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FORMATION OF EPITHELIAL SHEETS BY SERIALLY CULTIVATED HUMAN MUCOSAL CELLS AND THEIR APPLICATIONS AS A GRAFT MATERIAL

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ABSTRACT

A cultured epithelial sheet can be formed from living mucosal cells in vitro and used as a graft material. In this article, we describe our culturing methods for the preparation of mucosal epithelial sheets as well as the biological characteristics of these sheets compared with those of skin epithelial sheets. A cultured epithelial sheet has 5 to 8 cell layers and sufficient mechanical strength to be used as a graft material. It takes 12 days to form an epithelial sheet from small epithelial segments as compared with 14 days in the case of a skin epithelial sheet. Furthermore, viability of mucosal epithelial sheets was maintained for 30 days in vitro as opposed to 22 days for skin epithelial sheets. Based on the findings from an in vitro study, we applied this cultured mucosal epithelium to humans for reconstruction of skin and mucosal defects and succeeded in repairing the defects. This report also presents an overview of the problems relevant to the use of such methods.

INTRODUCTION

The method of keratinocyte culture employing 3T3 developed by Rheinwald and Green[1] as the feeder layer made it possible to culture keratinocytes in large amounts. Utilizing this method, O'Corner[2] and Galico et al.[3] reported on the use of a cultured epithelial sheet graft in a burn patient. The advantage of skin reconstruction using a cultured epithelial sheet graft is that a large epithelial sheet can be obtained from a small segment of skin. According to Green et al.[1] 6,000cm^2 of cultured epithelium was produced from 1cm^2 of skin in three weeks and was successfully transplanted. The entire grafted epithelium was reported to have survived without rejection. Since the report of Green et al. many reports[4-11] have been published on cultured epithelial grafts. Because this method of treatment has many advantages, it has gradually come into the general clinical practice of plastic surgery.

As more cases were treated with this method, some problems arose in this seemingly perfect method of treatment. About three weeks are required to make an epithelial sheet, making it difficult to meet the requirements of emergency surgery, which is the first problem. The second problem is that skin keratinocytes can easily make terminal differentiation and become keratinized in vitro, losing their viability.

On the other hand, the mucosal epithelial cell is well known to be at a lower stage of differentiation than the skin keratinocyte,[12] which provides the following potential advantages: 1) Short cell turnover time results in a shorter culture time required to fabricate an epithelial sheet. 2) Long-term maintenance of cells under culture conditions may be possible without keratiniza-

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tion. This means that long-term viability of mucosal epithelial sheets can be maintained in vitro.

Mucosal epithelial cells seem to be a potential material to solve the above-mentioned problems related to cultured epithelial grafting using skin keratinocytes.[13]

In the field of oral surgery, skin grafting is commonly performed to reconstruct an extensive mucosal defect produced in the oral cavity after, for example, the extirpation of a malignant tumor.[14] As reported by other researchers,[15] however, skin grafted into the oral cavity maintains its original structure and functions without changing into mucosa.[15),16] Such grafted skin is thus quite different in nature from mucosa, and both types of tissues retain their own characteristics in a stable manner because of the interaction between the epithelium and subepithelial connective tissue.[17] This is often uncomfortable for the patient because of hair regrowth and sweating from the grafted skin. With recent advances in the chemotherapy and surgical treatment of these tumors, patients often return successfully to their daily lives. Therefore, the ability to reconstruct mucosal defects in such individuals is desirable.

For reconstruction of mucosal defects, mucosal grafting is the ideal method. The use of this method, however, is extremely limited because of the scarcity of donor sites. Limitation in availability of graft materials has made such reconstruction difficult. We, however, have applied cultured epithelial sheets using human mucosal cells for such mucosal reconstruction. With this method, abundant mucosal epithelium can be made in vitro as transplantable material from a small piece of mucosal tissue.

This is a remarkable advancement in the field of maxillofacial reconstructive surgery. Here, we report on the results of our studies, describing laboratory and clinical aspects of the investigations.

CULTURED EPITHELIAL SHEET USING HUMAN MUCOSAL CELLS

Preparation of the Mucosal Epithelial Sheet[12]

The method for preparation of the mucosal epithelial sheet is illustrated in Fig. 1. Excessive oral mucosa was obtained from patients treated in the Department of Oral Surgery, Nagoya University Hospital, through biopsy during oral surgery. Submucosal tissues were removed with scissors, and the resulting samples were cut into small pieces, which were then immersed twice in phosphate-buffered saline solution (PBS) containing antibiotics (penicillin G, 1000 U/ml; kanamycin, 1 mg/ml; Fungizone, 2.5 μg/ml) for 30 min at 37°C. Afterward, these tissues were immersed in Dulbecco's modified Eagle's medium (DMEM) containing 1000 PU of dispase per ml for 16 h at 4°C. They were then treated with 0.25% trypsin solution for 30 min at room temperature to separate the cells. The enzyme activity was eliminated by washing with DMEM containing 10% fetal calf serum (FCS). Then the specimens were stirred in DMEM containing 5% FCS for 30 min. The suspension was filtered through nylon gauze (50 μm) to remove unsatisfactory segments, and a suspension of purified epidermal and mucosal cells was obtained. It was centrifuged twice for 5 min at 1500 rpm and the cell pellet was resuspended in culture medium.

3T3-J2 cells were used as the feeder layer. They were kindly provided by Dr. Howard Green (Department of Cellular and Molecular Physiology, Harvard Medical School, Boston, MA, USA) and treated with 4 μg of mitomycin C per ml in DMEM without FCS for 2 h before epithelial cell inoculation. The 3T3-J2 cells were rinsed with PBS (−) to remove mitomycin C and cultured for 24 h in DMEM.

