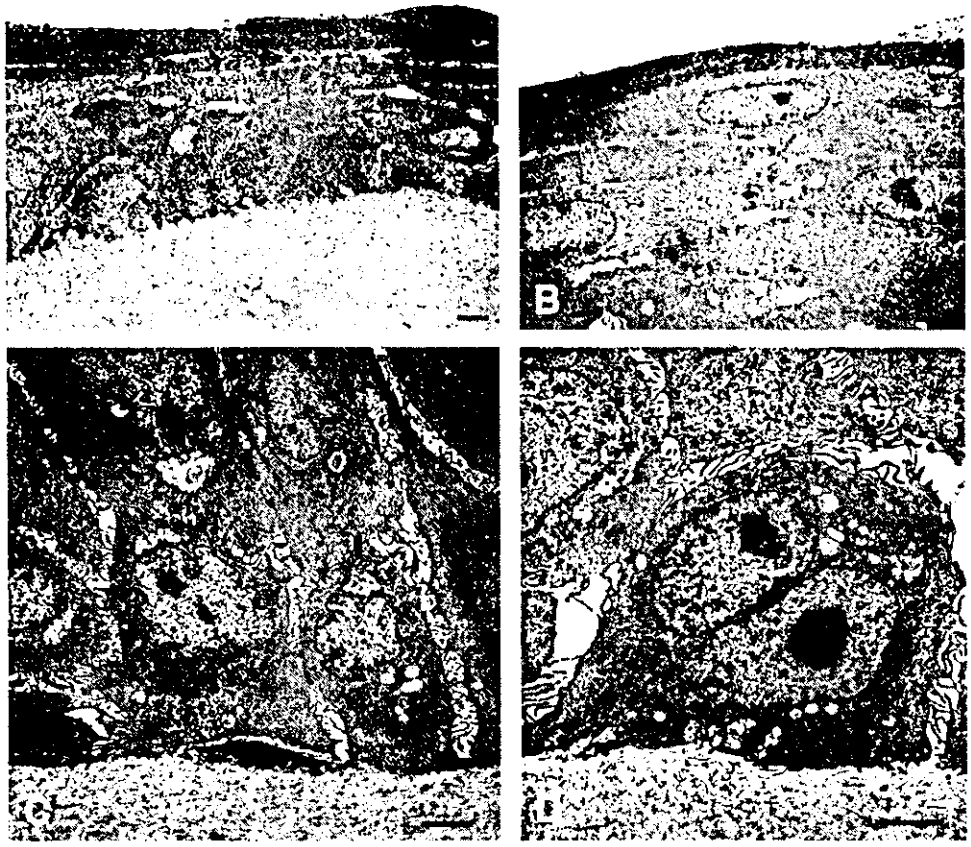

FIGURE 5. Transmission electron micrographs of rabbit cultivated oral epithelium on AM. The culture formed five to six layers of healthy, well-stratified epithelial cells (A, B). The epithelial cells in the basal cells layers were columnar (C). There was some evidence of basal cell proliferation (D). Scale bars, 2 μm.

cosa. Keratin-3 reportedly, is a reliable marker for corneal differentiation[45] and is positive for epithelial cells of the cornea, nose, and some oral mucosa,[46,47] and our results are consistent with this. Although the oral epithelial cells cultivated on AM could not become corneal epithelial cells, we suggest that they have the potential ability to become corneal-like epithelial cells under our culture conditions.

Our electron microscopy results from this study are of

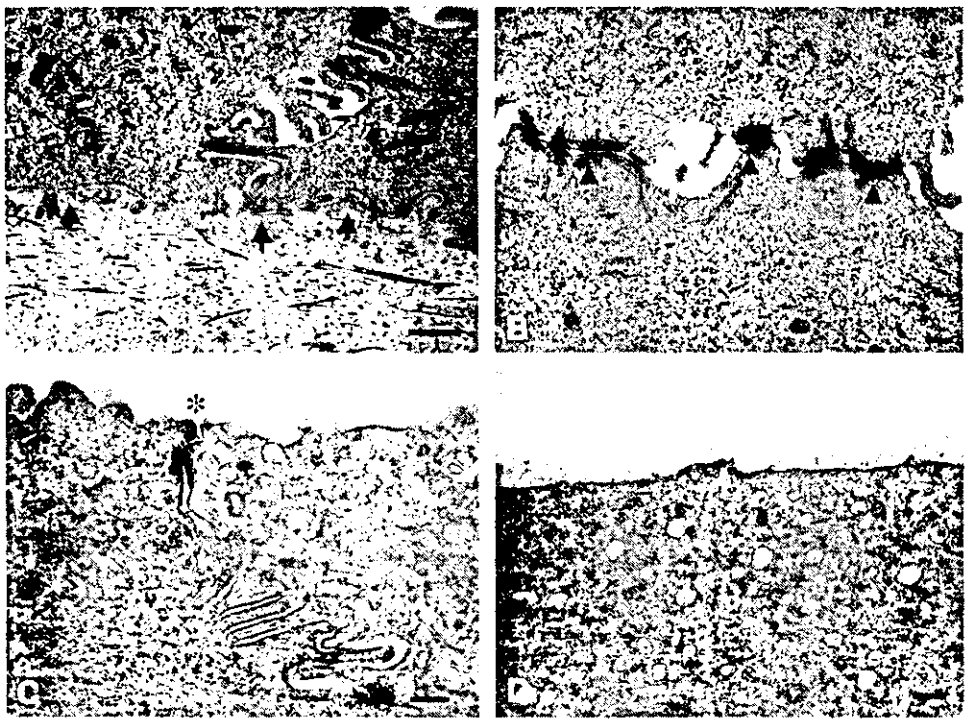

FIGURE 6. High-magnification transmission electron micrographs of rabbit cultivated oral epithelium on AM. The cells were attached to the basement membrane with hemidesmosomal junctions (*arrows*), and basement membrane extracellular matrix was evident (A). Adjacent cells were joined with numerous desmosomal junctions (*arrowheads*) (B). Apparent tight junctions (*) were occasionally evident between the most superficial cell layers (C). A glycocalyx layer was observed in the apical surfaces (D). Scale bars: (A, C) 500 nm; (B, D) 200 nm.

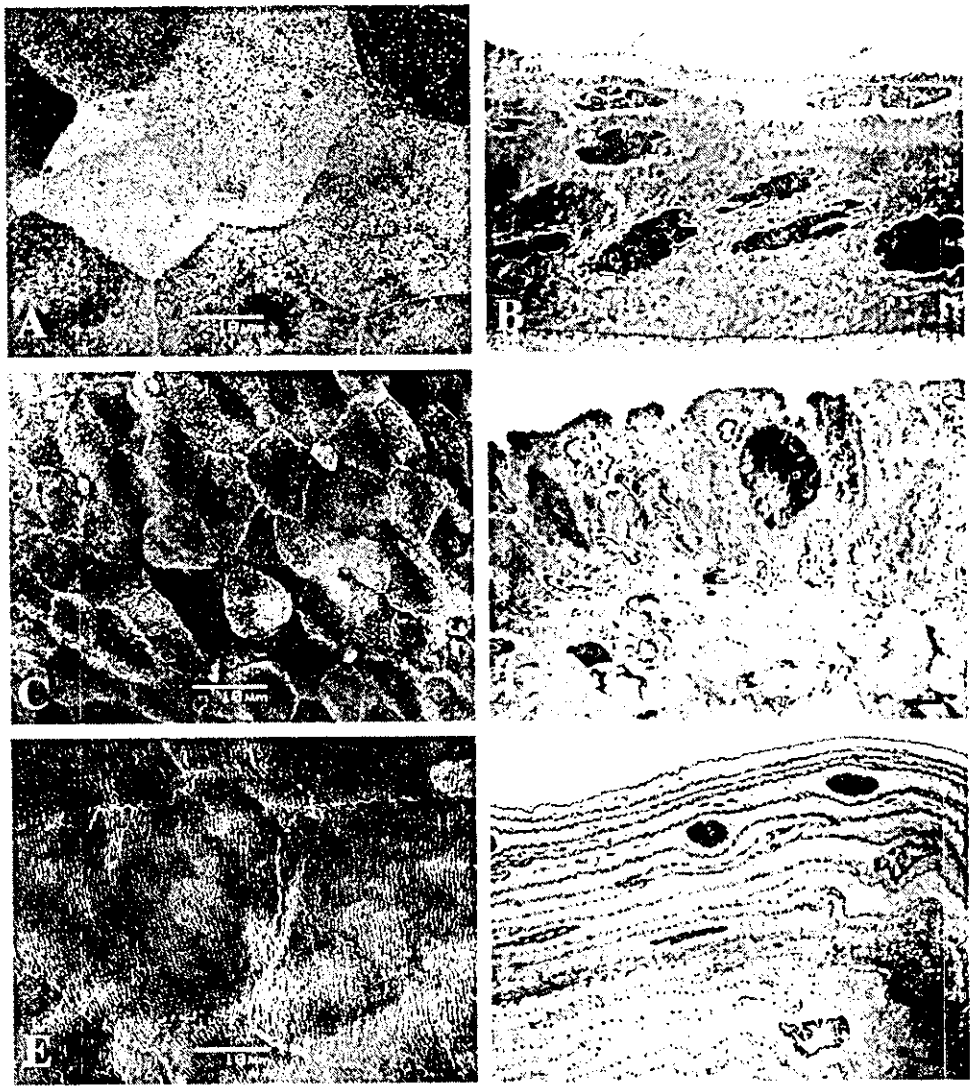

FIGURE 7. Scanning electron micrographs of normal rabbit corneal (A), conjunctival (C), and oral epithelial cell layers (E), showing the surface morphology of these cells. The superficial normal rabbit corneal epithelial cell layer was the most similar morphologically to the superficial oral mucosal epithelial cell layer cultivated on AM, as shown in Figure 4. Transmission electron micrographs of normal rabbit corneal (B), conjunctival (D), and oral epithelial cell layers (F) showing the ultrastructure of these cell layers. The normal rabbit corneal epithelial cell layer clearly had the ultrastructure most similar to that of the oral mucosal epithelial cell layer cultivated on AM, as shown in Figures 5 and 6. Scale bars: (B, D, F) 2 μm.

particular interest. SEM examination revealed that rabbit oral epithelial cells, after 3 weeks in culture, appeared healthy and well formed with tightly opposed cell junctions. Some superficial cells appeared to be undergoing the process of desquamation, as would be expected in a healthy stratified epithelial sheet. The cultivated oral cells were similar in size and appearance to rabbit corneal epithelial cells,[22] but were quite different from rabbit conjunctiva and oral mucosa. TEM confirmed that the cultivated oral epithelial sheet was very similar in appearance to that of the corneal epithelium and very different from the conjunctiva and oral mucosa. Similar to the corneal epithelium, it had four to five layers of stratified cells that were differentiated into columnar, wing, and squamous cells. We observed the evidence of rapid proliferation in the basal columnar cells. Both our SEM and TEM results show clearly that our oral mucosal cells, cultivated on AM, resembled normal corneal epithelial cells more closely than any other cell type.

