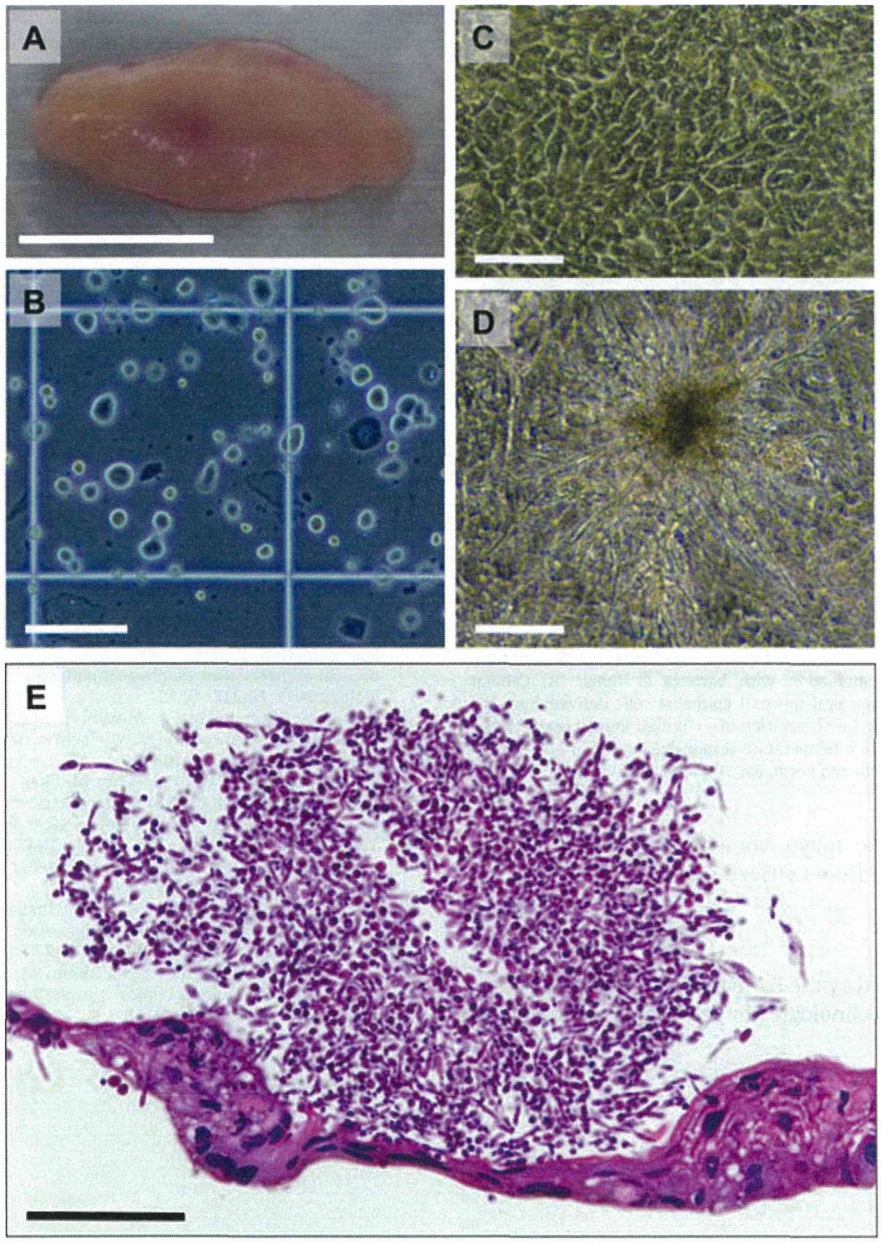

Fig. 1. *Candida albicans* (*C. albicans*) proliferating in the cell culture supernatant of human oral mucosal epithelial cells in this clinical study. (A) Human oral mucosal tissue of the patient. Bar = approximately 1 cm. (B) Oral mucosal epithelial cells derived from the patient after cell preparation. Bar = 100 μm. (C) Cellular morphology of the cultured human oral mucosal epithelial cells. Bar = 100 μm. (D) *C. albicans* observed on the cultured epithelial cells in a culture vessel. Bar = 100 μm. (E) Histological observation of the *C. albicans* adhering to a cultured epithelial cell sheet harvested from a temperature-responsive culture insert. The cell sheet and *C. albicans* were stained with hematoxylin and eosin. Bar = 50 μm.

