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# Chapter 1

## Skin

In the field of plastic and reconstructive surgery, many clinical reports have demonstrated the utility of cultured epithelial sheets which have been successfully cultured since the first report of Rheinwald and Green.

### **Cultivation of human epidermal keratinocytes**

Human diploid epidermis epidermal cells have been successfully grown in serial culture. To initiate colony formation, they require the presence of fibroblasts, but proliferation of fibroblasts must be controlled so that the epidermal cell population is not overgrown. Both conditions can be achieved by the use of lethally irradiated 3T3 cells at the correct density. When trypsinized human skin cells are plated together with the 3T3 cells, the growth of the human fibroblasts is largely suppressed, but epidermal cells grow from single cells into colonies. Each colony consists of keratinocytes ultimately forming a stratified squamous epithelium in which the dividing cells are confined to the lowest layer(s). Hydrocortisone is added to the medium, since in secondary and subsequent subcultures it makes the colony morphology more orderly and distinctive, and maintains proliferation at a slightly greater rate. Under these culture conditions, it is possible to isolate keratinocyte clones free of viable fibroblasts. Like human diploid fibroblasts, human diploid keratinocytes appear to have a finite culture lifetime. For 7 strains studied, the culture lifetime ranged from 20-50 cell generations. The plating efficiency of the epidermal cells taken directly from skin was usually 0.1-1.0%. On subsequent transfer of the cultures initiated from newborns, the plating efficiency rose to 10% or higher, but was most often in the range of 1-5% and dropped sharply toward the end of their culture life. The plating efficiency and culture lifetime were lower for keratinocytes of older persons.

### **Grafting of burns**

The cells from a small piece of epidermis can be grown into a large number of cultured epithelia. Such epithelia, generated from autologous skin, were grafted onto full-thickness burn wounds in two patients. The cultured epithelia acquired an epidermal structure resembling that achieved with conventional split-thickness skin grafts, and survived for the period of observation (up to 8 months). Since the method of cultivation can generate large amounts of epithelium, the procedure is applicable to the grafting of large areas, as in severe burns (Fig. 1). However, using the method of epithelial cell culture employing 3T3 cells as a feeder layer, the epidermal cells possess a lower growth potential than normal cells such as fibroblasts. Therefore, approximately 3 weeks are required to fabricate an epidermal sheet. This makes it difficult to meet the requirements of emergency surgery. There have been some clinical trials of cultured epithelial sheet freezing storage in the field of plastic surgery [1, 2]. Thus, various freezing methods were considered to maintain the activity of the

cultured epithelial sheets [3, 4]. These reports indicated that it is possible to store many cultured epithelial sheets long-term by using the freezing storage method and to meet the requirements of emergency surgery.

