Expression of Angiogenesis-Related Factors in Human Corneas after Cultivated Oral Mucosal Epithelial Transplantation

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Purpose. We analyzed the expression of angiogenesis-related factors in corneal tissues that had undergone previously autologous cultivated oral mucosal epithelial transplantation (COMET).

Methods. Six eyes from four chemically- and two thermally-injured patients with limbal stem cell deficiency who received COMET to promote wound healing were studied retrospectively. Immunoconfocal microscopy was performed on corneal specimens from the patients after COMET, as well on normal corneas, conjunctiva, and oral mucosa for keratin 8, fibroblast growth factor-2 (FGF-2), VEGF, collagen XVIII (endostatin), pigment epithelium-derived factor (PEDF), soluble fms-like tyrosine kinase-1 (sFlt-1), tissue inhibitor of metalloproteinase-3 (TIMP-3), thrombospondin-1 (TSP-1), and interleukin-1 receptor antagonist (IL-1ra).

Results. FGF-2, VEGF, endostatin, PEDF, and IL-1ra were detected in all the samples, with signals for FGF-2, VEGF, and IL-1ra localized to the full-thickness epithelial layer, as signals for endostatin limited to the basement membrane. Expression of PEDF varied in tissues, with a preferential expression in the suprabasal epithelial layer. FGF-2 and IL-1ra were abundantly expressed in the basal epithelial layer in specimens with increased stratification. Signals for sFlt-1, TIMP-3, and TSP-1 were detected in normal corneal epithelium, and in a specimen containing corneal epithelium, but were negative in all other specimens.

Conclusions. Expression of FGF-2, VEGF, PEDF, endostatin, and IL-1ra was similar in normal corneas, conjunctiva, oral mucosa, and corneas after COMET. Expression of sFlt-1, TIMP-3, and TSP-1 was limited to normal corneas and negative for other tissues. A lack of the aforementioned antiangiogenic factors may contribute to the peripheral corneal neovascularization seen after COMET. (*Invest Ophthalmol Vis Sci.* 2012;53:5615–5623) DOI:10.1167/iovs.11-9293

Over the past few decades, due to the major breakthroughs in limbal epithelial stem cell biology,[1] corneal transplantation has evolved into ocular surface reconstruction, a widespread surgical procedure for the treatment of severe ocular surface diseases.[2] Among the evolving ocular surface reconstruction methods, cultivated autologous limbal epithelial transplantation (CLET) represents a state-of-the-art tissue engineering technology in regenerative medicine.[3] Despite varying results,[4] long-term treatment success of up to 10 years in more than 75% of patients has been achieved recently.[5] However, due to the low success rate of allogeneic limbal stem cell transplantation (including CLET) in patients with bilateral limbal stem cell deficiency (LSCD) even under immunosupression,[6] cultivated autologous oral mucosal epithelial transplantation (COMET) has been proposed as an alternative for treating LSCD, and has achieved satisfactory long-term results for ocular surface stabilization and visual outcome.[7,8] Evidence has shown that after either CLET[9,10] or COMET,[11,12] transplanted cells can survive either to restore a normal corneal phenotype or to maintain ocular surface integrity. COMET also can be used as an alternative treatment to promote reepithelialization and reduce inflammation in acute corneal chemical or thermal injuries.[13] Furthermore, long-term existence of transplanted oral mucosal stem/progenitor cells on the ocular surface also supports the rationale for clinical use of cultivated epithelial transplantation.[3]

However, even after successful autologous COMET, various degrees of superficial corneal neovascularization (NV) can be detected beneath the transplanted epithelial sheet.[13–16] Clinically, corneal NV following total LSCD is the most severe type, and the molecular mechanisms regulating NV formation and restoration of corneal avascularity after CLET have been reviewed extensively.[17,18] Recently, in vitro studies using cultivated oral and corneal epithelial cells from either humans[19–20] or rabbits[20,21] have shown that decreased expression of thrombospondin-1 (TSP-1)[20] and soluble fms-

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like tyrosine kinase-1 (sFlt-1),[22] and increased secretion of fibroblast growth factor-2 (FGF-2)[21] may be responsible for the enhanced angiogenic potential after COMET. Until now, clinicopathologic studies focusing on angiogenesis-related factors in patients receiving COMET have been lacking. In our study, we aimed to analyze angiogenesis-related factors in corneal tissues that previously had undergone COMET to understand which factors are responsible for inferior anti-angiogenic activity after COMET.

Materials and Methods

Ethics Statement

No animal work was conducted in this study.

Subjects Recruitment and Clinical Assessment

The clinical trial and subsequent clinicopathologic study were approved by the Institutional Review Board of Chang Gung Memorial Hospital in 2004 and 2009 respectively (registry numbers 93-292A and 98-2148B). The clinical trial per se was approved further by Taiwan's Department of Health in 2006 and was executed under its supervision as a Phase I clinical trial (registry number 0950206914). Informed consents were obtained from all patients in accordance with the tenets of the Declaration of Helsinki. Written consents were obtained not only before COMET and subsequent surgeries, but also before use of the patients' tissues for histologic study. There were six eyes from six patients with total LSCD caused by chemical or thermal injuries used in this study.

Cultivation of Oral Mucosal Epithelial Cells (OMECs)

The protocol for cultivating OMECs adhered to our earlier report[13] and involved the use of denuded amniotic membrane as the carrier, mitomycin C-inactivated NIH/3T3 fibroblasts as the feeder cells, and SHEM with 5% fetal calf serum as the culture medium. The protocol differed from that reported previously by Nakamura et al[15] in the following ways: larger biopsy specimens of buccal mucosa (6×6 mm or larger) were harvested, serial separation by 0.25% trypsin-EDTA (every three minutes for at least 3 times) was used, and there was no air-lifting during the culture.

COMET and Subsequent Surgeries

The procedures of COMET complied with our earlier report.[13] For optical purposes, cataract surgery or ocular surface reconstruction procedures, for example keratolimbal allograft (KLAL), conjunctival limbal autograft (CLAU), penetrating keratoplasty (PKP), or deep anterior lamellar keratoplasty (DALK), were performed after COMET.

Immunohistochemistry and Confocal Laser Scanning Microscopy

Corneal tissues were obtained during PKP in patients 1 and 3; during CLAU in patients 2, 4, and 6; and during KLAL in patient 5. All aforementioned specimens were assessed under the effect of COMET, but not of the other subsequent surgeries. Normal corneas, conjunctiva (from donor corneal buttons unsuitable for keratoplasty), oral mucosa (redundant oral tissues from patients undergoing oral surgery), and cultivated OMECs were included as controls for comparative analysis. Freshly removed human tissues were embedded in OCT compound and then were snap frozen in liquid nitrogen. Immunofluorescent staining was performed in accordance with our previously described method.[12] Briefly, frozen sections were rinsed with PBS and then fixed with 100% methanol at 4°C for 10 minutes. To minimize nonspecific reactions, the sections were blocked by incubation at room temperature with 2.5% bovine serum albumin for 30 minutes. The slides then were incubated at 4°C overnight with the appropriate primary antibodies (see Supplementary Table S1 for a list of antibody information, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9293/-/DCSupplemental), which included markers for ocular surface epithelia[12] (keratin 8, also a negative marker for OMECs), for angiogenesis[17,18] (FGF-2 and VEGF), for anti-angiogenesis (collagen XVIII [endostatin], pigment epithelium-derived factor [PEDF], sFlt-1, tissue inhibitor of metalloproteinase-3 [TIMP-3], and TSP-1), and for anti-inflammation (interleukin-1 receptor antagonist [IL-1ra]). Sections incubated with irrelevant mouse or rabbit IgG were used as negative controls. After washing with Tris-buffered saline containing 0.5% Tween-20, the sections were incubated at room temperature for one hour with the appropriate secondary antibodies, including FITC-conjugated donkey anti-mouse IgG (for keratin 8, sFlt-1, PEDF, TSP-1, and VEGF), FITC-conjugated donkey anti-rabbit IgG (for endostatin, FGF-2, and TIMP-3), and FITC-conjugated donkey anti-goat IgG (for IL-1ra). Subsequent staining procedures and confocal microscopy were similar to our previous study.[12]

