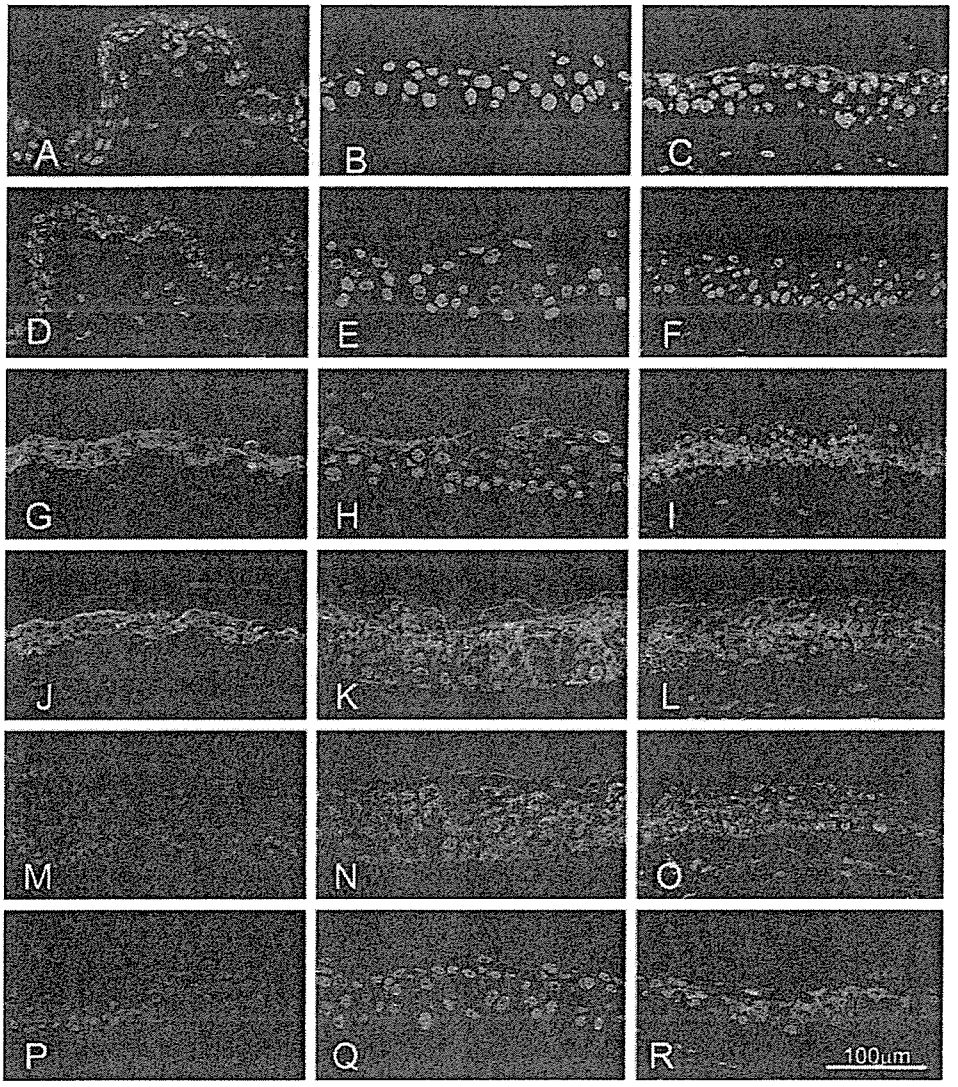

FIGURE 7. Immunohistochemical examination of the engrafted epithelium. In vivo HCjE (**A**, **D**, **G**, **J**, **M**, **P**), cultivated HCjE (**B**, **E**, **H**, **K**, **N**, **Q**), and engrafted epithelium (**C**, **F**, **I**, **L**, **O**, **R**) were immunostained (*green*) with MUC4 (**A**–**C**), MUC5AC (**D**–**F**), CK4 (**G**–**I**), CK13 (**J**–**L**), CK3 (**M**–**O**), or CK12 (**P**–**R**) and counterstained with propidium iodide (*red*).

ment. Because we were dealing with xenotransplantation, one of the limitations of this study is the short follow-up period of 14 days. With more prolonged follow-up, it may be that some conjunctival cells would differentiate into goblet cells and that progressive conjunctivalization and neovascularization would occur. More long-term studies are needed to investigate some of these questions.

In summary, ours is the first report that clearly demonstrates the potential of cultivated HCjE as an alternative tissue source for replacement of the corneal epithelium. Our animal study is a step toward the eventual transplantation of autologous cultivated HCjE to treat patients with ocular surface disorders, and studies are ongoing to resolve outstanding issues.

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Ocular Surface Reconstruction With Combination of Cultivated Autologous Oral Mucosal Epithelial Transplantation and Penetrating Keratoplasty

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• PURPOSE: To report an assessment of the two-step surgical combination of cultivated autologous oral mucosal epithelial transplantation (COMET) and penetrating keratoplasty (PKP) used to treat patients with severe limbal deficiency disorders, and to investigate the keratin expression patterns of transplanted surviving oral mucosal epithelium.

• DESIGN: Observational case series.

• METHODS: Two patients with Stevens-Johnson syndrome and chemical eye injury were treated by COMET followed, approximately six months later, by a PKP triple procedure. In the course of a mean follow-up period of 22.5 months, their clinical outcomes and the efficacy of this two-step surgical procedure were assessed. In addition, the keratin expression in corneal buttons excised during PKP were immunohistochemically examined to characterize the oral mucosal epithelium that survived ectopically on the cornea. In vivo laser confocal microscopy was used to investigate the structure of the epithelium on the corneal grafts.

• RESULTS: The ocular surfaces were successfully reconstructed with cultivated autologous oral mucosal epithelial sheets and PKP. No clinical complications, such as persistent epithelial defects, rejections, or recurrence of cicatrization, were encountered. Postoperative best-corrected visual acuity was 20/125 in one patient and 20/100 in the other. The surviving oral mucosal epithelium, distinguished by its fluorescence pattern, consisted of an irregular, nonkeratinized, stratified epithelium without goblet cells. Immunohistochemical study demonstrated that K3, but not K12, was expressed in the transplanted cultivated oral mucosal epithelium that was similar to oral mucosal tissue. In vivo, the epithelial structure and cell density in the basal cell layer of the corneal grafts were similar to normal cornea.

• CONCLUSIONS: This study presents a two-step surgical approach to treat severely scarred ocular surfaces by means of a combination of COMET and PKP. Clinical outcomes suggest that this treatment may be beneficial for the maintenance of the reconstructed ocular surface by providing oral mucosal epithelium around the corneal graft. (Am J Ophthalmol 2006;xx:xxx. © 2006 by Elsevier Inc. All rights reserved.)

BECAUSE SEVERE STEM CELL DEFICIENCY IS SOMETIMES accompanied by severe corneal stromal opacity and/or corneal endothelial dysfunction, most patients require penetrating keratoplasty (PKP) for visual rehabilitation. However, ocular surface reconstruction through corneal epithelial transplantation and PKP increases the risk for immunologic rejection and graft failure, and patients require long-term intensive immunosuppression and continuous care.[1,2]

Another clinical problem encountered in ocular surfaces reconstructed with PKP is the persistence of an epithelial defect after loss of the donor corneal epithelium. PKP without epithelial transplantation results in persistent epithelial defects as a result of the limited life span of the donor central corneal epithelium, especially in patients with limbal deficiency; the resultant graft-melting and conjunctival invasion severely compromises visual recovery. Therefore, to improve the clinical outcome and long-term

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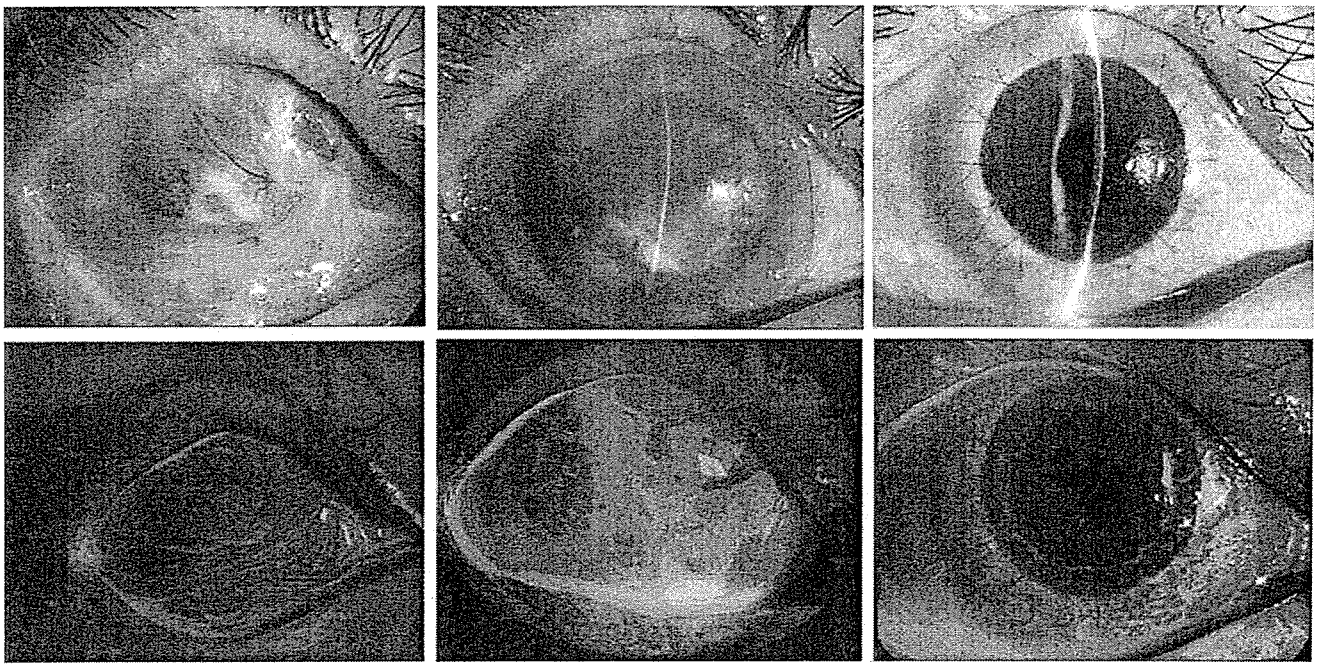

FIGURE 1. Clinical appearance before and after ocular surface reconstruction using cultivated autologous oral mucosal epithelial transplantation (COMET) and penetrating keratoplasty (PKP) in patient 1, a 70-year-old man with chemical injury. (Top left) Preoperatively, there is total conjunctivalization with severe scarring of both the cornea and conjunctiva. (Bottom left) Fluorescein staining. (Top center) Two months after initial surgery with COMET. (Bottom center) Uneven, hyperfluorescein staining pattern indicates survival of oral mucosal epithelium on ocular surface. (Top right) Status three months after PKP with cataract surgery. (Bottom right) Fluorescein staining demonstrated the slow invasion of oral mucosal epithelium surrounding the corneal graft.

prognosis of these patients, their reconstructed ocular surfaces must be provided with a more stable epithelial supply.