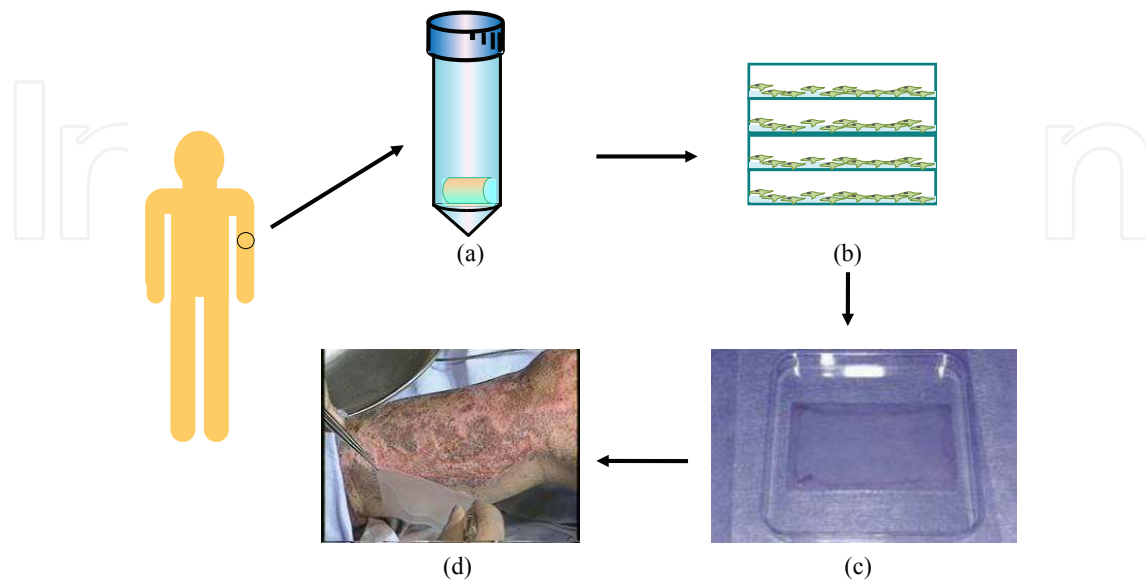


Fig. 1. Protocol of tissue engineered epithelium. (a) Extract skin tissue. (b) Cultured fibroblast on feeder layer. (c) Preparation of cultured skin. (d) Transplantation of cultured skin (From Ueda et al. 1995).

### Effects of freezing storage

Numerous clinical reports have shown the utility of cultured epithelial grafting in the field of plastic and reconstructive surgery. Recently, freezing storage of the cultured epithelium has been tried and has successfully grafted after thawing. It is clinically convenient if it is possible for cultured epithelium to keep its normal structure and viability. However, few papers have described the structural changes in cultured epithelium after freezing storage. In the present study, the morphological changes and cell viability of cultured mucosal epithelial sheets after freezing were studied in comparison with cultured epidermal sheets. Furthermore, we discuss the effect of storage temperature and cryoprotectants. As a result, there were some structural changes such as vacuolar degeneration in the cultured mucosal sheets using dimethyl sulphoxide (DMSO) as a cryoprotectant. Such changes were more clearly observed at  $-80^{\circ}\text{C}$  than at  $-196^{\circ}\text{C}$  with DMSO. However, little morphological change was observed in both epithelial sheets cultured with glycerin. The cell viability analyzed by flow cytometry showed that more than 62% of the cells kept their viability after freezing storage. These results suggest that the optimum conditions of freezing for cultured epithelium were  $-196^{\circ}\text{C}$  storage by slow cooling methods with glycerin as a cryoprotectant.

A telomere is a special base-sequence repeat at the end of a eukaryotic chromosome (TTAGGG repeat in humans) [5, 6]. Telomeres are required for protecting chromosomes against illegitimate fusion events, mediating chromosome location in the nucleus, and preventing the outermost end from being recognized as defective DNA [7, 8]. Thus, they function as important buffers to guarantee the stability and functionality of the chromosomes. Since a telomere does not replicate completely during cell division, it gradually shortens as cell

division proceeds [9-11]. Over a single lifetime, up to 10 kb of telomeres are reduced gradually to around 5 kb after repeated cell division, resulting in cellular senescence [12]. From investigations using fibroblasts, telomere length has been proven to have a close correlation with the possible number of cell divisions [12]. Telomeres shorten during cell divisions without telomerase activity. Telomerase is a specialized ribonucleoprotein complex that directs the synthesis of telomeric repeats at chromosome ends [13]. Telomerase activity maintains telomeric DNA within relatively constant ranges, allowing cells, e.g. some neoplastic cells, to proliferate indefinitely [14, 15]. Although no telomerase activity is observed in most somatic cells, it has been detected in regenerating cells such as basal cell populations in skin epithelium [16-19]. Recently, the loss of telomerase activity has reportedly been associated with the replicative senescence of normal human oral and skin keratinocytes [16, 20]. Thus, the change in telomerase activity during long-term cultivation is of great interest, since it might be involved in the mechanisms underlying cellular senescence in cultured epidermis.

