-cells (Ehrenreich et al., 1992) and PMNs (Cambiaggi et al., 2001; Massai et al., 2003), suggests involvement of the peptide in inflammatory processes.

ECE-1, a membrane-bound zinc metallopeptidase composed of two disulphide-linked subunits (Sawamura et al., 1993), is the key enzyme (Turner and Tanzawa, 1997) in the biosynthesis of ET-1. It converts the inactive intermediate big ET into the mature peptide. ECE-1 protein and mRNA have been detected in endothelial, epithelial and glandular cells of various organs (Korth et al., 1999). In the eye, immunoreactivity for ECE-1 is found in blood vessels of the retina, optic nerve, choroids (Wollensak et al., 2002) and in non-pigmented ciliary epithelial cells (Prasanna et al., 1999). Antagonists of endothelin system are currently utilized in treatment of several pathological conditions (Finsnes et al., 2001; Howard and Morrell, 2005; Kelland and Webb, 2006). Therefore, we examined the localization of ET-1/ECE-1 protein and mRNA in chalazia, in order to evaluate the role of the endothelin system in this granulomatous disorder and individuate possible therapeutic targets.

Materials and methods

Tissue specimens

Twenty surgically excised chalazia were provided by the Department of Ophthalmology and Neurosurgery (S. Maria alle Scotte, University Hospital, Siena, Italy). All research procedures involving humans were carried out according to the Helsinki Declaration for the use of human tissue in research. Written informed consent was obtained from all patients or their parents, in the case of minors. The patients age range was 14-66 years (6 women, 14 men). All patients underwent surgery after failure of conservative treatment by intralesional steroid injection (one injection in 16 cases, two with a three weeks interval in 4 cases). At the time of resection, the patients had been affected with chalazion for a period variable from six months to one year. None of the subjects was affected with glaucoma, retinal disorders or systemic pathology such as hypertension or atherosclerosis.

Chalazia were fixed in Bouin's solution and embedded in paraffin wax. Specimens of normal human eyelids, excised in corrective surgery for involutional senile ectropion, were analogously processed for examination of normal meibomian glands. Serial sections, 6-7 μm thick, were mounted on SuperFrost Plus microscope slides (Fisher Scientific; Pittsburgh, PA). For ISH the slides were coated with tissue-adhesive solution (Vectabond, Vector Laboratories, Burlingame, CA). For histological examination, deparaffinized sections of chalazia were stained with haematoxylin and eosin.

Immunohistochemistry

For all IHC procedures, sections were washed with 0.1 M phosphate buffer saline (PBS) (pH 7.4). For the immuno-peroxidase procedure, sections were incubated with 6% H_2O_2 in methanol for 20 min, in order to inactivate endogenous peroxidases. Proteolytic digestion was carried out by treatment with 0.1% trypsin (Sigma-Aldrich, St. Louis, MO) in PBS at 37°C for 10 min and, to prevent nonspecific binding of antibodies, slides were incubated in PBS containing 5% bovine serum albumin (BSA) (Sigma-Aldrich) for 30 min at room temperature (rt). Finally, they were incubated overnight at 4°C with specific primary antibodies and antisera as indicated in Table 1. Healthy human eyelids sections were also submitted to IHC for ET-1 and ECE-1.

The EnVision peroxidase (PO)/alkaline phosphatase (APase) detection system (EnVision,+ Labelled polymer, DAKO, Carpinteria, CA) was used according to the manufacturer's instructions and immunoreactivity was visualized with 3-amino-9-ethylcarbazole (AEC) or 3-3' diaminobenzidine (DAB)/Fast Red or Fuchsin (DAKO) as substrate. In negative control samples, the primary antibody was replaced by PBS-5% BSA.

We first carried out an immunophenotype analysis of infiltrates, in order to characterize the prevalence and distribution of inflammatory cells in chalazia, by use of T cells, PMN and macrophage markers. Subsequently we examined the distribution of ET-1 peptide and ECE-1 protein in structural components of eyelid tissue. Qualitative and semiquantitative analysis of

immunoreactivity was carried out by observation of slides under 400x and 1000x magnifications, allowing to discriminate cytoplasmic or membrane localization as well as proximity to nucleus. Intensity of stain was qualitatively evaluated by two observers. In order to confirm subjective evaluation and for comparison among different specimens, the acquisition of images was performed under constant parameters. Density of pixels was measured by Photoshop Software.

Images were acquired on a Carl Zeiss Axioplan 2 imaging microscope using an AxioCam HR CCD camera and AxioVision 3.1 software (Carl Zeiss, Göttingen, Germany).

Double immunofluorescence

In order to characterize the phenotype of inflammatory ET-1-positive cells, double immunofluorescence was performed. ET-1 rabbit antiserum and mouse monoclonal anti-CD68 or anti-lactoferrin antibodies, at dilutions listed in Table 1, were sequentially incubated 1 h at rt. After rinses in PBS, fluorescein isothiocyanate conjugated (FITC)-anti-rabbit IgG (Sigma-Aldrich) (1:80 in PBS-1% BSA) and tetramethyl rhodamine isothiocyanate conjugated (TRITC)-anti-mouse IgG (Sigma-Aldrich) (1:100 in PBS-1%BSA) were incubated 1 h at rt. Slides were mounted with anti fading medium glycerol-PBS with 1,4, Diazabicyclo[2.2.2]octane (DABCO, Sigma-Aldrich), and examined with the Zeiss Axioplan2 light microscope equipped with epifluorescence. Acquisition of images and merge of signals were obtained by AxioVision 3.1 software.

Probe preparation

The ET-1 and ECE-1 probes were prepared according to Klein et al. (1995) with minor modifications (Cambiaggi et al., 2001). Total RNA was extracted from cultured human endothelial cell line ECV304 (Takahashi et al., 1990), using "RNAeasy mini kit" (Quiagen, Hilden, Germany). Its integrity was checked on 1.6% weight/volume (wt/vol) agarose gel and the concentration was determined spectrophotometrically. To obtain cDNA, 5 μg of extracted RNA were used as a template in the reverse transcriptase (RT) reaction, at 37°C for 30 min, with the ET-1 antisense specific primer 5'-GCT CTC TGG AGG GCT TGC-3' or the ECE-1 antisense specific primer 5'-TTG GAA TCG GCA CTG ACA TAG A-3' (MWG Biotech/M-Medical, Milano, Italy). ET-1 and ECE-1 cDNA was then amplified by polymerase chain reaction (PCR), using ET-1 sense primer 5'-CAG TTT GAA CGG GAG GTT TTT-3,' ECE-1 sense primer 5'-GTA CTT CTG GCG GCA GGA CT-3'and the antisense primers used for the RT reaction. Amplifications were performed in a thermal cycler (PCR Sprint, Hybaid, UK) under the following conditions: 94°C for 5 min, then 30 cycles at 93°C for 30 sec, 56°C for ET-1 and 58.7°C for ECE-1, both for 45 sec, 72°C for 1 min, and finally 72°C for 5 min. The 645- base pairs (bp) ET-1 and 518-bp ECE-1 amplicons were then labelled with digoxigenin (Dig) in a repeated PCR with the same ET-1 and ECE-1 primers under the same conditions as before, apart from the addition of 70 mM Dig-labelled dUTP (Dig-11-dUTP, Boehringer Mannheim, Mannheim, Germany) (Fig. 1).

