The entrapment of inflammatory cells may explain the antiinflammatory effects often observed following AM patches. As presented in our previous study, in a clinical situation, infiltrating cells are found throughout the AM stroma and not only at the junction of AM and cornea/sclera.[12] Most of the infiltrating lymphocytes and/or monocytes were TUNEL positive in the AM obtained from clinical samples.[12] Fetal membranes express FasL, by which the fetus is afforded protection against cytolytic actions of lymphocytes from the mother.[29–32] Whether the apoptosis process is an active effect exerted by AM remains to be clarified because there is the possibility that lymphocytes simply underwent a physiologic course of apoptosis while being trapped within the AM. Because none of the histologic samples showed infiltrating cells invading the basement membrane, most of the cells seem to have been sequestered from the ocular surface lying directly beneath the AM.[12] There is also the possibility that the AM also acts as a physical barrier, as with contact lenses, to protect the ocular surface from inflammatory cells in the tear film.

AM has the ability to suppress allo-reactive T cells in vitro,[33] an effect that may be mediated by secretory factors such as $PGE_2$,[34] HLA-G,[35] and FasL.[29–32] Because high-molecular-weight HA is also associated with inhibition of cytokine production,[24] HA in AM stroma may regulate both $T_H1$(IL-2 and IFN-γ) and $T_H2$ (IL-6 and IL-10) types of cytokine production.[33]

In summary, HA was present in high levels in the stroma of AM. Our data demonstrate that HA–CD44 interaction plays an important role in the adhesion of inflammatory cells, including lymphocytes, to AM stroma. Although further studies are required to elucidate the molecular events involved, entrapment of inflammatory cells may explain some of the clinical effects observed in the use of AM in ocular surface reconstruction.

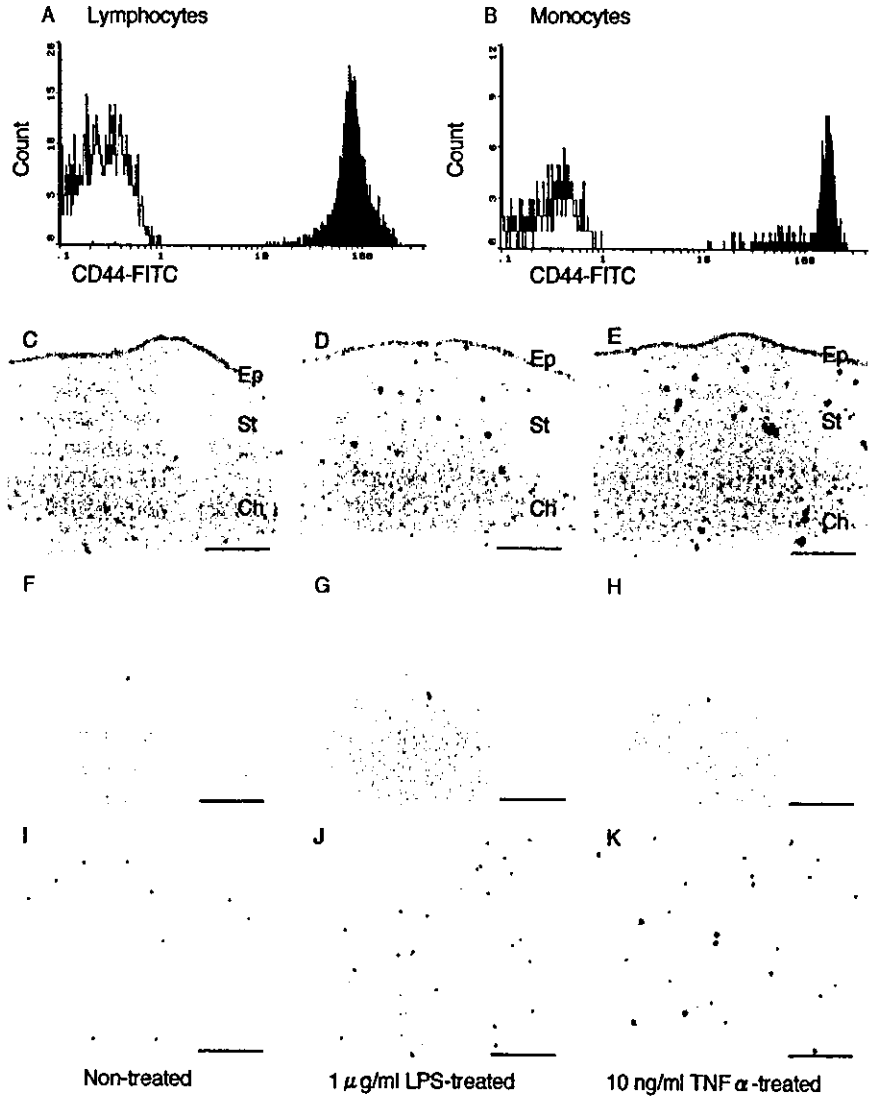


**FIGURE 4.** PBMC expressing CD44 (A, B) were used in an adhesion assay to AM (C, E), immobilized chondroitin sulfate (CS) (F, H), and immobilized HA (J, K). Activation of PBMC by preincubation with 1 μg/mL LPS or 10 ng/mL TNF-α for 72 hours caused increased adhesion in AM and HA-coated slides but not in CS-coated slides. Ep, amniotic epithelium; St, stroma of AM; Ch, chorion. The bar represents 100 μm.

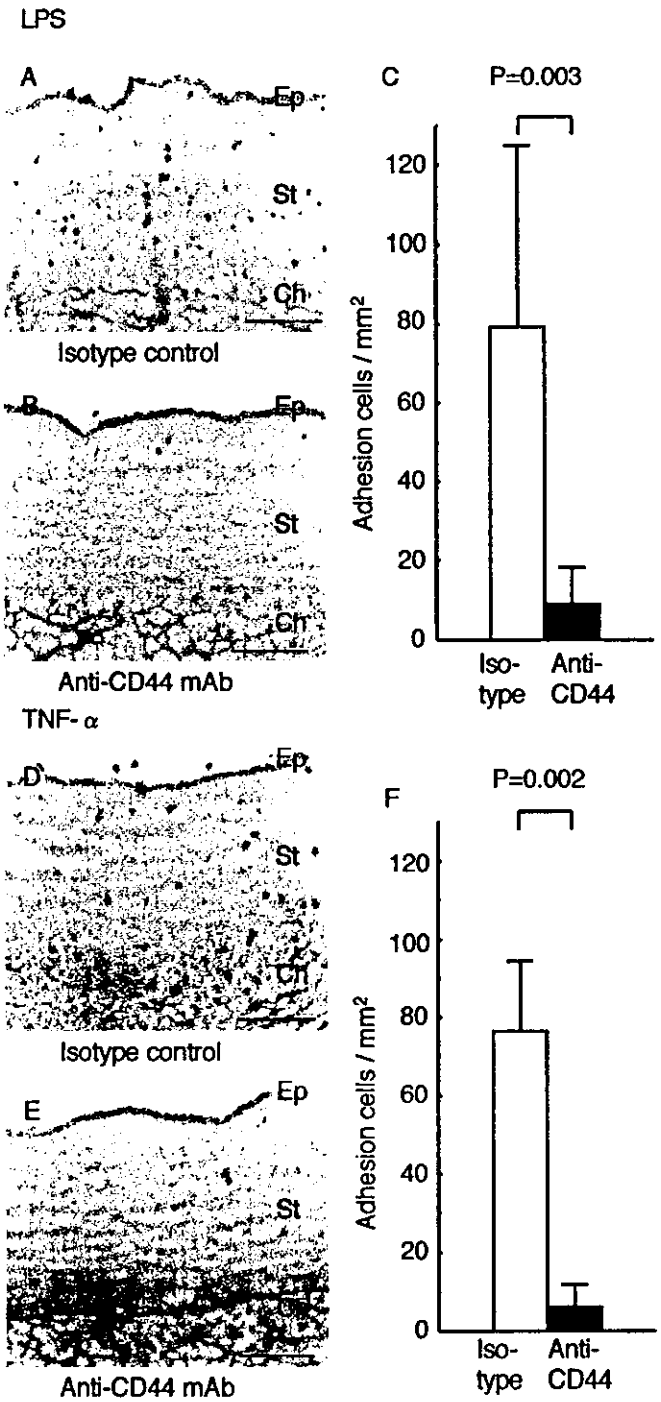


**FIGURE 5.** Adhesion of LPS-treated (A) and TNF-α–treated (D) PBMC to AM was inhibited by the anti-CD44 mAb (B, E). Anti-CD44 mAb significantly inhibited adhesion of PBMC activated by both LPS (C) and TNF-α (F) (n = 10 each). Ep, amniotic epithelium; St, stroma of AM; Ch, chorion. The bar represents 100 μm.

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# Characterization and Distribution of Bone Marrow–Derived Cells in Mouse Cornea

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**Purpose.** Bone marrow (BM)–derived stem cells are thought to possess extensive differentiation capacity. The present study was conducted to investigate the characteristics and distribution of these cells in the normal mouse cornea.

**Methods.** BM cells and BM-derived hematopoietic stem/progenitor cells (HSCs) from enhanced GFP (eGFP) transgenic mice ($lin^-$, Sca-$1^+$) were intravenously transplanted into irradiated wild-type C57BL/6 mice. At 4 to 6 months after transplantation, the mice were killed, and their whole corneas examined by histologic and immunohistochemical methods (CD11c, CD11b, and CD45).

**Results.** At 2 weeks after BM cell transplantation, $GFP^+$ cells gradually migrated into the cornea from the limbal area. At 2 to 6 months, they were distributed over the entire cornea. In cross sections of whole cornea, $GFP^+$ cells comprised 27.3% ± 11.1% (BM) and 24.0% ± 8.01% (HSC) of total cells in the peripheral corneal stroma. In the center of the corneal stroma, $GFP^+$ cells were 7.58% ± 2.63% (BM) and 8.06% ± 1.76% (HSC) of total cells. Immunohistochemistry showed that $GFP^+$ $CD11c^+$, $CD11b^+$, $CD11c^-$, and $CD11b^-$ cells occupied the entire corneal stroma.

**Conclusions.** The present study provides direct evidence of the distribution of BM-derived cells in the mouse cornea. Immunohistochemical study showed that some of these cells are BM-derived antigen-presenting cells such as dendritic cells and macrophages. Some elements of BM-derived cells may continue to exist in the corneal stroma. (*Invest Ophthalmol Vis Sci.* 2005;46:497–503) DOI:10.1167/iovs.04-1154

Adult somatic stem cells have been isolated from several tissue sources including neurons,[1,2] retina,[3] corneal limbal epithelium,[4,5] and bone marrow (BM).[6–8] It had been thought that somatic stem cells preferentially generate differentiated cells of the same lineage as their tissue of origin. However, recent studies suggest that tissue-specific stem cells can differentiate into lineages other than their tissue of origin and that, with respect to the developmental potential of different adult cell types, there is far more plasticity than previously thought. Particular attention has been focused on the plasticity of BM-derived stem cells. They are reported to possess extensive differentiation capacities and can differentiate into several epithelial types such as liver, lung, and skin.[9] Furthermore, BM-derived mesenchymal stem cells can differentiate in vitro not only into mesenchymal cells, but also into cells with visceral mesoderm, neuroectoderm, and endoderm characteristics.[10] These findings suggest that BM-derived stem cells may have the ability to transdifferentiate into a variety of tissues, including those of the eye.

Normal corneal tissue is located in the anterior segment of the eye, and it participates in several major functions. It is the gateway into the eye of visual images and plays a critical role in maintaining corneal transparency and avascularity. It is composed of three layers: the corneal epithelium, stroma, and endothelium. Corneal epithelial stem cells exist in the basal cell layer of the limbal region[4,5] and in the transitional zone between the cornea and conjunctiva. They are supported by the limbal vascular arcade. Little is known about stem cells of the corneal stroma and endothelium, and the origin of these cells is not well understood.

From an immunologic point of view, the normal avascular cornea was thought to be an immune-privileged site without functional antigen-presenting cells (APCs) and largely devoid of BM-derived cells. Therefore, higher success rates would be expected with corneal than other organ transplants. This notion has lost favor since the demonstration of large numbers of resident BM-derived cells of different lineages—for example, macrophages and dendritic cells—in both the epithelium and stroma of the normal cornea.[11–13] Until now, indirect evidence obtained by immunohistochemical studies has shown these cells to be present and important questions, such as the original cell type and the physiological and functional significance of these progenitors, remain unanswered.