### **The telomere length and morphology**

Cultured epidermis has been successfully used in clinical treatment such as burns and pigmentary disorders. Although the generation of wide cultured epidermis for clinical use may require repeated passages, especially for allografts, the effects of long-term cultivation on its quality and cell viability are not well known. To investigate the changes in morphology, telomere length, and telomerase activity during the passages of cultured epidermis and keratinocytes up to the passage limit, and to examine the usefulness of telomere length as a performance criterion for cultured epidermis. The keratinocytes obtained from five patients were used to generate cultured epidermis (Fig. 2). At the early passage and after cultivation up to the passage limit, morphology, telomere length and telomerase activity were investigated by using microscopes, southern blot analysis and telomeric repeat amplification protocol assay, respectively. The cultured cells started to show morphological changes when each passage was close to its limit and the cell sheets assumed an irregular stratification with various sizes of cytoplasm and nuclei. At the passage limit, the telomere length had decreased approximately 80-85%, and the average telomerase activity had declined under serum-free culture conditions. The results of this study showed the morphological change and telomere length reduction by long-term cultivation on cultured epidermis. Although the reduction in telomere length and telomerase activity may not be the major cause of the senescence, they could provide an useful information for the quality of the cultured epidermis. Dispase, a neutral protease from *Bacillus polymyxa*, is widely used to harvest multilayered keratinocyte sheets from culture dishes [16]. In clinical use, extensive washing to remove dispase from keratinocyte sheets is required before they can be applied to wounds, because residual dispase is harmful to the wounded site. In industrial production of keratinocyte sheets, this washing is laborious, and is a technological barrier to automation of the process. In the present study, we used ultralow-attachment plates with a surface composed of a covalently bound hydrogel layer that is hydrophilic and neutrally charged, to investigate whether keratinocytes could be harvested from the plate without enzymatic treatment after removing the magnet, because the keratinocytes did not adhere to the plate surface. The present results indicate that magnetic force and magnetite nanoparticles can be used to construct and harvest keratinocyte sheets.



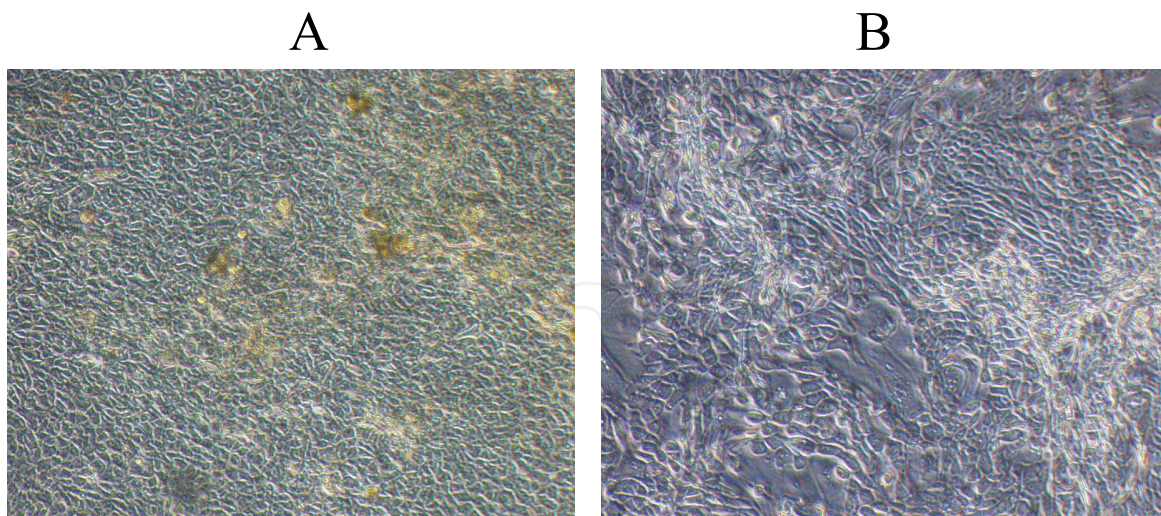


Fig. 2. A: The cultured epidermis in passage 1. During the first passage, the keratinocytes formed a sheet, and showing a uniform and typical cobble stone-like appearance. B: The cultured epidermis in passage 6. Around the terminal passage, the sheets displayed an uneven thickness with irregular cell shapes (From Miyata et al. 2004. Reprinted with permission).

