Table. Clinical Data and Surgical Outcome of Patients*

Patient No./ Sex/Age,y	Disease	Eye	Additional Procedures†	Preoperative Visual Acuity	Postoperative Visual Acuity	Follow-up, mo
1/F/68	SJS	Left	AMT + Phaco/IOL	HM	20/630	19
2/M72	SJS	Left	AMT	HM	20/1000	18
3/F/70	SJS	Right	Phaco/IOL	HM	20/2000	15
4/M/31	Thermal burn	Right	None	20/500	20/63	15
5/M/65	Chemical injury	Right	AMT + Phaco/IOL	20/500	20/500	12
6/M/19	SJS	Right	None	HM	20/50	11
7/M/75	OCP	Left	AMT + Phaco/IOL	HM	20/1000	10
8/F/63	SJS	Left	AMT + Phaco/IOL	HM	20/1000	10
9/M/53	SJS	Left	Phaco/IOL	HM	20/32	8
10/M/57	SJS	Right	AMT	HM	20/1000	8

Abbreviations: AMT, amniotic membrane transplantation; HM, hand motions; OCP, ocular cicatricial pemphigoid; Phaco/IOL, phacoemulsification of cataract and intraocular lens implantation; SJS, Stevens-Johnson syndrome.

*All patients had complete epithelialization within 2 to 5 days.

†All patients underwent removal of the corneal pannus and surrounding diseased tissue and transplantation of autologous serum–derived epithelial equivalent.

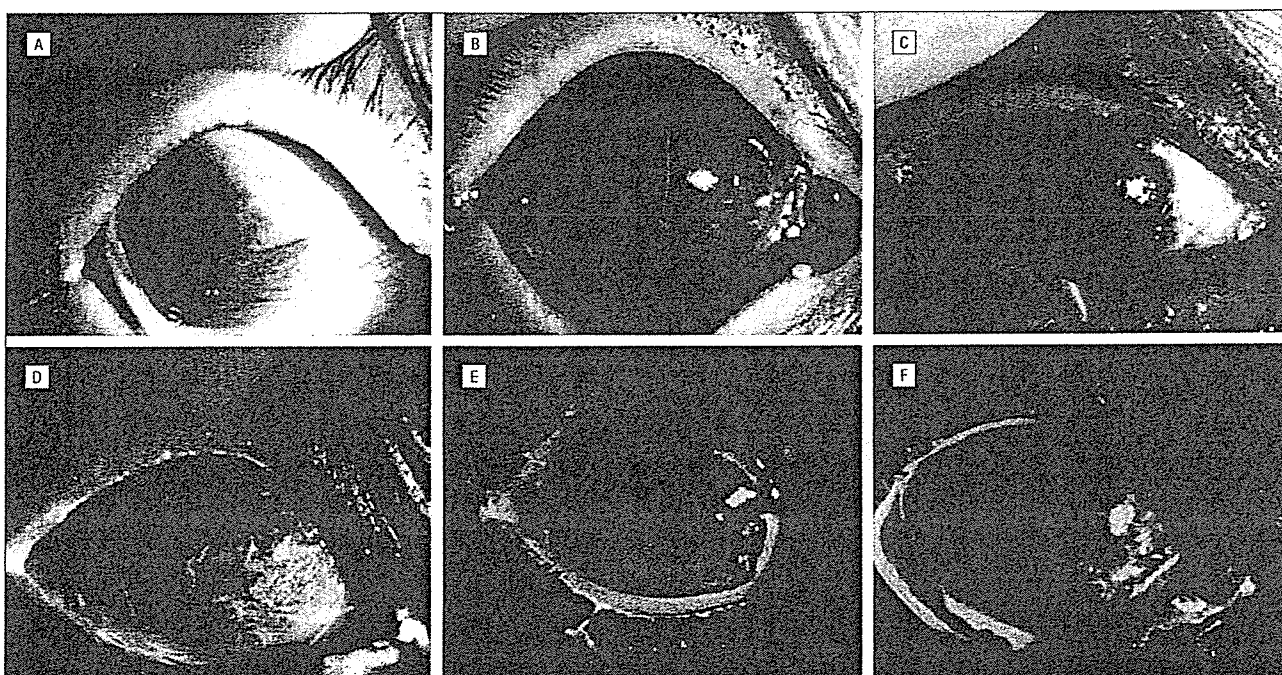

Figure 3. External appearance (A-C) with corresponding fluorescein staining (D-F) in a patient with Stevens-Johnson syndrome and total limbal stem cell deficiency (patient 3). The preoperative appearance (A) demonstrates extensive conjunctivalization, neovascularization, and scarring, with persistent epithelial defects noted on fluorescein staining (D). Two days after transplantation, the corneal surface was clear and smooth (B), and fluorescein staining confirmed that the entire corneal surface was covered by the cultivated epithelium (E). The postoperative appearance at 15 months shows a smooth, epithelialized corneal surface with minimal scarring and inflammation (C and F).

reports on cultivated epithelial transplantation used allogeneic tissue.[10,13-17] After allogeneic transplantation in these vascularized and inflamed eyes, patients require long-term medication, such as corticosteroids and immunosuppressive agents, to prevent allograft rejection. In addition, many of these severely damaged eyes require multiple ocular surgical procedures, such as penetrating or lamellar keratoplasty to remove significant corneal scarring, before vision can be satisfactorily restored.[6] Previous use of allogeneic transplantation may predispose these eyes to an increased risk of corneal graft rejection and failure.[30,31] In our study, the use of autologous oral epithelial transplantation for ocular surface epithelialization overcame the problems related to allogeneic transplantation because it helped reduce the risk of graft rejection and the need for long-term medication and immunosuppression. This is particularly important in conditions where multiple reconstructive procedures or transplantations are required for long-term restoration of vision.

Stevens-Johnson syndrome is a major cause of severe OSD, and afflicted patients often have multisystemic involvement. A high concentration of sFasL at the onset of SJS has previously been shown to play a role in keratinocyte apoptosis and in the pathophysiology of the disease.[22] In our study, we found that serum sFasL levels

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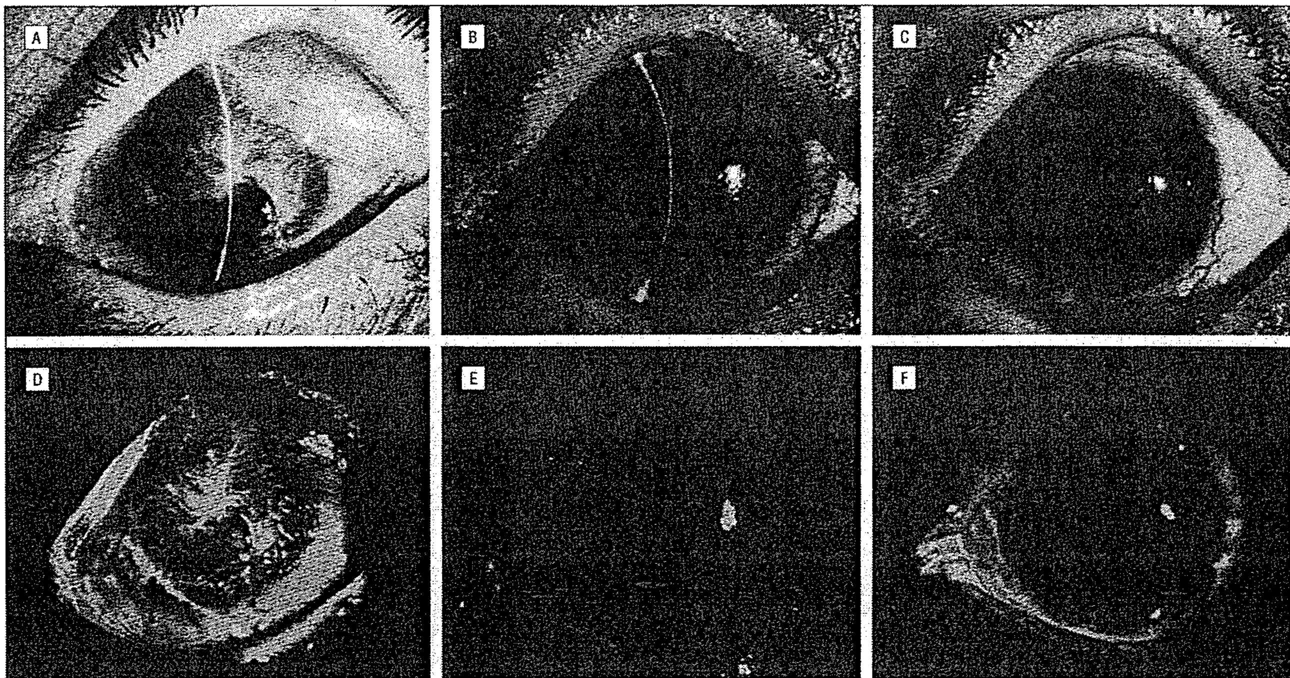

Figure 4. External appearance (A-C) with corresponding fluorescein staining (D-F) of a patient with ocular cicatricial pemphigoid and severe ocular surface disease with total limbal stem cell deficiency (patient 7). Preoperative appearance demonstrates superior symblepharon formation, extensive conjunctivalization, neovascularization, and scarring of the cornea (A), and severe epitheliopathy is demonstrated on fluorescein staining (D). Five days after transplantation, the corneal surface was clear and smooth (B), and fluorescein staining confirmed that the entire corneal surface was covered by the cultivated epithelium (E). Postoperative appearance at 8 months shows a smooth, epithelialized corneal surface with minimal scarring and inflammation (C and F).