We are the first to attempt the characterization and clarification of the distribution of BM-derived cells in the normal mouse cornea. In the current study, we sought to acquire a direct demonstration by transplanting BM cells from enhanced green fluorescence protein (eGFP) transgenic mice using our unique protocol.[11–16] We transplanted GFP-labeled BM cells and hematopoietic stem/progenitor cells (HSCs) into syngeneic C57BL/6 (wild-type) mice and found BM-derived cells distributed in the mouse cornea. We then evaluated the characteristics of these BM-derived cells by immunohistochemical studies.

## Materials and Methods

### Experimental Animals

The mice were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The experimental

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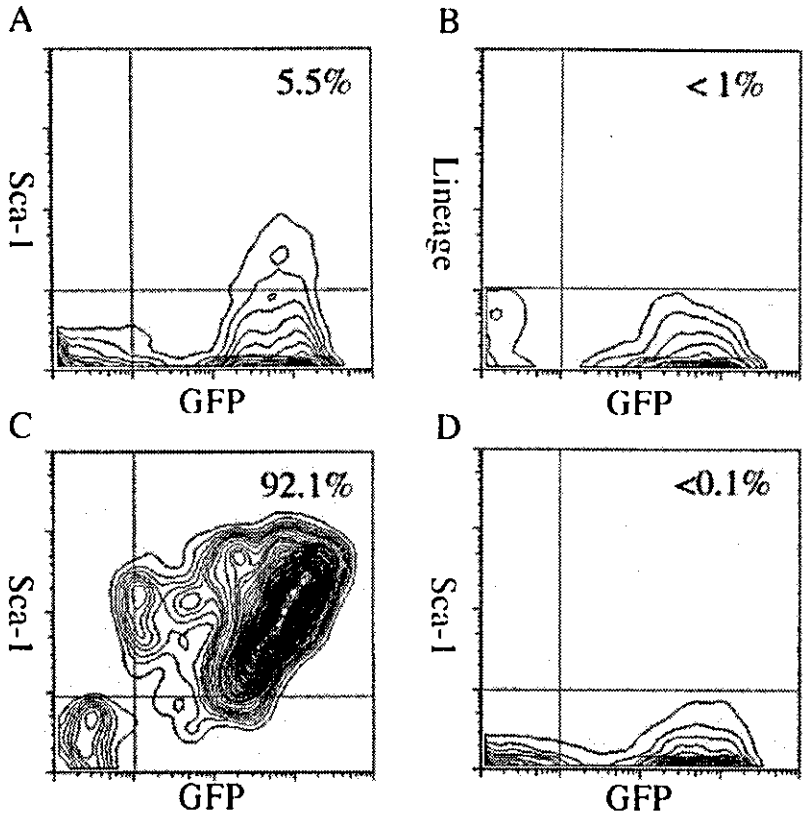


FIGURE 1. Enrichment of hematopoietic stem cells confirmed by flow cytometry. Original BM cells (A). After lineage depletion (B). Positive (C) and negative (D) selection for Sca-1$^+$ cells. After negative and positive selection, the lin$^-$ Sca-1$^+$ cell purity of all GFP$^+$ cells exceeded 95%. Each percentage represents the amount of double-positive cells among all nucleated cells.

procedures were approved by the Committee for Animal Research of Kyoto Prefectural University of Medicine and Kyushu University. Adult ($n$ = 3) and newborn ($n$ = 6) C57BL/6 mice were the recipients of BM cell and HSC transplants, respectively. BM cells were obtained from mice that transgenically express GFP, driven by the chicken $\beta$-actin promoter.[17]

## Bone Marrow Transplantation

To observe directly the migration of BM-derived cells into the mouse cornea, we used BM cell transplantation.[16] Female eGFP mice (8–10 weeks old) were killed by cervical dislocation while under deep ether anesthesia, and BM cells were obtained by flushing the femurs with sterile phosphate-buffered saline (PBS). The BM cells were washed several times in sterile PBS, filtered twice through a nylon mesh (pore size, 70 $\mu$m), counted, and resuspended in PBS at $5 \times 10^7$ cells/mL. To generate chimeric mice, all BM cells ($6 \times 10^6$ to $1 \times 10^7$) derived from eGFP transgenic mice were intravenously injected into 8-week-old C57BL/6 recipients that had been lethally x-irradiated with 9 Gy. Their eyes were protected with lead shields to prevent radiation retinopathy. These BM cell transplant recipients were then maintained under special pathogen-free conditions, and successful BM cell transplantation was confirmed by the identification of GFP$^-$ cells in peripheral blood at 2 weeks after transplantation. The corneas of three mice were carefully studied by fluorescence biomicroscopy until 6 months after transplantation. We also used these corneas for histologic and immunohistochemical studies.

## Hematopoietic Stem Cell Transplantation

To characterize BM-derived stem/progenitor cells in the mouse cornea, we performed HSC transplantation.[14,15] BM cells were harvested from femurs and tibias of 8- to 12-week-old eGFP mice. Single-cell suspensions of donor cells were prepared by repeated serial passage through a 23-gauge needle. To deplete mature hematopoietic cells, the BM cells were incubated with lineage-specific antibodies (B220, CD3, Gr-1, Mac-1, and TER 119) for 30 minutes at 4°C. After washing with PBS containing 2% fetal bovine serum, the cells were incubated with sheep anti-rat immunomagnetic beads (Dynabeads M-450 coupled to sheep anti-rat IgG; Dynal, Great Neck, NY). Cells not bound to the immunobeads were further purified for Sca-1$^+$ cells. The purity of lineage$^-$ cells was higher than 92% in all experiments. After negative selection of mature hematopoietic and immune cells, positive selection of Sca-1$^+$ cells was performed as just described. After negative and positive selection, the purity of lin$^-$ Sca-1$^+$ cells of all the eGFP$^+$ cells exceeded 95% (Fig. 1).[14,15] To obtain high cell purity, samples were applied twice to columns in each experiment. The resultant $10^4$ lin$^-$ Sca-1$^+$ cells were transplanted into C57BL/6 mice within 2 days of their birth. The HSC transplant recipients were maintained under special pathogen-free conditions for 4 weeks. Successful HSC transplantation was confirmed by the identification of GFP$^-$ cells in the peripheral blood at 4 weeks after transplantation. At 4 to 5 months after HSC transplantation, six mice were used for histologic and immunohistochemical studies.

## Antibodies

The primary antibodies (all from BD-PharMingen, San Diego, CA) used in this study were purified hamster anti-mouse CD11c (clone HL3), purified rat anti-mouse CD45 (clone 30-F11), and RPE-conjugated rat anti-mouse CD11b (clone M1/70). Secondary antibodies were Cy3-conjugated goat anti-hamster IgG and Cy3-conjugated donkey anti-rat IgG (Vector Laboratories, Inc., Burlingame, CA).

## Immunohistochemistry

Immunohistochemical studies of markers for APCs were performed by using a previously reported method[11–13] and a modified version of our method.[18,19] Briefly, freshly excised corneas were fixed for 60 minutes at 4°C in 4% paraformaldehyde, extensively washed with PBS, fast frozen in liquid nitrogen, and embedded in optimal cutting temperature (OCT) compound (Tissue-Tek II; Miles Laboratories, Elkhart, IN).

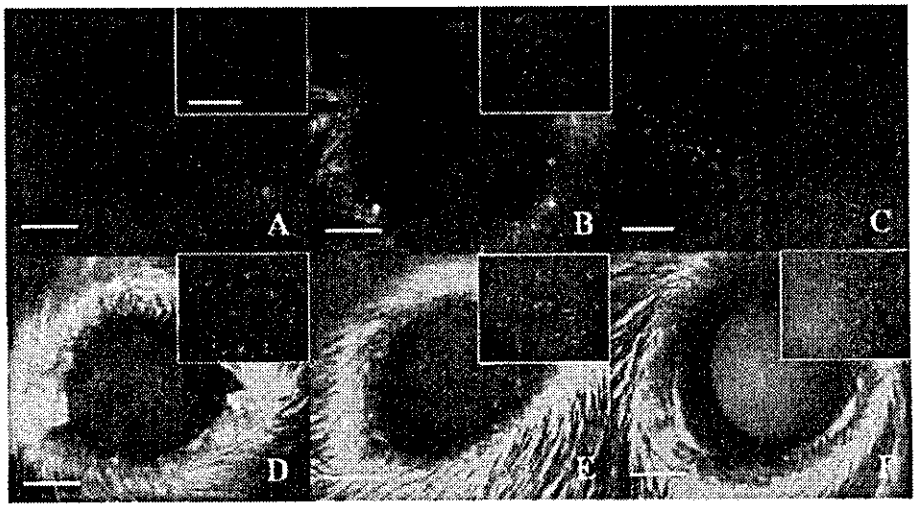


FIGURE 2. Representative time-course slit lamp photographs of murine eyes after BM cell transplantation. (A) One week, (B) 2 weeks, and (C) a high magnification of (B) at the limbal area; (D) 1, (E) 2, and (F) 6 months after transplantation. *Boxes* contain data from the center of the cornea. In the early stages (within the first week) after BM cell transplantation, we observed no $GFP^+$ cells in the recipient mouse cornea (A). Within 2 weeks, there was intense staining for $GFP^+$ cells in the periphery of the cornea (B, C). Within 2 months, $GFP^+$ cells appeared to migrate into the center of the cornea. Their numbers increased in both the periphery and center of the cornea (D, E). Starting at 2 months after BM transplantation, cell density reached a plateau that persisted up to 6 months (F). Scale bars: (A, B, D–F) 1 mm; (C and *insets*) 250 μm.

Cryostat sections (7 μm in thickness) were placed on gelatin-coated slides, air-dried, and rehydrated in PBS at room temperature for 15 minutes. To block nonspecific binding, the tissues were incubated with both anti-Fc receptor mAb (CD16/32; BD PharMingen, San Diego, CA) and 2% bovine serum albumin (BSA) for CD11c and CD11b and with 2% BSA and 10% donkey serum for CD45 at room temperature for 30 minutes. Then the sections were incubated at room temperature for 1 hour with the primary antibody and washed three times in PBS containing 0.15% Triton X-100 (PBST) for 15 minutes. The controls were incubated with the appropriate normal rat and hamster IgG (Dako, Kyoto, Japan) at the same concentration as, but without, the primary antibody. After staining with the primary antibody (CD11c, CD45), the sections were incubated at room temperature for 1 hour with appropriate secondary antibodies, Cy3-conjugated goat anti-hamster IgG, and Cy3-conjugated donkey anti-rat IgG. After several washes with PBS, the sections were coverslipped using antifade mounting medium, with or without propidium iodide (PI; Vectashield; Vector Laboratories) and examined under a confocal microscope (Fluoview; Olympus, Tokyo, Japan).

## Quantitative Evaluation

For statistical assessment of corneal cell distribution and characterization, four different fields and six different sections of each cornea were analyzed (24 areas/eye). For analytical purposes, each cornea was divided into central and peripheral areas. The central area was defined as the area within 1 mm of the center and the peripheral area as that within a 1- to 1.5-mm radial distance from the center.

# RESULTS

## Migration of BM Cells into the Cornea

In the early stages (first week) after BM cell transplantation, there were no $GFP^+$ cells in the recipient mouse cornea (Fig. 2A). Within 2 weeks of transplantation, some $GFP^+$ cells appeared in the periphery of the cornea. However, only a small number of $GFP^+$ cells were present in the center of the cornea (Figs. 2B, 2C). Within 2 months, the number of $GFP^+$ cells in both the periphery and center of the cornea gradually increased. From 2 months after BM cell transplantation, the cell density reached a relative plateau that persisted up to 6 months (Figs. 2D–F). Our quantitative analysis of $GFP^+$ cells in the mouse cornea is summarized in Figure 3.

## Distribution of BM Cells and HSCs

To determine whether there were BM-derived $GFP^+$ cells in the recipient cornea, we performed histologic analysis under a dual-channel fluorescence microscope. Cross-sections of recipient corneas showed that most of the $GFP^+$ cells were distributed in the peripheral corneal stroma and that cell density gradually decreased toward the center (Fig. 4A–D). In the entire corneal epithelium, we noted only a small number of

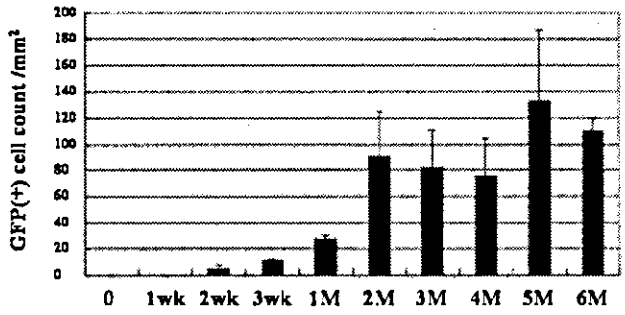


FIGURE 3. Quantitative analysis of $GFP^+$ cells in the mouse cornea at the indicated times after BM cell transplantation. During the first 2 months, the number of $GFP^+$ cells gradually increased. Thereafter, cell density reached a relative plateau that persisted up to 6 months after transplantation.