Pellegrini and associates[3] first reported the transplantation of cultivated corneal epithelium. Subsequent technical and surgical advances have made possible the grafting of cultivated corneal epithelial stem cell sheets.[4–8] Nakamura and associates[9] reported the successful transplantation of cultivated mucosal epithelial stem cell sheets derived from autologous cell sources. Autologous conjunctival epithelium[10,11] and nonocular (for example, oral mucosal) epithelium[12] have been used as a cell source for the cultivation of grafts to treat patients with bilateral ocular disorders. Because of its high proliferation potential, short cell-turnover time, and the safety of oral biopsy, oral mucosal epithelium has attracted attention as a cell source.[13,14] Initial clinical studies and midterm assessments of cultivated autologous oral mucosal epithelial transplantation (COMET) yielded favorable results from the perspective of ocular surface stabilization and visual recovery.[15–17] However, the cell biology and the longevity of surviving oral mucosal epithelium on the ocular surface require further investigation.

This study presents a two-step surgical strategy that uses a combination of COMET and PKP. The ocular surface was stable and the cornea remained transparent after the transplantation of cultivated oral mucosal epithelium when this two-step process is used. This surgical strategy reconstructs the ocular surface by transplanting a corneal graft that is surrounded by ectopically transplanted autologous oral mucosal epithelium just after the second PKP surgery, and the ectopically transplanted autologous oral mucosal epithelium may gradually cover the graft surface. This offers the potential for supplying mucosal epithelium for prolonged periods, and this high proliferation potential could possibly address the issue of wound healing. There is no direct evidence to date that oral mucosal epithelium would display a higher level of proliferation than ocular surface epithelium, but previous studies have demonstrated that oral mucosal epithelium has a high proliferation potential compared with epidermal cells.[13,14] On the basis of the condition of the oral mucosal epithelium, it is worth noting that this surgical concept and modality appear to have improved the clinical outcome of ocular surface disease that previously had a poor prognosis, although the follow-up period after PKP is relatively short.

METHODS

THIS STUDY WAS APPROVED BY THE INSTITUTIONAL REview Board for Human Studies of Kyoto Prefectural University of Medicine, and prior informed consent was obtained from all patients in accordance with the tenets of

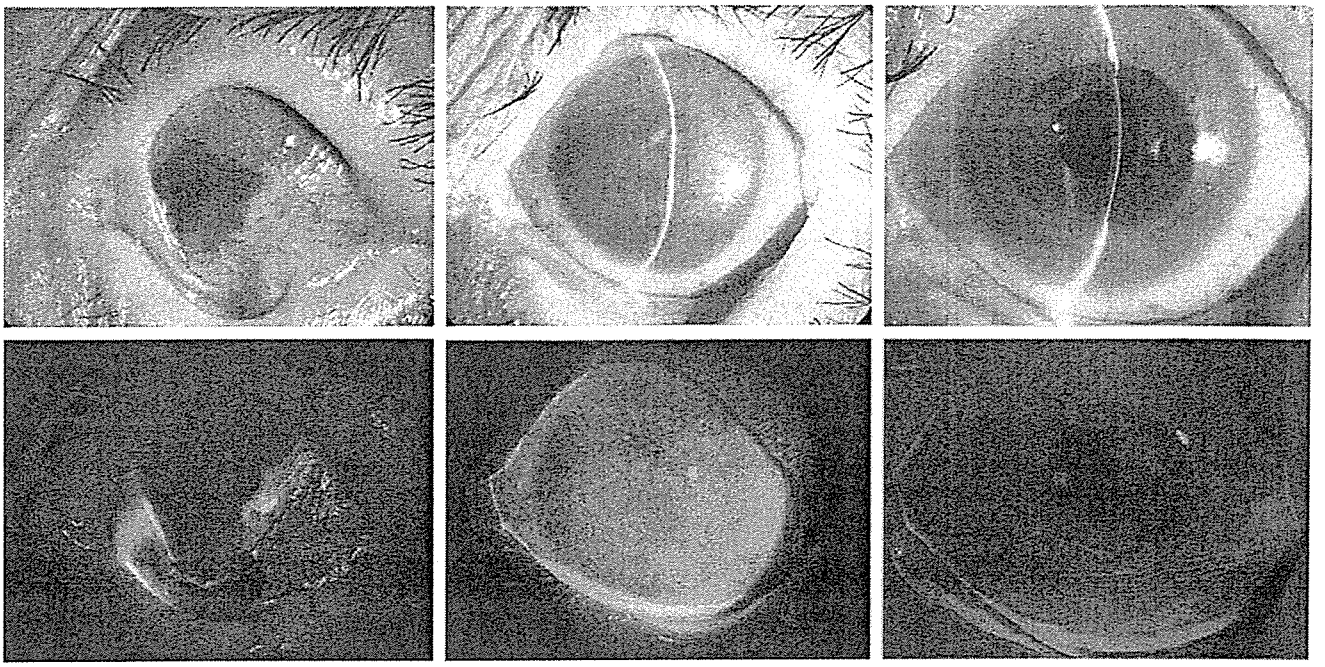

FIGURE 2. Clinical appearance before and after reconstruction using cultivated autologous oral mucosal epithelial transplantation (COMET) and penetrating keratoplasty (PKP) in patient 2, a 71-year-old man with Stevens-Johnson syndrome. (Top left) Preoperative total conjunctivalization with severe symblepharon and partial parakeratinization. (Bottom left) Fluorescein staining. (Top center) Two months after the initial surgery with COMET. (Bottom center) Fluorescein staining of surviving oral mucosal epithelium distinguishes between corneal and conjunctival epithelium. (Top right) Three months after PKP with cataract surgery. (Bottom right) Fluorescein staining demonstrated the presence of thicker oral mucosal epithelium surrounding the corneal graft.

the Declaration of Helsinki for research involving human subjects. This study involved two patients with bilateral total limbal deficiency; their ages were 70 and 71 years, respectively. The primary reason for their limbal deficiency and cicatrization was severe chemical injury and Stevens-Johnson syndrome. Both patients manifested severe destruction of the ocular surface; limbal deficiency was unequivocally diagnosed on the basis of the total replacement by scarred conjunctival tissue and the complete absence of the palisades of Vogt (Figures 1 and 2). Minimum reflex tearing was noted by slit-lamp examination and the Schirmer test, and there was sufficient meniscus height to maintain a wet mucous surface. Both patients presented severe scarring involving the full thickness of the cornea and restricted visibility of anterior chamber components. Patients 1 and 2 were followed for 26 and 19 months, respectively.

Human amniotic membrane (AM) was harvested at the time of elective caesarean section; preservation was at −80°C. Under sterile conditions, the membranes were deprived of their amniotic epithelium by two hours' incubation at 37°C with ethylenediamine tetraacetic acid (EDTA) 0.02% solution to loosen cell adhesion. This was followed by gentle scraping with a cell scraper.

The procedure for generating cultivated oral mucosal epithelial sheets has been reported by Nakamura and associates.[12,15] Under local anesthesia, 3 to 5 mm^2 oral mucosal biopsy specimens were obtained after proper treatment of the oral cavity. After removing submucosal connective tissues, small explants were immersed in phosphate-buffered (PBS) saline solution containing antibiotics (50 IU/ml penicillin-streptomycin and 5 μg/ml amphotericin B), incubated at 37°C for one hour with 1.2 IU dispase, and then treated with trypsin-EDTA 0.05% solution for 10 minutes at room temperature (RT) to separate the cells. The oral mucosal epithelium was then placed on denuded AM spread on the bottom of culture inserts and cocultured with mitomycin C–inactivated 3T3 fibroblasts. The culture was submerged in medium until cell confluence and then exposed to air by lowering the level of the medium for one to two days to promote epithelial differentiation. Cultures were incubated at 37°C in a 5% CO_2–95% air incubator; the medium was changed daily.

The initial surgical procedure for ocular surface reconstruction was as described in previous reports.[5,15] In brief, after a 360-degree conjunctival peritomy, conjunctivalized tissue on the corneal surface and thick, fibrotic subconjunctival tissues were removed. The subconjunctival spaces were treated with mitomycin C 0.04% for five minutes and then vigorously washed with saline solution. Then AM transplantation was carried out to reconstruct the conjunctival fornix. The preserved AM was placed with epithelial side up and then sutured with 10-0 nylon. After excising the AM covering the corneal surface, a

19-mm–diameter piece of cultivated autologous oral mucosal epithelial sheet was transplanted onto the corneal surface and sutured with 10-0 nylon. The integrity of the cultivated epithelium was confirmed by fluorescein staining at the end of surgery. Postoperatively, the transplanted epithelial sheet was protected with a medical-use contact lens.

PKP was performed five to six months after the initial COMET ocular surface reconstruction. PKP with cataract surgery was performed according to the usual procedures. In brief, a 7-mm–diameter trephination was performed on the host cornea, followed by continuous circular capsulorrhexis. The lens was removed by the regular phaco amelification and aspiration technique through the trephinated cornea. After inserting the intraocular lens, a 7.25-mm–diameter fresh donor cornea with epithelium was fastened with interrupted and continuous sutures. The corneal surface was then covered with a soft contact lens that was changed as appropriate during the follow-up period.

Immunohistochemical studies of keratin expression in the reconstructed ocular surface epithelium derived from cultivated oral mucosal epithelium were performed by using the previously described procedure.[12] Corneal buttons excised with a 7-mm–diameter trephine were examined at the time of the second surgery. Normal oral tissue was the control for immunohistochemical comparison studies; all tissues were stored at −80°C. Cryostat sections (7 μm in thickness) were placed on gelatin-coated slides, air dried, and rehydrated in PBS for 15 minutes at RT. The tissues were then incubated for 30 minutes at RT with bovine serum albumin 1% to block nonspecific bindings and further incubated (one hour, RT) with primary antibodies. Mouse monoclonal antibodies were used against keratin 1/10/4/13 (Novocastra, Newcastle, United Kingdom), keratin 3 (Progen, Heidelberg, Germany), and rabbit polyclonal antibodies against keratin 12 (Transgenic, Kumamoto, Japan). Control incubations were with appropriate normal mouse and rabbit IgG (Dako, Kyoto, Japan) at the same concentration as the primary antibody. After staining with the primary antibody, sections were incubated (one hour, RT) with the appropriate secondary antibodies; we used fluorescein isothiocyanate–conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, Pennsylvania, USA) and fluorescein isothiocyanate–conjugated donkey anti-rabbit IgG (Vector Laboratories, Burlingame, California, USA). After several washes with PBS, the sections were coverslipped with antifading mounting medium containing propidium iodide (Vectashield; Vector Laboratories) and examined under a confocal microscope (Fluoview; Olympus, Tokyo, Japan).

