Graefe's Arch Clin Exp Ophthalmol
(2007) 245: 123–134

DOI 10.1007/s00417-005-0095-3

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Comparison of intact and denuded amniotic membrane as a substrate for cell-suspension culture of human limbal epithelial cells

Received: 11 January 2005
Revised: 15 July 2005
Accepted: 18 July 2005
Published online: 13 April 2006
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Abstract *Background:* We have previously developed a limbal epithelial culture system using a cell-suspension method on denuded amniotic membrane (AM). However, other workers reported that intact AM is advantageous for limbal epithelial culture in that it preserves stem cell characteristics. In this study, we cultivated human limbal epithelial cell-suspensions on both intact and denuded AM and compared the morphology and adhesion of the limbal epithelial cells on these two substrates. *Methods:* Human limbal epithelial cells were dissociated from donor eyes using dispase and gentle pipetting and then seeded onto intact and denuded AM as cell suspension. Limbal epithelial cells on AM were co-cultured with a MMC-treated 3T3 fibroblast feeder layer and epithelial differentiation was promoted by air lifting. Cultures were examined by light, scanning and transmission electron microscopy and differences in cellular attachments and intercellular spacing were quantified. Basement membrane complexes were examined by indirect immunofluorescence. *Results:* Limbal cells grown on denuded AM were well stratified and differentiated. Cells were well attached to each other and to the basement membrane. In contrast, limbal cells cultured on intact AM failed to stratify and in places formed a monolayer.The culture on denuded AM had significantly ($P<0.001$) more desmosomal junctions as well as significantly ($P<0.001$) more junctional attachments to the carrier than the intact culture. In addition, the intercellular spaces between cells cultivated on denuded AM were significantly ($P<0.001$) smaller than those between cells grown on the intact substrate. In cultures on both denuded and intact AM, the basement membrane zone displayed a positive staining for collagen VII, integrins alpha-6 and beta-4 and laminin 5. *Conclusions:* We successfully cultivated well-stratified and -differentiated limbal cells on denuded AM, while on the intact AM limbal cells failed to stratify and in places formed only a monolayer of cells. The limbal cells cultivated on denuded AM were well attached to the AM stroma and were morphologically superior to the limbal epithelium cultivated on intact AM. We conclude that for purposes of transplantation of differentiated epithelial sheets, denuded AM is probably the more practical carrier for human limbal epithelial cell cultures when using our cell-suspension culture system.

Keywords Amniotic membrane · Limbal epithelial cell culture · Cell suspension

Introduction

Corneal epithelium is a self-renewing tissue maintained by the proliferation of stem cells, thought to be located at the limbus [2, 34]. A healthy corneal epithelium is essential for a clear cornea and good vision. However, severe ocular surface diseases (OSD) such as Stevens–Johnson syndrome can completely destroy the corneal epithelium, along with the limbal stem cells. In cases of severe OSD in which the limbal and central epithelia are both absent, the neighboring conjunctival epithelium invades the corneal surface, and visual acuity is severely obstructed [36, 45]. In order to reconstruct such damaged ocular surface, new surgical techniques, such as autologous conjunctival transplantation [40], keratoepithelioplasty and autografts or allografts of limbal transplantation [11, 41, 43] have been developed in the past 20 years. Following this progress on the ocular surface surgery front, Pellegrini et al. first reported the successful ocular surface reconstruction using autologous cultivated corneal epithelial stem cells in patients with severely affected unilateral OSD [31]. Since then, attention has been focused on the ex vivo expansion of corneal epithelial cells on appropriate substrates. One of these substrates is the amniotic membrane for use as a limbal epithelial cell carrier [16, 35, 44].

The amniotic membrane (AM) consists of a thick basement membrane and a adjacent stroma. Over the past several years, preserved human AM has been used by many investigators, with or without limbal transplantation, to reconstruct severely damaged ocular surface diseases such as chemical and thermal injuries [12, 37], severe pterygium [32, 38], persistent corneal ulcers [23, 26], ocular cicatricial pemphigoid and Stevens–Johnson syndrome [46, 48]. The AM has been reported to promote epithelialization after transplantation via growth factors such as EGF, KGF and HGF [17], and also to inhibit conjunctival fibrosis by suppressing the transforming growth factor beta signaling system and myofibroblast differentiation of normal fibroblasts [47, 25]. The AM stroma has anti-inflammatory effects produced by inducing the suppression of interleukin 1 alpha and interleukin 1 beta in epithelial cells [39]. Also, the AM blocks polymorphonuclear cells from infiltrating into the corneal stroma [30] and inhibits protease activity [13]. Furthermore, it is reported that the basement membrane components of AM resemble ocular surface epithelium; not only conjunctival epithelium [6], but also corneal epithelium [5]. Based on this knowledge, preserved human AM is considered to be one of the best carriers for transplantation of cultivated corneal epithelial cells.

With the aim of establishing an effective ocular surface reconstruction for severe ocular surface diseases, several groups of scientists, including ours, have tried to develop their original culture system using human AM as a carrier. We have developed a limbal epithelial culture system using denuded AM [15, 16]. Our culture system is also supported by the 3T3 fibroblast feeder layers and an air-lifting technique. Using co-culture system with 3T3 fibroblasts based on the keratinocyte culture system established by Rheinwald and Green [33], we obtain a well-stratified and morphologically differentiated cellular multilayer that closely resembles corneal epithelium. These stratified and differentiated cultivated epithelia are used to cover the severely damaged ocular surface in patients with total stem cell deficiencies such as those found in chemical injury, ocular cicatricial pemphigoid, and Stevens–Johnson syndrome, at the time of the surgery and remain in situ to maintain a clear corneal surface [14, 18, 19, 29]. Conversely, another established culture system using an AM carrier is explant culture on intact AM . This culture system is usually carried out without using a 3T3 fibroblast feeder layer and air-lifting. It is believed that the culture system is better for the preservation of stem cells or limbal epithelial progenitor cells in the culture epithelial sheet. Workers have demonstrated that such cultivated limbal epithelial cells showed slow cycling and label-retaining characteristics and did not express K3 and K12 keratins [7, 27] and connexin 43 [7]; these characteristics resemble those found in the stem cell-containing limbal basal epithelium in vitro. These epithelial sheets are normally epithelial monolayers and are expected to become stratified after transplantation. Their culture system is identical to the system used by Tsai et al. in clinical procedures [44].

Accumulated evidence from basic research and from clinical observations allows us to say that both of the culture systems, namely culture on denuded AM with 3T3 fibroblast feeder layers and air lifting and the culture on intact AM without 3T3 fibroblasts and air lifting, are promising systems for providing cultivated limbal epithelial sheets for ocular surface reconstruction. In this paper, to further the development of limbal epithelial cell culture systems, we sought to evaluate intact AM for use in cell-suspension culture for the purposes of successful transplantation. We cultivated human limbal epithelial cells on intact and denuded AM using a cell-suspension culture system [20] supported by a 3T3 fibroblast feeder layer and compared the ultrastructure of the cultivated epithelial cells.