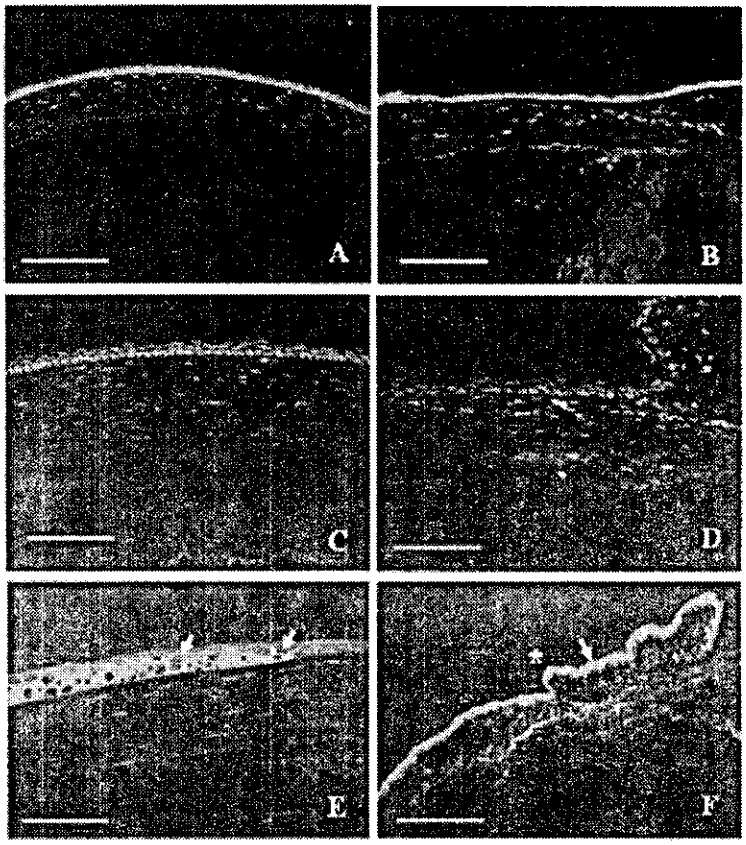


FIGURE 4. Representative cross sections of recipient corneas (A, B, E, BM transplantation; C, D, F, HSC transplantation) show that most of the $GFP^+$ cells were distributed in the peripheral corneal stroma and that the cell density gradually decreases toward the center (A, C, central area; B, D, peripheral area). Only a small number of $GFP^-$ cells were observed throughout the epithelium (E, *arrows*). In contrast, in conjunctival epithelium, several $GFP^-$ cells were noted (F, *arrow*; area to the right of the conjunctiva *). Cell nuclei were stained with PI (*red*). Scale bars: (A, B, F) 200 μm; (C, D, E) 100 μm.

these cells (Fig. 4E), whereas in the conjunctival epithelium we observed several $GFP^+$ cells (Fig. 4F). The percentage of $GFP^+$ cells per section was calculated as the number of $GFP^+$ cells divided by the total number of $PI^+$ cells × 100. In the peripheral cornea of mice receiving BM cell transplants, $GFP^+$ cells were 2.03% ± 1.87% (epithelium) and 27.3% ± 11.1% (stroma). At the center of the cornea, they were 0.93% ± 0.65% (epithelium) and 7.58% ± 2.63% (stroma). By contrast, in the peripheral corneas of mice transplanted with HSC, $GFP^+$ cells were 0.78% ± 0.51% (epithelium) and 24.0% ± 8.01% (stroma). At the center of the cornea, they were 0.58% ± 0.4% (epithelium) and 8.06% ± 1.76% (stroma; Fig. 5). The differences between epithelium and stroma in each category were statistically significant (Mann-Whitney test; $P < 0.01$).

## Immunohistochemical Analysis

To characterize BM-derived $GFP^+$ cells in corneal tissue, primarily the corneal stroma, we used fluorescence immunohistology with antibodies to the leukocyte markers CD11c, CD11b, and CD45. Negative control sections, incubated with normal rat and hamster IgG but without the primary antibody, exhibited no discernible specific immunoreactivity over the entire region.

$CD11c^+$ or $CD11b^+$ indicate cells coexpressing GFP and CD11c or GFP and CD11b, respectively. The percentage of $CD11c^+$ or $CD11b^+$ cells was calculated by dividing the respective number of cells by the total number of $GFP^+$ cells × 100. In the corneal peripheral stroma of BM cell recipients, we observed 19.4% ± 9.93% $CD11c^+$ cells and 38.7% ± 16.3% $CD11b^+$ cells. In the central stroma, 15.3% ± 8.94% were $CD11c^+$ cells and 48.7% ± 13.1% were $CD11b^+$ cells. In the corneal peripheral stroma of HSC recipients, there were 35.7% ± 14.0% $CD11c^+$ cells and 56.7% ± 22.4% $CD11b^+$ cells. In the central stroma, 41.5% ± 17.8% were $CD11c^+$ cells and 53.7% ± 13.9% were $CD11b^+$ cells (Figs. 6, 7, 8). Most $GFP^+$ cells in the cornea were immunostained with CD45 in both BM- and HSC-recipients (Fig. 9). Asterisks in Figure 8 indicate statistically significant difference between $CD11c^+$ and $CD11b^+$ (Mann-Whitney test; $^*P < 0.01$, $^{**}P < 0.05$).

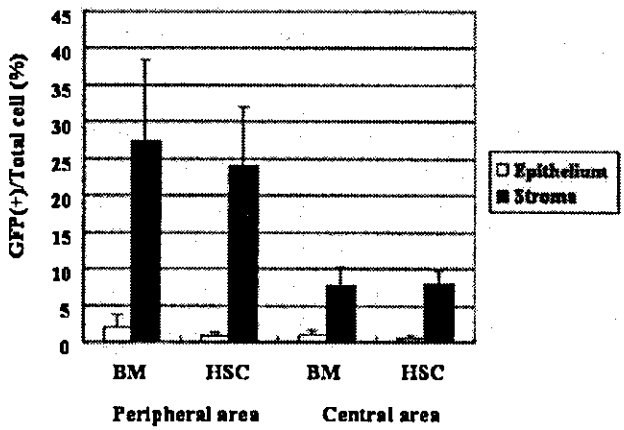


FIGURE 5. Distribution of $GFP^-$ cells in the cornea of mice transplanted with BM cells or HSCs. The percentage of $GFP^-$ cells per section was calculated as the number of $GFP^-$ cells divided by the total number of $PI^+$ cells plus $GFP^-$ cells × 100. Most of the $GFP^+$ cells were distributed in the peripheral corneal stroma. Cell density gradually decreased toward the center. In the entire area covered by epithelium, there were only a few $GFP^-$ cells. The differences between epithelium and stroma in each category were statistically significant (Mann-Whitney test; $P < 0.01$).

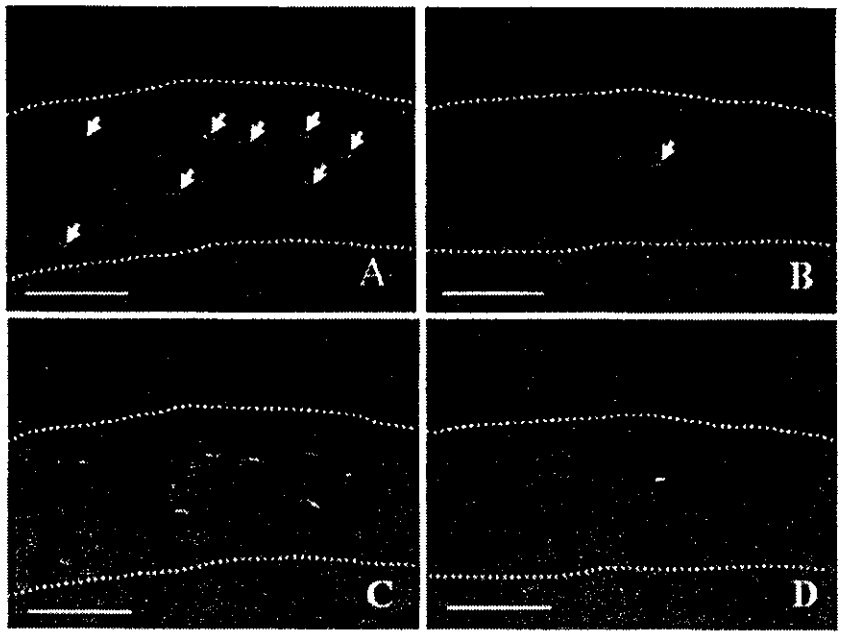


FIGURE 6. Representative immunohistochemical staining for CD11c (*red*) in the peripheral (A, C) and central (B, D) cornea of mice receiving HSC transplants. Some $GFP^+$ cells (*green*) immunostained with CD11c (*arrows*). Note the presence of $GFP^+CD11c^-$ cells throughout the transplant-recipient cornea. GFP (A, B), CD11c (C, D). *Dotted lines*: perimeter of whole corneal tissue. Scale bars, 100 μm.

## DISCUSSION

The cornea is a transparent, avascular tissue, with integrity maintained by various factors derived from the tear film and aqueous humor. Although the normal cornea does not contain vessels, there is indirect immunohistochemical evidence that it is endowed with a significant number of resident BM-derived APCs.[11–13] Hamrah et al.[11] reported that corneal epithelium contains major histocompatibility complex (MHC) class II-negative Langerhans' cells and corneal stroma a large number of resident BM-derived cells of different lineages. These cells were not only macrophages but also $CD11c^+$ dendritic cells. Brissette-Storkus et al.[12] also documented that the normal murine corneal stroma contains a significant number of $CD45^+$ leukocytes and that most of these cells are monocytes or macrophages. However, to date, there has been no direct demonstration of their existence. BM-derived stem cells, such as hematopoietic- and mesenchymal stem cells, have extensive differentiation capacity.[8,9] We considered two possible mechanisms of BM-derived cell differentiation: One is that BM-derived stem cells that have differentiated into APCs such as Langerhans' cells or macrophages migrate into corneal tissue. Alternatively, BM-derived stem cells transdifferentiate into corneal cells such as corneal keratocytes, and function in the cornea. We examined these possibilities using our unique protocol and found that some BM-derived cells were definitely distributed in the cornea. We also determined that these cells are partially of BM-derived APC lineage, a finding that directly confirms the cell origin of

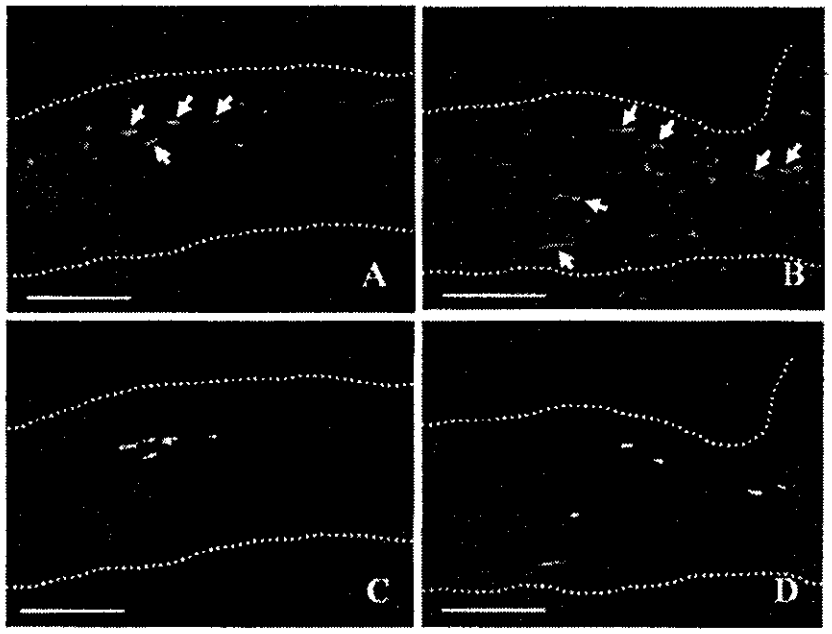


FIGURE 7. Representative immunohistochemical staining for CD11b (*red*) in the cornea of mice receiving BM cell (A, C) or HSC (B, D) transplants. Some $GFP^+$ cells (*green*) immunostained with CD11b (*arrows*). Note the $GFP^+CD11b^-$ cells dispersed throughout the transplant-recipient cornea. GFP (A, B), CD11b (C, D). *Dotted lines*: whole corneal tissue. Scale bars, 100 μm.

