Unique Distribution of Thrombospondin-1 in Human Ocular Surface Epithelium

Eiichi Sekiyama,1 Takahiro Nakamura,1,2 Leanne J. Cooper,3 Satoshi Kawasaki,1 Junji Hamuro,1 Nigel J. Fullwood,4 and Shigeru Kinoshita1

PURPOSE. The study was conducted to elucidate the detailed expression pattern of angiogenesis-related factors in human ocular surface epithelium. The focus was factors with significantly higher gene expression in corneal epithelium (CE) than in conjunctival epithelium (CJE).

METHODS. The relative gene expression of 36 angiogenesis-related factors was compared in human CE and CJE, by using the introduced amplified fragment-length polymorphism (iAFLP) method. Also examined were the expression patterns in the CE, limbal epithelium (LE), and CJE of factors with significantly higher expression in the CE, by using real-time PCR, in situ hybridization, immunohistochemistry, and immunoelectron microscopy.

RESULTS. Only thrombospondin (TSP)-1 exhibited significantly higher expression in the CE. In situ hybridization and real-time PCR showed TSP-1 transcripts in the basalmost cells of the CE and LE. Compared with the CJE, they were significantly upregulated at those sites. Immunohistochemistry revealed that TSP-1 was strongly expressed in the basalmost region of the CE. Its expression was faint in LE and absent in CJE. Immunoelectron microscopy revealed that the CE and LE demonstrated TSP-1 labeling just below the epithelium, in the basalmost region of basal cells, and occasionally in the basal cell membrane. There was little or no labeling in the CJE.

CONCLUSIONS. In the human ocular surface epithelium, basal cells of the CE and LE, but not of the CJE, synthesize TSP-1. High levels of TSP-1 are present only just below the CE. Its unique distribution may be related to corneal avascularity and integrity. (Invest Ophthalmol Vis Sci. 2006;47:1352–1358) DOI:10.1167/iovs.05-1305

The corneal epithelium (CE) and conjunctival epithelium (CJE) are continuous, and together they comprise the ocular surface. Although both are stratified, squamous, nonkeratinizing epithelia that originate from surface ectoderm, they differ considerably in their characteristics and functions. For example, although some keratins are expressed in the CE and CJE as cytoskeletal proteins, they are of different subtypes.1,2 In the CJE, but not the CE, goblet cells are interspersed between epithelial cells, and the tight junctions of the superficial cells render the CE impermeable to water-soluble substances. Consequently, compared with the CE, the CJE exhibits very poor barrier function.3,4

One of the major differences between the CE and CJE is the underlying vascular system. Although the connective tissue under the CJE is rich in vessels, the tissue under the normal CE is devoid of a vascular system. Corneal avascularity, necessary for good visual acuity, is attributable to the balanced expression of angiogenic and antiangiogenic factors.5–10 Although the cornea is thought to produce and contain numerous antiangiogenic factors including thrombospondins,3 pigment epithelial-derived factor,3 and endostatin,7 the mechanisms underlying the maintenance of corneal avascularity remain poorly understood.

To gain insight into angiogenesis-related corneal characteristics, we compared the gene expression patterns of 36 factors related to angiogenesis in the CE and CJE. We then investigated the detailed expression patterns in the ocular surface epithelium of factors with significantly higher expression in the CE than the CJE.

MATERIALS AND METHODS

RNA Extraction from CE, LE, and CJE

Our study adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine. Human conjunctival epithelial cells were obtained from donors who provided prior informed consent.