Results

Clinical Outcomes

Beginning in April 2006, COMET were performed either to reconstruct the corneal surface in chronic thermal burns (patients 1 and 3), or to promote reepithelialization in alkali (patient 2) and acid (patients 4, 5, and 6) burns with persistent epithelial defect (Figs. 1A1–1F1). There were one female and five male patients, with a mean age of 35.5 ± 14.3 (range 18–55) years. The mean interval between ocular injury and COMET was 11.0 ± 11.2 (range 2–31) months. Although the ocular surface became stabilized postoperatively, residual corneal stromal opacity and mild-to-marked corneal NV were evident (Figs. 1A2–1F2). Therefore, from 9–26 (average 15.7 ± 6.9) months after COMET, CLAU (patients 2, 4, and 6), KLAL, (patient 5), PKP (patients 1, 3, and 5), DALK (patient 6), or cataract surgery (patients 1, 3, 4, and 5) was performed to improve vision further.

After these subsequent surgeries, a stable ocular surface was maintained in all patients. The mean follow-up time after COMET was 36.7 ± 17.0 (range 16–56) months, and the only major complication was secondary glaucoma diagnosed in patient 5. All relevant demographic data and clinical features of the six patients are summarized in Figure 1 and Table 1.

Histologic Findings

Corneal buttons obtained during PKP or CLAU revealed either 5–10 stratified epithelial layers oriented similarly to normal corneal epithelium (patients 1–4 and 6) or more than 10 layers of epithelia resembling normal oral mucosa (patient 5). No epithelial papillary structures were observed, but basal keratinocytes in patients 1, 2, 3, and 5 were smaller and more compact (Figs. 1A4–1C4, 1E4), similar to those found in the papilla of oral mucosa. In patient 3, a clear junction (Fig. 1C4, arrow) between OMECs (left) and corneal epithelium (right) could be identified. On the other hand, superficial keratinocytes of patient 5 were organized loosely and extremely thickened, resembling parakeratinization (Fig. 1E4). The anterior membrane (AM) substrate was barely visible beneath the epithelium, except in patient 3, whose stroma was less-infiltrated with inflammatory cells. In addition, superficial peripheral NV was noted occasionally just under the AM in the anterior stroma (Figs. 1D4, 1E4; arrowheads).

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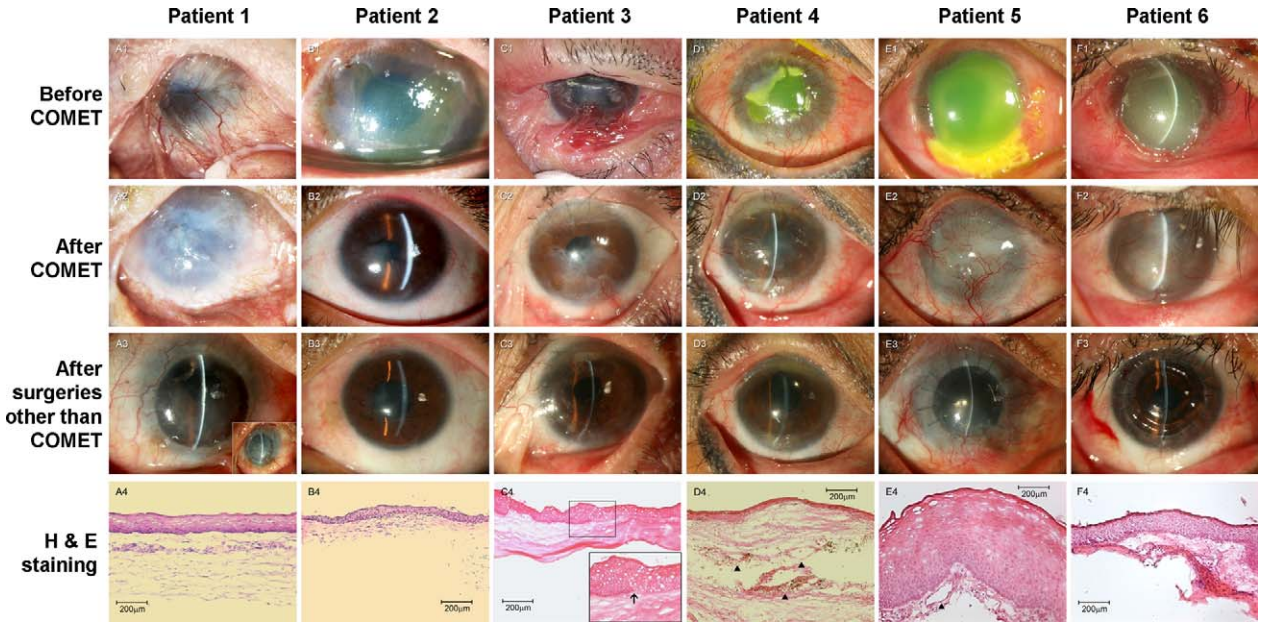

Figure 1. Representative external eye photographs (**A1–F1**, **A2–F2**, **A3–F3**) and hematoxylin-eosin staining (**A4–F4**) for patients 1 (**A1–A4**), 2 (**B1–B4**), 3 (**C1–C4**), 4 (**D1–D4**), 5 (**E1–E4**), and 6 (**F1–F4**). At 15 months after thermal injury, the ocular surface of patient 1 became stabilized, but still was covered by dense fibrovascular tissue (**A1**). Following COMET, although there was less inflammation, superficial NV persisted (**A2**). PKP combined with cataract extraction was performed 10 months after COMET, and the graft remained stable for 18 months (**A3**) before failing (**A3**, *insert*), requiring the patient to wait for a re-graft. Corneal inflammation and epithelial defect persisted in patient 2 one month after alkaline burn, and pannus began to invade the cornea (**B1**) despite repeated amniotic membrane dressing (AMD). Following COMET, the ocular surface became quiescent and reepithelialized soon, but superficial NV and residual opacity of cornea persisted one year thereafter (**B2**). At 22 months later, the patient received a CLAU, with the photo taken 30 months postoperatively (**B3**). Ankyloblepharon persisted despite repeated fornix reconstruction with amniotic membrane transplantation (AMT) in patient 3 after thermal injury (**C1**). Two years after COMET (six months after cataract surgery), quiescent ocular surface was achieved with residual central corneal opacity with lower NV ingrowth (**C2**). Three years after COMET (one year after PKP), corneal graft was clear with medial symblepharon not affecting ocular movement (**C3**). Despite repeated AMD and AMT, persistent epithelial defect and NV were noted in patient 4 (**D1**). COMET was performed successfully with residual corneal opacity and NV noted (**D2**), and necessitating CLAU later, which resulted in a clear and intact cornea (**D3**). One month after nitric acid injury and multiple AMD treatments, patient 5 still suffered from severe corneal inflammation, epithelial defect, and secondary glaucoma (**E1**). COMET performed four months after injury was effective to promote reepithelialization, but was unable to improve corneal opacity and NV (**E2**). Vision improved after keratolimbal allograft one year later, followed by PKP and cataract surgery (**E3**). Before referral for acidic injury, AMD had been done in patient 6, but in vain, with corneal inflammation and melting (**F1**). After correction of entropion, COMET was performed four months after injury with residual stromal opacity and NV (**F2**). After CLAU and later DALK, corneal NV regressed and the cornea became transparent (**F3**). The corneal tissues from patients 1–4 and 6 (**A4–D4**, **F4**) showed 5–12 stratified epithelial layers without papillary structures. The basal keratinocytes in patients 1, 2, 3, and 5 were smaller and more compact (**A4**, **B4**, **C4**, **E4**), similar to those found in the papillae of normal oral mucosa. Note that a clear junction zone between oral mucosal epithelium (*left*) and corneal epithelium (*right*) can be seen in patient 3 (**C4**, *insert*, *arrow*). In contrast, suprabasal keratinocytes of patient 5 were loosely organized intermediate layers with vacuoles and ambiguously cornified superficial layers with fewer nuclei in a parakeratinized pattern (**E4**). In addition, superficial peripheral NV was noted occasionally just under the AM in the anterior stroma (**D4**, **E4**; *arrowheads*).

