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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.

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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.

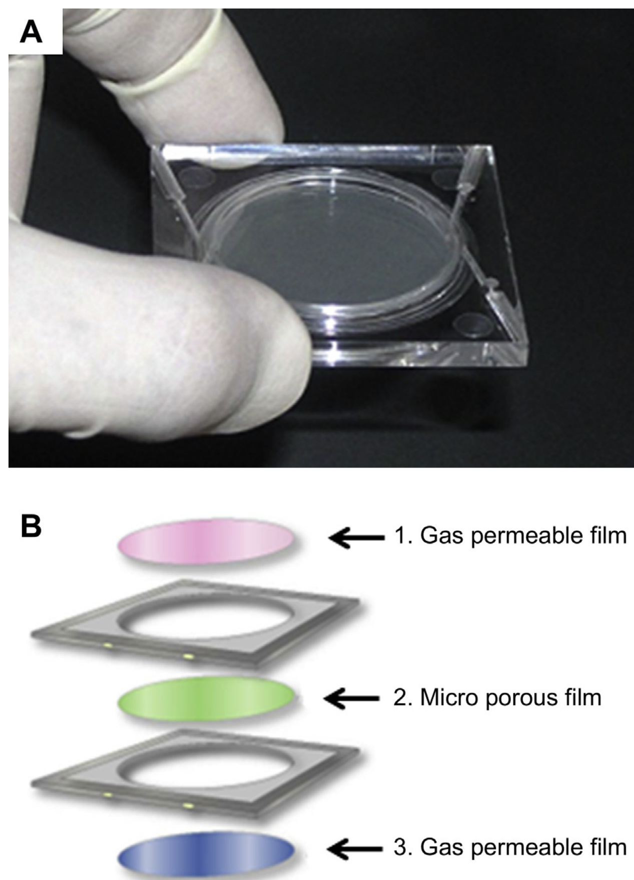

Fig. 1. A new culture vessel called cell cartridge. A, The slanting front view of the cell cartridge. B, Its inner structure. The cell cartridge had two rooms for epithelial cells and feeder layer cells separated by the microporous (0.4 μm) film on which a temperature-responsive polymer, poly(*N*-isopropylacrylamide)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

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