A 3:1 mixture of DMEM and Ham's F12 medium supplement was made with FCS, 5%; cholera toxin, 10 ng/ml; hydrocortisone, 0.5 μg/ml; transferrin, 5 μg/l; triiodothyronine, 2×10^{-9} M; insulin, 5 μg/ml; penicillin, 100 U/ml; kanamycin, 0.1 μg/ml; and Fungizone, 0.25

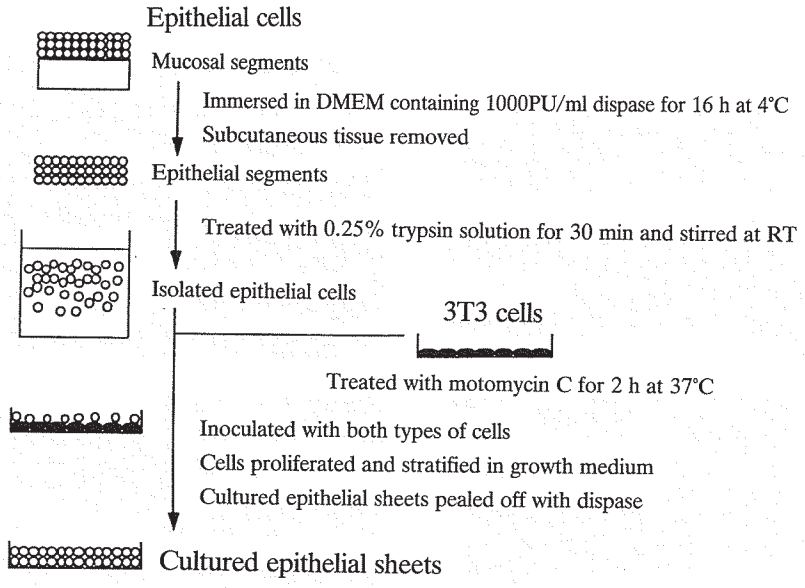

Fig. 1. Illustrative method for preparation of cultured epithelium using mucosa cells. DMEM: Dulbecco's modified Eagle's medium; RT: room temperature

μg/ml. Human recombinant epidermal growth factor was added at 10 ng/ml when cell adhesion was completed. 3T3-J2 cells treated with mitomycin C were inoculated into petri dishes (35 mm in diameter) at a density of 2×10^4. Then, epithelial cells were inoculated on the surface of the feeder layer (1×10^4 cells/cm^2). The petri dishes were kept in an atmosphere of 5% CO_2 in room air. The medium was changed every two days.

To compare the nature of a mucosal epithelial sheet with that of a skin epithelial sheet, skin karatinocytes were obtained in the same manner and cultured to form skin epithelial sheet.

Characteristics of Mucosal Epithelial Sheets

Cultured cells originating from both oral mucosa and skin were observed by phase contrast microscopy on days 5, 10, 15 and 20. The epithelial sheets were peeled off with 400 PU of dispase per ml and processed for histological examinations except on day 5 for both sheets, and on day 10 for mucosal cell sheets because of technical difficulty. The peeled epithelial sheets were immersed in 3% buffered formaldehyde for 3h, then dehydrated with graded alcohol. Five-micrometer sections were cut on a microtome and stained with hematoxylin and eosin for light microscopic observations.

Epithelial cells from both skin and mucosa started to form colonies within three days. Then the colonies expanded and appeared to eliminate the 3T3-J2 cells from day 5 (Figs. 2A and 2E). Both epidermal cells and mucosal cells became confluent on days 10 to 12 (Figs. 2B and 2F). Both kinds of epithelial cells became completely confluent and showed stratification on day 15 (Figs. 2C and 2G). At the same time, the thickness of the epithelial sheets was found to be uneven, showing a thicker part which was recognized as the stratifying region. Mean times until formation of the epithelial sheets were 12 days for the mucosal epithelial sheets and 14 days for the skin epithelial sheets. The stratified cultured epithelial sheets from mucosal cells maintained their morphology until day 30, and sheets from epidermal cells, until day 22 (Figs. 2D and 2H),