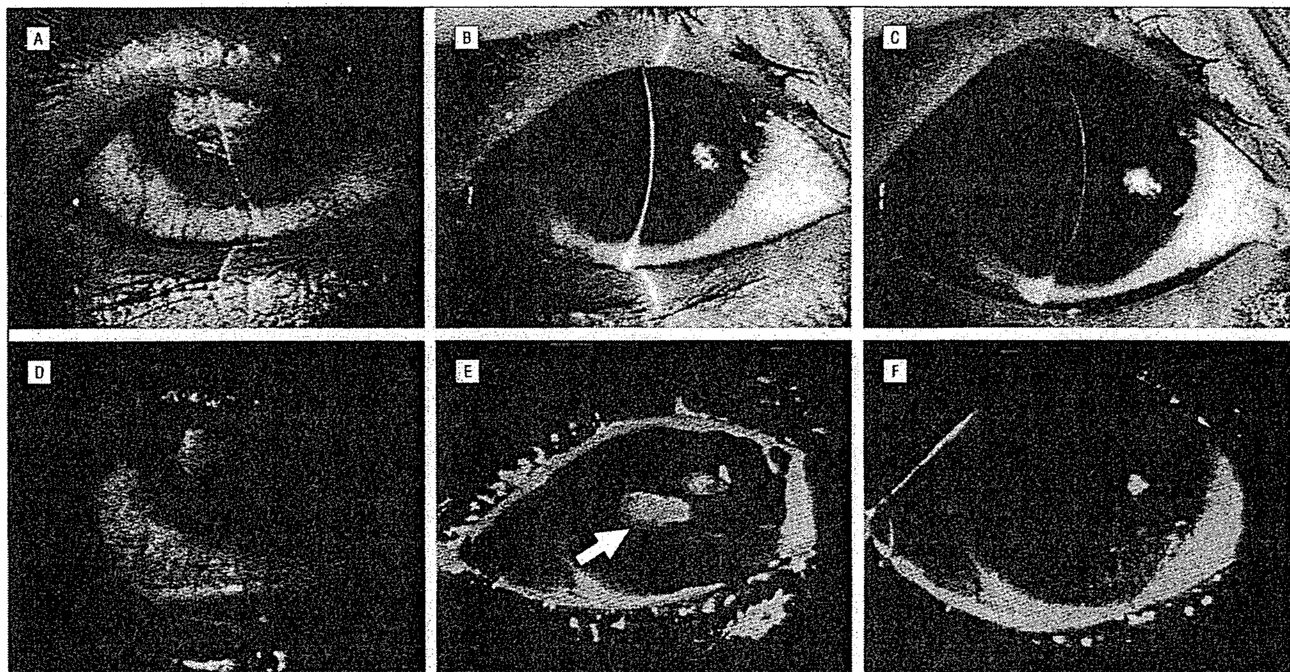

Figure 5. External appearance (A-C) with corresponding fluorescein staining (D-F) of a patient with Stevens-Johnson syndrome who underwent cultivated epithelial transplantation (patient 6). Preoperative appearance demonstrates severe keratinization of the cornea with superior symblepharon formation (A and D). A small corneal epithelial defect was noted 3 months after surgery. The arrow indicates the epithelial defect stained with fluorescein (B and E). The epithelial defect healed over from the adjacent oral epithelium, and the corneal surface was noted to be smooth and epithelialized, with minimal scarring and inflammation (C and F).

were too low to be detected in subjects with SJS, other subjects with severe OSD, and healthy controls, suggesting that the serum sFasL level would not be an impediment in our culture system. To our knowledge, our recent study is the first to evaluate the suitability of AS from patients with SJS in supporting in vitro epithelial cell proliferation.[32] We demonstrated that oral epithelial cells cultured in AS-supplemented medium had similar prolif-

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erative capacities compared with FBS-supplemented cultures, which ensures that the regenerative potential of these cells was similarly maintained in both culture systems.[32]

Most of the previous studies on bioengineered corneal epithelial equivalents for clinical transplantation have relied primarily on FBS-supplemented medium in the culture process.[11-20] In this study, we demonstrated that the morphological appearance of AS-derived cultivated oral epithelium was similar to that of normal in vivo cornea and FBS-derived cultures. Immunohistochemical analysis confirmed the presence of the keratin 4–keratin 13 pair, which is consistent with that of nonkeratinized, stratified epithelia. The cultivated oral epithelial cells also demonstrated positive staining for keratin 3, a marker for corneal differentiation,[33] suggesting that these epithelial sheets bore some similarities to normal corneal epithelium. The AS-derived oral epithelial equivalents also demonstrated the presence of basement membrane–related proteins and hemidesmosomes (integrins $\alpha6$ and $\beta4$),[34] which are important for ensuring graft integrity during surgical manipulation and after transplantation. The cultivated oral epithelial sheets demonstrated good cell-to-substrate adhesion, and graft integrity was maintained throughout the follow-up.

The ability of transplanted oral epithelial equivalents to continue to regenerate and replenish the corneal epithelial surface is of critical importance when evaluating their use for clinical transplantation. We demonstrated that AS-derived cultivated oral epithelial transplantation achieved complete corneal epithelialization within 2 to 5 days, which is similar to our previous results with cultivated oral epithelial transplantation using FBS-supplemented culture medium.[18,19] The corneal surface of all eyes remained clear and smooth and was covered with transplanted epithelium at the last follow-up visit, with the longest follow-up being 19 months. Although this was a noncomparative clinical study, the clinical results of transplanting AS- and FBS-derived cultivated oral epithelial equivalents were similar to those of our previous clinical experience,[19] suggesting that AS-cultivated epithelial transplantation is a safe and effective procedure for the treatment of severe OSD.

CONCLUSIONS

We have demonstrated the effective use of AS-derived cultivated autologous oral epithelial transplantation for the treatment of severe limbal stem cell deficiency. This novel treatment modality has important clinical implications because it eliminates the use of bovine material in the culture process, reduces the risk of allograft rejection and transmission of infection, and reduces the need for long-term corticosteroid and immunosuppressive therapy. This study has brought us one step closer toward developing safer xenobiotic-free autologous bioengineered products that are derived entirely from the patient's own tissue. The successful use of completely autologous bioengineered tissue equivalents for clinical transplantation represents a significant advancement in the field of ocular bioengineering and transplantation.

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An Investigation of Removed Cultivated Epithelial Transplants in Patients After Allocultivated Corneal Epithelial Transplantation

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Abstract

Objective:
To investigate the ultrastructural changes of removed cultivated corneal epithelial transplants using scanning and transmission electron microscopy.

Methods:
Allocultivated corneal epithelial transplantation, using an amniotic membrane carrier, was carried out on 3 patients. The primary diagnoses consisted of 1 with acute-phase chemical burn, one with drug-induced pseudopemphigoid, and 1 with Stevens-Johnson syndrome. After a period of several months the transplants were removed from these patients because of graft opacities. The removed transplants were then prepared for examination by scanning and transmission electron microscopy.

Results:
In all 3 cases there was a similar pattern of findings: the amniotic membrane remained intact, although it had become partially vascularized and invaded by keratocytes. Inflammatory cells were present in the epithelial layer and within the amniotic membrane. Most of the amniotic membrane was covered by conjunctival epithelial cells and goblet cells. Only a few areas of normal cultivated corneal epithelial cells were found.