### Multilayered keratinocyte sheets using magnetite nanoparticles

Novel technologies to establish three-dimensional constructs are desired for tissue engineering. In the present study, magnetic force was used to construct multilayered keratinocyte sheets and harvest the sheets without enzymatic treatment. Our original magnetite cationic liposomes, which have a positive surface charge in order to improve adsorption, were taken up by human keratinocytes at a concentration of 33 pg of magnetite per cell. The magnetically labeled keratinocytes ( $2 \times 10^6$  cells, which corresponds to 5 times the confluent concentration against the culture area of 24-well plates, in order to produce 5-layered keratinocyte sheets) were seeded into a 24-well ultralow-attachment plate, the surface of which was composed of a covalently bound hydrogel layer that is hydrophilic and neutrally charged. A magnet (4000 G) was placed under the well, and the keratinocytes formed a 5-layered construct in low-calcium medium (calcium concentration, 0.15 mM) after 24 hours of culture. Subsequently, when the 5-layered keratinocytes were cultured in high-calcium medium (calcium concentration, 1.0 mM), keratinocytes further stratified, resulting in the formation of 10-layered epidermal sheets. When the magnet was removed, the sheets were detached from the bottom of the plates, and the sheets could be harvested with a magnet. These results suggest that this novel methodology using magnetite nanoparticles and magnetic force, which we have termed “magnetic force-based tissue engineering” (Mag-TE), is a promising approach for tissue engineering.

### Mucosa

In the field of oral surgery, mucosal grafting has been carried out for reconstruction after tumor removal, preprosthetic surgery and so on. However, it is difficult to obtain enough oral mucosa for reconstruction of large defects, and in these cases, skin autografting has been employed. Skin grafting in the oral region has many disadvantages, such as

keratinization, secretion and hair growth, and it is inevitably accompanied by patient discomfort. Furthermore, a skin graft is associated with a new defect at the donor site. To solve these problems, the use of cultured gingival epithelium was reported.

### Formation of epithelial sheets

A cultured epithelial sheet can be formed from living mucosal cells *in vitro* and used as a graft material (Fig. 3). 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.

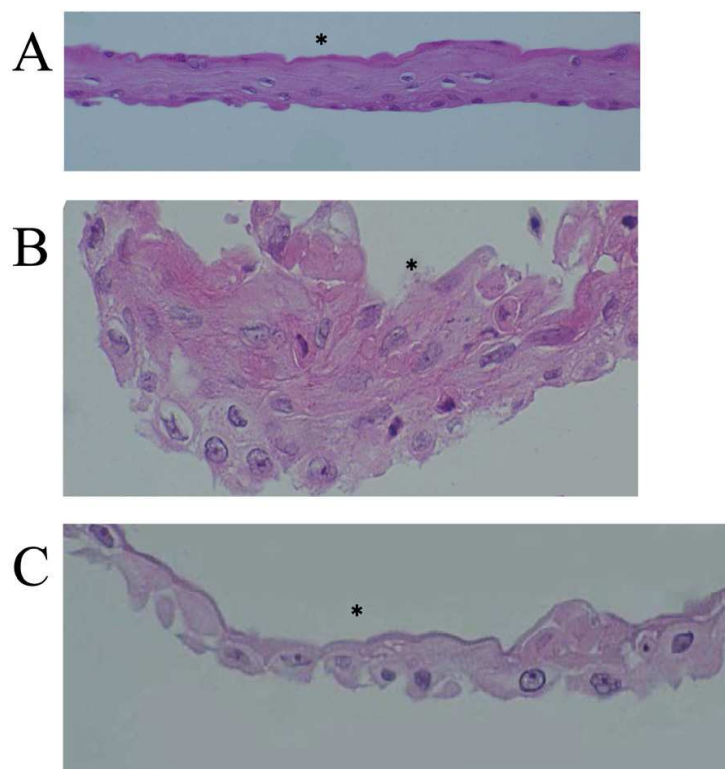


Fig. 3. A: The cultured epidermis in passage 2. The sheet showed 5 to 8 cell layers of even thickness including a single basal-cell layer. The polyhedral cells of stratum overlying the basal cells became flattened from the basal (plate) side toward to the surface (medium) side (indicated by an asterisk), and the cells in the surface layer showed an enucleation. B: The cultured epidermis in passage 6, 3 weeks after beginning of the culture. The cell sheets presented an irregular stratification with various sizes of cytoplasm and nuclei. C: When the cells reached the full passage limit, most areas of the sheet presented a single cell layer and only partial stratification (From Miyata et al. 2004. Reprinted with permission).

