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Corneal regeneration by transplantation of corneal epithelial cell sheets fabricated with automated cell culture system in rabbit model

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ABSTRACT

We have performed clinical applications of cell sheet-based regenerative medicine with human patients in several fields. In order to achieve the mass production of transplantable cell sheets, we have developed automated cell culture systems. Here, we report an automated robotic system utilizing a cell culture vessel, cell cartridge. The cell cartridge had two rooms for epithelial cells and feeder layer cells separating by porous membrane on which a temperature-responsive polymer was covalently immobilized. After pouring cells into this robotic system, cell seeding, medium change, and microscopic examination during culture were automatically performed according to the computer program. Transplantable corneal epithelial cell sheets were successfully fabricated in cell cartridges with this robotic system. Then, fabricated cell sheets were transplanted onto ocular surfaces of rabbit limbal epithelial stem cell deficiency model after 6-h transportation using a portable homothermal container to keep inner temperature at 36 °C. Within one week after transplantation, normal corneal epithelium was successfully regenerated. This automatic cell culture system would be useful for industrialization of tissue-engineered products for regenerative medicine.

1. Introduction

Recently, tissue engineering and regenerative medicine have become the focus of public attention, and some successful outcomes in the clinical settings have been reported. We have developed cell sheet-based regenerative medicine utilizing temperature-responsive culture surfaces [1–6]. By utilizing fabricated carrier-free cell sheets, we have successfully performed the clinical applications to treat human patients in skin [7], cornea [8,9], esophagus [10,11], heart [12], periodontal tissue [13], and knee cartridge [14].

In the clinical research, cell culture to fabricate transplantable cell sheets was manually performed in clean rooms of cell processing facilities according to the regulation of Good Manufacturing Practice (GMP) [15]. Therefore, the production cost is inevitably high. In particular, autologous tissue-engineered products are pretty expensive due to the small production. It has been pointed out that drastic reduction of the manufacturing costs should be necessary to provide these tissue-engineered products to many patients and establish regenerative medicine as a general treatment [16–18]. Therefore, a series of new technology would be necessary to support manufacturing processes from isolation of cells from tissues to shipping inspection of the final products [19,20]. For example, in order to improve the efficiency of cell culture process, the development of an automated cell culture system (ACCS) has been promoted [21].

So far, ACCSs which handle conventional open culture vessels with robotic arms have been reported [22–25]. To minimize the possibility of bacterial and/or viral contamination, ACCSs inevitably need special air-conditioning units with high-efficiency particle arrestor (HEPA) filters to use open culture vessels. Closed culture vessels connected to closed liquid circuits can reduce the size of ACCS by eliminating bulky air-conditioning units and robotic arms. For example, an ACCS using mono-layered closed culture vessels connected to a closed circuit was reported [26]. However, typical culture conditions for epithelial cells employ cell culture inserts and feeder layer cells [8–10]. Therefore, a closed culture vessel having two separate rooms and more complex closed circuits might be needed in these cases.

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2. Materials and methods

2.1. Cell culture

The animals were treated in accordance with experimental procedures approved by the Committees for Animal Research of Tokyo Women's Medical University and of Osaka University Medical School. Corneal limbus was excised from eyes of Japanese White rabbits by scissors, then treated with 200 U/mL of dispase II (Godo Shusei, Tokyo, Japan) in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) at 37 °C for 1 h. Epithelium was peeled off under a dissecting microscope, and cut into small pieces by scissors. These tissues were treated with 0.25% trypsin–0.1% ethylenediamine tetraacetic acid solution (GIBCO–Invitrogen, Carlsbad, CA) for 20 min at 37 °C, to scatter the epithelial cells. Disaggregated cells were suspended in a keratinocyte culture medium (KCM) composed of a basal mixture of 3 parts Dulbecco's modified Eagle's medium and 1 part nutrient mixture F-12 Ham (Sigma), and supplemented with 2 nM triiodothyronine (Wako Pure Chemicals, Osaka, Japan), 5 μg/mL transferrin (GIBCO–Invitrogen), 5 μg/mL insulin (Eli Lilly, Indianapolis, IN), 10 ng/mL epidermal growth factor (Invitrogen), 0.4 μg/mL hydrocortisone (Kowa Pharmaceutical, Tokyo, Japan), 1 nM cholera toxin (List Biological Laboratories, Campbell, CA), 1% penicillin–streptomycin (Invitrogen), and 5% fatal bovine serum (Moregate BioTech, Queensland, Australia) [27]. The rate of viable cells was obtained by Trypan blue exclusion test on a hemocytometer under a phase-contrast microscope (TE2000; Nikon, Tokyo, Japan). To evaluate putative progenitor cell populations in the cells, colony-forming assays (CFA) were performed. The cells were seeded at the density of 200 cells/35-mm well, and cultured with mitomycine C (MMC)-treated NIH/3T3 feeder cells seeded at the density of 2.0×10^4 cells/cm^2. After 2 weeks, the number of colonies was counted under a microscope.

2.2. Instrumentation

2.2.1. Cell culture vessel

In the present study, we used new culture vessels, cell cartridge (Fig. 1A) [28]. The cell cartridge had two rooms for epithelial cells and feeder layer cells separated by the microporous (0.4 μm) film on which a temperature-responsive polymer, poly(*N*-isopropylacrylamide) was covalently immobilized (Fig. 1B), and were connected to closed circuits. The lower gas permeable film was treated by O_2 plasma to improve cell adhesion. Corneal epithelial cells and MMC-treated 3T3 cells were cultured in an upper and lower culture rooms, respectively.