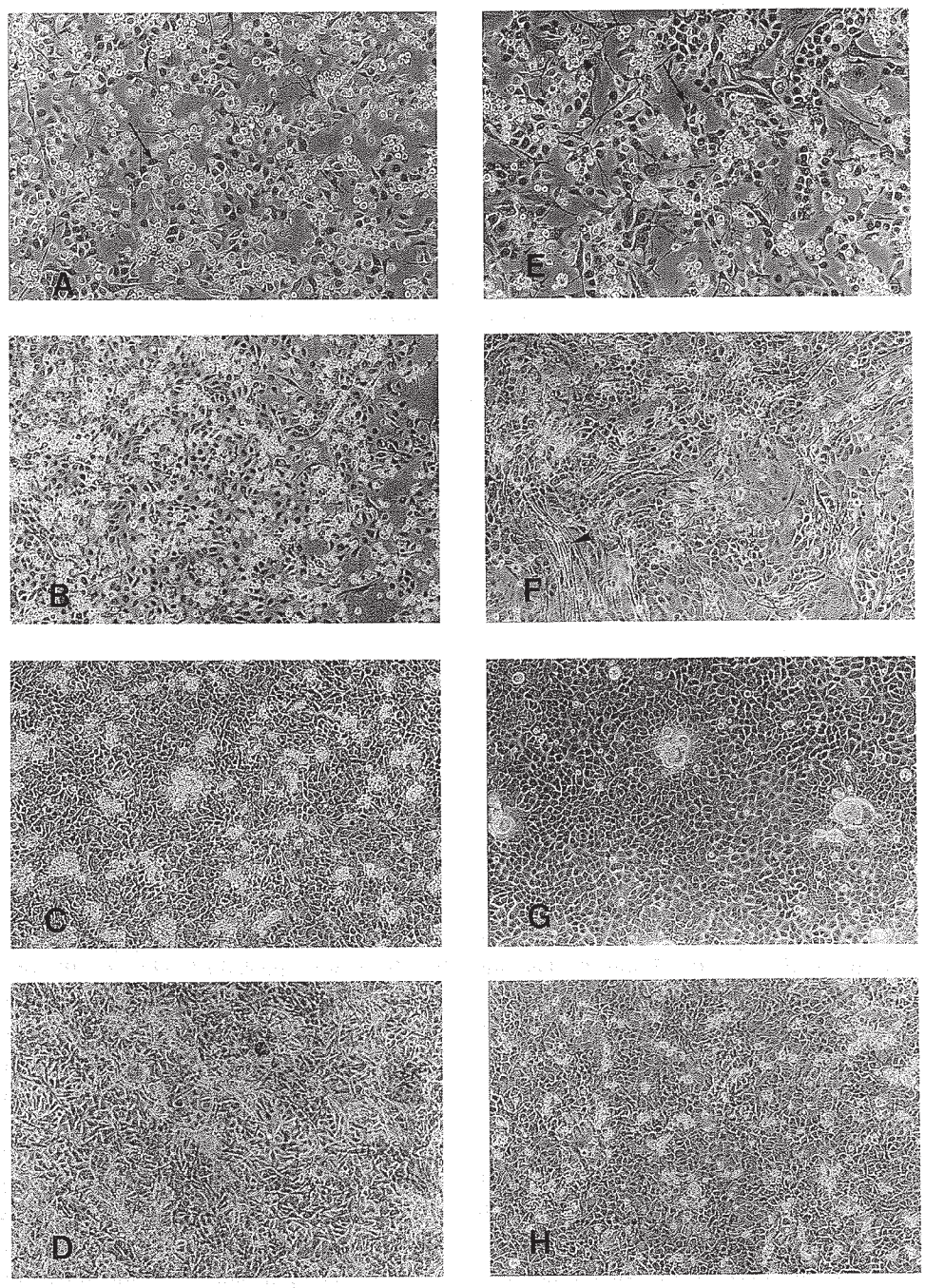

Fig. 2. Phase contrast micrographs of cultured epidermal and mucosal cells. No differences in proliferating pattern are seen between the two cell types. Epithelial cells demonstrate multiangular shape (arrow). 3T3 cells were eliminated as epithelial cells proliferated (arrowhead). A-D: epidermal cell sheets; E-H: mucosal cell sheets; A,E: day 5; B,F: day 10; C,G: day 15; E,H: 20. × 100

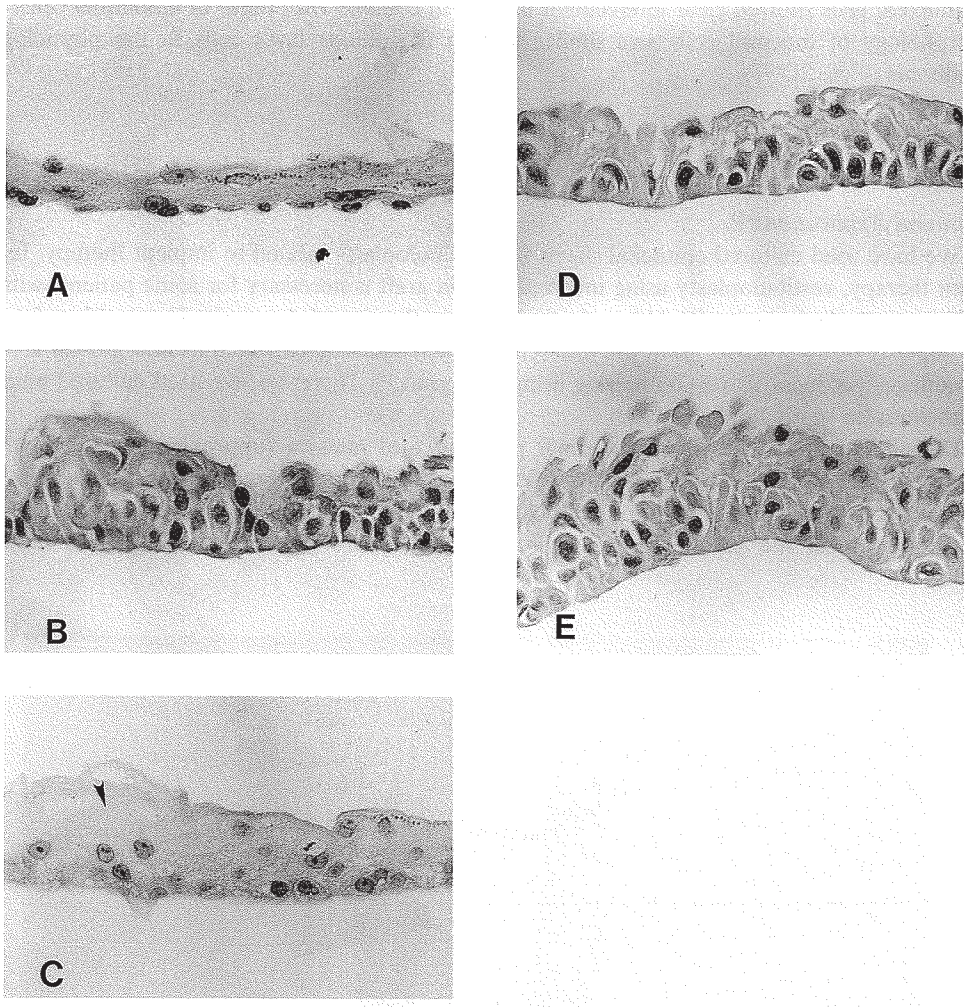

Fig. 3. Bright field photomicrographs of H-E-stained sections of cultured epithelial sheets from day 10 to day 20. Arrowhead indicates an enucleating cell. A-C: epidermal cell sheets; D,E: mucosal cell sheets; A: day 10; B,D: day 15; C,E: day 20. × 400

when epithelial fragments were detached from the sheets. The detached cultured sheets no longer possessed sufficient viability for transplantation.

In light photomicrographs of H-E-stained sections, cultured mucosal cell sheets on day 15 and cultured epidermal cell sheets on day 10 showed almost identical morphology (Figs. 3A and 3D). However, epidermal cells had a keratinizing tendency and showed enucleation on the surface in contact with the medium, from day 15 (Fig. 3B). On the other hand, mucosal cells seldom showed enucleation during stratification (Fig. 3D). Sections from cultured epithelial sheets of mucosal cells showed clear intercellular bridges in contrast with those of epidermal cells. The morphology of mucosal cells was similar to that of spinous layer cells in the physiological epidermis (Figs. 3D-E).

CLINICAL APPLICATIONS OF CULTURED EPITHELIAL SHEETS

Intraoral Applications[18)]

We have used cultured epithelial sheets for vestibuloplasty related to implant therapy. In implant therapy, vestibuloplasty using mucosal or skin graft is necessary for some patients without sufficient attached gingiva.[18)] Although, limitation in the size of donor site is a problem for vestibuloplasty using mucosal graft, the different characteristics of skin such as hair growth are disadvantages pertinent to vestibuloplasty using skin graft.[18)] Therefore, we used cultured mucosal epithelium for vestibuloplasty.

