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Chapter 8

Salivary gland

Selected salivary grand cell culture

Salivary gland atrophy is commonly seen in patients in whom those salivary glands have been irradiated for oral and facial cancer treatment. In addition, aging has been reported to induce histological changes in the parenchyma of the salivary glands such as the replacement with fat or connective tissue [1]. The atrophic gland leads to dry mouth, a condition frequently associated with various symptoms including active dental caries, a burning sensation in the mouth, and difficulty with eating, swallowing or speech. Moreover, an increased incidence of infection such as oral candidiasis has been reported [2]. Patients with dry mouth have been treated with salivary substitutes and/or medications such as pilocarpine or cevimeline hydrochloride [3]. These treatments temporarily relieve the patients' symptoms, inducing salivation from the residual tissue. However, no treatment is available for the purpose of regenerating the atrophic glands.

Recently, the possibility of re-engineering atrophic or damaged salivary glands has been explored with the aim of developing novel clinical treatments [4]. Gene therapy for the salivary gland shows promise as a future treatment adjunct, since gene transfer to an irradiated salivary gland has been shown to increase fluid secretion in an animal model [5]. However, the clinical application of gene therapy to the atrophic glands requires further development and validation of the new vehicles for gene delivery, since currently available viral or nonviral vectors still require some considerations regarding safety, efficiency or the duration of transgene expression. Tissue engineering is a promising new approach to the replacement of lost or damaged tissue. Therefore, we attempted to establish a method for culturing normal epithelial salivary gland cells. Monolayer culture of salivary-gland cells has the advantage of allowing investigation of the effects of individual biochemical substances without the influence of other modifying factors. However, the use of monolayer culture techniques on cells from the normal salivary gland requires the elimination of contaminating fibroblasts and the maintenance of cell proliferation for prolonged periods. To avoid contamination by fibroblasts and other cell types, Olivar tried to select specific cell types using Ficoll (Pharmacia) density-gradient centrifugation, but he reported only the suspension culture protocols [6]. The same aims were addressed by Yang et al., using low Ca^{2+} concentrations in his medium as well as a collagen gel matrix [7]. This method exhibited fibroblast proliferation and allowed salivary-gland cells to exhibit a duct-like appearance. While this technique permitted investigation of tissue morphology, it was difficult to investigate cellular proliferation. To inhibit contamination by fibroblasts, and to obtain a stable monolayer of cultured cells in a short time, Sabatini et al. used 3T3 cells as a feeder layer for salivary-gland cell culture [8]. The 3T3 cells inhibited the proliferation of fibroblasts while maintaining the proliferative activity of adjacent epithelial cells for prolonged periods of time without differentiation. The same group reported the presence of two types of cell in these cultures; one cuboidal and in close contact with the surrounding cells, the other rounded without

cell-to-cell contact. So, we modified their method to selectively culture acinar cells during the first and second passages. The method is described in detail in the following

Preparation of 3T3 cells

The 3T3 cells were kindly provided by Dr. Howard Green (Department of Cellular and Molecular Physiology, Harvard Medical School, Boston, MA, U.S.A.). To suppress cell proliferation, they were treated with 4 μg/ml mitomycin C in DMEM for 2 hours at 37°C without fetal bovine serum. Cells treated in this manner promote the proliferation of adjacent epithelial cells but do not proliferate themselves. After incubation, the 3T3 cells were rinsed three times with Hank's solution to remove the mitomycin C.

