The Use of Human Mesenchymal Stem Cell–Derived Feeder Cells for the Cultivation of Transplantable Epithelial Sheets

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PURPOSE. To report the efficacy of human bone marrow–derived mesenchymal stem cells as a source of feeder cells for the cultivation of transplantable corneal epithelial cell sheets.

METHODS. Human mesenchymal stem cells (marrow adherent stem cells; MASCs) were cultured in α-modified Eagle’s medium with 10% serum and were treated with mitomycin C. Expression of cytokines in MASCs was confirmed by reverse transcription-polymerase chain reaction. Human limbal epithelial cells were cocultured with MASCs or 3T3 feeder cells to compare colony-forming efficiency (CFE). Limbal epithelial cells were cultured on MASCs or 3T3 feeder cells at the air-liquid interface to allow stratification, and stratified epithelial sheets were analyzed by immunohistochemistry against cytokeratin 3 (K3), K15, p63α, and ABCG2. Rabbit limbal epithelial cell sheets were cultivated with MASC feeder cells and transplanted to the ocular surface of the limb-deficient rabbits. Epithelial grafts were observed by slit lamp microscopy for 4 weeks and then evaluated by histology and immunohistochemistry against K3 and K4.

RESULTS. MASC feeder cells expressed keratinocyte growth factor, hepatocyte growth factor, and N-cadherin. The CFE of human limbal epithelial cells was similar in MASC and 3T3 feeder groups. Stratified cell sheets were successfully cultivated with MASC feeder cells expressing K3, K15, p63α, and ABCG2. Transplanted epithelial sheets regenerated the corneal phenotype in limb-deficient rabbits.

CONCLUSIONS. MASC-derived feeder cells are suitable for the engineering of epithelial sheets, avoiding the use of potentially hazardous xenogenic feeder cells. (Invest Ophthalmol Vis Sci. 2009;50:2109–2115) DOI:10.1167/iovs.08-2262

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Tissue engineering to produce a fully stratified corneal epithelial cell sheet was first introduced as an alternative to transplanting corneal limbal tissue in which corneal stem cells reside.1 Cultivated epithelial sheets are often used with carriers such as amniotic membranes2–4 and fibrin.3 More recently, carrier-free sheets were introduced as a way to produce transplantable sheets that can be transplanted to the ocular surface without sutures.6,8 However, all techniques reported thus far require 3T3 feeder cells to produce a robust, stratified sheet that can withstand manipulation during surgery. The use of xenologic cells such as the 3T3 fibroblast entails ethical issues and safety issues because of the rare incidence of transmittable disease.

Engineering cell sheets without the use of feeder cells is the ideal solution to avoid transmissible disease. However, a drastic change in condition from the environment in vivo to culture media in vitro often drives a change in phenotype in many types of cells studied, including the corneal epithelium. This is especially true for the undifferentiated progenitor population, which requires an environment that mimics the stem cell niche in vivo. The use of 3T3 cells as feeder cells has a long history, and though the specific mechanisms involved are still not completely clear, colony-forming efficiency (CFE) by single clones is dramatically increased by coculture with 3T3 cells.9 We found that using two layers of feeder cells to maintain adhesion molecules and soluble factors recreated the basal limbal phenotype observed in the human cornea of cytokeratin 15 (K15)-positive basal, K12-positive suprabasal cells in cultivated epithelial sheets.10,11 Therefore, engineering epithelial sheets that contain progenitor cells requires the use of feeder cells.

Attempts have been made to use human tissue sources, such as fibroblasts and adipocytes, as feeder cells.12,13 Although such options offer the use of autologous cells, the heterogeneity of each cell lot may pose a problem in quality control. We therefore focused on mesenchymal stem cells extracted from human bone marrow that are relatively easy to manipulate in culture. Human mesenchymal stem cells (marrow adherent stem cells; MASCs) are nonhematopoietic lineage cells that can be cultivated and passaged on plastic as colony-forming fibroblastic cells.14 Bone marrow banks already provide donor cells for the treatment of life-threatening disorders such as leukemia and lymphoma. If MASCs can serve as efficient feeder cells, extracted from the marrow of donor bank stocks may serve as a source of high-quality human feeder cells for regenerative medicine.

