Mini Review

Application of cell-sheet engineering for reconstitution of functional thyroid tissue from isolated thyroid cells

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We have recently used a tissue-engineered cell sheet system with temperature-sensitive culture dishes to regenerate tissues such as corneal epithelial graft sheets, bioartificial tracheas, mucosal epithelial sheets for esophageal ulcers, and pulsatile cardiac tissue grafts, all of which can be used in clinical medicine. With regard to the thyroid gland, the three-dimensional follicular structure consists of polarized thyroid epithelial cells, and this structure is important for the production and secretion of thyroid hormones. In order to regenerate thyroid tissue from isolated cells, we used temperature-responsive culture dishes to harvest thyroid follicular cell sheets in the first step of reconstituting functional thyroid tissue. Initial trials demonstrated that the following conditions improved the recovery of cells sheets: 1) pretreatment of the dishes with serum, 2) use of medium free of thyroid stimulating hormone (TSH), and 3) co-culture with skin fibroblasts.

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Introduction

Thyroid glands possess unique structures called follicles which produce and secrete thyroid hormone[1)]. Within these structures, thyroid follicular cells function in a polarized manner[1)]. That is, the basal portion of follicular cells transports iodide from the blood via the Na^+/I^- symporter (NIS). Intracellular thyroid peroxidase (TPO) rapidly oxidizes iodide and binds it to tyrosyl residues of thyroglobulin. TPO also catalyzes iodotyrosine coupling in iodothyronines[1)]. These reactions take place at the apical cell membrane, where TPO is localized. TPO molecules contact the follicular lumen, where the iodinated thyroglobulin is stored as a colloid. In the presence of thyroid-stimulating hormone (TSH), there is a rapid endocytosis of thyroglobulin and its subsequent hydrolysis releases thyroid hormones into the blood stream at the basal portion of the cell. As mentioned above, these reactions are closely dependent upon the localization of

TPO, NIS, TSH receptors, and colloid relative to the blood stream. Therefore, the three dimensional (3D) structure as well as cell polarity are very important for proper function of the thyroid gland. For the reconstitution of functional thyroid grafts from isolated thyroid cells or thyroid stem cells, these structures and cell polarity should be reproduced. Reconstitution of the 3D structure of thyroid follicles has been reported using collagen gel culture[2,3]. However, it is not a suitable system for *in vivo* transplantation of functional tissue to animals or human patients because of the difficulties of manipulations and adverse effects of collagen itself.

Recently, we established a tissue reconstruction method using tissue-engineered cell sheets[4,5]. This method consists of harvesting cell sheets from thermo-responsive culture dishes, covalently grafted with the temperature-responsive polymer poly-(*N*-isopropylacrylamide) (PIPAAm). Using this method, the detached cell sheets maintained long-term differentiated functions within their extracellular matrix. We have already reported the application of this technique for reconstituting pulsatile myocardial tubes[6], bioartificial tracheas[7], grafts for esophageal ulceration[8], periodontal ligament tissues[9], and corneal epithelial sheets[10].

Our long-term goal is to achieve reconstitution of functional thyroid tissue using tissue-engineered cell sheets. Towards that end, and as a first step, we studied the effects of culture conditions using rat FRTL-5 thyroid cells as well as primary cultured cells from human thyroids. In this report, we describe the establishment of culture conditions for harvesting thyroid cell sheets from the temperature-responsive culture dishes.

Materials and methods

Specific procedures for the preparation of PIPAAm-grafted cell culture dishes were described previously[11]. Rat FRTL-5 cells were obtained form Interthyroid Corporation (Baltimore, MD, USA) and were cultured in Ham's F-12 medium, supplemented with 5% calf serum (CS) and a six-hormone mixture (6H), containing bovine TSH (1 mU/mL), insulin (10 μg/mL), cortisol (0.4 ng/mL), transferrin (5 μg/mL), glycyl-L-histidyl-L-lysine acetate (10 ng/mL), and somatostatin (10 ng/mL) at 37℃ in a humidified atmosphere of 5% CO_2 in air.