An important question regarding the growth of oral epithelial cells on AM is how the basal cells attach to the underlying AM and whether a normal barrier function develops in the superficial cells. We believe that these are key points for the successful transplantation of the cultivated oral epithelial sheet. The normal corneal epithelial cells have specialized junctions on their cell surfaces to ensure firm adhesion to neighboring cells and the extracellular matrix below.[48] Desmosomal junctions are present between the cell-to-cell surfaces and give the cell sheet structural integrity.[48-50] Hemidesmosomes present on the basal cell surfaces serve to attach the basal cells to the basement membrane and, as is true of desmosomes, are also linked to the intermediate filament scaffold.[48-50] Tight junctions are present between the superficial cells, form an impermeable barrier, and are essential for normal functioning of the corneal epithelium.[51-53] Our TEM results show that cultivated oral epithelium is attached to a basement membrane with hemidesmosomal junctions. Adjacent cells in the cultivated oral sheet are also joined with numerous desmosomal junctions, and what appear to be tight junctions are evident between the most superficial cell layers. From these results, we believe that oral epithelial cells cultivated on AM have junctional specializations similar to those of in vivo corneal epithelial cells. These findings encouraged us to perform the transplantation of cultivated oral epithelial cells on AM.

Another key point for success in performing cultivated oral epithelial sheet transplantation is how the most superficial cells contact the tear-ocular surface interface. The anterior surface of the normal corneal epithelium has numerous folds in the anterior epithelial cell membranes in the form of microvilli and microplications, together with a glycocalyx layer.[54,55] The role of these surface irregularities may be to increase cell surface area and therefore to aid in intra- and extracellular movement of nutritional and waste products across the cell membrane, in addition to stabilizing the corneal tear film that

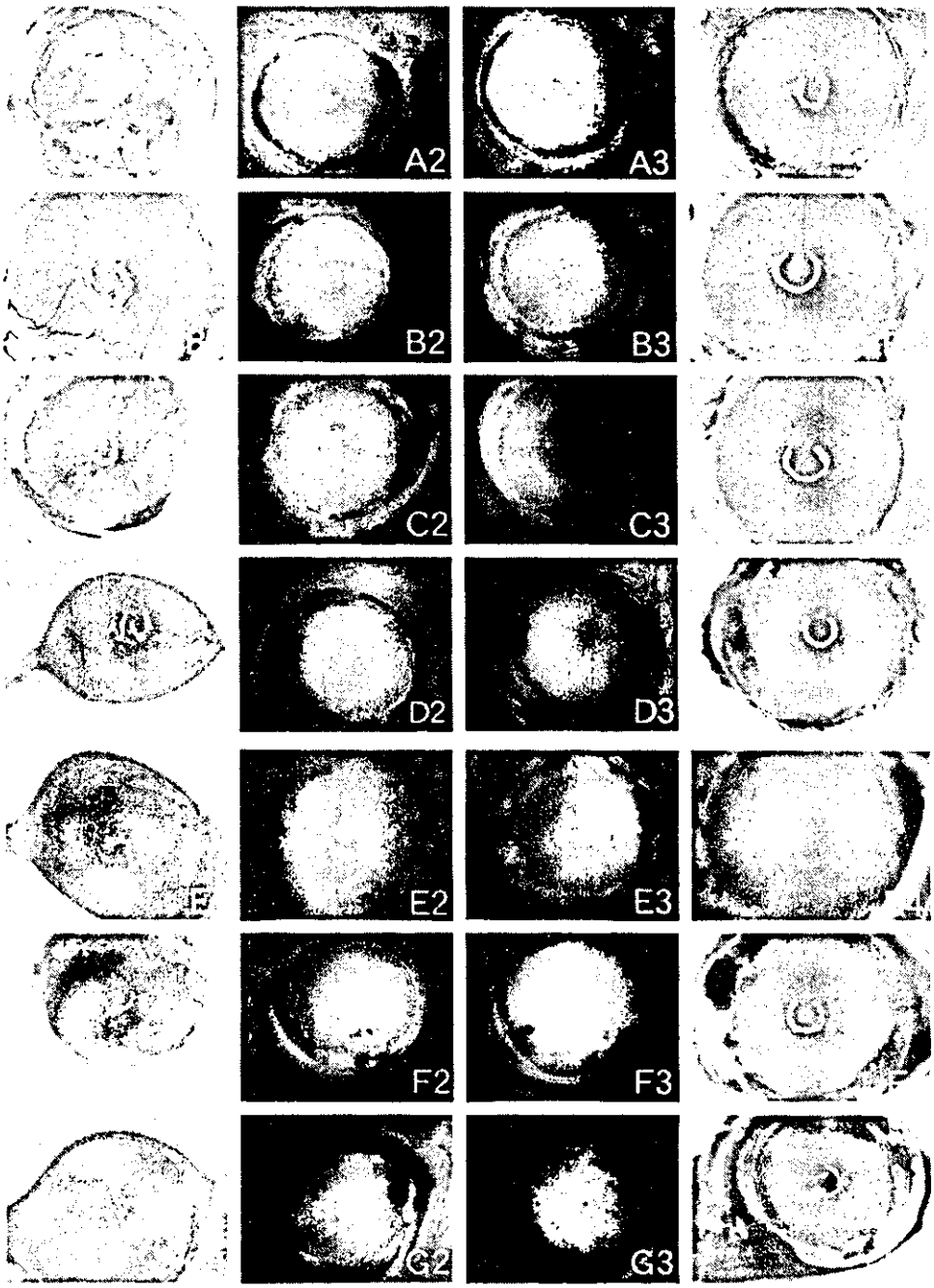

FIGURE 8. Representative slit lamp photographs of eyes of four rabbits taken before transplantation (A1-D1), 48 hours after transplantation with fluorescein (A2-D2), 10 days after transplantation with fluorescein (A3-D3), and without fluorescein (A4-D4). Before transplantation, all eyes showed total limbal stem cell destruction (A1-D1). Forty-eight hours after surgery, most of the corneal surfaces were covered with transplanted cultivated oral epithelial cells, which showed no fluorescein staining (A2-D2). The epithelialized, non-fluorescein-stained area was clearly separated from the surrounding conjunctival epithelium by an annular epithelial defect. Ten days after surgery, the central epithelialized area had spread outward (A3-D3). The epithelium at this time covered almost the entire corneal surface and in some areas was in contact with the inner part of the healing conjunctival epithelium. At this time, the corneal surface of all rabbits was covered with clear oral epithelium (A4-D4). Three representative slit lamp photograph series of a control eye that received no transplant (E1-E4), and then a transplant of acellular AM (F1-F4), and finally a transplant of oral mucosal tissue onto the keratectomized cornea in the limbal-corneal area (G1-G4). The time course of photographs 1 through 4 is the same as for those in (A) through (D).

is essential for good vision. Examination by SEM revealed that the apical surface of the cultivated oral epithelial cells is covered with numerous microvilli, almost identical with those found on corneal epithelial cells. We also found evidence of a cell surface glycocalyx, similar in appearance to the glycocalyx present on the surface of corneal epithelial cells. The apical surface of the corneal epithelium is normally covered by the tear film, which has a variety components, including the mucus layer on the epithelial surface.[56,57] Mucus is a highly hydrated gel, the most prominent components of which are glycoproteins termed mucins. The mucin MUC1 is the principally membrane-associated mucin and has been identified in the glycocalyx of many epithelium-lined organs including stomach, esophagus, oral mucosa, and ocular surface.[58,59] Our finding of a glycocalyx layer on the cultivated oral epithelium led us to consider the interesting hypothesis that this glycocalyx might act as a substitute for the glycocalyx on the ocular surface epithelium.

After the successful culture of rabbit oral epithelial cells on AM, we tried to reconstruct the damaged corneal surfaces by transplantation of autologous cultivated oral epithelial cells, to test the viability of using these cells as a substitute for cultivated corneal epithelial cells. Two days after the autologous surgical transplantation of oral epithelial cells, most of the corneal surfaces on which cultivated oral epithelium had been placed were free of epithelial defects. This indicated the complete survival of the transplanted oral epithelial cells. Moreover, the transplanted grafts were surrounded by a conjunctival epithelial defect at 360°, suggesting that there was no contamination of host conjunctival epithelium. Ten days after transplantation, the area covered by the oral epithelium had expanded outward and was in contact with healing conjunc-

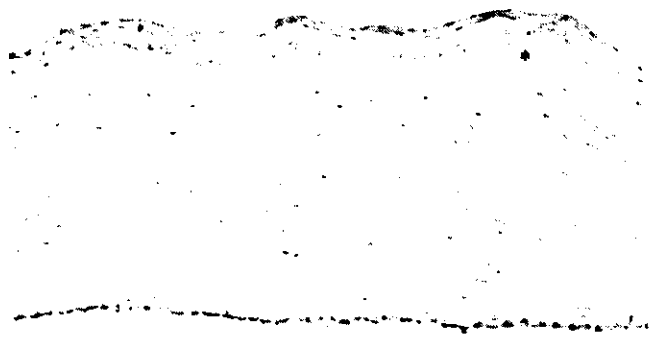

FIGURE 9. Light micrograph of cultivated oral epithelium 10 days after transplantation. The transplanted grafts adhered well to the host corneal stroma with no evidence of subepithelial cell infiltration or stromal edema. Superficial cells of the transplanted grafts had nuclei, suggesting that they were nonkeratinized mucosal epithelial cells. (*) Amniotic stroma. Original magnification, ×100.

tival epithelium in some areas. This suggests that, in the early days after oral epithelial transplantation, the oral epithelial cells on AM survive and spread onto the adjacent keratectomized cornea. We examined sections of these corneas, by the periodic acid-Schiff (PAS) reaction, which is reactive to conjunctival goblet cells, and confirmed that there was no contamination of the host conjunctival epithelium (data not shown). The corneal surfaces of all eyes were clear and smooth, and the entire corneal surfaces were completely covered with transplanted autologous oral epithelium. In addition, all rabbits could follow moving objects. We also observed, as expected, that the control animals that received no transplants or transplants of acellular AM onto a keratectomized cornea did not show any evidence of epithelialization at day 10. Finally, we clearly showed that direct transplantation of oral mucosal tissue cannot be used for ocular surface reconstruction.