2.2.2. Automated cell culture system

A prototype of the automated cell culture system was developed to perform cell seeding, medium change, cell culture, and microscopic monitoring of cells according to computer programs. Maximum three cell cartridges were cultured in the ACCS at one time. The ACCS had two small handling manipulators. One handled cell cartridges, the other was used only when cells were seeded into cell cartridges.

2.3. Transportation

All the cell cartridges were sealed with Parafilm (Pechiney Plastic Packaging, Chicago, IL) to avoid pouring of culture medium, packed within homothermal portable containers developed for cell sheet transportation (Hitachi Transport System, Tokyo, Japan) [29], and transported from Hitachi Central Research Laboratory (Saitama, Japan) to Osaka University Medical School (Osaka, Japan) by car and train. Temperature was continuously monitored with temperature sensors (T&D Corp., Nagano, Japan) during transportation.

2.4. Transplantation

Tissue-engineered cell sheets fabricated from cornea limbal epithelial cells were autologously transplanted onto keratectomized ocular surfaces of a rabbit limbal stem cell deficiency model surgically prepared 2 weeks before transplantation. Keratectomy was used to excise the entire corneal surface, including the limbus and the conjunctival tissue within 5 mm of the limbus, completely removing the corneal and limbal epithelium and exposing the stroma [8,30]. Six rabbits were used for epithelial cell sheet transplantation ($n = 3$) and control without cell sheet transplantation ($n = 3$). After two weeks of keratectomy, conjunctival scar tissue with some neovascularization covered the entire corneal stromal surface, including severe corneal opacity. Before cell sheet transplantation, the conjunctivalized ocular surface was surgically removed to reexpose the native transparent corneal stroma. Then, transplantable cell sheets fabricated in cell cartridges were harvested by temperature reduction (20 °C, 5% CO_2, 30 min) and transferred to a polyvinylidenedifluoride (PVDF) support membrane (23 mm in diameter with a 16-mm hole in the center) and placed over the transparent stromal bed immediately. A part of corneal epithelial cell sheets was cut and processed into paraffin-embedded sections respectively. Within 5 min, the cell sheets spontaneously produced stable attachment to the stroma, and PVDF membranes were removed with scissors. For healing protection, the corneal surface was finally covered with a soft contact lens, and a tarsorrhaphy was performed. Antibiotics (0.3% ofloxacin) and steroids (0.1% betamethasone) were topically applied three times a day after transplantation. One week after surgery, rabbits were sacrificed. The operated eyes were enucleated, and the each cornea of operated eyes was cut into three sections and processed into paraffin-embedded sections, respectively.

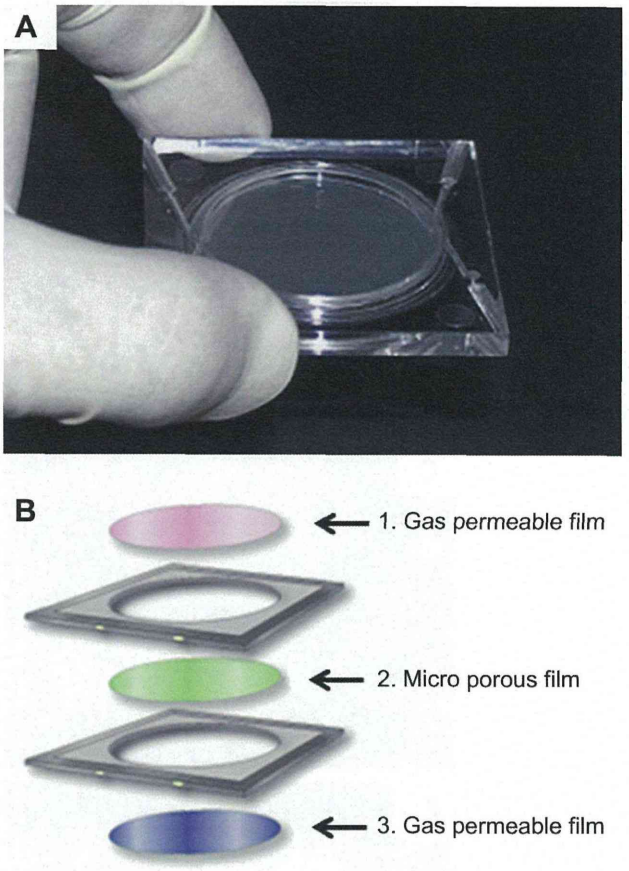

Fig. 1. A new culture vessel called cell cartridge. A, The slanting front view of the cell cartridge. B, Its inner structure. The cell cartridge had two rooms for epithelial cells and feeder layer cells separated by the microporous (0.4 μm) film on which a temperature-responsive polymer, poly(*N*-isopropylacrylamide) was covalently immobilized. The size was 46 × 46 × 4 mm (WDH).