Using donor tissues obtained from the Northwest Lions Eye Bank (Seattle, WA), we scraped the 4-mm diameter central region of the epithelium to obtain CE. For limbal epithelium (LE) we cut out the center part of the cornea and scraped the epithelium corresponding to the region of the palisades of Vogt (n = 11; mean age, 57.5 ± 6.4 years). After scraping the LE, we prepared sections of the remaining tissues and stained them with hematoxylin and eosin to ascertain that we harvested only LE and not CE. CJE cells from the bulbar conjunctiva of healthy volunteers were collected under topical anesthesia with 0.4% oxybuprocaine eye drops using a sterile nylon thread brush (Cytobrush Plus; Medscard Medical AB, Malmo, Sweden), as previously described (n = 6; mean age, 52 ± 5.8 years).11

After a 5-minute immersion of the CE, LE, and CJE at room temperature (RT) in acid phenol with guanidine isothiocyanate (TRIZOL reagent; Invitrogen Corp., Carlsbad, CA), total RNA was extracted and dissolved in distilled water according to the manufacturer’s guidelines. To check its integrity, 1 μL of the RNA solution was electrophoresed on 1% agarose gels, the gels were stained (SYBR Green; Molecular Probes Inc., Eugene, OR), and 18S and 28S ribosomal RNA band fluorescence was quantified with a luminescent image analyzer (LAS1000; Fuji Film Medical Systems Inc., Stamford, CT). The remaining RNA was stored at −80°C until use.

Gene Expression Analysis by iAFLP

We examined the gene expression patterns in the CE and CJE of 36 angiogenesis-related factors (Table 1), using the introduced amplified fragment length polymorphism (iAFLP) method.12,13 described else-

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TABLE 1. Angiogenesis-Related Factors and Gene-Specific Primers

<table>
<thead>
<tr>
<th>Factor</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Thrombospondin-1*</td>
<td>5'-GTATGAGTAAGGGGAGGGA-3'</td>
</tr>
<tr>
<td>Thrombospondin-5*</td>
<td>5'-GCTCCTGAGTAGTGCTTCAAT-3'</td>
</tr>
<tr>
<td>VEGF</td>
<td>5'-GCTATGACTTGGCCAGATTGAGT-3'</td>
</tr>
<tr>
<td>FGF-1</td>
<td>5'-GGCAATCAGCTGGCCACAGA-3'</td>
</tr>
<tr>
<td>FGF-4</td>
<td>5'-GGCCACACCAAGAAAGTGA-3'</td>
</tr>
<tr>
<td>Angiopoietin-1</td>
<td>5'-GAGTTAGGGATGAGGCTTCA-3'</td>
</tr>
<tr>
<td>Angiopoietin-2</td>
<td>5'-GAGATTGCGTCCTATGCAAT-3'</td>
</tr>
<tr>
<td>IL-1 alpha*</td>
<td>5'-GAGTACATGATTGCTTCAAT-3'</td>
</tr>
<tr>
<td>IL-1 beta*</td>
<td>5'-GCGCTGCTGGAGAACGGAGA-3'</td>
</tr>
<tr>
<td>IL-8</td>
<td>5'-GGTTGACAGGTCGAGGCTG-3'</td>
</tr>
<tr>
<td>IL-12 alpha</td>
<td>5'-GTCGACATGATAGCCGATCT-3'</td>
</tr>
<tr>
<td>TGF-beta 1</td>
<td>5'-GACGACTTGGACCTCCAGAGG-3'</td>
</tr>
<tr>
<td>TGF-beta 2*</td>
<td>5'-GGCTGATCCCCCTATATGATG-3'</td>
</tr>
<tr>
<td>TGF-beta 3*</td>
<td>5'-GTGTCGCAAGGGAAATATGA-3'</td>
</tr>
<tr>
<td>IGF</td>
<td>5'-GCAAATGCAAGCTTATCTGCTT-3'</td>
</tr>
<tr>
<td>Angiogenin*</td>
<td>5'-GCGAGGAAAAAGGTGTAAG-3'</td>
</tr>
<tr>
<td>Angiogenin-inhibitor*</td>
<td>5'-GACTGCGAGGAATATATCGCA-3'</td>
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*Factors yielding products of the expected sizes.