After more than one year of regular follow-up, an in vivo laser confocal microscope (Heidelberg Retinal Tomograph II/Rostock cornea module [HRT II]; Heidelberg Engineering, Heidelberg, Germany) was used for in vivo morphologic study of the reconstructed corneal epithelium on the corneal graft.[18] Confocal images in central regions were scanned from the apical layer to the basal epithelium. The density of the in vivo epithelium was measured by a computerized analysis system provided with the HRT II instrument.

RESULTS

ORAL MUCOSAL TISSUES WERE SAFELY EXCISED WITHOUT any complications. Approximately 1×10^5 oral mucosal epithelial cells were seeded onto the denuded AM and cultured for five to eight days until they reached confluence covering the entire AM. By two-week cultivation and air lifting, mature oral mucosal epithelium sheets that consisted of 5 to 6 cell layers were generated. Histologic examination confirmed that the sheets were comprised of well-differentiated stratified epithelium similar to that of the in vivo cornea; they consisted of a basal layer formed by cuboidal cells, several suprabasal cell layers, and flat apical cell layers. The condition of the cultivated epithelial sheet was confirmed by fluorescein staining at the end of the transplantation procedure. Both cases showed that the cultivated epithelial sheets were well stratified and without epithelial defect or any remarkable surface damage.

Patient 1 was a 70-year-old man who had experienced alkali burns to both eyes when he was 30 years old. Although history of previous surgeries was unavailable, slit-lamp examination showed round scarring in the peripheral cornea suggestive of earlier PKP. His right eye, chosen for ocular surface reconstruction, showed complete conjunctivalization on the corneal surface with extensive scarring and symblepharon formations (Figure 1). The intraocular status was unascertainable, yet ultrasound examination returned no abnormal vitreoretinal findings. His best-corrected visual acuity (BCVA) of the right eye was hand motion. On October 17, 2003, he underwent COMET and AM transplantation after the removal of scar tissue from both the cornea and subconjunctival space. Survival of the entire oral mucosal epithelium was confirmed on the second postoperative day, and it gradually covered the entire ocular surface. His visual acuity remained unchanged after the initial surgery. After the initial surgery, the reconstructed ocular surface showed uneven and irregular fluorescein staining absent of any epithelial defects (Figure 1). He experienced no recurrence of cicatrization or prolonged inflammation after the first operation. It is notable that the ocular surface before PKP was stable and uniform without inflammation. Because intraoperative observation showed the existence of a previous small PKP, PKP was selected to remove the corneal scar rather than lamellar keratoplasty. The second step, PKP combined with cataract surgery, was performed six months after the initial surgery; the graft had remained clear without any epithelial defect or rejection. There was minimal neovascularization along the sutures, but not in the corneal graft. A slow ingrowth of trans-

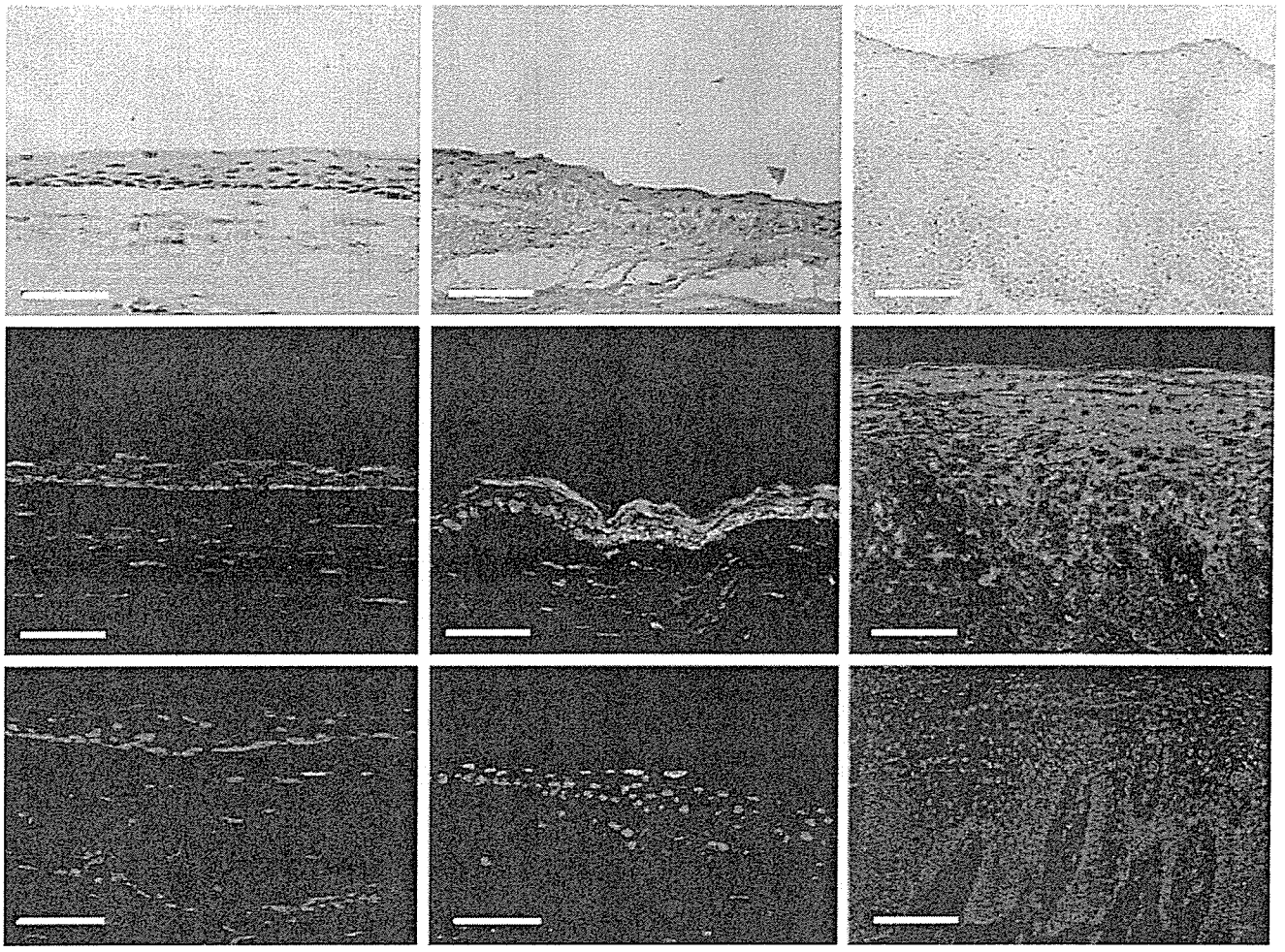

FIGURE 3. Immunohistological appearance of keratin 3 and 12 expressions in a cultivated oral mucosal epithelium sheet, surviving cultivated oral mucosal epithelium on the corneal button (subsequently resected at the time of penetrating keratoplasty [PKP]), and normal oral mucosal tissue. (Top left) Hematoxylin and eosin staining of a cultivated oral mucosal epithelial sheet from patient 1. (Second row, left) K3 expression in excised corneal button from patient 1. (Bottom left) There is no K12 expression in the excised corneal button from patient 1. (Top middle) Hematoxylin and eosin staining of cultivated oral mucosal epithelial sheet from patient 2. (Middle row, middle) K3 expression in excised corneal button from patient 2. (Bottom middle) K12 expression in excised corneal button from patient 2. (Top right) Hematoxylin and eosin staining of normal oral mucosal epithelium. (Middle row, right) K3 expression in normal oral mucosal epithelium. (Bottom right) Normal oral mucosal epithelium does not express K12. (Left and middle) Scale bars = 100 μm. (Right) Scale bar = 200 μm.

planted oral epithelium from the limbus was observed in the course of long-term follow-up (Figure 1). His BCVA improved to 20/100 and remained stable without reduction. Although his intraocular pressure (IOP) was occasionally high, he did not require glaucoma surgery. The occasional increase in IOP was managed by the topical application of carteolol hydrochloride 0.02% twice daily and latanoprost 0.05% once daily. Carbonic anhydrase inhibitor was also used to reduce IOP if the topical medication was not enough; however, no glaucoma surgery was required to control IOP.

Patient 2 was a 71-year-old man with no history of previous surgical treatment who had acquired Stevens-Johnson syndrome in his 40s. As shown in Figure 2, this patient had total conjunctivalization and severe scarring. He manifested minimal tear secretion and partial parakeratinization. Preoperatively, his visual acuity was hand movement. COMET was performed on this patient on May 26, 2004. There was an early epithelial defect in the center region during the two weeks after surgery; however, it healed without corneal melting or conjunctival invasion. His visual acuity remained unchanged after the initial operation. The second step, PKP with cataract surgery but not lamellar keratoplasty, was performed 5.5 months later by means of the standard procedure from the point of early visual rehabilitation. Subsequently, his BCVA improved to 20/125. He developed no postoperative complications except for a total corneal epithelial defect that originated