2.5. Histology

Harvested corneal epithelial cell sheets before grafting, and reconstructed ocular surfaces (one week after grafting) were fixed with 10% neutral buffered formalin (Wako Chemicals). The fixed specimens were then routinely processed into 4–5-μm thick paraffin-embedded sections. Hematoxylin and eosin staining (HE) was performed by conventional methods. For immunohistochemical analyses, deparaffinized sections were treated with either anti-cytokeratin 3 (CK3) (AE5, Invitrogen, Carlsbad, CA), or anti-p63 antibody (4A4, Invitrogen) at 4 °C overnight. Secondary antibody was horseradish peroxidase (HRP) conjugated anti-mouse IgG (1:1000 dilution) (Jackson Immuno Research Laboratories, West Grave, PA). All sections were detected by 3,3′-diaminobenzidine tetrahydrochloride and counterstained with hematoxylin. Stained cells were observed under a fluorescence microscope equipped with a digital camera.

3. Results

3.1. Development of automated cell culture system (ACCS)

The ACCS developed in the present study was a whole automated cell culture platform consisting of a main culture system (MCS) (Fig. 2A) and the other systems including a refrigerator, a liquid controller, a gas controller, and a PC to control the whole system. First, cell cartridges were placed inside the MCS through the cell cartridge pass box with an air lock, and inner liquid circuits

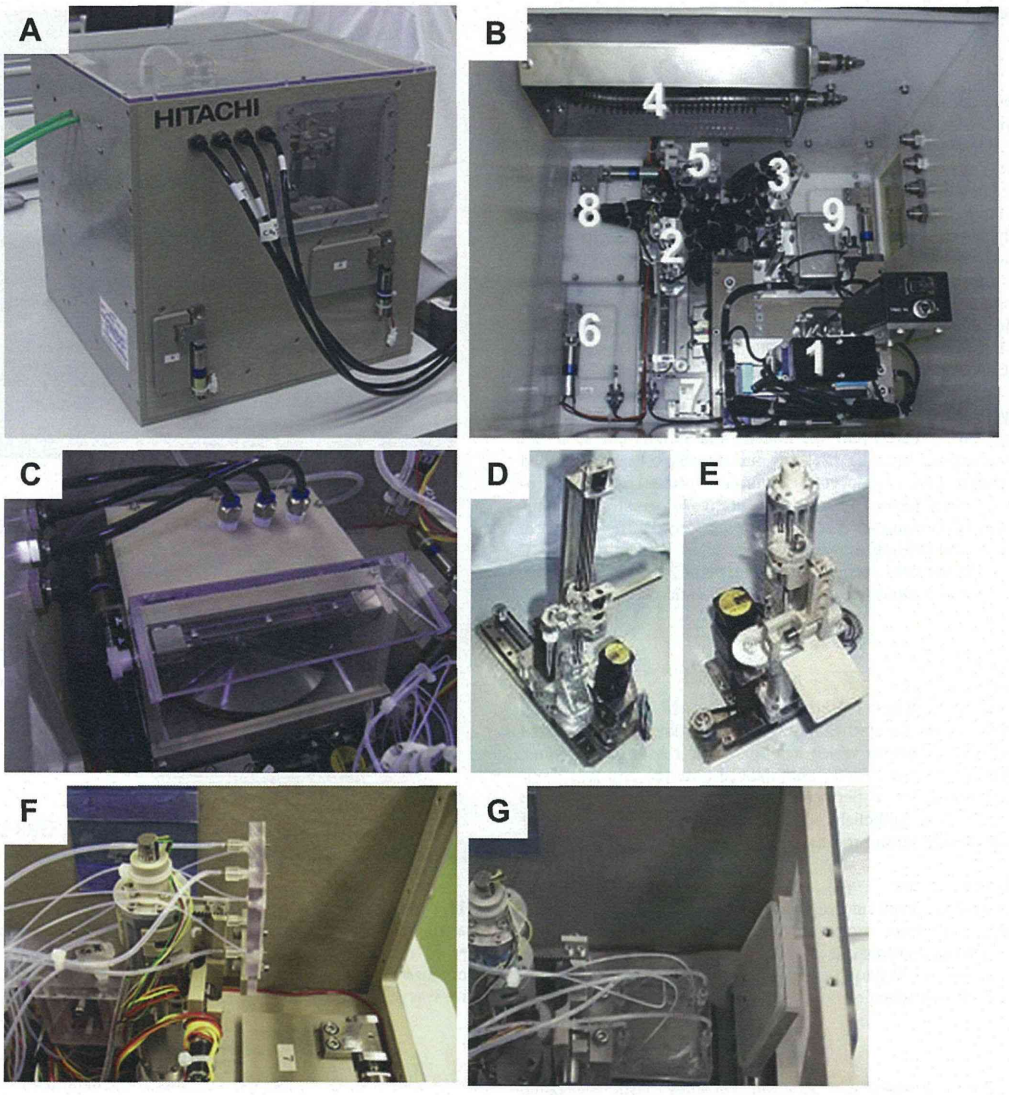

Fig. 2. The automated cell culture system. A, The front view of main culture system (MCS). B, The inside of the MCS. The main components are labeled: 1, an incubator with CCD camera; 2, a tip manipulator; 3, a handling manipulator with a connector; 4, a heater in environment controllers; 5, a liquid circuit including a tank; 6, a tip pass box with an air lock system; 7, a dust pass box with an air lock system; 8, a cell pass box with a centrifuge and an air lock system; 9, a cell cartridge pass box with an air lock system. C, The incubator. This incubator has a door and a turntable on which three cell cartridges were cultured at one time. The culture environment was kept at 37 °C, 5% CO_2, and over 95% humidity. D, The tip manipulator. E, The handling manipulator to connect with a cell cartridge and pour cells. F, The connector of the handling manipulator. G, The handling manipulator integrated with a cell cartridge in the cell cartridge pass box via its connector.

were connected with outer tubes inside a clean bench. Then, cell suspensions were injected to the MCS. Thereafter, cell culture was continued in the MCS even outside a clean bench, since the MCS was designed as airtight.