where (n = 6). For validation and normalization, we examined the gene expression patterns of four keratins, 19 ribosomal protein subunits, and GAPDH by the same method. We used a pUC 19-base vector primer14 to monitor the adequate synthesis and cleavage of cDNA on agarose gels. Extracted total RNA (1 µg) was annealed with a pUC 19-base vector primer (5 ng) in a total volume of 10 µL. After heat denaturation (70°C, 3 minutes), the reaction mixture that included 0.5 mM dNTP, 1× RT buffer (supplied with SuperScript II; Invitrogen), 15 mM dithiothreitol (DTT), and 0.1 U/mL of reverse transcriptase (SuperScript II; Invitrogen) was incubated for 60 minutes at 42°C. Second-strand synthesis then followed, in which 130 µL of second-strand reaction mixture was added, to yield a final concentration of 0.33 mM dNTP, 2.7 mM DTT, 1× Escherichia coli ligase buffer (supplied with E. coli ligase), 0.27 U/mL DNA polymerase 1, 0.15 U/mL E. coli ligase, and 0.013 U/mL E. coli RNase H (all from Invitrogen). After purification by phenol-chloroform extraction and ethanol precipitation, cDNA was dissolved in 20 µL of distilled water, and 2.5 µL of the solution was stocked for electrophoresis.

To digest cDNA, 2 µL of 10X NEB3 buffer (supplied with Mbol) and 5 units of Mbol (New England Biolabs Ltd., Herfordshire, UK) were added. This was followed by incubation at 37°C for 60 minutes and heat inactivation at 70°C for 20 minutes. A small aliquot of the digested cDNA was electrophoresed on agarose gels along with stocked undigested cDNA, to check the quality of cDNA synthesis and Mbol digestion. After checking, small aliquots (approximately 1:6) of all digested cDNAs were pooled to obtain a reference sample (control) to connect cDNAs were pooled to obtain a reference sample (control) to connect.

For RNase H-reverse transcriptase (SuperScript II; Invitrogen). Primers and probes for TSP-1 and β-actin were purchased from ABI. For real-time quantification we used the ΔCt method (ABD). The Ct value is the fractional cycle number at which the amplified target amount reaches a fixed threshold of detectable fluorescence. The threshold was set in the midlinear phase of the amplification plot. To standardize the amount of sample cDNA added to each reaction, the amount of target gene in each sample was normalized to the endogenous control (β-actin) by subtracting the Ct of β-actin from the Ct of the target gene. Analyses were performed in a sequence detector (Prism 7000; ABI) using the accompanying data-analysis software.

In Situ Hybridization

Human TSP-1 cDNA, corresponding to base pairs 3874-4375 of the previously reported human TSP-1 cDNA (GenBank accession number NM_003246; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD), was subcloned into the pGEM-T vector (Promega, Madison, WI) and used for the generation of sense and antisense RNA probes. Digoxigenin (DIG)-labeled RNA probes were prepared with DIG RNA labeling mix (Roche, Basel, Switzerland).

Fresh human corneal, limbal, and conjunctival epithelia obtained from the eye bank were dissected, fixed, and embedded in paraffin by using proprietary procedures, and 6-µm sections were cut (n = 3; eyes aged 36.7 ± 16.5 years). They were deparaffinized with xylene, rehydrated with an ethanol series and phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS (15 minutes), and then washed with PBS. Then the sections were treated with 10 µg/mL proteinase K in PBS (30 minutes, 37°C), washed with PBS, refixed with 4% paraformaldehyde in PBS, washed again with PBS, and placed in 0.2 M HCl for 10 minutes. After they were washed with PBS, the sections were acetylated by a 10-minute incubation in 0.1 M triethanolamine-HCl (pH 8.0) and 0.25% acetic anhydride. After they were again washed with PBS, they were dehydrated through an ethanol series. After 55°C, 16-hour hybridization to probes (100 ng/mL) in probe diluent (Geno- staff, Tokyo, Japan), the sections were washed in 5× SSC (HybriWash; Genostaff) at 55°C for 20 minutes and then in 5% formamide, 2× hybridization buffer (55°C, 20 minutes; HybriWash; Genostaff). This was followed by RNase treatment in 20 µg/mL RNase A in 10 mM Tris-HCl (pH 8.0), 1 M NaCl, and 1 mM EDTA. The sections were washed twice with 2× hybridization buffer (55°C, 20 minutes), twice with 0.2× of the buffer (55°C, 20 minutes), and once with TBST (Tris-buffered saline-0.1% Tween-20). After 30-
Immunohistochemistry for TSP-1