To the best of our knowledge, our study is the first to demonstrate the survival, on keratectomized corneas, of autologous epithelial cells derived from oral biopsy tissue and grown on human AM. We believe that this approach to treating severely damaged eyes with limbal stem cell deficiencies has great potential. However, development of this technique is at an early stage, and there are many questions that must be resolved, including questions about the longevity and mobility of the autologously transplanted oral epithelial cells on the host eye and whether the transplants contain stem cells or progenitor cells. It is very important to determine whether the oral mucosal cell type has the appropriate characteristics to act as a substitute for the corneal epithelium. We must also determine in which cases cultivated oral epithelial transplantation should be used, the best time to perform the grafts, and whether this procedure is superior to existing surgical techniques. We are currently investigating the characteristic of cultivated oral epithelial cells and comparing the gene expression profile of oral epithelial cells with that of corneal epithelial cells. We hope that this will help to answer some of these questions.

In conclusion, we have successfully generated on AM confluent cultures of oral epithelial cells expanded ex vivo from biopsy-derived oral mucosal tissues. We have successfully performed autologous transplantation of these cells onto keratectomized rabbit corneas. Finally, we believe that autologous transplantation of cultivated oral epithelium is a feasible method for ocular surface reconstruction, although, because the long-term outcome of such transplantation is not yet clear, its feasibility for clinical use should be evaluated further.

Acknowledgments

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Successful Regrafting of Cultivated Corneal Epithelium Using Amniotic Membrane as a Carrier in Severe Ocular Surface Disease

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Purpose. Our group performed cultivated allogeneic corneal epithelial transplantation in 13 eyes from 11 patients with severe ocular surface disorders. After the clinical application of this new surgical treatment, some patients experienced epithelial and subepithelial opacities. We applied our procedure again in these patients to achieve successful ocular surface reconstruction. **Methods.** The corneal limbal epithelial cells from donor corneas were cultivated for 4 weeks on denuded amniotic membrane (AM) carrier, with 3T3 fibroblast coculture and airlifting. The study subjects consisted of 3 patients. At 3 and 12 months after the first operation, the failed epithelial graft with AM was replaced with new allogeneic corneal epithelium cultivated on AM. **Results.** At 48 hours after transplantation, the corneal surfaces of the 3 eyes were clear and smooth; the entire corneal surfaces were evenly covered with the transplanted cultivated corneal epithelium, which did not stain with fluorescein. The ocular surface epithelia of these patients are all stable without epithelial defects. **Conclusions.** We have shown that, in cases where the initially transplanted cultivated epithelium becomes opaque, it is possible to repeat the transplantation process with new cultivated epithelium on AM.

Key Words: Amniotic membrane—Corneal epithelium—Ocular surface disease—Stem cell—Ocular surface reconstruction.

Severe ocular surface diseases (OSD), such as Stevens-Johnson syndrome (SJS) and chemical burns, are some of the most challenging problems facing the clinician today.[1,2] Recently, attention has focused on the use of cultivated corneal epithelial stem cell transplantation as a new approach for ocular surface reconstruction in severe OSD.[3–5] We have developed a surgical system for cultivated corneal epithelial stem cell transplantation using amniotic membrane (AM) as a carrier.[6,7] Our group performed cultivated allogeneic corneal epithelial stem cell transplantation in 13 eyes from 11 patients with acute and chronic phase severe OSD and recently reported the successful long-term results of this new surgical method.[8] From these 13 cases, 3 eyes experienced epithelial and subepithelial opacities. We applied our procedure again in these patients and achieved successful ocular surface reconstruction.

We used the method previously described[6–8] with minor modifications. Briefly, corneal limbal epithelial cells from donor corneas were cultivated for 4 weeks on denuded AM carrier, with 3T3 fibroblast coculture and airlifting. The cultivated cells showed 4–5 layers of stratification, were well differentiated, and stained with cornea-specific keratin 3 /12.[8]

CASE REPORT

The study subjects are shown in Table 1. Before this study, all 3 had undergone cultivated corneal epithelial stem cell transplantation on their damaged eyes. For the chronic phase patient with corneal stromal scarring (Patient 2), we also simultaneously performed lamellar keratoplasty using preserved donor graft without donor corneal epithelium to replace the corneal stroma. In all cases, the corneal surfaces were clear, smooth, and perfectly covered with transplanted graft at 48 hours after the first transplantation. Postoperatively, 0.3% ofloxacin and 0.1% dexamethasone were instilled 4 times a day, and betamethasone (1 mg/day), cyclosporin (150 mg/day), and cyclophosphamide (100 mg/day) were given to prevent postoperative inflammation and allograft rejection. Conjunctival inflammation subsided remarkably after surgery, but epithelial rejection occurred in 2 eyes (Patients 1 and 3). The definition of epithelial rejection used here was the sudden onset of corneal epithelial damage with conjunctival inflammation. In one eye, conjunctival invasion was induced by persistent corneal epithelial defect (Patient 2).

At 3 months (Patient 2) and 12 months (Patients 1 and 3) after the first operation, the failed epithelial graft was replaced with new allogeneic corneal epithelium sheet. Briefly, we removed the failed epithelial graft with AM by gently peeling it from the entire cornea. Subsequently, we could see that the bare corneal stroma had kept its transparency. We secured the new cultivated allogeneic corneal epithelium on AM onto the corneal surface with 10–0 nylon sutures, and then covered it with a therapeutic soft contact lens. Postoperatively, 0.3% ofloxacin and 0.1% dexamethasone were instilled 4 times a day. Betamethasone (1 mg/day), cyclosporin (150–200 mg/day), and cyclophosphamide (100 mg/day)

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TABLE 1. *Patient Profile*

Patient No.	Age/gender	Diagnosis	First operation	Visual acuity		Time to second operation (months)	Follow-up (months)
				Preoperative	Postoperative		
1	35/M	Chemical*	CCEST‡	HM‖	8/200	12	14
2	64/M	SJS†	CCEST + LK§	HM	6/200	3	12
3	21/M	SJS	CCEST	2/200	20/40	12	12

* Chemical, chemical burns.
† SJS, Stevens-Johnson syndrome.
‡CCEST, cultivated corneal epithelial stem cell transplantation.
§ LK, lamellar keratoplasty.
‖ HM, hand motion.

were given longer than before to prevent postoperative inflammation and allograft rejection. At 48 hours after transplantation, the corneal surfaces were clear and smooth, and the entire corneal surfaces were evenly covered with the transplanted graft. The observation periods after the second transplantation are shown in Table 1. The ocular surface epithelia of these patients are all stable, without epithelial defects, and postoperative visual acuity in all 3 cases has markedly improved (Fig. 1).

DISCUSSION

Recently, we reported that allogeneic cultivated corneal epithelial stem cell transplantation is a useful and effective means of ocular surface reconstruction for severe OSD.[6–8] This type of epithelial transplantation is generally thought to be more problematic than other types of transplantation, such as penetrating keratoplasty, because it is considered to have a high risk of allograft rejection. We administered intensive immunosuppressants during the postoperative period to all patients in this study, but despite our efforts, three of them experienced epithelial and subepithelial opacities. In these three cases, we resurfaced the ocular surface with new cultivated graft to improve vision. We learned two important things from this second transplantation. First, that it was comparatively easy to remove the failed epithelial graft from the host eyes. The transplanted graft, including amniotic stroma, did not strongly adhere to the host corneal stroma, so we could perform the second operation safely and easily. Second, we saw after removal of AM that the host corneal stroma had kept its transparency. It has been reported that AM matrix has an antiscarring effect in ocular surface reconstruction,[9,10] so we suspect that the AM might be protecting the corneal stroma from scarring. In conclusion, we have shown that, in cases where the initially transplanted cultivated epithelium becomes opaque, it is possible to repeat the transplantation process.

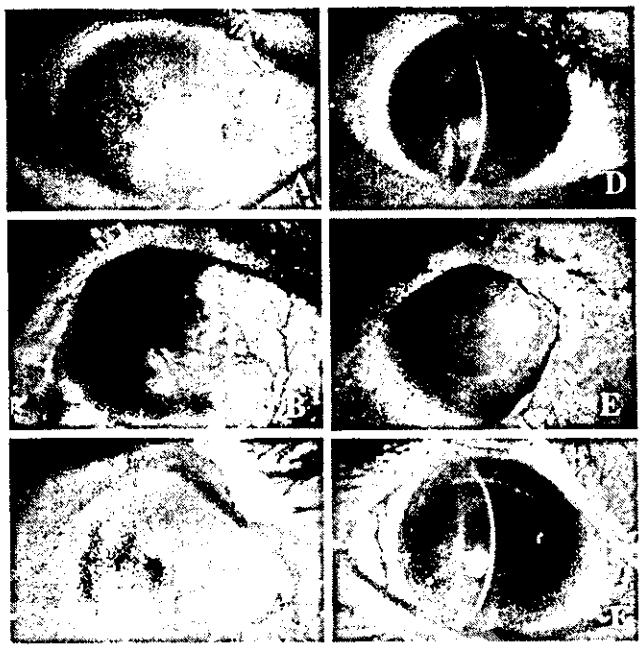

FIG. 1. Slit-lamp photographs of all regrafting patients taken before surgery **(A–C)**, and at the last follow up visit **(D–F)**. Corneal surfaces were clear and smooth, and the entire corneal surfaces were evenly covered with transplanted cultivated corneal epithelium. (A and D, patient 1; B and E, patient 2; C and F, patient 3).

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Tight junction-related protein expression and distribution in human corneal epithelium

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Abstract

Purpose. To investigate the expression and cellular distribution of the tight junction-related proteins occludin, claudin and ZO-1 in human corneal epithelium.

Methods. Light and electron immunohistochemistry was used to determine tissue distribution of occludin, claudin-1 and ZO-1 in the human corneal epithelium. Reverse transcription-polymerase chain reaction was used to reveal claudin mRNA expression in human corneal epithelium.

Results. In transverse sections, occludin and ZO-1 were localized at the apical cell–cell junctions between superficial cells in stratified corneal epithelium. Both basal and basolateral membranes of superficial cells were stained by the claudin-1 antibody, but no apical membrane staining was observed. In en face sections, claudin-1 and ZO-1 antibodies showed as bands that corresponded to the junctional complex. Claudin-1 staining of superficial cell cytoplasm was also observed. Occludin staining was seen at the junctional complex, where it was not continuous, but dotted along the cell junctions.