Conclusions:
We suggest that the process of allograft rejection is responsible for the corneal epithelial loss and that this is followed by conjunctival invasion onto the amniotic membrane.

Key Words: **allograft rejection, limbal epithelial cells, amniotic membrane, transplantation, ultrastructure**

(*Cornea* 2004;23:235–242)

The healthy ocular surface is composed of two different types of epithelial cells, namely, corneal epithelial cells and conjunctival epithelial cells. The corneal epithelial cells are essential for a clear cornea and good vision. However, in severe ocular surface diseases such as Stevens-Johnson syndrome (SJS) and chemical burns, the corneal epithelial cells are sometimes totally destroyed. This damage often extends to the limbal area of the cornea; this is the region where corneal epithelial stem cells are located.[1,2] In a severely injured cornea in which the limbal and central epithelia are both absent, the neighboring conjunctival epithelial cells invade onto the corneal surface, leading to inflammation and vascularization. Ultimately, visual acuity is severely obstructed.[3,4]

Cultivated corneal epithelial transplantation has been the focus of recent attention as a new approach for ocular surface reconstruction in limbal epithelial transplantation.[5,6] Our group first cultivated rabbit limbal epithelial cells on amniotic membrane (AM) in vitro and then, following transplantation onto the rabbit ocular surface, confirmed the viability of the transplanted cultivated epithelium in vivo.[7] Next, we showed that a denuded AM substrate (without amniotic epithelial cells) is more suitable for corneal epithelial cell culture than cellular amniotic membrane (with amniotic epithelial cells).[8] Most recently we have developed a suspen-

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sion culture system.[9] Currently, for clinical treatment of cases of severe stem cell deficiencies, we use cultivated corneal limbal epithelial transplantation with a denuded AM carrier. We use this treatment even for acute-phase SJS[10] patients, who are normally considered to be contraindicated for corneal transplantation.

Our group performed allocultivated corneal epithelial transplantation on 13 eyes with acute- and chronic-phase severe ocular surface disorders in the period between September and December 1999. In all 13 eyes, the ocular surface was covered with transplanted cultivated epithelium 48 hours after transplantation, and visual acuity improved more than 2 lines in 73% of eyes. However, during the long-term observation period, 3 eyes had suffered allograft rejection, and finally they were covered again with opaque epithelium. We carried out cultivated corneal epithelial transplantation again in these patients and achieved successful ocular surface reconstruction. This procedure is described by Nakamura et al.[11] We obtained the opaque epithelial samples from the patients' eyes at the time of the second transplantation and processed these for scanning and transmission electron microscopy. In this paper we report the ultrastructural findings of compromised allocultivated corneal epithelial transplants using scanning and transmission electron microscopy.

MATERIALS AND METHODS

Cultivation of Corneal Limbal Explants on Denuded Amniotic Membrane Carrier

In accordance with the tenets of the Declaration of Helsinki and with proper informed consent, human AMs were obtained at the time of cesarean section. The membranes were washed with sterile phosphate-buffered saline (PBS) containing antibiotics (5 mL of 0.3% ofloxacin) under sterile conditions and stored at −80°C in Dulbecco Modified Eagle Medium (GIBCO BRL, Rockville, MD) and glycerol (Nacalai Tesqu Co., Kyoto, Japan) at the ratio of 1:1 (vol/vol). Before use, membranes were deprived of their amniotic epithelial cells by incubation with 0.02% ethylene diamine tetraacetic acid (EDTA, Waco Pure Chemical Industries, Osaka, Japan) at 37°C for 2 hours to loosen cellular adhesion, followed by gentle scraping with a cell scraper (Nalge Nunc International, Naperville, IL).[8]

The explant culture was made using the same method we have previously reported.[8] Briefly, the limbal tissue was cut into 2 × 2 mm square, 100 μm thick explants. Three pieces of the explant were placed directly, epithelial side down, on a portion of denuded AM spread on a culture plate insert. The culture was submerged into the medium described above for 2 weeks and then exposed to air by lowering the medium level (airlifting) for 2 weeks. The explants were left in the culture dish for the whole duration of the incubation. We cocultured the corneal limbal epithelium with mitomycin C-inactivated 3T3 fibroblasts according to the keratinocyte culture methods.[12] The cultivated cells were 4–5 layers of stratified and well-differentiated epithelial cells, and they showed corneal-specific keratin expressions (keratin 3 and keratin 12).

Samples for Ultrastructural Examination

Samples were obtained from three patients whose primary diagnoses were acute-phase SJS (case 1), acute-phase chemical burn (case 2), and drug-induced pseudopemphigoid (case 3) (Table 1). Patients received cultivated corneal epithelial transplants, which were subsequently rejected. These transplants were removed and prepared for examination by scanning and transmission electron microscopy as detailed below.

Scanning Electron Microscopy

The samples were fixed in 4% glutaraldehyde in PBS. They were washed in PBS for 15 minutes and then postfixed in 2% osmium tetroxide for 2 hours. They were washed

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TABLE 1. Patient Profile

Case	Age/Gender	Diagnosis	First Operation	Time to Epithelial Rejection (M)	Time to the Second Operation (M)
1	21/M	SJS	CCET	6	12
2	35/M	Chemical	CCET	3	12
3	32/F	P-pemphigoid	CCET+LK	10	12

SJS, Stevens-Johnson syndrome; chemical, acute chemical burn; p-pemphigoid, drug-induced pseudopemphigoid; CCET, cultivated corneal epithelial transplantation; LK, lamellar keratoplasty.

again in PBS before being passed through an alcohol series. After 2 20-minute changes of 100% ethanol the samples were then transferred to hexamethyldilisane for 10 minutes and air dried. The samples were then mounted on aluminium specimen stubs and sputter coated with gold before being examined on a JEOL JSM 5600 scanning electron microscope.

Transmission Electron Microscopy

The samples were fixed in 4% glutaraldehyde in PBS. They were washed in PBS for 15 minutes and then postfixed in 2% osmium tetroxide for 2 hours. They were washed again in PBS before being passed through an alcohol series and embedded in Araldite resin (Agar Scientific, UK). Ultrathin sections were cut on a Reichert Ultracut E, collected on naked copper grids, and stained with aqueous uranyl acetate, phosphotungstic acid, and lead citrate before being examined on a JEOL JEM 1010 transmission electron microscope.

RESULTS

Clinical Details

All 3 eyes recovered clear corneal surfaces after the first transplantation, but subsequently all cases showed epithelial opacity following allograft rejection. Case 1 (Fig. 1A–D) is shown in detail. Figure 1A was taken 5 months after transplantation, before rejection. Figure 1B, taken 7 months after initial transplantation, and Figure 1C, taken 12 months after transplantation, show typical allograft rejection that was characterized by severe inflammation and conjunctivalization. Figure 1D was taken 1 month after the patient received a second transplant. At that time the corneal surface remained clear and

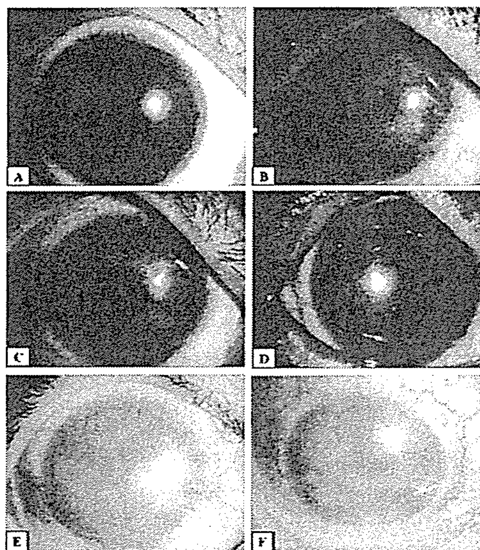

FIGURE 1. Clinical photographs of case 1 (A–D), case 2 (E), and case 3 (F). The progression of case 1 shows typical allograft rejection and is described in detail as follows: 5 months following the initial transplant, the corneal surface was covered with clear epithelium (A); 2 months later, allograft rejection occurred accompanied by large corneal defect and severe inflammation (B). One year after the original transplant, the corneal surface became vascularized and covered with opaque conjunctival epithelium (C). A month after the second transplant, the corneal surface remained clear and smooth (D). Figures E and F (cases 2 and 3, respectively) were taken 12 months after the first corneal epithelial transplantation and show typical allograft rejection characterized by severe vascularization and conjunctival invasion.

smooth. Cases 2 and 3 showed a similar pattern of rejection. Figure 1E shows case 2 a year after corneal epithelial transplantation. Case 2 experienced allograft rejection accompanied by sudden-onset epithelial defects and severe recurrence of the inflammation. Figure 1F was taken from case 3 12 months after the initial transplantation. Case 3 suffered microbacterial infection initially, and then loss of epithelial transparency. We speculate that the inflammation caused by the bacterial infection induced allograft rejection. Patients received new cultivated epithelial transplants, which were secured onto the corneal surface with 10-0 nylon sutures and then covered with a therapeutic soft contact lens. The clinical details of these patients have been described by Nakamura et al.[11] Patients have been closely monitored, and at this time their corneal surfaces remain clear and smooth (Fig. 1D).