During the first-stage surgery a small piece of oral mucosa (approximately 0.5cm^2) was removed from the patient (Fig. 4). Then, epithelial cells were isolated and inoculated on 3T3 cells. After two weeks, the cultured epithelium was fabricated and stored at −80°C for three to six months until the second-stage surgery. The procedure of vestibuloplasty with cultured

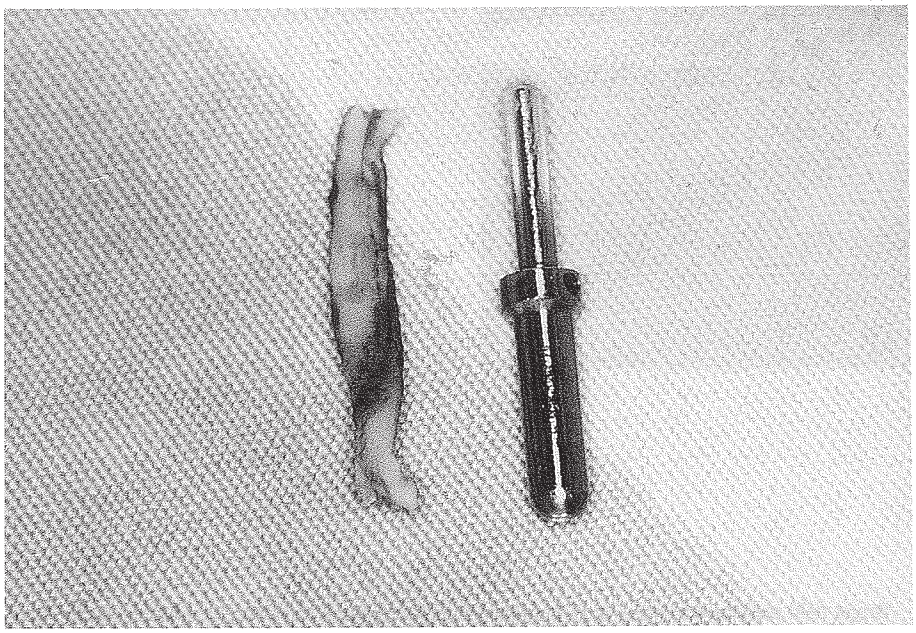

Fig. 4. Mucosal segment (0.5cm^2) obtained from the patient to form cultured epithelial sheet.

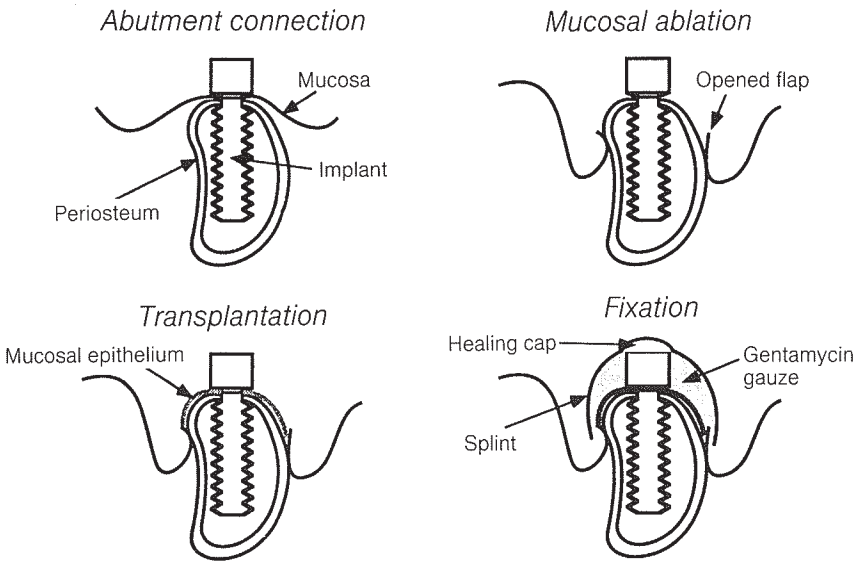

Fig. 5. Vestibuloplasty procedures using cultured mucosal epithelium.

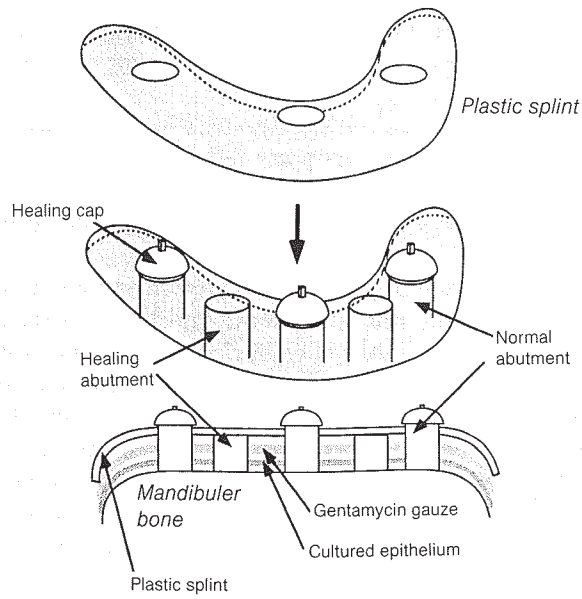

Fig. 6. Method of covering plastic splint.
Figure shows the relation of abutments and plastic splint. Normal abutments and healing abutments were located alternately. The space for cultured epithelium and gentamycin gauze was maintained by this method.

mucosal epithelium was as follows: The mucosa of the alveolar ridge was peeled off, leaving the periosteum on bone in the second-stage surgery of implant therapy. Cover screws on fixtures were removed, and the abutments were connected. The cultured oral mucosa was transplanted onto the periosteum or soft tissue around the bone and fixed by a plastic splint and gauze with tetracycline. In this procedure, normal abutments and healing abutments were connected alternately. The plastic splint was located over the healing abutments, and normal abutments penetrated the splint and were fixed by healing caps (Fig. 5, 6).

We present three cases in which patients underwent vestibuloplasty using cultured mucosal epithelium for implant therapy (Table 1).