patient, and the cultured epithelial cells were successfully harvested as cell sheets (Table 1, Fig. 2). To maintain a sterile environment, the temperature-responsive cell culture inserts to which the cultured epithelial cell sheets adhered were placed in transportable containers while in the safety cabinet of a clean room specialized for fabricating transplantable cell sheets for a clinical setting. The containers were then transported to Nagasaki University Hospital in the transportation box, which was mounted on a hot plate to keep the temperature at 37 °C. After transport, the epithelial cell sheets were finally transplanted onto the esophageal ulcer of the patient after endoscopic dissection to remove esophageal cancer.

Here, we have reported our experience of contamination with *C. albicans* during the fabrication of transplantable oral mucosal epithelial cell sheets derived from a patient who was not suffering from candidiasis. By adding 1 μg/mL amphotericin B to the transportation medium, fungal proliferation was completely inhibited and esophageal mucosal regeneration was successfully observed. Therefore, the method described in this report should be useful for preventing contamination with *C. albicans* without increasing the concentration of amphotericin B in the culture medium.

Disclosure statement

Teruo Okano is a founder and director of the board of CellSeed Inc., licensing technologies and patents from Tokyo Women's Medical University. Teruo Okano and Masayuki Yamato are

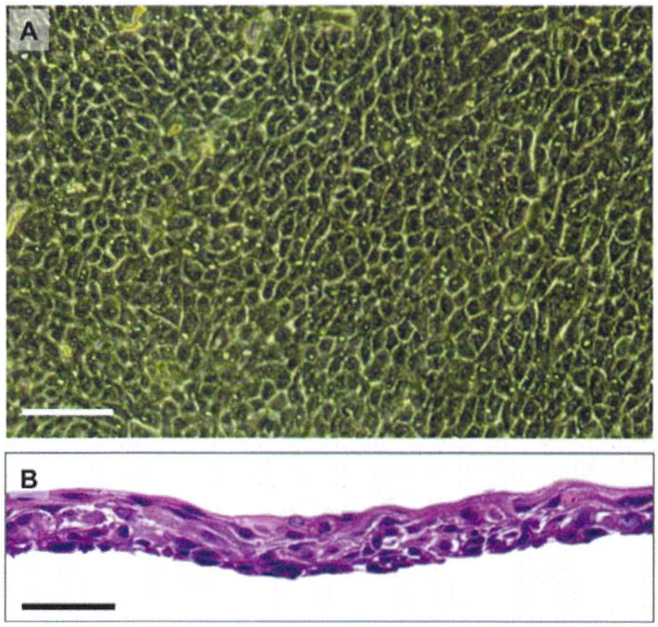

Fig. 2. Second trial of cultivation of human oral mucosal epithelial cells derived from the same patient, without contamination with bacteria or fungi. (A) Cellular morphology of the cultured human oral mucosal epithelial cells derived from the patient. Bar = 100 μm. (B) Histological observation of a cultured human oral mucosal epithelial cell sheet harvested from a temperature-responsive culture insert. The cell sheet was stained with hematoxylin and eosin. Bar = 50 μm.

shareholders of CellSeed Inc. Tokyo Women's Medical University is receiving research funding from CellSeed Inc.

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NEW METHODS

Endoscopic cell sheet transplantation device developed by using a 3-dimensional printer and its feasibility evaluation in a porcine model

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Background: To prevent severe esophageal stenosis after aggressive endoscopic submucosal dissection (ESD), our group previously reported an efficient treatment using cell sheets that had been fabricated from patient cells. However, this transplantation procedure had not been easy for every endoscopist and needed to be improved to derive the full effect of epithelial cell sheets.

Objective: To develop an endoscopic device that enables easy and effective cell sheet transplantation and to evaluate its performance and clinical feasibility.

Design: Animal study.

Setting: Animal experimentation laboratory.