The transcripts for claudin-1 and several other claudin isotypes, such as -2, -3, -4, -7, -9 and -14 were identified.

Conclusion. Not only occludin, but also some claudins act as integral transmembrane proteins in the corneal epithelium. ZO-1 is a component of the corneal epithelial tight junction, as it is in most epithelial cells.

Keywords: tight junction; corneal epithelium; occludin; claudin; ZO-1; intercellular adhesion molecules; immunocytochemistry; electron microscopy

1. Introduction

Corneal epithelium forms a barrier that isolates the eye from the outside environment and regulates passive movement of fluid, electrolytes, macromolecules and cells through the paracellular pathway. Tight junctions (TJs) create this barrier. Some reports observe that in corneal epithelium TJs are present only between the most superficial cells and are characterized by delicate P-face particle strands and complimentary E-face grooves by electron microscopy (Tanaka et al., 1983; Mclaughlin et al., 1985).

The junctional complex of simple epithelial cells, located at the most apical part of the lateral membrane, consists of three distinct components: TJs, adherence junctions and desmosomes. On electron micrographs of ultrathin sections, TJs appear as a series of apparent fusions (the kissing point), involving the outer leaflets of the plasma membranes of adjacent cells. In addition to the 'barrier function', TJs serve a 'fence function'. For vectorial transport of materials across cellular sheets, plasma membranes are functionally divided into apical and basolateral domains. TJs are the morphological counterpart of a localized diffusion barrier between those domains (Anderson et al., 1993).

Recent studies have revealed that the TJ complex includes two integral transmembrane proteins: claudin; occludin; and membrane-associated proteins such as ZO-1, ZO-2, and ZO-3 (Tsukita et al., 2001).

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Occludin (~60 kDa) was the first transmembrane protein identified (Furuse et al., 1993); current data indicate that it plays a regulatory rather than a structural function in TJs, a function that is controlled partially by phosphorylation (Sakakibara et al., 1997; Saitou et al., 1998). Claudin (~23 kDa) comprises a family of transmembrane proteins that form the strands of the tight junction; 24 claudins have been identified thus far. Claudins are the only junctional proteins known to have tissue specificity. Both occludin and claudins contain four transmembrane domains, with both N and C termini oriented into the cytoplasm, but these two proteins show no sequence similarity (Furuse et al., 1998a; Morita et al., 1999a; Tsukita and Furuse, 1999). Different mixtures of claudins and occludin create TJ strands that are associated laterally with strands of adjacent cells, forming paired strands that eliminate extracellular space (Furuse et al., 2001).

The membrane-associated guanylate kinase homologue (MAGUK) protein family is distinguished by a core cassette of protein-binding domains, which include one or more PSD95/dlg/ZO-1 (PDZ) domains, an SH3 domain and a region of homology to guanylate kinase (GuK). The domains assemble signaling, receptor, and transport complexes at a variety of membrane junctions. The junction scaffold in TJ is formed by three members of the MAGUK protein family: ZO-1, ZO-2 and ZO-3, which bind not only to occludin or claudin, but also to the cortical actin cytoskeleton (Stevenson et al., 1986; Gumbiner et al., 1991; Balda et al., 1993; Fanning et al., 1998; Haskins et al., 1998; Itoh et al., 1999a,b).

In the rabbit cornea, ZO-1 is found in the superficial layer of epithelium where TJs are located, as well as between wing cells and basal epithelial cells that have no TJ structure (Sugrue and Zieske, 1997). The expression and localization of other TJ proteins have been reported recently in rat corneas (Suzuki et al., 2000).

We recently developed human corneal epithelial cell culture on amniotic membranes for ocular surface reconstruction. The TJ formation between the superficial cells of culture sheets might influence the outcome of the surgery (Ban et al., 2003). In order to use TJ-related protein expression and distribution as landmark of TJ formation, it is important to know those of in vivo human corneal epithelium.

In this report, we examine the distribution of TJ proteins ZO-1, occludin and claudin-1 from in vivo human corneal epithelium.

2. Materials and methods

2.1. Tissue preparation—human corneas

The experiments in this paper used human corneal tissue supplied from USA eye bank or that extirpated from patients with corneal stromal opacity diseases during penetrative keratoplasty surgery, therefore the corneal epithelium was intact. All corneas were obtained with consent of the patients and the procedures followed the Tenets of the Declaration of Helsinki. All experiments were done immediately after obtaining the tissues.

2.2. Primary antibodies

The rabbit anti-ZO-1 polyclonal antibody and the rabbit anti-claudin-1 polyclonal antibody were purchased from Zymed Laboratories (South San Francisco, CA, USA). The goat anti-occludin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.3. Immunohistochemistry

TJ-associated proteins were studied via indirect immunohistochemistry. For the transverse images, 7 μm cryostat sections were placed on gelatin-coated slides; air dried and rehydrated in phosphate buffered saline (PBS) containing 1·0 mM $MgCl_2$, 0·1 mM $CaCl_2$ at room temperature for 15 min. The sections were fixed in 3·7% paraformaldehyde in PBS containing 1·0 mM $MgCl_2$, 0·1 mM $CaCl_2$ at room temperature for 10 min. After several washes with PBS, sections were incubated with 1% bovine serum albumin (BSA) at room temperature for 30 min, to block nonspecific binding. Following this, the sections were incubated at room temperature for 1 hr with primary antibody, then washed three times in PBS for 15 min. For negative controls, the equivalent serum was used. After staining with the primary antibodies, the sections were incubated at room temperature for 1 hr with suitable fluorescein (FITC)-conjugated secondary antibodies.

After several washes with PBS, the sections were coverslipped using anti-fading mounting medium containing propidium iodide (PI) (Vector Laboratories, Burlingame, CA, USA).

For the en face images, whole corneas were fixed in 3·7% paraformaldehyde in PBS containing 1·0 mM $MgCl_2$, 0·1 mM $CaCl_2$ at room temperature for 10 min. After several washes with PBS, tissues were permeabilized by incubation in PBS containing 0·1% Triton X-100 for 10 min. Tissues were developed to the first and second antibody steps, described above. During all steps, the epithelial side was kept upward to avoid damage. Tissues were mounted on the slide glass using antifading mounting medium containing PI. The slides were examined by confocal microscope (Fluoview; Olympus, Tokyo, Japan).

2.4. Immuno-electron microscopy

Tight Junction associated proteins in the superficial human epithelial cells were studied via immuno-electron

microscopy. The samples were fixed in 4% paraformaldehyde in phosphate buffer at pH 7.2. The samples were washed in phosphate buffer before being dehydrated through and ethanol series to 95% ethanol and embedded in LR white (London Resin, London, UK). Polymerization was carried out at 52°C. Ultrathin sections were cut and collected on gold-coated grids. Labeling for the primary antibodies (described above) was carried out by floating the grids in droplets in a moist chamber. The controls consisted of the primary antibody step being replaced by a non-specific antibody. The sections were first placed in droplets of 0·1 M glycine in PBS for two 10 min periods then incubated at room temperature on droplets of normal serum (goat or rabbit) for 20 min, after which the serum was flicked off and the grids transferred to a droplet containing the primary antibody at 1–50 dilution in PBS buffer at pH 7·4 containing 1% BSA and 1% Tween 20 overnight. This was followed by the grids being washed sequentially for 8 min each time in 5 droplets of buffer solution. The grids were then placed in droplets containing the secondary antibody at a dilution of 1–50 in PBS at pH 8·2 containing 1% BSA, 1% normal goat or rabbit serum and 1% Tween 20, for 2 hr. Secondary goat anti-rabbit IgG or rabbit anti-goat IgG 5 nm gold conjugated antibodies (British Biocell International, Cardiff, UK) were used to visualize the primary antibodies. After which the grids were washed for 8 min each time in 5 droplets of the buffer alone, followed by three 2-min washes in droplets of distilled water. After the final wash the sections were examined in a JEOL 10–10 transmission electron microscope (TEM), the grids were counter-stained with aqueous uranyl acetate and phosphotungstic acid.

2.5. RNA isolation and RT-PCR amplification of Claudin species

For reverse transcription-polymerase chain reaction (RT-PCR), human corneal epithelial sheets peeled from donor corneal buttons ($n = 4$), were directly lysed with reagent (Trizol; GIBCO BRL, Rockville, MD, USA); total cellular RNA was isolated according to the manufacturer's instructions. RT-PCR was performed in accordance with previous report by Yi et al. (2000). In brief, complementary DNA (cDNA) was generated in the presence of 0·5 μg oligo (dT) from 5 μg total RNA with reverse transcriptase (SuperScript II; Life Technologies, Rockville, MD, USA). We used PCR primers Yi et al. designed for human claudins-1 to -4, -7, -9, -10, -14 and -15 (Yi et al., 2000). PCR products were examined by 2% agarose gel and ethidium bromide staining. The observed PCR products corresponded to their expected molecular weights. We did the sequence to check the PCR products were those of we expected.

3. Results

3.1. Distribution of TJ components in human corneal epithelium

In human corneal epithelium transverse sections, we found that most apical cells of the epithelium exhibit ZO-1 immuno-reactivity at their lateral margins. We also found very fine staining at the border between basal and wing cells (Fig. 1(A)). On en face light microscope images, ZO-1 was found to form a continuous ring around the large superficial epithelial cells, whereas wing cell immuno-staining was not detected, partially because of weak reactivity (Fig. 1(B)). Immuno-electron microscopy showed labeling for ZO-1 in the TJ region of the superficial cells (Fig. 2(A)).

The staining pattern of occludin was concentrated at cell–cell borders only in the superficial layer of the corneal epithelium (Fig. 1(C)). On en face images, it was not continuous, but dotted along the cell junctions (Fig. 1(D)). Immuno-electron microscopy showed labeling for occludin associated with the cytoplasm and the lateral membranes of the superficial cells (Fig. 2(B)).

Both basal and basolateral membranes of superficial cells were stained by claudin-1 antibody, but apical membrane was not stained (Fig. 1(E)). In en face sections, claudin-1 antibodies showed as bands that corresponded to the basolateral membranes (Fig. 1(F)). Claudin-1 staining of superficial cell cytoplasm was also observed. Immuno-electron microscopy showed light labelling for claudin-1 associated with the lateral membranes of the superficial cells (Fig. 2(C)).

We did not find any staining in our negative controls (Fig. 1(G) and (H)).

3.2. Claudin subtype expression in human corneal epithelial cells detected by RT-PCR

We used PCR primers Yi et al. designed for human claudins-1 to -4, -7, -9, -10, -14 and -15 (Yi et al., 2000), and determined the presence of transcripts of these nine species in in vivo human corneal epithelial cells by RT-PCR method.