Culture medium, Dulbecco's phosphate buffer saline (PBS) to wash circuits, and waste were stored at 4 °C in the refrigerator. Aliquots of culture medium and PBS were prewarmed at 37 °C before used. The gas controller mixed air and CO_2 with valves, and supplied 5% CO_2 gas to an incubator in the MCS after filtration through a 0.22-μm filter. The PC was connected to all the sensors and actuators via the local area network, and controlled the ACCS with the operating software running on Windows XP. This PC allowed checking out the malfunction in the system for data logging of sensor values and system errors.

The MCS was composed of nine major components (Fig. 2B–E); an incubator with CCD camera, a tip manipulator, a handling manipulator having a connector with a cell cartridge, a liquid circuit including a tank and controllers, environment controllers, a tip pass box and a dust pass box, a cell pass box with a centrifuge, and a cell cartridge pass box. The shape of the MCS was a rectangular parallelepiped, and the size was 30 cm × 30 cm × 52 cm (WDH). The MCS had four air-rocks (tip pass box, dust pass box, cell pass box, and cell cartridge pass box) to take culture materials (cells, cell cartridges, pipette tips, and used tips) in and out of.

The liquid circuit was semi-closed and composed of disposable tubing, two liquid tanks, connectors, and liquid bags. The two liquid bags were manually poured with culture medium and PBS inside a clean bench, respectively, then put in the refrigerator. These bags were connected with a heater to prewarm culture medium and PBS. Prewarmed liquids ran into the tanks inside the MCS to eliminate air bubble. Liquid flow was controlled with tubing pumps and

electromagnetic valves regulated by the PC. The environmental controllers kept the inner temperature at 37 °C, and supplied fresh and clean air through filters inside the MCS. Pipette tips were cleanly stored in a tip pass box with the air lock.

The whole automatic cell culture processes were divided to several tasks, and controlled by computer programs (Table 1, Supplementary video). For cell seeding, corneal epithelial cells and MMC-treated 3T3 cells were separately injected into two 2-mL tubes suspended under centrifuge arms, and cells were centrifuged. The tip manipulator automatically attached a pipette tip from a tip pass box. Cells were resuspended in culture medium by the tip manipulator at an appropriate cell concentration. Then, each cell type was transferred to each tank with the tip manipulator. Used pipette tips were dumped in the dust pass box. In the tank, the cell density was adjusted by diluting culture medium. Then, the cell cartridge handling manipulator moved to the cell cartridge pass box (Fig. 2F). A connector was equipped on the handling manipulator and connected to the circuit including the tank. The inner door of the cell cartridge pass box opened, and the connector was attached firmly to the cell cartridge (Fig. 2G). The cell cartridge integrated with the connector was rotated up 90° to the upright position, cells in each tank were separately poured into each culture room in the cell cartridge, using pumps and valves [31]. After cell injection, the front door of the CO_2 incubator was opened, and the cell cartridge was transported to a turntable in the CO_2 incubator. The cell cartridge was disconnected from the connector, and the connector moved out of the CO_2 incubator. The front door of the CO_2 incubator was closed, and cell culture started. At the same time, three cell cartridges were incubated on the turntable. To fabricate transplantable corneal epithelial cell sheets, cells were cultured for two weeks. In the incubator, temperature, humidity, and CO_2 concentration were kept 37 °C, over 95%, and 5%, respectively. Cells were monitored with a CCD camera with x–z axis movement.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.07.065.

To exchange culture medium in cell cartridges, fresh medium from a liquid bag in the refrigerator was put into a tank through the circuit by the liquid controller with valves and pumps. Then, fresh medium was poured into each culture room in the cell cartridge integrated with the connector at the upright position. Waste liquid was collected in the waste bag in the refrigerator. Finally, the cell cartridge was transported back on a turntable in the CO_2 incubator, and disconnected from the connector.

After two-week culture, the cell cartridges were taken out of the ACCS by the following steps. The cell cartridge was integrated with the connector, and moved onto the tray in the cell cartridge pass box by the handling manipulator. After the cell cartridge pass box was separated from the rest of the ACCS by the air lock, the cell cartridge on the tray was ejected from the pass box to the outside of the ACCS.

Table 1
Automatic cell culture process.

Process	Device
Cell density adjustment	Centrifuge, tip manipulator, liquid controller
Cell cartridge transportation	Handling manipulator
Seeding cells into a cell cartridge	Handling manipulator, pump, liquid controller
Cell culture	Incubator
Medium change	Handling manipulator, pump, liquid controller
Monitoring cells	CCD camera
Environment control	Heater, gas controller