Intervention: Three pigs underwent circumferential esophageal ESD while under general anesthesia. A total of 12 cell sheets were endoscopically transplanted to the ESD site; 6 cell sheets were transplanted by using an endoscopic device that we developed, and 6 cell sheets were transplanted by using the conventional method.

Main Outcome Measurements: Procedure time, transplanted area on the ESD site, transplantation success rate, and monitoring of adverse events or incidents.

Results: The device allowed successful transplantation of all cell sheets with a shorter procedure time than with the conventional method (4.8 ± 0.8 minutes vs 13.3 ± 5.7 minutes, respectively) ($P = .005$) and onto a larger area (111.3 ± 56.3 mm^2 vs 41.8 ± 4.2 mm^2, respectively) ($P = .023$) with a higher success rate (100% vs 83%, respectively). No adverse incidents were monitored in each method.

Limitations: Animal study, small sample.

Conclusion: A newly designed endoscopic cell sheet transplantation device would be useful.

BACKGROUND

Esophageal stenosis is one of the major adverse events after aggressive endoscopic submucosal dissection (ESD) for early-stage esophageal cancer.[1-4] For treating stenosis, endoscopic balloon dilation has been widely used, although repeated stenosis is still an issue.[2] Clinical research involving steroid therapies[5-7] and stent treatments[2,8,9] have been studied to overcome esophageal stenosis after aggressive ESD.

Abbreviation: ESD, endoscopic submucosal dissection.

DISCLOSURE: *Dr Okano is a founder of CellSeed Inc, is a member of the Board of Directors of CellSeed Inc and has licensed technologies and patents from Tokyo Women's Medical University related to this study and is a shareholder in CellSeed Inc. Dr Yamato is a consultant and shareholder in CellSeed Inc. All other authors disclosed no financial relationships relevant to this article. This study was supported by Grant-in-Aid for Scientific Research (14483609 to Dr Kanai), Cell Sheet Tissue Engineering Center (CSTEC), The Creation of Innovation Centers for Advanced Interdisciplinary Research Areas Program" from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), and a Health Labor Scientific Research Grants (26220101 to Dr Okano and 26270801 to Dr Yamato).*

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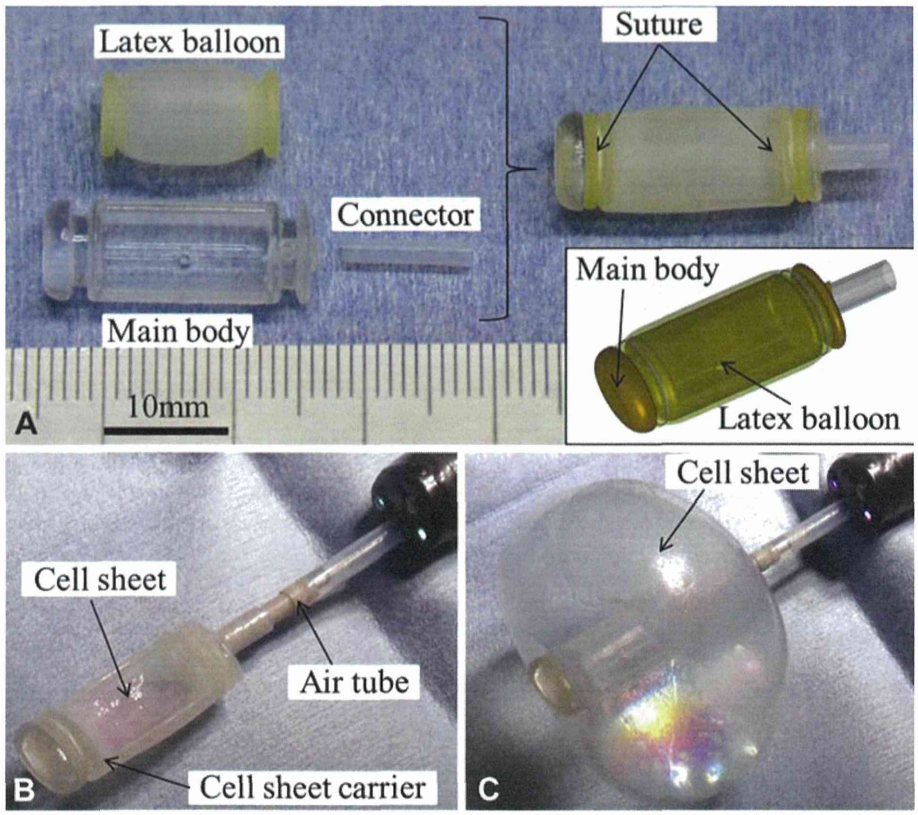