Materials and methods

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 the time of Caesarean section. The membranes were washed with phosphate-buffered saline (PBS) containing antibiotic (5 ml of 0.3% ofloxacin) under sterile conditions and stored at −80°C for 5 months in Dulbecco's Modified Eagle Medium (Gibco BRL, Rockville, MD,

USA) and glycerol (Sigma, St. Louis, MO, USA) at the 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 2.5×2.5 cm in size. Several pieces of AM were then deprived of their amniotic epithelial cells by incubation with 0.02% ethylene diamine tetraacetic acid (EDTA; Sigma) at 37°C for 2 h to loosen cellular adhesion, followed by gentle scraping using a cell scraper (Nalge Nunc International, Naperville, IL, USA). As reported previously [14, 15, 19, 30] limbal epithelial cells were co-cultured with mitomycin C (MMC) inactivated 3T3 fibroblasts. Briefly, confluent 3T3 fibroblasts were incubated with 4 μg/ml of MMC for 2 h at 37°C under 5% CO_2. These were then trypsinized and plated onto plastic dishes with a density of 2×10^4 cells/cm^2. Intact and denuded AMs were spread, basement membrane/amniotic epithelial side up, on culture plate inserts (Corning, NY, USA) in dishes containing the treated 3T3 fibroblasts.

Cell-suspension culture of human limbal epithelial cells

Human corneal tissue supplied from Northwest Lion Eye Bank (Seattle, USA) and Cologne University Eye Bank (Cologne, Germany) was used for epithelial cell culture. Immediately after the central corneal button had been used for corneal transplantation, the limbal ring was washed 3 times with sterile PBS. Following removal of excessive scleral and corneal stroma (approximately two thirds of the thickness of stroma), the limbal ring was incubated at 37°C for 1 h with 1.2 IU dispase (Roche Molecular Biochemicals, Mannheim, Germany). Following incubation of the limbal rings, limbal epithelium was probed and separated from the residual corneal stroma under a dissecting microscope using two fine forceps by means of gentle horizontal movements. Limbal epithelial cells were dissociated as small sheets of cells that had the folding structure and pigmentation that are consistent with the anatomical and histological characteristics of the palisades of Vogt. By probing and separating the basal epithelial cells carefully, we could remove limbal epithelial cells including basal cells. These small epithelial sheets were dissociated into single cells containing some fragments of cells composed of clumps of 5–10 epithelial cells and suspended into 3 ml of culture medium ($5–10\times10^4$ cells/3 ml medium). This suspension was divided onto six pieces of AM, specifically three pieces of intact AM and three pieces of denuded AM, placed on culture inserts in dishes as detailed above. The equal seeding of cells onto each membrane was achieved by gentle mixing of the cell-suspended medium before each seeding and confirmed by phase-contrast microscopy. The culture medium used was a 1:1 mixture of Dulbecco's Modified Eagle Medium and Ham's F12 medium and included fetal bovine serum (10%), insulin (5 μg/ml), cholera toxin (0.1 nmol/l), epidermal growth factor (10 ng/ml), and penicillin–streptomycin (50 IU/ml). The culture was submerged in the medium for 2 weeks and then exposed to air by lowering the medium level (air lifting) for 2 weeks to promote corneal epithelial differentiation. Cultures were incubated at 37°C in a 5% CO_2 –95% air incubator for up to 28 days, and the medium was changed every 2 days.

Light microscopy

The samples were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, routinely processed and examined after toluidine blue staining.

Scanning electron microscopy

The samples were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer. They were washed 3 times in PBS for 15 min and then post-fixed in 2% osmium tetroxide for 2 h. They were washed again in PBS before being dehydrated through a graded alcohol series. After two 20-min changes of 100% ethanol the samples were transferred to hexamethyldisilazane (HMDS) for 2×10 min and allowed to air dry. The samples were then mounted on aluminum 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 3% glutaraldehyde in 0.1 M cacodylate buffer. They were washed in PBS for 3×15 min and then post-fixed in 2% osmium tetroxide for 2 h. They were washed again in PBS before being passed through a graded alcohol series and embedded in Araldite resin (Agar Scientific, UK). Ultrathin sections were cut on a Reichert Ultracut E microtome, 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.

Quantitative analysis of denuded and intact cultures

The protocol for calculating intercellular space areas and to quantify desmosomal junctions between neighboring epithelial cells was as previously described [20]. Briefly, images of the cultured epithelial cells on intact and denuded AM were digitized. Regions of the interface of adjacent cells (corresponding to a 3μm distance) cultivated on both denuded AM (n=15) and intact AM (n=15) were selected at random by a person independent of this project. 'Optimas 6.0' Image Analysis Software (Optimas UK) was used to calculate the intercellular area (the area of

extracellular space between the adjacent cells), while the number of desmosomes in the same randomly selected area was counted manually. Statistical analysis of the differences in the intercellular areas and in the number of desmosomes was carried out for each substrate using the Mann–Whitney rank sum test (Sigma Stat. software; SPSS Science Software, UK) and a significance level of 0.001. The number of hemi-desmosomal junctions at the basement membrane in the denuded culture, and that of desmosomal junctions attaching cultivated cells to AM epithelial cells on the intact substrate were similarly quantified over randomly selected 3μm lateral distances. Statistical analysis was carried out for each substrate using a *t*-test at a significance level of 0.001.

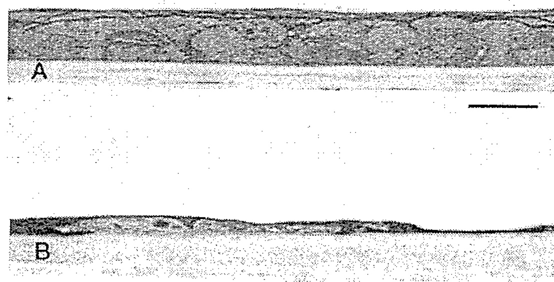

Fig. 1 The light microscopy showed limbal epithelial cells cultivated on denuded AM produced a well-stratified cell layer (**a**), while in contrast, limbal epithelial cells cultivated on intact AM formed only one or two strata of cells (**b**). *Scale bars* 10 μm

Immunofluorescence microscopy for basement membrane complexes

Immunohistochemistry was carried out for basement membrane components laminin 5, integrin-α6, integrin-β4 and collagen VII. Samples of both denuded and intact AM with and without limbal epithelial cells were examined. Specimens were fixed in 4% paraformaldehyde for 1 h then washed with PBS and dehydrated with 20% sucrase overnight. They were embedded in OCT compound (Miles, Elkhart, IN, USA). Frozen 6-μm sections mounted on a silanized slide were rinsed in PBS for 3×5 min and pre-incubated with 1% bovine serum albumin (BSA) in PBS for 30 min at room temperature to block nonspecific binding. Sections were then incubated overnight at 4°C with primary mouse monoclonal antibodies: against laminin 5 (MAB19562; Chemicon, Temecula, CA, USA, integrin–β4 (MAB 2058; Chemicon), collagen VII (MAB 1345; Chemicon) and integrin–α6 (HUMANCD49f; Cymbus Biotechnology, Hampshire, UK). For negative controls, the primary antibody was omitted. After four washes with PBS, the sections were then incubated at room temperature for 1 h with an FITC-conjugated secondary antibody (goat anti-mouse IgG) purchased from Molecular Probes (Eugene, OR, USA). After four washes with PBS, the sections were coverslipped using anti-fading mounting medium containing propidium iodide (Vectashield; Vector, Burlingame, CA, USA) and the slides were examined by means of confocal microscopy (Olympus Fluoview, Tokyo, Japan).