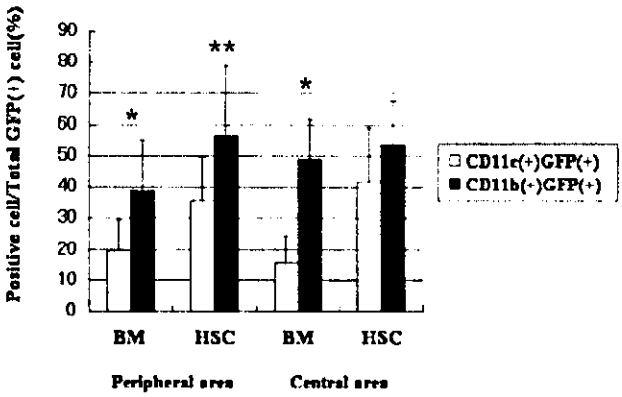


FIGURE 8. Quantitative analysis of the immunohistochemistry for CD11c and CD11b in the cornea of mice receiving transplants of BM cells or HSCs. The percentage of positive cells per section was calculated as the number of cells that coexpressed GFP and CD11c or GFP and CD11b, divided by the total number of $GFP^+$ cells × 100. The number of $CD11c^-$ cells in both the peripheral and central areas of the cornea was greater in mice receiving transplants of HSCs than in those receiving BM cells. Approximately 50% of $GFP^+$ cells were immunostained with CD11b in both BM cell and HSC recipients. There were statistically significant differences between $CD11c^-$ and $CD11b^+$ (Mann-Whitney test; $^*P < 0.01$, $^{**}P < 0.05$).

a significant number of resident dendritic cells in the corneal tissue.

Our observations in corneas receiving GFP-labeled BM cell transplants are of particular interest. Ours is the first report on the time course of the migration of GFP-labeled BM cells into the cornea. Within 2 months after BM cell transplantation, the density of GFP-labeled cells gradually increased; thereafter, cell density was comparatively stable, and finally, at 6 months, it reached a plateau. These findings led us to the interesting hypothesis that BM-derived cells continuously migrate into corneal tissue and contribute to corneal integrity. At present, we do not know the longevity of GFP-labeled BM cells in the mouse cornea. Using other experimental protocols, further cell biological study is needed to clarify this point.

There have been no reports on the distribution of hematopoietic stem/progenitor cells (not bone marrow cells) in the mouse cornea. Although the type of transplantation necessary to obtain these data is very difficult, our group has mastered the technique by using a unique protocol that facilitates our long-term observation of the eyes of transplant-recipient mice.

Our study demonstrates that most of the $GFP^+$ cells were distributed in the corneal stroma: Approximately 25% were found in the periphery and 7% in the center. In contrast, a small number, approximately 1%, were found in the corneal epithelium. The distribution rates of $GFP^+$ cells were similar in mice receiving with BM cells and HSCs. These results suggest that cells migrating into the corneal tissue may be definite populations of BM cells, such as HSCs or undifferentiated progenitor cells.

Based on our immunohistochemical results, we divided $GFP^+$ cells in the corneal tissue into four groups: $GFP^+CD11c^+$, $GFP^+CD11b^+$, $GFP^+CD11c^-$, and $GFP^+CD11b^-$ cells. $GFP^+CD11c^+$ cells (approximately 40% in the HSC transplantation experiment) are thought to express the dendritic cell phenotype[20-22] and $GFP^+CD11b^+$ cells (approximately 55% in HSCs) either the dendritic cell or macrophage phenotype.[23] Using a protocol similar to ours, Espinosa-Heidmann et al.[24] found that BM-derived progenitor cells contributed to experimental choroidal neovascularization. When they used the F4/80 antibody (monocyte marker), they observed $GFP^+F4/80^+$ cells in the limbus, ciliary body, and normal choroid and sclera, suggesting a high turnover and recruitment rate of infiltrated macrophages. Based on their findings and our observations, we postulate that some of the $GFP^+$ cells in the mouse cornea are BM-derived APCs.

Some of the $GFP^+$ cells were negative for cell-surface markers for APCs (CD11c and CD11b), and their origin is unclear. Corneal stroma is composed of both corneal keratocytes and a variety of extracellular matrices comprising collagen subtypes. In our experience, the morphology of $GFP^+$ cells in the corneal stroma and of corneal keratocytes is very similar. If BM-derived stem cells terminally transdifferentiate into corneal keratocytes, they can be expected eventually to lose surface CD45 expression. We posit that our immunologic experiment did not detect immature $Sca\text{-}1^+$ cells in the mouse cornea (data not shown), suggesting that transplanted hematopoietic stem/progenitor cells first homed to BM and engrafted in the recipient mice, and then provided mature BM-derived cells in the cornea. Based on our present results we cannot unequivocally claim that BM-derived $GFP^+$ cells can transdifferentiate into corneal cell phenotypes or neurons. Therefore, morphologic and immunohistochemical studies are under way to examine extracellular matrices and cell-surface markers that are uniquely synthesized by corneal keratocytes.

Several technical and conceptual issues deserve consideration in the interpretation of our results. It is important to note that even in eGFP mice significantly fewer than 100% of the cells express GFP. As this may be due to cell-cycle dependent expression of GFP, we suggest that our results underestimate the potential contribution of BM-derived cells in the mouse cornea. We are currently investigating whether the findings we made with our animal model are applicable to humans. Therefore, we are studying the distribution of BM-derived cells in human corneas.

In conclusion, ours is the first study that presents direct evidence for the migration into the cornea of GFP-labeled BM-derived cells. We provide immunohistochemical evidence that some of the migrating cells were BM-derived cells such as

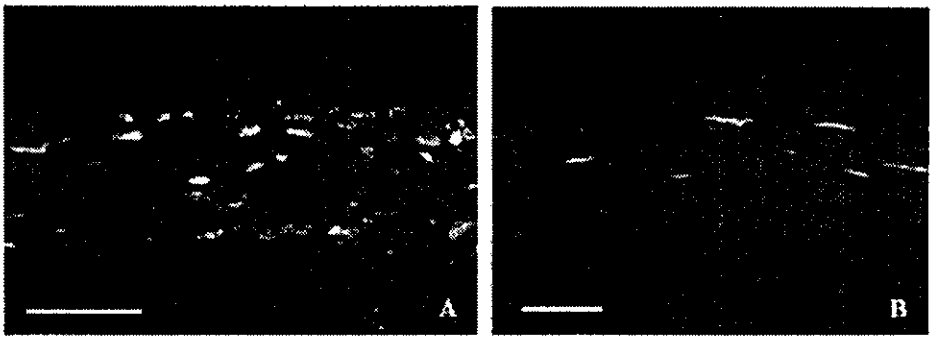


FIGURE 9. Representative immunohistochemical staining for CD45 (*red*) in the cornea of HSC-recipient mice. (A) Peripheral, (B) central retina. Most $GFP^+$ cells were immunostained with CD45 (*yellow*). Scale bars: (A) 100 μm; (B) 50 μm.

dendritic cells and macrophages. Cell biology studies will determine the lineage(s) of the other cells.

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# Sterilized, Freeze-Dried Amniotic Membrane: A Useful Substrate for Ocular Surface Reconstruction

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**Purpose.** To examine the feasibility of using sterilized, freeze-dried amniotic membrane (FD-AM) as a substrate for cultivating autologous corneal epithelial cells for ocular surface reconstruction.

**Methods.** Human AM deprived of amniotic epithelial cells by incubation with EDTA was freeze dried, vacuum packed, and sterilized with γ-irradiation. The resultant FD-AM was characterized for its physical, biological, and morphologic properties by stretch stress tests, immunohistochemistry, electron microscopy, and cell culture. In addition, 3 weeks after an ocular surface injury, the conjunctivalized corneal surfaces of eyes in eight rabbits were surgically reconstructed by transplantation of autologous cultivated corneal epithelial cells on FD-AM.

**Results.** A stretch stress test revealed no significant differences between sterilized FD-AM and cryopreserved AM. Immunohistochemistry for several extracellular matrix molecules and electron microscopic analysis of FD-AM revealed that the process of drying and irradiation did not affect its biological and morphologic properties. The corneal epithelial cells cultivated on FD-AM had four to five stratified, well-differentiated cell layers. Corneas that were grafted with the cultivated corneal epithelial cells on FD-AM were clear and were all epithelialized at 10 days after surgery.

**Conclusions.** The sterilized, freeze-dried AM retained most of the physical, biological, and morphologic characteristics of cryopreserved AM; consequently, it is a useful biomaterial for ocular surface reconstruction. (*Invest Ophthalmol Vis Sci.* 2004;45:93–99) DOI:10.1167/iovs.03-0752

The amniotic membrane (AM), the innermost layer of the placental membrane, has been used as surgical material in a variety of fields.[1–7] In ophthalmic applications, several researchers have reported limited success in the use of AM as a conjunctival graft in symblepharon in a variety of ocular surface disorders.[8,9] In 1995, Kim and Tseng[10] reported the transplantation of preserved human AM for corneal surface reconstruction in a rabbit model. These reports encouraged the use of preserved human AM for ocular surface reconstruction in patients with severe ocular surface diseases.[11–13]

A variety of characteristics make AM ideally suited for use in ocular surface reconstruction. It has an anti-inflammatory effect,[13,14] antifibroblastic activity,[15] antimicrobial[16] and antiangiogenic[17] properties, and very limited immunogenicity.[18–20] In addition, it provides a healthy new substrate suitable for reepithelialization by the corneal epithelium.[21] Recently, particular attention has been focused on the ex vivo expansion of corneal and oral epithelial cells on various substrates, including preserved human AM.[22–25] Our group has developed both cultivated corneal and oral epithelial transplantation using preserved AM as a carrier and has successfully achieved ocular surface reconstruction with this technique.[21–26]

Thus, AM has unique properties that can be helpful in treating a variety of ocular surface diseases; however, some biological and logistic problems remain. First, human AMs are obtained at the time of elective cesarean section and cryopreserved at −80°C under sterile conditions, using our previously reported protocol. However, this procedure does not guarantee a completely sterile AM because of its biological origins. In view of the attention focused on various pathogenic organisms in recent years, proper sterilization of the AM is vital. Second, cryopreservation of AM requires an expensive and bulky −80°C deep freezer. These problems are a barrier to the wider use of AM, particularly in developing countries. Ideally, for clinical use, AM should be sterile and free of contamination. It should also be easily to obtain, transport, and store for long periods without deterioration.

Therefore, in this study we investigated the possibility of producing AM that can be sterilized and preserved at room temperature. To the best of our knowledge, there have been no papers reporting the effectiveness of sterilized, freeze-dried amniotic membrane (FD-AM) for ocular surface reconstruction, although dehydrated AM has recently become commercially available in the United States. We have produced sterilized, FD-AM using our unique protocol and have successfully used this biomaterial as a substrate in ocular surface reconstruction.

## Materials and Methods

### Preparation of FD-AM

Human AM was prepared according to our previously reported standard method.[24–26] With proper informed consent in accordance with the tenets of the Declaration of Helsinki for research involving human subjects and on approval by the Institutional Review Board of Kyoto Prefectural University of Medicine, human AMs were obtained at the time of elective cesarean section in volunteers who were seronegative for human immunodeficiency virus, human hepatitis B and C, and syphilis. Under sterile conditions, the AM was washed with sterile

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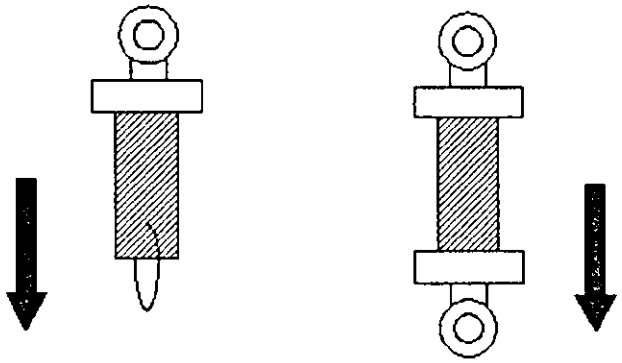

FIGURE 1. Diagrams illustrating the PSS test (*left*), and SS test (*right*). Each sample (*hatched area*) is held with a single clip and a 10-0 nylon suture (PSS) or with two clips (SS). Each sample was pulled vertically until it broke, to determine the maximum tear resistance.

phosphate-buffered saline (PBS) containing antibiotic-antimycotic liquid (penicillin, 10,000 U/mL; streptomycin, 10,000 μg/mL; and amphotericin B, 25 μg/mL), and cut into approximately 4 × 4-cm pieces. The AM was then deprived of amniotic epithelial cells by incubation with 0.02% ethylene diamine tetraacetic acid (EDTA; Nacalai Tesqu Co., Kyoto, Japan) at 37°C for 2 hours. Denuded AM was freeze dried under vacuum conditions and vacuum packed at room temperature as soon as possible. Finally, γ-irradiation (25 kGy) was used to sterilize the resultant FD-AM. Bacteriologic tests were performed on the cultures to confirm sterilization.