Scanning Electron Microscopy

Examination of the removed cultivated corneal epithelial transplants from host corneas by scanning electron microscopy revealed that in general all three cases showed similar findings. Only a few areas of normal cultivated corneal epithelial cells were found. The appearance of these cells has been previously reported.[9] Examination of the apical surface of these cells showed that they were 30–40 μm in diameter with tightly opposing cell junctions as described previously[9] (Fig. 2A). In some places there were also areas of bare AM. However, most of the AM was covered by conjunctival epithelial cells. These cells are slightly different in appearance from the corneal epithelial cells in that they are smaller, measuring approximately 5–15 μm in diameter in comparison to the remaining corneal epithelial cells. In addition, their cell boundaries were more prominent (Fig. 2B). The apical surfaces of the superficial cells were covered in microvilli, which showed gross clumping. In some places there appeared to be evidence of the remaining cultivated corneal epithelial cells being destroyed by inflammatory cells (Fig. 2C). Numerous inflammatory cells were found on the apical surface of the epithelial cells (Fig. 2D).

Transmission Electron Microscopy

Examination of the removed cultivated corneal epithelial transplants from host corneas by transmission electron microscopy showed that in general all samples had a similar appearance, although the chemical burn patient showed slightly less inflammation than the other cases. In all 3 cases the AM remained intact (Fig. 3A–C) and showed no attachment to the corneal stroma (Fig. 3C), allowing the transplants to be easily removed from the host cornea. The AM had become partially vascularized (Fig. 3D), although the blood vessels were relatively small in size and were distributed throughout the AM and were generally found directly beneath the conjunctival epithelium. The AM also became invaded by keratocyte-like cells (Fig. 3B) that formed layers similar to those found in the scleral or corneal stroma. Numerous inflammatory cells including lymphocytes, plasma cells, macrophages, and granulocytes were present in the epithelial layer (Fig. 3E) and within the AM itself (Fig. 3F). They were more numerous in the patients with SJS and drug-induced pseudopemphigoid. Most of the AM was covered by conjunctival epithelial cells characterized by the presence of goblet cells (Fig. 3G). Only a few areas of normal cultivated corneal epithelial cells were found (arrow), and in some regions these cells appeared necrotic and under attack by inflammatory cells. In places, it appeared that necrotic corneal epithelial cells were being replaced by the invading conjunctival epithelial cells migrating underneath (Fig. 3H). Although all 3 cases showed a similar pattern of rejection, the severity of the rejection process appeared less extreme in case 2, the chemical burn cornea (Fig. 3H). There were few inflammatory cells, and those that were present were usually confined to the apical surface of the epithelium. There was much less infiltration of inflammatory cells into the epithelial layer and stroma and little or no vascularization of the stroma.

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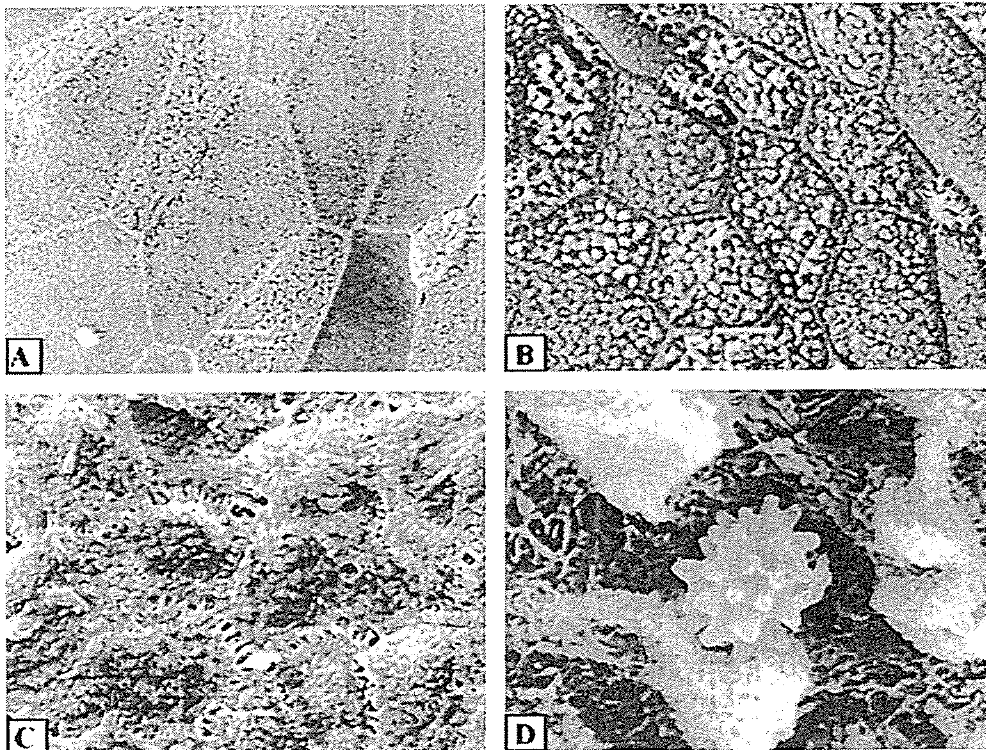

FIGURE 2. Scanning electron micrographs of removed cultivated corneal epithelial transplants from host corneas (A–D). Figure A was taken from the drug-induced pseudopemphigoid patient (case 3), figure B from the SJS patient (case 1), and figures C and D from the chemical burn patient (case 2). In some places areas of normal corneal epithelial cells were found in which the cell junctions are well developed and tightly opposed (A). However, most of the AM is covered by conjunctival epithelial cells (B) in which the cell boundaries are more prominent in comparison to the remaining corneal epithelial cells. A transitional zone was also found in which the remaining cultivated corneal epithelial cells were becoming necrotic (C). Inflammatory cells were found in all cases (D). Scale bars 5 μm (A–C) and 2 μm (D).

DISCUSSION

Cultivated corneal epithelial transplantation is a very new surgical treatment of severe stem cell deficiencies. This new approach has often proved very successful in maintaining good vision in SJS and chemical burn patients by transplanting the cultivated epithelial sheet during the acute phase.[10]

To date, it has not been possible to determine exactly what happens to these transplants on the human cornea. Histopathologic changes in excised corneal buttons from patients following amniotic membrane and limbal transplantation have been previously reported.[13] However, this ultrastructural investigation of the removed rejected transplants gives us the first opportunity to study transplanted epithelium and the AM carrier that has been removed from a patient's eye and provide insight into the mechanism of rejection.

In all 3 cases the AM remained intact, allowing it to be easily removed from the patient's ocular surface and replaced with another graft. We found that the ultrastructural changes occurring were very similar in all 3 cases, suggesting that a common mechanism of rejections was occurring.