Results

Light microscopy

Limbal epithelial cells cultivated on denuded AM produced a well-stratified thick cell layer (Fig. 1a), while in contrast those limbal cells cultivated on intact AM formed only one or two layers (Fig. 1b).

Scanning electron microscopy

Examination of the apical surface of the limbal cells cultivated on denuded AM showed a continuous layer of flat polygonal epithelial cells (Fig. 2a). The cells averaged about 50–60 μm in diameter and were similar in appearance to normal human corneal epithelial cells. In some areas, epithelial cells appeared to be undergoing the process of desquamation, as would be expected in a healthy epithelial sheet. Cultivated cells were closely attached to each other with tightly opposed cell junctions and distinct cell boundaries (Fig. 2c). The apical surface of the cells was covered in short microvilli (Fig. 2e).

With regard to cells cultured on intact AM, we found that not all of the AM was covered with limbal epithelial cells. The AM epithelial cells appeared disrupted and had large intercellular spaces and long, distended microvilli (Fig. 2d, f). Where limbal cells were present on top of the AM epithelial cells, they did not appear to be well attached to the cells beneath them (Fig. 2g). Adjacent limbal cells were not tightly attached to each other, and there were prominent intercellular spaces (Fig. 2b,d). In places the limbal cells appeared to be burrowing beneath the AM epithelial cells and making direct contact with the basement membrane (Fig. 2h).

Transmission electron microscopy

Examination at low magnification confirmed the light-microscopic findings. There were considerable morphological differences between cells cultured on intact and denuded AM. Limbal epithelial cells grown on denuded AM were well stratified and differentiated into four to five distinct cell layers (Fig. 3a). Adjacent cells were joined by numerous desmosomes and intercellular spaces were minimal (Fig. 3c). Basal epithelial cells adhered well to

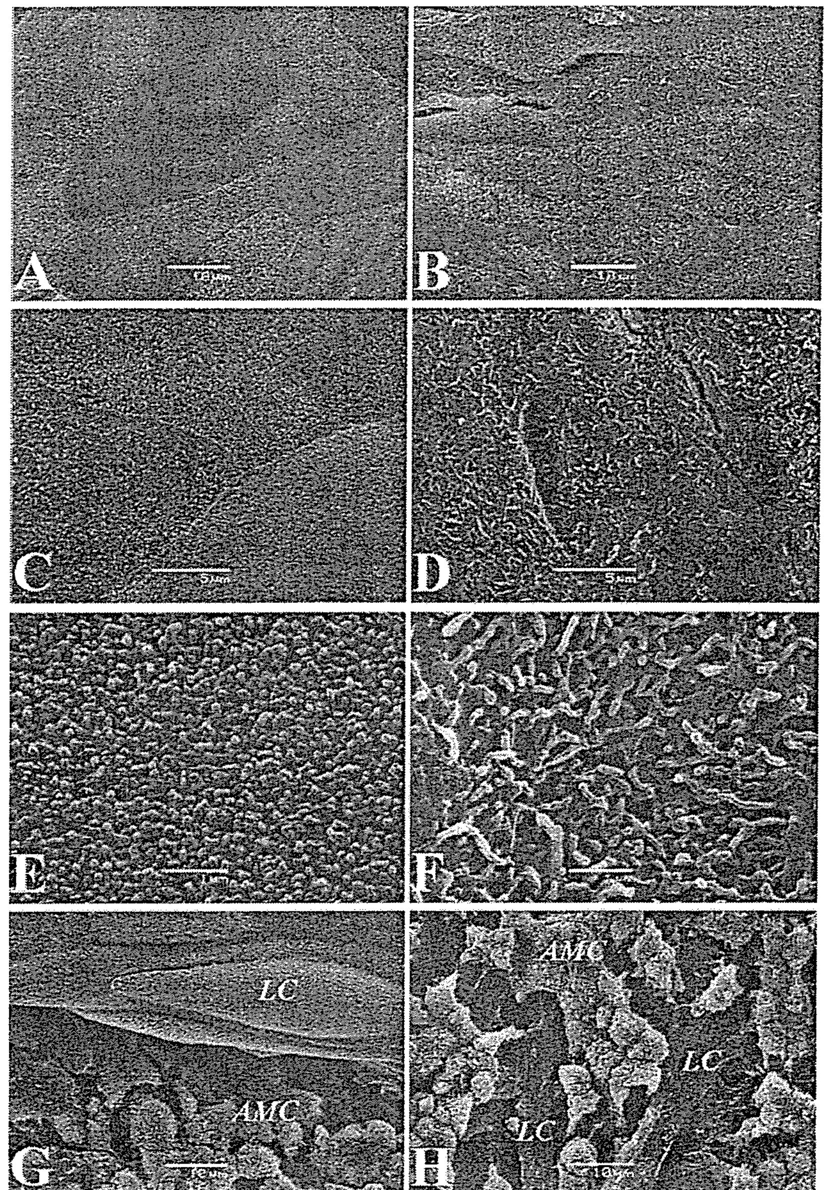

Fig. 2 Scanning electron micrographs of human limbal epithelial cells cultured on denuded (**a**, **c**, **e**) and intact (**b**, **d**, **f–h**) AM. Limbal cells grown on denuded AM formed a confluent layer (**a**). Epithelial cells were closely attached to each other with tightly opposed cell junctions and distinct cell borders (**c**). Apical microvilli were short and regular in appearance (**e**), closely resembling those of normal corneal epithelium. In contrast, human limbal cells grown on intact AM had less distinct cell borders (**b**, **d**). The surface of these cells was covered in abnormally long and distended microvilli (**f**). In some areas, the limbal cells (*LC*) appeared to grow over the top of the amniotic epithelial cell debris (*AMC*) with poor basal attachment (**g**). In other places, cultivated limbal cells spread beneath the degraded AM epithelial cells, apparently making contact with the basement membrane itself (**h**). *Scale bars*: **a**, **b**, **g**, **h** 10μm; **c**, **d** 5μm; **e**, **f** 1μm

the AM substrate with hemi-desmosomal attachments and produced basement membrane material (Fig. 3e).

In contrast, limbal epithelial cells cultivated on the intact AM did not look healthy. Not all of the AM was covered with limbal cells, and in places there were no cells at all (Fig. 3h). The amniotic epithelial cells were necrotic, with degraded external membranes and large intercellular spaces between adjacent cells of the monolayer. These cells were not well adhered to the AM matrix, and there was little evidence of any basement membrane material (Fig. 3g). The limbal epithelial cells were at most three layers thick and in some areas formed a monolayer; as such, they were neither well stratified nor differentiated (Fig. 3b). Spaces between the cells were large and there were very few desmosomal attachments (Fig. 3d). Attachment of the basal limbal cells to the AM epithelial cells was via desmosomal junctions (Fig. 3f), though these were infrequent.