In situ hybridization

ISH was performed as previously described (Klein et al., 1995). Briefly, sections were dewaxed, rehydrated, fixed in 4% paraformaldehyde for 20 min and treated with 3 μg/ml proteinase K (Boehringer) in Tris-ethylenediaminetetracetic acid (TE) for 20 min at 37°C. The sections were then acetylated in 0.25% acetic anhydride in triethanolamine, permeabilized with PBS-Triton x100, and dehydrated in an ethanol series.

The hybridization mixture contained: 30 μl 100% deionized formamide (Boehringer), 20 μl x20 saline sodium citrate, 20 μl TE, 10 ml denatured salmon sperm DNA (10 mg/ml, Boehringer), 1 ml tRNA (100 mg/ml; Boehringer), 25 μl Dig-labelled PCR product for pp-ET-1 mRNA or 30 μl Dig-labelled PCR product for ECE-1 mRNA and 14 ml H_2O. The hybridization solutions were incubated at 100°C for 5 min, cooled on ice for 10 min and incubated at 42°C overnight. Subsequently the sections were incubated with mouse anti-digoxigenin antibodies (2 μg/ml; Boehringer) for immunoperoxidase or Fab fragments from sheep anti-Dig alkaline phosphatase conjugated antibodies (7.5 U/ml; Boehringer) in PBS-3% BSA for 1 h. After washing in PBS/Tween 20, the sections for the immunoperoxidase were incubated in rabbit anti-mouse antibodies coupled to peroxidase (13 μg/ml; DAKO) in PBS-3% BSA for 30 min. To enhance staining, sections were washed and incubated with swine antibodies against rabbit immunoglobulins coupled to peroxidase (26 μg/ml; DAKO). The immunoreactivity was visualized by DAB for immunoperoxidase and by Fast Red solution with levamisole (DAKO) for immunoalkaline-phosphatase. Four negative control procedures were performed to assess the specificity of the ISH signal: 1) omitting Dig-

Table 1. Specificities, dilution, and sources of primary antibodies used in this study.

Antibody/ Antiserum	Host	Dilution	Source	Cell expression
ET-1	Rabbit	1 :700	Peninsula*	
ECE-1	Rabbit	1:700	Dr.Yanagisawa†	
CD68	Mouse	1:25	DAKO‡	Macrophages
CD3	Rabbit	prediluted	DAKO‡	T-lymphocytes
Lactoferrin	Mouse	1:110	OXIS§	Neutrophils

*Peninsula Laboratories, Belmont, CA; †: Kind gift of Dr. M. Yanagisawa (Emoto and Yanagisawa, 1995) (Institute of Molecular Embryology and Genetics, Kumamoto University, Honjo, Kumamoto, Japan); ‡: Dako, Carpinteria, CA; §: Oxis International Inc., Portland, OR.

labelled probes; 2) omitting anti-Dig antibody; 3) omitting Dig-labelled probe and anti-Dig antibody; 4) incubating, before ISH, the sections in Ribonuclease (RNase) A (Boehringer) 300 μg/ml and 300 IU RNase T1 (Boehringer) in Tris-HCl at 37°C overnight.

Results

Histopathological findings showed the coexistence of various stages of inflammatory and degenerative changes. In areas affected in a more advanced stage, large lipogranulomas enclosed lipid vacuoles and gland remnants. Inflammatory cells were predominantly macrophages, with recurrent multinucleate giant cells, lymphocytes and neutrophils. Degeneration of glands occurred, with disruption of epithelial acinar layers and deposition of abundant secreted material. Inflammation also spread to the surrounding tissue, so that scattered foci of inflammatory cells invaded the stroma, surrounded and partially infiltrated both normal meibomian adenomers and ducts or glands showing cystic dilatation. Abundant collagen deposition was observed among gland acini (Figs. 2A,B).

Immunophenotype analysis of infiltrates showed a large number of CD68+, CD3+ and lactoferrin-positive cells around and within the basal epithelial layer, both in normal and degenerating glands (Figs. 2C,D), in stroma, within lipogranulomas, and in areas bordering meibum accumulations (Figs. 2E,F).

Immunohistochemistry led to detection of ET-1 peptide in the outer periphery of meibomian adenomers, i.e. in metabolically active epithelium, and in central ducts, both in normal glands (Fig. 3A) and in degenerating ones (Fig. 3B). Pericellular diffusion of stain, indicative of mature peptide secretion, occurred in lipogranulomas and glands (Fig. 3C,D). Mature epithelial cells, in secretory discharge phase, did not immunostain for ET-1. No ET-1 stain was observed in the endothelium of blood and lymphatic vessels in specimens fixed in Bouin's solution.

Table 2. Localization and relative frequencies of ET-1 immunoreactive cells and ET-1 mRNA- expressing cells in chalazia.

	ET-1+ cells	pp-ET-1mRNA+ cells
Morphologically unaffected MB*		
- Adenomers:		
basal cells	+++	+++
central sebum laden cells	-	-
- Ducts	+++	+++
Degenerating MB*		
- Adenomers:		
basal cells	+++	+++
central sebum laden cells	-	-
- Ducts	+++	+++
Blood and lymphatic vessels endothelium	-§	+++++
Lipogranulomas	+++++	+++++
Stromal scattered cells		
- Macrophages	+++++	++++
- Neutrophils	+++++	++++

MB*: meibomian gland. §: Lack of immunoreactivity due to processing method (see discussion). Values are expressed as ranges of percentage of stained cells in a structure: -: no staining; +: 1-9%, ++: 10-19%, +++: 20-49%, ++++: 50-79%, +++++: >80%.

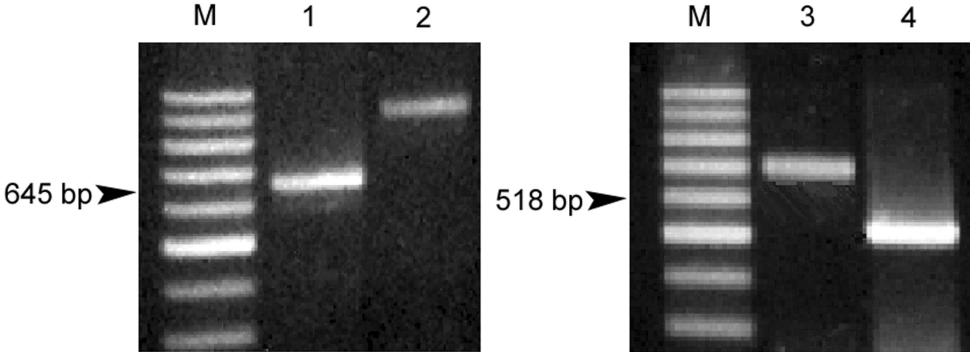

Fig. 1. DNA probes for pp-ET-1 and ECE-1. Repeated PCR with 645-bp pp-ET-1 and 518-bp ECE-1. Lane M, the marker (0.39 μg) 100 bp DNA Ladder (Promega); lane 1, 645-bp pp-ET-1 product; lane 2, 645-bp pp-ET-1 product in presence of digoxigenin (Dig)-11-dUTP (as Dig-labelled UTP counts for 2 nucleotides, therefore the molecular weight of the Dig-labelled PCR fragments is higher); lane 3, 518-bp ECE-1 PCR product in presence of digoxigenin (Dig)-11-dUTP; lane 4, 518-bp ECE-1 PCR product.