Immunofluorescent Confocal Microscopy

To identify the source of epithelial cells in the corneal tissues, we first performed a screening staining of keratin 8,[12] which was positive in normal corneal and conjunctival epithelia, but was negative in normal oral mucosal epithelium, and treated corneal specimens from patients 1, 2, 4, 5, and 6 (Fig. 2). Interestingly, keratin 8 was positive in the superior half of the specimen from patient 3 (Fig. 2E, right), suggesting a mixed

Table 1. Demographic Information and Clinical Characteristics

Patient/Sex/ Age/Eye	Cause of LSCD	Initial BCVA	Pre-COMET Surgeries	INT-1 (mo)	Post-COMET Surgeries	INT-2 (mo)	Final BCVA	Follow-Up Post-COMET (mo)	Complications
1/M/27/OD	Thermal	HM	AMD, AMT, SK	17	PKP, E	9	CF/30 cm*	56	
2/M/18/OS	Alkaline	20/600	AMD	2	CLAU	22	20/40	55	
3/M/55/OS	Thermal	CF/60 cm	AMD, AMT, FR	31	E + L, PKP	26	20/40	50	
4/F/49/OD	Acidic	20/600	AMD	8	CLAU, E + L	9	20/100	33	
5/M/27/OD	Acidic	HM	AMD, CT	4	KLAL, PKP, E + L	14	20/200	29	Secondary glaucoma
6/M/37/OD	Acidic	CF/30 cm	AMD, AMT, LS	4	CLAU, AMD, LS, DALK	14	20/60	18	

AMD, amniotic membrane dressing; AMT, amniotic membrane transplantation; BCVA, best corrected visual acuity; CF, counting fingers; CT, conjunctival tenoplasty; E, extracapsular cataract extraction; FR, fornix reconstruction; HM, hand movement; INT-1, interval from injury to COMET; INT-2, interval from COMET to CLAU or PKP; L, posterior chamber intraocular lens (implantation); LS, lid surgery; LP, light perception; SK, superficial keratectomy.

* Was 20/200, now pending re-graft due to endothelial failure.

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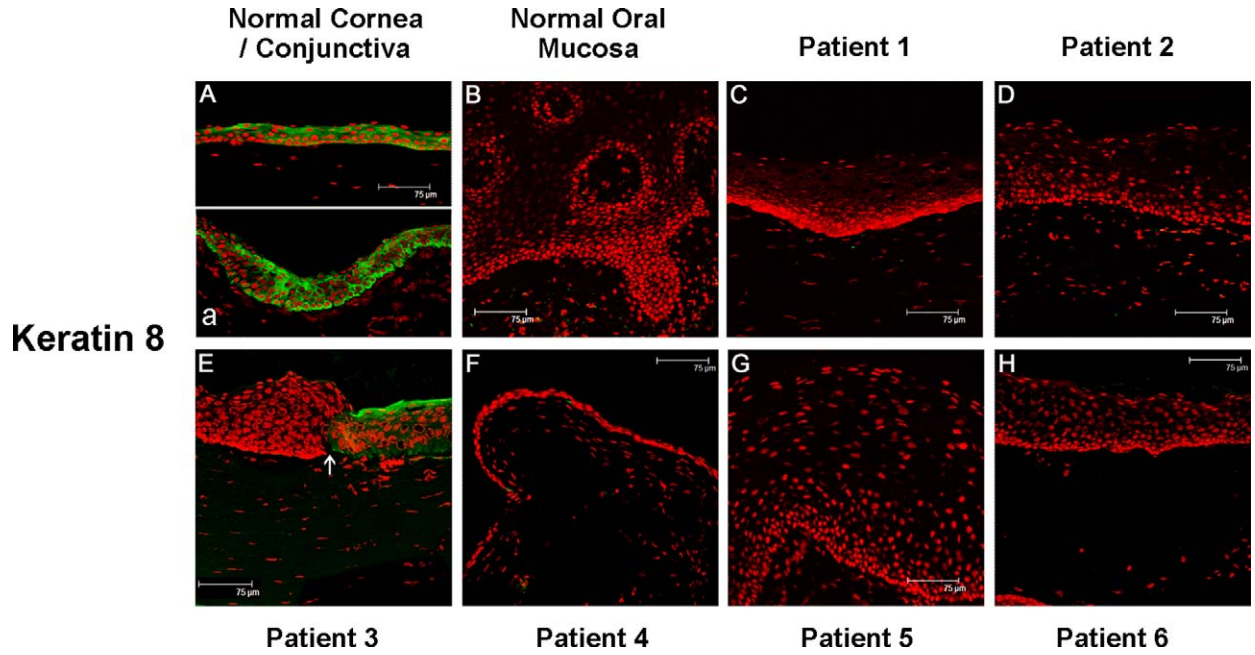

Figure 2. Immunoconfocal microscopy of keratin 8 in normal cornea, conjunctiva, oral mucosa, and corneal tissues from patients 1–6. Cell nuclei were counterstained with PI (*red*). Keratin 8 (*green*) staining was positive in full-thickness corneal (**A**) and conjunctival (**a**) epithelia, but completely negative in the oral mucosal epithelium (**B**). In corneal specimens from patients 1, 2, 4, 5, and 6, keratin 8 was universally negative (**C**, **D**, **F**–**H**). Interestingly, keratin 8 staining was positive in the superior portion of the specimen from patient 3 (**E**, *green*). *Arrow*: indicates the junction of oral mucosal (*left*) and corneal epithelia (*right*).

origin of oral mucosal (inferior side) and corneal epithelia (superior side).

Expression of Angiogenic Factors

FGF-2 was expressed universally in all tissues examined. Specifically, it was expressed in full-thickness normal corneal and conjunctival epithelia, and in specimens from patients 1–4 and 6, while the signal was strong in the basal layer of normal oral mucosal epithelium and specimens from patient 5 (Figs. 3B1, 3G1). Moreover, VEGF also was expressed in full-thickness normal corneal, conjunctival, and oral mucosal epithelia, and in the specimens from all six patients. In all patients, the signal for VEGF also could be detected in the stromal cells and blood vessels (Figs. 3B2, 3C2, 3G2; arrows).