The transcripts for claudin-1 and several other claudin isotypes, such as -2, -3, -4, -7, -9 and -14 were identified from in vivo human corneal epithelium (Fig. 3). The result was different from that for THCE cells, from which claudins-1, -2, -3, -7, -9, -14, and -15 were amplified (Yi et al., 2000). No amplified claudin mRNA was observed without reverse transcriptase (RT).

4. Discussion

In the present study, using fresh corneas and gentle treatment throughout our protocol, we investigated the distribution of ZO-1, occludin and claudin in in vivo human corneal epithelium.

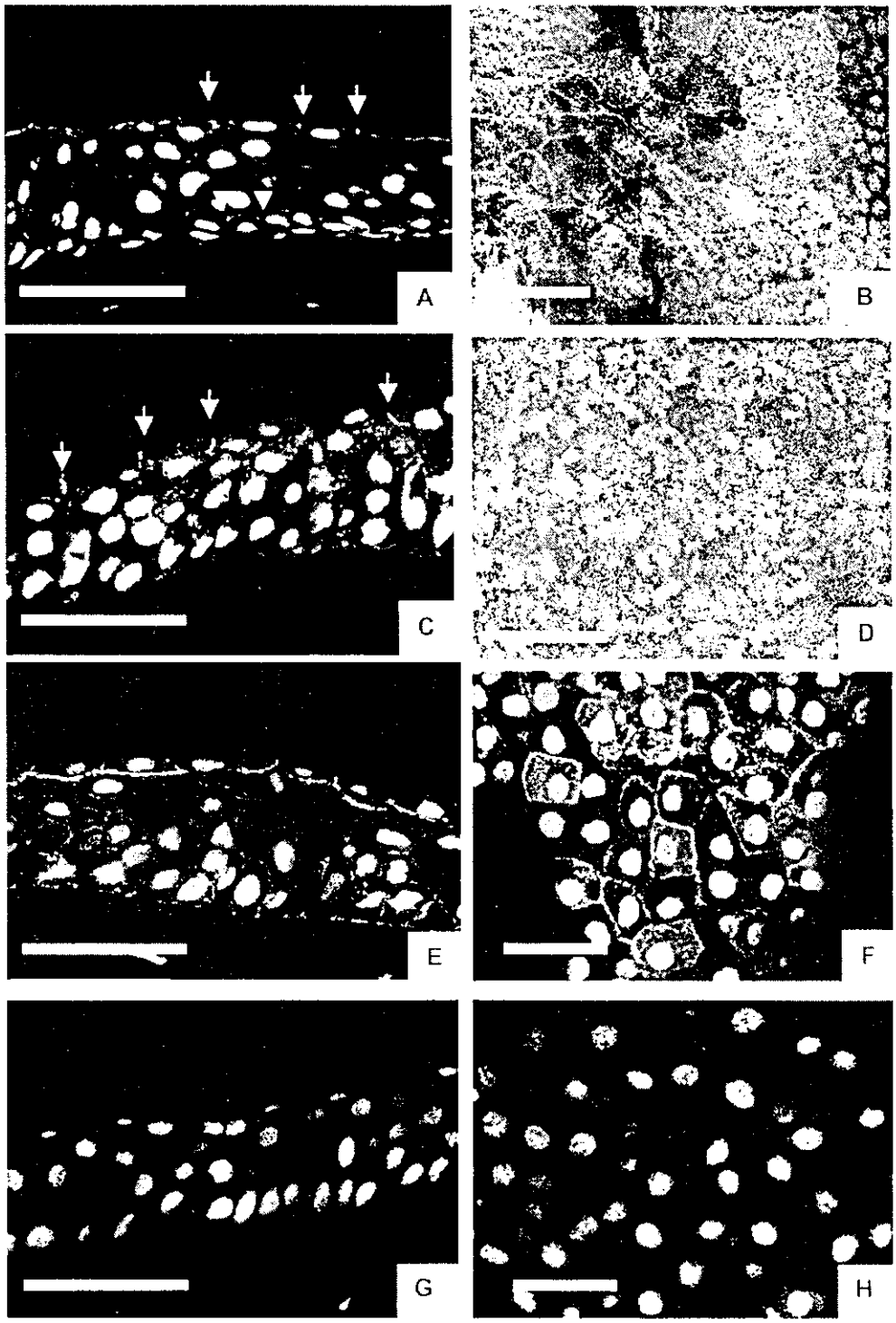

Fig. 1. Immuno-histochemical analysis of TJ-related protein expression; ZO-1 (A and B), occludin (C and D) and claudin-1 (E and F), on transverse images (A, C, E and G) or en face images (B, D, F and H). G and H: negative control. Most apical cells of the epithelium exhibited ZO-1 immunoreactivity at their lateral margins (A; arrow). There was very fine staining at the border between basal and wing cells (A; arrowhead). On en face images, ZO-1 was found to form a continuous ring around the large superficial epithelial cells (B). The staining pattern of occludin was concentrated at cell–cell borders only in the superficial layer of the corneal epithelium (C). On en face images, it was not continuous, but dotted along the cell junctions (D). Both basal and basolateral membranes of superficial cells were stained by claudin-1 antibody, but apical membrane was not stained (E). In en face sections, claudin-1 antibodies showed as bands that corresponded to the basolateral membranes (F). Claudin-1 staining of superficial cell cytoplasm was also observed. This section was taken from deeper layer than the section for ZO-1 and occludin staining, therefore nucleus were stained by PI more in en face sections of claudin-1 than those of ZO-1 and occludin. No staining was observed in negative controls (G and H). (Scale bar = 100 μm).

Fig. 2. Transmission electron micrographs showing immuno-electron microscopic localization of TJ proteins in human superficial corneal epithelium. The 5 nm immuno-gold particles appear as black dots. (A) Shows that the labeling for ZO-1 is localized at the most apical region of the superficial cell to cell junction. (B) Shows that the labelling for occludin was found in the cytoplasm of the superficial cells (arrow) and at the lateral interface of these cells. (C) Shows labelling for claudin-1 is located at the lateral interface of the superficial cells. Scale bars = 100 nm.

Two different groups reported the freeze-fracture appearance of cell junctions in the rabbit, rat and monkey corneal epithelium. According to those reports, tight junctions are observed only between the superficial cells and are characterized by delicate P-face particle strands and complimentary E-face grooves (Tanaka et al., 1983; Mclaughlin et al., 1985).

There are several reports describing TJ-related protein expression and distribution in in vivo rabbit and rat corneal epithelial cells (Sugrue and Zieske, 1997; Suzuki et al., 2000; Wang et al., 2001) or human corneal epithelial cell lines (Yi et al., 2000). However, we could find only one report describing ZO-1 distribution in human corneal epithelial cells in vivo (Crewe and Armitage, 2001), but no other tight junction components were described, because of the difficulty of getting intact human corneal epithelium.

Wang et al. (2001) examined ZO-1 spatial and quantitative distribution in the rabbit corneal epithelium. His immuno-histotology showed: (a) no detectable ZO-1 in basal cells; (b) incipient punctuate accumulations in the wing cells; (c) numerous foci and diffuse cytosolic staining in the squamous cells; and (d) strong staining at the apical tight junction locations in the superficial squamous layer. Immuno-blot analysis of separated layers showed a squamous: basal cell ZO-1 concentration ratio in excess of 100. Sugrue and Zieske (1997) also resolved two distinctive patterns of ZO-1 expression in the rabbit corneal epithelium, one being the lateral boundary of the apical cell, appearing as a true zonula around the cell. The second pattern of expression for ZO-1 was as punctuate spots corresponding to the most apical portion of the basal corneal epithelial cells, 'with the above wing cells'. Immuno-electron microscopy revealed that the mid-epithelial accumulations of ZO-1 were not tight junctions, but rather a form of adherence junction. ZO-1 expression in the mid-epithelial level of the cornea is neither correlated with the presence of tight junction, nor with established barrier functions. Interestingly, at this level ZO-1 co-localizes with paxillin, a focal adhesion-associated phosphoprotein. Sugrue et al. postulated that ZO-1/paxillin may function to reinforce attachments at the level of the basal cell-wing cell junction and may be regulated by reversible phosphorylation. Yi et al. (2000) found occludin, ZO-1 and ZO-2 at the cell borders of the superficial layer, whereas claudin-1 was localized mainly in the basal and wing cell layers of rat corneal epithelium. Crewe and Armitage (2001)) examined human cornea as excised directly from the eye or as organ-cultured for 1 to >28 days. In both, they found that ZO-1 labelling was restricted to the most superficial cells, where it was localized at the cell borders, completely encircling the cells. No ZO-1 labelling was seen in the wing to basal epithelial cells. Suzuki et al. (2000) detected occludin in the wing and superficial cell layers of the intact rat corneal epithelium. Our study on the localisation and nature of TJ-related proteins agrees well with the results from other laboratories.

Tight Junction strands can be formed without occludin, as vascular endothelial cells differentiated from occludin-deficient embryonic stem cells still have well-developed networks of TJ strands (Saitou et al., 1998). While, on immuno-replica electron microscopy, anti-occludin antibody reaction product is directly incorporated into TJ

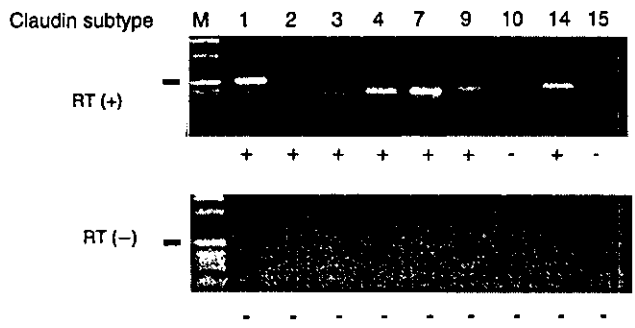

Fig. 3. Claudins expressed in in vivo human corneal epithelial cells detected by RT-PCR. The transcripts for claudin-1, -2, -3, -4, -7, -9 and -14 were identified from in vivo human corneal epithelium ($n = 4$). No amplified claudin mRNA was observed without reverse transcriptase (RT). M lane: molecular weight marker, Bar: 500 bp.

strands (Saitou et al., 1997). When occludin cDNA was introduced into mouse L fibroblast lacking TJs, no well-developed TJ strand network was identified. However, when occludin was co-transfected with claudin-1, it was concentrated into well-developed claudin-1 based strands. These findings suggested that claudins are mainly responsible for TJ strand formation, and that occludin is also the component of TJ strands together with claudins (Furuse et al., 1998b). The dotted distribution of occludin in our immunohistochemistry accords with this hypothesis.