Quantitative analysis of denuded and intact cultures

There was a highly significant ($P<0.001$) difference in the area of intercellular spaces between adjacent limbal

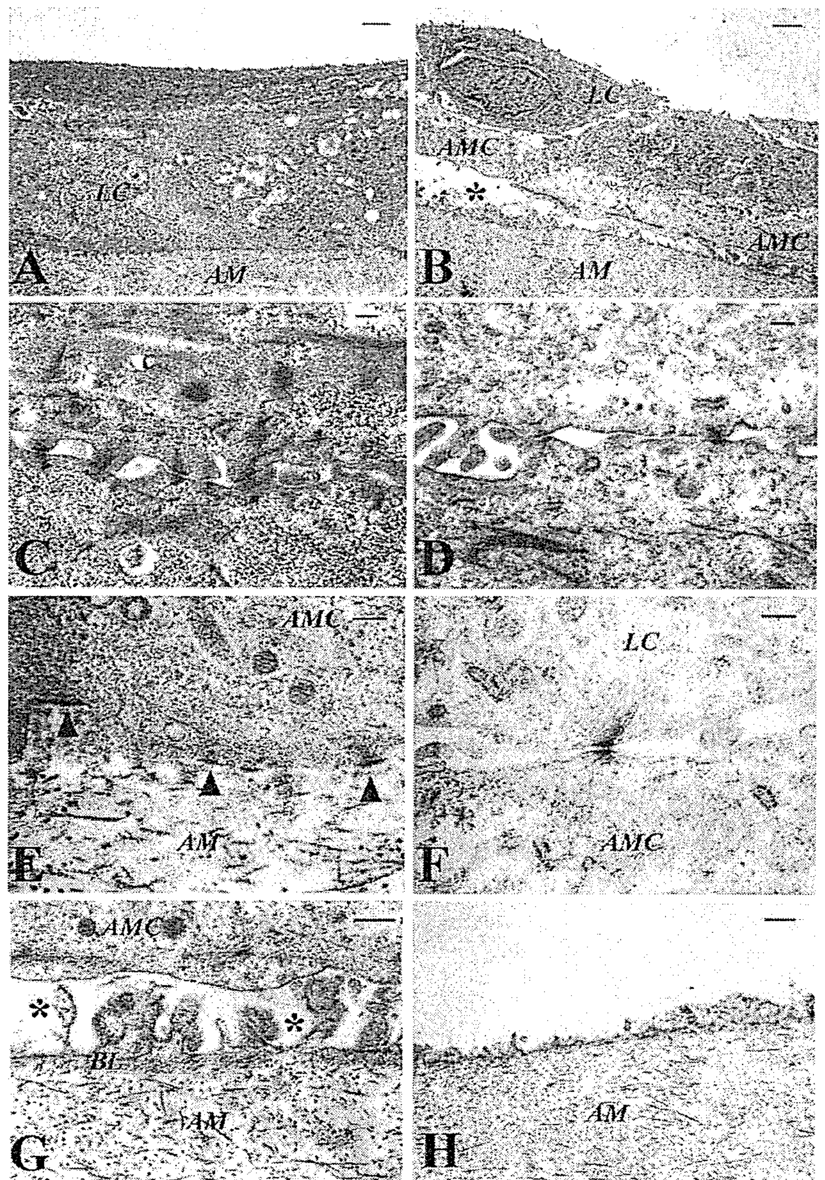

Fig. 3 Transmission electron micrographs illustrating the morphology of human limbal cells (*LC*) cultivated on denuded and intact AM. The culture on denuded AM produced a well-stratified and differentiated cell layer, similar in appearance to normal corneal epithelium (**a**). In contrast, limbal cells on intact AM often formed a monolayer (**b**). There were large spaces between neighboring cells, and the AM epithelium (*AMC*) was poorly attached to the basement membrane. There were numerous desmosomal contacts between adjacent cells in the culture on denuded AM (**c**), while limbal cells cultured on intact AM were not closely attached to each other and very few desmosomes were observed (**d**). Basal limbal cells appeared to be well attached to the denuded amniotic membrane via hemi-desmosomal junctions and the secretion of basement membrane material (**e**). On the intact AM, limbal cells were joined to underlying amniotic epithelial cells via infrequent desmosomes (**f**). Similarly, the necrotic amniotic epithelial cells were themselves not well attached to the basal lamina (*BL*), with little evidence of any hemi-desmosomal junctions (**g**). As a result, there were areas of 'intact' amniotic membrane completely devoid of an epithelial cell layer (**h**). *Scale bars*: **a**, **b** 2μm; **c–f** 200 nm; **g** 500 nm; **h** 1μm

epithelial cells cultured on denuded and intact AM. The average intercellular area was calculated as 0.19 $\mu m^2 \pm 0.11$ SD (n=15) for the cells cultured on denuded AM, while limbal cells cultivated on intact AM had an average intercellular area of 1.10 $\mu m^2 \pm 1.06$ SD (n=15; Fig. 4a). There was also a highly significant ($P<0.001$) difference in the number of desmosomes between neighboring epithelial cells between cultures. Average numbers of desmosomes between adjacent cells were 2.20±1.28 SD (n=15) and 0.40±0.61 SD (n=15) for cells cultivated on denuded and intact AM respectively (Fig. 4b). The averages for intercellular area and number of desmosomes are given for a 3 μm long interface between adjacent cells. In terms of basal attachments of the cultivated epithelial cells to the substrates, there was again a highly significant ($P<0.001$) difference between the number of hemi-desmosomes (1.87±1.19 SD, n=15) at the basement membrane in the denuded culture and that of desmosomal junctions (0.47±0.52 SD, n=15) at the basal limbal cell–AM epithelial cell interface in the culture on intact AM (Fig. 4c).

Immunofluorescence microscopy

Laminin 5

Both intact and denuded AM without limbal epithelial cells evinced laminin-5 localized in the basement membrane

