Dental Regenerative Therapy using Oral Tissues

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Abstract

Anti-Aging Medicine is a theoretical and practical science which aims to ensure the achievement of a long and healthy life. Dental medicine plays an important role in its practice. Given the substantial influence of dental/oral diseases on general health, the maintenance and improvement of oral function promotes not only dental/oral Anti-Aging but also systemic Anti-Aging as well.

The current target of Anti-Aging dental medicine is the prevention or slowing down of the age-related decline in oral function by evaluating indicators of oral function, such as dental age, periodontal age, occlusion age, swallowing age, and salivary age. In this symposium, Dr. Kenji Mishima (Department of Dentistry, Tsurumi University), speaking on “Application of Cell Transplantation Therapy to Salivary Gland Dysfunction”, Dr. Masahiro Saito (Research Institute for Science and Technology, Tokyo University of Science), speaking on “Role of Tooth Regeneration in Anti-Aging Medicine” and myself, Dr. Narisato Kanemura (Dental Medicine, Graduate School of Medical Science, Kyoto Prefectural University of Medicine), speaking on “Development of a New Periodontal Tissue Regeneration Method Aimed at Anti-Aging Use”, delivered presentations about the current status and future prospects of regenerative dentistry, which aims not only to prevent a decrease in oral function but also to restore it when function is lost, and introduced the latest in regenerative dentistry involving the salivary glands, teeth, oral mucosal epithelia, and periodontal ligaments. In addition, to describe collaboration between dental medicine and ophthalmology, Dr. Takahiro Nakamura (Faculty of Life and Medical Sciences, Doshisha University), speaking on “Current Status and Future Prospects of Corneal Regenerative Therapy using Oral Tissue”, introduced the current status and future prospects of corneal regenerative therapy using periodontal mucosal epithelium. Summaries of these lectures are presented here. In the “Dental Regenerative Therapy using Oral Tissues” symposium at the 2011 11th Scientific Meeting of the Japanese Society of Anti-Aging Medicine, the experts were invited to report recent findings on maintenance.

KEY WORDS: oral tissue, regenerative therapy, saliva, tooth regenerative therapy, periodontal ligament

1. Application of Cell Transplantation Therapy to Salivary Gland Dysfunction

The causes of salivary gland dysfunction include refractory diseases such as Sjogren’s syndrome and Stevens-Johnson syndrome, radiation therapy against head and neck cancer, and a variety of drugs 1). Current treatments include the use of artificial saliva and oral therapy with muscarinic acetylcholine receptor agonists, which stimulate salivary secretion from residual acinar cells. Severe cases may be resistant to these treatments, and patients may develop oral cavity lesions such as mucositis, caries, or periodontal disease. In addition, as salivary gland dysfunction is a pathogenic factor in aspiration pneumonia in the elderly, serious concerns have been expressed about infection treatment methods. The possibilities of regenerative medicine have therefore been investigated, specifically the reconstruction of lost gland tissues using transplantation of exogenous salivary gland stem cells. However, cell surface markers specific to salivary gland stem cells are difficult to isolate and thus remain unknown. We have therefore focused on a cell population called “side population (SP)” cells, which can be isolated without using a cell surface marker. Since their first isolation from bone marrow as a fraction containing a high frequency of stem cells, SP cells have therefore focused on a cell population called “side population (SP)” cells, which can be isolated without using a cell surface marker. Since their first isolation from bone marrow as a fraction containing a high frequency of stem cells, SP cells have been analyzed in a variety of organs 2-6). In the present study, we investigated the effects of experimental treatment with SP cells using a mouse model of irradiation-induced salivary gland dysfunction, and the possibility of establishing a treatment approach with a specific factor expressed in SP cells.
Investigation of effects of treatment with SP cells

Salivary gland tissue collected from green fluorescent protein (GFP) transgenic mice was digested with collagenase and hyaluronidase to remove interstitial tissue, and epithelial clusters were isolated using a filter mesh. The isolated epithelial clusters were then treated with trypsin to disperse the cells. 