3.2. Transportation of automatically fabricated cell sheets

Often, cell processing facilities where the ACCS can be installed are far away from surgical operating rooms where tissue-engineered products fabricated with the ACCS are transplanted. In order to demonstrate the feasibility of the developed ACCS, we performed transplantation of fabricated cell sheets to an animal model after transportation by car and train. Because of temperature-responsive culture surfaces, the temperature control is a critical issue to avoid the detachment of cell sheets during transportation. Immediately after the automated cell culture, rabbit corneal epithelial cell sheets fabricated with the ACCS in Hitachi Central Research Laboratory (Saitama, Japan) were transported to the Medical School of Osaka University (Osaka, Japan) to transplant them. The distance of the two sites was 581 km. A portable homothermal container (30 cm in diameter of the top, 27.5 cm in height) that allowed a constant inner temperature of 36 °C during transportation was used [29]. The wall of the container had vacuum heat insulation to minimize heat loss. At the core of the homothermal container, a small inner chamber was installed, and the top and bottom of the inner chamber were directly contacted with cupper plates to make heat distribution uniform. Within the inner chamber was filled with a heat shortage material, n-eicosane, in plastic bags. Cell cartridges were placed at the center of the inner chamber during transportation and surrounded by the heat storage material. This container didn't need batteries or energy supply to keep inner temperature at 36 °C. The extra space inside the portable container was occupied by an adiabator to reduce heat loss. With the portable container, cell sheets fabricated on temperature-responsive culture surfaces were transported for 6 h and 20 min by car and train. The portable container kept the inner temperature at 36.1 ± 0.1 °C, although the outer temperature was significantly changed (Fig. 3).

Primary rabbit limbal epithelial cells were seeded at the density of 2.0×10^4 cells/cm^2 into the cell cartridges inside the ACCS (Fig. 4A), and cultured for two weeks. The rate of the viable cells was 93.6%. The rate of putative progenitor cells was 8.9%. During two weeks, the ACCS automatically changed culture medium totally ten times. Cultured cells grew to confluency, and were multi-layered (Fig. 4B). Even after transportation in the container, all the cells in cell cartridges still intactly adhered on the temperature-responsive culture surfaces and no cell morphological changes were observed (Fig. 4C).

After the transportation, cell cartridges were transferred to CO_2 incubator at 20 °C and 5% CO_2 in an operation room for the transplantation. Within 30 min, all the cells were detached from the

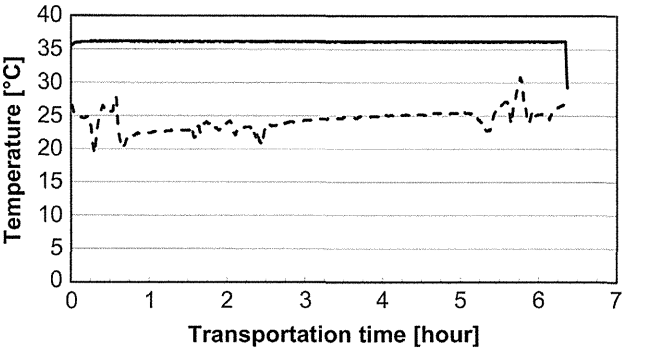

Fig. 3. Temperature change of the inside and outside of the homothermal container. Cell cartridges were transported by train and car for 6 h and 20 min. Each line shows the temperature inside (solid line) or outside the container (dashed line).

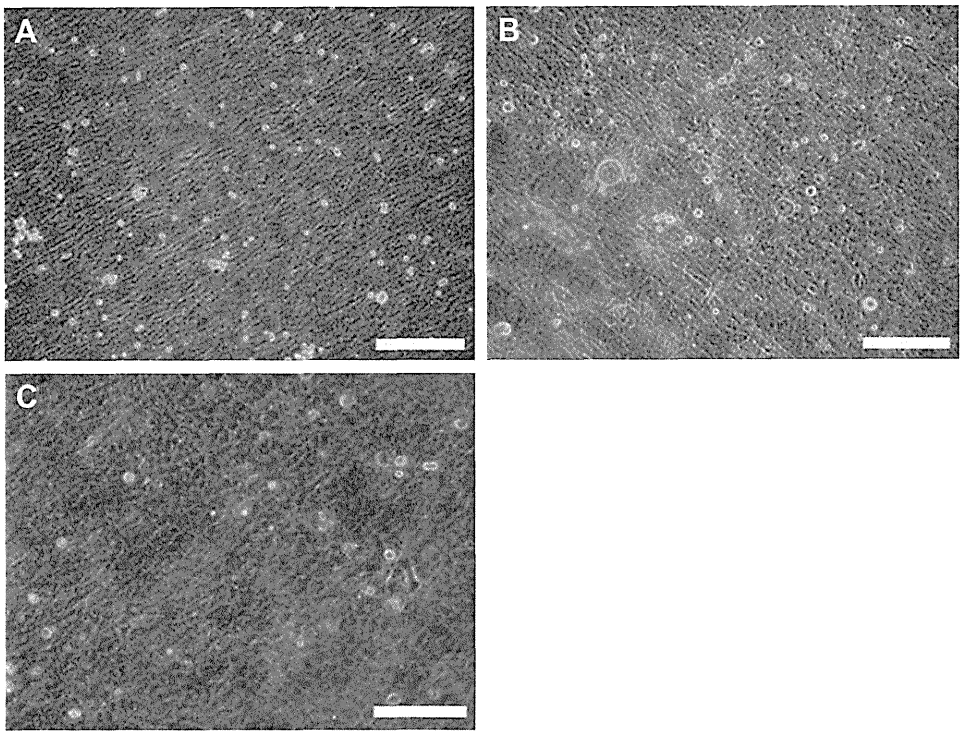

Fig. 4. Phase-contrast microscopic images of cultured rabbit corneal epithelial cells. A, Seeded cells into the cell cartridges using the ACCS. B, Cells cultured for two weeks in the ACCS. C, Cells transported for 6 h and 20 min in the transport container. Bars = 250 μm.

temperature-responsive culture surface as a single continuous cell sheet [8].