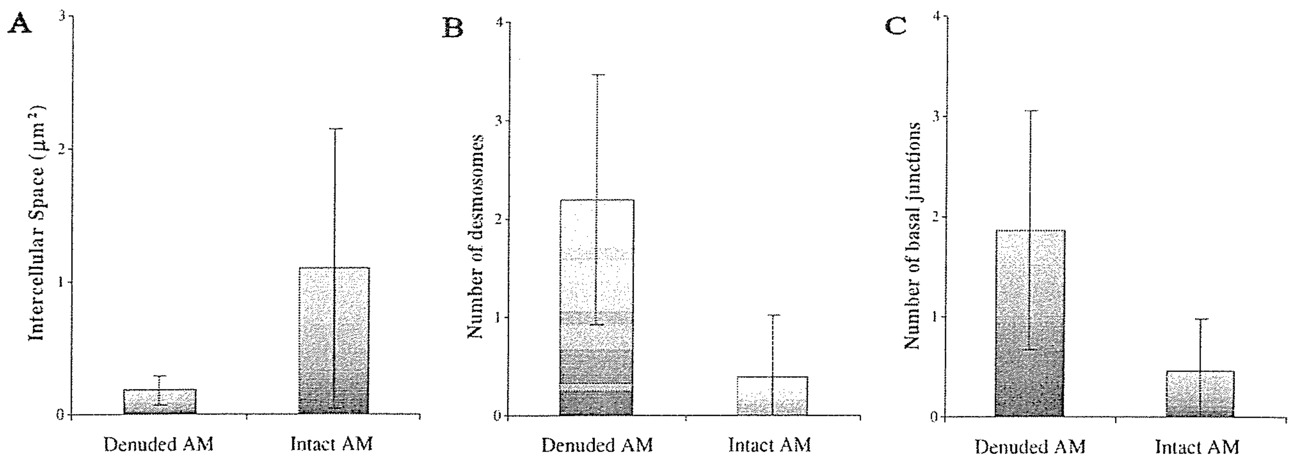

Fig. 4 (**a**) Histogram showing the differences in intercellular spaces between adjacent human limbal epithelial cells cultured on denuded AM (average 0.19μm²±0.11 SD, *n*=15) and intact AM (average 1.10μm²±1.06 SD, *n*=15). (**b**) Histogram showing comparative average numbers of desmosomes between adjacent cells. These were 2.20±1.28 SD (*n*=15) and 0.40±0.61 SD (*n*=15) for cells cultivated on denuded and intact AM respectively. (**c**) Histogram depicting the difference in numbers of basal attachments of the cultivated epithelial cells to the substrates. There were more hemi-desmosomes (average 1.87±1.19 SD, *n*=15) at the basement membrane in the denuded culture than desmosomes (average 0.47±0.52 SD, *n*=15) at the basal limbal cell–AM epithelial cell interface in the culture on intact AM. All data were collated using random 3μm interfaces, and differences in intercellular spacing and numbers of junctions were highly significant ($P<0.001$)

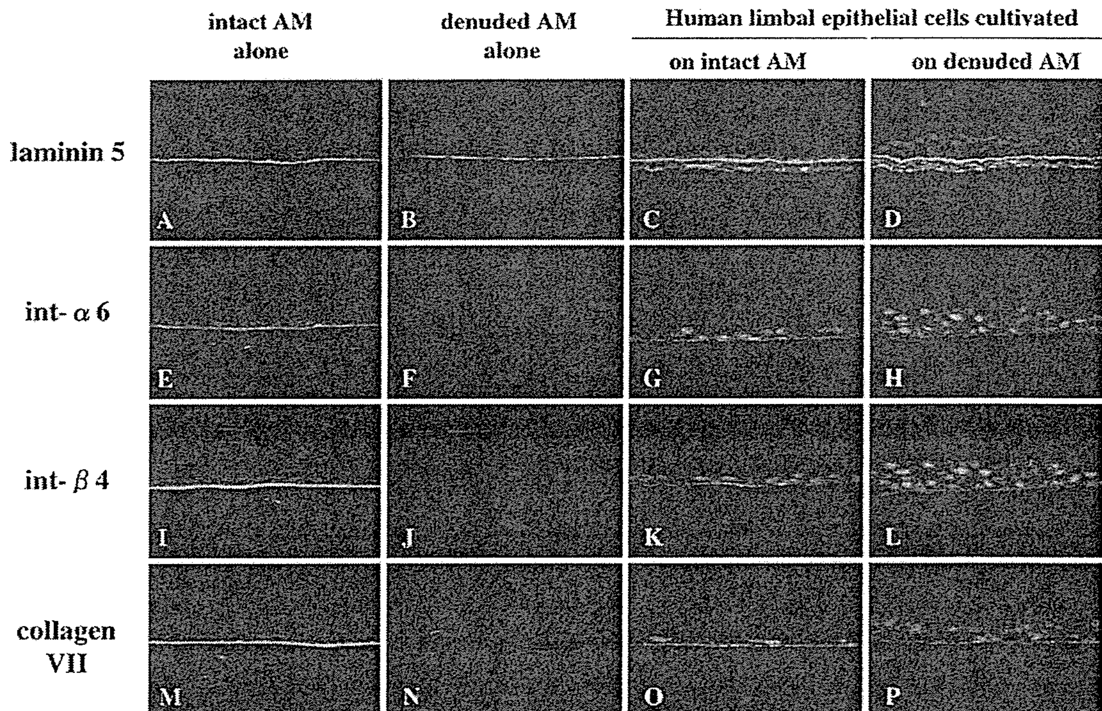

Fig. 5 Distribution of basement membrane complexes in cryopreserved AM alone (cultivated for 28 days without limbal epithelial cells) (**a**, **b**, **e**, **f**, **i**, **j**, **m**, **n**) and AM cultivated for 28 days with limbal epithelial cells (**c**, **d**, **g**, **h**, **k**, **l**, **o**, **p**). Intact AM showed positive staining for laminin 5 (**a**), integrin-α6 (**e**), integrin-β4 (**i**) and type VII collagen (**m**) in the basement membrane area. After removal of amniotic epithelial cells, laminin 5 was retained (**b**) but integrin-α6 (**f**), integrin -β4 (**j**) and type VII collagen (**n**) were not detected. Where limbal epithelial cells were cultivated on intact AM, the basement membrane area stained positive for laminin 5 (**c**), integrin-α6 (**g**), integrin-β4 (**k**) and collagen VII (**o**). Positive staining was also seen in the cultivated limbal epithelial cells on denuded AM (**d**, **f**, **l**, **p**). Cell nuclei were stained with propidium iodide (*red*)

area (Fig. 5a,b). After 28 days in culture with limbal epithelial cells, thicker bands of immunofluorescence were observed in both intact and denuded AM cultures (Fig. 5c,d). Laminin 5 was also positive in the AM stroma after cultivation of limbal cells.

Integrin-α6, -β4

Integrin-α6 and integrin-β4 were located in the basement membrane area of the intact AM without limbal epithelial cells (Fig. 5e,i). Integrin-α6/β4 is one of the major ligands to the basement membrane component laminin 5 and also participates in hemi-desmosome formation. The expression was lost after the denudation process (Fig. 5f,j) but after 28 days' culture of limbal epithelial cells integrin-α6 and -β4 were again expressed along the basement membrane area (Fig. 5h,l).

Collagen VII

Positive staining for type VII collagen, which is a component of anchoring fibrils, was observed in intact AM without limbal epithelial cells (Fig. 5m) and expression was lost after the amniotic epithelial cells were removed (Fig. 5n). After cultivation of limbal epithelial cells, both intact (Fig. 5o) and denuded AM (Fig. 5p) expressed collagen VII in the basement membrane area.

Discussion

Our primary objective in this study was to quantitatively compare human limbal epithelial cells cultured on denuded AM (in which the basement membrane is exposed by the enzymatic removal of host epithelial cells) with those cultivated on intact AM (where amniotic epithelial cells are retained) using our cell-suspension culture method supported by a 3T3 fibroblast feeder layer and air lifting. We sought to determine which would be the more suitable substrate for the cultivation of human limbal epithelial cells and subsequent use in transplantation, in terms of outgrowth rates, morphological appearance and overall mechanical strength.