Figure 1. The endoscopic cell sheet transplantation device composed of a cell sheet carrier and an air tube. **A,** The structure of a cell sheet carrier. A latex balloon for US endoscope (MH-525; Olympus) was covered on the main body and secured at both sides with surgical sutures, and a connector (polytetrafluoroethylene tube) was glued at the proximal end of the main body. **B,** Delivery mode. After a cell sheet carrier was connected to an air tube that was passed through a biopsy channel (>2.8 mm), a cell sheet was loaded on a balloon and stored inside of the device by balloon deflation for easy delivery. **C,** Transplantation mode. The cell sheet could be expanded and adhered to an ulcer site for transplantation by balloon inflation.

Our group previously reported a regenerative approach in which autologous epithelial cell sheets were transplanted onto an ESD site to promote structural and functional re-epithelialization.[10,11] Cell sheets are fabricated from a patient's cells in our original designed temperature-responsive culture dishes,[12] and the thickness is usually less than 0.1 mm. A cell sheet can be grafted by just placing it on an ESD site without suturing or glue due to the existence of extracellular matrix underneath the cell sheet.[13] Several clinical studies have had good results.[14] To date, the cell sheets have been transplanted as previously reported.[10,11,14] Briefly, after a cell sheet was harvested on a round plastic membrane (~8 mm in diameter), it was delivered and placed on an ESD site by using conventional endoscopic forceps. Because the current transplantation procedure has not been easy for all endoscopists, we developed a novel device that allows easy delivery and transplantation of cell sheets. In this study, the clinical feasibility and the superiority of the device were evaluated in an in vivo porcine model.

METHODS AND MATERIALS

All animal experiments were performed according to the Guidelines of Tokyo Women's Medical University on Animal Use.

Prototyping for a novel endoscopic device

The device was composed of a cell sheet carrier (Fig. 1A) and an air tube. A cell sheet carrier was made of a main body fabricated by a 3-dimensional printer with a biocompatible plastic material (Objet 350 and MED610; Stratasys, Eden Prairie, Minn), a latex balloon for a US endoscope, a polytetrafluoroethylene tube, and surgical sutures. A balloon was covered on a main body and tied at both ends with a suture. A polytetrafluoroethylene tube was glued at the proximal end for a connector to an air tube. The cell sheet carrier was connected to an air tube which was passed through an endoscope's biopsy channel (>2.8 mm). An operator can supply the air into the balloon for expanding or can suck the air from the balloon. The air tube

TABLE 1. Study results

Transplantation order	Animal	Transplantation method	Name of cell sheet	Procedure time, min:s	Transplanted area, mm^2	Adverse events
1	1	Membrane	M-1	15:03	38.0	None
2		Membrane	M-2	9:01	40.7	None
3		Membrane	M-3	23:43	46.0	None
4	2	Device	D-1	4:45	163.6	None
5		Device	D-2	6:16	32.6	None
6		Device	D-3	4:33	175.5	None
7		Membrane	M-4	10:10	38.0	None
8		Membrane	M-5	13:30	— (detached)	None
9		Membrane	M-6	8:32	46.4	None
10	3	Device	D-4	4:29	131.4	None
11		Device	D-5	3:47	100.6	None
12		Device	D-6	4:52	63.8	None

could transfer linear rotation torque to the cell sheet carrier.

A harvested cell sheet was loaded and kept in place on the balloon with a little water by water surface tension. The cell sheet was then stored inside the device by air suction because the balloon with the expandable cell sheet was adhered to the inner wall of the device (Fig. 1B) and easily delivered through an overtube into an esophagus. Conversely, the cell sheet could be attached to an ESD site for transplantation by balloon inflation (Fig. 1C).