Tissue preparation and salivary gland epithelial cell culture

The submandibular glands were excised from Wistar rat and rinsed in PBS containing 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B. Using curved scissors, the tissues were cut into cubes of at least 1 mm^3. Salivary gland cells were dissociated by digestion with a solution of 0.1 mg/ml collagenase in PBS for 30 minutes at room temperature. Cells were then stirred for 30 minutes with a magnetic stirring bar, filtered through a 70-μm nylon mesh, and collected by centrifugation for 5 minutes at 1500 rpm. The separated salivary gland cells were cultured in 75-cm plastic flasks, in a humidified atmosphere of 10% CO_2 at 37°C in a 3: 1 mixture of DMEM and Ham's F-12 containing 5% fetal bovine serum, 5.0 μg/ml insulin, 5.0 μg/ml transferrin, 2×10^{-9} M tri-iodothyronine, 1×10^{-9} M cholera toxin, 0.4 μg/ml hydrocortisone, 0.1 μg/ml epidermal growth factor, 10 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B (Fig. 37). To confirm the origin of these cultured cells, amylase production was examined by electron microscopy and periodic acid Schiff staining, together with immunocytochemical analysis of myosin, anticytokeratin (CK-1, CK 10/13, CK-MNF116, CK-LMW, CK-HMW and CK-19) and amylase antibody.

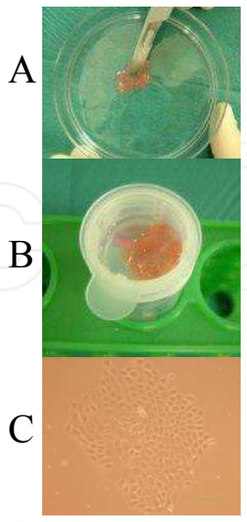

Fig. 37. Protocol of salivary gland epithelial cell culture. A: Excise salivary glands, and cut into cubes of at least 1 mm^3. B: Dissociation by digestion with a solution of 0.1 mg/ml collagenase in PBS for 30 minutes and filtered through a 70-μm nylon mesh. C: Cell culture (From Horie et al. 1996. Reprinted with permission).

The cultured cells demonstrated secretion granules containing amylase and presented features characteristic of acinar cells, which they retained until passage two. By using a feeder layer in conjunction with a newly formulated culture medium, the selectability of these cells was improved. Changes in proliferation of cultured salivary-gland cells in the presence of selected neurotransmitters were also examined, Isoproterenol enhanced cellular proliferation.
Thus, we established a culture method for normal epithelial salivary gland cells. However, tissue engineering of a whole organ is still not feasible since it requires the regeneration of a complex ductal system, blood supply and renervation, none of which are possible with the current technology. Cell transplantation is also a possible treatment option to restore the function of lost or damaged tissue, and has been extensively studied in cases of type I diabetes [9-12], Parkinson's disease [13-15], and liver dysfunction [16]. Most such studies have aimed to restore the function of a specific cell type or cell groups in an organ. The possibility of regenerating an entire organ by cell transplantation has not been demonstrated except for the liver, which possesses enormous regenerative capacity [17]. Recent studies have shown that transplanted bone marrow cells might replace tissue in many more organs than was previously thought possible. If this is the case, transplanted salivary gland stem cells could eventually restore at least part of the organ after extensive cell proliferation and differentiation. Since the cell therapy approach can avoid some of the technical difficulties posed by tissue engineering, it is worthwhile investigating its potential for the treatment of atrophic salivary glands.

Cell transplantation into an atrophic salivary gland

Next, we investigated the feasibility of a cell therapy for an atrophic salivary gland using cultured salivary gland cells, we established the culture method. First, we investigated the survival and distribution of transplanted cells in the salivary gland. Second, we demonstrated a possible differentiation of the transplanted cells. Third, we examined the possible drawbacks of cell transplantation on normal salivary glands since the cells could be harmful if they attach and remain within a normal tissue structure. Below is how to generate an atrophic salivary gland model and transplant the cultured cells into atrophic glands.

Generation of atrophic submandibular gland by ductal ligation

The atrophic submandibular gland was generated by a ductal ligation according to Tamarin with modifications [18, 19]. Briefly, the main ducts of the left submandibular-sublingual glands of male Wistar rats (each weighing about 200 g) were exposed surgically. The ducts with some surrounding connective tissue investments were isolated by blunt dissection under a surgical microscope. A specially prepared stainless steel tube and a nylon ligature were looped around each duct and tied. The tissues were then reapposed and the skin was sutured. The ligation period lasted one week for all experiments. One week after ductal ligation, atrophy of the acinar cells was observed. Ducts were dilated and fibrous connective tissues were prominent.