Residual tissues from penetrating keratoplasty (PKP) were obtained from the eye bank (n = 5, mean age: 49 ± 17.5 years). They were embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Miles, Inc., Elkhart, IN) and 8-μm-thick cryostat sections were cut so that the sections contained continuous epithelium from the central cornea to the limbal conjunctiva. The samples were examined immunohistochemically using our previously described method. Briefly, after a 10-minute fixation in cold acetone, the sections were incubated for 20 minutes with 10% goat serum and 1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO), incubated again at RT for 1 hour with two types of mouse anti-human primary antibody (catalog no. BA18, dilution: ×100; Oncogene, San Diego, CA; catalog no. T2905; dilution, ×100; Sigma-Aldrich), and washed three times for 10 minutes each in PBS. In control experiments, we replaced the primary antibody with identical concentrations of appropriate nonspecific normal IgG (Dako, Kyoto, Japan).

After incubation with the primary antibody, the sections were washed with PBS containing 0.15% Triton X-100 and then incubated for 1 hour at RT with the appropriate secondary antibodies, FITC-conjugated anti-mouse antibodies (Molecular Probes, Inc.). After several washes with PBS, the sections were coverslipped with antifade mounting medium containing propidium iodide (Vectorshield; Vector, Burlingame, CA) and examined by confocal microscope (Fluoview; Olympus, Tokyo, Japan).

Immunoelectron Labeling with TSP-1

Samples of tissues obtained from the U.S. Eye Bank were fixed in 2.5% paraformaldehyde in phosphate buffer (pH 7.2), washed three times in phosphate buffer containing 0.1 M glycine, and dehydrated through a graded series of ethanol (50%, 70%, 80%, and 90% ethanol solutions, 20 minutes at each concentration; n = 3, mean age, 37 ± 16.5 years). The samples were then transferred to resin (London Resin White; TAAB Laboratories, Aldermaston, UK) for infiltration, embedded in molds containing fresh resin, and polymerized at 50°C for 24 hours. Ultrathin sections were cut on an ultramicrotome (Ultracut E; Reichert, Vienna, Austria) and collected on gilded copper grids (G-100; Agar Scientific, Stansted, UK). We labeled for the primary mouse monoclonal antibody by first placing the tissue-bearing grids in droplets of 0.1 M glycine in PBS (<2, 10 minutes each) and then incubating them in droplets of normal goat serum (20 minutes at RT). After the excess goat serum was removed, the grids were incubated overnight at 4°C with the primary antibody (catalog no. BA18, dilution, ×50; Oncogene) in PBS buffer (pH 7.4) containing 1% BSA and 1% Tween-20 (buffer 1). In the control experiments, the primary antibody was replaced with nonspecific antibody.

The grids were washed three times for 8 minutes each in five droplets of buffer 1 and then five times (8 minutes each) in distilled water. They were subsequently transferred to the appropriate secondary antibody (1:50 dilution in PBS; pH 8.2) containing 1% BSA, 1% Tween-20, 1% normal goat serum, 1% fish gelatin, and 2% sodium chloride (buffer 2). Secondary goat anti-rabbit IgG or goat anti-mouse IgG gold-conjugated (5 nm) antibodies (British Biocell International, Cardiff, UK) were used to visualize the primary antibodies. The samples were incubated for 3 hours at room temperature, and the grids were washed three times for 8 minutes each in five droplets of buffer 2 and then five times (8 minutes each) in distilled water. After the final wash, the sections were counterstained in aqueous uranyl acetate for 1 hour and examined under a transmission electron microscope (JEM 1010; JEOL, Tokyo, Japan).