Fabrication of human skin cell sheets

Normal human epidermal keratinocytes (Lonza, Basel, Switzerland) were cultivated on temperature-responsive cell culture inserts (CellSeed, Tokyo, Japan) for 14 days at 37°C.[15] They were harvested as a cell sheet (~18 mm in diameter) by reducing the temperature to 20°C for 30 minutes.

Animal experiments: transplantation procedure time

Three miniature pigs (6 months old, 16-20 kg, female, from the Nippon Institute for Biological Science) got artificial ulcerations (270°-360° in range, 5 cm in length) in the lower esophagus by applying the hook-knife method of ESD, by using a GI endoscope (GIF-XQ260; Olympus, Tokyo, Japan) and a hook-knife (KD-620 LR; Olympus). An overtube (EMR tube; Create Medic, Tokyo, Japan) was then inserted into the esophagus. Twelve cell sheets were individually transplanted to the ESD sites by using the device or a conventional method as shown in Table 1. Procedure times (endoscope insertion time) were monitored.

TABLE 2. Study results: statistical analysis

Outcome	Device method (n = 6)	Membrane method (n = 6)	*P* value
Transplantation success rate, no. (%)	6 (100)	5 (83)	.341
Procedure time, mean ± SD, min	4.8 ± 0.8	13.3 ± 5.7	.005
Transplanted area, mean ± SD, mm^2	113.0 ± 56.3	41.8 ± 4.2	.023

Macroscopic analysis: transplantation success rate and area

All animals were killed 6 hours after transplantation. The esophagi were extracted and cut open. The macroscopic views were photographed, and the transplanted cell sheets were counted to calculate the transplantation success rate (%) (number of cell sheets adhered to the esophagus/number of cell sheets attempted to be transplanted × 100). Each area was then covered by a cell sheet was calculated by using image analysis software (Image J; U.S. National Institutes of Health, Bethesda, Md), and the statistical analysis was performed by using a 2-tailed unpaired Student *t* test.

Histological analysis

To confirm the basal layer of the transplanted cell sheets adhered to host tissues, immunohistochemistry was performed with antibodies anti-cytokeratin 14 (MAB9766; Abnova Corporation, Taipei, Taiwan) and anti-p63 and anti-integrin β4 (SC-8431, SC-9090; Santa Cruz Biotechnology, Dallas, Tex).