Table 1. Intraoral Applications of Cultured Mucosal Epithelium for Vestibuloplasty

Case No.	Age (Years)	Sex	Primary disease	Grafts Applied (cm^2)	Site grafted	Result of grafting (%)
1.	60	M	Atrophy of mandible	15	Mandible	100
2.	61	M	Ca.[a] of tongue	20	Mandible	100
3.	40	M	Ameloblastoma	20	Mandible	70

[a]Ca.: Squamous Cell Carcinoma

Report of Cases

Case 1: A sixty-year-old male complained of masticatory dysfunction due to poor stability and retention of a conventional denture. Clinical investigation revealed advanced atrophy of the mandible and an approximately 3 mm-wide attached gingiva (Fig. 7A). Although the narrow attached gingiva was a negative factor for implants, implant therapy was considered necessary to improve masticatory function. Therefore, new vestibuloplasty using cultured mucosal epithelium was applied to implant therapy.

Fixtures were placed according to the method described by Brånemark.[19] Simultaneously, a small mucosal segment (approximately $0.5cm^2$) was removed from the oral mucosa. Mucosal cells were isolated and inoculated as previously described. After 14 days, we obtained two circular cultured mucosal sheets (f35cm, approximately $20cm^2$). The cultured mucosal sheets were kept frozen for three months in liquid nitrogen.

The second-stage surgery and vestibuloplasty were performed three months after the first-stage surgery. The cultured mucosal sheets were thawed rapidly and washed sufficiently in normal saline solution. After abutment connection, vestibuloplasty was done with a cultured mucosal epithelium graft (Fig. 7B). The cultured mucosa was tightly fixed with a plastic splint and tetracycline gauze (Fig. 7C). After one week, the fixation was removed and the region of cultured mucosal graft was covered with epithelium. After one month, the attached gingiva had widened from 3mm to 8mm in almost all areas except the gingiva around the central abutment, where slight inflammation was observed (Fig. 7D). However, this inflammation was controlled with suitable oral hygiene control when a bone-anchored bridge was constructed (Fig. 7E). Twenty-two months after the vestibuloplasty, the attached gingiva remained, and there was no inflammation around abutments.

Case 2: A sixty-one-year-old male with tongue carcinoma underwent partial resection of the tongue and oral floor, and marginal resection of the mandible. Reconstruction of the tongue and oral floor was performed by a deltopectoral skin flap (D-P flap), and that of the mandible was done by onlay bone graft from the iliac crest (Fig. 8A). Due to wide and thick grafted skin on

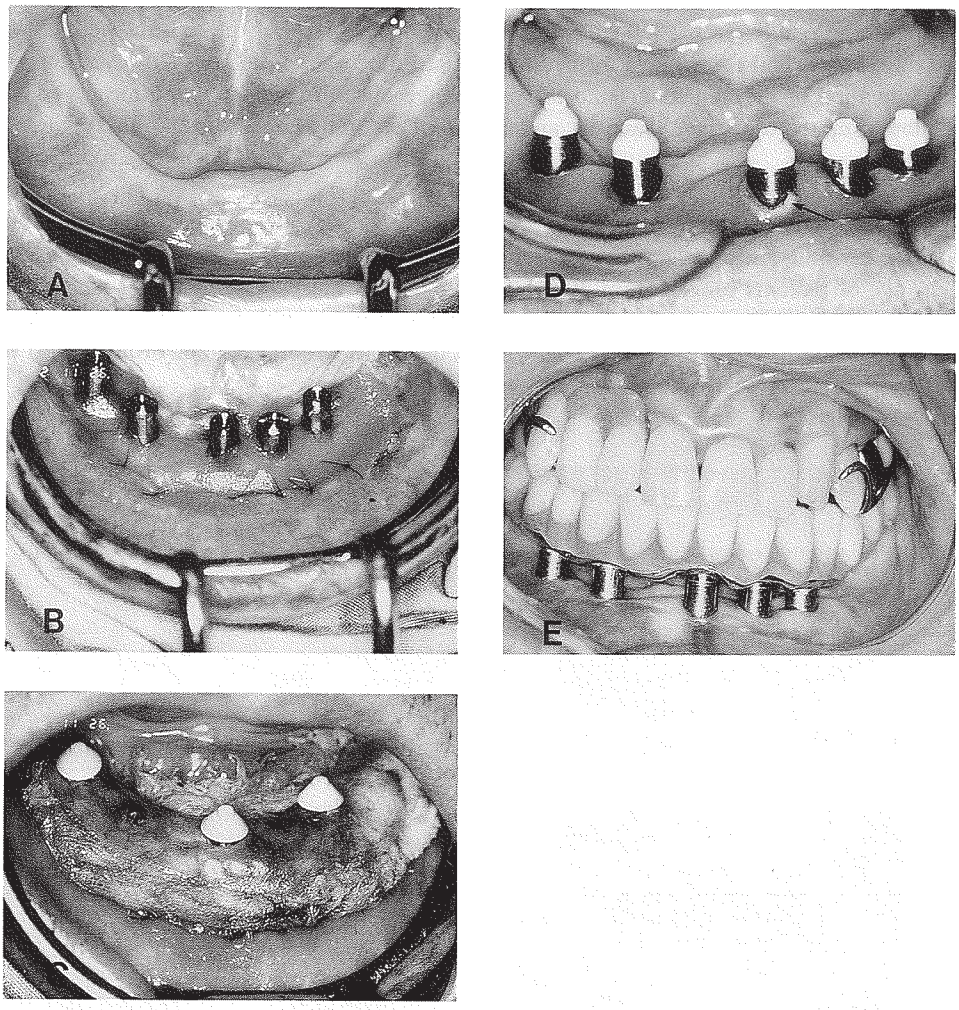

Fig. 7. Case 1 A: Preoperative view of mandibular gingiva and vestibule.
B: Transplantation of cultured mucosal epithelium.
C: Plastic splint for the covering and fixation of the cultured epithelium.
D: Intraoral view after 1 month. Some inflammation was found around abutment (arrow).
E: Intraoral view after prosthesis fabrication.

the bone, fabrication of a conventional denture was difficult. Therefore, implant therapy was employed for this patient. Since the thick grafted skin was not suitable for abutment penetration because of thickness and mobility, vestibuloplasty using cultured mucosal epithelium was performed at the time of abutment connection. Incision was made in the margin of the D-P flap and the flap was reflected and moved to the tongue side. In this case there was no periosteum on the grafted bone. Consequently, thin soft tissue remained on the bone, and the cultured mucosal epithelium was grafted on to that tissue (Fig. 8B).