RESULTS

iAFLP Analysis

We compared the gene expression patterns of 36 major angiogenesis-related factors in human CE and CJE by using the iAFLP method.
method. Of the 36 primers tested, 18 yielded products of the expected sizes, suggesting that the signals represented concentrations of target transcripts.

Among 36 factors, only TSP-1 was significantly upregulated in the CE (Fig. 1, yellow box). When we examined the expression pattern of keratins and ribosomal protein subunits used for validation, we found significant upregulation of CK3 and -12 in the CE, and of CK4 and -13 in the CJE and found similar expression patterns of 19 ribosomal protein subunits (Fig. 1). These results support the validity of the method used.

**Real-Time PCR for TSP-1**

The expression of TSP-1 mRNA in the CE, LE, and CJE (n = 5) was determined by real-time PCR. Compared with the CJE, mean TSP-1 mRNA was significantly upregulated in the CE and LE (P < 0.01, Mann-Whitney test). TSP-1 mRNA was slightly higher in the LE than the CE, although the difference was not statistically significant (Fig. 2).

**In Situ Hybridization**

In situ hybridization revealed the spatially restricted distribution of TSP-1 transcripts in the CE and LE, particularly in the basal epithelial cells (Figs. 3A, 3B, black arrowheads). We did not detect TSP-1 transcripts in the CJE (C). (D-F) Sense RNA probes showed negligible levels of reactivity. Scale bars, 50 μm.

**Immunohistochemistry for TSP-1**

There was clear evidence of TSP-1 on the basement membrane side of the CE (Fig. 4A). Some patchy staining was detected in fibrous tissues and the corneal stroma below the LE (Fig. 4C, white arrows). Although we could detect moderate staining in the sclera distant from CJE, no staining was found in and just below the CJE (Fig. 4A). The area of TSP-1 expression in the ocular surface epithelium corresponded with Bowman’s layer (Figs. 4A, 4C).
Immunoelectron-Labeling with TSP-1

In the CE, Bowman’s layer was strongly labeled for TSP-1 (Fig. 5A, black arrowheads). TSP-1 labeling was also found in the basal region of the basal cells (Fig. 5A, white arrowheads) and occasionally in the basal membrane of the epithelial cells (Fig. 5B, black arrowheads). In the LE, there was slight labeling just below the epithelial cells (Fig. 5C, white arrowheads), in the basal region of the basal cells, and occasionally in the basal membrane of the epithelial cells (Fig. 5C, black arrowheads). In contrast, the basal region of the CJE exhibited little or no labeling (Fig. 5D).

DISCUSSION

The cornea is one of the few avascular tissues in the adult body. Its avascularity is essential for corneal transparency and visual acuity. The mechanisms that maintain corneal avascularity and the characteristic angiogenesis-related differences between the cornea and conjunctiva remain poorly understood. Ours is the first report of the gene-expression patterns of major angiogenesis-related factors in the CE and CJE. We found that among 36 angiogenesis-related factors, TSP-1, one of the major antiangiogenic factors, was expressed at significantly higher levels in the CE.

TSP-1 was first identified as a platelet protein secreted on platelet activation17,18 with a role in platelet aggregation.18,19 TSP-1 has been described as a multifunctional protein that affects cell adhesion, migration, and proliferation20,21 and plays a role in angiogenesis by exerting inhibitory effects on corneal neovascularization.5,22,25 In mice, a deficit in a single antiangiogenic factor did not result in spontaneous corneal neovascularization.24,25 We postulate that human corneal avascularity is also regulated by multiple antiangiogenic factors rather than a single one. Of interest, TSP-1, one of the major antiangiogenic factors contributing to corneal avascularity5,6 was expressed at significantly higher levels in the human CE than in the CJE.