The results of treatment with SP cells, including the observation of salivary gland dysfunction induced by UV laser irradiation and measurement at two wavelengths (450 nm and 675 nm), demonstrated a restoration of secretion volume at 1 month after transplantation. In addition, examination of the removal of tissues by fluorescence microscopy indicated the restoration of secretion volume at 1 month after transplantation.

Functional analysis of SP cell-specific expression gene

Hoechst 33342-negative SP cells and Hoechst 33342-positive non-SP (main population, MP) cells were collected using FACS, and RNAAs were extracted from the collected SP and MP cells using a PicoPureRNA isolation kit (Arcturus). The amplified RNAs derived from SP and MP cells were then used to synthesize cDNAs, which were fluorescence-labeled with Cy3 or Cy5 and subjected to competitive hybridization on NIA 15K mouse cDNA array (Version 2) to compare their gene expression profiles based on the detected signals. This method identified multiple genes specifically expressed in SP cells, among which we selected clusterin for functional analysis. Specifically, clusterin gene was introduced into STO cells, a mouse embryonic fibroblast cell line, by lipofection to prepare a cell line that stably expresses clusterin following drug selection. We next investigated the possible function of clusterin in reducing damage caused by reactive oxygen species (ROS), on the basis that irradiation-induced cell damage is mediated by ROS. Specifically, we counted viable cells stained with trypan blue 24 hours after stimulation of clusterin-expressing and control cells with different concentrations of hydrogen peroxide solution. The results showed that soluble factor(s) secreted from SP cells may be involved in the secretory mechanism of residual acinar cells.

Verification of treatment effects of clusterin using a mouse model with salivary gland dysfunction

To determine whether clusterin directly contributes to the reversal of cellular dysfunction of the salivary gland, clusterin-expressing recombinant lentivirus (Lenti-Clu, 5 x 10^6 TU) was injected into one submandibular gland of mice with irradiation-induced salivary gland dysfunction 4 days after irradiation, and GFP-expressing lentivirus (Lenti-GFP, 5 x 10^6 TU) was injected into the other. Gene transfection efficiency and time-dependent change in saliva volume were then measured to assess restoration of secretory ability. The results indicated that Lenti-GFP transfection led to GFP positivity in approximately 16% of cells. In contrast, Lenti-Clu-injected mice showed an improvement in pilocarpine-stimulated salivation at 4, 8, and 16 weeks after virus injection compared to Lenti-GFP-injected mice. These results suggested that clusterin, which is expressed in SP cells, is involved in the functional restoration of glandular secretion.

These results revealed that SP cells or clusterin, a specific factor expressed in SP cells, is effective in the treatment of irradiation-induced salivary gland dysfunction. We are planning to examine the possible clinical application of these factors in the future.

2. Role of Tooth Regeneration in Anti-Aging Medicine

Introduction

Teeth possess not only a masticatory function (i.e., “chewing”) but also act as sensory receptors, sending masticatory stimulation to centers in the brain. Caries and tooth loss secondary to periodontal disease, the incidence of which is increasing in the elderly, are known to cause significant problems with masticatory function and to affect systemic condition. Thus, the development of dental regenerative medicine that can essentially restore the physiology of natural teeth will be useful in preventing a decline in oral function and in promoting Anti-Aging. Here, we discuss the current status of R&D in dental regenerative therapy against tooth loss, as well as its potential role in Anti-Aging Medicine.

Tooth regeneration by a bioengineered organ germ method

Previously, functional complementary therapies with artificial devices such as dentures, dental bridges, and dental implants have been used as dental support for tooth loss. Although these complementary therapies are effective in the restoration of masticatory function, they cannot restore the innate physiological aspects of teeth, such as tooth movement associated with aging and response to masticatory stimulation.
Thus, a more biological treatment approach to tooth regeneration has been sought.

Teeth develop through continuous interaction between odontogenic epithelial cells and odontogenic mesenchymal cells which together constitute the embryonic tooth germ. For this reason, technologies that enable the regeneration of tooth germ from epithelial and mesenchymal cells through three-dimensional cell manipulation techniques have been developed with the goal of regenerating third teeth, in addition to primary and permanent teeth. To date, however, the ability to produce highly effective and normal tooth development has not been reached. In 2007, we developed the “bioengineered organ germ method,” in which epithelial and mesenchymal cells derived from the tooth germ are compartmentally arranged at high cellular density (Fig. 1, top). When ectopically transplanted in vivo, the bioengineered tooth bud cells develop with structurally normal regenerated teeth as well as periodontal tissue (periodontal ligament, cementum, alveolar bone), indicating the potential use of bioengineered tooth bud cells in tooth regenerative therapy.