Expression of Antiangiogenic Factors

Endostatin and PEDF are potent endogenous factors opposing angiogenesis, and have been immunolocalized to corneal basement membranes[22] and epithelia.[23] Paradoxically, collagen XVIII (precursor of endostatin) was positive in the basement membrane of all the tissues examined, and the signal also was positive in the blood vessels (Figs. 4A1–4I1). On the other hand, PEDF was expressed in the basal epithelial layer of oral mucosa, and the suprabasal epithelial layer of normal corneal and conjunctival epithelia and specimens from patients 1–4 and 6 (Figs. 4A2–4G2, 4I2). Except for specimens from patients 2, 4, and 6 (Figs. 4E2, 4G2, 4I2), all the other specimens stained rather weakly. From the aforementioned results, it is apparent that endostatin and PEDF were not deficient in vascularized corneas after COMET.

TIMP-3 and TSP-1 are potent antiangiogenic factors abundant in the basement membrane,[17,18] and sFlt generally is accepted as the single most important antiangiogenic factor that maintains corneal avascularity.[18] All of them have been reported in normal corneal epithelium,[22–25] but not in conjunctival and oral mucosal epithelia. In our study, only normal corneal epithelium and samples from patient 3 (superior side) were stained positively for the three markers (Figs. 4A3–4A5, 4F3–4F5). Consistent with the previous reports, the signal for sFlt-1 was positive in the whole layer of corneal epithelium (Figs. 4A3, 4F3), while it was only positive in the basement membrane zone for TIMP-3 and TSP-1 (Figs. 4A4–4A5, 4F4–4F5).

Expression of Anti-Inflammatory Factors

Finally, to explain the late onset (3–6 months) and limited corneal NV after COMET,[13–16] we chose to study the expression of IL-1ra, which previously was found to be expressed constitutively in the human cornea[26] or cultivated OMECs,[27] and has been shown to inhibit corneal NV in mice.[28] Signaling for IL-1ra was positive strongly in the normal corneal epithelium, but only mildly positive in normal conjunctival and oral mucosal epithelia. Overall, signaling for IL-1ra was more prominent in OMECs in the corneal specimens compared to oral mucosa, where the signal was limited only to the basal epithelial layer (Fig. 5B). In patient 3, signal intensity was similar in the corneal epithelium (Fig. 5D) and the OMECs (Fig. 5d). Interestingly, IL-1ra also was expressed very strongly in the basal epithelial layer in that patient (Fig. 5d).

Collectively, expression of all markers used in the specimens from all patients resembled more strongly that of normal oral mucosa or cultivated OMECs (not shown), while expression in the specimens from patient 3 (superior side) was similar to that of normal cornea. Table 2 summarizes the immunostaining pattern of all the studied markers.

Discussion

The advantages of COMET are that it is repeatable (multiple biopsies on oral mucosa possible) and free of rejection, while

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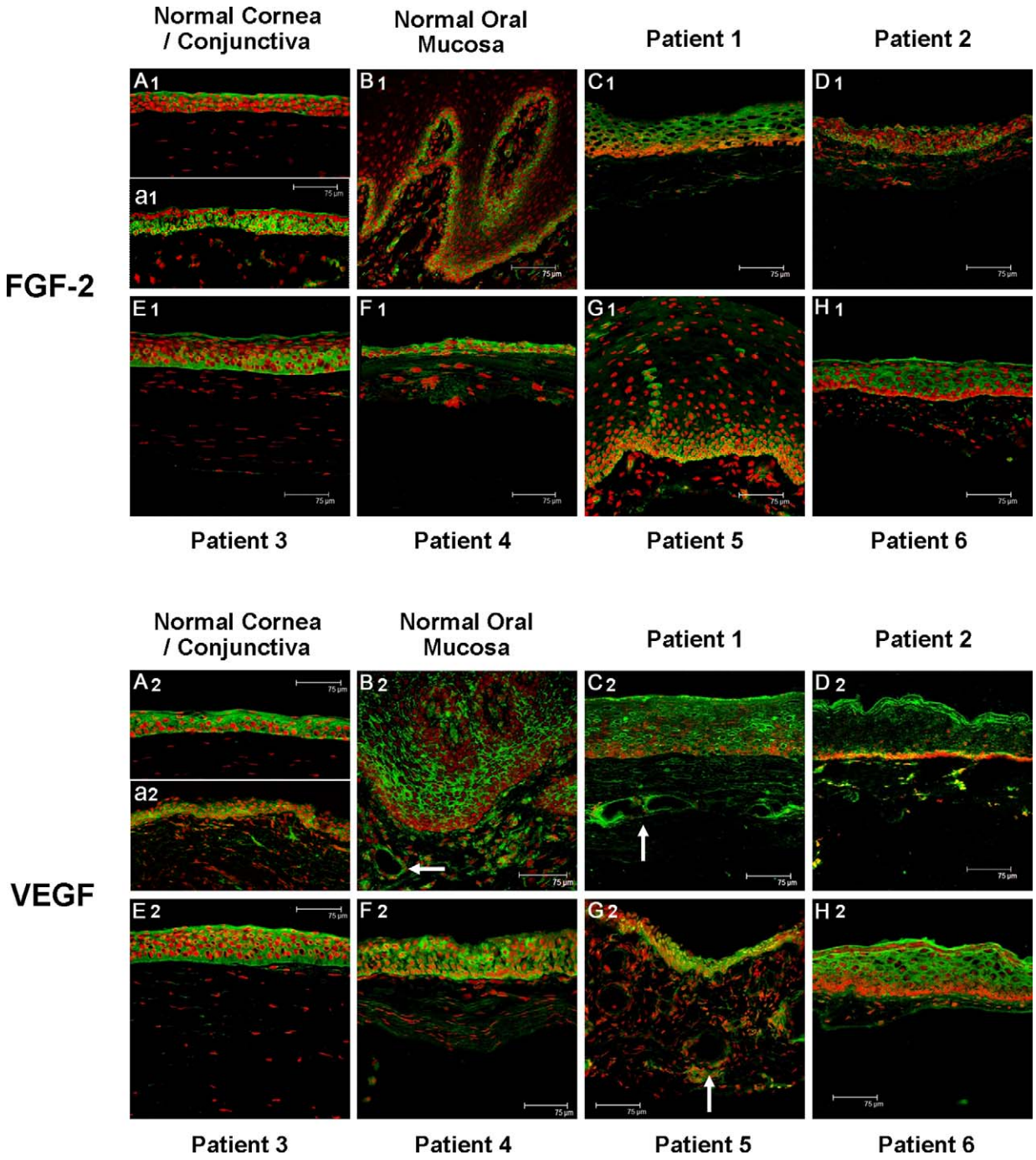

Figure 3. Immunoconfocal microscopy of angiogenic factors in normal cornea, conjunctiva, oral mucosa, and corneal tissues from patients 1–6. Cell nuclei were counterstained with PI (*red*). FGF-2 staining (*green*) was positive in the whole layer of normal corneal (**A1**) and conjunctival epithelia (**a1**), and in specimens from patients 1–4 and 6 (**C1–F1**, **H1**), but was positive only in the basal layer of normal oral mucosal epithelium (**B1**) and specimens from patients 5 (**G1**). VEGF staining (*green*) invariably was positive in the whole layer of normal corneal (**A2**), conjunctival (**a2**), and oral mucosal (**B2**) epithelia, and in all specimens from patients 1–6 (**C2–H2**). In addition to stromal cells, VEGF signaling also was detected in blood vessels in normal oral mucosa, and in specimens from patients 1 and 5 (**B2**, **C2**, **G2**; *arrows*).

