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A Comparison of Lyophilized Amniotic Membrane with Cryopreserved Amniotic Membrane for the Reconstruction of Rabbit Corneal Epithelium

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Abstract Many researchers have employed cryopreserved amniotic membrane (CAM) in the treatment of a severely damaged cornea, using corneal epithelial cells cultured on an amniotic membrane (AM). In this study, two Teflon rings were made for culturing the cells on the LAM and CAM, and were then used to support the AM, which is referred to in this paper as an Ahn's AM supporter. The primary corneal epithelial cells were obtained from the limbus, using an explantation method. The corneal epithelium could be reconstructed by culturing the third-passage corneal epithelial cells on the AM. A lyophilized amniotic membrane (LAM) has a higher rate of graft take, a longer shelf life, is easier to store, and safer, due to gamma irradiation, than a CAM. The corneal epithelium reconstructed on the LAM and CAM, supported by the two-Teflon rings, was similar to normal corneal epithelium. However, the advantages of the LAM over that of the CAM make the former more useful. The reconstruction model of the corneal epithelium, using AM, is considered as a good *in vitro* model for transplantation of cornel epithelium into patients with a severely damaged cornea.

Keywords: cornea, epithelium, amniotic membrane, teflon ring

INTRODUCTION

Tissue engineering is an interdisciplinary field that makes bioartificial tissues to regenerate damaged tissues, *e.g.* skin [1] or to support metabolic functions, *e.g.* liver [2,3] using cells, biomaterials, and signaling molecules. Especially tissue engineered cornea to treat various corneal diseases has been developed by culturing corneal epithelial stem cells on natural or synthetic biomaterials.

The normal ocular surface is composed of corneal, limbal and conjunctival epithelial cells. The cornea is a transparent, avascular tissue, the structure of which allows it to serve as both a barrier to the outside environment and as an optical pathway [4-9]. The corneal tissue is comprised of five layers; the epithelium, Bowman's layer, stroma, Descemet's membrane and endothelium [10,11]. The corneal epithelium, located in the outermost layer of the eyeball, can be easily and directly damaged by thermal or alkali burns. With severe damage to the limbus, it is the neighboring conjunctival epithelial cells that finish up covering the corneal surface. In this case, injury to the corneal epithelium may result in the loss of sight [4,12,13].

The AM, which is the innermost layer of the placenta, is a thin, elastic and transparent tissue, which protects the fetus from maternal immune attack and inflammation [14,15]. The AM has been used in various medical applications, such as wound dressing [14-16], tissue adhesion barriers [17] and ophthalmologic fields. In addition, the AM is used in the reconstruction of nerve tube [18], tracheal epithelium [19] and mucosal epithelium [20]. Kim and Tseng [21] first demonstrated the various uses of AM in the ophthalmology field. AM patching has been used to treat Stevens-Johnson syndrome [22] and alkali burns [23], and has also been used as a substrate [4,6, 8,24,25] for the transplantation of the corneal epithelium in both experimental reconstruction models and patients with a severely damaged ocular surface.

Many researchers and clinicians have mainly used the CAM to treat severely damaged corneas, with corneal epithelial cells cultured on the amniotic membrane [4-8]. However, the LAM has a higher rate of graft take, a longer shelf life, is easier to store and safer, due to gamma irra-

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diation, than a CAM [26]. The advantage of reconstructing the corneal epithelium on a LAM was presented in an international conference in 2002 [27-31]. Recently, the LAM was confirmed as a suitable substrate for cultivating autologous corneal epithelial cells for ocular surface reconstruction [32].

In this study, the AM was supported by two teflon rings (Ahn's supporter), which enhanced the submerged and air liquid interface culture. Corneal epithelium could be reconstructed by culturing the third-passage of the corneal epithelial cells on the AM. The aim of this study was to compare the effects of the LAM with CAM, using Ahn's supporter, on the reconstruction of rabbit corneal epithelium.

MATERIALS AND METHODS

The study followed tenets of the Declaration of Helsinki.