The wider aim of any such project is to optimize a procedure for successful ocular surface reconstruction and to find the best possible carrier for corneal cells in transplantation. In the pursuit of an ideal carrier a number of criteria have to be fulfilled, including biocompatibility, flexibility, mechanical strength, permeability for nutrient transport and support of surface epithelial cell growth. Mechanical or tensile strength is essential for any graft to survive the rigors of transplantation, not least onto the ocular surface. Following a transplant operation, the cultured epithelium must be able to withstand the physical forces of blinking and eyeball movement, as well as potential blows and straining effects. Patients with OSD (such as Stevens–Johnson syndrome, chemical burns and ocular cicatricial pemphigoid) are often troubled with severe dry eye, abnormal mucin secretion in tears (due to fewer goblet cells) and deformed lids with trichiasis [4, 19, 48].

Equally important in a corneal graft is the provision of a source of stem cells for adequate self-renewal and long-term graft survival, especially in patients presenting total limbal stem cell deficiencies. Precise functioning of the cornea is essential for good vision and relies upon the continual production of new epithelial cells, their appropriate differentiation, and eventual desquamation from the ocular surface. Functional properties need to be maintained through differentiation as cells constantly stream towards the surface. Hence the need for a supply of specialized stem cells [3, 22]. A delicate balance is required between supplying sufficient stem cells for renewal and providing enough terminally differentiated corneal-like cells to maintain essential corneal functions.

Since 1997, many investigators have endeavored to find the ideal substrate for corneal/limbal epithelial cell culture and to refine already existing procedures for ocular surface reconstruction. AM has been used successfully in surgery for many years, and there is no question as to the benefits of its use on the eye. Issues do, however, arise with regard to optimal conditions for use in cell culture. AM can be used fresh or frozen, cryopreservation being the most commonly employed method of preserving AM for use in ocular surface reconstruction. The process involves placing tissue on nitrocellulose paper, in a part-glycerol solution, with subsequent storage in liquid nitrogen fumes at −80°C until surgery. Ice crystals form, causing irreversible damage to the cell organelles; thus, the amniotic epithelial cells are not viable after preservation. Kruse and coworkers, in a study on the effects of cryopreservation on membranes, found that cells removed from preserved AM were not capable of proliferation in culture [24]. They concluded that amniotic membrane grafts seem to function primarily as matrix and not by virtue of transplanted functional cells, and our SEM and TEM findings support this.

Several different culture systems have been developed with the aim of successful reconstruction of severely damaged ocular surfaces. We have developed an explant and cell-suspension culture system of limbal epithelial cells on denuded AM for clinical use. We use denuded AM to promote epithelial growth and adhesion to the amniotic basement membrane and to promote epithelial stratification [15]. We also enhance the epithelial barrier function by air lifting [1]. In addition, we include 3T3 fibroblast feeder layers in our culture system. Our culture method is more focused on developing a stratified and functionally differentiated cellular multilayer that can be securely attached and can survive on the ocular surface after transplantation. We first established this culture system using limbal explants [15, 16, 19] and then moved on to develop cell-

suspension culture of mucosal epithelial cells in order to transfer more stem cells [14, 20, 28, 29].

Conversely, another established culture system for culturing using an AM carrier is explant culture on intact amniotic membrane. This culture system is usually performed without using a 3T3 fibroblast feeder layer and air lifting. It is thought that this culture system is better at preserving the stem cells or limbal epithelial progenitor cells in the cultured epithelial sheet. Workers have demonstrated that such cultivated limbal epithelial cells have slow cycling and label-retaining characteristics and do not express K3 and K12 keratins [7, 27] and connexin 43 [7], all features resembling those found in the stem cell-containing limbal basal epithelium in vitro. Meller and coworkers also showed the ability of intact AM to support ΔNp63 protein expression in human limbal epithelial cells and maintain a higher resistance against phorbor ester-induced differentiation, indicating that characteristic signs of limbal epithelial progenitor cells may by preserved during ex vivo expansion on intact AM [10]. Epithelial sheets cultivated on intact AM were intended to minimize the promotion of the differentiation process and consisted of structurally undifferentiated monolayer or two to four layers with poor formation of desmosomal junctions and hemi-desmosomes. It is intended that they would become stratified after transplantation. The culture system employed by Meller et al. is identical to that used by Tsai et al. in clinical procedures [44].

Our findings in rabbit experiments [16], alongside clinical studies of cultivated limbal epithelial transplantation [14, 18, 19], have illustrated the need for good cellular attachment to the AM extracellular matrix. Without this, we cannot expect early survival of the transplanted graft on the corneal surface. We have previously shown in a rabbit model that limbal cells cultivated on intact AM do not attach well to the amniotic epithelial cells, since they are badly degraded by the cryopreservation process and this obviously has detrimental effects on the integrity of the transplanted epithelium [16]. This is substantiated by the animal experiments reported by Ti and Tseng recently [42]. These investigators described long-term observations of limbal epithelial cells cultivated on intact AM without using 3T3 fibroblast feeder layers and air lifting in rabbits and suggested that insufficient adhesion complexes are formed on the intact substrate, resulting in susceptibility to such trauma as exposure, blinking and explant removal at the time of transplantation.

To the best of our knowledge, previous studies to compare substrates have involved the use of animal models [15, 16]. While the rabbit has some advantages in such experiments, it does not accurately represent the human anatomy and findings cannot be reliably extrapolated to man. Rabbits have a nictitating membrane (third eyelid), which means the corneal surface is not subjected to the same physical forces in blinking. They also have a thinner, more permeable cornea. In this paper, we compared intact and denuded human AM for the cultivation of human limbal epithelial cell suspension in order to accurately ascertain the resulting morphological differences.

Our observations with the SEM showed that there was a very striking difference in morphology between the two culture systems. Limbal cells cultivated on denuded AM were confluent, formed tightly opposed junctions and had very distinct cell borders. In contrast, those cultured on intact AM had less well defined cell boundaries and exhibited larger intercellular spaces. Interestingly, there were large areas of the culture sheet devoid of any cells. This is an indication of the weak attachment of the necrotic AM epithelial cells to the substrate. Limbal cells were often in direct contact with the basement membrane and appeared to be only loosely attached to the underlying epithelial cells—again, most likely as a result of their poor condition.

TEM observations were consistent with those on SEM. We found that limbal cells grown on denuded AM were well stratified and differentiated, very similar in appearance to normal corneal epithelium. The basal cells were attached directly to the basement membrane with numerous hemidesmosomal junctions and appeared to be secreting basement membrane material. On the intact AM, we found at most three cell layers. Limbal cells were attached to each other and, interestingly, also to the underlying amniotic epithelial cells via infrequent desmosomal junctions. This has also been observed in rabbit cultures [15]. The amniotic epithelium appeared to have been severely disrupted by the process of cryopreservation, and the cells were clearly dead. The poor structural condition of these necrotic cells no doubt accounts for their weak attachment to the superior limbal epithelial cells; however, we were surprised that junctions were formed at all. Though the complexes were very few in number, the exact mechanism through which desmosomes are able to form between viable and non-viable cells remains a mystery.