Harvested cell sheets were examined by histological evaluation with HE staining and immunofluorescence. The cell sheets comprised of three to five stratified and well-differentiated cell layers (Fig. 5A). p63, a putative epithelial stem/putative marker [32] was detected in the nuclei of the basal cell layers (Fig. 5C). CK3 served as a negative marker for epithelial stem cells [33] was absent from the basal layers (Fig. 5E). These results suggest that harvested cell sheets contain putative stem/progenitor cells in the basal cells.

3.3. Transplantation

Automatically cultured, and transported cell sheets were successfully transplanted onto ocular surfaces in a rabbit corneal stem cell deficiency model [34]. Transplanted corneal epithelial cell sheets readily resisted displacement under tension with forceps, implying stable adhesion to the corneal stroma. Damaged ocular surfaces in the model became clouded (Fig. 6A) and failed to repel fluorescein penetration into the stroma before cell sheet transplant (Fig. 6C). However, corneal surfaces became clear and smooth (Fig. 6B) and were completely protected from fluorescein penetration by grafted cell sheets immediately after a week from transplant (Fig. 6D). Ocular surfaces were completely reconstructed with faint or no observable defects. After observation, rabbits were sacrificed under an anesthetic, and the eyes were examined by histological evaluation with HE staining and immunofluorescence. The transplanted cell sheets existed on stroma surface and gaps between regenerated epithelium and stroma, underlying stromal vascularization inflammatory cells, and goblet cells were not observed in corneas receiving cell sheet-transplants (Fig. 5B). p63 was detected in the nuclei of the basal cell layers (Fig. 5D). CK3 was absent from the basal layers, but expressed in the mature corneal epithelium (Fig. 5F). These results imply that reconstructed ocular surfaces had basal layers containing putative stem/progenitor cells, and multi-layered epithelial structure resembling native corneal epithelium morphologically.

4. Discussion

Conventionally, the culture operation to fabricate transplantable epithelial cell sheets was manually performed using open culture vessels such as 6-well plates with culture inserts [9]. Nowadays, in order to improve the efficiency and safety of cell culture process, several ACCSs have been developed. ACCS which has a robotic arm is already commercially available [22–25]. These ACCSs are designed to use conventional open culture vessels, and mimic the manual process as closely as possible to avoid process modifications based on assumptions about the sources of process output variation. Continuous operation of the robotic arm achieves scale-up of cell culture and fabrication of tissue-engineered products [23]. However, it is quite difficult for ACCSs which has a single robotic arm to handle culture inserts in 6-well plates that are commonly used to fabricate epithelial cell sheets. When an additional robotic arm is installed, the size of ACCSs should be bigger, and the ACCSs need larger air-conditioning units with HEPA filter. In addition, the use of conventional open vessels such as culture inserts in 6-well plates would not be appropriate for transportation in clinical settings, and require a special container to prevent contamination.

An ACCS using a closed culture vessel connected to a closed circuit was also reported [26]. This system was designed to expand bone marrow-derived mesenchymal stem cells. Since this system uses its original culture vessels, it doesn't accept epithelial cell culture using feeder layer separated by microporous membrane. An FDA-approved, tissue-engineered product, Dermagraft is fabricated