Primary Culture of Rabbit Corneal Epithelial Cells

The primary rabbit corneal epithelial cells culture was performed on the eyes of New Zealand white male rabbit (2 kg). Biopsy specimens of the anterior rabbit cornea, 2 × 2 mm^2 in size, were excised from limbal areas under anesthesia. The limbal explants were placed epithelial side down on a culture dish. The medium used was Epi-Life (Cascade Biologics, Catalog No.: M-EPIch-5000, USA), supplemented with a human corneal growth supplement (HCGS, Cascade Biologics, Catalog No.: S-009-5, USA). The medium contained 0.2% bovine pituitary extract, 5 mg/L bovine insulin, 0.18 mg/L hydrocortisone, 5 mg/L bovine transferrin and 1 μg/L mouse epidermal growth factor. Normal rabbit corneal epithelial cells were cultured from the limbus. Outgrown corneal epithelial cells were noted, as shown in the lower part of Fig. 1.

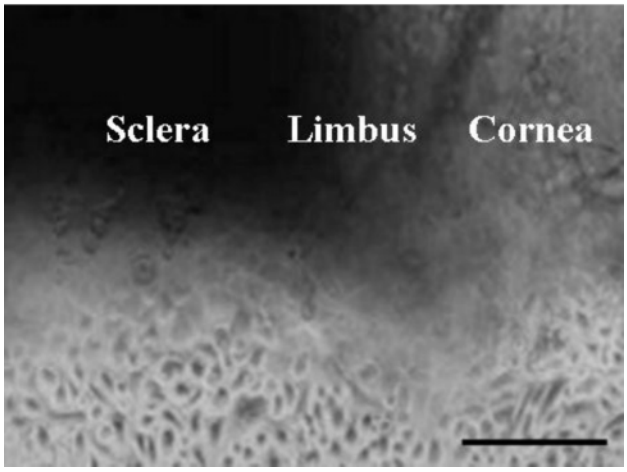

Fig. 1. Primary rabbit corneal epithelial cells from the limbal explantation culture. The upper left part is the sclera, the upper right part the cornea and the lower part the outgrown rabbit corneal epithelial cells. (Bar = 200 μm)

Preparation on amniotic membrane (AM)

The AM was collected from the normal full term placenta, after the completion of spontaneous labor only, from healthy mothers [20]. Under sterile conditions, the AM was separated from the placenta at the time of the cesarean section. The AM was washed 3 times in phosphate buffered saline (PBS, Invitrogen, USA), with 1% Antibiotic-Antimycotic (Invitrogen, USA), and stored using two methods; cryopreservation and lyophilization.

CAM

After washing, the AM was placed on a nitrocellulose membrane (NC, Osmonics, USA) and cut into approximately 3 × 3 cm^2 in sized pieces, and then stored at -80°C in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) and glycerol (Sigma, USA), at a 1:1 (vol/vol) ratio. Prior to culturing, the AM was thawed and washed 3 times with PBS. The AM was then treated with 0.05% trypsin (Sigma, T-4799, USA)/0.02% ethylene diamine tetra acetic acid (EDTA, Invitrogen, Cat. No. 11266-012, USA), at 37°C for 30 mins, in order to loosen the cellular adhesion. This was followed by gentle scraping with a cell scraper (Nalge Nunc, 179693, USA) to remove the amniotic epithelial cells. The AM was washed 3 times with PBS and placed in the tissue-holding two compartment apparatus (Ahn's supporter) under aseptic conditions.

LAM

After first washing with PBS, the AM was treated with 0.05% trypsin/0.02% EDTA at 37°C for 30 min, which was followed by gentle scraping with a cell scraper. The AM was washed 3 times with PBS, and attached to a nitrocellulose membrane. The AM was placed in a deep freezer, at -80°C for 24 h, after which it was lyophilized in a freeze dryer (SFDSM06, Samwon Freezing Engineering Co., Korea), at -80°C for 48 h, to obtain the LAM. An Ahn's supporter was used to support this LAM. The LAM was used in the culture after γ irradiation (25 kGy, Greenpia Technology, Korea).

Ahn's AM Supporter

Two Teflon rings were manufactured in order to culture and transplant the corneal cells on the AM. The Ahn's supporter (Bioland Ltd., Korea) contained two Teflon rings, which were used to support the AM. The inner diameter of the Ahn's supporter was 14 mm. For culturing at the air-liquid interface; the supporter has three legs, 2 mm in height, with the total height of the AM supporter being 9 mm. Fig. 2(a) shows the schematic diagram of Ahn's AM supporter and Fig. 2(b) shows the LAM with the Ahn's supporter, respectively.