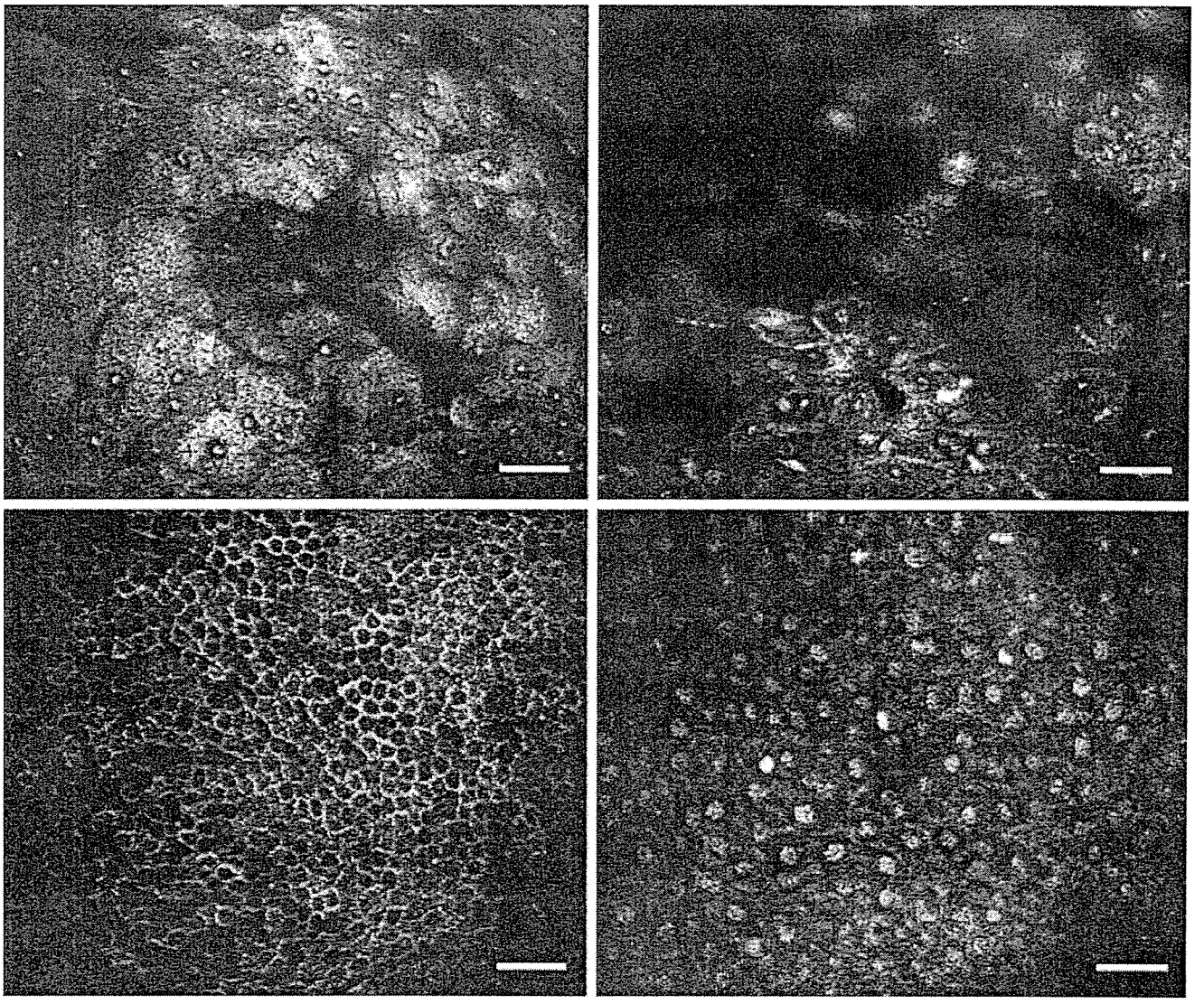

FIGURE 4. In vivo confocal micrographs demonstrate the appearance of the epithelium in the region of the transplanted central corneal surface. (Top left) Apical surface in patient 1. (Top right) Apical surface in patient 2. (Bottom left) Basal cell layers in patient 1. (Bottom right) Basal cell layers in patient 2. Note that the cell shape and density in each region are similar to normal cornea. Scale bars = 50 μm.

in the donor cornea after the medical contact lens fell off. However, the defect was gradually reepithelialized from the surrounding oral mucosal epithelium after rewear of the medical contact lens.

Immunohistochemical analysis was performed on the surviving transplanted cultivated oral mucosal epithelium on the cornea excised during PKP (Figure 3). Both patients demonstrated nonkeratinized stratified epithelium on the AM covering the cornea. Notably, in different regions, the stratified epithelium consisted of three to 10 layers; this finding was consistent with the results of slit-lamp examination. None of the specimens contained goblet cells. Immunohistochemistry confirmed the presence of K4 and K13; these keratins are specific for mucosal epithelium (data not shown). The expression of K1, which is specific for keratinized epithelium, was not detected (data not shown). As expected, K3 was expressed in surviving epithelium on the cornea as well as in oral mucosal epithelium. Conversely, K12, which is specific for corneal epithelium, was not expressed in the surviving epithelium, except for faint, occasional staining in the apical region.

We used the HRT II instrument for in vivo laser confocal scanning to study the histologic structure of the transplanted epithelium (Figure 4). The presence of a large, flat epithelium with small cell nuclei in the apical surface was noted in both patients; this is consistent with the normal corneal surface. The average cell density in the apical layer of the corneal graft was 840 ± 295 cells/mm^2 (mean ± SD) and not markedly different from a normal cornea.[18] The basal cells were smaller, denser, and aligned

in regular fashion, this also is similar to the normal corneal structure. The density of basal cells in the two patients was 8075.3 and 1492.0 cells/mm^2, respectively; in patient 1 it was within the range reported for normal central cornea (8996 ± 1532 cells/mm^2), whereas in patient 2 it was below the normal range.[18]

DISCUSSION

THIS STUDY PRESENTS A TWO-STEP SURGICAL APPROACH to treat patients with severe limbal deficiency disorder and corneal opacity. It consists of a combination of COMET and the conventional PKP triple-procedure. The two patients were followed for a mean of 22 months and encountered no immunologic rejection or persistent epithelial defect, common critical complications after combined surgical treatment consisting of corneal epithelial transplantation and PKP.

Cultivated autologous corneal epithelial transplantation that used AM was first introduced by Tsai and associates[4] and Koizumi and associates.[5] This tissue-engineered procedure promotes a strategy for reconstructing the corneal surface with autologous oral mucosal epithelium. This histologic study of the central cornea of two patients documents that transplanted cultivated oral mucosal epithelium on the corneal surface remained intact for at least the first six months after transplantation. Immunohistochemically, the surviving transplanted cultivated oral mucosal epithelium on the cornea was positive for K3 and K4 (data not shown) and negative for K10 (data not shown) and K12, indicating that it was neither corneal nor conjunctival. Rather, it resembled cultivated oral mucosal epithelium grown on AM. Thus, the intrinsic characteristics of the ectopically transplanted epithelium did not change. This finding coincides with observations made when cultivated oral mucosal epithelial sheets were transplanted onto rabbit eyes.[12] Because epithelial differentiation largely depends on the substrate, transplanted cultivated oral mucosal epithelial sheets do not resemble the in vivo oral mucosal epithelium, probably because of modifications induced by the external environment—that is, the corneal stroma or AM. The absence of neovascularization into the cornea after the grafting of oral mucosal epithelium may also be attributable to interaction with the corneal stroma. Studies are currently underway to elucidate biologic factors, such as mucin expression by surviving oral epithelium, to gain an understanding from the perspective of corneal function.

To improve the success rate of ocular surface reconstruction with PKP, allogenic recognition by the host immune system must be minimized. Therefore, limbal transplantation was avoided and a two-step approach was used instead. Tsubota and associates[19] demonstrated better graft survival when a two-step procedure was used to treat severe ocular surface disorders. The survival rate of limbal transplants and PKP grafts after combined surgery is relatively poor.[1,2,19] Because the limbal region contains allogenic antigens such as antigen-presenting cells and major histocompatibility complex class 2 molecules, allogenic limbal transplantation may be inappropriate in patients with severe limbal deficiency disorders. Although the findings of this study must be considered preliminary, they suggest that the mucosal epithelium covering the cornea, because it was derived from autologous oral mucosal epithelium, is not subject to allosensitization. Therefore, the results of this study indicate that this two-step procedure involves a risk for endothelial rejection that is no greater than that encountered with conventional PKP.

This two-step procedure features another improvement: the continuous, prolonged supply of epithelium, which compensates for the limited survival of corneal epithelium on the central corneal graft. However, although no epithelial defect was observed during the 22-month follow-up period, additional long-term observations are necessary to determine whether oral mucosal epithelial cells will offer continuous replacement on the transplanted cornea. Patients with limbal stem cell–deficient eyes often manifest persistent epithelial defects on their grafts after PKP. The proliferation potential of conjunctival epithelium is relatively low, and this may partly explain the persistence of the epithelial defects. Oral mucosal epithelium is thought to be less well differentiated, and this may be an advantage in terms of short cell turnover time and a quicker wound-healing response after transplantation.[13,14] However, no comparison of the relative rates in epithelial healing for ocular surface epithelial cells compared with oral epithelial cells was attempted in this study. Hayashida and associates[20] used a rabbit model to demonstrate that in vivo and in cultivated sheets, p63- and integrin 1–positive cells manifested the higher proliferation characteristics of oral mucosal epithelial cells. Inatomi and associates[17] previously reported positive midterm results in patients who had undergone ocular surface reconstruction by COMET. This in vivo laser confocal microscopic study demonstrated that the stratified epithelium existed in the central zone of the two patients. The shape of apical cells and size and density of the basal cells were similar to normal cornea, suggesting the maintenance of a well-differentiated structure of graft after the ocular surface reconstruction. At present, it is unclear whether the epithelium examined in this study was transplanted allogenic corneal epithelium or regenerated epithelium derived from autologous cultivated oral mucosal epithelium on the peripheral cornea.

The results of experimental and clinical studies suggest that COMET is a promising and advantageous alternative to mucosal epithelium transplants for ocular surface reconstruction. This study documented the survival of ectopically transplanted oral mucosal epithelium and showed that the transplantation of autologous oral mucosal stem cells to donor corneal grafts avoids common epithelial complications. At present, the long-term survival of both

the transplanted oral mucosal epithelia and allogenic corneal grafts in this study continue to be monitored.

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Proteomic analysis of soluble factors secreted by limbal fibroblasts

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Purpose: To identify soluble factors selectively secreted by limbal fibroblasts as possible regulators of limbal basal epithelium.

Methods: Limbal, corneal, and conjunctival fibroblasts were first expanded in vitro in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, and then maintained in serum-free medium for two weeks. Proteomic analysis of culture supernatants was done to compare differences in secreted matricellular proteins. Real time PCR and western blots were done to confirm the expression of secreted protein acid and rich in cysteine (SPARC), a protein found in abundance in extracellular proteins secreted by limbal fibroblasts. Immunohistochemistry of SPARC was done in human limbal tissue to show the spatial distribution of the protein. An adhesion assay was designed to demonstrate the effects of SPARC on an SV40 immortalized human corneal epithelial cell line (HCEC).

Results: Proteomic analysis revealed several proteins selectively secreted by limbal fibroblasts. The particular spots were identified as SPARC, vimentin, serine protease, collagen alpha 2 precursor, tissue inhibitor of metalloproteinase 2 (TIMP-2), and 5,10-methlenetetrahdrofolate reductase (FADH2). The expression of SPARC was confirmed by western blot analysis, and mRNA expression was significantly higher in limbal fibroblasts compared to central corneal fibroblasts when analyzed by real time PCR. Immunohistochemistry revealed higher distribution of SPARC in the subepithelial stroma of the limbus compared to the central cornea. The addition of 10 μg/ml murine SPARC in HCEC significantly reduced cell spreading at three h.

Conclusions: The matricellular protein SPARC is preferentially secreted by limbal fibroblasts, and may modulate intercellular adhesion of basal limbal epithelial cells.

The limbal basal epithelium has distinct characteristics compared with the corneal epithelium in the expression of several genes including increased α-integrin, ATP binding cassette protein 2 (ABCG2), and decreased keratin 3 (K3) and connexin 43 [1]. The differential expression of these markers is often raised as evidence for the presence of stem cells in the basal limbal epithelium. Accumulating evidence from clinical studies also support the limbal stem cell hypothesis, with successful ocular surface reconstruction reported by several laboratories following limbal transplantation [2,3], and more recently, cultured limbal epithelial sheet transplants [4,5]. The stromal niche is believed to modulate the phenotype of overlying epithelium, which probably involves soluble factors as well as regulation by direct contact. The plasticity of epithelial cells according to the underlying stroma was demonstrated by the use of amniotic membranes, and also by reversing epithelium/stroma combinations [6].