Regeneration of functional teeth

Successful tooth regenerative therapy requires not only the histological normality of the regenerated tooth but also its eruption/occlusion within the recipient’s intraoral environment, and the full spectrum of normal tooth physiology, such as functional regeneration of the periodontal ligament and response to external noxious stimuli. When bioengineered tooth bud cells were transplanted into sites of tooth loss in adult mice, the bioteeth erupted and grew, and occlusion of the regenerated teeth was established with hardness comparable to that of natural teeth (Fig. 1, top). Further, the tissue structure of the regenerated teeth was similar to that of natural teeth, and a fully matured periodontal ligament structure was also observed, including alveolar bone. The periodontal ligament of the regenerated teeth was shown to retain the physiological capacity to remodel surrounding alveolar bone in response to experimental orthodontic force and to move teeth in a similar manner to natural teeth. In addition, similarly to natural teeth, the dental pulp and periodontal ligament of the regenerated teeth had multiple peripheral nerves, including sympathetic and sensory nerves. Upon application of mechanical stress through dental pulp exposure or dental makeover, upregulation of c-Fos expression in response to intraoral noxious stimuli in some neurons in the spinal trigeminal nucleus was observed for both regenerated and natural teeth, revealing restoration of physiological response to external noxious stimuli in the regenerated teeth. These results demonstrated that not only masticatory function but also the full spectrum of normal tooth physiology could be restored in a tooth regenerated by means of transplantation of bioengineered tooth bud cells, and clearly indicate the clinical applicability of the approach to regenerative therapy against tooth loss.

Tooth regeneration using bioengineered tooth units

Elderly people often have severe progressive periodontal disease presenting with extensive destruction of periodontal tissue essential for mastication, and tooth loss has been known to lead to the absorption of surrounding alveolar bone resulting in severe bone defects. For tooth regeneration in the elderly, an approach based on the regeneration of teeth with finished components (e.g., dental prostheses) together with periodontal tissues for immediate functional recovery after implantation would be more appropriate than transplantation of bioengineered tooth germ. On this basis, we developed the “bioengineered tooth unit,” which includes tooth and periodontal tissue (i.e., functional unit of tooth), with the aim of developing tooth regenerative technology that allows for immediate functioning.

Because the culture of three-dimensional organs ex vivo is not currently possible, we demonstrated our concept by constructing bioengineered tooth units suitable for transplantation by transplanting bioengineered tooth bud

Fig. 1 Development of tooth regenerative therapy aimed at Anti-Aging.
Regeneration of functional teeth using bioengineered tooth bud cells and treatment of extended bone defects by bioengineered tooth unit transplantation are expected to aid progress in anti-aging regenerative medicine technologies which enable the transduction of masticatory stimulation to be restored. (Scale bar: 200 µm)
cells into kidney capsules. When the bioengineered tooth unit was implanted into sites of tooth loss to secure normal occlusion, osseointegration was seen between the alveolar bone of the bioengineered tooth unit and that of the recipient, as was the restoration of a functional periodontal ligament and responsiveness to external noxious stimuli \( \text{(Fig. 1, bottom)} \). In addition, when the bioengineered tooth unit was implanted into an extensive bone defect model in mice, osseointegration was seen between the alveolar bone of the bioengineered tooth unit and jaw bone of the recipient, indicating the ability of alveolar bone to regenerate vertically. These results indicate the potential of transplantation of bioengineered tooth units in regenerative therapy to generate an immediately functioning tooth and in patients who experience tooth loss with major bone defect \(^{15}\).