## Physical Characteristics of FD-AM

To investigate the physical characteristics of FD-AM, one-point suspension stretch stress (PSS) tests and stretch stress (SS) tests were performed on 10 × 30-mm samples, using our previous protocol.[27] Samples examined were cryopreserved AM, FD-AM with γ-irradiation, and FD-AM without γ-irradiation ($n = 6$). The PSS and SS tests are outlined in Figure 1. In the PSS test, one end of each sample is held with a clip and the other end with a 10-0 nylon suture. In the SS test, each end is held with a clip. Each sample is then pulled vertically with a uniaxial stretching device. Cross-head speed was set at 10 mm/min.

## Immunohistochemistry for Extracellular Matrix Molecules

Immunohistochemical studies of several extracellular matrix molecules in the FD-AM were performed, using our previously described method.[28,29] Cryopreserved AM was also examined for comparison. Briefly, semithin (6 mm) cryostat sections were obtained from unfixed tissue embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Miles, Inc., Elkhart, IN). After fixation with cold acetone for 20 minutes, the sections were incubated with 10% goat serum for 30 minutes. Subsequently, the sections were incubated at room temperature for 1 hour with the primary antibody (Table 1) and then washed three times in PBS containing 0.15% TritonX-100 (PBST) for 15 minutes. The controls consisted of replacing the primary antibody with the appropriate nonspecific normal mouse and rabbit IgG (Dako, Kyoto, Japan) at the same concentration. As an additional control, the primary antibody was omitted. After incubation with the primary antibody, the sections were then incubated at room temperature for 1 hour with appropriate secondary antibodies, Alexa Fluor 488–conjugated anti-mouse and rabbit IgG antibody (Molecular Probes Inc., Eugene, OR). After several washings with PBS, the sections were coverslipped using antifade mounting medium containing propidium iodide (Vectashield; Vector, Burlingame, CA) and examined by confocal microscopy (Fluoview; Olympus, Tokyo, Japan).

## Intracorneal Transplantation of FD-AM

To investigate the compatibility of FD-AM with corneal tissue, we transplanted it into the intracorneal stroma ($n = 4$). This was performed by marking the rabbit cornea 3.0 mm inside the limbus, after which a semilayer incision of the corneal stroma was performed with Vannas scissors. The FD-AM was then inserted into the intrastromal layer. One suture of 10-0 nylon was placed around the corneal wound. After surgery, a topical antibiotic (revofloxacin) was applied three times daily. Corneal transparency and neovascularization were assessed by slit lamp microscopy. The transplanted cornea was stained with hematoxylin and eosin (HE).

## Primary Culture of Rabbit Corneal Epithelial Cells on FD-AM

We cultured rabbit corneal epithelial cells using a previously reported system.[24–26] Briefly, confluent 3T3 fibroblasts were incubated with 4 μg/mL of mitomycin C (MMC) for 2 hours at 37°C under 5% $CO_2$ to inactivate proliferation. They were then rinsed with PBS, trypsinized, and plated onto plastic dishes at a density of $2 \times 10^3$ cells/cm$^2$. The FD-AM was spread, epithelial basement membrane-side up, on the bottom of culture plate inserts (Corning Inc., Corning, NY) placed in dishes containing treated 3T3 fibroblasts. We also used an air-lifting technique to promote epithelial differentiation and epithelial barrier function. Limbal biopsy specimens, each 4 mm$^2$ in size, were taken from eight adult albino rabbits (2–2.5 kg) anesthetized by intramuscular injection of xylazine hydrochloride (5 mg/mL) and ketamine hydrochloride (50 mg/mL). Animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with the experimental procedure approved by the Committee for Animal Research at Kyoto Prefectural University of Medicine. Corneal endothelium and half the corneal stroma were removed with scissors to the extent possible, and the resultant samples were then incubated at 37°C for 1 hour with 1.2 IU dispase. The medium consisted of DMEM and Ham's F12 (1:1 mixture) with 10% fetal bovine serum (FBS), insulin (5 μg/mL), cholera toxin (0.1 nmol/L), human-recombinant epidermal growth factor (10 ng/mL), and penicillin-streptomycin (50 IU/mL). This was centrifuged twice for 5 minutes at 1000 rpm, and the resultant cell pellet was resuspended in culture medium. The suspension of limbal epithelial cells was then seeded onto FD-AM spread on the bottom of culture inserts, and cocultured with MMC-inactivated 3T3 fibroblasts. The culture was submerged in medium for 2 weeks and then exposed to air by lowering the medium level (air-lifting) for 1 week. Cultures were incubated at 37°C in a 5% $CO_2$-95% air incubator for up to 21 days, with the medium changed

TABLE 1. Primary Antibodies and Sources

| Antibodies | Category | Dilution | Source |
|---|---|---|---|
| Collagen 1 | Rabbit polyclonal | ×300 | LSL, Tokyo Japan |
| Collagen 3 | Rabbit polyclonal | ×300 | LSL |
| Collagen 4 | Rabbit polyclonal | ×300 | LSL |
| Collagen 5 | Rabbit polyclonal | ×300 | LSL |
| Collagen 7 | Mouse monoclonal | ×100 | Chemicon, Temecula, CA |
| Fibronectin | Mouse monoclonal | ×100 | Novocastra, Newcastle-upon-Tyne, UK |
| Laminin 5 | Mouse monoclonal | ×100 | Chemicon |

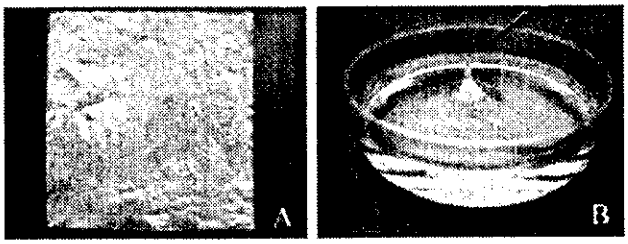

FIGURE 2. The sterilized, freeze-dried amniotic membrane was waferlike, very light and thin (**A**). It became smooth and flexible on hydration, similar to cryopreserved AM (**B**).

every day. We also checked the immunohistochemical staining of cornea-specific keratin-3 and -12 in the cultivated corneal epithelial sheet on FD-AM, using our previously reported method.[28,29]

## Electron Microscopy

Both FD-AM and rabbit corneal epithelial cells cultured on FD-AM were examined by scanning (SEM) and transmission (TEM) microscopy. Specimens were fixed in 4% glutaraldehyde in 0.1 M PBS, washed three times for 15 minutes each in PBS, and postfixed for 2 hours in 2% aqueous osmium tetroxide. They were washed three more times in PBS before being passed through a graded ethanol series. For SEM preparation, specimens were transferred to hexamethyldisilazane (TAAB Laboratories Equipment Ltd., Aldermastron, UK) for 10 minutes and allowed to air dry. When dry, specimens were mounted on aluminum stubs and sputter coated with gold before examination in a digital scanning electron microscope (JSM 5600; JEOL, Tokyo, Japan). For TEM preparation, the specimens were embedded in agar 100-epoxy resin (Agar Scientific, Stansted, UK). Ultrathin (70 nm) sections were collected on copper grids and stained for 1 hour each with uranyl acetate and 1% phosphotungstic acid and then for 20 minutes with Reynolds lead citrate before examination on a transmission electron microscope (JEM 1010; JEOL).

## Autologous Transplantation of Cultivated Corneal Epithelial Cells on FD-AM

To simulate the conditions found in stem cell deficiencies,[21,26] an ocular-surface injury was created in one eye of each of eight adult albino rabbits by excising all the conjunctival tissue within 5 mm of the limbus and performing a superficial keratectomy of the entire corneal surface, including the limbal epithelial cells. Antibiotic eye drops (revofloxacin) and intramuscular gentamicin (1 mg/kg) were administered after surgery. At 3 to 4 weeks after the ocular surface injury, the conjunctivalized ocular surfaces of all eight rabbits were surgically reconstructed by transplanting autologous corneal epithelial cells cultivated on FD-AM. In all cases, the damaged corneal surface, including the 5-mm zone of adjacent conjunctival tissue, was carefully excised in animals under anesthesia. All animals, whose corneal epithelial cells had been placed in culture 3 weeks earlier, received autologous cultivated corneal epithelial cells on FD-AM. The sheets were sutured to the keratectomized corneal surface with 10-0 nylon sutures and covered with a therapeutic soft contact lens. After surgery, topical antibiotics (revofloxacin) and steroids (betamethasone) were applied three times daily. For experimental controls, four eyes received FD-AM only on keratectomized corneas.

# Results

## Appearance and Morphologic Features of FD-AM

The FD-AM used in our study was waferlike, very light and thin (Fig. 2A), easy to handle, and suturable without tearing. It became smooth and flexible on hydration, similar to preserved AM (Fig. 2B). The results of the bacteriology tests performed were all negative.

Examination of the FD-AM by SEM revealed a continuous flat layer of smooth basement membrane (Fig. 3A). The basal lamina was clearly present and intact, forming a continuous flat and generally smooth layer above the fibrous collagen stroma. The AM epithelial cells had been successfully removed. Examination of the FD-AM by TEM also confirmed that the AM was well preserved and there were no cells remaining on the surface. The basal lamina was also clearly visible (Fig. 3B). Figure 3C shows that the stroma of the FD-AM also appeared normal and the collagen fibers making up the AM stroma are well preserved by the freeze-drying technique.

## Physical Strength of FD-AM

The one-point suspension PSS and SS tests were performed on 10 × 30-mm samples to determine the maximum tear resistance of all membranes. Under wet conditions, preserved AM (control) showed an average tearing strength of 14.3 gram-force (gf) (PSS) and 257.7 gf (SS). FD-AM without γ-irradiation showed an average tearing strength of 15.4 gf (PSS) and 286.7 gf (SS). Finally, FD-AM with γ-irradiation showed an average tearing strength of 11.7 gf (PSS) and 221.9 gf (SS) (Figs. 4A, 4B). There were no statistically significant differences in the physical strength between cryopreserved AM, FD-AM without γ-irradiation, and FD-AM with γ-irradiation (*t*-test, $n = 6$).

## Immunohistochemistry of Extracellular Matrix Molecules

Six individual cryopreserved AM and FD-AM samples were examined. The patterns of extracellular matrix molecule expression in the samples were investigated with immunohistochemistry. Negative control sections, incubated with normal mouse and rabbit IgG, and without primary antibody, exhib-

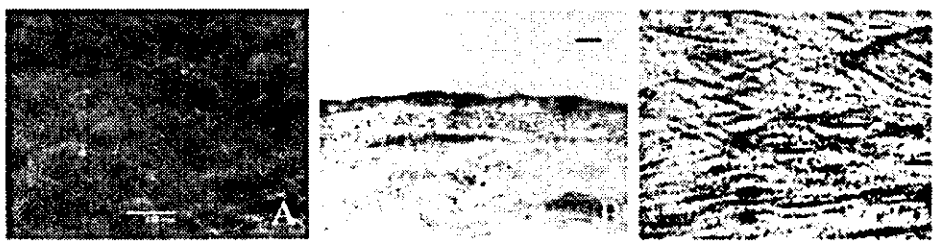

FIGURE 3. Scanning and transmission electron micrographs of the sterilized, freeze-dried amniotic membrane. SEM micrograph showing a continuous flat layer of smooth basement membrane (**A**). TEM micrograph showing that the amniotic membrane was denuded of cells and that apically the basal lamina remained intact (**B**). TEM micrograph showing that the collagen fibrils making up the stromal matrix appeared to be well preserved by the freeze-drying process (**C**). Scale bar: (**A**) 10 μm; (**B**) 200 nm; (**C**) 100 nm.