Reconstruction of Rabbit Corneal Epithelium on AM

The third passage rabbit corneal epithelial cells were plated

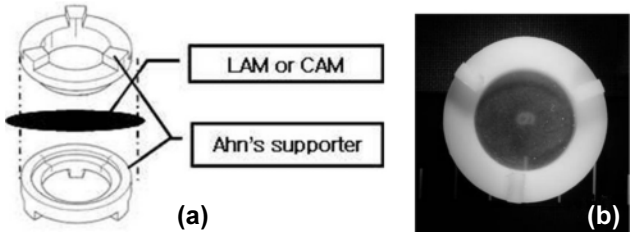

Fig. 2. Ahn's AM supporter. Schematic diagrams of the Ahn's AM supporter (a) and photograph of LAM supported by the Ahn's supporter (b).

onto the epithelial side of the AM of the Ahn's AM supporter, at a density of 5×10^5 cells/cm^2. Two days submerged culture later, the air-liquid interface culture was maintained for 6 days. All the cultures were incubated at 37°C in a 5% CO_2/95% air incubator, with the medium changed every 3 days.

Histological Preparation for Light Microscopy

The reconstructed corneal epithelium was fixed with 10% buffered neutral formalin solution, followed by the standard histological procedures for paraffin embedded tissue. The sections were cut in 4~5 μm slices and stained with hematoxylin (Sigma, Catalog No.: h9627, USA) and eosin (Sigma, Catalog No.: E6003, USA). Immunohistochemical stains were performed as follows: cornea-specific keratins (K3) were used to examine the differentiation of the reconstructed corneal epithelium. The p63, a marker of limbal stem cells, was stained in order to examine the growth ability of the corneal epithelial cells on the AM. The primary antibodies used in this study were monoclonal mouse anti-epithelial keratin-AE5 (1:100, ICN Biomedicals, Inc., USA) and monoclonal mouse anti-p63 (1:50, DAKO, Copenhagen, Denmark) antibodies. The immunohistochemical staining of the paraffin embedded tissue was carried out using a dextran polymer system, Envision+ kit (DAKO, Copenhagen, Denmark). The antigen-antibody reaction was visualized using diaminobenzidine (DAB) as the chromogen, with Mayer's hematoxylin counterstaining.

Ultrastructural Preparation for Electron Microscopy

The surface, and sections of the AM, both with and without the amniotic epithelial cells, as well as a section of the reconstructed corneal epithelium were examined by electron microscopy. After the reconstructed tissue had been fixed with 2.5% glutaraldehyde, it was post-fixed with 1% osmium tetroxide. The samples were dehydrated and double stained with uranyl acetate-lead citrate for the transmission electron microscopy (TEM, JEM-200CX, JEOL, Japan) and coated with gold for the scanning electron microscopy (SEM, JSM-35CF, JEOL, Japan), respectively.

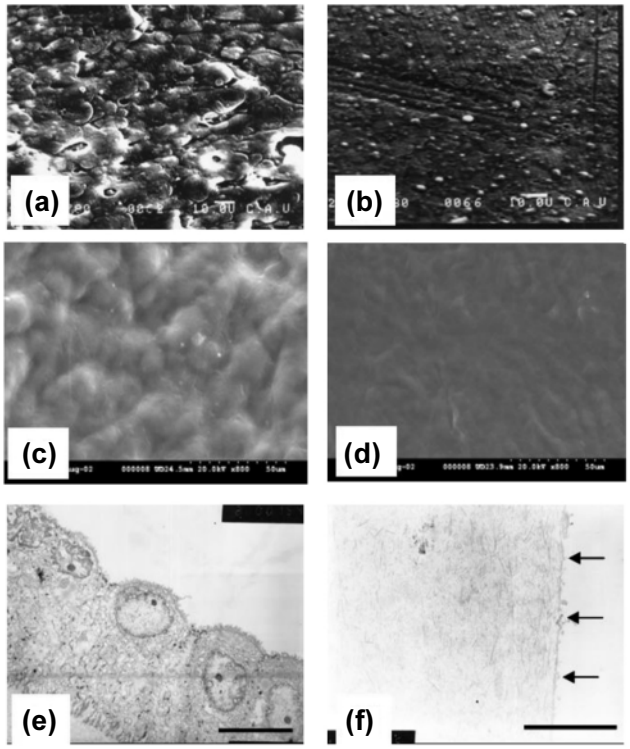

Fig. 3. Preparation of the AM. SEM images of CAM and LAM before (a, c) and after removal of amniotic epithelial cells (b, d). TEM images of AM before ((e), bar = 10 μm) and after ((f), Bar = 5 μm) removal of amniotic epithelial cells.