We found that the AM became vascularized with small blood vessels forming directly beneath the basement membrane. The stroma of the AM also contained long flat-

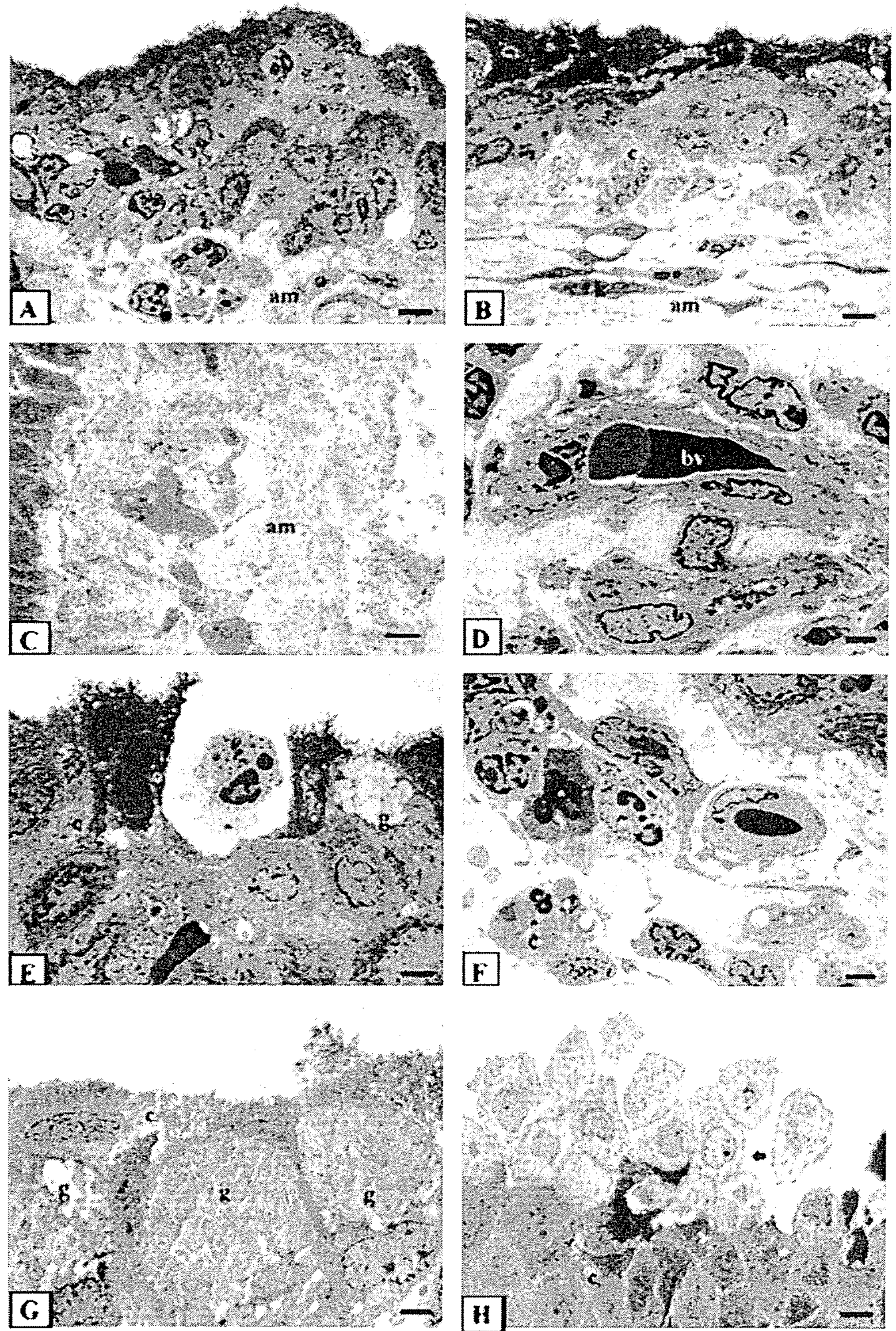

FIGURE 3. *(Legend on facing page).*

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tened cells similar in appearance to scleral keratocytes. The majority of the removed AM was covered by conjunctival cells. This was confirmed by their characteristic morphology and the presence of goblet cells, which are found only in the conjunctiva and are not present in corneal epithelium. Numerous inflammatory cells including lymphocytes, plasma cells, macrophages, and granulocytes were found throughout the epithelium and within the AM itself. These ultrastructural and clinical findings suggest that the process of allograft rejection is responsible for the corneal epithelial cell loss and that this is followed by conjunctival invasion onto the AM.

The findings follow the classic pattern of epithelial rejection as reported by previous workers.[14,15] Particularly characteristic is the process of lymphocyte infiltration.[16] We observed the presence of various inflammatory cells including lymphocytes, plasma cells, and macrophages as well as vascularization. The rejection process appears to have resulted in the loss of almost all of the cultivated corneal epithelial cells and allowed the invasion of conjunctival cells onto the AM.

Allograft rejection is one of the biggest problems of corneal epithelial cell transplantation, both in limbal transplantation and in keratoepithelial transplantation. Normally our allocultivated corneal transplantation technique together with a regimen of topical application of antiinflammatory drugs is effective in suppressing rejection. However, in these patients epithelial rejection has taken place. This is possibly because these 3 patients have a particularly severe history of inflammatory ocular surface disease. These patients were therefore already predisposed to inflammation. For example, case 3, who had drug-induced pseudopemphigoid, suffered corneal opacity and epithelial rejection as a result of bacterial infection. In this case it is probable that there were residual inflammatory cells already present as a result of the underlying inflammation caused by psuedopemphigoid as well as those caused by the infection.

It is well documented that patients with underlying immunologically mediated diseases, such as SJS or ocular phempigoid, who undergo limbal stem cell transplantation have much lower success rates than do those patients with noninflammatory ocular surface diseases.[17] In addition, the pattern of limbal stem cell rejection appears similar to the rejection of the allocultivated epithelial transplants reported in this paper.[17] Interestingly the rejection process appeared less severe in case 2, the chemical burns victim, and this might possibly be explained by the fact that there was no underlying immunologically mediated disease in this case.

Another explanation as to why the transplants reported in this paper have been rejected while other similar cases have not[10] could be unfavorable HLA matches. Other workers have shown that use of best-matched HLA conjuntival limbal allografts have a much better success rate than nonmatched tissue.[18] It might be worth considering tissue matching in the future, although limited tissue supply makes this problematic.

However, one great advantage of our cultivated epithelial transplantation technique is that if a graft fails it can be removed and replaced. This study has shown that even after several months on the patient's eye the AM remains intact, allowing the rejected transplant to be easily peeled off the patient's eye. In fact, several months following their original surgery, these patients underwent

FIGURE 3. *(continued)* Transmission electron micrographs of removed cultivated corneal epithelial transplants from host corneas (A–H). Figures A–F were taken from the SJS patient (case 1), figure G from the patient with drug-induced pseudopemphigoid (case 3), and figure H from the chemical burn patient (case 2). In all cases the AM (am) remained intact (A–C) although it had become vascularized (bv) (D) and invaded by keratocytes (k) (B). Abundant inflammatory cells (*) are found within the epithelium (E) and within the AM (F). Conjunctival epithelial cells (c) interspersed with goblet cells (g) (G) cover most of the AM. In some areas the conjunctival cells (c) appear to be displacing residual corneal epithelial cells (arrow) (H). Scale bars 5 μm (A,B,H) and 2 μm (C–G).

further cultivated corneal epithelial cell transplantation. The progress of these patients is being closely monitored, and at this time the ocular surfaces of these patients remain clear and smooth.

We hope that this investigation has provided new information about cultivated corneal transplantation and why these transplants occasionally fail. We hope to use this information to develop more effective postoperative treatments for these patients.

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Serum-Free Spheroid Culture of Mouse Corneal Keratocytes

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Purpose. To develop a serum-free mass culture system for mouse keratocytes.

Methods. Corneas of C57BL6/J mice were enzyme digested after the epithelium and endothelium were removed. Stromal cells were cultured in serum-free DMEM/F12 (1:1) containing epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), and B27 supplement. Primary spheres were dissociated by trypsin and subcultured as suspended secondary spheres. Cells from postnatal day (P)6 to P10 spheres were subcultured onto plastic dishes or type I collagen gels for phenotype analysis. The expression of the keratocyte markers keratocan, aldehyde dehydrogenase (Aldh), and CD34, were analyzed by RT-PCR, and vimentin and α-smooth muscle actin (α-SMA) were examined by immunocytochemistry.

Results. Primary keratocytes formed spheres, which were cultured for over 12 passages. Suspended sphere cells expressed vimentin, keratocan, CD34, and lumican, but were negative for cytokeratin K12 (K12) and Pax6. Sphere cells subcultured on plastic exhibited a dendritic morphology characteristic of keratocytes, and maintained keratocan, Aldh, and CD34 expression in serum-free medium. Sphere cells subcultured with 10% serum became fibroblastic, and expressed α-SMA when stimulated by transforming growth factor (TGF)-β. α-SMA-positive cells demonstrated contractile properties on collagen gels, compatible with the myofibroblast phenotype.