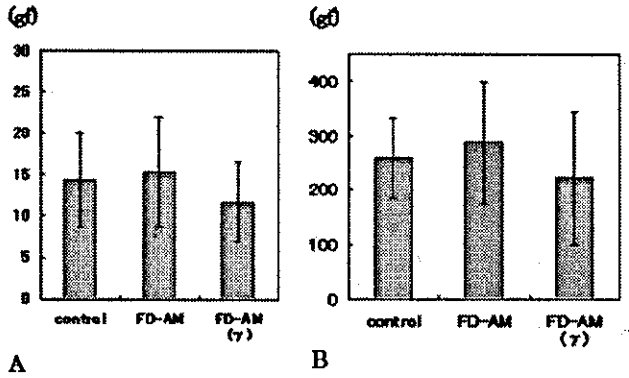


FIGURE 4. The graphs show the results of the PSS test (A) and SS test (B). In both tests, there were no statistically significant differences in physical strength between control freeze-dried amniotic membrane without γ-irradiation and freeze-dried amniotic membrane with γ-irradiation ($n = 6$, $t$-test).

ited no discernible specific immunoreactivity over the entire region. The immunoreactivity observed in each specimen was compared with these controls. Immunohistochemistry showed the presence of collagen (types I, III, IV, and V) and fibronectin throughout the whole FD-AM. In contrast, collagen VII and laminin-5 were expressed on the basement membrane side of FD-AM (Figs. 5A1–A7). As previously reported, these immunoreactivities were similar to those in cryopreserved AM (Figs. 5B1–B7).[30,31]

## Intracorneal Transplantation

One month after intracorneal FD-AM transplantation, we observed the transplanted rabbit corneal surface by slit lamp microscopy. All the transplanted membranes adapted well to the host corneal stroma, with no evidence of subepithelial cell infiltration or stromal edema (Fig. 6). There was no neovascularization on the corneal surface. The FD-AM clarity was also markedly improved (Fig. 6B) and some host keratocytes appeared inside the matrix of the transplanted FD-AM (Fig. 6C).

## Cultivated Corneal Epithelial Sheet

Corneal epithelial cells began to form colonies on the FD-AM within 3 days. After 7 days in culture, a confluent primary culture of corneal epithelial cells had been established that covered the entire FD-AM (Fig. 7A). At 3 weeks, the cultivated corneal epithelial cells showed four to five layers of stratification, were well differentiated, and appeared very similar to normal corneal epithelium (Fig. 7B). These sheets showed immunoreactivity for cornea-specific keratin-3 and -12 (Figs. 7C, 7D).

SEM examination of the cultivated corneal epithelial cells revealed a continuous layer of flat squamous polygonal epithelial cells. These cells appeared healthy and well formed, and the apical surfaces were covered in short, regular microvilli (Fig. 8A). TEM examination of the corneal epithelial culture sheet showed that the cells produced five to six layers of well-stratified epithelium, appeared healthy, and were differentiated into basal columnar cells, suprabasal cuboid wing cells, and flat squamous superficial cells (Fig. 8B). The epithelial cells in the basal cell layers were well attached to the FD-AM substrate with hemidesmosome attachments, and produced basement membrane material (Fig. 8C). In all cell layers the epithelial cells were closely attached to neighboring cells by numerous desmosomal junctions (Fig. 8D).

## Autologous Transplanted Epithelium

At 3 to 4 weeks after the ocular surface injury, conjunctival epithelium completely covered the damaged corneal surface in all eight albino rabbits, with considerable neovascularization and subconjunctival inflammation evident (Figs. 9A1–D1). The extent of injury was similar in all animals. After the conjunctivalized tissue was removed, we then reconstructed the ocular surface with a cultivated corneal epithelial sheet on 12-mm diameter discs of FD-AM (day 0). No signs of infection, bleeding, or sheet detachment were observed. At an early stage (day 2) after transplantation, the eight eyes that had received autologous cultivated corneal epithelial cells on FD-AM all possessed an epithelialized area (Figs. 9A2–D2), most of which was not stained by fluorescein and was separated from the outer rim of healing conjunctiva by an annular epithelial defect, which stained with fluorescein. At 10 days after transplantation, the area covered by the epithelium had expanded outward and was connected with healing conjunctival epithelium in some areas (Figs. 9A3–D3). Moreover, the corneal surfaces of all eyes were clear and smooth, and the entire corneal surfaces were completely covered with transplanted autologous corneal epithelium (Figs. 9A4–D4). The control animals, which had received FD-AM only, showed no evidence of epithelialization at day 10 (Figs. 9E1–E4).

## Discussion

Most ophthalmologists currently use cryopreserved AM under conditions that are as sterile as possible; however, complete sterilization cannot be achieved with present procedures. We believe that complete sterilization of AM is very important and would make for safer and more frequent use of AM. Previously, several methods have been used to preserve AM, including hypothermic storage and freezing. However, these methods require expensive and bulky equipment such as low-temperature freezers. If AM could be preserved at room temperature, it would be extremely convenient, especially for people in developing countries. We attempted to achieve this by preserving AM in the dry state and using γ-irradiation for sterilization.

Our examinations of the physical properties were of particular interest. PSS and SS tests disclosed no significant differences in the mechanical properties of cryopreserved AM, FD-AM without γ-irradiation, and FD-AM with γ-irradiation. Our system for producing FD-AM is unique in several important respects. We subjected the AM to freeze drying under vacuum conditions. Under these conditions, AM can maintain its flexibility and strength. If the AM is dried under ambient conditions, it loses both its smoothness and flexibility and is quite different from cryopreserved AM. Our group previously reported that AM dried under ambient conditions had an average tearing strength of 4.5 gf (PSS) and 48.7 gf (SS), much weaker than the FD-AM reported herein.[27] After freeze-drying the AM, we vacuum-packed it as soon as possible to prevent oxidation. FD-AM kept in ambient conditions invariably became biodegraded. These findings are all consistent with a previous report.[27]

The organization of the extracellular matrix macromolecules plays an important role in the physical and biological properties of AM. We used immunohistochemistry in this study to demonstrate that collagen (types I, III, IV, and V) and fibronectin are expressed in the whole FD-AM, whereas collagen-VIII and laminin-5 were observed in the basement membrane side of FD-AM. These results are similar to those of cryopreserved AM.[30,31] Moreover, our electron microscopic results for FD-AM showed that a continuous flat layer of smooth basement membrane and basal lamina was clearly present and intact, indicating that AM was well preserved by

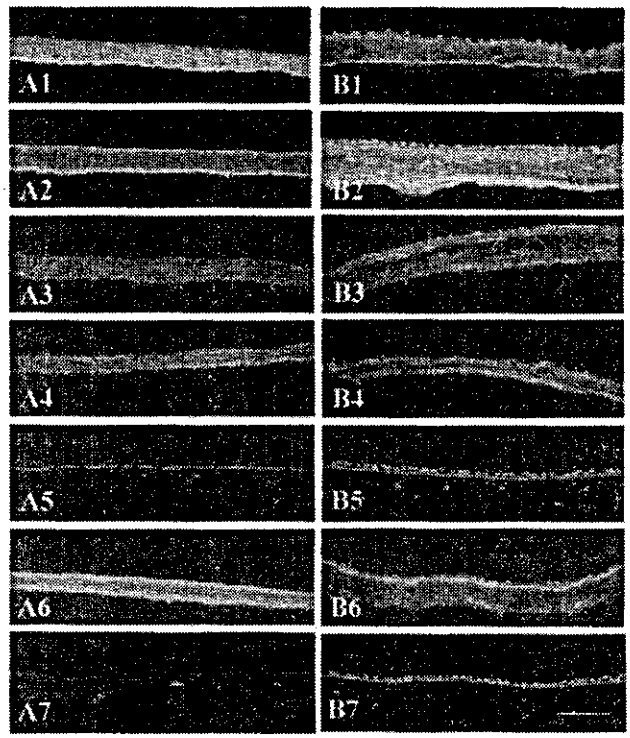


FIGURE 5. Representative immunohistochemical staining of collagen I (A1, B1), collagen III (A2, B2), collagen IV (A3, B3), collagen V (A4, B4), collagen VII (A5, B5), fibronectin (A6, B6), and laminin-5 (A7, B7) in the sterilized, freeze-dried amniotic membrane (A) and cryopreserved intact AM (B). Collagen (types I, III, IV, and V) and fibronectin were expressed in the whole AM. In contrast, collagen VII and laminin-5 were expressed in the basement membrane side of AM. Nuclei were stained with propidium iodide (*red*). Scale bar, 200 μm.

the freeze-drying technique. On the basis of these physical, immunohistochemical, and microscopic examinations, we strongly believe that the process of drying and irradiation does not affect the physical or biological properties of AM.

To use FD-AM as a biomaterial, it is important to examine its biocompatibility. We did this by intracorneal transplantation. All the transplanted FD-AM examined in this study adapted well in the host corneal stroma, with no evidence of subepithelial cell infiltration, stromal edema, or neovascularization. Nor was there evidence of infection or rejection on the corneal surface. These results are consistent with the previous report regarding cryopreserved human AM.[32] From these results, we are confident that the FD-AM we produced has excellent biocompatibility with ocular surface tissues.

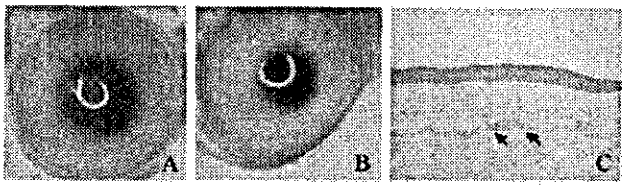


FIGURE 6. Representative slit-lamp photographs of one rabbit taken just after transplantation (A) and 1 month after transplantation (B) of freeze-dried amniotic membrane (FD-AM). A cross-section of the cornea 1 month after FD-AM transplantation (C). One month after intracorneal FD-AM transplantation, there was no evidence of neovascularization or stromal edema on the corneal surface (B) and the clarity of FD-AM was markedly improved. (C) The membrane adapted well to the corneal stroma and some host keratocytes appeared inside the matrix of the transplanted FD-AM (*arrows*). Original magnification: ×200.

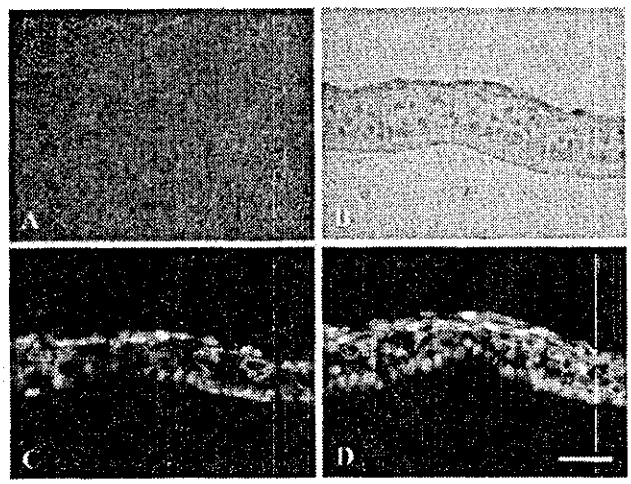


FIGURE 7. Shows a confluent primary culture of corneal epithelial cells on freeze-dried amniotic membrane (A). Light micrograph showing a cross-section of the cultivated corneal epithelial cells on freeze-dried amniotic membrane stained with hematoxylin and eosin (B). The cultivated corneal epithelial sheet had four to five layers of stratified, well-differentiated cells and appeared very similar to in vivo normal corneal epithelium (A, B). Light micrographs showing immunohistochemical staining for keratin-3 (C) and -12 (D). Keratin-3 and -12 were expressed in the superficial and intermediate layers of the cultivated corneal epithelial sheet (C, D). Original magnification, (A) ×100. Scale bar: (B, C, D) 50 μm.