RESULTS AND DISCUSSION

Preparation of AM

In the SEM images of the AM with the amniotic epithelial cells, irregular surfaces of both the CAM and LAM are shown in Figs. 3(a) and (c), respectively, as the surfaces are lined by amniotic epithelial cells. It was difficult to culture the corneal epithelial cells on the AM due to an epithelial cell barrier and the irregular surface. Practically, there is a slim chance of success in reconstructing corneal epithelium on the LAM with amniotic epithelial cells (data not shown). The SEM images of the AM without amniotic epithelial cells were smoother than those with epithelial cells on both the CAM and LAM. As shown in Figs. 3(b) and (d), the AM without the amniotic epithelial cells was better in the primary cell culture for the attachment and culturing of the corneal epithelial cells. The TEM images of the AM with amniotic epithelial cells show a monolayer of amniotic epithelial cells firmly attached to the basement membrane of the AM, as showed in Fig. 3(e), and without amniotic epithelial cells, as shown in Fig. 3(f). In Fig. 3(f), the area strongly stained in the right part is the basement membrane (arrow) of the AM. It was confirmed that the basement membrane of the AM was well preserved, even though the amniotic epithelial cells had been removed by trypsin treatment.

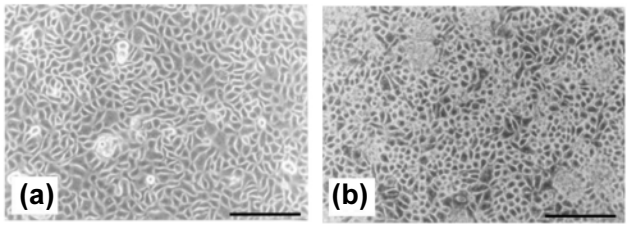

Fig. 4. Photograph of corneal epithelial cells at third passage on plastic dish (a) and the inoculated third passage corneal epithelial cells on LAM (b). (Bar = 200 μm)

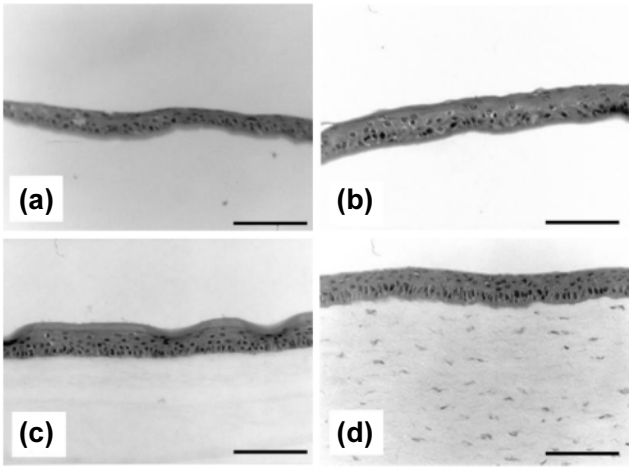

Fig. 5. Histology of the reconstructed bioartificial corneal epithelium on the AM (Hematoxylin & eosin staining). The reconstructed corneal epithelium on the LAM for 3 days (a), the reconstructed corneal epithelium on the LAM (b) and CAM (c) for 6 days, normal rabbit corneal epithelium (d). (Bar = 100 μm)

Culture of Corneal Epithelial Cells on LAM

The optical microscopic images of the third-passage corneal epithelial cells, before their inoculation on the AM, are showed in Fig. 4(a), and the corneal epithelial cells, cultured on the LAM for 2 days before the air liquid interface culture, are showed in Fig. 4(b). The third-passage corneal epithelial cells were confirmed to be well attached and cultured on the LAM.