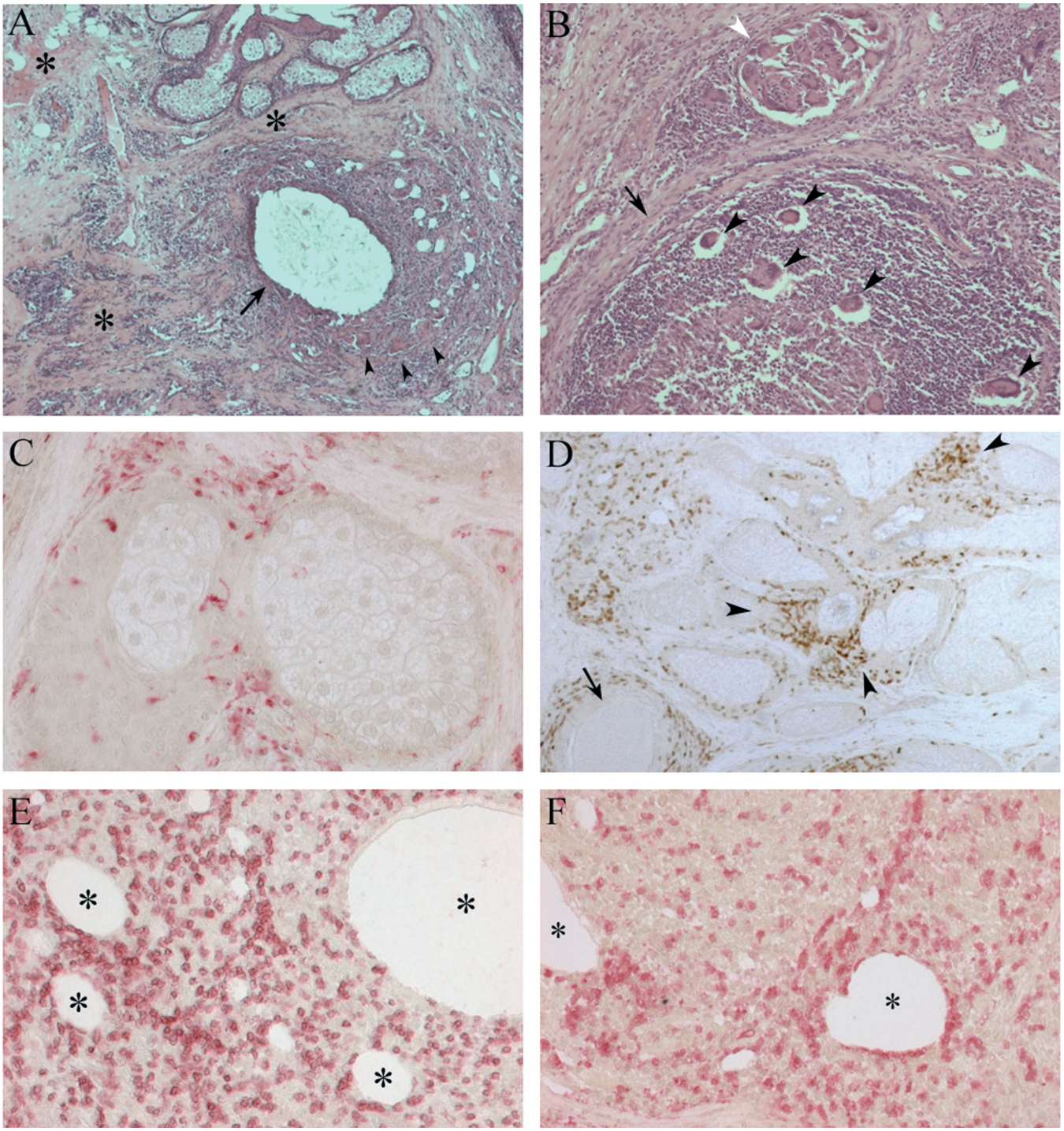

Fig. 2. A, B. Histopathological analysis of hematoxylin-eosin-stained chalazion sections. **A.** Degenerated adenomer (arrow) with mononuclear cells and MGCs (arrowheads) enclosing small lipid vacuoles. Adjacent normal adenomers and ducts, partially surrounded by inflammatory cells. Collagen proliferation (asterisks) around glands and infiltrates. x 50. **B.** Disrupted adenomer (white arrowhead) invaded by MGCs and a large lipogranuloma (arrow) with mononuclear cells and MGCs (arrowheads). x 100. **C-F.** Immunohistochemical phenotypic characterization of inflammatory cells in chalazia. **C.** Localization of CD68+ macrophages around and within meibomian adenomers of normal morphology (substrate: Fast Red). x 200. **D.** Large deposits of CD3+ T cells at the periphery of morphologically normal meibomian glands (arrowheads) and degenerating adenomer (arrow), with focal invasion of basal epithelial layers (s.: DAB). x 50. **E.** High density of CD3+ T cells within lipogranulomas enclosing sebum droplets (asterisks) (s.: Fast Red). x 200. **F.** Many lactoferrin+ cells within lipogranuloma, with higher density around deposits of sebum (asterisks) (s.: Fast Red). x 200