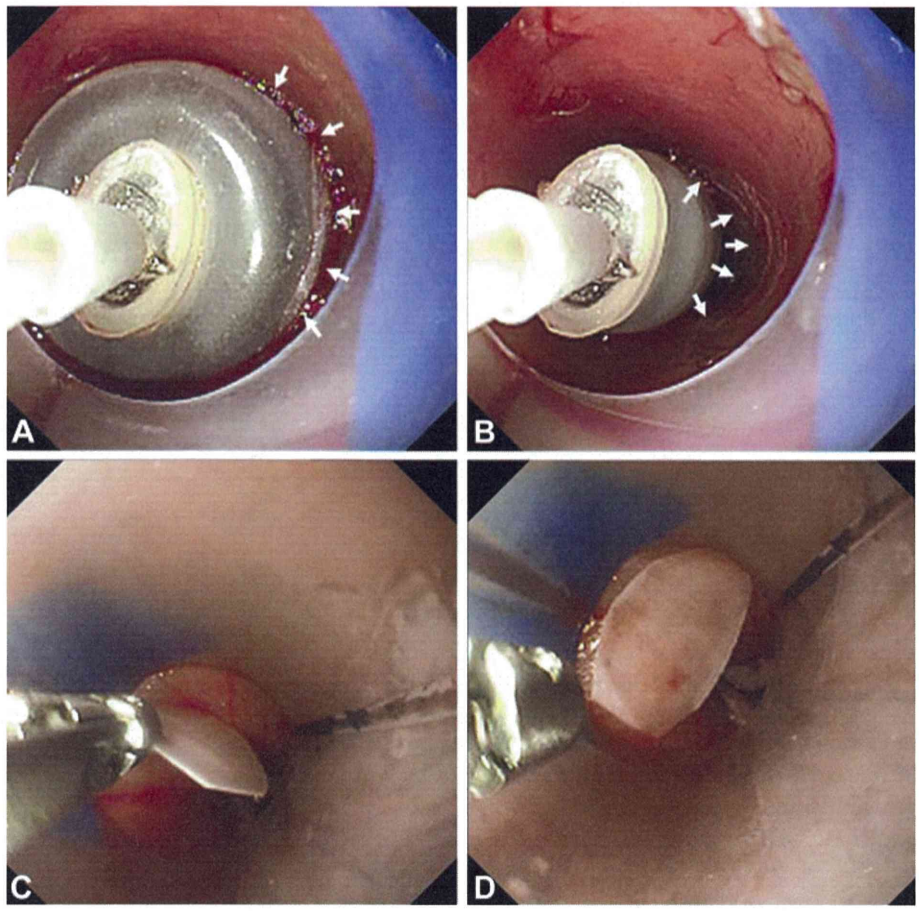

Figure 2. Endoscopic views of a cell sheet transplantation. **A, B**, The cell sheet transplantation device method. After delivery of the cell sheet through an overtube to the ulcer site, the positions of the cell sheet and ulcer site were aligned by insertion/extraction and rotation of the device. The balloon was then inflated and attached the cell sheet to the ulcer site for approximately 30 seconds. The balloon was then deflated and the cell sheet was successfully transplanted. **C, D,** The conventional support membrane method. The support membrane with a cell sheet was carefully attached to the ulcer site by using conventional endoscopic forceps. The transplantation procedure was completed after pushing several points of the back side of the support membrane and waiting for more than 5 minutes to confirm that the support membrane did not become detached.

RESULTS

The results of this study are summarized in Tables 1 and 2.

Cell sheet transplantation

The cell sheet transplantation device method is shown in Figures 2A and 2B. After a cell sheet was stored inside the device by vacuuming approximately 1.5 mL of air, the device was then passed through an overtube to the ESD site. The balloon was inflated slowly by supplying approximately 15 mL air, and the cell sheet was kept attached for approximately 30 seconds. After the balloon deflation, the cell sheet was confirmed to be transplanted. Five other cell sheets were also successfully transplanted in a similar manner with no adverse events.

The conventional support membrane method is shown in Figures 2C and 2D. A round support membrane with a cell sheet was grasped and delivered to the ESD site through an overtube; it was then attached and the membrane was immediately pushed several times. After confirming no detachment for more than 5 minutes, the endoscope was withdrawn.

Macroscopic analysis: transplantation success rate and area

The statistical analysis of the transplantation success rate and area is summarized in Table 2.

Histological analysis

The histology of the cell sheets transplanted by the device is shown in Figure 3. The basal layers of the cell sheets, which expressed integrin β4, p63, and CK14, were adhered to the host tissue.

DISCUSSION

The cell sheet transplantation device provides stable and easy transfer of therapeutic materials by storing them inside by balloon deflation, and is assumed to deploy