Summary

Against the background of the aging society, dental therapy requires the development of dental therapeutic techniques that allow for the promotion of Anti-Aging. We consider regenerative therapy aimed at achieving a functional tooth is a desirable option. Our previous studies demonstrated that transplantation therapy against tooth loss using either bioengineered tooth bud cells or a bioengineered tooth unit can regenerate teeth that have similar physiological functions to natural teeth \( \text{(Fig. 1)} \). Realization of these therapies requires the identification of patient-derived stem cell seeds for use in regeneration of tooth germ \(^{16,17}\), development of technique to prepare bioengineered tooth bud cells using iPS cells \(^{18}\), and development of size control techniques to achieve suitable tooth size for the transplantation site \(^{19}\). Solving these problems would result in the realization of tooth regenerative therapy as an Anti-Aging dental therapy.

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3. Development of a New Periodontal Tissue Regeneration Method Aimed at Anti-Aging Use

Introduction

Periodontal tissue, which consists of the gingiva, periodontal ligament (PDL), cementum, and alveolar bone, is a collective term for the tissues that surround the tooth and play a role in supporting its function. Periodontal disease (periodontal disorder), which destroys these periodontal tissues, is a chronic inflammatory disorder which ultimately leads to tooth loss. Periodontal disease affects approximately 80% of adults and is the most common cause of tooth loss in the aged. Thus, the regeneration of periodontal tissue that has been lost due to causes such as periodontal disease is a major goal of dental care. Recently, associations between periodontal disease and diabetes (a lifestyle-related disease), cardiovascular disease \( \text{(i.e., heart disease, arteriosclerosis)} \), and systemic disorders \( \text{(e.g., aspiration pneumonia)} \) have been suggested \(^{20}\), indicating that periodontal disease represents an aging factor that is associated not only with oral health but also general health. With the rapid transition into the era of an aging society, the regeneration of periodontal tissue destroyed by periodontal disease is a critically important research area in Anti-Aging Medicine, because of its systemic Anti-Aging effects, rather than simply the restoration of oral function, such as chewing.

The PDL, a periodontal tissue, is a fibrous connective tissue with a thickness of approximately 200 μm that surrounds the tooth root and connects the tooth with the supporting alveolar bone. It lies between the alveolar bone and cementum, and fixes the tooth to the jaw bone \(^{21}\). The whole periodontal ligament is made up of a mixture of various types of cells, including fibroblasts, osteoblasts, cementoblasts, osteoclasts, undifferentiated mesenchymal cells, and epithelial cells derived from epithelial cell rests of Malassez. Of these, PDL-derived fibroblasts play an important part in the remodeling of PDL fibers \(^{20}\). However, when periodontal disease occurs, periodontal pockets are formed and the lysis/disappearance of PDL fibers occurs, leading to the destruction of periodontal tissue and finally to tooth loss.

Regeneration of periodontal tissue using periodontal ligament-derived cells

Thanks to recent progress in dental medicine, the pathology and mechanism of periodontal disease have become increasingly clear. Previous studies have strongly suggested that the presence of newly formed PDL is important for the regeneration of periodontal tissue \(^{21}\). As such, researchers have investigated the regeneration of periodontal tissue using autologous transplantation of fibroblasts derived from PDL and grown in vitro \(^{22}\). Van Dijl \text{et al.} reported that the regeneration of periodontium-like hard tissues (newly formed cementum-like hard tissues) was possible using the autologous transplantation of PDL-derived cells into experimentally-created periodontal tissue defects in an experimental animal (beagle dog) \(^{23}\). In addition, Dogan \text{et al.} reported that periodontal tissues (newly formed cementum and new bone) could be regenerated using blood clots as carriers of cultivated PDL-derived cells \(^{24}\), and Nakahara \text{et al.} reported that differentiation of newly formed cementum was enhanced using collagen sponge as a cell culture substrate to facilitate close contact between cultivated cells and the tooth root surface \(^{25}\). These reports indicate that
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Periodontal tissue can be regenerated using PDL-derived cells, and that a carrier (substrate) is important for the transplantation of cultivated cells. We have performed studies using amniotic membrane (AM), a biomaterial that has attracted interest as a cell culture substrate in various medical fields, and from these developed the idea of using this as a substrate for PDL-derived cells.