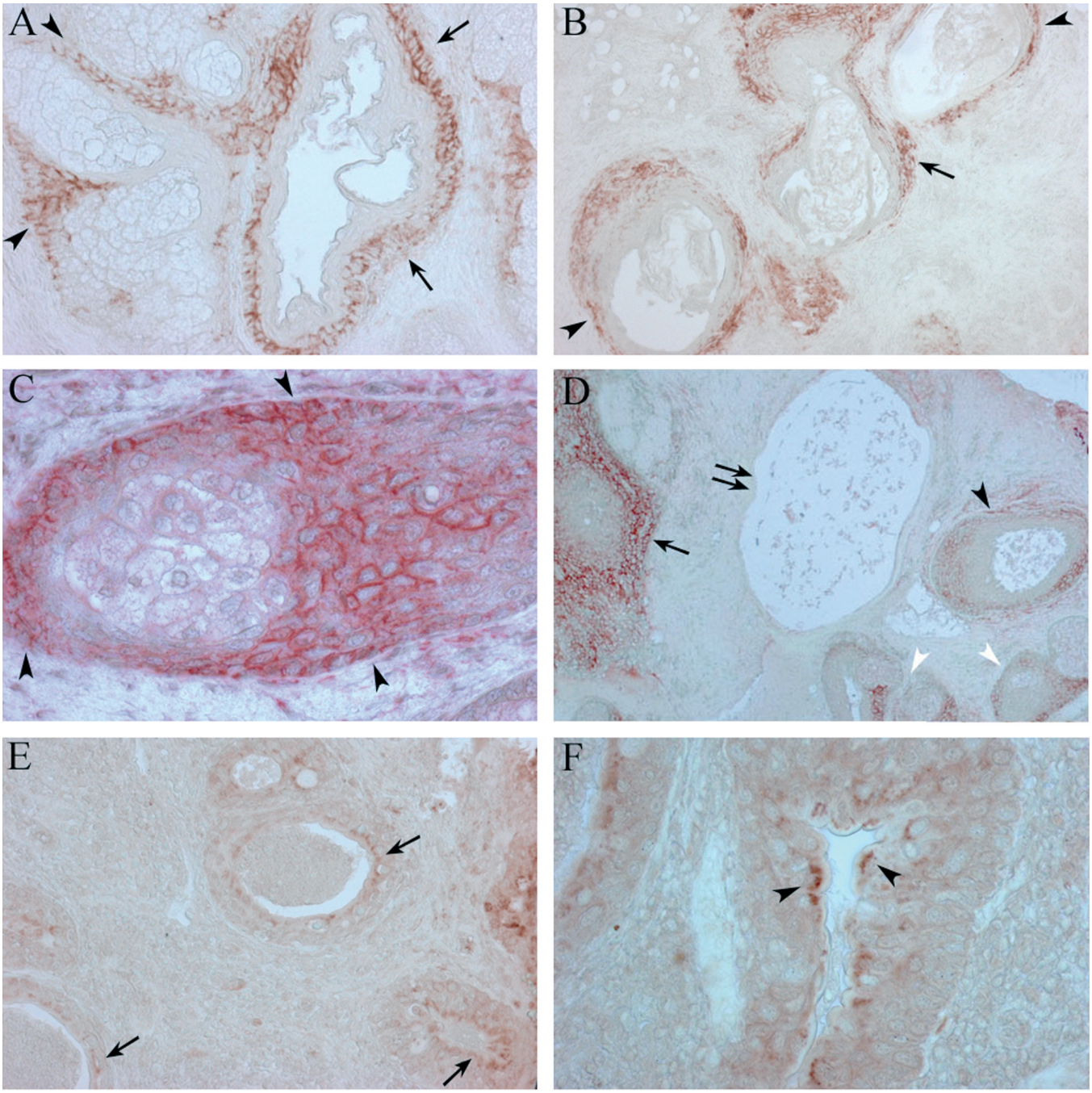

Fig. 3. Immunohistochemical localization of ET-1 and ECE-1 in chalazia. **A.** High occurrence of ET-1+ cells in the outer periphery of meibomian adenomers (arrowheads) and central duct (arrows) not showing degenerative changes (s.: AEC). x 200. **B.** Degenerating meibomian gland immunostained for ET-1 in external districts of alveoli (arrowheads) and origin of duct (arrow) (s.: AEC). x 200. **C.** Metabolically active epithelial ET-1+ cells (arrowheads) in a meibomian adenomer of normal morphology. Pericellular stain suggest extracellular secretion (s.: Fast Red). x 400. **D.** ET-1 reactivity in basal epithelial cells of meibomian glands in different stages of degeneration (double arrows: fully disrupted adenomer; black arrowhead: degenerating adenomer; white arrowheads: morphologically normal adenomers). The adjacent lipogranuloma is also strongly reactive (arrow) (s.: Fast Red). x 100. **E.** ECE-1 immunoreactivity in basal cells of degenerating adenomers (arrows) with signs of secrete engorgement. (s.: AEC). x 200. **F.** Longitudinal section of a crypt of Henle immunostained for ECE-1. Cytoplasmic localization with juxtanuclear intense stain is observed (arrowheads) (s.: AEC). x 400

Immunoreactivity for ECE-1 protein was detected on eyelid glands affected by chalazion. A diffuse peripheral positivity was found in the secretory region of normal and degenerating meibomian glands (Fig. 3E). The crypts of Henle, mucous glands for eyelid lubrication, and their ducts, opening into the free surface of conjunctiva, were also immunostained for ECE-1. Stain was especially evident in juxtanuclear cytoplasmic regions of glandular cells (Fig. 3F). Within lipogranulomas and in scattered foci of inflammatory cells, ECE-1 immunoreactive elements were also detected.

Phenotype characterization of ET-1+ inflammatory cells, by double staining with macrophage and neutrophil markers, revealed that macrophages (Fig. 4A-C), giant cells (Fig. 4D-F) and PMNs (Fig. 4G-I) consistently expressed ET-1.