Fresh human thyroid tissues were obtained during thyroidectomy for Graves' disease. Each patient gave informed consent, approved by the intramural committee of Ito Hospital (Shibuya-ku, Tokyo, Japan). The tissues were minced with scissors and surgical knives, washed with ice-cold Ca^{2+}- and Mg^{2+} -free Hanks' balanced salt solution (HBSS), and digested with 5 mg/mL Dispase (Godo-Shusei Co., Tokyo, Japan) and 1 mg/mL collagenase (Class II, Worthington Biochemical, Lakewood NJ, USA) in HBSS at 30℃ for 1 hr. After washing with HBSS, the isolated cells and fragmented follicles were resuspended in DMEM supplemented with 10% fetal calf serum (FCS). The cells were seeded on PIPAAm dishes using experimental conditions described in *Results and Discussion*. Unless otherwise noted, the cells were cultured at 37℃ in a humidified atmosphere of 5% CO_2 in air.

Results and Discussion

1) Attachment of Rat FRTL-5 cells to PIPAAm dishes

In preliminary experiments, FRTL-5 cells hardly attached to PIPAAm dishes after cell seeding. This response had been observed previously with other types of cells, and FCS or medium treatment of the dishes facilitated attachment. We, therefore, treated the dishes with FCS for 18 hours at 37℃ in the CO_2 incubator. After removal of FCS the dishes were filled with 6H medium with 5% CS and left in the incubator for one hour before cell seeding. FRTL-5 cells were seeded with 6H medium with 5% CS prewarmed to 37℃. Using this method, the cells attached to the PIPAAm dishes after 24 hours of culture. We carried out the following experiments using dishes treated by the same procedures.

2) Recovery of FRTL-5 cell sheets from PIPAAm dishes

In an effort to recover FTRL-5 cell sheets from the temperature sensitive PIPAAm dishes, we first treated the cells under normal growing condition (6H medium with 5% CS). Even after shifting the temperature of the dishes to 20℃, the cells remained firmly attached. After mild agitation of the medium by pipetting, they detached as small pieces but not as a sheet. TSH is the most important regulator of thyroid tissue and it affects cellular functions, morphology and growth. For example, it changes the morphological appearance of the cells by increasing cell height. We speculated that it might also affect cell adherence. We, therefore, employed cells preincubated without TSH. After incubation in medium without TSH for 24 hours, the temperature of the cells was shifted to 20℃. Under these conditions, the cells detached as an intact sheet from the dish after several hours (Fig.1A,B). These observations suggested that TSH modulates the attachment of thyroid cells to the surrounding extracellular matrix.

3) Rat FRTL-5 cells co-cultured with human skin fibroblasts

To study alternative approaches to cell harvesting, we investigated the influence of cell co-culture. FRTL-5 cells were seeded first. After one day of culture, human skin fibroblasts were seeded in 6H medium supplied with 5% CS. After one day's culture, the

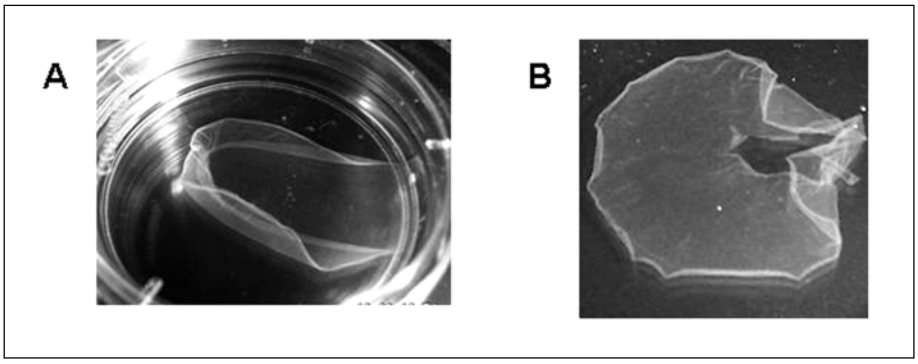

Fig.1 Recovery of rat FRTL-5 cell sheets using temperature-sensitive culture dishes

FRTL-5 cells were cultured and seeded on PIPAAm dishes as described in Materials and Methods. When the cells grew to confluence, the medium was shifted to 6H medium containing 5% CS, which lacks TSH, and cultured for one day. The temperature of the cells was then shifted to 20℃. The cells started to detach from the margin after one hour (A) and the cells were recovered as a sheet within 4 to 6 hours (B).