Our observation that TSP-1 transcripts were detected primarily in the basal cells of the CE and LE and that the protein was present in and just below the basal cells of both epithelia led us to postulate that TSP-1 was synthesized by basal corneal and limbal epithelial cells and that it was secreted primarily in the basal direction. TSP-1 is secreted by many types of cells including fibroblasts,26,27 vascular endothelial cells,26,27 smooth muscle cells,26 macrophages, and monocytes.28 Ours is the first documentation of its secretion in human ocular surface epithelium. In the current study, we provide evidence that in the human CE and LE, TSP-1 is secreted by basal cells, mainly toward the basal side.

Our immunoelectron microscopic study disclosed the detailed distribution of TSP-1 in the CE. Hiscott et al.29 detected TSP-1 in the basal epithelial cell cytoplasm and basement membrane, but not within Bowman’s layer. The presence of TSP-1 at the cell–matrix interface is not unexpected, because there is evidence that TSP-1 exists both as a secreted protein and as an insoluble extracellular matrix molecule30,31 that binds to several macromolecules, such as heparin32 and fibronectin,33 and that it acts as an integrator of cell–matrix interactions.34 We detected high levels of TSP-1 in Bowman’s layer. TSP-1 has a high affinity for some collagens, particularly collagen type V,35,36 and the main constituents of Bowman’s layer are collagens I, III, and V.37,38 We postulate that this explains its presence in Bowman’s layer. The function of TSP-1 as an adaptor and modulator of cell–matrix interactions results in tissue remodeling and accelerated wound healing20,21 and the transformation of latent TGF-β1 into an active form39 that prompts the migration of corneal epithelial cells. Thus, TSP-1 in Bowman’s layer may contribute to corneal epithelial cell migration and corneal avascularity. Studies are under way in our laboratory to elucidate the physiological implications of the observed TSP-1 distribution.

FIGURE 5. Transmission electron micrographs of the basal region of the CE (A, B), the LE (C), and the CJE (D). Significant labeling for TSP-1 was found in Bowman’s layer (A, black arrowheads), inside basal cells (A, white arrowheads), and in the basal cell membrane (B, black arrowheads). There was some labeling just below the LE (C, black arrowheads) and inside basal cells (C, white arrowheads), but little or no labeling in the basal region of the CJE (D). The 5-nm gold particles appear as black dots. Scale bars, 100 nm.
Although our immunohistochemical and immunoelectron microscopic studies detected TSP-1 in the CE, only slight immunohistochemical staining for TSP-1 was detected just below the LE. Immunoelectron labeling for TSP-1 in the LE confirmed that it was present in low amounts (Fig. 3C). We posited that although limbal basal epithelial cells secrete TSP-1 mainly toward the basal side, it diffuses rapidly into fibrous tissue, corneal stroma, or sclera because the area directly below the LE is devoid of Bowman’s layer with an affinity for TSP-1. Regarding the fact that normal keratocytes do not secrete TSP-1, we postulate that the staining in the sclera (Fig. 4A) below the CE may have diffused from basal cells of the LE and/or fibroblasts in the connective tissues below the CE. We thus suggest that TSP-1 located below the CE accumulates in Bowman’s layer, whereas TSP-1 located below the LE diffuses into peripheral tissues.

In summary, in the human ocular surface epithelium, TSP-1 was secreted by corneal and limbal basal epithelial cells toward the basal side. In this respect, the LE and CE exhibited similar characteristics. In contrast to the LE, TSP-1 accumulated in Bowman’s layer just below the CE. The expression pattern of TSP-1 in the CE is different from the pattern noted in the CE and LE. The presence of TSP-1 in Bowman’s layer may be related to corneal avascularity and the migration of corneal epithelial cells; however, the physiological relevance of its unique distribution remains to be elucidated. Investigations of the role of TSP-1 in the ocular surface epithelium are under way in our laboratory.

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References