The morphologically normal meibomian glands

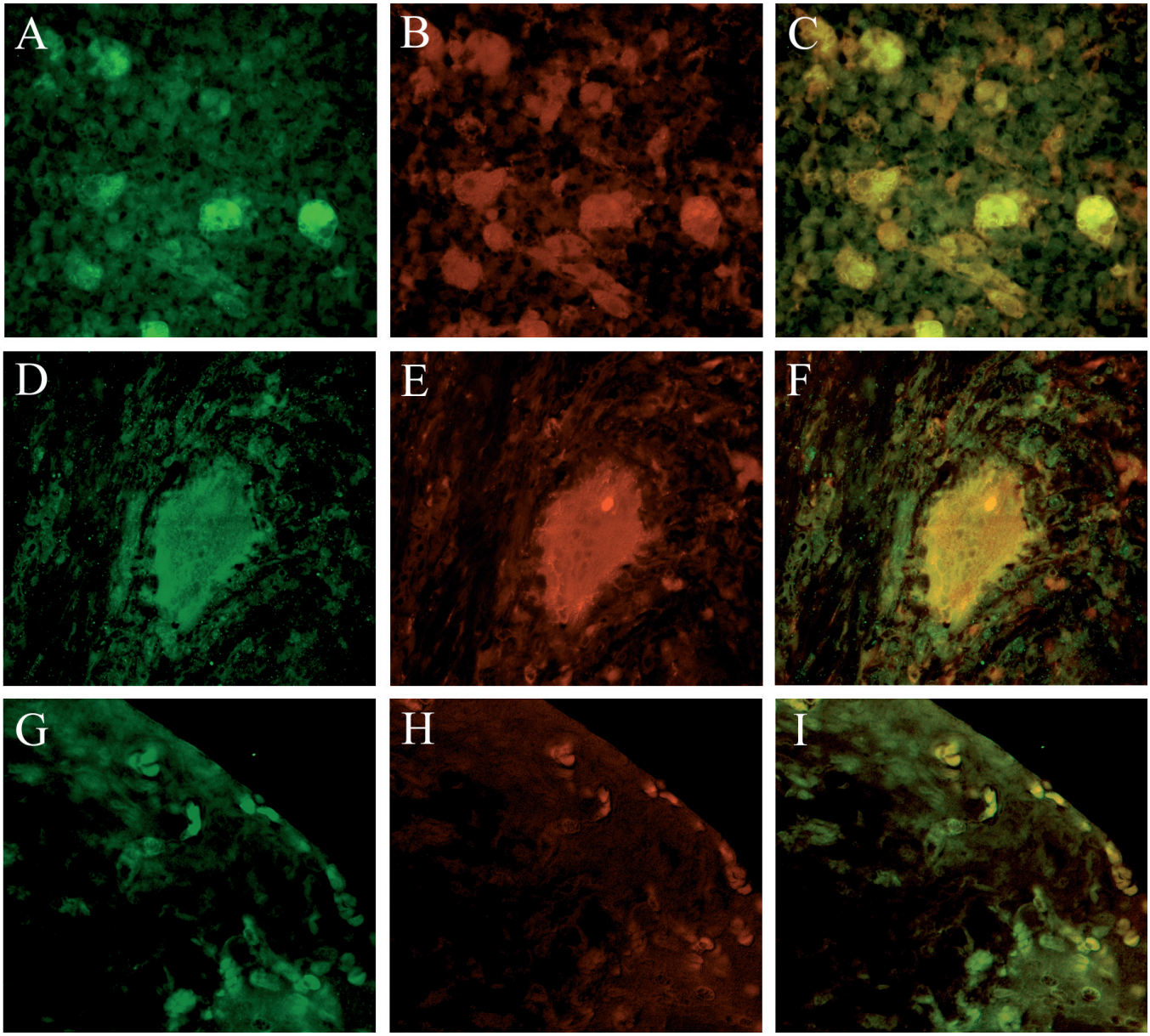

Fig. 4. Double immunofluorescence staining. **A-C.** Sequential detection of ET-1 (**A**, green) and CD68 (**B**, red) immunoreactivity showed substantial co-localization (**C**, merge) in macrophages within a lipogranuloma. x 400. **D-F.** Sequential detection showing ET-1 expression on a giant cell (**D**, green), the CD68 marker (**E**, red), and co-localization (**F**, merge). x 400. **G-I.** Sequential detection of ET-1 (**G**, green), the lactoferrin marker (**H**, red), co-localization (**I**, merge) in neutrophils infiltrating the conjunctival and tarsal connective tissue. x 400

from ectropion, where no inflammation was present, expressed a fainter immunoreactivity for ET-1 and ECE-1 (data not shown).

In contrast to the lack of ET-1 immunostain, pp-ET-1 mRNA was expressed in vascular endothelial cells. Basal epithelium of the healthy meibomian glands,

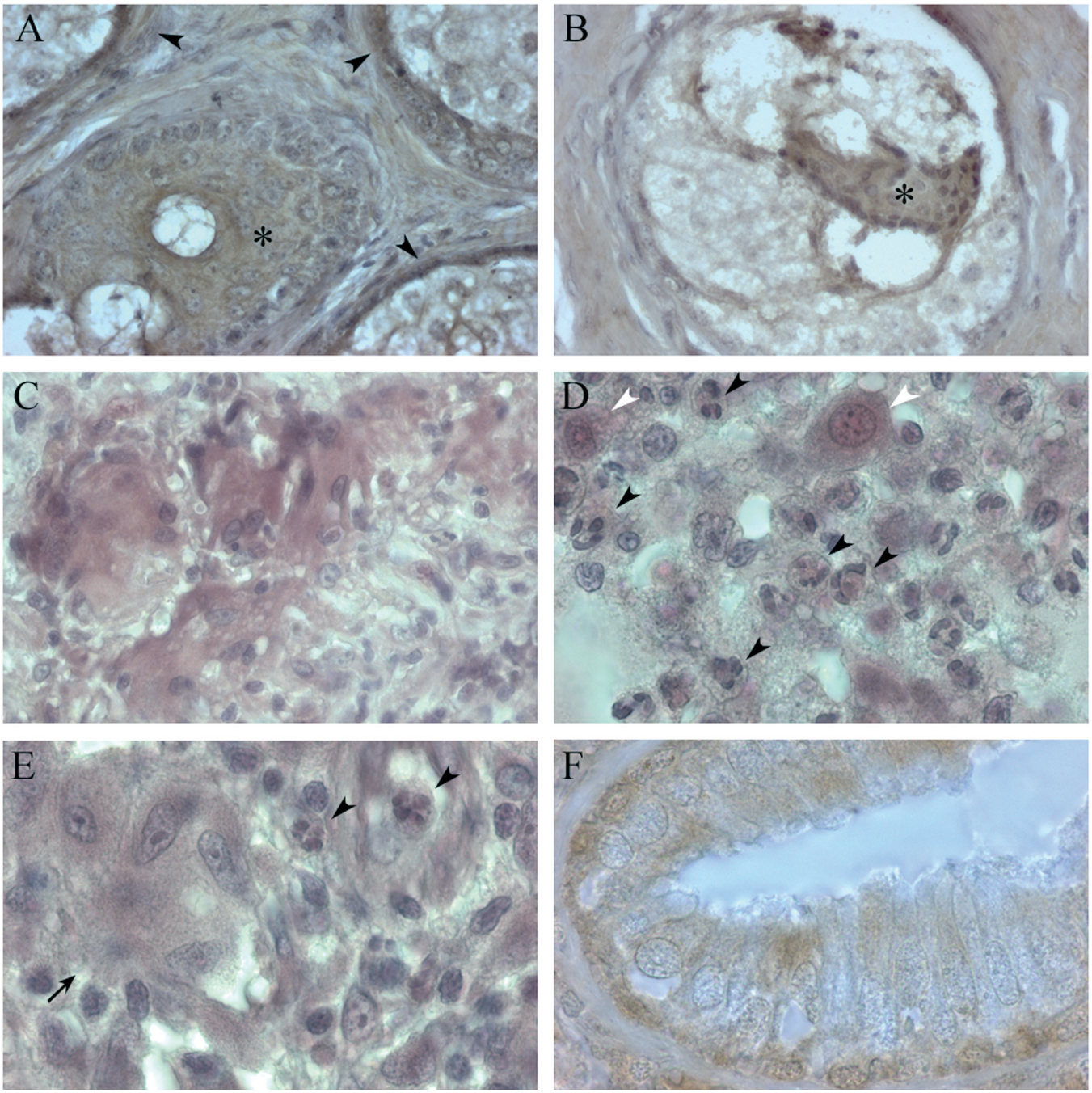

Fig. 5. In situ hybridization: pp-ET-1 mRNA and ECE-1 mRNA in chalazia. **A.** Peripheral metabolically active epithelial cells positive for pp-ET-1 mRNA in meibomian adenomers in transverse (arrowheads) and oblique (asterisk) view (s: DAB, haematoxylin counterstain). x 400. **B.** Partially disrupted adenomer of the meibomian gland with detached parietal epithelium (asterisk) positive for pp-ET-1 mRNA (s.: DAB, haematoxylin counterstain). x 400. **C.** Multinucleate giant cells positive for pp-ET-1 mRNA. (s.: Fuchsin; haematoxylin counterstain). x 1000. **D.** Pp-ET-1 mRNA positivity in macrophages (white arrowheads) and PMNs (black arrowheads) in a granuloma (s.: Fuchsin, haematoxylin counterstain). x 1000. **E.** ECE-1 mRNA positivity in macrophages both isolated and fusing to form a MGC (arrow), and PMNs (arrowheads). (s.: Fuchsin, haematoxylin counterstain). x 1000. **F.** ECE-1 mRNA positive epithelial cells in a Henle crypt (s.: DAB). x 1000

degenerating adenomers and gland ducts were also reactive (Fig. 5A,B). Most MGCs, macrophages and PMNs observed within disrupting alveolar tissue and lipogranulomas, were positive for pp-ET-1 mRNA (Figs. 5C,D). Analytical classification and semiquantitative frequency evaluation of ET-1 immunoreactive cells and pp-ET-1 mRNA expressing cells are presented in Table 2.