Development of a new periodontal tissue regeneration method using amniotic membrane

The AM is a thin membrane that covers the outermost surface of the placenta and consists of parenchymal tissue with a specific thickness. The tissue is normally discarded after parturition, can be collected from the placenta almost aseptically, and can be obtained without ethical or technical problems. It has unique characteristics, including anti-inflammatory and infection-reducing effects, and has been utilized as a biomaterial in various surgical therapies for purposes such as the prevention of adhesion/scarring in skin transplantation/abdominal surgery, healing acceleration as a skin burn wound dressing, and ocular surface reconstruction in ophthalmology. In addition to its use as a transplantation material, it has also attracted attention for its high usefulness and effectiveness as a culture substrate. We have previously shown the effectiveness of new amniotic membrane-based regenerative therapy to oral healthcare through successful preparation of a cultured oral mucosal epithelial cell sheet on AM and the establishment and clinical application of an autotransplantation technique for various types of oral mucosal defects in dental oral surgery. Recently, we applied this AM-based cell-culture system to culture PDL-derived cells for regenerative therapy for periodontal tissue. Below, we present progress to date and future prospects of our investigation for the development of cell sheets aimed at regeneration of periodontal tissue.

Preparation of periodontal ligament-derived cell sheets cultured on amniotic membrane

We have previously confirmed that PDL-derived cells can be successfully cultured to form a sheet using an AM-based cell-culture system. In addition, we have reported that PDL-derived cell sheets cultured on AM could potentially regenerate periodontal tissue, based on the observation that periodontal tissues (i.e., newly formed cementum and new bone) were regenerated by autologous transplantation of these sheets into periodontal tissue defects in an experimental animal (beagle dog). Growth factor, cell type, and substrate are important aspects of transplantation and regenerative therapies which are expected to act in combination in the regeneration of tissues, including in periodontal tissue defects. Among them, a variety of culture substrates have been investigated for PDL-derived cells, but an ideal substrate for periodontal tissue regeneration has not yet been developed. In addition, PDL-derived cells on substrate have not been evaluated, and a wide review of the literature reveals that the proliferation and differentiation abilities of PDL-derived cells on AM is poorly understood. Considering that the preparation of cultured human PDL-derived cell sheets will have clinical applications, we also performed an immunohistochemical study of the cell kinetics of PDL-derived cells on AM.

Human AM collected from placenta obtained during cesarean section was used. PDL tissues were collected from tooth roots after tooth extraction, etc., as appropriate. The collected PDL tissues were subjected to primary culture, and cells derived from them were used after 3-4 passages. The cells were seeded onto AM and cultured for approximately 2 weeks, then subject to immunostaining for Ki-67 (cell proliferation marker), vimentin (mesenchymal marker), desmoplakin (desmosomal marker), and ZO-1 (tight junction marker). In addition, to investigate adhesion between the AM and these cultured cells (i.e., cell-substrate adhesion), immunostaining for laminin 5/10 and collagen IV/VII (components of basement membrane) and scanning electron microscopic (SEM) observation were performed.
Tissue collection from extracted teeth and the use of AM for experimental purposes were conducted after obtaining informed consent from patients following sufficient explanation. Experimental use of PDL tissues, PDL-derived cells, and AM was approved by the Medical Ethical Review Board of Kyoto Prefectural University of Medicine (RBMR-R-21).

Results showed that the PDL-derived cells formed a monolayer on the AM after approximately 2 weeks of culture. Immunofluorescence showed the localization of Ki-67- and vimentin-positive cells and expression of desmoplakin and ZO-1. These cells were considered capable of proliferation and potentially maintaining their PDL-like properties even on AM. In addition, strong cell-cell adhesion structures, namely desmosomes and tight junctions, were shown to be present between cells (32). Laminin 5/10 and collagen IV/VII were expressed at the basal region of the PDL-derived cells (i.e., cell-AM boundary), and SEM images showed that the cells had differentiated and proliferated on AM with lateral conjugation and adhesion to the AM, indicating strong adhesion between PDL-derived cells and AM.

**Summary**

These results confirm the proliferation of PDL-derived cells on AM and the presence of strong cell-cell adhesion structures and basal membranes. AM was shown to be a potentially suitable culture substrate, and PDL-derived cells were considered to form a sheet on AM, and not to be in the form of disparate individual cells. PDL-derived cell sheets cultured on AM can be considered to represent a novel material for a new periodontal tissue regeneration method, provided its ability to regenerate periodontal tissue is confirmed and some AM-specific effects are demonstrated.