Conclusions. The phenotype of mouse keratocytes can be maintained in vitro for more than 12 passages by the serum-free sphere culturing technique. (*Invest Ophthalmol Vis Sci.* 2005;46:1653–1658) DOI:10.1167/iovs.04-1405

The corneal stroma is characterized by a well-organized extracellular matrix consisting of a dense network of collagen fibrils and proteoglycans that are produced by keratocytes, the principal stromal mesenchymal cell of cranial neural crest origin.[1,2] In adult tissue, keratocytes are mitotically quiescent cells with a flat, dendritic morphology. Keratocytes form a three-dimensional network of cells through their extensive dendritic processes, linked via gap junctions,[3-10] and secrete collagens and keratan sulfate proteoglycans such as lumican, mimecan, and keratocan.[11-15] The corneal stroma is rich in total keratan sulfate proteoglycan content,[16] but contain relatively small amounts of dermatan sulfate proteoglycans.[17]

During corneal wound healing, the quiescent keratocytes are activated and transform into fibroblasts and/or myofibroblasts, losing their characteristic dendritic morphology. Keratan sulfate proteoglycans are downregulated,[11,18] whereas keratocytes proliferate and migrate to the site of injury, causing scar formation.[19-22] The conversion to myofibroblasts, characterized by intense expression of the contractile protein α-smooth muscle actin (α-SMA),[21,23,24] is induced by endogenous and exogenous transforming growth factor (TGF)-β.[25-27]

Ex vivo expansion of keratocytes is often performed to investigate keratocytes in vitro, and various culture techniques have been reported involving the use of plastic substrates. However, when cultured in serum-containing medium, collagenase-isolated keratocytes from bovine[28] and rabbit[27,29] corneas readily lose their in vivo quiescent phenotype and acquire a fibroblastic phenotype with altered physiological properties.[28,30,31] In the presence of 2% to 10% serum, keratan sulfate proteoglycan production is greatly reduced or absent in keratocyte-derived fibroblasts,[28,32,33] whereas production of dermatan sulfate proteoglycans is upregulated. Furthermore, TGF-β stimulation or culture at low densities[30] causes corneal fibroblasts to differentiate further into myofibroblasts with a more spread-out morphology.[26,29,32,34] Serum-free cultures have been reported to be effective in the maintenance of the dendritic morphology of keratocytes and the production of keratan sulfate proteoglycans.[27,28,30-33,35,36] However, the cultivation of a large quantity of cells by subculturing has been difficult.

In this report, we introduce our method for subculturing mouse corneal keratocytes in large quantities, using a modified version of a suspension culture method originally described for neural stem cells.[37-39] In our study, the sphere culture of keratocytes did not require serum, and the dendritic keratocyte phenotype was restored when subcultured on plastic substrate in serum-free medium.

Materials and Methods

Cell Culture

All animals were handled in full accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Stromal cells were dissociated from adult C57BL6/J mice (7–8 weeks old) and then cultured as described previously[40] with modifications. In brief, cornea tissue was excised in Hanks' balanced salt solution (HBSS) supplemented with 10% fetal bovine serum (FBS) by circular incision outside the limbus. The iris, ciliary body, and Descemet's membrane including the endothelium were bluntly dissected from the cornea. The remaining stroma with epithelium was incubated in 5 mg/mL of Dispase II (Roche Diagnostics, Indianapolis, IN) at 4°C overnight. Loose epithelial sheets were then removed, and corneal stromal discs were cut into small segments and digested in 0.05% trypsin (Sigma-Aldrich, St. Louis, MO) for 30 minutes at 37°C, followed by 78 U/mL

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TABLE 1. PCR Primers

Gene	Primer Sequence (5′–3′)	Product Size (bp)	GenBank Accession ID
Cytokeratin K12	Forward: TCCTCCTGCAGATTGACAACG Reverse: TTCCAGGGACGACTTCATGG	511	NM_010661
Pax6	Forward: AGTTCTTCGCAACCTGGCTA Reverse: TGAAGCTGCTGCTGATAGGA	500	NM_013627
Keratocan	Forward: AGGATGCCTTCATTCACGGAC Reverse: GCTCATTGTGGTGCTTATGGGG	491	NM_008438
Lumican	Forward: TGCTGTCTCGGCTTCTCTGAAAG Reverse: AACATCCCCCACATTCCCAACC	567	NM_008524
CD34	Forward: CCTTATTACACGGAGAATGGTGGAG Reverse: AAGAGGCGAGAGAGGAGAAATGGG	477	NM_133654
Vimentin	Forward: GAACGGAAAGTGGAATCCTTGC Reverse: GGTTGGCAGAGGCAGAGAAATC	591	NM_011701
Aldh	Forward: CTTCCAGCGGGTCATAAATCTG Reverse: AGCCAGCAAAACAAGTGTCAGG	528	NM_007436
Gapd	Forward: GACCACAGTCCATGCCATCAC Reverse: TCCACCACCCTGTTGCTGTAG	453	NM_008084

collagenase (Sigma-Aldrich) and 38 U/mL hyaluronidase (Sigma-Aldrich) for 30 minutes at 37°C.

Stromal cells were mechanically dissociated into single cells, and cultured in DMEM/F12 (1:1) supplemented with 20 ng/mL epidermal growth factor (EGF; Sigma-Aldrich), 10 ng/mL of fibroblast growth factor 2 (FGF2, Sigma-Aldrich), B27 supplement (Invitrogen, Carlsbad, CA), and 10^3 U/mL leukemia inhibitory factor (LIF; Chemicon International Inc., Temecula, CA) at a density of 5×10^5 cells/mL in a 37°C 5% CO_2 incubator. Initial culture was performed in 24-well plates or 35-mm dishes and then subcultured to 25-cm^2 culture flasks. The spheres were then further subcultured in 75 cm^2 culture flasks after 7 to 14 days, which was repeated every 7 to 14 days. Medium was changed every 5 to 7 days. All dishes and flasks used for sphere culture were polystyrene, noncoated vessels obtained from Asahi Techno Glass (Tokyo, Japan). Stromal sphere cells were examined by immunocytochemistry and RT-PCR. To allow cells to differentiate, cells dissociated from corneal spheres were cultured in serum-free or DMEM/F12 medium (10% FBS) supplemented with or without 2 ng/mL TGF-β (Sigma-Aldrich) for 4 days. Subcultured cells were stained by calcein-AM (Dojindo Laboratories, Tokyo, Japan), as described,[41] to visualize cell morphology. Primary stromal discs of mouse cornea were cultured in keratinocyte-serum free medium (K-SFM; Invitrogen) or DMEM/F12 with 10% FBS for 10 days (37°C, 5% CO_2), to identify any contamination by epithelial cells.

Immunocytochemistry

Immunocytochemistry was performed as described previously.[42] In brief, mouse corneal sphere cells and cells freshly isolated from mouse cornea were attached to glass slides by cytospin preparation (Auto Smear CF-120; Sakura, Tokyo, Japan) and then fixed in 4% paraformaldehyde for 15 minutes at 4°C. Cells were incubated in blocking serum for 30 minutes and then incubated with primary antibodies for 60 minutes. Primary antibodies used were anti-cytokeratin K12 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Pax6 (1:500, Chemicon International, Inc.), anti-vimentin (1:100, Santa Cruz Biotechnology), and anti-αSMA (1:200, Laboratory Vision, Fremont, CA). Immunoreactivity of primary antibodies was visualized with secondary antibodies conjugated with Cy3 or FITC (Jackson ImmunoResearch Laboratories, West Grove, PA).

Reverse Transcription–Polymerase Chain Reaction

Sphere cells and freshly dissociated corneal cells were collected and immediately frozen in liquid N_2. cDNAs were synthesized with a cDNA synthesis kit (Life Sciences, Inc., St. Petersburg, FL) from total RNA also prepared with a kit (RNeasy; Qiagen, Hilden, Germany). Gene-specific primers used for cytokeratin K12 (K12), Pax6, vimentin, keratocan, lumican, CD34, aldehyde dehydrogenase (Aldh), and Gapd are shown in Table 1. PCR was then performed (GeneAmp 9700; Applied Biosystems, Foster City, CA). The PCR products were analyzed by agarose gel electrophoresis.