### The characteristics of cultured mucosal cell sheet

The characteristics of cultured mucosal cells from the oral mucosa were investigated and compared with those of cultured epidermal cells. Total cell counts showed that mucosal cells possessed greater proliferating ability than epidermal cells. The results of 3(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assay confirmed this observation and also suggested that the mucosal cells maintained biological activity longer than epidermal cells. The most important morphological characteristics of mucosal cells in culture were their low grade of differentiation. Interestingly, the epidermal cells showed enucleation and keratinization progressively during culture, whereas the mucosal cells showed no obvious enucleation when examined by light microscopy. Transmission electron microscopy showed a smaller number of desmosomes in cultured mucosal cells than epidermal cells. The results of this study reveal cultured mucosal cell sheets to be a possible material for grafting in addition to cultured epidermal cell sheets.

### Transplantation of cultured mucosal epithelium

We investigated morphological changes after transplantation of cultured mucosal epithelium using a modified Barrandon's method (1988). Serially cultivated human mucosal epithelium was transplanted onto the reverse side of rectangular dorsal skin flaps in hairless mice. The morphological changes in the epithelium were studied using paraffin sections. The modified Barrandon's method used in this study has advantages such as minimum external trauma and less chance of infection. The cultured epithelium was taken within 1 week and gradually increased its epithelial thickness (Fig. 4). Keratinized epithelium arises after 3 weeks. At 4 weeks after grafting, the grafted epithelium comprised 7 to 10 cell layers. The structure of transplanted tissue, in conjunction with surrounding connective tissues, showed dermis-like features at day 7 after transplantation. From these results, it was confirmed that cultured mucosal epithelium could be successfully transplanted and its morphology was similar to that of normal mucosal tissue.

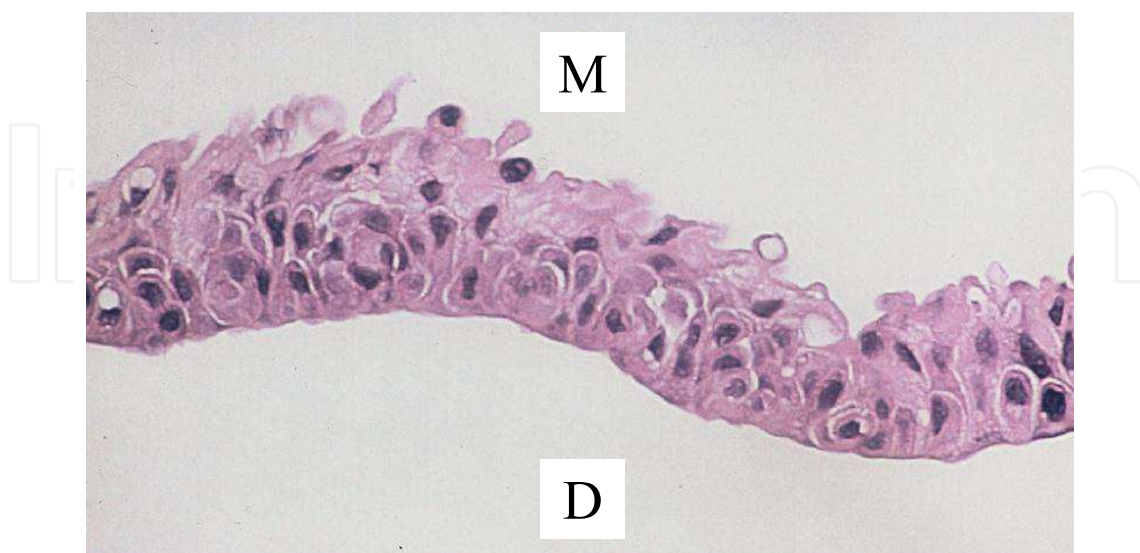


Fig. 4. Bright field photomicrographs of H-E stained sections of cultured mucosal epithelium of day 14 ( $\times 250$ ) (M: medium side, D: dish side) (From Sugimura et al. 1997. Reprinted with permission).



**Grafting in the oral and maxillofacial region**

Cultured epithelium has proven to be a good grafting material for skin defects. In our experience two kinds of epithelial cells, skin keratinocytes and mucosal cells, have been used to fabricate cultured epithelial sheets and autografted to the patients. Traumatic scars of the face were treated by cultured epidermal epithelium (CEE). The skin graft in the oral cavity was replaced by mucosa using cultured mucosal epithelium (CME). Also, the CME was applied to the skin defects at the donor sites of split-thickness skin grafts. Postsurgical follow-up showed good results. As a result, CME was useful in improving the biological environment around the abutments of dental implants, and it also promoted the re-epithelialization of skin defects. From our investigations, CEE/CME are promising treatment modalities which can reduce pain and speed up the healing process in burn patients. Therefore, cultured epithelium banks are worth establishing for autografting and allografting of skin/mucosal defects.