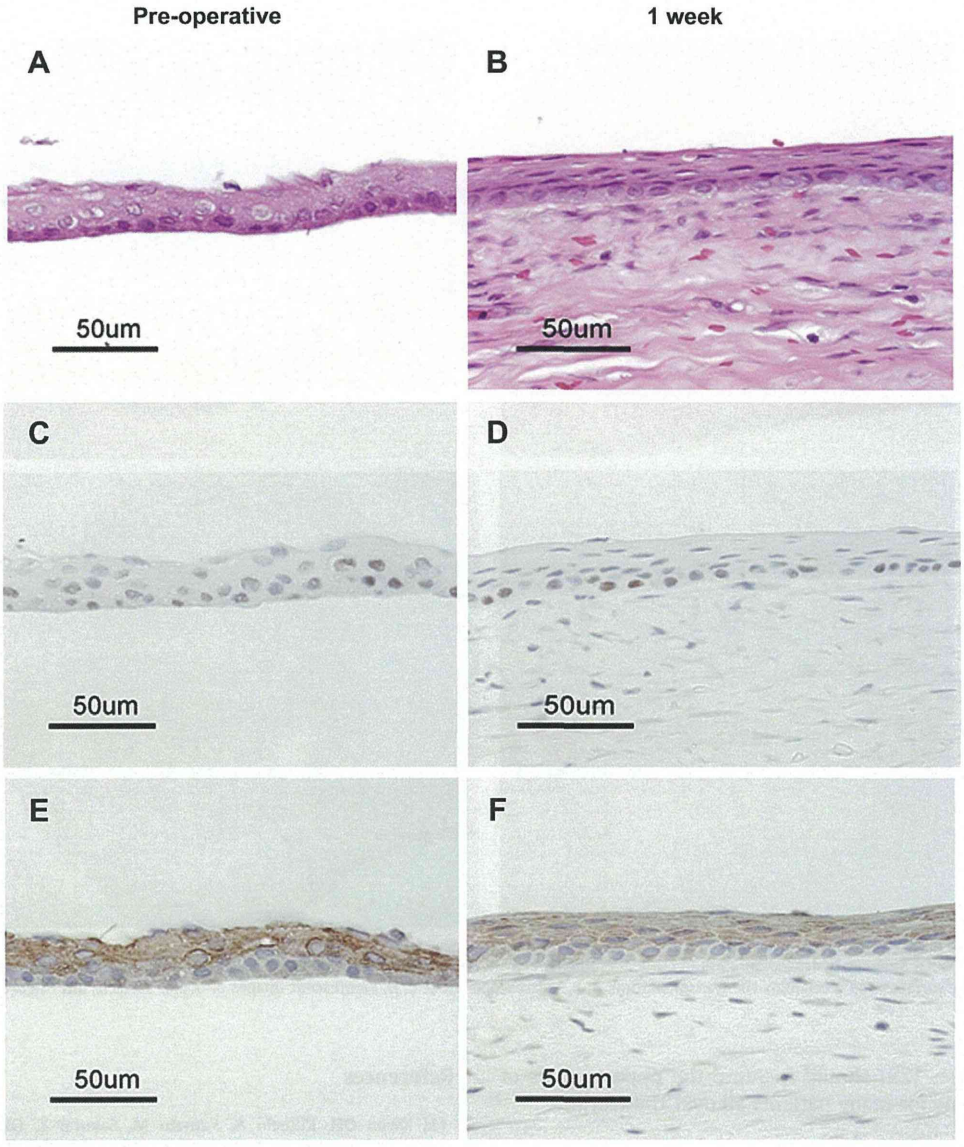

Fig. 5. Histology. Left panels show transplantable epithelial cell sheets fabricated with the ACCS. Right panels show rabbit cornea one week after the transplantation. A, B, HE staining. C, D, Immunohistochemistry with anti-p63 antibody. E, F, Immunohistochemistry with anti-CK3 antibody. Bars = 50 μm.

with its customized bioreactor using closed culture vessels connected to a closed circuit [35]. After culture of foreskin dermal fibroblasts on biodegradable scaffolds in the vessels, each vessel containing an individual piece of Dermagraft was sealed, and then cut out of the bioreactor. This individual piece of a closed culture vessel is safe to transport, and shipped to the customer. Since Dermagraft is an allogeneic product and mass production in each lot is performed, the design of the closed culture vessel is appropriate. Since transplantable epithelial cell sheets to treat limbal stem cell deficiency are fabricated with patients' own cells in an autologous manner, small production for each patient is performed.

In the present study, a prototype of the ACCS based on a concept using double-layered closed cell culture vessels [28] and miniaturization of MCS was developed. The cell cartridge was connected to a closed circuit only in cell seeding and medium change, then disconnected and cultured [31]. The closed culture vessels were tiny and easy to transport. Actually, we successfully performed automatic fabrication of transplantable corneal epithelial cell sheets with the ACCS, transported them with a portable homothermal container [29], and finally transplanted to a rabbit corneal stem cell deficiency model. The ocular surfaces were reconstructed one week after transplantation as previously shown with manually fabricated cell sheets [8].

Since the size of MCS in the present ACCS was small, a number of MCSs culturing cells of different patients can be connected with a single refrigerator and a single controller. Therefore, autologous products for different patients can be fabricated at one time in a single clean room of CPF. Since the employment and training of operators can be reduced to the minimum by using ACCSs, the production cost can be saved, and the safe and stable mass production of tissue-engineered products can be achieved.

In the future, manual productions of tissue-engineered products in expensive and large facilities would be completely replaced with automated factories full of ACCSs as observed with automobile and semiconductor industries. New technologies such as ACCSs and