ECE-1 mRNA was detected both in structural components of eyelids and in inflammatory cells (Fig. 5E). Hybridization signal was localized in the meibomian acini in conjunctival epithelial cells and in basal layers of crypts of Henle (Fig. 5F). Vascular walls also resulted ECE-1 mRNA positive: endothelium was strongly reactive in all classes of vessels; in larger vessels, localization was also evident in smooth muscle cells .

Discussion

In the present study, using two different approaches, IHC and ISH, we demonstrated significant expression of the ET-1 and ECE-1 mRNA and protein in inflammatory cells and structural eyelid components in chalazia.

The timing of pathological events in chalazia, as suggested either by our histological findings and previous data (Hogan and Zimmerman, 1962; Read and Lucas, 2001), evidentiates that the primary event is a lipid engorgement in acini and ducts. A gland rupture follows, with release of lipids, foreign body inflammatory reaction and lipogranuloma formation. By migration of inflammatory cells, which release cytokines and proinflammatory mediators, inflammation spreads to the adjacent tissue and other healthy meibomian glands. In contrast with chalazia, our specimens of eyelids from ectropion showed no inflammatory deposits in association with degenerative changes, in accordance with an extensive autoptic study of human meibomian glands (Obata, 2002). Therefore, in chalazia, the presence of inflammatory cells, surrounding and partially infiltrating healthy meibomian acini, is to be considered as an early sign of the inflammation diffusion from the lesion initial site.

The abundance of macrophages in our cases is in accordance with previous non morphological data. In fact the chromatographic pattern (Nicolaides et al., 1988) of chalazion lipids is more similar to phagocyte membrane than to the healthy meibomian gland secrete. Macrophages also contribute to the genesis of chalazion by metabolizing lipid droplets into toxic fatty acids, which in turn attract other macrophages in lipogranuloma. ET-1-positivity of stromal and periglandular macrophages indicates an even more complex role of these mononuclear cells in the setting of lipogranuloma.

Previously regarded as only involved in cleavage of exogenous big ET to the biologically active peptide (Sessa et al., 1991; Kaw et al., 1992), PMNs were also identified as ET-1-producing cells in this and our previous studies (Cambiaggi et al., 2001; Massai et al., 2003). Whereas the role of this class of leucocytes in acute inflammation is well defined, the spectrum of their activities within the setting of granulomatous chronic inflammatory lesions is not yet completely elucidated. We showed that ET-1 is a component of the PMN cytokine set engaged in granuloma formation.

It is to be pointed out that, both in chalazia and in control eyelids, morphologically normal meibomian glands show ET-1 immunoreactivity. ET-1 mRNA is reported in other human gland types, such as serous nasal submucosal glands (Mullol et al., 1993) and bronchial epithelial cells (Blouquit et al., 2003). Both mucus and Cl^- secretion of these glands are stimulated via ETB receptor, demonstrating a physiological role of ET-1 in exocrine secretion. ET-1 constitutive expression in the human eye has been investigated in anterior and posterior segments (Ripodas et al., 2001; Fernandez-Durango et al., 2003) but, to the best of our knowledge, no specific reports focusing on ET-1 localization in human meibomian glands were available so far. The basal ET-1 synthesis in meibomian epithelium would provide, via ET glandular receptors, release of meibum. We hypothesize that the enhancement of this physiological activity, by inflammatory induction, contributes to the spreading of tissue lesion by a massive release of meibum, causing further inflammation.

Paracrine and autocrine induction of ET-1 synthesis is a biological event well documented in different cell types (Oie et al., 1997; Ahmed et al., 2000; Felks et al., 2006). Active ET-1 secretion by lipogranulomas and scattered inflammatory cells, besides stimulating the very producer cells by autocrine induction, is likely to trigger the paracrine enhancement of ET-1 synthesis by adjacent healthy gland epithelium. Autocrine enhancement by gland acini would follow. Therefore in chalazia ET-1 might represent a local inflammatory mediator, as assessed in early stages of human appendicitis by our previous results (Massai et al., 2003).

The lack of immunoreactivity for ET-1 peptide in endothelial cells of our samples, in the presence of pp-ET-1 mRNA, probably reflects the tissue-processing method, as previous data in human colon (Inagaki et al., 1991) and appendix (Massai et al., 2003) point out. In fact, the selection from our laboratory of antiserum and of Bouin's fixative was optimal for ET-1 immunostain of inflammatory cells rather than endothelium.

Strong expression of ECE-1 in inflammatory infiltrate and gland epithelium in our samples is in accordance with experimental studies, showing ECE-1 upregulation in immunologically mediated disorders such as allograft rejection (Lattmann et al., 2006). ECE-1 exists in four distinct isoforms (Valdenaire et al., 1999): a or α, c, and d, mainly expressed on the cell surface, b or ß, with juxtanuclear localization in the trans-Golgi network, as proved by colocalization with a trans-Golgi marker (Schweizer et al., 1997). Cell surface isoforms are ectoenzymes for cleavage of exogenous big

ET-1, whereas intracellular ECE-1b cleaves endogenous precursor (Ahmed et al., 2000). In our study, neither our anti ECE antiserum, nor our probe allowed us to discriminate ECE-1 isoforms. The juxtanuclear strong immunoreactive rim that we observed in meibomian and Henle's glands is however consistent with increase of ß subunit within Golgi apparatus.

In our series a male prevalence (14 vs 6) was observed, without any significant histopathological difference between genders. Though androgens are known to strongly influence functions of meibomian glands (Sullivan et al., 2000; Krenzer et al., 2000), male and female genders turned out to be equally susceptible to chalazia.

Experimental data evidentiate an influence of steroids on ET-1 expression: a cultured line from human ciliary epithelium shows induction of ET-1 synthesis, directly following addition of dexamethasone to culture medium (Zhang et al., 2003). All our patients had undergone a local triamcinolone treatment and surgery was carried out after a minimum time lapse of three months from steroid injection. Clinical uneffectiveness of steroids in these cases was confirmed by histopathological active inflammation; therefore we consider that a role of medical treatment in our findings can be ruled out.

In conclusion, the endothelin system might be involved in the genesis of chalazia by different pathways: 1) ischaemia from vasoconstriction; 2) cytokine autocrine/paracrine activity in support of granulomatous structures and in induction of gland secretion and lipid deposition; 3) loops of reciprocal inductions ET-1/other cytokines and inflammatory mediators; 4) collagen synthesis by direct proliferative stimulus on fibroblasts (Shi-Wen et al., 2001) and induction of metalloproteases. In analogy with the biological or pharmacological inactivation of other cytokines, selective inhibitors of ECE-1 and antagonists of ET-1 receptors are already employed in clinical treatment and trials in human pathology (Finsnes et al., 2001; Howard and Morrell, 2005; Kelland and Webb, 2006). These molecules seem to provide an effective blockade of pathogenic activity of the endothelin system. As modulators of inflammatory response and exocrine gland secretion, ECE and ET could therefore be of interest as targets in medical treatment of chalazia.

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References

Ahmed S.I., Thompson J., Coulson J.M. and Woll P.J. (2000). Studies on the expression of endothelin, its receptors subtypes, and converting enzymes in lung cancer and in human bronchial epithelium. Am. J. Respir. Cell Mol. Biol. 22, 422-431.