After one month, attached gingiva had formed around the abutments (Fig. 8C). Bone resorption around the fixtures in the left side was observed, and threads of the fixtures were exposed. These threads touched the gingiva formed by cultured mucosal epithelium. However, there was no inflammation of the gingiva around the fixtures, because the gingiva was attached and thin. We followed up the case for 25 months, and no complication had occurred (Fig. 8D).

Extraoral Applications[13)]

To confirm the usefulness of mucosal epithelial sheets for skin repair, six patients with a donor site for split-thickness skin grafts were treated with cultured mucosal epithelium (Table 2). The age of patients ranged from 40 to 79 years. All of the patients had oral malignant tumors and underwent tumor resection and reconstructive surgery with free skin grafting. A split-thickness skin graft of 18/1000 inches was taken from the femoral region. From two to three

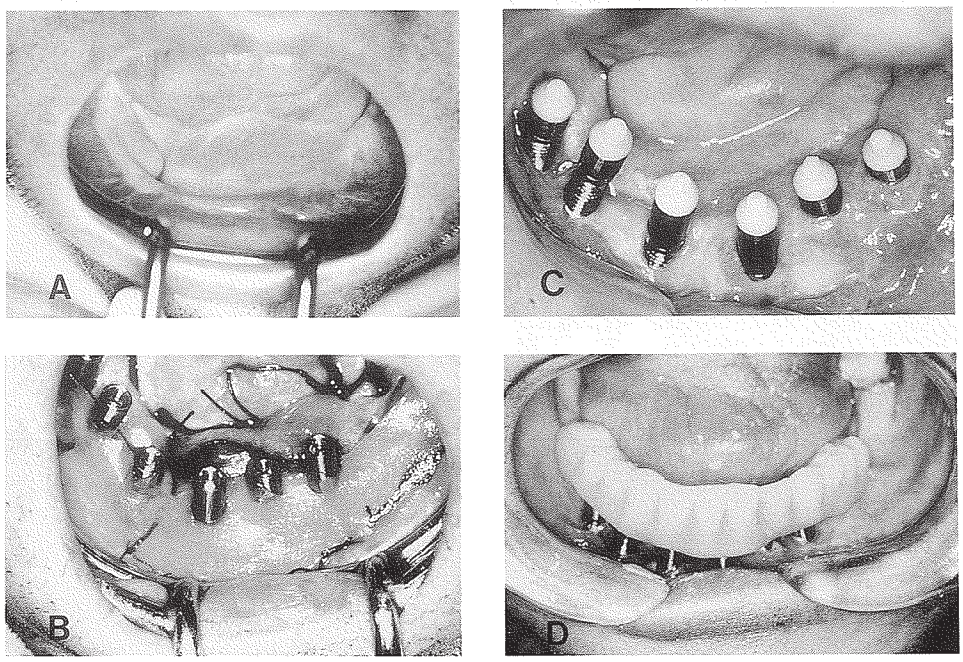

Fig. 8. Case 2
A: Preoperative view of mandibular gingiva and vestibule.
B: Transplantation of cultured mucosal epithelium.
C: Intraoral view after 1 month. Some inflammation was found around abutments.
D: Intraoral view after prosthesis fabrication.

weeks before the surgery, a mucosal segment (1cm^2) was taken from the oral cavity, and an epithelial sheet was prepared. Half of the wound area was covered with an ointment dressing, and used as control.

Table 2. Extraoral Applications of Cultured Mucosal Epithelium: Skin Defects Repaired with Cultured Mucosal Epithelial Sheets.

Case No.	Age (Years)	Sex	Primary disease	Grafts applied (cm^2)	Site grafted	Result of grafting (%)
1.	40	F	Ca.[a] of cheek	175	Thigh	100
2.	59	F	Ca. of mouth floor	175	Thigh	100
3.	65	M	Ca. of maxilla	175	Thigh	95
4.	79	F	Ca. of tongue	175	Thigh	85
5.	64	F	Ca. of tongue	175	Thigh	100
6.	71	M	Ca. of tongue	175	Thigh	100

[a]Ca.: Squamous Cell Carcinoma

Mucosal epithelial sheets (175cm^2) were prepared from mucosal segments (1cm^2) within 14 to 18 days in all cases.

The mucosal epithelial graft was applied to one half of the wound area utilizing the collagen membrane as the carrier. The remaining half was covered with gentamycin ointment gauze as the control area.

The dressing was removed on the fifth postoperative day to observe the graft site. All of the grafted epithelial sheet survived completely and adhered to the wound bed, and was thick enough to be manipulated. A thin transparent cultured epithelial sheet was found to be alive on the graft site. Dermis of the donor site was seen through the corium. On the other hand, the control areas did not re-epithelize until the 13th to 18th day postoperatively. In all patients, the graft side dried earlier than the control side with less hemorrhage and pain. Furthermore, exudate was remarkably reduced and there was less itching as compared with the control area. More than three to four weeks after grafting, epithelialization had proceeded on both the graft and control sides, which were indistinguishable. Normal keratinization occurred on the site of mucosal epithelial grafting.

Report of Cases

Patient 5: A 64-year-old housewife had squamous cell carcinoma (T1N0M0) in the right side of her tongue. On August 13, 1992, five days after her admission to the Nagoya University Hospital, a 1-cm^2 full thickness mucosal sample was excised from her lower lip and the donor site was closed primarily. Chemotherapy with pepleomycin (30mg) was performed before surgery. The tumor remarkably reduced in volume and almost disappeared from macroscopic observation. However, an induration (1cm ϕ) was detected in the center where the tumor had existed.

Hemiglossectomy was performed, and free skin was grafted on to the resected tongue surface on September 5, 1992. The split-thickness skin (18/1000 inches thick) was obtained from the right thigh. A cultured mucosal sheet (175cm^2) was placed on half of the donor site (Fig. 9A). The split-thickness skin graft took completely, and the cultured epithelial graft on the donor site survived completely. However, the control side did not epithelize and had hemorrhage and

exudate (Fig. 9B). Ten days after surgery, the recipient site of the cultured epithelial graft was completely reepitheliarized and dried up.