MATERIALS AND METHODS

MASC Cell Culture

Human mesenchymal stem cells (marrow adherent stem cells; MASCs) were supplied by SanBio Inc. (Mountain View, CA) and fed with α-modified Eagle’s medium (α-MEM; Invitrogen, Carlsbad, CA) with...
10% FBS twice a week. NIH/3T3 cells were cultured in Dulbecco’s MEM (DMEM; Invitrogen) containing 10% FBS. To make the feeder cells, confluent MASCs or NIH/3T3 cells cultured in 75-cm² flasks were treated with mitomycin C (MMC) at 37°C for 2 hours, and dissociated cells were cryopreserved until use. One day before epithelial cell seeding, feeder cells were thawed and seeded in culture vessels at a density of 2.5 × 10⁴ cells/cm². To determine the optimal concentration of MMC, MASCs were treated with 1, 2, 4, 6, 8, and 16 μg/mL MMC (Nacalai Tesque, Inc., Kyoto, Japan) at 37°C for 2 hours. Cells treated in each concentration were dissociated and seeded in six-well plates at a density of 10⁵ cells/well, and cells from three wells were harvested on day 1; the rest were harvested on day 3. Cell counts were determined at each time point to investigate the inhibitory effects of MMC. To confirm the continuous effect of MMC on growth inhibition, MASCs with or without MMC treatment (4 μg/mL) were also compared on day 7.

**Epithelial Cell Culture**

Human limbal epithelial cells (LEC) were obtained from cornneas in United States eye banks after the central buttons were used for corneal transplantation. Rabbit LECs were obtained from rabbits killed by intravenous injection of excess volume of pentobarbiturate (50 mg/kg body weight) followed by injection of 1 M KCl (1 mL/kg body weight). All animal experimental procedures and protocols conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research. After excess tissue was removed, the limbus was immersed in 1.2 U dispase II (Roche Diagnostics, Basel, Switzerland) in F12/DMEM (Invitrogen) at 37°C for 1 hour. Epithelium was separated from the stroma and treated with 0.05% trypsin EDTA at 37°C for 30 minutes to disperse cells. Human and rabbit LECs were seeded immediately after isolation and cultured in supplemental hormone epithelial medium (SHEM) consisting of F12/DMEM, 10% FBS, 10 ng/mL EGF, 5 μg/mL human recombinant insulin (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 500 ng/mL hydrocortisone (water soluble, H-0396; Sigma-Aldrich, St. Louis, MO), 5 μg/mL transferrin (Sigma-Aldrich), 250 ng/mL isoproterenol hydrochloride (Wako), and antibiotics. Medium was replaced three times a week.

**RT-PCR**

MASC feeder cells (5 × 10⁴ cells) were seeded in 60-mm dishes, and the medium was changed to SHEM the following day. Total RNA was extracted with the use of a commercial kit (RNaseasy; Qiagen, Hilden, Germany) on day 2, followed by cDNA synthesis (RevTara Ace-kit; Toyobo Co. Ltd., Osaka, Japan). PCR was performed (GeneAmp 9700; Applied Biosystems, Foster City, CA) with Advantage 2 PCR Enzyme System (Clontech, Takara Bio Inc., Shiga, Japan) as follows: 95°C for 5 minutes, 2 cycles of 95°C for 90 seconds, 57°C for 30 seconds, and 72°C for 30 seconds, and 28 cycles of 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds). Sequences for primers and PCR product size are listed in Table 1. PCR products were analyzed by gel electrophoresis.

**Colony-Forming Efficiency**

MASCs and NIH/3T3 feeder cells were seeded in a 100-mm dish at a density of 2.5 × 10⁴ cells/cm² (n = 3) for each feeder. The next day, 1000 human limbal epithelial cells were seeded on the dish. After 2 weeks, dishes were fixed with formalin and stained with 1% rhodamine B (Wako) in distilled water. CFE was calculated by the following formula: CFE = number of colonies/number of cells seeded. CFE was compared with use of the Student’s t-test, and P < 0.05 was considered statistically significant.

**Engineering of Epithelial Cell Sheets**

Cell culture inserts (Transwell; catalog number 3450; Corning, Corning, NY) were coated with 300 μl fibrin (5.5 mg/mL, Bolheal; Fugisawa, Osaka, Japan) as described previously, and 5 × 10⁴ feeder cells were seeded in the bottom of the paired well. The next day, human or rabbit limbal epithelial cells (2 × 10⁶ cells/cm²) were inoculated in the culture inserts. Aprotinin (666 KIU/mL; Wako) was added in SHEM to prevent the digestion of fibrin glue during culture. After the epithelial cells reached confluence, cells were air-lift cultured for an additional 3 days without aprotinin. Medium was changed daily during air-lift culture. Cultivated rabbit epithelial cell sheets were used for transplantation, and human epithelial sheets were used for immunohistochemistry.