Recently, preserved AM has been widely used as a substrate for cultivating corneal, conjunctival, and oral mucosal epithe-

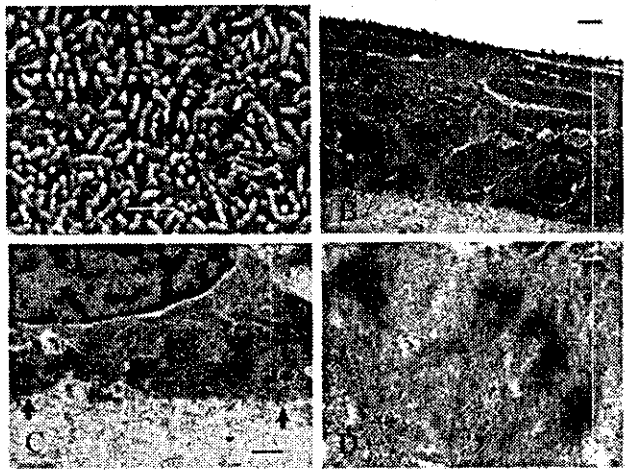


FIGURE 8. Scanning (A) and transmission (B, C, D) electron micrographs of rabbit cultivated corneal epithelial cells on freeze-dried amniotic membrane. The cultivated corneal epithelial sheet appeared healthy and well-formed and the apical surface of the cells was covered in short, regular microvilli (A). TEM examination showed that the cells produced four to five layers of well-stratified epithelium, appeared healthy, and were differentiated into basal columnar cells, suprabasal cuboid wing cells, and flat squamous superficial cells (B). The epithelial cells in the basal cell layers were columnar, well joined to the FD-AM substrate with hemidesmosome attachments (*arrows*), and produced basement membrane material (C, *). In all cell layers, the epithelial cells were closely attached to neighboring cells by numerous desmosomal junctions (D). Scale bar: (A) 1 μm; (B) 2 μm; (C) 500 nm; (D) 100 nm.

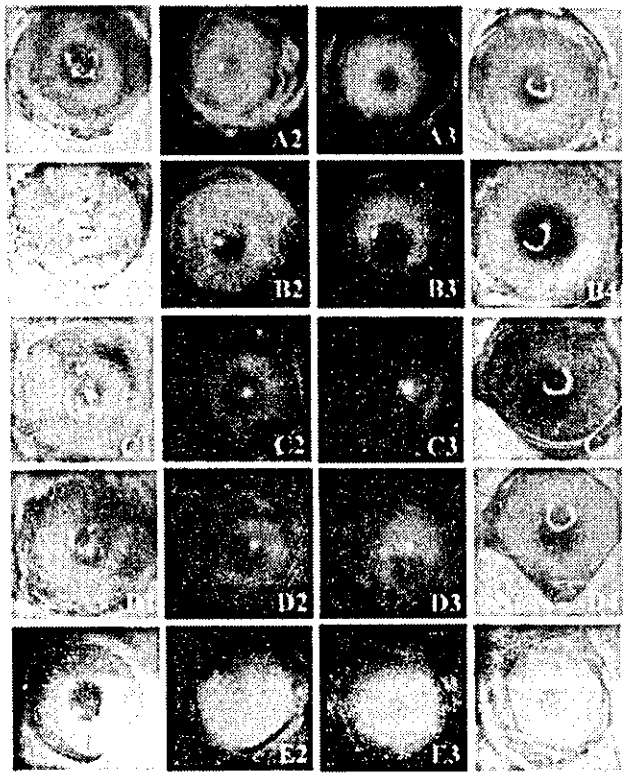


FIGURE 9. Representative slit-lamp photographs of four rabbits taken before transplantation (A1–D1), 48 hours after transplantation with fluorescein (A2–D2), and 10 days after transplantation with (A3–D3) and without (A4–D4) fluorescein. Before transplantation, all cases showed total limbal stem cell destruction (A1–D1). Forty-eight hours after surgery, most of the corneal surfaces were covered with transplanted cultivated corneal epithelial cells and so 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. At 10 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 corneal epithelium (A4–D4). Representative slit-lamp photographs of a control rabbit that received a freeze-dried AM transplant only (E1–E4) onto its keratectomized cornea. The time course of these photographs is the same as detailed above.

lium.[22–26] After successfully producing FD-AM, we tried to cultivate rabbit corneal epithelial cells on it to investigate its effectiveness as a substrate. Fortunately, we could use the experience we gained from cultivating corneal and oral epithelial cells on cryopreserved AM in rabbits and humans.[24–26] We adopted this system for culturing rabbit corneal epithelial cells (with some modifications) and successfully generated confluent cultures of cells on FD-AM. At 3 weeks, the cultivated corneal epithelial cells showed four to five layers of stratification, were well differentiated, and demonstrated immunoreactivity for cornea-specific keratin-3 and -12, indicating that FD-AM supported normal corneal differentiation.

Important points regarding corneal epithelial cell growth on FD-AM include how the basal cells attach to the underlying FD-AM and how the most superficial cells contact the tear-ocular surface interface. We believe these to be key factors in the successful transplantation of cultivated corneal epithelial sheet on FD-AM. Our TEM results showed that cultivated corneal epithelium was firmly attached to the basement membrane with hemidesmosomal junctions. Adjacent cells in the cultivated corneal epithelium were also joined by numerous desmosomal junctions. Examination by SEM also revealed that the apical surface of the cultivated corneal epithelial cells was covered with numerous microvilli, almost identical with those on in vivo corneal epithelial cells. From these results, we believe that corneal epithelial cells cultivated on FD-AM have morphologic properties similar to those of in vivo corneal epithelial cells.

Using FD-AM as a culture substrate, we reconstructed damaged corneal surfaces by transplanting autologous cultivated corneal epithelial cells. Two days after transplantation, most of the corneal surfaces on which cultivated corneal epithelium had been placed were intact without epithelial defects, and were surrounded by a conjunctival epithelial defect at 360°, suggesting no contamination of host conjunctival epithelium. There were no biological immunoresponses to the transplanted FD-AM. Shortly after the transplantation procedure, all conjunctival inflammation in the rabbits rapidly subsided. At 10 days after transplantation, the area covered by the cultivated corneal epithelium had expanded outward and was in contact with healing conjunctival epithelium in some areas. We examined sections of these transplanted corneas using the periodic acid-Schiff reaction and confirmed that there was no contamination of the host conjunctival epithelium (data not shown). From our experiments, we conclude that corneal epithelial cells cultivated on FD-AM can survive and spread onto the adjacent keratectomized corneal surface.

In conclusion, our study is the first to demonstrate the usefulness of sterilized FD-AM for ocular surface reconstruction, on the basis of several experiments evaluating physical, morphologic, and biological properties. We have shown that the sterilized, FD-AM we produced retains the characteristics of cryopreserved AM. On the basis of these results, we are in the process of using this biomaterial for ocular surface reconstruction in patients with severe ocular surface diseases and are carefully evaluating the long-term clinical usefulness of FD-AM.

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# Amniotic Membrane as a Carrier for Cultivated Human Corneal Endothelial Cell Transplantation

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**Purpose.** It would be advantageous if cultivated human corneal endothelial cells (cHCECs) could be transplanted for the treatment of diseases caused by corneal endothelial disorders. To achieve this, a matrix that can serve as a carrier for cHCECs is needed. The present study was conducted to examine the feasibility of using amniotic membrane (AM) as a carrier for this application.

**Methods.** HCECs obtained from peripheral corneal tissue were cultivated, passaged, and transplanted onto denuded AM. The cell density and morphology of the resultant cHCECs on AM were examined by light, scanning electron, and transmission electron microscopy. To determine whether these cHCEC sheets on AM carrier were functional in vivo, the cHCEC sheets on AM were transplanted onto rabbit corneas whose Descemet's membrane and endothelial cells had been completely removed. After transplantation, the corneal appearance was examined by slit lamp biomicroscopy, and corneal thickness was measured daily by pachymetry. At 7 days after surgery, the grafts were examined by light, scanning electron, and transmission electron microscopy.

**Results.** The density of the cHCECs on AM was greater than 3000 cells/mm$^2$. Morphologically, the cHCEC sheets consisted of a fairly continuous layer of flat squamous polygonal endothelial cells that appeared uniform in size with tightly opposed cell junctions in vitro and in vivo after transplantation. The corneas that received transplanted cHCEC sheets had little edema and retained their thinness and transparency.

**Conclusions.** The cell density and morphology of cHCECs on AM were similar to those of normal corneas, and cHCECs on AM were functional in vivo. These results indicate that AM maintains HCEC morphology and function and could serve as a carrier for cHCEC transplantation. (*Invest Ophthalmol Vis Sci.* 2004;45:800–806) DOI:10.1167/iovs.03-0016

Corneal endothelium is essential for the maintenance of normal corneal hydration, thickness, and transparency.[1] Corneal endothelium of human has essentially no regenerative capacity in vivo.[2,3] Serious corneal endothelial cell loss causes irreversible corneal edema. The type of corneal transplantation performed depends on which corneal layers are damaged. Penetrating keratoplasty is generally the treatment of choice for eyes with a damaged endothelial layer such as in Fuchs' endothelial dystrophy and iatrogenic bullous keratopathy. However, there are several concerns with penetrating keratoplasty: rejection; suture problems, which can cause astigmatism and infection; and denervation, which affects corneal function. In addition, in many countries, including Japan, the supply of donor corneas is insufficient. Therefore, the ideal situation would be if only corneal endothelial cells could be transplanted to treat corneal endothelial diseases. Furthermore, it would be highly desirable if small numbers of corneal endothelial cells could be expanded in culture, and these cultivated human corneal endothelial cells (cHCECs) could be transplanted into eyes of many patients.

For cHCEC transplantation in vivo, some type of carrier is obviously necessary. Up to now, for corneal endothelial cell transplantation, gelatin membranes (Schwartz BD, et al. *IOVS* 1980;21:ARVO Abstract 100; McCully JP, et al. *IOVS* 1981;22: ARVO Abstract 230)[4,5] and coated hydrogel lenses[6] have been used as synthetic carriers for these cells. Human amniotic membrane (AM), widely used as a surgical material,[9] has been used successfully as a carrier for cultivated corneal epithelial cell transplantation.[8–10] In this study, we sought to examine whether AM could also serve as a carrier for cHCECs, both in vitro and in vivo.

## Materials and Methods

### Human Corneal Tissue

A donor cornea (47 years old, male, endothelial cell density: 3065 cells/mm$^2$) obtained from Northwest Lions Eye Bank was preserved (Optisol GS; Chiron Vision, Irvine, CA) and transported to our hospital on ice. After using its center for human corneal transplantation the residual limbal tissue was used for this study at day 7 after death.

### HCEC Culture

To cultivate HCECs, corneal limbal tissue was placed in a Petri dish containing Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corp., Carlsbad, CA), 50 U/mL penicillin, and 50 μg/mL streptomycin. Under a dissecting microscope, Descemet's membrane with its attached corneal endothelium 1 mm apart from trabecular meshwork was stripped from the stroma and placed in a 35-mm dish containing 1.2 U/mL dispase in phosphate-buffered saline (PBS). The tissue was incubated for 1 hour at 37°C, and the cells were rinsed gently with a sterile pipette. The dispase was then inactivated by suspending the cells in a medium containing DMEM, 50 U/mL penicillin, and 50 μg/mL streptomycin. After gentle centrifugation (3 minutes at 180*g*), the cells were resuspended in culture medium containing DMEM, 50 U/mL penicillin, 50 μg/mL streptomycin, 10% fetal bovine serum (ICN Biomedicals, Inc., Aurora, OH), and 2 ng/mL basic fibroblast growth factor (Invitrogen Corp.).

The cells were incubated in wells of a collagen IV–coated 24-well plate at 37°C in 5% carbon dioxide-95% humidified air. The medium was changed every other day. Cells reached confluence in 10 to 20

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Investigative Ophthalmology & Visual Science, March 2004, Vol. 45, No. 3

days and were then subcultured by treatment with trypsin-EDTA (Invitrogen Corp.) and seeded at a ratio of 1:2 to 1:8.

## Preparation of AM

In accordance with the tenets of the Declaration of Helsinki and with proper informed consent, human AMs were obtained at the time of cesarean section. The method of removing the amniotic epithelial cells from the AM has been reported.[11] Briefly, human AM was stored at −80°C in DMEM and glycerol (Nacalai Tesque, Kyoto, Japan) after the AM was washed with PBS containing antibiotics (5 mL of 0.3% ofloxacin). Immediately before use, the thawed AM was deprived of amniotic epithelial cells by incubation with 0.02% EDTA (Wako Pure Chemical Industries, Osaka, Japan) at 37°C for 2 hours, followed by gentle cell scraping with a cell scraper (Nalge Nunc International, Naperville, IL). The tissues were then washed twice with sterile PBS. To confirm whether epithelium was completely removed from AM, light microscopy was used to examine the AM (Fig. 1A).

## Seeding cHCECs on Denuded AM

Denuded AMs were spread, basement membrane side up, on the bottom of polyester culture inserts (Corning Corp., Corning, NY), and the inserts were placed in wells of a 12-well plate. Confluent monolayers of cHCECs from passage 5 were trypsinized, centrifuged, and resuspended at a final cell-seeding concentration of $6.0 \times 10^4$ cells/mm$^2$ ($1.2 \times 10^6$ cells/mL × 0.5 mL of cell suspension per 100 mm$^2$ of culture area). Resuspended cells were gently seeded on denuded AM spread on culture inserts in wells of a 12-well plate, centrifuged gently (3 minutes at 180*g*) to increase the cell density of the cHCEC sheets and to increase uniform attachment to the denuded AM, and incubated at 37°C in 5% carbon dioxide-95% humidified air. The culture medium was changed 3 days later and then every other day for 2 weeks.

