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Enhanced cell survival of melanocyte spheroids in serum starvation condition

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Abstract

Autologous melanocyte transplantation for vitiligo treatment by use of melanocyte suspension has drawbacks including cell damage in cell preparation and transportation, difficult manipulation and low engraftment rate in acral vitiligious lesions. We have proposed the concept of cellular patch as an alternative solution. In the development of melanocyte patches, we have shown that chitosan membrane supports the growth and phenotype expression of melanocytes. Surprisingly, melanocytes spontaneously grow into three-dimensional spheroids on chitosan-coated surface. In this work, we demonstrate that, compared with monolayered melanocytes, melanocyte spheroids show a better survival in growth factor and serum-deprived condition. Survival of melanocytes is further ameliorated when a greater portion of melanocytes is precultured into spheroidal morphology. Melanocyte spheroids disintegrate and the cells return to a physiological dendritic morphology after they are reinoculated on collagen I-coated surface. Our results show that melanocytes are morphologically transformable depending on the substratum used and spheroidal melanocytes have a superior survival to that of monolayered dendritic melanocytes in stringent conditions. Preculturing melanocytes into spheroids can provide melanocytes a survival advantage. Chitosan-based melanocyte patch can be a promising method to enhance the engraftment rate and facilitate the cell preparation and transplantation procedures in melanocyte transplantation for vitiligo treatment.

Keywords: Melanocytes; Spheroids; Chitosan; Vitiligo; Cellular patch; Cell survival

1. Introduction

Vitiligo, a common acquired depigmented disease is characterized by destruction of functional melanocytes in the epidermis [1,2]. Non-invasive treatment for vitiligo includes topical steroid, psoralen plus UVA (PUVA), narrow-band UVB, and low energy laser irradiation [1–3]. These treatments are aimed at reactivating dormant melanocytes, presumably located in the hair follicle, to repopulate the depigmented epidermis [1,4–6]. In difficult cases, autologous melanocyte transplantation using cultured melanocytes has been used [2,7–10]. In the preparation of cells for transplantation, the cells are trypsinized into suspension. The cell suspension is then applied on the dermabraded depigmented lesions during transplantation. However, difficulty in handling the melanocyte suspension and variable successful repigmentation rates is often encountered. For example, it takes efforts to evenly spread melanocytes on the lesions during transplantation. When the lesional skin is rugged, seeding and secure attachment of melanocytes onto the skin is very challenging. Further, the success rates for repigmentation for lesions on acral

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parts and periorificial areas are very disappointing [2,10]. Correction of depigmentation on these areas is of invaluable cosmetic, psychological and social significance.

To overcome drawbacks using melanocyte suspension, the concept of using tissue-engineered "cellular patch" for melanocyte transplantation has been proposed in our previous publication [11]. The cellular patch is made by preculturing melanocytes on a biomaterial membrane and the biomaterial-melanocyte patch can be used to cover the lesional sites with an upside-down orientation during transplantation. It can simplify the process in preparing cells for transplantation and also facilitate the transplantation procedures. In the development of melanocyte patch, we have shown that chitosan is a good candidate for use as the membranous part of the patch [11]. It supports the growth and phenotype expression of human melanocytes [11]. Similar to neuron cells, human melanocytes adopt a dendritic morphology both in vivo and in conventional culture condition [12]. Surprisingly, human melanocytes formed three-dimensional multicellular spheroids on chitosan-coated surface when an appropriate seeding density was used [11]. Up to this point, the biological function of the melanocyte spheroid was unknown.

In this work, we demonstrate that human melanocytes have a better survival in growth factor and serum deprived condition when they are precultured into multicellular spheroids on chitosan-coated surface. The enhanced survival is further ameliorated when a greater portion of melanocytes is cultured into spheroidal morphology before growth factor and serum are deprived. Further, spheroidal melanocytes grow back into physiological dendritic melanocytes when they are re-inoculated on collagen I-coated surface. Hence, chitosan-based melanocyte patch can be a promising method to enhance the engraftment rate and facilitate the cell preparation and transplantation procedures in autologous melanocyte transplantation for vitiligo treatment.