In order to screen for differences in secreted proteins by limbal and corneal fibroblasts, we performed 2-D PAGE (proteomic analysis) of condensed supernatants of serum-free cultured cells. After six proteins were identified, we further analyzed the distribution and function of secreted protein acidic and rich in cysteine (SPARC), also known as osteonectin. SPARC is a 43 kDa protein that contains a COOH-terminal extracellular (EC) module with two Ca^2-binding domains and a follistatin-like module shared by a family of SPARC-related genes [7]. SPARC is also expressed by corneal epithelial cells, and is believed to be involved in the wound healing process of both the epithelium and stroma of the cornea [8,9]. In addition, SPARC modulates cell growth and adhesion of vascular endothelial cells [10], and has been reported to promote cancer cell migration and invasion [11]. Several of these physiological functions reported in SPARC are consistent with properties expected of soluble factors in the stromal niche of the limbus. Epithelial cell precursors are believed to be less dependent on intercellular communication, which in turn maintain these cells in an undifferentiated state. Evidence for this is given by the limited expression of the gap junctional protein, connexin 43, in basal limbal epithelial cells [1]. We therefore hypothesized that SPARC secreted constitutively by limbal fibroblasts can regulate epithelial cell adhesion.

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METHODS

Materials: Mouse recombinant SPARC and fibronectin were purchased from Sigma-Aldrich (St. Louis, Mo). Chemicals for proteomic analysis were obtained from Wako Pure Chemical Industries (Osaka, Japan) unless otherwise noted. The SV40 transformed immortalized human corneal epithelial cell line (HCEC) was a kind gift from Dr. Kaoru Araki-Sasaki (Kagoshima Miyata Eye Clinic, 1-5-1, Nishida, Kagoshima, Japan) [12].

Cell culture and adhesion assay: Human donor corneas not suitable for transplantation were obtained from the Northwest Lions Eye Bank. The epithelium and endothelium were bluntly removed with a gill knife, and stromal tissues were cut into small segments (approximately 2 mm x 2 mm) to allow fibroblasts to migrated during culture. Fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM) medium containing 10% fetal bovine serum until confluent, and then in serum-free DMEM for two weeks prior to proteomic analysis. HCECs were maintained in supplementary hormonal epithelial medium (SHEM), a 1:1 mixture of DMEM and Ham's F12 medium (DMEM/F12; Gibco, Invitrogen Corporation, Carlsbad, CA) containing 15% fetal bovine serum, insulin (5 µg/ml; Sigma-Aldrich, St. Louis, MO), cholera toxin (0.1 µg/ml; EMD Biosciences, San Diego, CA), human recombinant epidermal growth factor (10 ng/ml; Gibco), dimethyl sulfoxide (0.5%; Sigma-Aldrich), penicillin (0.7 mg/ml; Wako Pure Chemical Industries), and streptomycin (1.39 mg/ml; Wako Pure Chemical Industries).

Proteomic analysis: Supernatants from limbal, central corneal, and conjunctival fibroblasts cultured in serum-free DMEM for two weeks were collected by centrifugation. In brief, supernatants were placed in ultrafiltration tubes (Vivaspin 20; Sartorius, Goettingen, Germany) and centrifuged (MX-300; Tomy Seiko Co., Tokyo Japan) to remove proteins with molecular weights of less than 3 kDa. Lysis buffer (8 M urea, 2% NP-40, 2% ampholine (pH3.5-10), 5% 2-ME, protease inhibitor) was then added to the supernatant, and centrifugation was repeated. Two-dimensional PAGE was performed as previously described in the literature [13]. In brief, the first dimension was based on isoelectric focusing (pH 3.5-10) using a disk gel (Nihon Eido, Tokyo, Japan), followed by the second dimension done by SDS-PAGE in a 16.8% acrylamide gel (Bio-Rad Laboratories, Hercules, CA). Protein spots were visualized by Coomassie brilliant blue (CBB). Selected spots were dissected and digested with trypsin in 0.1 M ammonium hydrogen carbonate containing 10% acetonitrile for 16 h at 37 °C. Peptides were extracted from the gels with 60% acetonitrile containing 0.1% trifluoroacetic acid and then vortexed for 30 min. Peptide fragments were separated by C18 column (Magic C18 P/N 902-61260-00; AMR Inc., Tokyo, Japan) in a linear gradient (5-60%) of acetonitrile containing 0.1% formic acid. Separated peptides were analyzed by ion-trap mass spectrometry (MS, LCQ DECA; Thermoquest Corp., San Jose, CA) using a nanospray ionization apparatus. MS data analysis was done using Sequest (Thermoquest) and the Mascot Internet version [14].

Western blot: Western blot was used to confirm the expression of SPARC by cultured limbal and corneal fibroblasts, as well as primary cultured corneal epithelial cells. Culture supernatants were collected after two weeks of culture in DMEM containing 10% FBS and stored without condensation for western blot analysis. Cell pellets were dissolved with lysis buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1% Nonidet P-40) and homogenized. Samples were incubated for 40 min at 4 °C, and then centrifuged at 15,000 rpm for 30 min

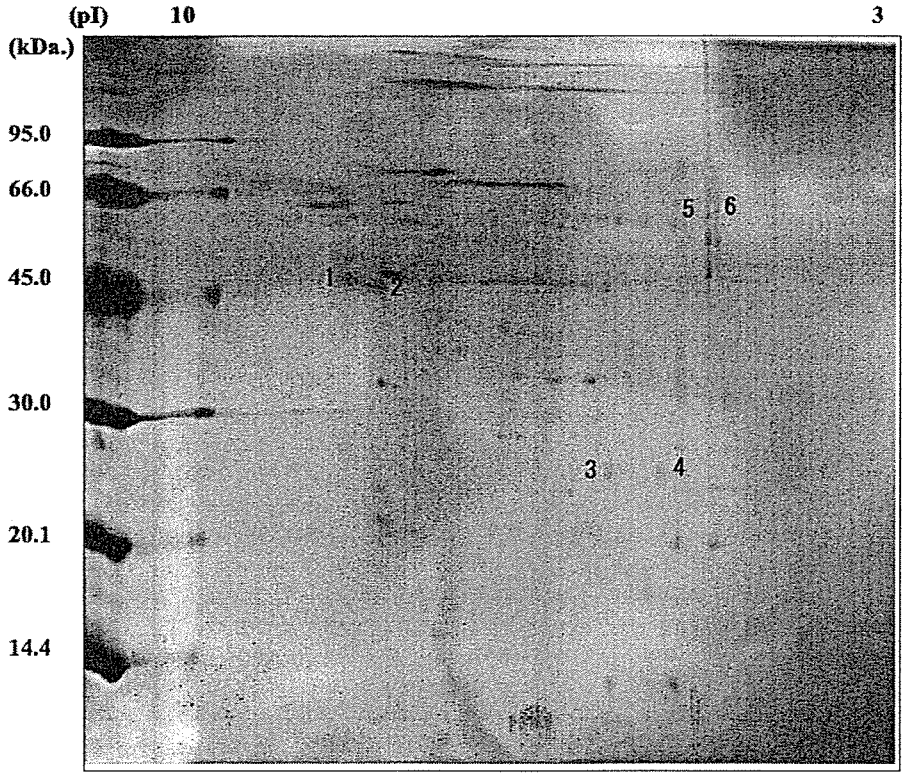

Figure 1. 2-D PAGE of proteins concentrated from the supernatant of limbal fibroblasts. Spots were visualized with Coomassie brilliant blue. Six spots (numbered) were found to be distinctively expressed by limbal fibroblasts and were identified by ion-trap mass spectrometry. The name and GenBank accession number for each protein are listed in Table 1.

at 4 °C. Protein concentration of the supernatant was determined by the DC protein assay (Bio-Rad Lab). All samples were then diluted in 2X sample buffer (100 mM Tris-HCl, pH 6.8, 4% SDS; Gibco, Invitrogen, Carlsbad, CA), 20% Glycerol (Wako), 12% 2-mercaptoethanol (Wako) and boiled. Ten µg of each sample (5 µg for β-actin) were loaded on a Novex NuPAGE 10% Bis-Tris gel (Invitrogen) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). Membranes were blocked with 5% skim milk (Difco Laboratories, Detroit, MI) and 1.5% normal donkey serum in PBS for 60 min at room temperature. Membranes were reacted with an anti-SPARC antibody (J.B.789; US Biological, Swampscott, MA) for 60 min at room temperature. After three washes in TBST, donkey biotinylated antimouse IgG (Jackson ImmunoResearch) was added for 30 min at room temperature. Protein bands were visualized by the Vectastain ABC Elite Kit (Vector Laboratories, Bulingame, CA) using DAB (Vector Laboratories) as a substrate.

Real-time polymerase chain reaction: Total RNA was isolated from cultured limbal and corneal fibroblasts using the SV total RNA isolation system (Promega Co., Madison, WI) according to the manufacturer's recommendations. cDNA was prepared from total RNA with oligo (dT) priming and AVM reverse transcriptase XL (Takara, Bio Inc., Shiga, Japan) by incubation of a 25 µl mixture at 41 °C for 1 h. cDNA was subjected to PCR using the gene specific oligonucleotide primers and probe (5'-ACC CCA TTG ACG GGT ACC TCT CCC A-3'). Real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR) using TaqMan chemistry (Applied Biosystems, Foster City, CA) and the ABI Prism 7700 Sequence Detection System (Applied Biosystems) was used to semiquantitate SPARC expression in limbal, corneal, and conjunctival fibroblasts. PCR products were detected during the exponential phase of the reaction in order to semiquantitate SPARC expression by each cell type (n=3).

Immunohistochemistry: Frozen sections prepared from a donor human cornea embedded in 4% carboxymethyl cellulose (CMC; Finetec Co., Ltd., Japan) were fixed in 4% paraformaldehyde (PFA) for 10 min. The fixed sections were

Table 1.

Protein	Accession number	Spot number
secreted protein, acidic, cysteine-rich (SPARC); Osteonectin(secreted protein, acidic, cysteine-rich)	NP_003109	1
Vimentin	AAA61279	2
tissue inhibitor of metalloproteinases-2	AAC50729	3
Human Collagen alpha2(I) chain precursor	P08123	4
serine protease, Homo sapiens	AAC97211	5
5,10-methylenetetrahydrofolate reductase (FADH2; EC 1.7.99.5)	S46454	6

Proteins that were detected in the supernatant of limbal fibroblasts, but not from central corneal fibroblasts in the proteomic analysis.