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**4. Current Status and Future Prospects of Corneal Regenerative Therapy using Oral Tissue**

**Introduction**

Human ocular surfaces consist of corneal epithelium and conjunctival epithelium. These are uniquely differentiated surface ectoderm-derived mucosal membranes which maintain homeostasis of the ocular surface in cooperation with lacrimal fluid. The tissue structure can be divided into three cellular layers: an outermost corneal epithelium layer, a corneal stroma layer, and an inner corneal endothelium layer. The corneal epithelium consists of stratified squamous epithelia with a thickness of approximately 50 μm which provides physical/biological protection from the external environment to the ophthalmus. Thanks to progress in a variety of fundamental research programs, corneal epithelial stem cells have been found to exist in the basal layer of the corneal limbus, which is positioned at the periphery of the cornea (42,43). When the corneal limbus (i.e., corneal epithelial stem cell) is lost for various reasons, biological reactions occur in which the surrounding conjunctival epithelia cover the corneal surface with accompanying inflammation or vascularization, etc., thereby resulting in significant visual disorder. Diseases associated with abnormalities of the corneal epithelial stem cell like the example above are called “refractory ocular surface disease,” and have been extensively investigated in both fundamental and clinical studies to elucidate the condition and develop treatment methods.
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Development of ocular surface reconstruction

To date, surgical reconstruction after refractory ocular surface disease has usually consisted of corneal epithelial cell transplantation (keratoepithelioplasty, corneal limbal transplantation) using donor tissue 44,45) and cultivated corneal epithelial cell transplantation 46-49). However, because these involve allotransplantation, heavy long-term use of immunosuppressive agents is required after operation. The problems of postoperative rejection, infection, and decreased quality of life in these patients indicate the need for a safer and more effective transplantation technique. Because many refractory ocular surface diseases are binocular diseases, autologous corneal epithelium cannot be used, making it important to select a cell source that has no risk of postoperative rejection. We investigated the possibility of ocular surface reconstruction using autologous oral mucosal epithelium, with the aim of developing a novel surgical technique that uses mucosal epithelium other than ocular surface mucosal epithelium (Fig. 4).

Development of a cultivated oral mucosal epithelial sheet using amniotic membrane

Cultivated oral mucosal epithelial sheet

Regeneration of a living tissue in vitro requires the establishment of an extracellular environment that facilitates the differentiation and proliferation of cells (i.e., scaffold for cells). Particularly in the case of refractory ocular surface diseases, normalization of the substrate, including the extracellular matrix, is considered essential, in addition to reconstruction of the epithelium. Amniotic membrane, a biomaterial, is a thin membrane over a thick basal membrane devoid of vasculature that covers the fetus and placenta within the uterus. It has been reported to have a variety of biological effects, including suppression of scarring and inflammation, suppression of neovascularization, and acceleration of wound healing 51-53).

First, we initiated the development of a cultivated oral mucosal epithelial sheet using amniotic membrane. Our research team first investigated the suitability of amniotic membrane with the epithelium scraped off as a culture substrate for oral mucosal epithelium in an animal study in rabbits 39). An oral mucosal cell suspension was prepared from oral mucosa collected from white rabbits, and cultured on amniotic membrane for about 3 weeks. During the culture process, cocultivation with 3T3 fibroblasts by culture insert was performed using air-lifting for differentiation induction of epithelial cells. As a result, oral mucosal epithelial cells cultured on amniotic membrane adhered to and grew on the amniotic membrane substrate and reached confluence after 1 week. On culture for 2-3 weeks, they were found to stratify, forming 5-6 layers of cells, and to have a morphology comparable to that of the basal cells, wing cells, and superficial cells of normal corneal epithelium. Morphological investigation by electron microscopy revealed that the cultivated oral mucosal epithelial sheet has desmosomes, hemidesmosomes, and tight junctions, all of which are involved in cell adhesion between epithelial cells. Numerous microvilli were observed on the cell surface, showing properties of mucosal epithelium. Immunostaining for keratin, an epithelial cytoskeletal protein, showed the expression of keratin 4/13, a mucosal-specific keratin, but not keratin 1/10, which are epidermis-specific keratinizing type keratins. In addition, among keratinizing-type keratins 3/12, immunostaining was observed only for keratin 3. While normal oral mucosal epithelium is a unique mucosal membrane in the body that expresses keratin 3, our cultivated oral mucosal epithelial sheet was found to have the cytoskeleton characteristics of non-keratinized mucosa and cornea.