2. Materials and methods

2.1. Preparation of culture wells coated with chitosan

A 15 mg/ml (W/V) solution of chitosan (C-3646, Sigma, USA, Mn = 810, 000 gm/mole, degree of deacetylation = 85%) was prepared by dissolving chitosan in 1 M acetic acid. For preparing chitosan-coated wells, 0.5 ml of chitosan solution was added into each well of 24-welled tissue culture polystyrene plates (Costar, USA). The solution was then allowed to dry at 50 °C for 2 days to form a thin membrane. Each well was then neutralized by 0.1 N NaOH aqueous solution for 15 min and washed thoroughly with double-distilled water. Before cell culture, the prepared chitosan-coated wells were sterilized in 70% alcohol overnight and rinsed extensively with phosphate buffered saline (PBS), followed by treatment under ultraviolet light overnight. As controls, uncoated polystyrene wells were treated by the same way as chitosan-coated wells.

2.2. Cell culture, cell death assays and cell survival assays

Human melanocytes were provided by cell bank of Department of Dermatology, National Taiwan University Hospital. The study protocol was approved by our Institutional Review Board. We conformed to the Helsinki Declaration with respect to human subjects in biomedical research. Human melanocytes are cultured using modified melanocyte medium (MMM) as previously described [11]. Passage 1 or 2 melanocytes are detached by trypsinization before seeding. Cells were seeded evenly in the wells at the density of $25 \times 10^3/cm^2$ in the following experiments unless stated otherwise.

To investigate the survival of melanocytes in serum starvation condition, the cells were first cultured in the chitosan-coated wells using MMM. In control groups, uncoated tissue culture polystyrene wells were used. The culture medium was aspirated and the cells were rinsed twice with PBS before the medium was changed to starvation medium DMEM (DMEM, BIOSOURCE INC., USA) at indicated times described in the result.

It has been shown that growth factor withdrawal induces death of human melanocytes [13]. Qualitative and quantitative determination of melanocyte death during growth factor and serum deprivation was performed using 7-Aminoactinomycin D (7-AAD) staining and lactate dehydrogenase efflux assay at indicated times [14,15]. For 7-AAD staining, cells in the supernatant were collected by centrifugation and cells attached to the culture wells were obtained by trypsinization. The total cells collected from the same well were mixed and rinsed with PBS twice and then incubated in 7-AAD (Sigma, USA) solution (1 mg/ml in PBS) for 20 min at 4 °C in the dark. The cells were collected by centrifugation and then rinsed thoroughly with PBS twice. Then the cells were transferred to a precoated glass slide. The cells were inspected and photographed with a Nikon fluorescence microscopy. For determination of lactate dehydrogenase released by dead cells into the medium, lactate dehydrogenase efflux assay using the supernatant was performed by use of a cytotoxicity detection kit at indicated times according to the product manual (Cytotoxicity Detection Kit, Cat. No. 1644793, Roche, Germany). The lactate dehydrogenase efflux was determined by the optical density at 490 nm obtained from an enzyme-linked immunosorbent assay (ELISA) plate reader (ELx800, BIOTEK).

Quantitative analysis of cell survival was determined by MTT assays at the indicated times [11,16], because counting of cells in the spheroids involves vigorous mechanical dissociation which damages the cells and results in a lower accuracy than automated quantification. The 300-μl MTT (Sigma, USA) solution (2 mg/ml in PBS) was added to each well. After 3 h incubation at 37 °C, the supernatant was discarded and dimethyl sulfoxide (Merck, Germany) of 200 μl was added to dissolve the formazan crystals. The dissolvable solution was jogged homogenously for 15 min at room temperature by the shaker. The solution of each well was transferred to an eppendorf and was centrifuged at 1500 r.p.m. for 1 min to remove any possible melanocytes contaminated in the solution. The optical density of 100 μl of the formazan solution was read on an ELISA plate reader (ELx800, BIOTEK) at 570 nm. For morphological observation, the cells are also photographed by digital camera coupled to a phase contrast microscopy.

2.3. Behavior of melanocyte spheroids on collagen-coated surface

Because transplanted melanocytes make direct contact with dermabraded dermis which is composed mainly of collagen I, we investigated the behavior of melanocyte spheroids on the collagen I-coated surface. Glass coverslips were coated with collagen I as previously described [17]. After cultured on chitosan-coated wells for 5 days when melanocytes formed compact spheroids, the melanocyte spheroids were removed from chitosan-coated wells by use of a pipette carefully without disturbing the spheroids. The spheroids were transferred to a tissue culture well containing a coverslip pre-coated with collagen I and then cultured in MMM. The morphology of the cells were photographed under phase contrast microscope at indicated times.

3. Results

3.1. Survival advantage of melanocyte spheroids in growth factor and serum deprived condition

When melanocytes were cultured on chitosan-coated wells, they formed multicellular spheroids on day