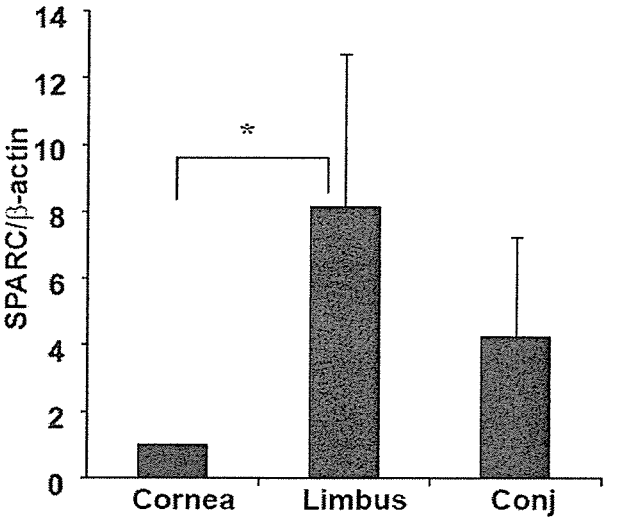

Figure 2 . Real time PCR comparing mRNA expression of SPARC in cultured limbal, corneal, and conjunctival fibroblasts. Data is expressed as SPARC expression by corneal fibroblasts as 1 unit. Limbal fibroblasts expressed a signficantly higher level of SPARC compared to the central cornea (n=3). The asterisk indicates a $p<0.05$.

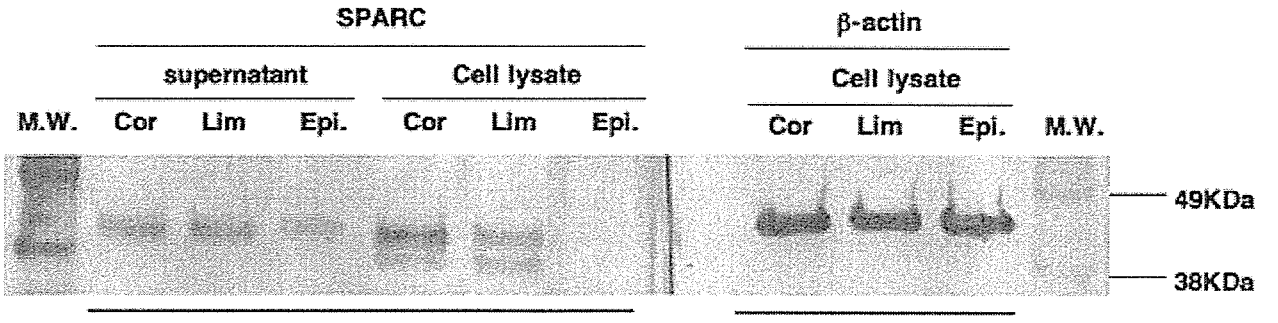

Figure 3. Western blot analysis of SPARC in culture supernatants and cell lysates of corneal and limbal fibroblasts, and primary corneal epithelial cells. Limbal fibroblasts secreted higher levels of SPARC protein compared with corneal fibroblasts. SPARC was also detected from primary epithelial cell cultures.

treated with liberate antibody binding solution (L.A.B.; Polyscience, Inc., Warrington, PA.) at room temperature for 15 min for antigen retrieval. Antibodies used were antiosteonectin (Haematologic Technologies, Inc. Essex Junction, VT) and Cy3-labeled antimouse IgG secondary antibody. Isotype rat IgG (Chemicon) was used as control. The sections were incubated with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI; Dojindo Laboratories, Tokyo, Japan) at room temperature for 5 min. Finally, sections were washed three times in Tris-buffered saline tween (TBST) and coverslipped using an antifading mounting medium (50 mM Tris buffer saline, 90% glycerin; Wako), and 10% 1,4-diazabicyclo (2,2,2) octane (Wako).

Adhesion assay: One of the established physiological effects of SPARC is the suppression of vascular endothelial cell growth and adhesion [10]. In order to pursue the possibility that SPARC may have similar effects on corneal epithelial cells in vitro, we performed a modified version of a cell adhesion study reported previously [15]. Nontreated 96 well plates (260887, Nalge Nunc Int, Rochester, NY) were coated with 100 µl of fibronectin in phosphate-buffered saline plus (PBS+; 1 µg/ml) at 4 °C overnight, and washed with PBS. Serum-free DMEM with or without murine SPARC (final 10 µg/ml) were added to the wells. HCEC were trypsinized, neutralized, resuspended in serum-free DMEM, and a 50 µl sample was added to each well (10^4/well). After 3 h incubation at 37 °C, the central area of each well (856 µm x 678 µm) was photographed using the Axiovert 200 microscope (x10, Carl Zeiss, Gottingen, Germany). Cells were scored as previously described [16]. Round cells with no apparent signs of spreading were given a score of 3. Rounded cells with short cellular processes were assigned a score of 2. Spread, flattened cells were given a score of 1. Adhesion score for each well was calculated by the average score of all visible cells in a randomly selected field of view.

RESULTS

Proteomic analysis: 2-D PAGE of supernatant from limbal fibroblasts is shown in Figure 1. Total protein levels were low, in general, since this was an analysis of culture supernatants and not of homogenized cells. Although samples were condensed prior to electrophoresis, only blots that were dense enough to allow sequence analysis were further investigated. The six proteins specifically identified in the supernatant of limbal fibroblasts along with their accession numbers are listed in Table 1.

Constitutive expression of SPARC by limbal fibroblasts: We further pursued the possible role of SPARC as a major matricellular protein in the limbal stroma. Real-time PCR was done to semiquantitate SPARC mRNA transcription in cultured cells, and the result was consistent with the higher protein content in limbal fibroblasts observed in the proteomic analysis (Figure 2). Western blot results confirmed SPARC protein secreted in the supernatant of limbal and corneal fibroblasts (Figure 3). SPARC was also expressed by primary corneal epithelial cells, however, the expression levels were lower compared to limbal and corneal fibroblasts.

Immunohistochemistry: The cumulative data show higher expression of SPARC in limbal fibroblasts in vitro, but does not necessarily reflect that this applies in vivo. We therefore performed immunohistology using an anti-SPARC monoclonal antibody in fresh donor limbal tissue to observe the distribu-

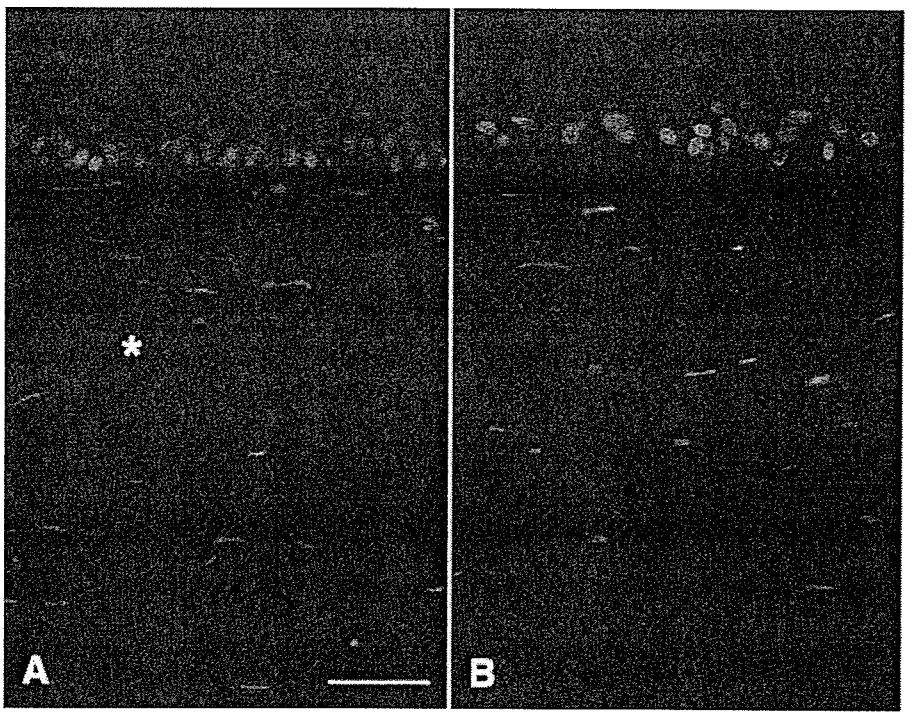

Figure 4. Immunohistology of a corneolimbal segment using an anti-SPARC monoclonal antibody. The subepithelial tissue in the limbus (**A**) showed SPARC distributed in the interstitial space (asterisk). SPARC-associated signals were much lower in the central cornea (**B**). The scale bar represents 50 µm.

tion of SPARC in situ. As shown in Figure 4A, a higher level of SPARC-associated Cy-3 fluorescence was observed in the subepithelial regions of the limbus compared with the central cornea. The difference can be appreciated when compared with the uniform fluorescence observed in the overlying epithelial cells (Figure 4B). Thus, SPARC is constitutively expressed in the limbal stroma by resident fibroblasts without the stimulation of a wound healing process.

Cell adhesion assay: In order to observe the effects of SPARC on corneal epithelial cells in vitro, an immortalized cell line (HCEC) were used to observe for changes in cell adhesion and morphology. The addition of SPARC in the culture supernatant resulted in rounding of individual HCEC after 3 h (Figure 5). The difference was statistically significant using a rounding index originally described by Lane and Sage [16] (n=5).

DISCUSSION

SPARC is a 43 kDa protein that contains a COOH-terminal EC module with two Ca^{2+}-binding domains, a follistatinlike module, and an NH_2-terminal acidic module [7]. The expression of SPARC by corneal stromal cells has been reported to play a role in the wound healing response, evidenced by the upregulation of SPARC by the fibroblast and myofibroblast phenotype [9]. However, SPARC was not detected in quiescent corneal stromal cells, and hence, the major function of the protein was speculated to be related to wound healing. Conversely, SPARC secreted by epithelial cells was shown to induce contraction of stromal fibroblasts in vitro, suggesting that SPARC is a key protein in epithelial/stromal interaction of the cornea [17]. SPARC has also been proposed to be involved in corneal epithelial migration and stratification following mechanical ablation [8].