the poorer antiangiogenic effect after transplantation is its major drawback. Despite corneal NV developed after COMET, we considered the results in all our patients to be a clinical success, because without COMET, the healing process would have taken a much longer time, and there is a high risk of corneal melting or even perforation. Numerous studies have focused on the mechanisms of corneal NV following LSCD[17,18] or other etiologies,[29] while others have reported differential expression of angiogenesis-related factors in cultivated corneal (CCE) and oral mucosal epithelial cells (COE).[20–22] Additionally, miscellaneous angiogenesis-related factors have been localized immunohistochemically in human corneas,[22–25,30,31] oral mucosa,[22,32–35] and vascularized corneas[23,36,37] (see Supplementary Table S2 for a literature review, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9293/-/DCSupplemental). In our study, we presented the immunohis-

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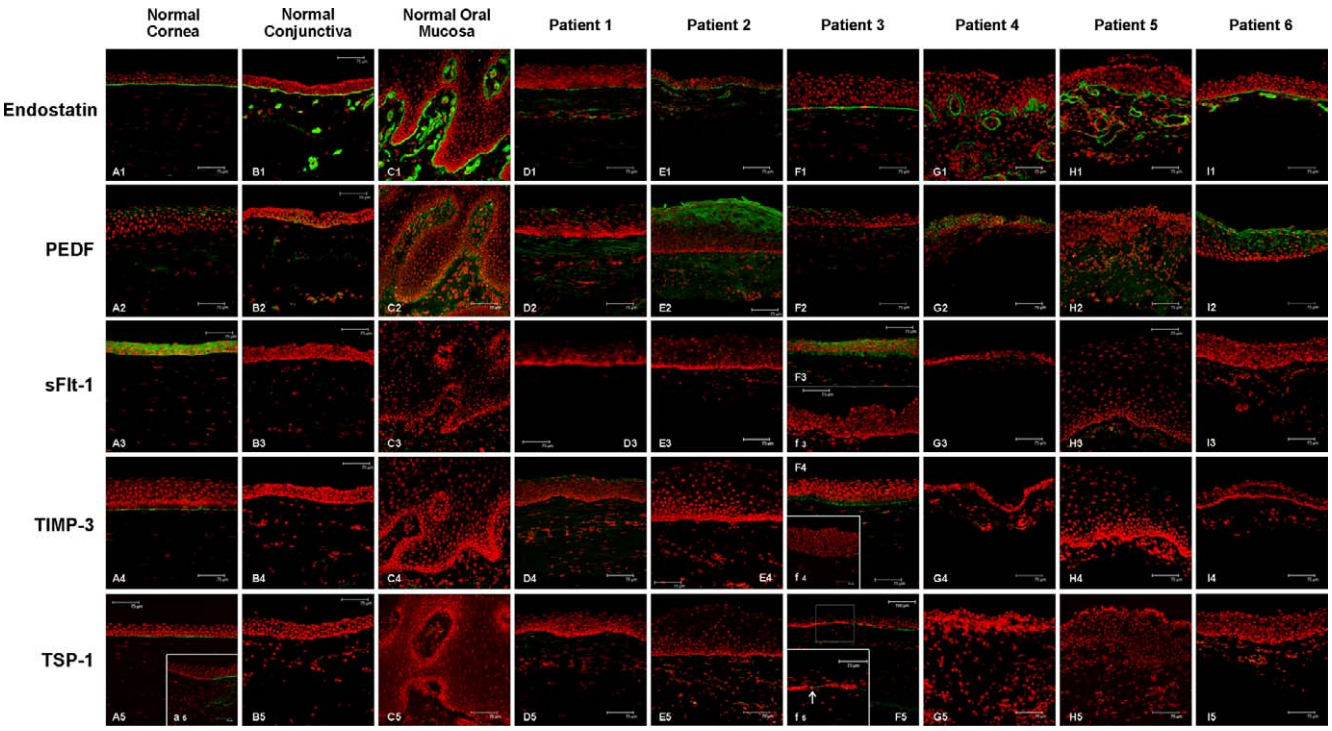

Figure 4. Immunoconfocal microscopy of antiangiogenic factors in normal cornea, conjunctiva, oral mucosa, and corneal tissues from patients 1–6. Cell nuclei were counterstained with PI (*red*). Endostatin staining (*green*) was positive in the basement membrane of normal corneal (**A1**), conjunctival (**B1**), and oral mucosal (**C1**) epithelia, and specimens from patients 1–6 (**D1–I1**). PEDF staining (*green*) was weakly positive in the suprabasal layer of normal corneal (**A2**) and conjunctival (**B2**) epithelia, and specimens from patients 1–6 (**D2–I2**), as well as in the basal layer of oral mucosal epithelium (**C2**). Paradoxically, PEDF staining was stronger in specimens from patients 2, 4, and 6 (**E2**, **G2**, **I2**). sFlt-1 staining (*green*) was positive only in the whole layer of normal corneal epithelium (**A3**) and the superior part of the specimen from patients 3 (**F3**), while it was negative in normal conjunctiva (**B3**), oral mucosa (**C3**) and specimens from patients 1–6 (**D3**, **E3**, **G3–I3**, **f3**, inferior cornea in patient 3). TIMP-3 staining (*green*) was positive only in the basement membrane of normal corneal epithelium (**A4**) and the superior part of the specimen from patient 3 (**F4**), while it was negative in normal conjunctiva (**B4**), oral mucosa (**C4**), and specimens from patients 1–6 (**D4**, **E4**, **G4–I4**, **f4**, inferior cornea in patient 3). TSP-1 staining (*green*) was positive only in the basement membrane of normal corneal epithelium (**A5**), and the corneal epithelium in the specimen from patient 3 (**F5**, **f5**, *right*; *arrow* in *insert* points to the junction of oral mucosal and corneal epithelia), while the signal was negative in the conjunctival side of normal limbus (*insert* **a5**, *left*), normal conjunctival (**B5**) and oral mucosal epithelia (**C5**), and treated corneal specimens from patients 1–6 (**D5–I5**), including the oral mucosal epithelium in patient 3 (**F5** and **f5**, *left*).

tochemical result of a panel of angiogenesis-related factors in chemically- and thermally-injured corneas after COMET. To our knowledge, this is the first clinicopathologic report of this type and will extend the body of evidence on the formation of corneal NV after COMET.

Seikiyama et al. reported that endostatin, PEDF, and TSP-1 stain more intensely in CCE than COE, and that there is no obvious difference in the expression of angiostatin, FGF-2, Flt-1, KDR (VEGFR-2), and VEGF between CCE and COE. Additional Western blot analysis confirmed the expression of TSP-1 to be significantly higher in CCE than COE.[19] The same group indicated that TSP-1 also is the only antiangiogenic factor expressed solely by the corneal, but not conjunctival epithelia.[20] In our study, TSP-1 was not detected in normal conjunctiva and oral mucosa, but was detected beneath normal corneal epithelium, consistent with the findings of Sekiyama et al.[20] and others.[24] Negative staining of keratin 8 and TSP-1 in almost all specimens suggested an oral mucosa-originated epithelia, and explains the inferior antiangiogenic property of COMET.