**Epithelial Sheet Transplantation**

Recipient rabbits were anesthetized with intravenous injection of diazepam (1.5 mg/kg body weight) and pentobarbiturate (20 mg/kg body weight), and the left eye of each rabbit was rendered total limbal stem cell deficient by 1-n-heptanol (Sigma-Aldrich) and mechanical debridement of the corneal epithelium. Additional surgical removal of the limbal and conjunctival epithelium was performed up to 2 mm from the limbus. Carrier-free sheets were transplanted as described previously. Cell sheets were allowed to attach for 5 minutes without sutures. Rabbits with denuded cornneas without sheet transplants (SHAM) served as control. Each rabbit was fitted with a bandage contact lens and treated with topical antibiotic (levofloxacin) and steroids (betamethasone) after surgery. Rabbits were examined weekly for epithelialization and inflammation and were killed at 4 weeks for histologic and immunohistochemical examination.

**Histology and Immunohistochemistry**

Cultivated epithelial cell sheets or enucleated cornneas were cut in half. One half was fixed with formalin and embedded in paraffin, and the other half was embedded in optimum cutting temperature compound and frozen in liquid nitrogen. Paraffin sections or formalin-fixed cryosections were stained with hematoxylin and eosin (HE) for histologic examination. For immunohistochemistry, cryosections were fixed with ice-cold acetone or 4% paraformaldehyde for 5 minutes and were reacted with anti-K3 (mouse, clone AE5; Progen, Heidelberg, Germany), anti-K4 (mouse, clone 6B10, Mob302; Diagnostic Biosystems Inc., Pleasanton, CA), anti-K12 (rabbit, sc-25,722; Santa Cruz Biotechnology, Santa Cruz, CA), anti-K15 (mouse, clone LHIK15, MS-1068; Laboratory Vision, Thermo Fisher Scientific Inc, Fremont, CA), anti-p63α (rabbit, sc-8544; Santa Cruz Biotechnology), and anti-ABCG2 (mouse, clone BXP-21, MAB4146; Millipore, Billerica, MA) antibodies. Sections were then treated with Cy3- or Alexa Fluor 488-conjugated secondary antibodies (Chemicon International, Temecula, CA). Cell nucleus was counterstained with DAPI (1 μg/mL; Dojindo Laboratories, Tokyo, Japan).

**RESULTS**

**Characteristics of MASC Feeder Cells**

MASC cells used as feeder cells are usually treated with 4 μg/mL MMC at 37°C for 2 hours to inhibit growth. To determine the optimal concentration of MMC for MASC feeders, cells were treated with 1 to 16 μg/mL MMC at 37°C for 2 hours and were subcultured to six-well plates. Cells were harvested on day 1 and day 3. Nontreated MASCs increased in number

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**Table 1. Primer Sequences**

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Bone Marrow–Derived Mesenchymal Feeder Cells 2111

Cultivated Human Epithelial Sheets

Human limbal epithelial cells were cultivated with 3T3 feeders and MASC feeders on fibrin sealant to form stratified epithelial cell sheets. HE staining of cultivated sheets showed a robust epithelial sheet of five to six layers with cuboid basal cells and flattened superficial cells when MASC feeders were used (Fig. 2A). The morphology of MASC feeder sheets was similar to or more robust than that of 3T3 feeder sheets, depending on the epithelial donor source (Fig. 2B). Both sheets diffusely expressed K3 (Figs. 2C, 2D). K12 stained sparsely in cultivated sheets, whereas K15, a keratin expressed by basal limbal cells, was observed in MASC feeder and 3T3 feeder sheets (Figs. 2E, 2F). The progenitor cell markers p63α (Figs. 2G, 2H) and ABCG2 (Figs. 2I, 2J) were also expressed at similar levels in both sheets.

Cultivated Sheet Transplantation

Stratified, carrier-free corneal epithelial sheets were successfully engineered using MASC feeders (Fig. 3A) and 3T3 feeders (Fig. 3B) with almost identical morphology and layers of stratification. Rabbits without cell sheet transplants (SHAM) characteristically had epithelial defects up to 1 week after surgery, as shown by the positive staining with fluorescein dye in Figure 3C. A smooth intact epithelial layer was observed in eyes transplanted with MASC feeder (Fig. 3D) and 3T3 feeder cell (Fig. 3E) sheet transplants. Irregular fluorescein staining persisted in the SHAM-operated group at 4 weeks, suggesting a conjunctivalized surface (Fig. 3F). Injection of conjunctival blood vessels was also more prominent in the SHAM group than on the MASC (Fig. 3G) and 3T3 (Fig. 3H) cultivated sheets.