Reconstruction of Rabbit Corneal Epithelium

The histological images of the well reconstructed corneal epithelium were compared with normal rabbit corneal epithelium (Fig. 5). The reconstructed epithelium of the corneal epithelial cells on the LAM cultured for 3 days had 3~4 layers of corneal epithelium, which was well attached to the LAM (Fig. 5(a)). Those of the corneal epithelial cells on the LAM (Fig. 5(b)) and CAM (Fig. 5(c)) cultured for 6 days had similar properties to those of the normal rabbit corneal epithelium in their cell stratification. The horny layer was more abundant in the reconstructed corneal epithelium on the CAM than on the LAM. The histology of the reconstructed corneal epithelium on the LAM, compared to that on the CAM, was more similar to the normal corneal epithelium. The immunohistochemical stain of the anti-keratin 3 antibodies (AE5), known as a specific differentiation marker of corneal epithelium, showed a positive reaction in the cultured corneal epithelium (Fig. 6). Both superficial cells of the corneal epithelium in an air liquid interface culture for 3 and 6 days showed similar staining reactions to that of normal rabbit corneal epithelium. The immunohistochemical staining of p63, a limbal stem cells marker [8], is shown in Fig. 7. The basal layer of normal limbal epi-

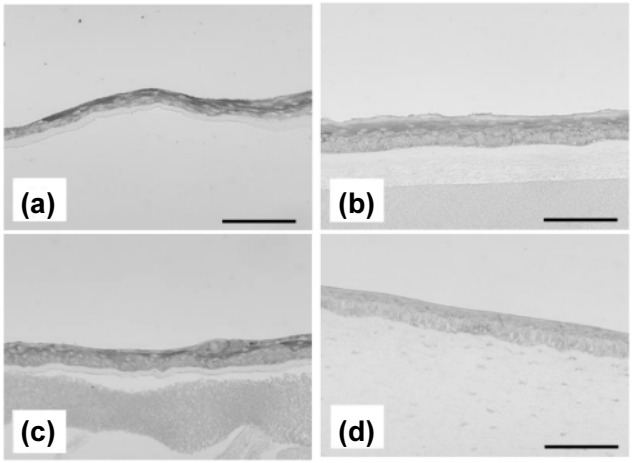

Fig. 6. Immunohistology of the reconstructed bioartificial corneal epithelium on the AM (K3 staining). The reconstructed corneal epithelium on the LAM for 3 days (a), the reconstructed corneal epithelium on the LAM (b) and CAM (c) for 6 days, normal rabbit corneal epithelium (d). (Bar = 100 μm)

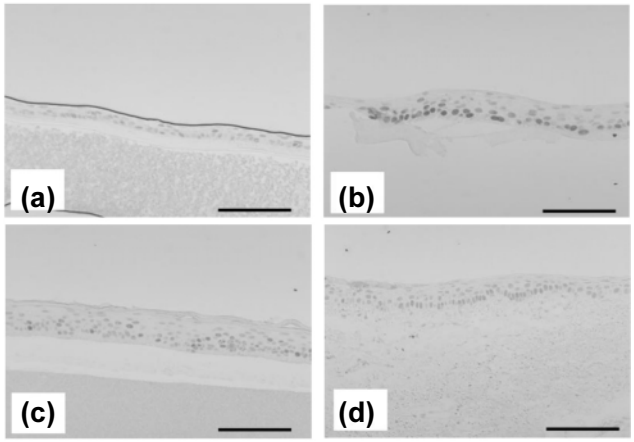

Fig. 7. Immunohistology of the reconstructed bioartificial corneal epithelium on the AM (p63 staining). The reconstructed corneal epithelium on the LAM for 3 days (a), the reconstructed corneal epithelium on the LAM (b) and CAM (c) for 6 days, normal rabbit corneal epithelium (d). (Bar = 100 μm)