Cell transplantation

The procedure and protocol for our cultured salivary gland epithelial cell transplantation are summarized in figure (Fig. 38). Prior to transplantation, the cultured salivary gland epithelial cells were detached from culture dishes by enzymatic treatment with 0.05% trypsin-EDTA. The cells were then labeled with a fluorescent cell linker PKH26 at a concentration of 2×10^{-6} M for 5 minutes. The cell pellet was resuspended in DMEM containing 10% fetal bovine serum to yield a density of 50,000 cells/μl. India ink was added

to the cell suspension at a 1:200 dilution in order to locate the injection site. Twenty microlitters of the cell suspension containing 2 × 10^6 PKH26-labeled cultured cells was injected through a 25-gauge needle into the atrophic submandibular gland. When the injections were properly placed, India ink was visible below the capsules. In the control group, the same cell suspension was injected into normal submandibular glands.

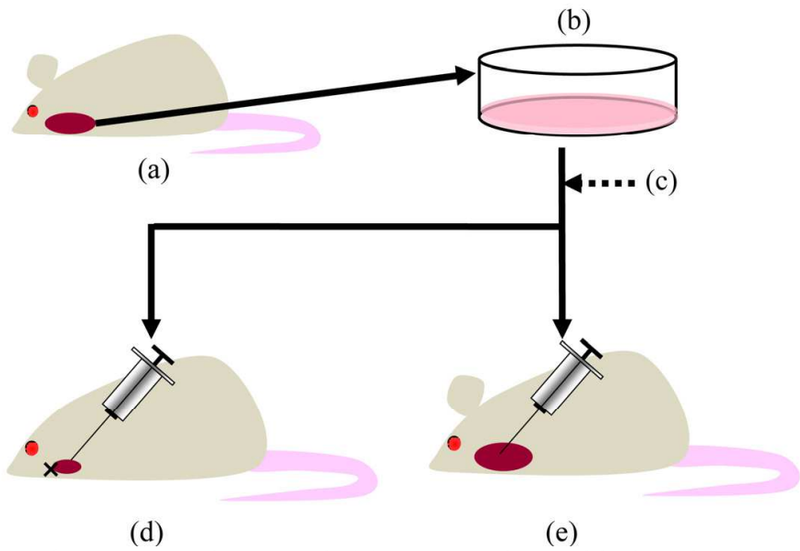

Fig. 38. Schema ofexperimental protocol. (a) Excise submandibular glands. (b) Cell culture. (c) Cell labeling with PKH. (d) Cell transplantation intoatrophic submandibular glands. (e) Cell transplantation into normal submandibular glands (From Sugito et al. 2004).

The submandibular glands were excised at 2 and 4 weeks after transplantation. At least four animals were used at each of the above time points in both the experimental and control groups. The excised glands were fixed in 10% buffered formalin and embedded in OCT Compound on dry ice. The samples were then stored at -80°C until the time for cryosectioning. Two weeks after cell transplantation, the transplanted cells were detectable in both the experimental and control groups. The cells were clustered in the connective tissue between the lobules. Four weeks after transplantation, the labeled cells were detectable in the experimental group but not in the control group. In the atrophic glands, the scattered transplanted cells were observed over a broad area of the gland but localized mainly around the acini and ductal region. Immunostaining results showed a possible involvement of the transplanted cells in ductal regeneration, while neither myoepithelial nor acinar differentiations were observed within the four weeks since transplantation. This study demonstrated that cell transplantation to the salivary gland is feasible, and that the transplanted cells were selectively attracted to and remained in the damaged area without affecting normal tissue.

The present study described the possibility of cell therapy for the salivary gland by use of cultured normal salivary epithelial cells. However, these results are very preliminary since the period of observation was short, and the longer-term fate of the transplanted cells is not known. Long-term observations as well as investigations into a suitable cell source are areas for future studies.

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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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