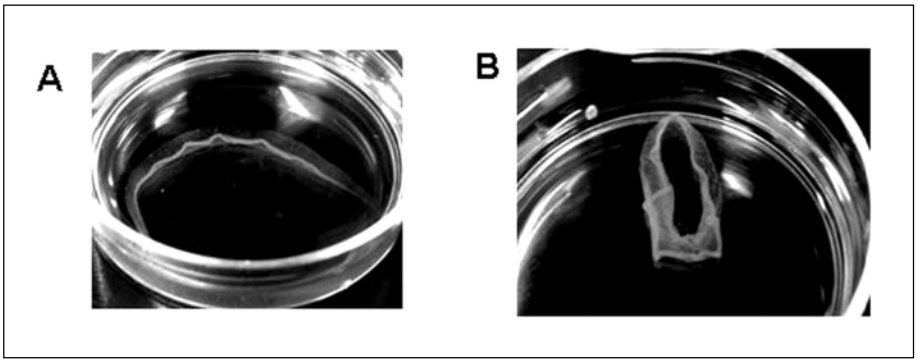

Fig.2 Recovery of human thyroid cell sheets using temperature-sensitive culture dishes

Isolated human thyroid cells were prepared and seeded on PIPAAm dishes as described in Materials and Methods. The next day, the medium was shifted to fresh medium with 10% FCS and the temperature of the cells was shifted to 20℃. Within 20 minutes, the cells started to detach from the margins (A) and the cells were recovered as a sheet within 60 minutes (B).

concentration of CS was reduced to 0.2% to prevent fibroblast proliferation. On the following day, the temperature of the cells was shifted to 20℃. After two hours, the cell sheet composed of FRTL-5 cells and skin fibroblasts could be recovered even when TSH was present in the medium (data not shown). Therefore, co-cultivation with other types of cells facilitates recovery of fragile cell sheets even when the growth medium is suboptimal for recovery.

4) Human thyroid cell culture on PIPAAm dishes and cell sheet recovery

Based on the preceding trials using rat FRTL-5 cells, PIPAAm dishes were pretreated as described above using preincubation of plates with FCS at 37℃. Then, the dishes were filled with DMEM with 10% FCS. After one hour, isolated human primary thyroid cells were seeded with DMEM with 10% FCS. Within 24 hours of culture, the cells attached onto the PIPAAm dishes.

After the cells reached confluence the old medium was replaced with fresh medium with 10% FCS and the temperature was shifted to 20℃. The human thyroid cells spontaneously detached after one hour (Fig.2A) and the cells were recovered as a contiguous cell sheet (Fig.2B).

Our observations indicated that FCS pretreatment facilitated cell attachment. However, for clinical use, avoidance of FCS would be preferable. Our findings suggested that fibroblasts and endothelial cells may reinforce the structure of the cell sheet. Therefore, co-cultivation with own fibroblasts may allow cell sheet recovery of immature stem cells or precursor cells.

We used cells cultured in medium containing 10% FCS without TSH for human thyroid cells. Previous reports[12,13], however, showed that higher concentrations of FCS without TSH inverts cell polarity and reduces the response to TSH. Therefore, TSH after cell sheet recovery may be necessary for reconstitution of

functional thyroid cell sheet. Thus, the culture conditions used before and after cell sheet recovery should be studied in greater detail to optimize reconstruction of functional thyroid grafts for use in clinical medicine.

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