In some experiments, to investigate the extent of the survival of cHCECs on AM after transplantation in vivo, we labeled the cHCECs with the fluorescent membrane dye DiI (1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate).[12] The stock solution of DiI was made by dissolving 5 mg dye in 5 mL 70% ethanol. The DiI labeling solution was made by diluting the stock solution by 10. Immediately before seeding the HCECs on to the AM, the cells were labeled in vitro by adding DiI solution to the cell suspension. After incubation for 5 minutes at 37°C, the cell suspension was cooled on ice for 15 minutes. Excess dye was then removed by washing the cells twice in PBS. Cells were resuspended at a final cell-seeding concentration of $6.0 \times 10^4$ cells/mm$^2$ and seeded on denuded AM, cultured for 7 days, and transplanted. We conducted this experiment with two cHCEC sheets on AM carriers transplanted into rabbit corneas. At 7 days after transplantation, we killed the animals, removed the grafts, and observed them under the fluorescence microscope. We stained same tissues with alizarin red and examined them under the microscope.

## Light and Electron Microscopy

Cultures of cHCECs on denuded AM were examined by light, scanning electron, and transmission electron microscopy.

For light microscopy, tissues were stained with alizarin red and hematoxylin and eosin. For alizarin red staining, day-14 cultures on AM were placed endothelial side up on glass slides. Tissues were briefly rinsed in 0.9% sodium chloride followed by a 1-minute staining with 1% alizarin red in deionized water. Cell density (cells per square millimeter) was calculated by averaging cell density of three cHCEC sheets. For cell density of one sheet, five areas (each area equaled 0.25 mm$^2$) per one sheet were examined and averaged.

For hematoxylin and eosin stain, day-14 cultures on AM were embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura Fine Technical, Tokyo, Japan). After freezing, the resultant blocks were cut into 8-μm-thick sections with a cryostat. The sections were stained with hematoxylin and eosin for examination.

For scanning electron microscopy, day 14 cultures on AM were fixed in 2.5% glutaraldehyde in 0.1 M PBS, washed three times for 15 minutes in PBS, postfixed for 2 hours in 2% osmium tetroxide, and washed three more times in PBS. After dehydration through a graded ethanol series (50%, 70%, 80%, 90%, 95%, and 100%) specimens were transferred to hexamethyldisilazane (Agar Scientific, London, UK) for 2 × 10 minutes and allowed to air dry. When dry, specimens were mounted on aluminum stubs and sputter coated with gold before examination on a scanning electron microscope (model JSM 5600; Japanese Electron Optical Limited [JEOL], Tokyo, Japan).

For transmission electron microscopy, day-14 cultures on AM were fixed in 2.5% glutaraldehyde in 0.1 M PBS, postfixed in 2% osmium tetroxide, dehydrated through a graded ethanol series, and embedded in epoxy resin (Agar 100; Agar Scientific). Ultrathin (70 nm) sections

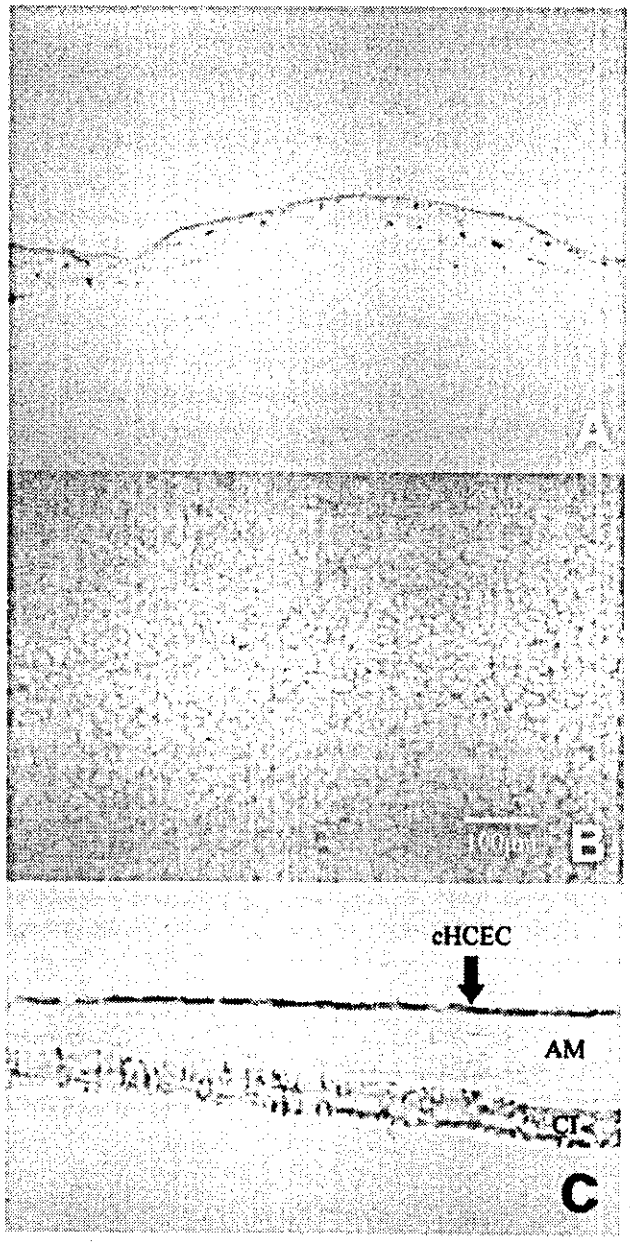


FIGURE 1. (A) Denuded AM before cHCECs were seeded. No amniotic epithelial cells were observed. (B) Light micrograph of cHCECs cultivated on denuded AM for 2 weeks. Flatmount stained with alizarin red. The cHCECs appeared as a fairly continuous monolayer with a cell density of 3340 cells/mm$^2$. (C) Cross section of the cHCECs on denuded AM on a culture insert (CI). A continuous intact monolayer of cHCECs is evident (*arrow*). Magnification: (A) ×100; (C) ×200.

TABLE 1. Transplantation Groups

| Group | Stripping DM | Transplantation |
|---|---|---|
| HCEC | (+) | cHCEC sheet |
| SD Control | (+) | (−) |
| AM Control | (+) | Acellular AM only |
| TO Control | (−) | (−) |

were collected on copper grids and stained for 1 hour each with uranyl acetate and 1% phosphotungstic acid and for 20 minutes with Reynolds lead citrate before examination on a transmission electron microscope (JEM 1010; JEOL).

## Animals

Male Japanese white rabbits weighing 2 to 3 kg were obtained from Shimizu Laboratory (Kyoto, Japan). All animals were treated in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. The Committee for Animal Research, Kyoto Prefectural University of Medicine, approved all animal studies.

## cHCEC Transplantation

Transplantation was performed on the right eye only. Corneal buttons and graft beds were prepared by excising a 7.00-mm site in the central cornea. Descemet's membranes together with the endothelium were stripped from the corneal buttons. cHCEC sheets, using AM as a carrier, on culture inserts were trephined to a diameter of 6.25 mm, and they were separated gently from inserts with fine forceps. These sheets were placed on the stroma of the corneal buttons, left for a few minutes until dry to secure the sheet to the stroma. The corneal buttons with cHCEC sheets were then placed on the graft bed of the same animal and sutured with eight interrupted sutures and a continuous suture (10-0 nylon). Four groups were prepared in this experiment (Table 1). Each group had three animals. In the first group (the cHCEC group), after Descemet's membrane was stripped, the cHCEC sheet was placed on the corneal button and transplanted as just described. In the second group (the SD control group), Descemet's membrane was stripped and the button transplanted as described. In the third group (the AM control group), after Descemet's membrane was stripped, just the acellular AM was transplanted, as described. In the last group (the TO control group), the host cornea was trephined only and transplanted as described, without stripping Descemet's membrane and transplanting any sheet. All grafted eyes were examined every day after transplantation. Grafts with technical difficulties (e.g., hyphema, infection, or loss of the anterior chamber) were excluded from further consideration. At day 4 after transplantation, the interrupted sutures were removed.

## Evaluation of Corneal Appearance, Thickness, and Histology after Transplantation

Each day after transplantation, corneal appearance was examined by slit lamp biomicroscopy, and corneal thickness was determined with an ultrasonic pachymeter (model SP-2000; Tomey, Nagoya, Japan). Corneal thickness is a measure of corneal endothelial function.[15] The mean of 10 measured values was calculated. At 7 days after transplantation, we killed the cHCEC sheet transplant recipients, removed the grafts, and observed them by light and electron microscopes.

# RESULTS

## Light and Electron Microscopy of the cHCECs Sheet In Vitro

The density of cHCECs seeded on AM was 3285.3 ± 62.0 cells/mm$^2$, the cHCECs appeared as a fairly continuous monolayer (Figs. 1B, 1C). Scanning electron microscopic images of HCECs cultivated on AM revealed a continuous layer of flat, squamous, polygonal endothelial cells that appeared uniform in size. The interdigitations at the cell boundaries were not as distinct as those in normal endothelium. These cells had a few pits and vacuoles, but appeared to be healthy, well developed, and closely attached to one another, with tightly opposed cell junctions (Fig. 2). Transmission electron microscopic images showed a monolayer of flat endothelial cells, healthy and well formed, with tightly opposed cell junctions. Adjoining cells overlapped each other slightly to maintain maximum contact, as would be expected. At the base of the cHCEC basement membrane, material was clearly being produced (Fig. 3).

## Evaluation of Corneal Appearance and Thickness after Transplantation

At 4 days after the operation, the control grafts, consisting of only stripped Descemet's membrane, became highly edematous (Fig. 4A) as did the control grafts with only acellular AM (Fig. 4B). However, grafts with cHCECs on AM had little edema and excellent transparency, and we could see the transplanted cHCEC sheets clearly through the transparent corneal button (Fig. 4C). No signs of rejection or neovascularization were observed. This situation continued for at least 7 days after transplantation (Fig. 5). Corneal grafts with cHCECs were sig-

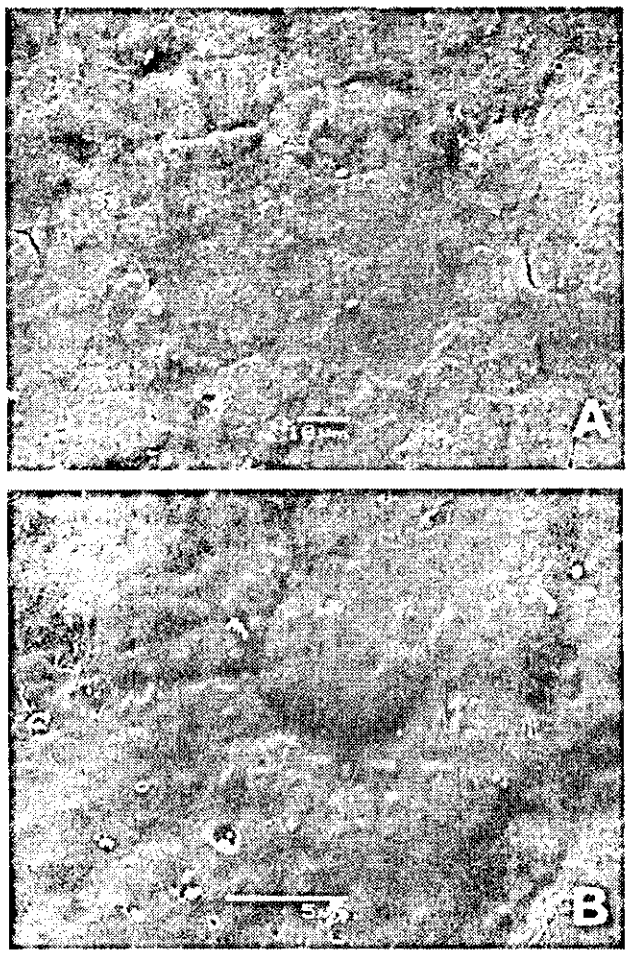


FIGURE 2. Scanning electron micrograph of cultured HCECs. The cells were cultivated on denuded AM for 2 weeks. (A) Cells formed a continuous monolayer layer on the AM. (B) At higher magnification, it was evident that the cells were polygonal, fairly uniform in size, and in close contact with each other with tightly opposed cell junctions.