Collagen Gel Contraction Assay

Collagen gel contraction assay was performed as described previously,[43–47] with some modifications. Collagen gels were prepared according to instructions provided by the manufacturer (Cellmaterix Type I-A; Nitta Gelatin, Osaka, Japan). In brief, collagen was mixed with 10-fold concentrated DMEM/F12 medium and 50 mM NaOH containing 260 mM $NaHCO_3$ and 200 mM HEPES at a proportion of 8:1:1 (vol/vol/vol) at 4°C. Then a 0.2-mL aliquot of the solution was placed in the center of each well of a six-well cell culture cluster (Corning Inc., Corning, NY) and allowed to polymerize at 37°C for 30 minutes in a cloning ring 10 mm in diameter (Asahi Techno Glass). Cells cultured in medium containing 10% FBS were harvested and suspended at 2×10^5 cells /mL. Eighty-five micrometers of the cell suspension was applied to a polymerized collagen gel and incubated overnight in a 37°C 5% CO_2 incubator. On day 1, the cloning ring was removed, and 2.5 mL of 10% FBS-containing medium was added to each well to submerge the cells. To examine TGF-β-dependent collagen gel contraction, TGF-β was added at a 0.1- or 1-ng/mL final concentration. As an inhibitor, an anti-TGF-β antibody (0.1 ng/mL) was also added in the medium for selected dishes. FBS-containing media with or without TGF-β and/or TGF-β antibody were changed on day 3. Gel thickness was measured on day 5 with an inverted phase-contrast microscope, by adjusting the plane of focus from the bottom to the top of the gel and recording the distance that the stage had been moved.

Data are expressed as the mean ± SD. Post hoc comparisons between groups was performed with the Tukey procedure. Differences were considered significant at $P < 0.01$.

RESULTS

Sphere Formation from Stromal Cells

More than five mice were used to prepare corneal stromal cells in each experiment. From 10 corneas, $1.32 \pm 0.16 \times 10^4$ cells ($n = 3$) were isolated, and subcultured cells proliferated into spheres, to yield an average of $7.97 \pm 0.35 \times 10^7$ cells per 75 cm^2 flask ($n = 6$) after four passages (P4). Sphere cells were propagated for >12 passages through 5 months without loss of viability. To avoid contamination of epithelial and endothelial cells, stromal discs were carefully prepared as described in the Materials and Methods sections. Dissociated cells from mouse stromal discs formed spheres when cultured in serum-free

medium containing EGF and FGF2 (Fig. 1A, left). To exclude the possibility that spheres may have originated from contaminating epithelial cells, we first performed primary cultures of mouse corneal discs, with or without dispase treatment, followed by epithelium separation. K-SFM with low Ca^{2+} was used to examine epithelial expansion.[48,49] When untreated discs were cultured, migration of epithelial and stromal cells was observed in K-SFM and in DMEM/F12 containing 10% FBS, respectively (Fig. 1B, left). There were no epithelial cells migrating from dispase-treated discs in both media, whereas fibroblasts migrated from the discs in DMEM/F12 with serum (Fig. 1B, right). We further cultured dissociated epithelial cells under conditions that allowed stromal spheres to form by 14 days. As a result, no spheres were observed in the epithelial cell culture (Fig. 1A, right). To demonstrate whether the spheres were hollow or solid, confocal microscopy of 4',6'-diamino-2-phenylindole (DAPI)-stained spheres was performed. Imaging in different focal planes showed that the inside of spheres was filled with cells, not hollow (Fig. 1C).

We then examined the expression of epithelial and stromal markers in primary and subcultured spheres (P10) by RT-PCR. Stromal markers examined were the proteoglycans, keratocan, and lumican,[13,14,50,51] as well as CD34, which was recently reported to be expressed in keratocytes.[52-54] As shown in

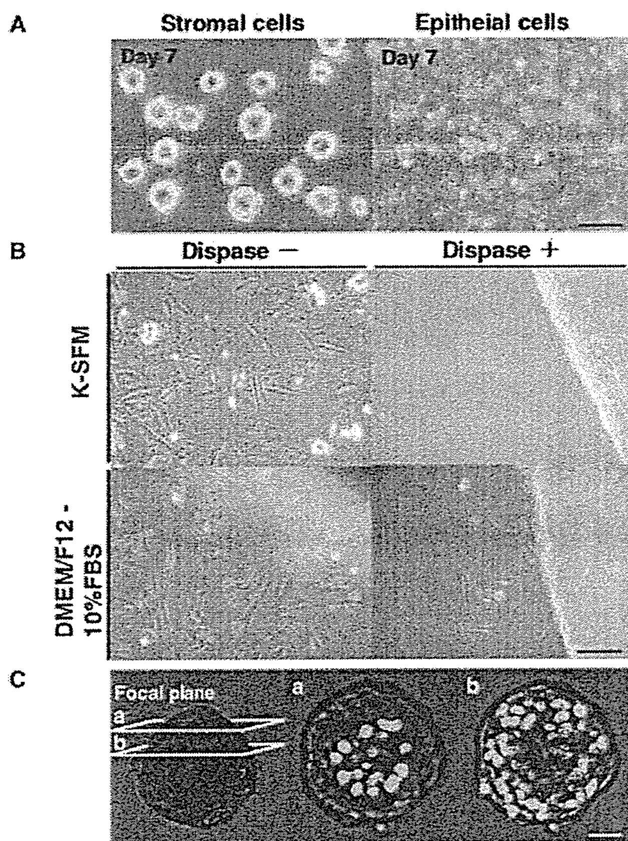

FIGURE 1. Sphere cells derived from the mouse corneal stroma. (A) Mouse corneal stroma and epithelium were separated by dispase treatment. Cells were cultured in DMEM/F12 supplemented with EGF and FGF2. After 7days' culture, spheres formed from stromal cells, but not from epithelial cells. (B) Mouse corneal discs were cultured in K-SFM or DMEM/F12 with 10% FBS. Epithelial cells migrated from intact corneal discs in K-SFM (*top left*) but not from dispase-treated (denuded) discs (*top right*). Expanding fibroblastic cells were still observed after dispase treatment. (C) Confocal images of the sphere in two different focal planes, a and b, as shown schematically (*left*). *Blue*: DAPI-stained nuclei. Scale bar: (A) 50 μm; (B) 100 μm; (C) 10 μm.

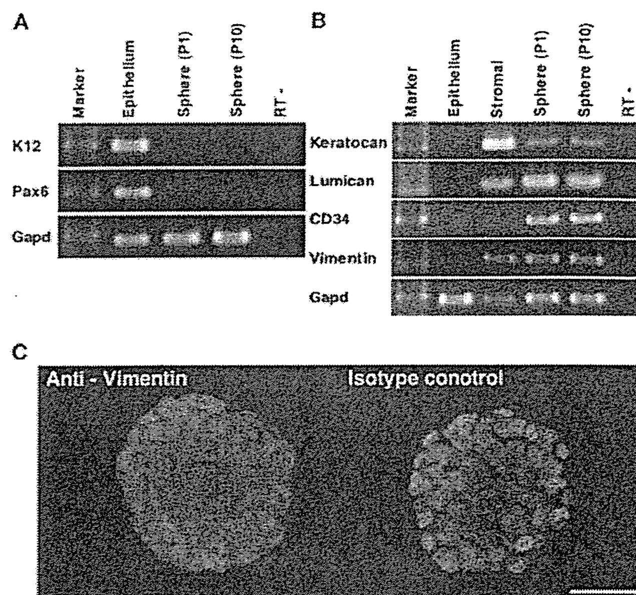

FIGURE 2. Sphere cells express keratocyte markers. (A, B) Total RNA was prepared from epithelial, stromal, and sphere cells. RT-PCR was performed with gene-specific primers. The epithelial markers K12 and Pax6 were not detected in the spheres (A). In contrast, the keratocyte markers keratocan, lumican, and CD34 were detected (B). Immunocytochemical analysis showed expression of the mesenchymal marker, vimentin, in spheres (C). *Blue*: nuclei of cells counterstained with DAPI. Scale bar, 50 μm.

Figures 2A and 2B, in addition to the mesenchymal intermediate filament vimentin, the expression of the genes described earlier were detected in the stromal spheres. On the contrary, K12 and Pax6, both of which are expressed in corneal epithelium,[40,42,55-58] were not detected in sphere cells (Fig. 2A).

Immunocytochemical analysis of spheres did not detect K12 and Pax6 expression (not shown), whereas vimentin staining was positive (Fig. 2C). These results show that sphere cells were of stromal, not epithelial, origin.