Transplanted rabbits were killed after 4 weeks for histologic examination. Macroscopic and microscopic HE staining samples are shown in Figure 4. Healthy rabbit corneas with no surgical intervention have a stratified epithelium of five to six layers with relatively cuboid cells in the basal layer (Figs. 4A, 4B). Stromal cells are equally distributed beneath the epithelium. Limbal-deficient eyes that received epithelial sheets cultivated with MASC feeders also showed a morphology similar to that of control cornea (Figs. 4C, 4D), except for a band of acellular stromal tissue beneath the epithelium, possibly because of the loss of keratocytes during epithelial debridement. 3T3 sheets showed histologic findings similar to those of the MASC group (Figs. 4E, 4F), whereas SHAM-operated eyes had a thin epithelium with relatively dense cellular infiltration beneath the regenerated epithelium (Figs. 4G, 4H).

Immunohistochemistry

Immunohistochemical examination against the corneal epithelium-specific K5 showed that MASC feeder epithelial sheets maintained a robust K5-positive layer of epithelial cells in the central cornea that tapered toward the conjunctival epithelium (Figs. 5C, 5D). Again, these findings were similar to those in healthy control corneas (Figs. 5A, 5B). Similar findings were observed in the 3T3 feeder sheets (Figs. 5E, 5F). SHAM-operated eyes did not show any K5-expressing cells, indicating that the thin epithelium in these eyes was of conjunctival origin (Figs. 5G, 5H). In rabbits, K4 can be used to differentiate conjunctival and corneal epithelia. Normal conjunctiva showed K4 staining spanning the entire epithelial layer (Fig. 5I).

FIGURE 1. MASC feeder cells supported the clonal growth of human limbal epithelial cells. (A) RT-PCR analysis of KGF, HGF, and N-cadherin expression by MASC feeder cells. (B) Human limbal epithelial cells formed colonies in MASC and 3T3 feeder cells. (C) No statistically significant difference was observed in CFE in MASC or 3T3 feeder cells. (D–G) Phase-contrast micrograph of epithelial colonies. Epithelial cells invaded beneath feeder cells in MASC feeders (D, F) and 3T3 feeders (E, G). Scale bars: 500 μm (D, E); 100 μm (F, G).
6B), whereas the superficial corneal epithelium was also stained with anti–K4 antibodies (Fig. 6A). Examination of the central cornea in MASC feeder and 3T3-cultivated sheets showed the corneal epithelial phenotype (Figs. 6C, 6D), whereas the SHAM-operated cornea presented the conjunctival phenotype (Fig. 6E). This figure confirms that total debridement of the corneal epithelium extending beyond the limbus renders the ocular surface devoid of corneal epithelial cells and that carrier-free MASC feeder–cultivated sheet transplantation rescued the eye from limbal stem cell deficiency.

**Figure 2.** Histology and immunohistochemistry of human limbal epithelial cell sheets. Epithelial cell sheets were prepared with MASC feeder cells (A, C, E, G, I) and 3T3 feeder cells (B, D, F, H, J) on fibrin sealant. Sections were stained with HE (A, B), anti-K3 (C, D), anti-K12 and anti-K15 (E, F), anti-p63α (G, H), and anti-ABCG2 (I, J). K12 (green) was observed mainly in the MASC feeder group, whereas K3 and K15 (both red) were expressed by both sheets. The progenitor cell markers p63α (red) and ABCG2 (red) were expressed similarly in both sheets. Scale bar, 100 μm.