Histological examination on the 7th day after grafting showed formation of thick epithelial layers. However, the superficial layer of the epithelium did not keratinize and exhibited parakeratosis (Fig. 9C). By twenty-eight days after the grafting, epithelialization had finished on both the graft and control sides, which were indistinguishable. At this stage, the histological specimen taken from the grafted area showed that the epithelial layer had differentiated enough to form normal skin epidermis. The superficial layer of the epithelium had keratinized (Fig. 9D).

Patient 6: A 71-year-old man had mouth floor carcinoma (T2N0M0) in the right side and was referred to the Department of Oral Surgery, Nagoya University Hospital on December 13, 1992. He also had such severe liver cirrhosis that he could not undergo heavy chemotherapy. Two days after admission to the Hospital, a mucosal sample ($1cm^2$) was taken from his lower lip. The sample was carried to the culture room immediately and preparation of the mucosal epithelium was initiated. Eighteen days after admission, January 6, 1993, he underwent tumor resection and total neck dissection in the right side. The remaining raw surface in the mouth floor was reconstructed with split-thickness skin graft taken from the right thigh. Half of the

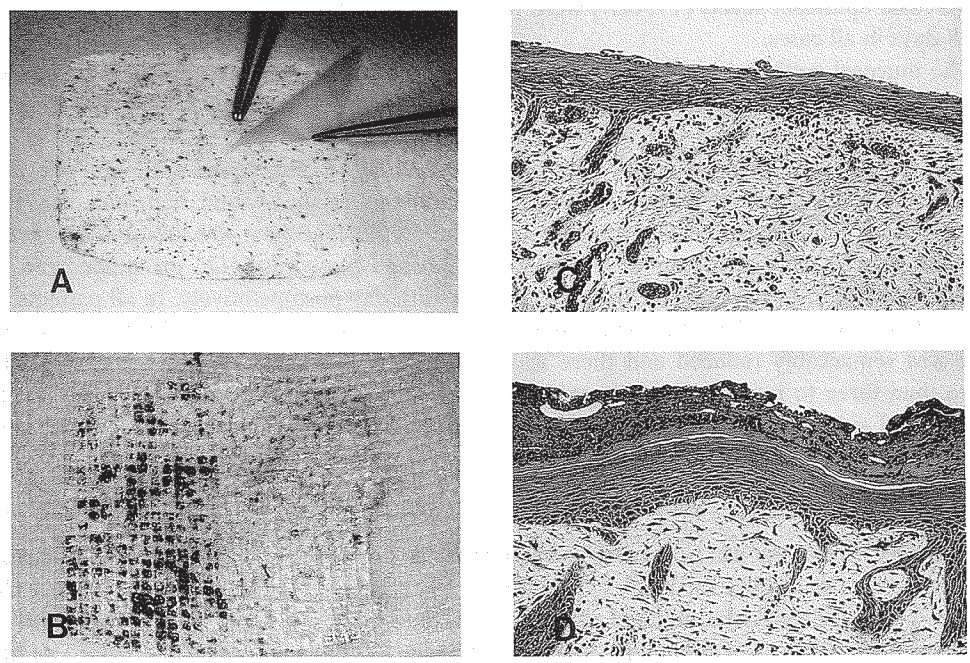

Fig. 9. A: Patient 5: 64-year-old woman treated with cultured mucosal epithelial grafting. The cultured mucosal epithelial sheet was placed on the donor site of split-thickness skin graft.
B: Three days after grafting, the cultured epithelium took completely. However, the control side did not epithelize and had hemorrage and exduate.
C: Histological findings on 7th day after grafting. They show formation of a thick epithelial layer. However, the superficial layer of the epithelium did not keratinize and kept the characteristics of mucosa.
D: Histogical findings on 28th day after grafting. The specimen shows normal keratinization of the epithelium.

donor site was covered with cultured mucosal epithelium. Five days after grafting, dressings were replaced, and complete epithelialization of the wound by grafted cultured epithelium was confirmed (Fig. 10A).

Eight days after the operation, the grafts continued to merge with the natural epidermis growing from the ungrafted area. Complaints of pain and tenderness at the grafted area were remarkably reduced, and exudate from the grafted area was much less than that from the control area. Four weeks after grafting, epithelialization had proceeded on both sides. However, the color of the grafted side became fainter and its texture was softer and smoother than the control side (Fig. 10B).

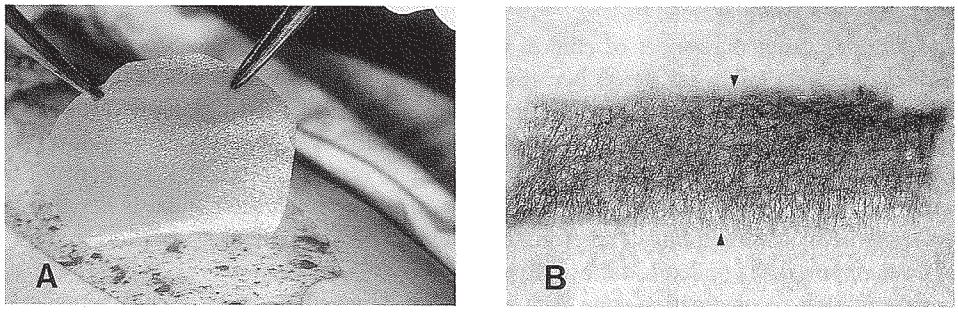

Fig. 10. A: Patient 6: 71-year-old man with mouth floor cancer. He was treated with tumor resection and split-thickness skin graft. The left side of the donor site was covered with cultured epithelium.
B: The 28th day after grafting. Epithelialization has proceeded on both sides.

DISCUSSION

Previously, attempts to make cultured epithelial sheets were not successful, because the epithelial cells possess lower growth potential than the mesenchymal cells and do not show stratification in the usual culture methods. Rheinwald and Green[1] reported a new method of epidermal cell culture using 3T3 cells as a feeder layer. The proliferating ability of these cells is known to be suppressed by irradiation or chemical treatment, and the treated cells only promote proliferation and stratification of adjacent epidermal cells. This property of 3T3 cells as a promoting agent made possible the formation of an epidermal sheet of sufficient size within a relatively brief time.