Kanayama et al. had reported that significantly more mRNA and protein of FGF-2 can be secreted by COE than CCE, and that anti-FGF-2 neutralizing antibody can reverse significantly the in vitro angiogenesis promoted by COE.[21] On the other hand, normal corneal epithelial basement membrane is rich in antiangiogenic factors[18] (e.g., endostatin, TIMP-3, and TSP-1) and may sequester major angiogenic factors (VEGF and FGF-2) that have high affinity to heparan sulfate proteoglycan.[38] The ubiquitous expression of FGF-2 in normal corneal, conjunctival, and oral mucosal epithelia, as well as in specimens from all our patients indicated that FGF-2 may not be a determining factor for the angiogenic tendency after COMET. Intriguingly, the full-thickness staining of FGF-2 in our normal corneas, conjunctiva, and samples from patients 1, 2, 3, 4, and 6 (Figs. 3A1, 3a1, 3C1–3F1, 3H1) differs from the basal staining in one report,[20] but the basal staining of FGF-2 in our normal oral mucosa and sample from patient 5 (Figs. 3B1, 3G1) is consistent with that in other reports.[32,34] Taken together, these findings indicated that FGF-2 alone may not have a major role in the angiogenesis after COMET.

VEGF is a highly potent angiogenic factor that acts to increase vascular permeability and endothelial growth, proliferation, migration, and differentiation[17,18] It has been reported that there is no difference in the expression of VEGF mRNA[21] and protein[20,21] between CCE and COE, while immunostaining of VEGF has been documented in normal corneas.[20,31] oral mucosa,[20,35] and vascularized corneas.[36,37] On the other hand, subconjunctival injection of bevacizumab (Avastin, a humanized anti-VEGF antibody) has shown short-term beneficial effects for corneal NV secondary to graft rejection,[39] lipid keratopathy,[40] or other causes.[41] We, thus, postulate that postoperative Avastin injection might be an effective way to

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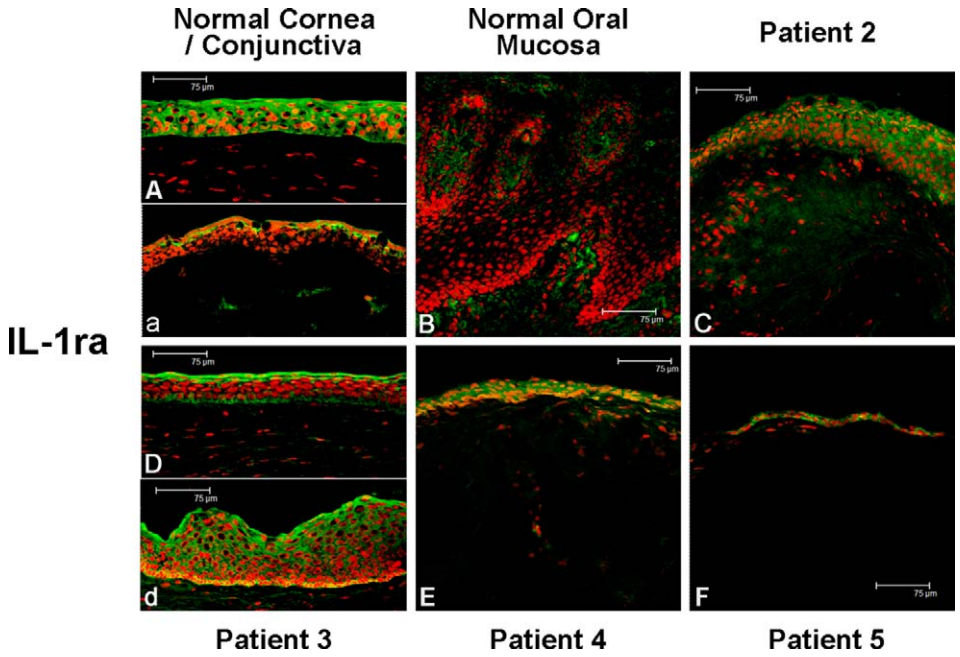

Figure 5. Immunoconfocal microscopy of IL-1ra in normal cornea, conjunctiva, oral mucosa, and corneal tissues from patients 2, 3, 4, and 5. Cell nuclei were counterstained with PI (*red*). IL-1ra staining (*green*) was positive markedly in the whole layer of normal corneal epithelium (**A**), moderately-to-markedly positive in normal conjunctival epithelium (**a**), and specimens from patients 2, 3, 4, and 5 (**C–F**), while it was only weakly positive in the basal epithelial layer of normal oral mucosa (**B**). Intensity of IL-1ra signaling was not different in either superior (**D**) or inferior part (**d**) of the specimen from patient 3; however, the signal was strongly positive in the basal epithelial layer (**d**).

treat NV after COMET. Nevertheless, the universal existence of VEGF even in normal corneas (Fig. 3A2) suggests that the mechanism controlling VEGF activity may have a more important role in the NV formation after COMET. It now is well recognized that sFlt-1, the soluble VEGF-A receptor-1 secreted by the corneal epithelium, perhaps is the single most important antiangiogenic factor that regulates corneal avascularity.[42] In our study, the total lack of signal for sFlt-1 in conjunctiva and oral mucosa is in sharp contrast with the prominent staining in corneal epithelia in normal corneas and the treated sample of patient 3 (Fig. 4F3). The significantly higher level of sFlt-1 secreted by CCE than by COE as shown by Kanayama et al.[21] may suggest that as a decoy for VEGF, sFlt-1 limits the activity of VEGF in normal corneal epithelia.

Despite potent antiangiogenic capacity,[17,18] the expression patterns of endostatin in all of our specimens were quite similar, being localized to the basement membrane zone, which is compatible with previous studies.[24,33] This again suggests that endostatin is only a minor determining factor in angiogenesis after COMET. Likewise, the expression patterns of PEDF, both a neurotrophic and antiangiogenic factor,[17,18] in our study and that of Sekiyama et al.[20] do not suggest that it is a critical element contributing to the differing levels of angiogenic activity. However, one intriguing observation is that although signals for FGF-2, PEDF, and IL-1ra all were

Table 2. Immunohistochemical Localization of Angiogenesis- and Inflammation-Related Factors

Related Markers	Normal Cornea	Normal Conjunctiva	Normal Oral Mucosa	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Ocular surface									
Keratin 8	+	+	-	-	-	+*	-	-	-
Angiogenic									
FGF-2	+	+	-/+	+	+	+	+	-/+	+
VEGF	+	+	+	+	+	+	+	+	+
Antiangiogenic									
Endostatin	BMZ	BMZ	BMZ	BMZ	BMZ	BMZ	BMZ	BMZ	BMZ
PEDF	+/-†	+/-†	-/+†	+/-†	+/-	+/-†	+/-	-	+/-
sFlt-1	+	-	-	-	-	+*	-	-	-
TIMP-3	BMZ	-	-	-	-	BMZ*	-	-	-
TSP-1	BMZ	-	-	-	-	BMZ*	-	-	-
Anti-inflammatory									
IL-1ra	+	+	+†	NA	+	+/++	+	+	++

BMZ, basement membrane zone; NA, not available; +, positive in full-thickness epithelium; -, negative in full-thickness epithelium; +/-, positive in superficial epithelial layers; -/+, positive in basal epithelial layer; +/++, more prominent in the basal than in the suprabasal layer.