We found that limbal fibroblasts secreted higher levels of SPARC compared to central corneal fibroblasts in vitro without stimulation by serum or cytokines, and also in vivo without any wound-healing stimuli. SPARC was one of only a few proteins detectable by proteomic analysis in the limbal fibroblast supernatant, suggesting a functional role in the homeostasis of the limbal structure. Although it can be argued that corneal fibroblasts cultured in vitro are not the same as keratocytes in vivo, experiments requiring large quantities of cells would not be possible without in vitro expansion. All cells used in the current study were first expanded in vitro using serum containing 10% serum, therefore, the phenotype of these cells at the time of analysis is not necessarily consistent with the normal phenotype. The results of preotomics alone, therefore, have limits without further analysis. Inter-

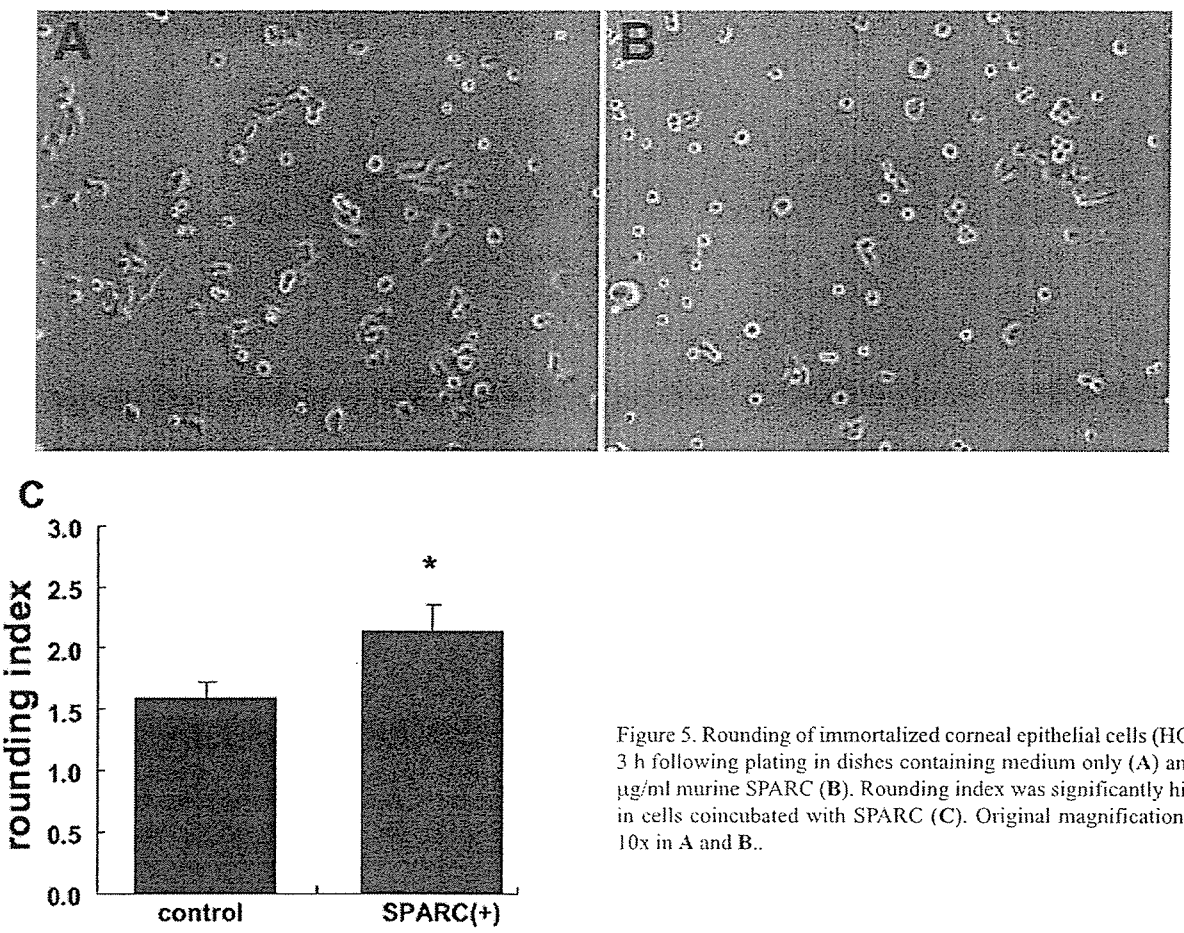

Figure 5. Rounding of immortalized corneal epithelial cells (HCEC) 3 h following plating in dishes containing medium only (**A**) and 10 µg/ml murine SPARC (**B**). Rounding index was significantly higher in cells coincubated with SPARC (**C**). Original magnification was 10x in A and B..

estingly, one of the proteins detected in the limbal cell supernatant was vimentin, an intracellular intermediate fiber, suggesting that some of the proteins in the supernatant may have been the result of apoptosis. We did not pursue this issue further, and chose to focus on SPARC which is a secreted protien.

Figure 4 shows the immunohistochemistry of SPARC in donor cornea tissue. The result shows that SPARC is expressed more in the limbal stroma, reflecting the results of real time RT-PCR and western blots of cell supernatants. We have also found through immunohistochemistry that epithelial cells were positive for SPARC, while western blots only detected trace levels of SPARC from cell lysates and supernatant of primary cultured epithelial cells. This may be explained by the fact that SPARC is secreted by corneal epithelial cells during wound repair [8,17], and that primary cells in vitro may be in a state similar to epithelial cells undergoing wound healing.

We focused on the function of SPARC, since the matricellular protein has been reported to regulate the adhesion of bovine aortic endothelial cells [10]. Using a previously described adhesion assay, we found that exogenous SPARC inhibited adhesion of a human corneal epithelial cell line, which may be due to the Ca^{2+}-binding ability of SPARC. Espana et al. [6] have previously reported that limbal stroma, and not corneal stroma, was required to maintain an undifferentiated phenotype (K3 negative, Cx 43-low) in corneal epithelial cell sheets. This implies that soluble factors expressed by limbal fibroblasts may be involved in this phenomenon. The extracellular matrix and basement membrane components of the limbal area are distinct from the central cornea, as reported by several studies [18,19]. SPARC is also involved in the migration and invasion of prostate cancer cells [11] and breast cancer cells [20] through the activation of matrix metalloproteinase 2 (MMP2). These are several properties that are expected of matricellular proteins in the putative limbal stem cell niche. Interestingly, the MMP2-specific inhibitor, TIMP2 was also preferentially detected in the supernatant of limbal fibroblasts, suggesting that an intricate network based on a balance of effectors and inhibitors may be involved in the homeostasis of the limbal stem cell niche.

Growth factors such as keratinocyte growth factor and hepatocyte growth factor are also mediators of fibroblast/epithelial interaction involved in epithelial proliferation and migration [21]. Although the network of epithelial/mesenchymal interaction in the corneal limbus is sure to involve a wide variety of matricellular proteins, cytokines, and growth factors, the inhibition of cellular adhesion and cell/cell interaction by SPARC may be a major component of the limbal microenvironment. We found that the human amniotic membrane (AM) also contains SPARC (data not shown), which may partially explain the ability of AM to preserve the undifferentiated state of limbal epithelial cell in vitro [22]. While further studies are required to elucidate the interactions of soluble factors involved in the limbal niche, a combination of such components may be used to enrich limbal stem cells in vitro.

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Ocular Surface Epithelial Cells Up-Regulate HLA-G When Expanded In Vitro on Amniotic Membrane Substrates

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Purpose: To study the modulation of immunoregulatory genes in ocular surface epithelial cells cultured on amniotic membrane (AM).

Methods: Microarray analysis was performed in a conjunctival epithelial cell line (CCL20.2) expanded on denuded AM. Among the genes that were upregulated by an AM substrate compared with collagen-coated dishes, the fetal nonclassic major histocompatibility complex molecule, HLA-G, was found to be the only immunoregulatory gene up-regulated by more than 2.5-fold. Because CCL20.2 is contaminated by HeLa cells, expression of HLA-G mRNA was confirmed in primary-cultured limbal (LE) and conjunctival epithelial (CE) cells by reverse transcriptase-polymerase chain reaction (RT-PCR), semiquantitative real-time PCR, immunocytochemistry, and Western blot analysis. A functional assay was performed using an HLA-G–transfected K-562 human erythroleukemia cell line.

Results: Freshly dissociated limbal epithelial cells express HLA-G mRNA; however, protein levels were low. Western blots and immunocytochemistry showed that both LE and CE cells upregulated the HLA-G protein when cultured on collagen-coated dishes and on AM. HLA-G mRNA levels were significantly higher in CE cultured on AM compared with collagen. Natural killer (NK) cell–induced cell lysis of an HLA class I–negative K-562 human erythroleukemia cell line was slightly reduced when transfected with LE-derived HLA-G mRNA.

Conclusion: CE and LE cells express functional HLA-G when expanded ex vivo, which may affect inflammation and immune reaction when transplanted to the ocular surface.

Key Words: HLA-G, amniotic membrane, corneal epithelium, conjunctival epithelium, cornea immune responses

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Numerous investigators have reported the use of amniotic membrane (AM) in ocular surface reconstruction since Kim and Tsengl[1] reinstated the technique in the modern era. AM is used mainly as either a graft, intended to function as a substrate for overlying epithelium, or as a patch to temporarily cover the ocular surface while the host tissue undergoes wound healing. The use of AM was integral in our experience with allograft limbal transplantation (ALT) for reconstructing the ocular surface in severe cicatricial disease.[2–4] AM is also used as a carrier for transplanting ex vivo cultured sheets of corneal epithelium[5] and oral mucosa epithelium.[6]

The effects of AM on the ocular surface were initially perceived as somewhat of a "black box," with many undefined characteristics of both soluble and nonsoluble components of AM cells and stroma. The anti-inflammatory actions of AM have been associated with the production of various cytokines,[7] regulation of growth factor expression,[8,9] and the release of proteinase inhibitors.[10] Direct interaction of AM with invading inflammatory cells may be involved in the elimination of leukocytes from the ocular surface.[11,12] Another important aspect of using AM on the ocular surface is its antiangiogenic property,[13–15] which is vital in maintaining a transparent ocular surface, and may work in suppressing immunologic rejection of allogenic cells and tissue.

To further explain the effects of AM, we hypothesized that AM may have direct immune-regulatory functions on surrounding cells when transplanted to the ocular surface. It is well documented that placental tissue, including AM, suppresses the semi-allo-immune response of the mother against the fetus.[16,17] Ueta et al[18] indicated that human AM is capable of inhibiting alloreactive T-cell response including cell division, proliferation, and T_H1/T_H2 cytokine synthesis in vitro. On the basis of these findings, we sought to study the expression and function of HLA-G, an immunoregulatory protein found to be upregulated in an initial screening by microarray analysis of cells cultured on AM.

MATERIALS AND METHODS

Cell Culture

The JAR (HLA-G$^-$) choriocarcinoma cell line, the JEG-3 (HLA-G$^+$) choriocarcinoma cell line, and the CCL20.2 conjunctival cell line were purchased from ATCC (American Type Culture Collection, Rockville, MD). Denuded AM was prepared as previously described.[19] Preserved AMs were rinsed in phosphate-buffered saline (PBS; 3 times), spread onto culture dishes, frozen at –80°C, and air-dried at room temperature. AM-coated dishes were stored at –80°C until use. Corneoscleral tissue from human donor eyes was obtained from Northwest Lions Eye Bank, and limbal rims were

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preserved for experiments after the central corneal button was used for corneal transplantation. After careful removal of excess sclera, iris, and corneal endothelium, limbal segments were placed in either collagen-coated dishes (Iwaki: Asahi Technoglass, Funabashi, Japan) or on AM-coated dishes. Limbal explants were cultured for 2 weeks at 37°C, 5% CO_2 in supplemented hormonal epithelial medium (SHEM), made of an equal volume of HEPES-buffered Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 (Invitrogen, Carlsbad, CA) containing bicarbonate, 0.5% dimethylsulfoxide (Sigma, St. Louis, MO), 10 ng/mL human epidermal growth factor (EGF; Invitrogen), 5 μg/mL insulin (Sigma), 100 ng/mL cholera toxin (Invitrogen), 15% fetal bovine serum (FBS), 70 μg/mL penicillin (Wako Pure Chemical Industries, Osaka, Japan), and 140 ng/mL streptomycin (Wako). Expanded cells were cultured serum-free for 3 days in Epilife containing HCGS (Kurabo Co., Osaka, Japan) at 37°C, 5% CO_2.