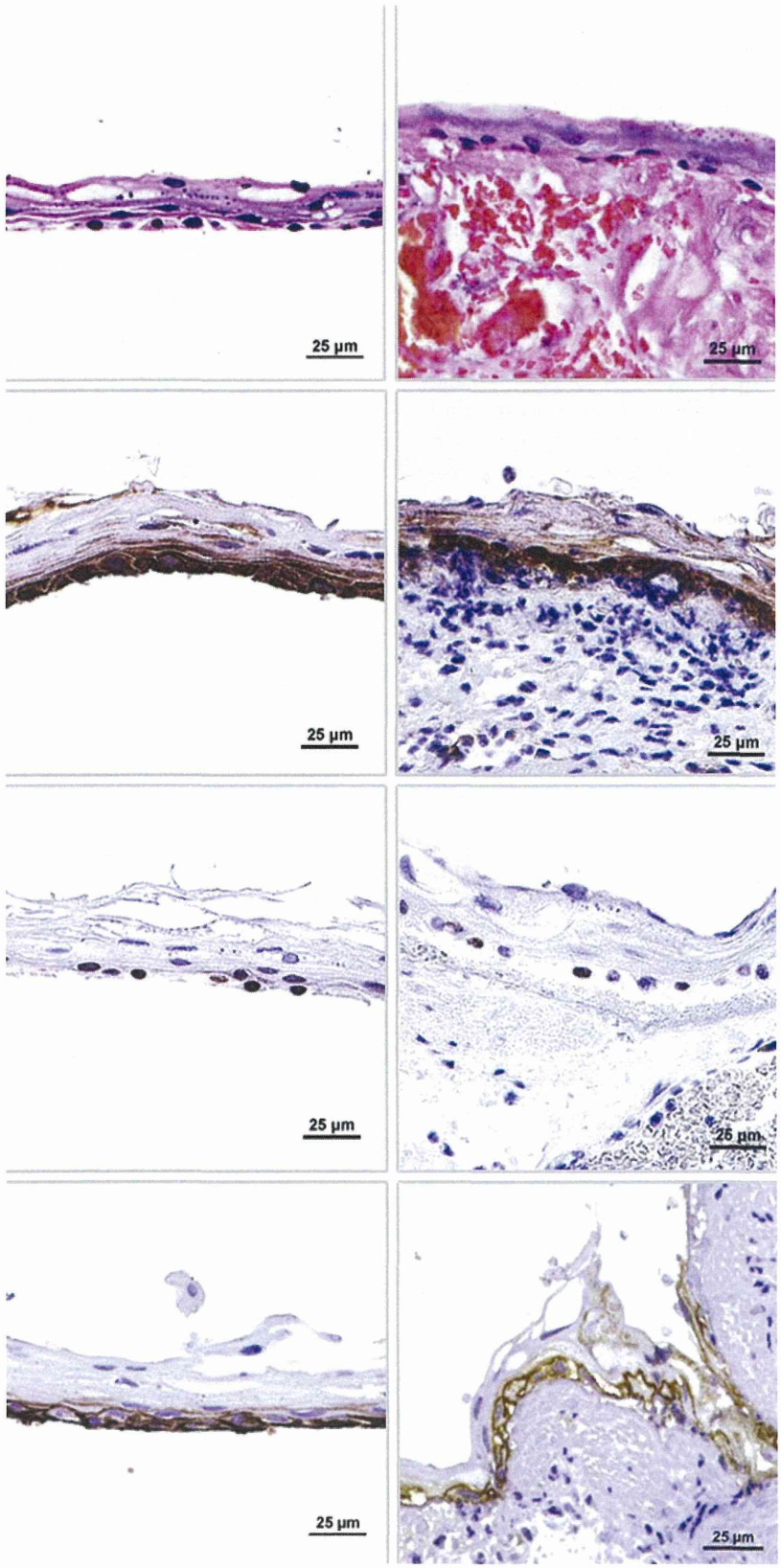

Figure 3. Histology of fabricated cell sheets and transplanted cell sheets by using the transplantation device 6 hours after transplantation (H&E, cytokeratin 14, p63, β4; orig. mag. ×40). The basal cell epithelium of a transplanted cell sheet using the device was confirmed to adhere to the host tissue while maintaining the basic components of cell sheets, such as cell–cell junctions and integrin expression.

conventional polyglycolic acid sheet[16,17] or gel[18,19] for preventing perforation or bleeding after ESD.

In this first feasibility study, the device enabled successful transplantation of almost all cell sheets onto a larger area in a shorter time with no adverse events than with the conventional method. Adhesion of the basal layers of transplanted cell sheets onto the ulcer site was histologically confirmed. These results support the feasibility of the device. Furthermore, the device might improve the effectiveness of cell sheet therapy because transplantations in larger areas would be more efficient for preventing stenosis in our experience (Kanai et al, manuscript in preparation).

We used a 3-dimensional printer that allowed a quick turnaround for improving the device with biocompatible material. We believe some devices that are fabricated by using a 3D printer could be available in human clinical trials.

In conclusion, a novel endoscopic device for cell sheet transplantation was designed. Improvements are now in progress for a human clinical study.