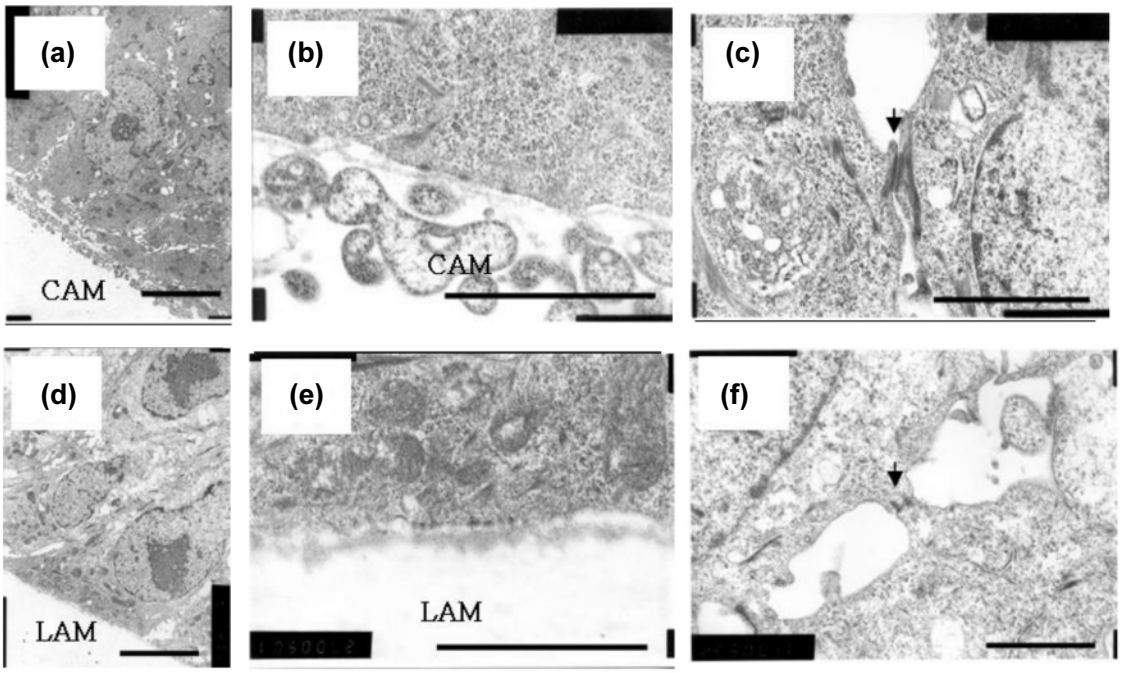

Fig. 8. TEM of the reconstructed bioartificial corneal epithelium on the AM. The basal cells of the reconstructed corneal epithelium on the CAM ((a), Bar = 5 μm) and LAM ((d), Bar = 5 μm). Hemidesmosomal cell-cell contacts (arrow) in the corneal epithelium reconstructed on both the CAM ((b), Bar = 1 μm) and the LAM ((e), Bar = 1 μm), and the desmosomal cell-cell contact (arrow head) in the corneal epithelium reconstructed on both the CAM ((c), Bar = 1 μm) and the LAM ((f), Bar = 1 μm).

thelium has many excellent proliferating cells; the corneal epithelium reconstructed on the LAM and CAM also had excellent proliferating cells, with more in the 6 day cultures (Figs. 7(a) ~ (c)). These results show that the LAM can also serves as a stem cell niche, in a similar or slightly better manner than the CAM [33]. The TEM images of the reconstructed corneal epithelium on the AM are showed in Fig. 8. Both the reconstructions of the corneal epithelium on the CAM (Figs. 8(a) ~ (c)) and the LAM (Figs. 8(d) ~ (f)) were similar to normal corneal epithelium in terms of ultrastructure, cell differentiation and cell junction formation. The basal cells of the reconstructed corneal epithelium on the CAM (Fig. 8(a)) and LAM (Fig. 8(d)) were columnar, which is the specific shape of the basal layer, and the corneal epithelial cells were well attached to both the CAM and LAM. The hemidesmosomal cell-cell contact was restored in the reconstructed corneal epithelium, on both the CAM (Fig. 8(b)) and LAM (Fig. 8(e)), between the corneal epithelial cells and AM, but showed slightly better adhesion and basal cell differentiation on the LAM. The desmosomal cell-cell contact was also restored in the reconstructed corneal epithelium on the AM (Figs. 8(c) and (f)). The above results (Figs. 5~8) suggest that the LAM had slightly better or similar properties to the CAM in terms of the differentiation and stratification of corneal epithelial cells. The corneal epithelia reconstructed on both the LAM and CAM were similar to that of the normal cornea.