To date, 24 subtypes of claudin have been identified (Tsukita et al., 2001).Claudin expression patterns vary considerably among tissues, and some claudins are known to be expressed in specific cells (Furuse et al., 1998a; Morita et al., 1999a).

Three claudin subtypes (claudins-6, -8, and -13) have no human expressed sequence tags (ESTs), and most, if not all, murine ESTs for these three claudins are from embryonic DNA libraries, which suggest that these genes may not be expressed in adult tissues. Claudin-11 has been found only in oligodendrocytes and Sertoli cells in the testis. No information was available for claudin-12 (Morita et al., 1999b; Yi et al., 2000). Morita et al. (1999c) reported that claudin-5/TMVCF is only expressed in endothelial cells of blood vessels. Recently, Kojima et al. pointed out claudin-5 is transiently expressed during the development of the retinal pigment epithelium of chick, but not in that of adult (Kojima et al., 2002). Claudin-16/parecellin-1 is exclusively expressed in the thick ascending limb of Henle and might form aqueous pores that function as Mg^{++} paracellular channels (Simon et al., 1999). We eliminated those subtypes from our experiment.

Nowadays, a TJ is thought not as a complete seal but as a kind of gate, which restricts passive movement between the cells. These movements are partly regulated in response to extracellular stimuli (Ban and Rizzollo, 2000a; Ban et al., 2000b, 2003). Other investigations have suggested that claudins form not only the backbone of TJ strands, but also function as extracellular aqueous pores. The results of our investigation into the expression and distribution of TJ proteins tends to support this hypothesis and it is tempting to speculate that the combination and ratio of the expressed claudins determine the complexity of the TJ strand network in human corneal epithelial cells.

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Comparison of ultrastructure, tight junction-related protein expression and barrier function of human corneal epithelial cells cultivated on amniotic membrane with and without air-lifting

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Abstract

Purpose. To evaluate the usefulness of the air-lifting technique for culturing corneal limbal epithelial cells on amniotic membrane (AM) for use in ocular surface reconstruction. A cultured sheet that has a good barrier function should be better for this purpose. In corneal epithelium, tight junctions (TJ) play a vital role in the barrier function. The TJ complex includes the integral transmembrane proteins occludin and the claudins, and some membrane-associated proteins such as ZO-1. In this paper, we investigated the barrier function and the expression of TJ related proteins.

Methods. Corneal limbal epithelium obtained from donor corneas and cultivated on acellular AM was divided into two groups. These were the non-air-lifting (Non-AL) group, which was continuously submerged in medium, and the air-lifting (AL) group, which was submerged in medium for 3 weeks, then exposed to air by lowering the medium level. Morphology and the permeability to horseradish peroxidase (HRP) were determined by electron microscopy. Tight junction (TJ)-related protein and mRNA expression changes were assessed by immunoblotting and reverse transcription-polymerase chain reaction.

Results. The cultures of both groups formed 4–5-layer-thick, well-stratified epithelium. The AL cultures had tightly packed epithelial cells with all the HRP/diaminobenzidine (DAB) reaction product accumulated on the apical surface of the superficial cells. The Non-AL culture, by contrast, had more loosely packed epithelial cells with larger intercellular spaces. The HRP/DAB reaction product penetrated the intercellular space to a depth of 3–4 cell layers. Statistically, there was a significant difference in intercellular spaces and desmosome count in the superficial cells between the groups. With AL, TJ-related proteins localized at the apical portion of the lateral membrane. TJ-related protein and mRNA amounts were not changed by AL while claudin subtype expression became more consistent and closer to that of in vivo corneal epithelium.

Conclusions. The AL technique reduces intercellular spaces in the superficial cells and promotes the formation of the barrier function. It is useful in culturing corneal epithelial cells for use in ocular surface reconstruction.

Keywords: ocular surface reconstruction; cell culture; corneal epithelium; corneal transplantation; tight junction; air-lifting

1. Introduction

Corneal epithelium functions as a barrier that isolates the eye from the outside environment; epithelial tight junctions (TJ) play a vital role in this function.

TJ encircle the cells just below the apical surface and constitute the principal regulatory barrier to passive movement of fluid, electrolytes, macromolecules and cells through the paracellular pathway. The TJ complex includes the integral transmembrane proteins occludin and the claudins, and some membrane-associated proteins such as ZO-1, ZO-2, and ZO-3 (Tsukita et al., 2001; Ban et al., 2003). Current data indicate that occludin, the first transmembrane protein identified, plays a regulatory rather than a structural role in TJ, a role that is controlled by

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phosphorylation (Furuse et al., 1993; Sakakibara et al., 1997; Saitou et al., 1998). The claudins are the transmembrane proteins that form the TJ strands. Thus far, 24 claudins have been identified; collectively they are referred to as the claudin family. Claudins are the only junctional proteins known to have tissue specificity. Occludin and the claudins contain four transmembrane domains with both N and C termini oriented into the cytoplasm (Furuse et al., 1998; Morita et al., 1999; Tsukita et al., 1999, 2001). Different mixtures of claudins and occludin create TJ strands that associate laterally with strands of adjacent cells, forming paired strands that close up the intracellular space (Furuse et al., 2001). ZO-1, ZO-2, and ZO-3, members of the membrane-associated guanylate kinase homologue (MAGUK) family, are located at the TJ-membrane contact points in epithelial and endothelial cells. The MAGUK proteins, which contain a series of protein-binding domains such as PDZ domains, are able to interact with each other, with occludin and claudins, and with the actin cytoskeleton. The MAGUK proteins allow the formation of a TJ-cytoskeleton complex (Stevenson et al., 1986; Gumbiner et al., 1991; Balda et al., 1993; Fanning et al., 1998; Haskins et al., 1998; Itoh et al., 1999a,b). Recently, many studies have pointed out that TJ cellular structures and barrier function are partly regulated in response to extracellular stimuli (Ban et al., 2000a,b).

In severe ocular surface diseases such as Stevens–Johnson syndrome and chemical burns, the corneal epithelial cells, including those of the limbal area (where corneal epithelial stem cells are located) (Schermer et al., 1986), are sometimes totally destroyed. In a severely injured cornea, with limbal and central epithelia both absent, the neighbouring conjunctival epithelial cells invade the corneal surface and visual acuity is severely obstructed. In ocular surface disorders with stem cell deficiencies, the conventional treatments are limbal transplantation and keratoepithelioplasty, but the results are not always satisfactory. Therefore, many scientists and clinicians have been trying to develop new treatments.

Pellegrini et al. (1997) reported the transplantation of autologous corneal epithelial cells cultivated on soft contact lens for severe ocular surface diseases. Subsequent to that report, the next advance of this new surgical technology was the use of human amniotic membrane (AM) as a substrate for in vitro epithelial cell culture (Koizumi et al., 2000a). Taking this a step further, the present authors began to use the air-lifting (AL) method to make sheets of cultured corneal epithelium that are similar to in vivo corneal epithelium (Koizumi et al., 2001).

Originally, AL was developed to make skin cell culture sheets for transplantation. The culture is submerged in the medium, then exposed to air by lowering the medium level. In general, the research of many investigators has shown that the closer the culture conditions are to the natural tissue environment, the more closely the cultured epithelium mimics in vivo tissue. Therefore, epidermal cell differentiation is further promoted when the cells are raised to an air–liquid interface (Prunieras et al., 1983; Williams et al., 1988; Nolte et al., 1993). We adapted this method for use in corneal epithelium culture.

In this report, we examined AL-related changes in morphology and TJ-related protein expression, and in barrier function in the corneal epithelium sheets. We conducted this study to determine whether AL acts as an extracellular stimulus of TJ function, and whether it is useful as a means of culturing corneal limbal epithelial cells so that they approximate in vivo corneal epithelium.

2. Materials and methods

2.1. Preparation of amniotic membrane and 3T3 fibroblast cells

In accordance with the tenets of the Declaration of Helsinki and with proper informed consent, human AMs were obtained at Caesarian 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's Modified Eagle Medium (GIBCO BRL, Rockville, MD, USA) and glycerol (Nacalai Tesqu Co., Kyoto, Japan) at a ratio of 1:1 (vol/vol). Immediately prior to use, the AM was thawed, washed three times with sterile PBS and cut into pieces approximately 2·5 cm × 2·5 cm in size. The AM was then deprived of amniotic epithelial cells by incubation with 0·02% ethylene diamine tetraacetic acid (EDTA, Waco Pure Chemical Industries, Osaka, Japan) at 37°C for 2 hr to loosen cellular adhesion, followed by gentle scraping using a cell scraper (Nalge Nunc International, Naperville, IL, USA).

Confluent 3T3 fibroblasts were incubated with 4 mg ml^{-1} of Mitomycin C (MMC) for 2 hr at 37°C under 5% CO_2, then trypsinized and plated onto plastic dishes to a density of 2×10^4 cells cm^{-2}. Denuded AMs (measuring approximately 2·5 cm × 2·5 cm) were spread, epithelial basement membrane side up, on the bottom of culture plate inserts (Millipore corporation, Cambridge, MA, USA) that were then placed in dishes containing treated 3T3 fibroblasts.

2.2. Explant culture of corneal limbal epithelial cells

Human corneal limbal tissue supplied from USA eye bank was used for explant cultures. The transplantation of AM-cultivated corneal epithelial cells was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine. The tissue was cut into 2 mm × 2 mm square, 100 μm thick explants. Three explants were placed directly, epithelial side down, on a portion of denuded AM spread on a culture plate insert and co-cultured with MMC-inactivated 3T3 fibroblasts. The culture medium used was Dulbecco's Modified Eagle Medium and Ham's F12 media (1:1 mixture), and included fetal bovine serum (10%), insulin

(5 μg ml^{-1}), cholera toxin (0·1 nmol l^{-1}), epidermal growth factor (10 ng ml^{-1}), and penicillin-streptomycin (50 IU ml^{-1}). Cultures were incubated at 37°C in a 5% CO_2—95% air incubator for up to 28 days, the medium being changed every day. The explants were left in the culture dish for the full duration of incubation.

2.3. Air-lifting

Cultures were divided into two groups: the Non-AL and the AL. The Non-AL cultures were continuously submerged in medium for 28 days. The AL cultures were submerged in medium for 21 days and then exposed to air by lowering the medium level for 7 days. It was important that the medium level was lowered to just meet the surface of the culture, allowing the medium to wet the surface and so allowing the tissue construct to remain moist on its apical surface.