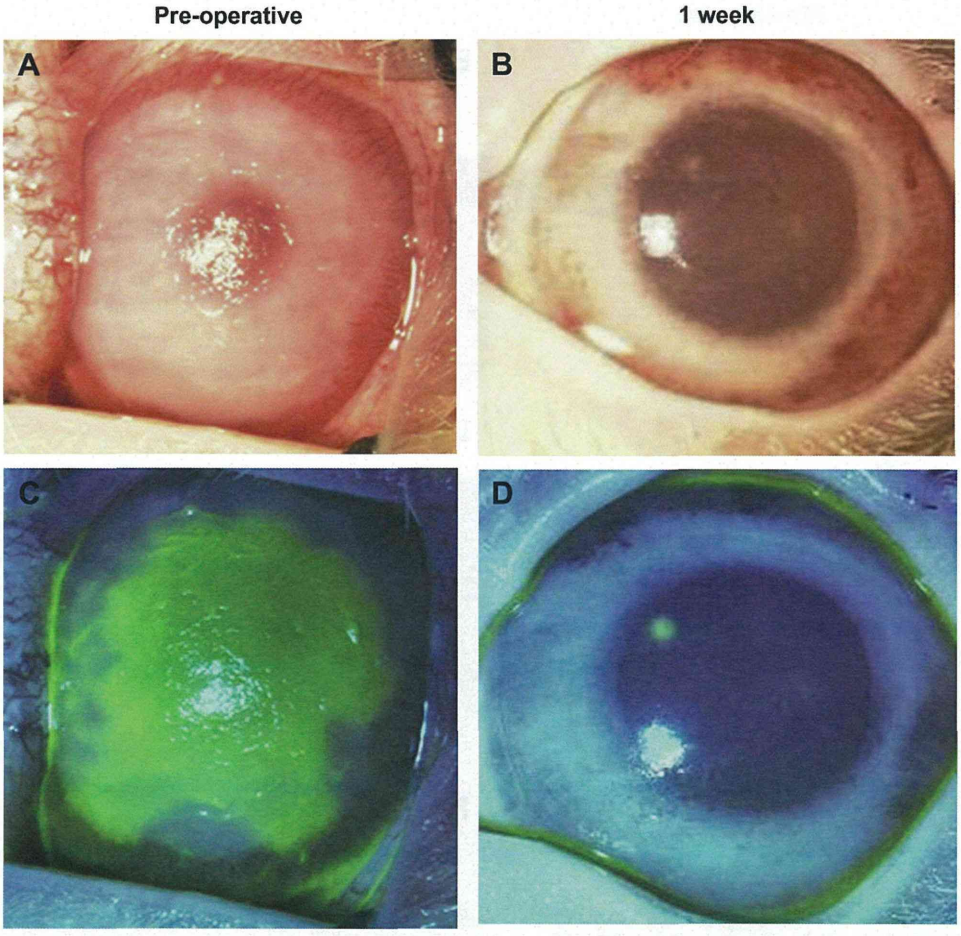

Fig. 6. Reconstruction of ocular surfaces by transplantation of corneal epithelial cell sheets fabricated with the ACCS. Left panels show preoperative ocular surfaces. Right panels show reconstructed ocular surfaces one week after the transplantation. A, B, Macroscopic view. C, D, Fluorescent images of ocular surfaces after fluorescein staining.

transportation devices [29] should increase the opportunities of regenerative medicine for many patients all over the world.

5. Conclusions

Here, we developed a prototype of a small automated cell culture system with closed culture vessels called cell cartridge. Transplantable corneal epithelial cell sheets were automatically fabricated with the system, transported, and successfully transplanted rabbit corneal stroma. Such the devices would promote regenerative medicine in the future clinical settings.

Acknowledgments

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Splitting culture medium by air-jet and rewetting for the assessment of the wettability of cultured epithelial cell surfaces

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ABSTRACT

This study found that the phenomenon of rewetting after squeezing culture medium varied in different culture conditions for rat oral mucosal epithelial cells. When culture medium covering over cultured cells was squeezed by an air-jet application, the motion of squeezed culture medium was able to be observed by using a commercially available movie camera. Squeezed width on cells cultured in keratinocyte culture medium (KCM), which contained with fetal bovine serum, was one-sixth of that in FBS-free KCM. This result corresponded to the mucous layer staining statuses of cultured cells in both cases; positive in KCM and negative in FBS-free medium. Furthermore, the gene expression of mucous glycoprotein MUC4 in KCM was 100 times higher than that in FBS-free medium, and the expression of MUC4 protein only showed on the apical surface of cells cultured in KCM. The relative gene expression levels of MUC1, 13, 15, and 16 in both the normal and FBS-free medium were found to be no more than one-thirtieth of that of MUC4 in KCM. The main factor of the wettability difference between KCM and FBS-free medium was speculated to be the difference of MUC4 expression between both media. This method can be a simple technique for testing not only the surface wettability but also the mucous formation of cultured cells.

1. Introduction

Generally, the surfaces of digestive tract [1], eye [2], etc. are covered with mucosal epithelium, which secretes and retains mucous layer. Mucous layer is a valuable structure for keeping an epithelial barrier function against pathogens and chemical irritants [3–5]. Mucous layer is composed of mainly mucin proteins, which is a glycoprotein with a structure where core protein is conjugated with hydrophilic glycans [6–8]. Therefore, the surface of mucous epithelium is a high wetting characteristic. The lost of the mucous layer on the surface of epithelia immediately gives infections, an ulcer, and the diseases of poor surface lubrication such as dry eye to the layer of mucous epithelium [9,10]. For dry eye syndrome, a noninvasive system for assessing the stability of tear on cornea has been investigated and applied to clinical use [11,12].

To date, regenerative medicine using cell sheets has been successfully established for replacing a dysfunction tissue such as the skin [13], cornea [14,15], esophagus [16], heart [17,18], and periodontal tissue [19]. Cell sheets are a thin membrane composed of cultured cells at 37 °C and harvested from a temperature-responsive cell-culture surface by reducing temperature to 20 °C. For preparing various types of cell-sheets, various types of cells are cultured at 37 °C on the surfaces [20]. Especially, oral mucosal epithelial cell sheets have been transplanted on the lost part of mucosal epithelia surface such as the damaged cornea epithelia and the esophageal ulcer after cancer dissection. In mucosal epithelial cell culture, the gene expression of some glycoprotein composing a mucous layer has changed between primary (uncultured) and cultured tissues [21,22]. Because the appearance of mucous protein affects the wettability of cell surface, wettability characterization by the measurement of contact angle on the surface of corneal epithelial explants has been reported [23,24]. However, it is difficult to measure the contact angle of cultured-cell surface in culture status. Therefore, a new method for assessing the wetting characteristic of cultured-cell surface has been strongly demanded.

This study developed an assessing method for evaluating the wetting characteristic of culture-cell surface (Fig. 1). After an air-jet application, which is inspired by our previous study [25], to the

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