This study aimed to reconstruct corneal epithelium on the LAM, which has advantages over the CAM. In order to evaluate the possibility that the LAM could be used as a substratum for the reconstruction of corneal epithelium, the corneal epithelia reconstructed on the LAM and CAM were compared. This culture system is different from that used by other researchers, for the following reasons: firstly, the use of third passage corneal epithelial cells and a serum free medium; secondly, the lack of need for a 3T3 fibroblast feeder layer; and thirdly, the use of an Ahn's AM supporter.

The rabbit corneal epithelium was reconstructed using third-passage corneal epithelial cells and serum free medium. In previous studies, the corneal epithelium of a toxicological test kit was reconstructed using immortalized corneal epithelial cells [34-36], and that for transplantation was reconstructed using normal limbal cells, which were obtained from the limbus and medium added serum [4-8,10]. It has been shown that normal cells became senescent after several passages *in vitro,* regardless of whether they had been maintained with fetal bovine serum. In addition to the restriction of the life span of normal corneal epithelium, the availability of donor corneal material is unpredictable. Therefore, models that use continuous cell lines have been proposed for ocular toxicology studies [34]. It is advisable to use a defined medium in the reconstruction of the corneal epithelium for the toxicological test kit because the serum can influence the results of the toxicological test, as the test material can react with the serum protein. As the reconstructed epithelium was mainly an autologous graft in the transplantation, primary cells obtained from the limbus were

used to reconstruct the corneal epithelium because a large number of cells were not necessary [4,6,8]. In previous study, it has been shown that in the reconstruction of the epidermis, the ultrastructural features of epidermal differentiation as well as the reconstitution of the basement membrane occurred similarly in both the serum-containing medium and the defined medium [37].

In addition, fully differentiated epithelium, with features of the epidermis, has been obtained *in vitro* by culturing second-passage normal human keratinocytes in a chemically defined medium, MCDB 153 [38]. Neurosphere forming cells have been shown to proliferate and maintain their pluripotency in defined media for more than a year [39]. As reported earlier [38,39], corneal epithelial stem cells can proliferate with the pluripotency of stem cells in serum free medium. In addition, the third-passage corneal epithelial cells obtained from the limbus were used to reconstruct the corneal epithelium. The cultured third-passage corneal epithelial cells had the typical shape of normal corneal epithelial cells. Moreover, tenth-passage corneal epithelial cells, which had a typical shape, could also be cultured. As stated earlier, an autologous transplantation of the other tissue and allograft would be possible if the primary cells are corneal epithelial stem cells from the limbus and the tenth-passage or more corneal epithelial cells are cultured with the typical corneal epithelial cell shape. In previous study, it has been shown that the autologous transplantation of cultivated oral epithelium is a feasible method for ocular surface reconstruction [20]. In contrast, if epithelial stem cells are obtained from the small limbal tissue, which contains concentrated stem cells, it is believed that the autologous transplantation of the corneal epithelial cells will be possible in patients with damaged skin or mucosa. In this study, we focused on the availability not of autograft, but allograft reconstructed corneal epithelial tissue using AM. Therefore, it was necessary to expand and subcultured corneal epithelial cells *in vitro* for an allograft that was different from an autograft using only primary cells. A sufficient cell number for this study was obtained after three passages, and it was also possible to reconstruct corneal epithelial tissue using the cells after four or five passages (data not shown). The superficial cells of the reconstructed corneal epithelium on the AM, expressing K3, were similar to those of the normal rabbit corneal epithelium, which was affected by the basement membrane of the AM and the high calcium concentration in the serum-free medium. This suggests that third passage corneal epithelial cells, cultured in serum-free medium, have similar properties to those of normal corneal epithelial cells.

Although the corneal epithelial tissue was reconstructed using serum free media, the problem with the use of the xenogenic protein, BPE, must be solved for its application to toxicological or clinical use. Further studies on the optimization of advanced media will allow for a chemically defined culture media in the near future.