* Only in the corneal epithelium in superior part of the specimen.

† Weak staining.

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localized to the basal layer of oral mucosa, distribution of these factors after transplantation was more similar to that of corneal epithelium as long as the OMECs stratification was cornea-like, while the staining for FGF-2 and IL-1ra was limited to the basal layer when the stratification was oral mucosa-like. These phenomena are demonstrated best by the samples from patients 5 (Fig. 3G1) and 3 (Fig. 5d). We still do not know whether this implies that OMECs in the cornea undergo a partial transformation to adapt to the new environment, nor do we know the mechanism explaining why the stratification of OMECs varied greatly among patients, as during cultivation the cell sheets were submerged just to prevent stratification and differentiation. Presumably, hyper-stratification may be related to a more severe corneal NV formation, as was seen in patients 3 (lower part of the specimen) and 5.

Like endostatin and TSP-1, TIMP-3, one of the four natural inhibitors that control the activity of matrix metalloproteinases,[17] is abundant in corneal basement membrane.[18,25] To our knowledge, there is no prior report of TIMP-3 in normal oral mucosa, but it has been reported in oral cancer[43] and normal corneas,[25] and to a lesser degree[44] or not at all in normal conjunctiva.[45] Given the positive staining for sFlt-1, TSP-1, TIMP-3, and also keratin 8 (positive in the ocular surface epithelia[12]), we postulated that the epithelium in the superior region of the sample from patient 3 is of corneal lineage, and that a deficiency in these antiangiogenic factors may be responsible for the inferior antiangiogenic effect after COMET evidenced by NV ingrowth into the inferior part of the cornea (Fig. 1C2).

IL-1ra is a potent endogenous inhibitor of inflammatory cytokine IL-1, and is thought to be responsible for the anti-inflammatory activity of normal corneal epithelium.[26] Using RT-PCR or cDNA microarray, IL-1ra has been found to be expressed by human amniotic cells[46] and significantly upregulated in limbal epithelial cells expanded on intact human amniotic membrane.[47] Expression of IL-1ra in oral mucosa also has been reported,[27] but we found only weak staining in the basal layer of oral mucosal epithelia. However, IL-1ra was expressed abundantly by the OMECs in the corneal specimens, especially in the basal epithelium, which presumably contains the stem cells (Fig. 5d). Coxon et al. reported the inhibitory effect of IL-1ra on bFGF- and VEGF-induced corneal angiogenesis.[48] Presumably, IL-1ra in OMECs helps to reduce corneal inflammation after transplantation and alleviate corneal neovascularization, which otherwise would be more extensive.

For the regulation of corneal angiogenesis, the interaction between transplanted OMECs and preexisting ocular surface epithelial cells cannot be neglected. Since severe inflammation is detrimental to the survival of corneal epithelial cells,[49] and since COMET provides an alternative source of epithelial cells that express IL-1ra, it is possible that transplanted OMECs help to reduce corneal inflammation, which in turn revives the remaining limbal stem cells. With the abundant expression of sFlt-1, TSP-1, and TIMP-3, these remaining corneal epithelial cells may contribute to the antiangiogenic effect. Alternatively, epithelium-trophic factors (such as FGF-2) secreted by OMECs may provide another possible mechanism by which to improve the viability of the residual corneal epithelial cells.

The major drawback of the study is that, by its very design, our study is limited to be a descriptive phenotypic study. Nevertheless, our study uses rare tissue samples, and the results do give some insight into the mechanism by which COMET may fail to prevent postoperative corneal neovascularization. In summary, we demonstrated the expression of angiogenesis-related factors in corneas after COMET. The lack of sFlt-1, TIMP-3, and TSP-1 may be responsible for inferior antiangiogenic activity after COMET. However, the implication of preferential expression of IL-1ra in the basal OMECs, and the question of whether or not OMECs also can express other anti-inflammatory factors, like IL-4 or IL-10, still await further investigation.

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References

1. Notara M, Alatza A, Gilfillan J, et al. In sickness and in health: corneal epithelial stem cell biology, pathology and therapy. *Exp Eye Res*. 2010;90:188–195.
2. Nishida K. Tissue engineering of the cornea. *Cornea*. 2003;22: S28–S34.
3. Shortt AJ, Tuft SJ, Daniels JT. Ex vivo cultured limbal epithelial transplantation. A clinical perspective. *Ocul Surf*. 2010;8:80–90.
4. Cauchi PA, Ang GS, Azuara-Blanco A, Burr JM. A systematic literature review of surgical interventions for limbal stem cell deficiency in humans. *Am J Ophthalmol*. 2008;146:251–259.
5. Rama P, Matuska S, Paganoni G, Spinelli A, De Luca M, Pellegrini G. Limbal stem-cell therapy and long-term corneal regeneration. *N Engl J Med*. 2010;363:147–155.
6. Miri A, Al-Deiri B, Dua HS. Long-term outcomes of autolimbal and allolimbal transplants. *Ophthalmology*. 2010;117:1207–1213.
7. Satake Y, Higa K, Tsubota K, Shimazaki J. Long-term outcome of cultivated oral mucosal epithelial sheet transplantation in treatment of total limbal stem cell deficiency. *Ophthalmology*. 2011;118:1524–1530.
8. Nakamura T, Takeda K, Inatomi T, Sotozono C, Kinoshita S. Long-term results of autologous cultivated oral mucosal epithelial transplantation in the scar phase of severe ocular surface disorders. *Br J Ophthalmol*. 2011;95:942–946.
9. Kawashima M, Kawakita T, Satake Y, Higa K, Shimazaki J. Phenotypic study after cultivated limbal epithelial transplantation for limbal stem cell deficiency. *Arch Ophthalmol*. 2007;125:1337–1344.
10. Pauklin M, Steuhl KP, Meller D. Characterization of the corneal surface in limbal stem cell deficiency and after transplantation of cultivated limbal epithelium. *Ophthalmology*. 2009;116:1048–1056.
11. Nakamura T, Inatomi T, Cooper LJ, Rigby H, Fullwood NJ, Kinoshita S. Phenotypic investigation of human eyes with transplanted autologous cultivated oral mucosal epithelial sheets for severe ocular surface diseases. *Ophthalmology*. 2007;114:1080–1088.
12. Chen HC, Chen HL, Lai JY, et al. Persistence of transplanted oral mucosal epithelial cells in human cornea. *Invest Ophthalmol Vis Sci*. 2009;50:4660–4668.
13. Ma DH, Kuo MT, Tsai YJ, et al. Transplantation of cultivated oral mucosal epithelial cells for severe corneal burn. *Eye*. 2009;23:1442–1450.
14. Nishida K, Yamato M, Hayashida Y, et al. Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. *N Engl J Med*. 2004;351:1187–1196.
15. Nakamura T, Inatomi T, Sotozono C, Amemiya T, Kanamura N, Kinoshita S. Transplantation of cultivated autologous oral mucosal epithelial cells in patients with severe ocular surface disorders. *Br J Ophthalmol*. 2004;88:1280–1284.
16. Inatomi T, Nakamura T, Koizumi N, Sotozono C, Yokoi N, Kinoshita S. Midterm results on ocular surface reconstruction using cultivated autologous oral mucosal epithelial transplantation. *Am J Ophthalmol*. 2006;141:267–275.