Blouquit S., Sari A., Lombet A., D'herbomez M., Naline E., Matran R. and Chinet T. (2003). Effects of endothelin-1 on epithelial ion transport in human airways. Am. J. Respir. Cell Mol. Biol. 29, 245-251.

Cambiaggi C., Mencarelli M., Muscettola M. and Grasso G. (2001). Gene expression of endothelin-1 (ET-1) and release of mature peptide by activated human neutrophils. Cytokine 14, 230-233.

Clozel M. and Salloukh H. (2005). Role of endothelin in fibrosis and antifibrotic potential of bosentan. Ann. Med. 37, 2-12.

Dallinger S., Dorner G.T., Wenzel R., Graselli U., Findl O., Eichler H.G., Wolzt M. and Schmetterer L. (2000). Endothelin-1 contributes to hyperoxia-induced vasoconstriction in the human retina. Invest. Ophthalmol. Vis. Sci. 41, 864-869.

Ehrenreich H., Anderson R.W., Fox C.H., Rieckmann P., Hoffman G.S., Travis W.D., Coligan J.E., Kehrl J.H. and Fauci A.S. (1990). Endothelins, peptides with potent vasoactive properties, are produced by human macrophages. J. Exp. Med. 172, 1741-1748.

Ehrenreich H., Burd P.R., Rottem M., Hultner L., Hylton J.B., Garfield M., Coligan J.E., Metcalfe D.D. and Fauci A.S. (1992). Endothelins belong to the assortment of mast cell-derived and mast cell-bound cytokines. New Biol. 4, 147-156.

Elferink J.G. and de Koster B.M. (1994). Endothelin-induced activation of neutrophil migration. Biochem. Pharmacol. 48, 865-871.

Emoto N. and Yanagisawa M. (1995). Endothelin-converting enzyme-2 is a membrane-bound, phosphoramidon-sensitive metalloprotease with acidic pH optimum. J. Biol. Chem. 270, 15262-15268.

Felxs M., Guyot M.C., Isler M., Turcotte R.E., Doyon J., Khatib A.M., Leclerc S., Moreau A. and Moldovan F. (2006). Endothelin-1 (ET-1) promotes MMP-2 and MMP-9 induction involving the transcription factor NF-kappaB in human osteosarcoma. Clin. Sci. 110, 645-654

Fernandez-Durango R., Rollin R., Mediero A., Roldan-Pallares M., Garcia Feijo J., Garcia Sanchez J., Fernandez-Cruz A. and Ripodas A. (2003). Localization of endothelin-1 mRNA expression and immunoreactivity in the anterior segment of human eye: expression of ETA and ETB receptors. Mol. Vis. 9, 103-109.

Fernandez-Patron C., Zouki C., Whittal R., Chan J.S., Davidge S.T. and Filep J.G. (2001). Matrix metalloproteinases regulate neutrophil-endothelial cell adhesion through generation of endothelin-1[1-32]. FASEB J. 15, 2230-2240.

Finsnes F., Lyberg T., Christensen G. and Skjonsberg O.H. (2001). Effect of endothelin antagonism on the production of cytokines in eosinophilic airway inflammation. Am. J. Physiol. Lung Cell. Mol. Physiol. 280, L659-L665.

Guruli G., Pflug B.R., Pecher S., Makarenkova V., Shurin M.R. and Nelson J.B. (2004). Function and survival of dendritic cells depend on endothelin-1 and endothelin receptor autocrine loops. Blood 104, 2107-2115.

Haller H., Schaberg T., Lindschau C., Lode H. and Distler A. (1991). Endothelin increases $[Ca^{2+}]_i$, protein phosphorylation, and O2--production in human alveolar macrophages. Am. J. Physiol. 261, L478-484.

Hogan M.J. and Zimmerman L.E. (1962). Lids and lacrimal drainage apparatus. In: Ophthalmic pathology. 2nd ed. WB Saunders Company (eds). Philadelphia. London. pp 189-192.

Howard L.S. and Morrell N.W. (2005). New therapeutic agents for pulmonary vascular disease. Paediatr. Respir. Rev. 6, 285-291.

Inagaki H., Bishop A.E., Escrig C., Wharton J., Allen-Mersh T.C. and Polak J.M. (1991). Localization of endothelinlike immunoreactivity

and endothelin binding sites in human colon. Gastroenterology 101, 47-54.

Ishizuka T., Takamizawa-Matsumoto M., Suzuki K. and Kurita A. (1999). Endothelin-1 enhances vascular cell adhesion molecule-1 expression in tumor necrosis factor alpha-stimulated vascular endothelial cells. Eur. J. Pharmacol. 369, 237-245.

Kaw S., Hecker M. and Vane J.R. (1992). The two-step conversion of big endothelin-1 to endothelin-1 and degradation of endothelin-1 by subcellular fractions from human polymorphonuclear leukocytes. Proc. Natl. Acad. Sci. USA 89, 6886-6890.

Kelland N.F. and Webb D.J. (2006). Clinical trials of endothelin antagonists in heart failure: a question of dose?. Exp. Biol. Med. 231, 696-699.

Klein S.C., van Wichen D.F., Golverdingen J.G., Jacobse K.C., Broekhuizen R. and de Weger R.A. (1995). An improved, sensitive, non-radioactive in situ hybridization method for the detection of cytokine mRNAs. APMIS 103, 345-353.

Korth P., Bohle R.M., Corvol P. and Pinet F. (1999). Cellular distribution of endothelin-converting enzyme-1 in human tissues. J. Histochem. Cytochem. 47, 447-462.

Krenzer K.L., Dana M.R., Ullman M.D., Cermak J.M., Tolls D.B., Evans J.E. and Sullivan D.A. (2000). Effect of androgen deficiency on the human meibomian gland and ocular surface. J. Clin. Endocrinol. Metab. 85, 4874-4882.

Kuhlmann A., Amann K., Schlotzer-Schrehardt U., Kruse F.E. and Cursiefen C. (2005). Endothelin-1 and ETa/ETb receptor protein and mRNA expression in normal and vascularized human corneas. Cornea 24, 837-844.

Lamas S., Michel T., Collins T., Brenner B.M. and Marsden P.A. (1992). Effects of interferon-gamma on nitric oxide synthase activity and endothelin-1 production by vascular endothelial cells. J. Clin. Invest. 90, 879-887.

Lattmann T., Ortmann J., Horber S., Shaw S.G., Hein M. and Barton M. (2006). Upregulation of endothelin converting enzyme-1 in host liver during chronic cardiac allograft rejection. Exp. Biol. Med. 231, 899-901.

Lederman C. and Miller M. (1999). Hordeola and chalazia. Pediatrics Rev. 20, 283-284.

Lees D.M., Pallikaros Z. and Corder R. (2000). The p55 tumor necrosis factor receptor (CD120a) induces endothelin-1 synthesis in endothelial and epithelial cells. Eur. J. Pharmacol. 390, 89-94.

Malendowicz L.K., Macchi V., Brelinska R., Trejer M., Gottardo G. and Nussdorfer G.G. (1998). Endothelin-1 enhances thymocyte proliferation in monolaterally adrenalectomized rats with contralateral adrenocortical regeneration. Histol. Histopathol. 13, 721-725.