2.4. HRP permeability assay

The polycarbonate culture inserts supporting explant cultures were placed in a humid chamber partially filled with PBS. Excess culture medium was pipetted from the surface of the liquid cultured cells; 200 μl of horseradish peroxidase (HRP) at a concentration of 100 mg ml^{-1} in PBS was then pipetted onto the apical surface of both cultures. After 45 min, the specimens were transferred to 2·5% glutaraldehyde in 0·1 M PBS overnight at 4°C. Both cultures were then washed for 30 min in 0·2% hydrogen peroxide followed by 15 min in 0·02% diaminobenzidine (DAB) in 0·05 M TRIS buffer (pH 7·5) containing 0·012% hydrogen peroxide. They were then post-fixed in 1% osmium tetroxide for 90 min and dissected into pieces a few millimeters in diameter before being dehydrated through an ethanol series to 100% ethanol. They were then subjected to two 20 min changes of propylene oxide before being embedded in Polybed 812 (Polysciences inc., Warrington, PA, USA). Ultrathin sections (50–70 nm thick) were cut and examined on a JEOL 10-10 transmission electron microscope (TEM). The sections were not counterstained, so as to enable easier visualization of the electron-dense HRP/DAB reaction product.

2.5. Quantitative ultrastructural comparison

One AL and one non-AL culture, initially prepared for the permeability study, was embedded in Agar 100-epoxy resin (Agar Scientific, UK) after being subjected to two 20 min changes of propylene oxide. Ultrathin sections (50–70 nm thick) were collected on naked copper grids and counterstained for 1 hr each with uranyl acetate and 1% phosphotungstic acid, then for 20 min with Reynolds lead citrate prior to examination on a JEOL 10-10 TEM.

A quantitative comparison of intercellular spaces and desmosome numbers between neighboring superficial epithelial cells was carried out as previously described by Koizumi et al. (2002). Briefly, images of the cultured epithelial cells were digitized using an Epson Perfection 1240U scanner. Regions of the interface of adjacent superficial cells from cultures ($n = 14$) were selected in a pseudo-random way by an individual independent of the project and who did not have knowledge of the experimental groups. Each selected region corresponded to a 3 μm long interface between adjacent cells. Optimas 6 Image Analysis Software (Optimas UK, UK) was used to determine the intercellular space (the area of extracellular space between the adjacent cells). The number of desmosomes between adjacent cells within the same pseudo-randomly selected regions was counted manually. Statistical analysis of the difference in intercellular volume and number of desmosomes by Mann-Whitney Rank Sum Test was carried out using Sigma Stat. Software (SPSS Science Software Ltd, UK).

2.6. Scanning electron microscopy

After being dehydrated through an ethanol series to 100% ethanol, the samples initially prepared for permeability study were transferred to hexamethyldilisane for 10 min and air-dried. They were then mounted on aluminum specimen stubs and sputter coated with gold before being examined on a JEOL JSM5600 scanning electron microscope (SEM).

2.7. Primary antibodies

Rabbit anti-ZO-1 polyclonal antibody and rabbit anti-claudin-1 polyclonal antibody were purchased from Zymed Laboratories (South San Francisco, CA, USA). Goat anti-occludin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.8. Immunohistochemistry

For indirect immunohistochemical study of tight junction-associated proteins, 7 μm cryostat sections were placed on gelatin-coated slides, air dried and rehydrated in PBS at room temperature for 15 min. For ZO-1 and claudin-1 staining, the sections were fixed in 3·7% paraformaldehyde for 10 min. After several washings with PBS, sections were incubated with 1% bovine serum albumin (BSA) at room temperature for 30 min, to block nonspecific binding. Following this, the sections were incubated at room temperature for 1 hr with the primary antibodies, then washed three times in PBS for 15 min. For negative controls, the equivalent serum was used. After staining with the primary antibodies, the sections were incubated at room temperature for 1 hr with suitable fluorescein (FITC)-conjugated secondary antibodies.

After several washings with PBS, the sections were coverslipped using antifading mounting medium containing

propidium iodide (PI) (Vector Laboratories, Burlingame, CA, USA). The slides were examined by confocal microscopy (Fluoview; Olympus, Tokyo, Japan).

2.9. Immunoblotting

The corneal epithelial sheets from AL and Non-AL culture were peeled mechanically and solubilized in PBS containing 100 mM EDTA, 100 mM leupeptin, 10 U ml^{-1} aprotinin, 4 mM pepstatin, and 4% sodium dodecyl sulfate (SDS). Three pairs of cultures from the same donor were used. Samples were incubated for 2 min in a boiling water bath, and protein concentrations were determined using the Micro BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of proteins were electrophoresed on 10% polyacrylamide gel for occludin and 15% polyacrylamide gel for claudin-1. Samples were immunoblotted as described by Rizzolo et al. (1994). Signals were visualized with ECL (Amersham, Arlington Heights, IL, USA) immunoblotting reagents according to the manufacturer's instructions, developed on X-ray film (Hyperfilm; Amersham), scanned with Image Master VDT-CL and analyzed with image Master Total Lab ver 1·00 (Amersham). Statistical analysis was carried out by *t*-test.

2.10. Semiquantitative RT-PCR

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed in accordance with our previous method (Nakamura et al., 2001). In brief, total RNA was isolated from AL and non Non-AL cultured corneal epithelium sheets using Trizol reagent (GIBCO BRL) in accordance with the manufacturer's protocol; five pairs of cultures from the same donor were used. Complementary DNA (cDNA) was synthesized from total RNA using an oligo (dT) primer and SuperScript II (Life Technologies, Rockville, MD, USA). To investigate relative levels of tight junction-associated protein mRNA expression in both cultures, G3PDH gene was used as internal control for this semiquantitative RT-PCR. Primer sequences used were ACCACAGTCCATGCCATCAC (sense) and TCCACCACCCTGTTGCTGTA (anti-sense) for G3PDH, TATGAGACAGACTACACAACTGGCG-GCGAGTCC (sense) and ATCATAGTCTCCAAC-CATCTTCTTGATGTG (anti-sense) for occludin, and TCAGCACTGCCCTGCCCCAGT (sense) and TGGTG-TTGGGTAAGAGGTTGT (anti-sense) for claudin-1. The PCR amplification parameters were as follows: denaturation at 95°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 1 min. The linear ranges of the amplification reaction for occludin, claudin-1 and G3PDH were determined by checking amplification reaction after each cycle from cycles 26 to 35 for occludin, from 23 to 32 for claudin-1 and from 19 to 23 for G3PDH. This showed that 30th cycle for occludin, 27th cycle for claudin-1 and 21st for G3PDH were in the midlinear phase, respectively. A 5 μl aliquot of the reaction mixture was then electrophoresed on 2% agarose gel (Sigma, St Louis, MO, USA) containing ethidium bromide to evaluate amplification and fragment size. The amplified product was quantified for each sample using a computing densitometer (420OE scanner; PDI, NY) and software (Quantity One; PDI, NY, USA). The final amount of PCR product was expressed as the ratio of the occludin and claudin-1 gene amplified to that of the G3PDH gene, to account for any differences in the beginning amounts of RNA. Statistical analysis was carried out by *t*-test.

2.11. RT-PCR amplification of Claudin species

For RT-PCR, cultured corneal epithelium sheets from AL and Non-AL groups ($n = 5$) were directly lysed with reagent (Trizol; GIBCO BRL); total cellular RNA was isolated according to the manufacturer's instructions. RT-PCR was performed in accordance with previous report by Yi et al. (2000). In brief, complementary DNA (cDNA) was generated in the presence of 0·5 μg oligo (dT) from 5 μg total RNA with reverse transcriptase (SuperScript II; Life Technologies, Rockville, MD, USA). We used PCR primers Yi et al. designed for human claudins-1 to -4, -7, -9, -10, -14 and -15 (Yi et al., 2000). PCR products were examined by 2% agarose gel and ethidium bromide staining. The observed PCR products corresponded to their expected molecular weights.

The sample numbers correspond to the same donor tissue from the same eye.

3. Results

3.1. TEM HRP permeability assay

Both cultures formed 4–5 layer-thick, well-stratified epithelium (Fig. 1). In the AL group, the epithelial cells had little or no intercellular spaces present in the superficial layer. The HRP/DAB reaction product had all accumulated on the apical surface of the superficial cells. No reaction product was visible between the cells (Fig. 1(A) and (C)).

In contrast, the epithelial cells of the Non-AL group had large intercellular spaces. The HRP/DAB reaction product was visible between the superficial cells and had penetrated the intercellular spaces to a depth of 3–4 cell layers (Fig. 1(B) and (D)).

3.2. Quantitative ultrastructural comparison

Both cultures formed 4–5 layers of thick healthy well-stratified epithelium. However, there were some differences between the culture groups, most obvious in the superficial cells. In the AL group, the superficial epithelial cells were tightly attached to each other with many well-formed desmosomal junctions (Fig. 2(B)). Furthermore,

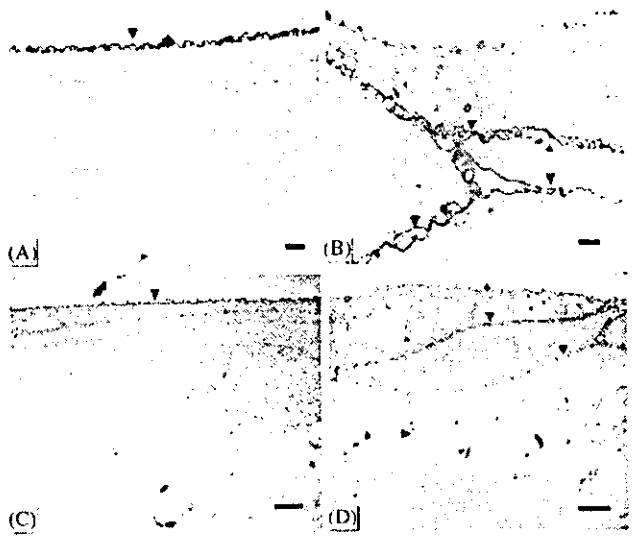

Fig. 1. TEM micrographs of AL (A and C) and Non-AL (B and D) cultured epithelial cells after HRP treatment. At high magnification it is evident that the AL sample (A) shows no penetration of HRP below apical surface (arrow), while the Non-AL sample (B) shows clear penetration of HRP between the apical cells (arrows) [scale bars = 500 nm]. At lower magnification it is evident that in the non-AL sample (D) the HRP has penetrated to a depth of 3–4 cell layers (arrows), while in the AL sample (C) the HRP is only seen on the outer surface of the superficial cells (arrow) (scale bars = 2 μm). Sections were not counter-stained, so as to visualize electron dense HRP product clearly.

the intercellular spaces between the superficial cells were small (Fig. 2(A)). In the Non-AL group, by contrast, the superficial epithelial cells had fewer desmosomes and large intercellular spaces were found between the cells (Fig. 2(C) and (D)).