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17. Ma DH, Chen JK, Zhang F, Lin KY, Yao JY, Yu JS. Regulation of corneal angiogenesis in limbal stem cell deficiency. *Prog Retin Eye Res*. 2006;25:563–590.
18. Ma DH, Chen HC, Lai JY, et al. Matrix revolution: molecular mechanism for inflammatory corneal neovascularization and restoration of corneal avascularity by epithelial stem cell transplantation. *Ocul Surf*. 2009;7:128–144.
19. Sekiyama E, Nakamura T, Cooper LJ, et al. Unique distribution of thrombospondin-1 in human ocular surface epithelium. *Invest Ophthalmol Vis Sci*. 2006;47:1352–1358.
20. Sekiyama E, Nakamura T, Kawasaki S, Sogabe H, Kinoshita S. Different expression of angiogenesis-related factors between human cultivated corneal and oral epithelial sheets. *Exp Eye Res*. 2006;83:741–746.
21. Kanayama S, Nishida K, Yamato M, et al. Analysis of angiogenesis induced by cultured corneal and oral mucosal epithelial cell sheets in vitro. *Exp Eye Res*. 2007;85:772–781.
22. Kanayama S, Nishida K, Yamato M, et al. Analysis of soluble vascular endothelial growth factor receptor-1 secreted from cultured corneal and oral mucosal epithelial cell sheets in vitro. *Br J Ophthalmol*. 2009;93:263–267.
23. Ambati BK, Patterson E, Jani P, et al. Soluble vascular endothelial growth factor receptor-1 contributes to the corneal antiangiogenic barrier. *Br J Ophthalmol*. 2007;91:505–508.
24. Schlotzer-Schrehardt U, Dietrich T, Saito K, et al. Characterization of extracellular matrix components in the limbal epithelial stem cell compartment. *Exp Eye Res*. 2007;85:845–860.
25. Kenney MC, Chwa M, Alba A, Saghizadeh M, Huang ZS, Brown DJ. Localization of TIMP-1, TIMP-2, TIMP-3, gelatinase A and gelatinase B in pathological human corneas. *Curr Eye Res*. 1998;17:238–246.
26. Kennedy MC, Rosenbaum JT, Brown J, et al. Novel production of interleukin-1 receptor antagonist peptides in normal human cornea. *J Clin Invest*. 1995;95:82–88.
27. Perrier S, Kherratia B, Deschaumes C, et al. IL-1ra and IL-1 production in human oral mucosal epithelial cells in culture: differential modulation by TGF-beta1 and IL-4. *Clin Exp Immunol*. 2002;127:53–59.
28. Dana MR, Dai R, Zhu S, Yamada J, Streilein JW. Interleukin-1 receptor antagonist suppresses Langerhans cell activity and promotes ocular immune privilege. *Invest Ophthalmol Vis Sci*. 1998;39:70–77.
29. Ellenberg D, Azar DT, Hallak JA, et al. Novel aspects of corneal angiogenic and lymphangiogenic privilege. *Prog Retin Eye Res*. 2010;29:208–248.
30. Karakousis PC, John SK, Behling KC, et al. Localization of pigment epithelium derived factor (PEDF) in developing and adult human ocular tissues. *Mol Vis*. 2001;7:154–163.
31. van Setten GB. Vascular endothelial growth factor (VEGF) in normal human corneal epithelium: detection and physiological importance. *Acta Ophthalmol Scand*. 1997;75:649–652.
32. Partridge M, Kiguwa S, Luqmani Y, Langdon JD. Expression of bFGF, KGF and FGF receptors on normal oral mucosa and SCC. *Eur J Cancer B Oral Oncol*. 1996;32B:76–82.
33. Vaananen A, Ylipalosaari M, Parikka M, et al. Collagen XVIII modulation is altered during progression of oral dysplasia and carcinoma. *J Oral Pathol Med*. 2007;36:35–42.
34. Wakulich C, Jackson-Boeters L, Daley TD, Wysocki GP. Immunohistochemical localization of growth factors fibroblast growth factor-1 and fibroblast growth factor-2 and receptors fibroblast growth factor receptor-2 and fibroblast growth factor receptor-3 in normal oral epithelium, epithelial dysplasias, and squamous cell carcinoma. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2002;93:573–579.
35. Johnstone S, Logan RM. Expression of vascular endothelial growth factor (VEGF) in normal oral mucosa, oral dysplasia and oral squamous cell carcinoma. *Int J Oral Maxillofac Surg*. 2007;36:263–266.
36. Philipp W, Speicher L, Humpel C. Expression of vascular endothelial growth factor and its receptors in inflamed and vascularized human corneas. *Invest Ophthalmol Vis Sci*. 2000;41:2514–2522.
37. Cursiefen C, Rummelt C, Küchle M. Immunohistochemical localization of vascular endothelial growth factor, transforming growth factor alpha, and transforming growth factor beta1 in human corneas with neovascularization. *Cornea*. 2000;19:526–533.
38. Iozzo RV. San Antonio JD. Heparan sulfate proteoglycans: heavy hitters in the angiogenesis arena. *J Clin Invest*. 2001;108:349–355.
39. Vassileva PI, Hergeldzhieva TG. Avastin use in high risk corneal transplantation. *Graefes Arch Clin Exp Ophthalmol*. 2009;247:1701–1706.
40. Chu HS, Hu FR, Yang CM, et al. Subconjunctival injection of bevacizumab in the treatment of corneal neovascularization associated with lipid deposition. *Cornea*. 2011;30:60–66.
41. Bahar I, Kaiserman I, McAllum P, Rootman D, Slomovic A. Subconjunctival bevacizumab injection for corneal neovascularization. *Cornea*. 2008;27:142–147.
42. Ambati BK, Nozaki M, Singh N, et al. Corneal avascularity is due to soluble VEGF receptor-1. *Nature*. 2006;443:993–997.
43. Sutinen M, Kainulainen T, Hurskainen T, et al. Expression of matrix metalloproteinases (MMP-1 and -2) and their inhibitors (TIMP-1, -2 and -3) in oral lichen planus, dysplasia, squamous cell carcinoma and lymph node metastasis. *Br J Cancer*. 1998;77:2239–2245.
44. Ng J, Coroneo MT, Wakefield D, Di Girolamo N. Ultraviolet radiation and the role of matrix metalloproteinases in the pathogenesis of ocular surface squamous neoplasia. *Invest Ophthalmol Vis Sci*. 2008;49:5295–5306.
45. Terai N, Schlötzer-Schrehardt U, Lampel J, et al. Effect of latanoprost and timolol on the histopathology of the human conjunctiva. *Br J Ophthalmol*. 2009;93:219–224.
46. Hao Y, Ma DH, Hwang DG, Kim WS, Zhang F. Identification of antiangiogenic and antiinflammatory proteins in human amniotic membrane. *Cornea*. 2000;19:348–352.
47. Sun CC, Su Pang JH, Cheng CY, et al. Interleukin-1 receptor antagonist (IL-1RA) prevents apoptosis in ex vivo expansion of human limbal epithelial cells cultivated on human amniotic membrane. *Stem Cells*. 2006;24:2130–2139.
48. Coxon A, Bolon B, Estrada J, et al. Inhibition of interleukin-1 but not tumor necrosis factor suppresses neovascularization in rat models of corneal angiogenesis and adjuvant arthritis. *Arthritis Rheum*. 2002;46:2604–2612.
49. Tsai RJ, Tseng SC. Effect of stromal inflammation on the outcome of limbal transplantation for corneal surface reconstruction. *Cornea*. 1995;14:439–449.

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