**Figure 3.** Cultivated limbal epithelium transplantation in a limbal stem cell deficiency rabbit model. (A, B) HE-stained sections of rabbit LECs cultured with MASC feeder cells (A) and 3T3 feeder cells (B) on fibrin sealant. (C–H) Fluorescein-stained eyes of limbal stem cell deficiency model rabbit at 4 days (C–E) and 4 weeks after transplantation (F–H). SHAM-operated group typically had epithelial defects on day 4 (C) that eventually healed with rough conjunctival epithelium (F). Smooth epithelial surfaces were observed in MASC cultivated sheets (D, G) and 3T3 cultivated sheets (E, H).
DISCUSSION

Cultivated epithelial sheet transplantation is an effective means to restore an intact epithelium to the patient with limbal stem cell deficiency. Epithelial sheets already in clinical use are engineered with epithelial cells from the corneal limbus or oral mucosa. However, irrespective of cell source, the preparation of epithelial sheets requires “supporting cells” or feeder cells to expand progenitor cells and to produce stratified sheets. Although epithelial sheets can stratify without coculture with feeder cells, we found that the number of K15-expressing progenitors and secondary CFE was higher when cultivated epithelial cells were in contact with feeder cells. Therefore, the use of feeder cells seems to be vital in maintaining larger amounts of progenitor cells in transplanted sheets.

FIGURE 4. HE-stained sections of transplanted corneas. (A, B) Healthy rabbit cornea control. Limbal-deficient cornea transplanted with MASC-cultivated sheets (C, D) showed a robust stratified epithelium similar to that of 3T3-cultivated sheets (E, F). SHAM-operated corneas had epithelial defects or were covered with thin epithelium (G, H), and infiltrating cells were observed under the regenerated epithelium (H, arrows). Scale bar, 100 μm.

FIGURE 5. Immunohistochemistry of transplanted corneas with anti–K3 antibody. K3 (red) was expressed in the healthy rabbit epithelium (A, B) and in limbal-deficient corneas that received MASC-cultivated (C, D) and 3T3-cultivated (E, F) sheets. SHAM-operated corneas were negative for K3 (G, H). Scale bar, 100 μm.
Most, if not all, methods for producing epithelial sheets have used murine-derived 3T3 cells as feeders. Although no known adverse reactions from xenologic cells have been reported thus far, ethical and safety issues have raised the need for a safer source of feeder cells. This is especially true if the feeder cells were to be used in direct contact with donor cells. Jang et al. reported the use of autologous fibroblasts as feeder cells in a rabbit alkali burn model and found that peripheral neovascularization was suppressed, in contrast to results with sheets using amniotic membrane alone. Fibroblasts from an autologous source may be a safer alternative to xenogenic cells. However, similar to culture-grade serum, feeder cells from different sources may have different properties that may affect the quality of the engineered sheet. MASCs can proliferate indefinitely from a single source; therefore, a well-characterized lot can be repeatedly used to manufacture several sets of sheets. One point worth noting is that colonies tended to be smaller with MASC feeder cells, possibly because MASCs (Figs. 1D, 1F) were more adhesive than 3T3 cells (Figs. 1E, 1G), and epithelial cells in individual colonies migrated beneath MASC feeder cells. Given that there was no significant difference in CFE, the difference in size may reflect a difference in adhesive- ness, not a difference in the ability to promote cell proliferation.

Although we have shown that MASCs can be used as feeder cells for the quality of the transplanted sheets, why feeder cells are required remains to be shown. Adult stem cells require strict control of the microenvironment, or niche, in which they reside in vivo. The stem cell niche of hematopoietic stem cells (HSCs) has been extensively studied in the bone marrow, where a subpopulation of osteoblasts that express N-cadherin play a key role in maintaining HSCs in a quiescent state. Recently, Hayashi et al. reported that N-cadherin is expressed by putative stem cells of the limbal epithelium, suggesting that N-cadherin is a key component of the limbal stem cell niche as well. We confirmed that MASCs express N-cadherin, in addi- tion to soluble growth factors, required for the proliferation of epithelial cells such as HGF and KGF (Fig. 1). Because feeder cells have long been known to support colony formation and epithelial cell proliferation through several passages, feeder cells probably provide an artificial niche in vitro that inhibits the drive toward differentiation in progenitor cells.

The use of the amniotic membrane (AM) has been proposed as a means to inhibit differentiation. AM is also a popular carrier for the cultivation of stratified sheets. We observed the efficacy of MASC feeders in cultivated sheets using AM carriers and found that the number of cell layers and the expression of keratins were similar to those when 3T3 cells were used (data not shown). Although more severe cases may require an AM substrate to support the epithelium, recent reports have shown that carrier-free sheets are as effective as AM sheets in selected cases. Given that procedures in regenerative medicine, such as cultivated epithelial sheets, are on the verge of global use, minimizing variables that may pose potential hazards is vital. We believe that using high-quality, human-derived MASC feeder cells with carrier-free technology will further improve the safety of epithelial sheet transplantation in the treatment of ocular surface disease.

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References