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Chapter 1

Cell Sheet Engineering for Periodontal Regeneration

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Isao Ishikawa, Tomohiro Ando,
Masayuki Yamato and Teruo Okano

Additional information is available at the end of the chapter

1. Introduction

Periodontitis is a world-wide infectious disease that destroys the tooth-supporting attachment apparatus, which consists of alveolar bone, cementum, and periodontal ligament. Recent studies have reported numerous associations between periodontitis and systemic diseases, such as cardiovascular disease (de Oliveira et al., 2010) and diabetes mellitus (Lalla and Papapanou, 2011), as well as a higher risk of preterm low birth-weight babies (Offenbacher et al., 1996). Furthermore, researches have recently shown that Bisphosphonate-Related Osteonecrosis of the Jaws (BRONJ) is also associated with severe periodontitis (Vescovi et al., 2011). Therefore, periodontal treatment may not only contribute to oral hygiene but also improvement of systemic conditions (Seymour et al., 2007). Conventional treatments, such as scaling, root-planing, and surgical cleaning, have been performed to remove the bacteria and contaminated tissue. However, these procedures frequently result in the formation of a weak attachment, a condition termed "long junctional epithelium (LJE)" (Caton et al., 1980), wherein the patients tend to present with a recurrence of disease without maintenance therapies (Axelsson and Lindhe, 1981). To overcome this problem, various regenerative therapies, such as guided tissue regeneration (GTR) and enamel matrix derivative, have been introduced in clinical practice. The use of cell-occlusive membranes for GTR is regarded as the first generation of periodontal regeneration, whereas the development and use of growth factors and endogenous regenerative technology for periodontal regeneration is regarded as the second generation of periodontal regeneration (Ishikawa et al., 2009). However, the outcomes of these studies were limited and associated with poor clinical predictability (Esposito et al., 2009). Therefore, stem cell-based approaches for periodontal regeneration have been studied and translated into clinical settings as the third

generation. In this chapter, we would like to describe the principles of "Cell Sheet Engineering" and its application of clinical settings, featuring our recent translational research for periodontal regeneration.

2. "Cell Sheet Engineering (CSE)"

The cell delivery for periodontal regeneration is usually performed with the combination use of cells and scaffolds, although the location and the differentiation of transplanted is difficult to control. In contrast to approaches that utilize scaffolds, we have developed an alternative technology for cell transplantation using temperature responsive culture dishes, which we call "Cell Sheet Engineering".

2.1. Intelligent surface of *N*-isopropylacrylamide (PIPAAm) and fabrication of cell sheets

Poly(*N*-isopropylacrylamide) (PIPAAm) is a temperature responsive polymer that has been widely utilized for novel biomedical applications. We have developed a PIPAAm-grafted surface as a smart biointerface wherein cell attachment/detachment can be easily controlled by simply changing the temperature (Okano et al., 1995; Yamada et al., 1990). This surface is slightly hydrophobic under cell culture conditions of 37 °C, but readily becomes hydrated and hydrophilic below its lower critical solution temperature (LCST) of 32 °C. Cells can adhere, spread, and proliferate similarly to that on ungrafted tissue culture grade polystyrene surfaces at 37 °C (Figure 1A), and cells detach from the surface by reducing temperature below LCST, making it possible to harvest the cells from the culture surfaces without the use of proteolytic enzymes (Figure 1B).

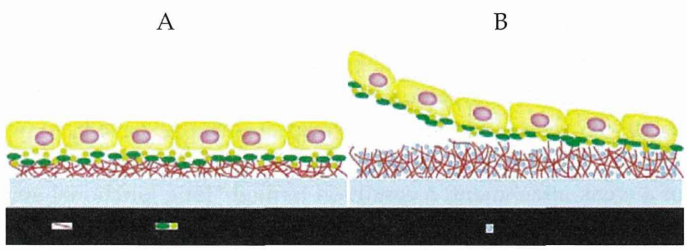

Figure 1. The principle of "Cell Sheet Engineering".

A: Cells can attach and proliferate on grafted surface of the temperature responsive polymer (poly (N-isopropylacrylamide: PIPAAm) at 37 °C, wherein PIPAAm is extensively dehydrated and compact. B: At temperatures below 32 °C, cells with extracellular matrix proteins spontaneously detach from the temperature responsive culture dishes, wherein PIPAAm is fully hydrated with an extended-chain conformation. A simple temperature change can control cell attachment/detachment without any damages. Modified and reprint from Iwata et al., 2013.