Fig. 4. Diagram showing the concept of cultivated oral mucosal epithelial transplantation for refractory ocular surface disease.
Ocular surface reconstruction using autologous cultivated oral mucosal epithelial sheet

After examining the biological characteristics of the resulting cultivated oral mucosal epithelial sheet, it was autologously transplanted into the ocular surface of rabbits \(^{39}\). A rabbit ocular surface disease model was created by superficial keratectomy. At 48 hours after transplantation, the mucosal epithelial sheet was confirmed by fluorescein staining to have remained transparent and on the ocular surface without defects. At 10 days after transplantation, it was observed to have remained on the ocular surface and to have extended outward compared to its position at 48 hours. In addition, histological examination of all corneal layers at this time point showed that the cultivated oral mucosal epithelial sheet had engrafted onto the ocular surface without stromal edema or cell infiltration, and with excellent biocompatibility with the ocular surface. These results indicate that our cultivated oral mucosal epithelial sheet has characteristics of corneal epithelium-like differentiation and stratified non-keratinized mucosal epithelium in terms of its histological and cell biology characteristics. In addition, oral mucosal epithelial sheet cultured on amniotic membrane was shown to engraft and survive even on the ocular surface, a unique environment in the body, suggesting its possible use as an alternative to corneal epithelium which maintains transparency after operation.

Clinical study of cultivated oral mucosal epithelial sheet transplantation

Based on the above basic data obtained from animal studies, a clinical study of autologous cultivated oral mucosal epithelial transplantation against refractory ocular surface disease was initiated in 2002 after approval by the Institutional Review Board for Human Studies of Kyoto Prefectural University of Medicine \(^{54,55}\). Of 17 eyes of 19 patients who underwent transplantation for corneal reconstruction at the Department of Ophthalmology, Kyoto Prefectural University of Medicine, up to January 2007 with long-term follow up for 3 years or more, approximately 53\% showed a visual improvement of 1 grade or more at 3 years after operation. Postoperative complications included prolonged corneal epithelium disorder observed in approximately 37\% during the follow-up period. During long-term follow-up, some patients showed ongoing reconstruction of the ocular surface with the transplanted cultivated oral mucosal epithelial sheet at 71 months after operation, revealing that oral mucosal epithelial cells, representing ectopic mucosal epithelial cells, can engraft and function on the ocular surface when applied using this surgical procedure. Considering that refractory ocular surface diseases have not been approved as an indication for corneal transplantation, the efficacy of cultivated oral mucosal epithelial sheet transplantation using autologous tissue was clinically adequate.

Future prospects

In the history of corneal transplantation, recent progress in regenerative medicine/regenerative therapy research has produced significant innovation. Cell transplantation therapy from the \textit{in vitro} to \textit{in vivo} environments has produced a paradigm shift to corneal transplantation techniques in which replacement is limited to the defect site. The major challenges at present are the conduct of a comprehensive clinical examination of the long-term results of previous cultivated epithelium transplantation procedures, and ensuring the safety and improving the quality of the cultivated epithelial sheets. Research tasks required to meet these challenges include the identification of stem cells in the cultivated epithelial sheet and the establishment of a culture environment, including niche. In addition, problems such as serum and feeder cells, which are used in the preparation process of the cultivated epithelial sheet, have to be resolved. Furthermore, in 2006, the Ministry of Health, Labour and Welfare (MHLW) implemented its “Guidelines for clinical research using human stem cells”, which mandates review by the MHLW in addition to review by an academic ethics committee when clinical research using tissue stem cells is conducted. Further development of culture techniques will need to conform with these guidelines. In any case, our responsibility is the development of safer and more evidence-based regenerative therapy.

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Conflict of interest statement:

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