It has been reported that the reconstruction of corneal epithelium on the AM was possible using medium with supplemented serum and a 3T3 feeder layer. It has been shown that a well stratified and differentiated corneal epithelial multilayer could not be obtained without a 3T3 fibroblast feeder layer [5]. Moreover, it has been reported that the function of the fibroblasts was not only to synthesize and degrade the extracellular matrix, but also to regulate the epidermalization, on one hand by remodeling the collagen fibers, and on the other by secreting diffusible factors that promote epidermal growth [40]. However, several disadvantages, such as long culture periods (3~4 weeks), xenotypic gene transfection, and other technical obstacles, have prevented its wider use in a 3T3 fibroblasts culture system [41]. Therefore, the corneal epithelium was reconstructed on the AM without culturing the 3T3 fibroblasts. It is believed that the supplement of serum free medium contained the corresponding diffusible factor secreted by the 3T3 fibroblasts, which had positive effects on the differentiation and stratification of the corneal epithelium. As shown in Figs. 5~8, the corneal epithelium reconstructed on the AM using the serum free media could form in such a way as to reassemble normal rabbit corneal epithelium. The advantages of this system in the reconstruction of corneal epithelium are that it is safe from serum and mitomycin c, without culturing 3T3 fibroblasts.

The Ahn's supporter, composed of two Teflon rings, was manufactured in this study, and is useful for the culturing, differentiation and transplantation of reconstructed tissue. In addition, it can be used as a support AM for the reconstruction of a bioartificial corneal epithelium, lamellar and full thickness cornea. Other groups have used commercially available culture plate inserts (Corning Inc., Corning, NY, USA), whereas this method was to used to suture the AM to a synthetic membrane of culture plate inserts. However, the need for a suture to hold the AM was inconvenient. Another group used a polycarbonate ring to hold the AM for the culturing of tracheal epithelial cells [19]. In choosing a material to support the AM the following was taken into account: the material must have no adverse effect on the cells or AM; the material must be able to be cultured using dynamic methods, as the specific gravity of the material is larger than that of water, and the material must recyclable. The AM was supported by two Teflon rings, and was designed to be tight. The Teflon rings were assembled so that the epithelial side of the AM was in the direction of the lower ring, and have 3 legs to allow for culturing on both sides of the AM, with the interval between the legs allowing for the smooth passage of the medium at the time of the air liquid interface culture. This study examined the possibility that two corneal cells could be cultured, one on either side of the AM, and the reconstructed lamellar and full thickness bioartificial cornea could be grown using the Teflon ring, LAM and collagen scaffold (data not shown).

However, in our culture system, the submersion followed by air-liquid interface culturing, which has already been adopted for the reconstruction of bioartificial skin [42-44], resulted in the problem of keratinization of the corneal epithelium. Keratinization of the reconstructed corneal epithelium occurred on the AM after 6 days. This keratinized epithelium appeared after 4~5 days in the air

liquid interface culture, which was different from the condition of healthy cells. In both the CAM and LAM, the keratinized epithelium appeared in the corneal epithelium reconstructed on both the CAM and LAM at the air liquid interface culture after 6 days. The keratinized epithelium of the corneal epithelium reconstructed on the LAM was removed during the staining process (Fig. 5(b)), but that on the CAM was not removed (Fig. 5(c)). Keratinized epithelium did not appear at the air liquid interface culture after 3 days (Fig. 5(a)). While the direction of nutrition supply in our culture system was vertical across the AM, the nutrition supply of the culture system described by Kinoshita [4] and Tsai [8] was in both the vertical and horizontal directions. Therefore, the latterly described culture system was believed to have a more adequate nutrition supply than ours. Besides, keratinization appeared because the evaporation rate was higher than that of the water supply to this culture system. If keratinization of the corneal epithelium causes problems with transplantation, the transplantation of the corneal epithelium reconstructed with 3~4 layers on the LAM at the air-liquid interface culture for 3 days might be a potential solution.

CONCLUSION

The application of tissue engineering using the AM is a recent innovation. As shown by our results, if there was little difference between the corneal epithelium reconstructed on the CAM compared to that on the LAM, the use of the LAM is recommended due to its many advantages. The Ahn's supporter developed in this study is a useful instrument for tissue reconstruction using the AM, a small intestine membrane and a collagen membrane. The reconstructed corneal epithelium model in this experimental study is expected to be a good *in vitro* model for transplantation in patients with a severely damaged cornea.

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