Characteristics of Sphere Cells

Sphere cells plated on collagen I-coated dishes in serum-free medium exhibited a dendritic morphology consistent with keratocytes (Fig. 3A).[3-10] RT-PCR showed that expression of keratocan and Aldh were retained under these conditions (Fig. 4). In contrast, the morphology of sphere cells subcultured in 10% serum were fibroblastic, and the expression of these genes was not detected (Fig. 4). Corneal sphere cells further differentiated to express α-SMA after exposure to TGF-β, which is consistent with the myofibroblast phenotype (Figs. 3C, 3D). Furthermore, when cells were subcultured on collagen gels in the presence of TGF-β, fibroblast-mediated gel contraction was observed (Fig. 5). Without TGF-β, contraction to 68.5% ± 1.75% of the original gel thickness was observed, whereas contraction was enhanced to 50.2% ± 3.96% or 29.4% ± 1.96% of the original thickness in the presence of 0.1 ng/mL or 1 ng of TGF-β, respectively ($P < 0.01$). TGF-β-dependent contraction was reduced to control levels when anti-TGF-β antibody was added to the medium.

DISCUSSION

We successfully isolated and subcultured sphere-forming cells from the mouse corneal stroma, yielding a multifold increase in available cells for further experiments. Zhao et al.[40] have re-

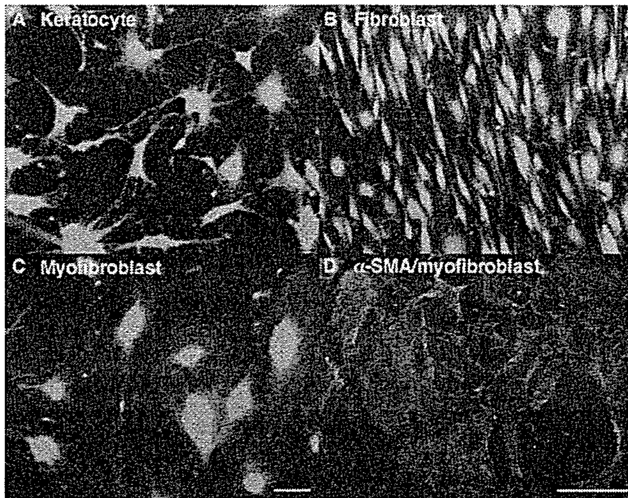

FIGURE 3. Phenotype of corneal stromal sphere cells stained by calcein-AM. Dissociated sphere cells were dendritic in SFM (A) and fibroblastic in adherent culture with medium containing 10% FBS (B). In the presence of TGF-β, morphology of adherent cells became myofibroblastic (C), and the cells expressed α-SMA, detected by immunocytochemistry (D, *green*). *Blue*: nuclei of cells counterstained with DAPI. Scale bar, 50 μm.

ported that cells present in limbus-derived spheres are derived from the limbal epithelium but not the stroma. However, the corneal sphere cells that we isolated did not express the epithelial markers K12 or Pax6 throughout the study, and furthermore, exhibited properties of corneal keratocytes when subcultured in serum-free medium. The morphology of the subcultured cells as shown in Figure 3 was similar to that of keratocytes in situ, and together with the expression of keratocan, lumican, Aldh, and CD34 in the subcultured cells, the

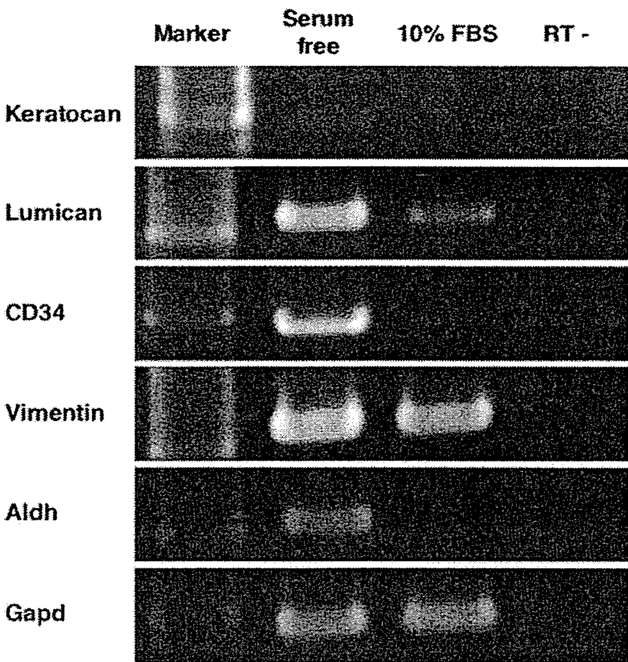

FIGURE 4. RT-PCR analysis of keratocyte markers expressed in sphere cells subcultured on plastic. Keratocan and Aldh were expressed only in cells in SFM, whereas lumican, CD34, and vimentin were also detected in cells cultured in the presence of 10% FBS.

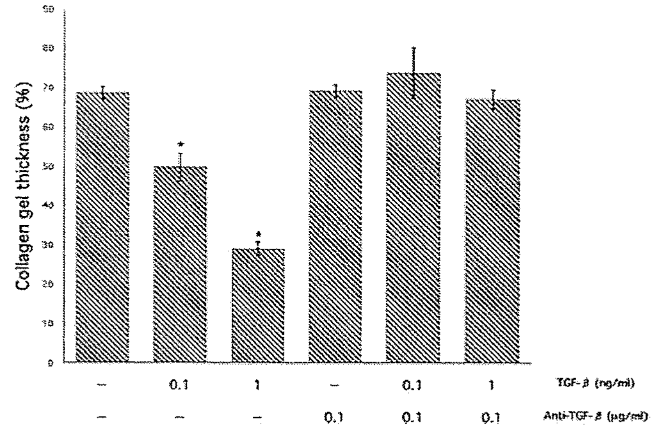

FIGURE 5. Collagen gel contraction assay of fibroblasts. Mouse corneal spheres were allowed to differentiate in 10% serum-containing medium. Dose-dependent TGF-β-induced collagen gel contraction was observed, which was inhibited by an anti-TGF-β antibody (*$P < 0.01$).

collective evidence shows that these cells were of keratocyte origin. Although most genes were expressed during sphere cultures and maintained after adhesion to plastic dishes, keratocan, and Aldh were exclusively expressed in the keratocyte phenotype in serum-free medium (Fig. 4). Although the biological role of Aldh is not known, abundant expression of the water-soluble enzyme was shown to be expressed in the keratocyte phenotype, but not by the fibroblasts or myofibroblasts.[32,59]

Plated sphere cells can further be induced to differentiate into the fibroblast and myofibroblast phenotypes. Sphere cells seeded onto plastic in the presence of 10% serum exhibited the morphology and properties of stromal fibroblasts.[28] The transition to α-SMA-positive myofibroblasts by exposure to TGF-β, causing collagen gel contraction (Fig. 5), is also a functional property of stromal fibroblast primary cultures.[29,43,60] Therefore, subcultured sphere cells can be conditioned to express all three known phenotypes of keratocytes after expansion by sphere culture. Berryhill et al.[36] reported that the fibroblast phenotype can be partially restored to the keratocyte phenotype in terms of extracellular matrix production and morphology. However, biological functions, including Aldh activity, were not restored, suggesting that reversal to keratocyte phenotype after mass culture in serum-containing medium is not practical. Espana et al.[41] have reported the use of amniotic membrane (AM) as a substrate for keratocyte cultures in the presence of serum. They have shown that even with the use of serum, primary keratocytes maintained dendritic morphology on AM. The expression of keratocan and lumican was also present for up to five passages, which is a significant improvement over previous reports using artificial substrates. Still, the scarcity of keratocytes in tissue usually necessitates the use of human tissue or cells from larger animals, such as cows,[28,36] rabbits,[30] and rhesus monkeys.[61] Biochemical and molecular analysis of such cells are difficult due to the lack of available antibodies and genomic information.

During subcultures of spheres, cells that failed to form spheres were found attached to the dish as nondividing, fibroblast-like cells (data not shown). Although these cells may have had low viability, there may be a selection process that allows only cells with high growth potential to propagate as spheres. Once secondary spheres are successfully initiated, subsequent passages continue to produce spheres for at least 12 passages, the longest that we observed. To our surprise, cells from later passages continued to show the keratocyte phenotype when subcultured on plastic, suggesting the possible presence of

committed progenitor cells during the sphere culture stage. Many aspects of the keratocyte are still not understood, and the availability of cells from the mouse cornea should be a powerful tool in studying the biology of these cells.

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