It is generally accepted that outgrowth is slower on intact AM than on epithelium-denuded membrane [8, 15]. We speculate that the AM cells directly hinder growth, and impair attachment by forming a barrier between the membrane and the limbal cells. Intact AM used on its own has been shown to be beneficial when spread on bare sclera, in inhibiting conjunctival overgrowth [19]. As such, it seems that intact AM does not preferentially encourage cell growth.

We also examined several components of the basement membrane complex by immunohistochemistry. Cellular/intact AM alone expressed all of the basement membrane-related molecules we examined; laminin 5, integrin-α6 and -β4 and collagen VII. The molecules appeared to be localized at the basement membrane area of the amniotic epithelial cells. Cell nuclei of amniotic epithelial cells were not stained by propidium iodide because, as reported previously [24], they had lost viability after cryopreservation. Following denudation of the amniotic epithelial cells, the expression of laminin 5 was preserved in the residual

basement membrane but integrin-α6, integrin-β4 and collagen VII were not detected. When limbal epithelial cells were cultured on denuded AM, the basement membrane area again stained positive for laminin 5, integrin-α6, integrin-β4 and collagen VII. It seems the basement membrane materials are produced by the stratified limbal epithelial cells; the expression patterns of the components were similar to those of normal corneal tissue. These results are consistent with our TEM findings that limbal epithelial cells made good attachments to the underlying denuded AM via numerous hemi-desmosomes. In contrast, human limbal epithelial cells cultivated on intact AM showed relatively weak expression of integrin-α6 and integrin-β4 but, interestingly, a similar expression of collagen VII. In skin culture, it is reported that epithelial–mesenchymal interactions are necessary for efficient synthesis of collagen VII [21]. In our study, the collagen VII deposition might have been enhanced by the 3T3 fibroblast co-culture. Since we have not studied limbal epithelial cell culture on AM without a 3T3 fibroblast feeder layer, further study is necessary to elucidate the influence of 3T3 fibroblast feeder layer on type VII collagen synthesis in limbal epithelial cell culture. TEM enabled us to quantify some of the morphological differences. Denuded AM produced significantly smaller intercellular spacing and an increase in desmosomal junctions between adjacent limbal cells. Both of these factors help to maintain the integrity of the epithelial layer and are important for visual acuity. Basal attachments were also significantly better in the denuded culture, with more hemi-desmosomal junctions at the basal cell–basement membrane interface than desmosomes joining the limbal cells to the AM epithelium in the intact culture. As such, we would expect limbal cells on denuded AM to be better equipped to survive transplantation in the long term.

Having established superior mechanical strength in the denuded culture, we address the issue of differentiation. As mentioned earlier, there is concern that stripping the membrane of its epithelial cells may cause limbal stem cells to terminally differentiate in culture, hence reducing the life span of the cell sheet once transplanted onto a stem cell-deficient eye [7, 8]. We have not shown the existence of stem cells or progenitor cells in our cultivated limbal epithelial sheets in this study. However, in our series of patients suffering from total stem cell deficiencies, the ocular surface of some patients is covered with clear corneal epithelium over 5 years after allo-cultivated limbal epithelial transplantation. These clinical results suggest the possible survival of limbal stem cells or progenitor cells on the corneal surface after transplantation. In addition, the necrotic AM epithelial cells are in such poor condition that any abilities they may have once had in terms of influencing "stemness" will be short-lived. This is substantiated in a recent paper comparing AM culture systems [9], in which the authors found that just 7 days after xenotransplantation the dead amniotic epithelial cells of the cryopreserved cellular AM, upon which limbal epithelium had been expanded, were no longer discernible.

In conclusion, since our culture system using 3T3 fibroblast and air lifting is different from the previously reported culture system using intact AM without a feeder layer or air lifting we are not able to directly compare the two culture systems. However, when we compare denuded and intact AM in our culture system, which has a 3T3 fibroblast feeder layer and air lifting to promote cellular differentiation, we see a faster outgrowth rate, smaller intercellular spaces and overall better mechanical strength. Thus, we believe that denuded AM is a more practical substrate for human limbal epithelial cell culture not only with explant culture but also when using the cell-suspension culture method.

Acknowledgements This study was supported in part by grants from the Alexander von Humboldt Foundation (Bonn, Germany), the EPSRC (UK), the Wellcome Trust (UK), the TFC Frost Trust (UK), the Japanese Ministry of Health and Welfare and the Japanese Ministry of Education (12557149: Tokyo, Japan).

References

1. Ban Y, Cooper LJ, Fullwood NJ, Nakamura T, Tsuzuki M, Koizumi N, Dota A, Mochida C, Kinoshita S (2003) 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. Exp Eye Res 76:735–743
2. Cotsarelis G, Cheng SZ, Dong G, Sun TT, Lavker RM (1989) Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. Cell 57:201–209
3. Dua HS, Azuara-Blanco A (2000) Limbal stem cells of the corneal epithelium. Surv Ophthalmol 44:415–425
4. Dua HS, Saini JS, Azuara-Blanco A, Gupta P (2000) Limbal stem cell deficiency: concept, aetiology, clinical presentation, diagnosis and management. Indian J Ophthalmol 48:83–92
5. Endo K, Nakamura T, Kawasaki S, Kinoshita S (2004) Human amniotic membrane, like corneal epithelial basement membrane, manifests the alpha5 chain of type IV collagen. Invest Ophthalmol Vis Sci 45:1771–1774
6. Fukuda K, Chikama T, Nakamura M, Nishida T (1999) Differential distribution of subchains of the basement membrane components type IV collagen and laminin among the amniotic membrane, cornea, and conjunctiva. Cornea 18:73–79