In this method, epithelial cells are cultured with irradiated 3T3 cells in the same petri dish. Irradiated 3T3 cells do not proliferate and only promote epithelial cell multiplication. Adjacent epithelial cells can stratify in low-calcium culture medium. Actually, the role of 3T3 cells in this system has not been clarified. The following roles of the 3T3 cells have been proposed: inhibition of the fibroblasts,[1),20)] improvement of the function of growth factors including epidermal growth factor,[21)] and regulation of epithelial cell differentiation.[22)] The method using 3T3 feeder cells has been successfully applied to mucosal epidermal cell culture.[23),24)]

With this culture method, Hata et al.[12)] formed a culture epithelium using skin keratinocytes and mucosal epithelial cells, and compared their characteristics.

As a result, the period for fabricating mucosal cell sheets was 12 days and that for skin epidermal sheets was 14 days. This difference was not significant. However, it does suggest that the mucosal cell possesses relatively greater proliferating ability, and indicates the usefulness of

mucosal cells in making cultured epithelial sheets.

Another result showed that mucosal epithelial sheets maintained their viability in vitro longer than skin epithelial sheets. In general, a cultured epithelial sheet loses its viability during culture. It is necessary to maintain the ability of the cells to proliferate until the sheet is transplanted. Reduction of viability seems to depend on the differentiation of epithelial cells. The differentiated epithelial cells, especially epidermal cells, showed enucleation and keratinization on the surface in contact with the medium. The enucleated cells might have had reduced viability and prevented medium transmission or transportation to basally located cells. On the other hand, mucosal cell sheets seldom showed enucleation or keratinization on the surface or at the base. This property of mucosal cell sheets may be an advantage for maintaining viability. In our studies, viability of cultured epithelial sheets was maintained for 30 days in mucosal cells, but for only 22 days in epidermal cells.

Furthermore, from the aspect of clinical availability, mucosal epithelial sheets have a great advantage over skin epithelial sheets because locating a donor site from which to obtain tissue for the preparation of cultured epithelium may be difficult, especially in patients with extensive burns covering a large portion of the body surface area. However, the oral cavity still remains a possible site under such circumstances. Also, it is easy to obtain an epithelial segment for making cultured epithelium without forming new scars, especially when the patient is nervous about a new scar due to skin sampling. Therefore, oral mucosa must be useful as a cell source for cultured epithelium grafts applied to tattoos, hypertrophic scars, or giant nervus.

In case of skin reconstruction, one area of concern is whether mucosal cells actually keratinize and become skin thereby substantiating use of mucosal epithelium for skin reconstruction. In our six patients, the graft reached a stage where it became indistinguishable from skin epithelium. This phenomena can be explained by the biological mechanism called "epitheliomesenchymal interactions." Mackenzie et al.[25] stated that differentiation and maintenance of epithelial specificity is directly dependent on the influence of the underlying mesenchymal tissue. Ueda et al.[15] applied an experimental dermal graft in the oral cavity in dogs and reported that the mucosal epithelium covering the dermis became naturally keratinized within eight weeks. In our clinical cases, at seven days after grafting, the transplanted mucosal epithelium still maintained its morphological characteristics. However, within 28 days the mucosal epithelium was differentiated enough to become keratinized.

The mechanism of the keratinization is clearly demonstrated by these facts. These findings also confirm that the existence of connective tissue or dermis is one of the most important factors for success in cultured epithelial grafting.

However, some problems still remain in this seemingly perfect method of treatment. Firstly, cultured epithelial sheets appear incapable of keeping the whole structure of skin intact because they lack connective tissue and basement membrane.[26] Therefore, attempts to use epithelium alone as a wound covering have not proved satisfactory because they failed to inhibit wound contraction. A different skin culture technique was used by Burke and associates[27] to produce an epithelial layer combined with a spongy collagen membrane. This method attempted to produce dermis (connective tissue) from spongy collagen and fibroblasts, with endothelial cells invading from the recipient bed. This technique has gained relatively general acceptance because of the mild degree of wound contraction. Further research should be continued to establish a complete method for adequate wound coverage without severe wound contraction.

Secondly, the cultured epithelial sheet is not strong enough to resist mechanical stress and bacterial infections. Especially in the application of cultured epithelial sheets for intraoral reconstruction, it is difficult to fix the cultured epithelial sheet on the recipient site tightly. Defense against microbacteria is also important for the success of grafting.

However, in vestibuloplasty using cultured mucosal sheets as part of implant therapy, these problems can be solved.[18] The fixation of the graft can be done easily using abutments and a plastic splint. After the second-stage surgery of implant therapy and grafting of cultured epithelium, these materials can firmly fix the grafted cultured epithelium.

The third problem concerns the mutation potential in cultured cells that have been prepared for cultured epithelium. Ebner and co-workers[28] reported on the possibility of morphological changes in cultured cells during culturing. Therefore, the safety of cells should be checked when tissue using cultured cells is planned for use in transplantation to humans. In our clinical study, the chromosomes of culture fibroblasts were tested by the air-dry method[20] to confirm the safety of cells preparatively.

There is an interesting aspect in regard to the improvement of the quality of cultured epithelium. With our method, there is a possibility that the mucosal epithelium contains stem cells because the full thickness of the mucosal epithelial segment in which the stem cells exist is used to make the epithelial sheet. The stem cells in the epithelium have the potential to differentiate not only into keratinocytes but also into cells that constitute the appendages of skin or mucosa, ie, sweat glands, salivary glands and hair follicles. Cultured to differentiate into varied cell types in vitro, stem cells fail to recreate mucosal or skin appendages when they are grafted in vivo. Stem cells appear to lose some of their potential by prolonged culture in vitro in the absence of a truly physiological microenvironment. In the future, this problem may be answered by analyzing the microenvironment that makes the keratinocyte stem cells differentiate into appendages. This is one of the most interesting facets of cultured epithelial sheets.

CONCLUDING REMARKS

We have developed cultured mucosal epithelium, which serves as a graft material. The biological characteristics of cultured mucosal epithelium have been studied in comparison with skin epithelium. The cultured mucosal epithelium can be formed faster than skin epithelium in vitro. Also, viability of the mucosal epithelial sheet was maintained longer than that of skin epithelium. We applied cultured mucosal epithelium for mucosal repair in three patients who required implant surgery and for skin repair in six patients. All grafts survived successfully and formed normal mucosal tissue and skin. We believe that this type of cultured tissue has a great potential to improve various kinds of reconstructive surgery in the future.

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