Mangahas C.R., dela Cruz G.V., Friedman-Jimenez G. and Jamal S. (2005). Endothelin-1 induces CXCL1 and CXCL8 secretion in human melanoma cells. J. Invest. Dermatol. 125, 307-311.

Massai L., Carbotti P., Cambiaggi C., Mencarelli M., Migliaccio P., Muscettola M. and Grasso G. (2003). Prepro-endothelin-1 mRNA and its mature peptide in human appendix. Am. J. Physiol. Gastrointest. Liver Physiol. 284, G340-G348.

Matsushima H., Yamada N., Matsue H. and Shimada S. (2004). The effects of endothelin-1 on degranulation, cytokine, and growth factor production by skin-derived mast cells. Eur. J. Immunol. 34, 1910-1919.

Mullol J., Chowdhury B.A., White M.V., Ohkubo K., Rieves R.D., Baraniuk J., Hausfeld J.N., Shelhamer J.H. and Kaliner M.A. (1993). Endothelin in human nasal mucosa. Am. J. Respir. Cell Mol. Biol. 8, 393-402.

Narayan S., Prasanna G., Krishnamoorthy R.R., Zhang X. and Yorio T. (2003). Endothelin-1 synthesis and secretion in human retinal pigment epithelial cells (ARPE-19): differential regulation by cholinergics and TNF-alpha. Invest. Ophthalmol. Vis. Sci. 44, 4885-4894.

Narayan S, Brun A.M. and Yorio. (2004). Endothelin-1 distribution and basolateral secretion in the retinal pigment epithelium. Exp. Eye Res. 79, 11-19.

Nazir S.A., Murphy S., Siatkowski R.M., Chodosh J. and Siatkowski R.L. (2004). Ocular rosacea in childhood. Am. J. Ophthalmol. 137, 138-144.

Nicolaides N., Flores A., Santos E.C., Robin J.B. and Smith R.E. (1988). The lipids of chalazia. Invest. Ophthalmol. Vis. Sci. 29, 482-486.

Nicolela M.T., Ferrier S.N., Morrison C.A., Archibald M.L., LeVatte T.L., Wallace K., Chauhan B.C. and LeBlanc R.P. (2003). Effects of cold-induced vasospasm in glaucoma: the role of endothelin-1. Invest. Ophthalmol. Vis. Sci. 44, 2565-2572.

Obata H. (2002). Anatomy and histopathology of human meibomian gland. Cornea 21 (Suppl.2), S70-S74.

Oie E., Vinge L.E., Tonnessen T., Grogaard H.K., Kjekshus H., Christensen G., Smiseth O.A. and Attramadal H. (1997). Transient, isopeptide-specific induction of myocardial endothelin-1 mRNA in congestive heart failure in rats. Am. J. Physiol. 273, H1727-H1736.

Prasanna G., Dibas A., Finkley A. and Yorio T. (1999). Identification of endothelin converting enzyme-1 in human non-pigmented ciliary epithelial cells. Exp. Eye Res. 69, 175-183.

Prasanna G., Hulet C., Desai D., Krishnamoorthy R.R., Narayan S., Brun A.M., Suburo A.M. and Yorio T. (2005). Effect of elevated intraocular pressure on endothelin-1 in a rat model of glaucoma. Pharmacol. Res. 51, 41-50.

Read R.A. and Lucas J. (2001). Lipogranulomatous conjunctivitis: clinical findings from 21 eyes in 13 cats. Vet. Ophtalmol. 4, 93-98.

Ripodas A., de Juan J.A., Roldàn-Pallarès M., Bernal R., Moya J., Chao M., Lopez A., Fernandez-Cruz A. and Fernandez-Durango R. (2001). Localisation of endothelin-1 mRNA expression and immunoreactivity in the retina and optic nerve from human and porcine eye. Evidence for endothelin-1 expression in astrocytes. Brain Res. 912, 137-143.

Ruetten H. and Thiemermann C. (1997). Endothelin-1 stimulates the biosynthesis of tumour necrosis factor in macrophages: ET-receptors, signal transduction and inhibition by dexamethasone. J. Physiol. Pharmacol. 48, 675-688.

Sawamura T., Shinmi O., Kishi N., Sugita Y., Yanagisawa M., Goto K., Masaki T. and Kimura S. (1993). Characterization of phosphoramidon-sensitive metalloproteinases with endothelin-converting enzyme activity in porcine lung membrane. Biochim. Biophys. Acta 1161, 295-302.

Schramek H., Coroneos E. and Dunn M.J. (1995). Interactions of the vasoconstrictor peptides, angiotensin II and endothelin-1, with vasodilatory prostaglandins. Semin. Nephrol. 15, 195-204.

Schweizer A., Valdenaire O., Nelbock P., Deuschle U., Dumas Milne Edwards J.B., Stumpf J.G. and Loffler B.M. (1997). Human endothelin-converting enzyme-1 (ECE-1): three isoforms with distinct subcellular localizations. Biochem. J. 328, 871-877.

Sessa W.C., Kaw S., Hecker M. and Vane J.R. (1991). The biosynthesis of endothelin-1 by human polymorphonuclear leukocytes. Biochem.

Biophys. Res. Commun. 174, 613-618.

Shi-Wen X., Denton C.P., Dashwood M.R., Holmes A.M., Bou-Gharios G., Pearson J.D., Black C.M. and Abraham D.J. (2001). Fibroblast matrix gene expression and connective tissue remodeling: role of endothelin-1. J. Invest. Dermatol. 116, 417-425.

Sullivan D.A., Sullivan B.D., Ullman M.D., Rocha E.M., Krenzer K.L., Cermak J.M., Toda I., Doane M.G., Evans J.E. and Wickham L.A. (2000). Androgen influence on Meibomian gland. Invest. Ophthalmol. Vis. Sci. 41, 3732-3742.

Takahashi K., Sawasaki Y., Hata J., Mukai K. and Goto T. (1990). Spontaneous transformation and immortalization of human endothelial cells. In Vitro Cell. Dev. Biol. 25, 265-274.

Turner A.J. and Tanzawa K. (1997). Mammalian membrane metallopeptidases: NEP, ECE, KELL and PEX. FASEB J. 11, 355-364.

Valdenaire O., Lepailleur-Enouf D.,Egidy G., Thouard A., Barret A., Vranckx R., Tougard C. and Michel J.B. (1999). A fourth isoform of endothelin converting enzyme (ECE-1) is generated from an additional promoter. Eur. J. Biochem. 264, 341-349.

Wollensak G., Loffler B., Beyermann B. and Ihling C. (2002). An immunohistochemical study of endothelin-1 converting enzyme in the human eye. Curr. Eye Res. 24, 6-11.

Yanagisawa M., Kurihara H., Kimura S., Tomobe Y., Kobayashi M., Mitsui Y., Yazaki Y., Goto K. and Masaki T. (1988). A novel potent vasoconstictor peptide produced by vascular endothelial cells. Nature 332, 411-415.

Zhang X., Krishnamoorthy R.R., Prasanna G., Narayan S., Clark A. and Yorio T. (2003). Dexamethasone regulates endothelin-1 and endothelin receptors in human non-pigmented ciliary epithelial (HNPE) cells. Exp. Eye Res. 76, 261-272.

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