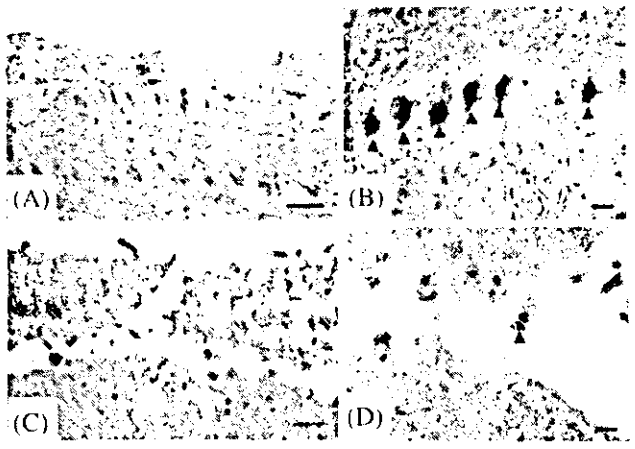

Fig. 2. TEM micrographs of AL (A and B) and Non-AL (C and D) cultured epithelial cells. At low magnification it is evident that Non-AL cultured epithelium (C) has large spaces between its cells. In contrast, AL cultured epithelium (A) has smaller intercellular spaces between neighboring superficial cells [scale bars = 500 nm]. At higher magnification only a few desmosomal junctions (arrowhead) are evident between the superficial cells in the Non-AL sample (D) while the AL sample (B) has numerous desmosomal junctions (scale bars = 200 nm).

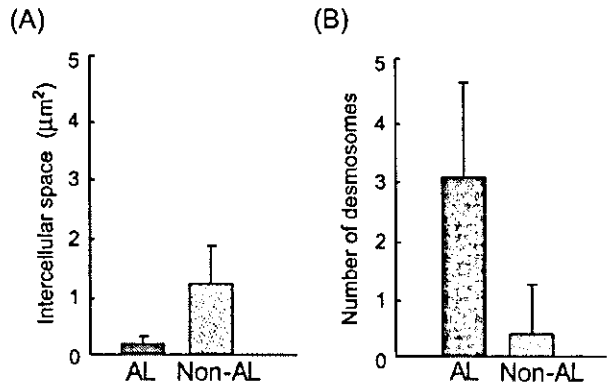

Fig. 3. (A) Histogram showing the differences in intercellular area between adjacent superficial epithelial cells of the two different experimental groups. AL averaged 0·15 μm^2 ± 0·12 S.D. (n = 14) and Non-AL averaged 1·15 μm^2 ± 0·66 S.D. (n = 14). These values for intercellular area were calculated for a 3 μm long interface between adjacent cells. (B) Histogram showing the differences in desmosome numbers between adjacent superficial epithelial cells of the two different experimental groups. AL averaged 3·00 ± 1·62 S.D. (n = 14) and Non-AL averaged 0·64 ± 1·01 S.D. (n = 14). These average values were calculated for a 3 μm long interface between adjacent cells.

The intercellular spaces of the Non-AL group were statistically significantly larger than in the AL groups ($P < 0·001$) (Fig. 3(A)).

The number of desmosomes found in Non-AL group was statistically significantly less than those found in the AL group ($P < 0·001$) (Fig. 3(B)).

These results suggest that AL significantly influences the size of the intercellular spaces and number of desmosomal junctions in the superficial layer.

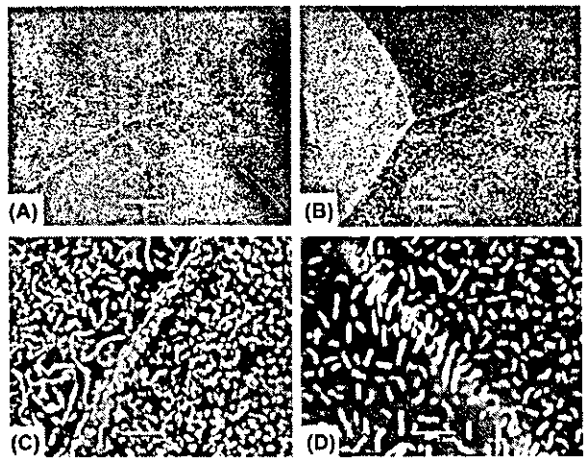

Fig. 4. SEM micrographs of AL (A and C) and Non-AL (B and C) cultured epithelial cells. At low magnification there is little difference evident between AL (A) and Non-AL (B) cultures. [scale bar = 5 μm]. However, at high magnification there are sometimes gaps evident between the cellular junctions of the Non-AL epithelial cells (D). In contrast, junctions between superficial cells in AL cultured epithelium appear more prominent and more tightly opposed (C). (Scale bar = 1 μm.)

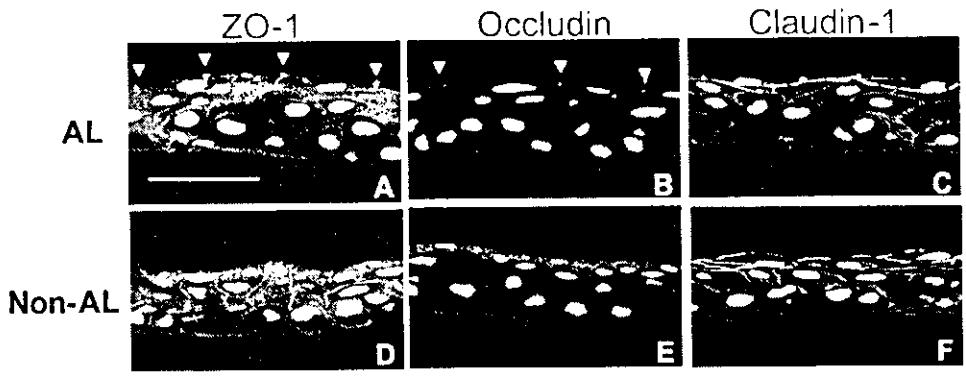

Fig. 5. Immunohistochemical analysis of TJ-related proteins; ZO-1 (A and D), occludin (B and E) and claudin-1 (C and F). Two different groups of corneal limbal epithelial cell culture were compared; AL (A, B and C) and Non-AL (D, E and F). In AL group, both ZO-1 and occludin localized in apical cell-cell junctions between superficial cells in stratified corneal epithelium (A and B). Non-AL group showed strong ZO-1 and occludin stainings in the cytoplasm of superficial cells than that of basal cells, but there was no localization (B and E). In Non-AL group, both apical and basolateral membranes was stained diffusely by claudin-1 antibody, whereas in the AL group, basolateral membrane is stained more than apical membrane (C and F). (Scale bar = 100 μm.)

3.3. Scanning electron microscopy

Scanning electron microscopic examination of the AL and non-AL cultures revealed that both groups were similar in appearance, with a continuous layer of flat polygonal epithelial cells (Figs. 4(A) and (B)). However, in some regions at high magnification, we observed differences between the culture groups. In the Non-AL group, the epithelial cell junctions appeared less tightly opposed with small gaps evident (Fig. 4(D)). In the AL group, by contrast, the epithelial cells were closely attached to each other and had distinct cell boundaries (Fig. 4(C)).

3.4. Immunofluorescence

In the AL group both ZO-1 and occludin were localized at the apical cell–cell junctions between superficial cells in stratified corneal epithelium (Fig. 5(A) and (B)). This localization was close to that in vivo (Ban et al., 2003). The Non-AL showed stronger ZO-1 and occludin staining in the cytoplasm of superficial cells than in that of basal cells, but there was no localization of staining at the apical cell junctions (Fig. 5(D) and (E)).

In the Non-AL, both apical and basolateral membranes were stained diffusely by claudin-1 antibody, whereas in the AL group, basolateral membrane was stained more than apical membrane (Fig. 5(C) and (F)).

These results indicate that AL is involved in tight junction-related protein localization similar to that in vivo.

3.5. Change in occludin and claudin-1 protein expression with air-lifting

Immunoblotting occludin and claudin-1 expression showed that the ratio of occludin or claudin-1 to actin did not change with AL (Fig. 6).

3.6. Change in occludin and claudin 1 mRNA with air-lifting

Semiquantitative analysis of occludin mRNA and claudin-1 mRNA expression showed the ratio of occludin or claudin-1 to G3PDH mRNA did not change with AL (Fig. 7).

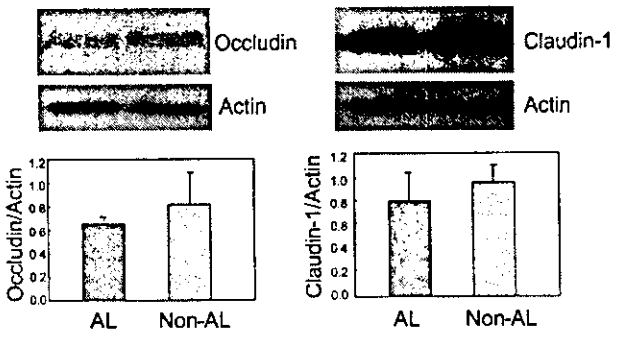

Fig. 6. Expression of occludin and claudin-1 in corneal limbal epithelial cell sheets. The ratio of occludin or claudin1 to actin did not change significantly with AL. (Occludin: $P = 0.3919$, Claudin-1: $P = 0.4082$).

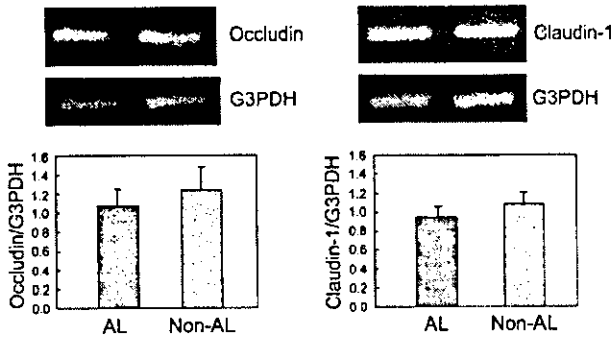

Fig. 7. Expression of occludin and claudin-1 mRNA in corneal limbal epithelial cell sheets. The ratio of occludin or claudin1 mRNA to G3PDH mRNA did not change significantly with AL. (Occludin: $P = 0.2390$, Claudin-1: $P = 0.1018$).