**Peri-implant soft tissue management**

In implant therapy, peri-implant soft tissue management through use of mucosal grafting or skin grafting is necessary in some patients who do not have enough attached gingiva around the abutment. However, limitation of donor site size is a problem for the mucosal graft, and the different characteristics of skin, such as hair growth, are disadvantages in treatment that involves the use of skin graft. On the other hand, cultured epithelium fabricated with living mucosal cells has proved to be a good grafting material for any kind of mucosal defect. In this study, we used cultured mucosal epithelium for soft tissue management in implant therapy. In the first surgical procedure of the implant therapy, a small segment of oral mucosa was sampled from a patient. The cultured epithelium was fabricated and then stored until it was grafted in the second surgery. Twelve cases in which patients underwent peri-implant soft tissue management through use of cultured mucosal epithelium for implant therapy are presented, and the usefulness of this technique in the making of attached gingiva is analyzed. From this study it was concluded that cultured mucosal epithelium can serve as a proper material for peri-implant soft tissue management.

**Gene-modified mucosal epithelium**

Human oral mucosal cells are attractive sites for tissue engineering because they are the most accessible cells in the body and easy to manipulate *in vitro*. They thus have possibilities for targeting by somatic gene therapy. We examined the efficiency of retrovirus-mediated gene transfer and the construction of mucosal epithelium *in vivo*. Human oral mucosal cells were transduced with a retroviral vector carrying the *lacZ* gene at high efficiency and constructed epithelium after G418 selection with 3T3 cells *in vitro*. The cultured oral mucosal epithelium membrane was then grafted onto immunodeficient mice.  $\beta$ -Gal expression was detected histochemically *in vivo* 5 weeks after grafting. Furthermore, we transduced factor IX cDNA into the mucosal epithelium membrane, and it was then transplanted into nude mice. Between 0.6 and 1.8 ng of human factor IX per milliliter was found in mouse plasma, and the production was continued for 23 days *in vivo*. These results confirmed that the oral mucosal epithelium is an ideal target tissue for gene therapy or tissue engineering.



### The use of gingival fibroblasts for soft-tissue augmentation

Fibroblasts were obtained from the patient's buccal gingival tissue and maintained in DMEM plus 10% autologous human serum, and incubated at 37°C with 5% CO<sub>2</sub>. Autologous patient serum was prepared from 100–150 ml of peripheral blood. The characteristics of the cultured cells were checked by immunofluorescent microscopy for known fibroblast markers. The cells were washed twice with sterile saline, and resuspended in sterile saline to a final concentration of  $1.0 \times 10^7$  cells/ml. The cell suspension was stored in 1.0 ml syringes until use. All satisfactory assessments were performed by the patient, and the following grading scale was used at 3, 6 and 12 months after the first injection (4: Completely satisfied, 3: Satisfied, 2: No remarkable change observed, 1: Not satisfied, 0: Exacerbation). Several patients were treated with live gingival fibroblast injections. The population was 100% female. Mean age at the time of the first injection was 50.29 years. At 3-month follow-up, the satisfactory rate among fibroblast-treated patients was 3.0 in nasolabial and 3.00 in lip. At 6 months, the satisfactory rate increased to 3.29, whereas the lip increased relatively at 4.00. At 12 months, the rates were 3.36 versus 4.00. No serious adverse events considered related to the study treatment were reported. Our results indicate that autologous fibroblast injections may provide an acceptable treatment for patients, especially those desiring better aesthetic results. Our initial experience with the autologous gingival fibroblast injection process indicates that it is probably capable of producing ongoing improvements in perioral lip wrinkles without the hypersensitivity complications and harvesting challenges associated with other treatments (Figs. 5, 6).