7. Grueterich M, Espana E, Tseng SC (2002) Connexin 43 expression and proliferation of human limbal epithelium on intact and denuded amniotic membrane. Invest Ophthalmol Vis Sci 43:63–71
8. Grueterich M, Tseng SC (2002) Human limbal progenitor cells expanded on intact amniotic membrane ex vivo. Arch Ophthalmol 120:783–790
9. Grueterich M, Espana EM, Tseng SC (2003) Modulation of keratin and connexin expression in limbal epithelium expanded on denuded amniotic membrane with and without a 3T3 fibroblast feeder layer. Invest Ophthalmol Vis Sci 44:4230–4236
10. Hernandez Galindo EE, Theiss C, Steuhl KP, Meller D (2003) Expression of Delta Np63 in response to phorbol ester in human limbal epithelial cells expanded on intact human amniotic membrane. Invest Ophthalmol Vis Sci 44:2959–65
11. Holland EJ, Schwartz GS (1996) The evolution of epithelial transplantation for severe ocular surface disease and a proposed classification system. Cornea 15:549–56
12. Kim JC, Tseng SC (1995) Transplantation of preserved human amniotic membrane for surface reconstruction in severely damaged rabbit corneas. Cornea 14:473–484
13. Kim JS, Kim JC, Na BK, Jeong JM, Song CY (2000) Amniotic membrane patching promotes healing and inhibits proteinase activity on wound healing following acute corneal alkali burn. Exp Eye Res 70:329–337
14. Kinoshita S, Koizumi N, Nakamura T (2004) Transplantable cultivated mucosal epithelial sheet for ocular surface reconstruction. Exp Eye Res 78: 483–491
15. Koizumi N, Fullwood NJ, Bairaktaris G, Inatomi T, Kinoshita S, Quantock AJ (2000) Cultivation of corneal epithelial cells on intact and denuded human amniotic membrane. Invest Ophthalmol Vis Sci 41:2506–2513
16. Koizumi N, Inatomi T, Quantock AJ, Fullwood NJ, Dota A, Kinoshita S (2000) Amniotic membrane as a substrate for cultivating limbal corneal epithelial cells for autologous transplantation in rabbits. Cornea 19:65–71
17. Koizumi N, Inatomi T, Sotozono C, Fullwood NJ, Quantock AJ, Kinoshita S (2000) Growth factor mRNA and protein in preserved human amniotic membrane. Curr Eye Res 20:173–7
18. Koizumi N, Inatomi T, Suzuki T, Sotozono C, Kinoshita S (2001) Cultivated corneal epithelial transplantation for ocular surface reconstruction in acute phase of Stevens-Johnson syndrome. Arch Ophthalmol 119:298–300
19. Koizumi N, Inatomi T, Suzuki T, Sotozono C, Kinoshita S (2001) Cultivated corneal epithelial stem cell transplantation in ocular surface disorders. Ophthalmology 108:1569–1574
20. Koizumi N, Cooper LJ, Fullwood NJ, Nakamura T, Inoki K, Tsuzuki M, Kinoshita S (2002) An evaluation of cultivated corneal limbal epithelial cells, using cell-suspension culture. Invest Ophthalmol Vis Sci 43:2114–2121
21. Konig A, Bruckner-Tuderman L (1991) Epithelial-mesenchymal interactions enhance expression of collagen VII in vitro. J Invest Dermatol 96:803–808
22. Kruse FE (1994) Stem cells and corneal epithelial regeneration. Eye 8 (Pt 2): 170–183
23. Kruse FE, Rohrschneider K, Volcker HE (1999) Multilayer amniotic membrane transplantation for reconstruction of deep corneal ulcers. Ophthalmology 106:1504–1510; discussion 1511
24. Kruse FE, Joussen AM, Rohrschneider K, You L, Sinn B, Baumann J, Volcker HE (2000) Cryopreserved human amniotic membrane for ocular surface reconstruction. Graefes Arch Clin Exp Ophthalmol 238:68–75
25. Lee SB, Li DQ, Tan DT, Meller DC, Tseng SC (2000) Suppression of TGF-beta signaling in both normal conjunctival fibroblasts and pterygial body fibroblasts by amniotic membrane. Curr Eye Res 20:325–334
26. Lee SH, Tseng SC (1997) Amniotic membrane transplantation for persistent epithelial defects with ulceration. Am J Ophthalmol 123:303–312
27. Meller D, Pires RT, Tseng SC (2002) Ex vivo preservation and expansion of human limbal epithelial stem cells on amniotic membrane cultures. Br J Ophthalmol 86:463–471
28. Nakamura T, Inatomi T, Sotozono C, Amemiya T, Kanamura N, Kinoshita S (2004) Transplantation of cultivated autologous oral mucosal epithelial cells in patients with severe ocular surface disorders. Br J Ophthalmol 88:1280–1284
29. Nakamura T, Inatomi T, Sotozono C, Koizumi N, Kinoshita S (2004) Successful primary culture and autologous transplantation of corneal limbal epithelial cells from minimal biopsy from unilateral severe ocular surface disease. Acta Ophthalmol Scand 82:468–471
30. Park WC, Tseng SC (2000) Modulation of acute inflammation and keratocyte death by suturing, blood, and amniotic membrane in PRK. Invest Ophthalmol Vis Sci 41:2906–2914
31. Pellegrini G, Traverso CE, Franzi AT, Zingirian M, Cancedda R, De Luca M (1997) Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. Lancet 349:990–993
32. Prabhasawat P, Barton K, Burkett G, Tseng SC (1997) Comparison of conjunctival autografts, amniotic membrane grafts, and primary closure for pterygium excision. Ophthalmology 104:974–985
33. Rheinwald JG, Green H (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell 6:331–343
34. Schermer A, Galvin S, Sun TT (1986) Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. J Cell Biol 103:49–62
35. Schwab IR, Reyes M, Isseroff RR (2000) Successful transplantation of bioengineered tissue replacements in patients with ocular surface disease. Cornea 19:421–426
36. Shapiro MS, Friend J, Thoft RA (1981) Corneal re-epithelialization from the conjunctiva. Invest Ophthalmol Vis Sci 21:135–142
37. Shimazaki J, Yang HY, Tsubota K (1997) Amniotic membrane transplantation for ocular surface reconstruction in patients with chemical and thermal burns. Ophthalmology 104:2068–2076
38. Shimazaki J, Shinozaki N, Tsubota K (1998) Transplantation of amniotic membrane and limbal autograft for patients with recurrent pterygium associated with symblepharon. Br J Ophthalmol 82:235–240
39. Solomon A, Rosenblatt M, Monroy D, Ji Z, Pflugfelder SC, Tseng SC (2001) Suppression of interleukin 1alpha and interleukin 1beta in human limbal epithelial cells cultured on the amniotic membrane stromal matrix. Br J Ophthalmol 85:444–449
40. Thoft RA (1977) Conjunctival transplantation. Arch Ophthalmol 95:1425–1427
41. Thoft RA (1984) Keratoepithelioplasty. Am J Ophthalmol 97:1–6

42. Ti SE, Anderson D, Touhami A, Kim C, Tseng SC (2002) Factors affecting outcome following transplantation of ex vivo expanded limbal epithelium on amniotic membrane for total limbal deficiency in rabbits. Invest Ophthalmol Vis Sci 43:2584–2592
43. Tsai RJ, Tseng SC (1994) Human allograft limbal transplantation for corneal surface reconstruction. Cornea 13:389–400
44. Tsai RJ, Li LM, Chen JK (2000) Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. N Engl J Med 343: 86–93
45. Tseng SC (1996) Regulation and clinical implications of corneal epithelial stem cells. Mol Biol Rep 23:47–58
46. Tseng SC, Prabhasawat P, Barton K, Gray T, Meller D (1998) Amniotic membrane transplantation with or without limbal allografts for corneal surface reconstruction in patients with limbal stem cell deficiency. Arch Ophthalmol 116:431–441
47. Tseng SC, Li DQ, Ma X (1999) Suppression of transforming growth factor-beta isoforms, TGF-beta receptor type II, and myofibroblast differentiation in cultured human corneal and limbal fibroblasts by amniotic membrane matrix. J Cell Physiol 179: 325–35
48. Tsubota K, Satake Y, Ohyama M, Toda I, Takano Y, Ono M, Shinozaki N, Shimazaki J (1996) Surgical reconstruction of the ocular surface in advanced ocular cicatricial pemphigoid and Stevens-Johnson syndrome. Am J Ophthalmol 122:38–52