Microarray Analysis

The Atlas Glass Total RNA Isolation Kit (BD, Franklin Lakes, NJ) was used to isolate total RNA from CCL20.2 cells cultivated on either collagen or AM-coated dishes. After RNA was isolated, genomic DNA was removed using DNAse (Qiagen, Hilden, Germany). The targets were prepared using the Atlas Glass Fluorescent Labeling Kit (Clontech Laboratories). Aminoallyl-dUTP was incorporated during first-strand cDNA synthesis. Fluorescent dye (Cy3 or Cy5) was covalently coupled to aminoallyl-dUTP in the first-strand cDNA. Absorbance of each target was determined by optical density measurements at 260 nm (DNA) and either 550 (Cy3) or 650 nm (Cy5). The total dye content (pmoles), amount of probe (nanograms), and specific activity (number of Cy molecules incorporated per number of bases) was calculated for each target synthesized. Once the target quality was determined to be appropriate, targets were hybridized to probes immobilized on glass slides. The slides were hybridized overnight at 50°C using the GlassHyb Hybridization Solution (BD). After we quantified gene expression with Atlas Glass Microarrays, analysis and visualization of data were done by the Atlas Navigator software (BD). The fluorescence intensity of each spot was calculated using the histogram quantitation method, which has the major advantage of being simple and stable.

Reverse Transcriptase-Polymerase Chain Reaction and Real-Time Polymerase Chain Reaction

Total RNA was isolated from cells using the SV total RNA isolation system (Promega, Madison, WI) according to the manufacturer's recommendations and verified by electrophoresis in denaturing 1.0% agarose gel. cDNA was prepared from total RNA with oligo(dT) priming and AVM reverse transcriptase (RT) XL (Takara, Bio, Ptsu, Shiga, Japan) by incubation of a 25-μL mixture at 41°C for 1 hour. The cDNA was subjected to polymerase chain reaction (PCR) by using the following HLA-G–specific oligonucleotide primers: forward primer 5′-cgcggaccaaccctcttcctgctactctcgg-3′ and reverse primer 5′-cggggtaccgcctcgctctggttggtagtagcc-3′. PCR amplifications (1 μL of cDNA in a total reaction volume of 50 μL) were run at 98°C for 10 seconds, at 56°C for 20 seconds, and at 72°C for 30 seconds (30 cycles). The amplification of β-actin was performed in the same manner to check cDNA quality. JAR cells were used as negative control, and JEG-3 cells were used as positive control. PCR·amplification products were separated by electrophoresis on a 2% agarose gel.

Real-time RT-PCR was used to semiquantify HLA-G expression in primary cultured limbal and conjunctival epithelium. The method allows for the direct detection of PCR products during the exponential phase of the reaction, combining amplification and detection using TaqMan chemistry (Applied Biosystems, Foster City, CA) and the ABI Prism 7700 Sequence Detection System (Applied Biosystems). The TaqMan probe was designed to anneal to the target sequence, HLA-G α−1 domain, between the classic forward and reverse primers.

Immunocytochemistry

Cytospin preparations of limbal epithelial cells (5.0×10^4 cells/slide) were prepared by Auto Smear CF-12D (Sakura Finetechnical, Tokyo, Japan). Samples were fixed in cold acetone for 10 minutes and washed with PBS. Cytospin preparations were blocked with 10% normal donkey serum (Chemicon International, Temecula, CA) for 1 hour. Sections were incubated for 60 minutes at room temperature with a monoclonal antibody (MEM-G/9, 1/100 dilution; Abcam, Cambridge, UK) that reacts with the native form of human HLA-G on the cell surface, as well as with soluble HLA-G molecules. Isotype normal mouse immunglobulin G1 (IgG1) (Dako Cytomation, Glostrup, Denmark) was used as control. After washing with TBST (0.825 mmol/L Tris, 136.9 mmol/L NaCl, 1.34 mmol/L KCl, 0.1% Tween 20; Sigma), the section was reacted with rhodamine-conjugated donkey anti–mouse IgG secondary antibody (Jackson Immuno Research, West Grove, PA) for 30 minutes at room temperature. After 3 washes with TBST, the sections were incubated with 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI; Dojindo Laboratories, Tokyo, Japan) at room temperature for 5 minutes. Finally, sections were washed 3 times in TBST, and a coverslip was fixed using an antifading mounting medium (50 mmol/L Tris buffer saline, 90% glycerin [Wako], 10% 1,4-diazabicyclo (2,2,2) octane [Wako]).

Western Blot Analysis

Samples were dissolved with lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% Nonidet P-40; Calbiochem, Darmstadt, Germany) and homogenated. Samples were incubated for 40 minutes at 4°C and centrifuged at 15,000 rpm for 30 minutes at 4°C. Protein concentration of the supernatant was determined by the DC protein assay (Bio-Rad Laboratory, Hercules, CA). All samples were diluted in 2× sample buffer (100 mmol/L Tris-HCl [pH 6.8], 4% sodium dodecyl sulfate [Invitrogen], 20% glycerol [Wako], 12% 2-mercaptoethanol [Wako]) and boiled. Twenty micrograms of each sample was loaded on a Novex NuPAGE 10% Bis-Tris gel (Invitrogen) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). Membranes were blocked with 5% skim milk (Difco Laboratories, Detroit, MI), 1.5% normal goat serum, and PBS for 60 minutes at room

temperature. Membranes were reacted with an anti–HLA-G (MEM-G1) antibody (Serotec, Oxford, UK) for 60 minutes at room temperature. After 3 washes in TBST, donkey biotinylated anti–mouse IgG (Jackson ImmunoResearch) was added for 30 minutes at room temperature. Protein bands were visualized by the Vectastain ABC Elite Kit (Vector Laboratories, Bulingame, CA) and DAB (Vector Laboratories) as substrate.

Transfectants

The K-562 human erythroleukemia cell line (ATCC) was maintained in IMDM (Invitrogen) supplemented with 100 IU/mL penicillin (Wako)/100 μg/mL streptomycin (Wako) and FCS (Sanko Zyunyaku Co. Ltd., Tokyo, Japan). After successful amplification of the full-length human *HLA-G* gene from corneal epithelium, HLA-G plasmids were generated by cloning HLA-G cDNA into a green fluorescent protein (GFP) construct (phrGFP1-Puromycin; Stratagene, La Jolla, CA). GFP vector transfectants (phrGFP1-Puromycin) were used as control. Transfection was done using the Effectene Transfection Reagent (Qiagen) and the Nucleofector electroporation device (Amaxa, Cologne, Germany). Cells were selected in media containing 100 μg/mL Puromycin (Sigma).

Peripheral Blood Mononuclear Cells and Purified Natural Killer Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from normal healthy volunteers by density gradient centrifugation using Ficoll-Paque PLUS (Amersham Biosciences, Uppsala, Sweden). $CD56^+$ natural killer (NK) cells were purified using the NK cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of the isolated populations used in the experiments was greater than 97% $CD3^-CD56^+$.

Cytotoxic Assay

The cytolytic action of NK cells against K-562 HLA-G transfectants was measured by bromodeoxyuridine (BrdU) release using the Cellular DNA Fragmentation enzyme-linked immunosorbent assay kit (Roche, Mannheim, Germany), in which effector cells (NK cells) were mixed with 1×10^5 BrdU-labeled target cells (K-562 transfectants) at the same effector:target ratio. After a 24-hour incubation at 37°C in a humidified 5% CO_2 incubator, DNA fragments by cell-mediated cytotoxicity were measured in the supernatant using a BrdU-specific monoclonal antibody. The percentage of cell lysis was calculated as follows: Percent specific lysis = [(OD_{450} experimental well − OD_{450} spontaneous release)/(OD_{450} maximum release − OD_{450} spontaneous release)] × 100.

RESULTS

Microarray Analysis

To screen for changes in gene expression by ocular surface epithelial cells under different culture conditions, CCL20.2 cells were cultured on either collagen- or AM-coated dishes and compared by microarray. Table 1 shows a list of genes that were upregulated by an AM substrate. There was a 2.6-fold increase in HLA-G expression, which was the only gene with a known immunoregulatory function. Because ATCC warns of a possible contamination of CCL20.2 cells by HeLa cells, we further studied the expression of HLA-G in freshly dissociated limbal epithelial cells, as well as primary cultures of limbal and conjunctival epithelial cells.

Cultured Conjunctival and Limbal Epithelial Cells Express HLA-G mRNA

HLA-G mRNA was expressed by primary conjunctival and limbal epithelial cells when cultured on collagen, as well as AM (Fig. 1). Freshly dissociated limbal epithelial cells also expressed HLA-G by RT-PCR. JAR cells (HLA-G negative) and K562 cells (HLA class 1 negative) were used as a negative control and JEG-3 cells were used as a positive control. No mRNA was detected from AM samples without seeded cells (data not shown), ruling out the possibility of contamination by AM mRNA.

Real-time PCR was used to semiquantitate HLA-G expression by each cell type when cultured on AM. In accordance with microarray results, CCL20.2 showed a 2.0-fold increase ($P < 0.05$) in HLA-G expression when cultured on AM (Fig. 2A). Similarly, primary conjunctival epithelial cells underwent a 1.9-fold increase ($P < 0.05$). Primary cultured limbal epithelial cells also had a tendency for upregulating HLA-G, but the difference was not statistically significant compared with collagen (Fig. 2A).

HLA-G Protein Expression by Limbal Epithelial Cells

Immunocytochemistry was done on cytospin samples of cultured primary limbal cells, JAR cells, and JEG-3 cells. Conjunctival cells were not available because of the scarcity of surgically removed tissue. Although freshly dissociated limbal cells did not express appreciable levels of HLA-G on the cell surface (Fig. 3A), primary limbal epithelial cells cultured on AM showed clusters of positive-staining cells (Fig. 3B). The specificity of HLA-G staining was confirmed by negative (JAR) and positive (JEG-3) controls (Fig. 3C and D). HLA-G protein was also compared by Western blot (Fig. 4). Similar to immunocytochemistry, HLA-G was detected in cultured limbal epithelial cells, but not by freshly dissociated cells from limbal tissue.

Inhibition of Cell Lysis by HLA-G–Transfected K-562 Cells

To show the immunosuppressive function of the HLA-G molecule, we transfected the *HLA-G* gene derived from limbal epithelium into the HLA class 1–negative K-562 cell by using a GFP vector. HLA-G was successfully transfected into K-562 as shown by RT-PCR (Fig. 5A). When activated NK cells isolated from fresh peripheral blood were cocultured with transfected K-562, cell lysis detected by BrdU release was slightly lower in HLA-G–transfected cells than in control (Fig. 5B).

DISCUSSION

The absence of a harmful maternal immune response against the semiallogenic fetus has long been a major enigma in current biology. During mammalian pregnancy, fetal cells invade the uterine structures and survive without immunologic rejection.[20] It has now become evident that trophoblast cells