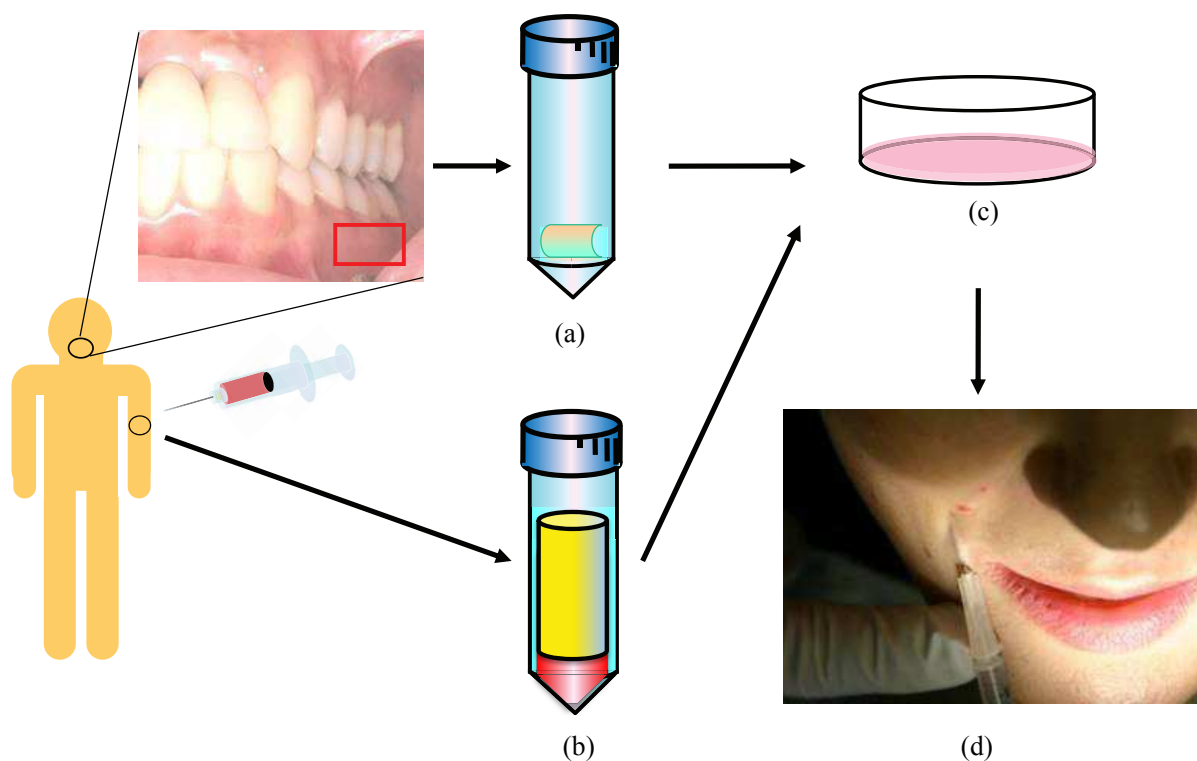


Fig. 5. Protocol of autologous fibroblast injections for soft-tissue augmentation. (a) Oral mucosa. (b) Autologous serum. (c) Cultured fibroblast with autologous serum. (d) Cell transplantation (From Ebisawa et al. 2008. Reprinted with permission).



Fig. 6. A: Before treatment. B: Three years after treatment (From Ebisawa et al. 2008. Reprinted with permission).

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## **Applied Tissue Engineering**

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Tissue engineering, which aims at regenerating new tissues, as well as substituting lost organs by making use of autogenic or allogenic cells in combination with biomaterials, is an emerging biomedical engineering field. There are several driving forces that presently make tissue engineering very challenging and important: 1) the limitations in biological functions of current artificial tissues and organs made from man-made materials alone, 2) the shortage of donor tissue and organs for organs transplantation, 3) recent remarkable advances in regeneration mechanisms made by molecular biologists, as well as 4) achievements in modern biotechnology for large-scale tissue culture and growth factor production.

This book was edited by collecting all the achievement performed in the laboratory of oral and maxillofacial surgery and it brings together the specific experiences of the scientific community in these experiences of our scientific community in this field as well as the clinical experiences of the most renowned experts in the fields from all over Nagoya University. The editors are especially proud of bringing together the leading biologists and material scientists together with dentist, plastic surgeons, cardiovascular surgery and doctors of all specialties from all department of the medical school of Nagoya University. Taken together, this unique collection of world-wide expert achievement and experiences represents the current spectrum of possibilities in tissue engineered substitution.

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