Development and validation of a simple method for the extraction of human skin melanocytes

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Abstract Primary melanocytes in culture are useful models for studying epidermal pigmentation and efficacy of melanogenic compounds, or developing advanced therapy medicinal products. Cell extraction is an inevitable and critical step in the establishment of cell cultures. Many enzymatic methods for extracting and growing cells derived from human skin, such as melanocytes, are described in literature. They are usually based on two enzymatic steps, Trypsin in combination with Dispase, in order to separate dermis from epidermis and subsequently to provide a suspension of epidermal cells. The objective of this work

was to develop and validate an extraction method of human skin melanocytes being simple, effective and applicable to smaller skin samples, and avoiding animal reagents. TrypLETM product was tested on very limited size of human skin, equivalent of multiple 3-mm punch biopsies, and was compared to Trypsin/Dispase enzymes. Functionality of extracted cells was evaluated by analysis of viability, morphology and melanin production. In comparison with Trypsin/Dispase incubation method, the main advantages of TrypLETM incubation method were the easier of separation between dermis and epidermis and the higher population of melanocytes after extraction. Both protocols preserved morphological and biological characteristics of melanocytes. The minimum size of skin sample that allowed the extraction of functional cells was 6×3 -mm punch biopsies (e.g., 42 mm²) whatever the method used. In conclusion, this new procedure based on TrypLETM incubation would be suitable for establishment of optimal primary melanocytes cultures for clinical applications and research.

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Introduction

In human skin, melanocytes are specialized cells of the epidermis and hair follicles that produce the pigment melanin. In normal epidermis, they constitute approximately 3–7% of the cells, contacting with several neighboring keratinocytes and forming an epidermal melanin unit. Melanocytes are localized in the basal layer of the epidermis at the junction of the dermis, and their dendrites allow melanin transfer to keratinocytes. Establishment and characterization of melanocyte cultures are important for understanding and treating pigment-related disorders, or developing in vitro pigmented skin models (Guerra et al. 2003; Duval et al. 2003; Yoon et al. 2003; Verma et al. 2015; Kumar et al. 2012). Although a number of cell lines originated from healthy and lesional skin are used for studying cellular and molecular biology, testing cosmetic and chemical products, primary culture remains as a more reliable model that mimics in vivo skin. Several methods for the isolation and the culture of melanocytes from human skin have been described (Hsu et al. 2005; Godwin et al. 2014; Tang et al. 2014a, b; Nielsen and Don 1984; Gilchrest et al. 1984; Yarani et al. 2013; Tsuji and Karasek 1983). The first human melanocyte culture from skin was published by Hu et al. (1957). Unlike basal keratinocytes that are proliferative and capable of regenerating the epidermal layers, melanocyte cultures have low proliferation potential and short life span. To obtain high-density primary cultures of melanocytes, different selective media are supplemented with mitogens as Phorbol 12-myristate 13-acetate (PMA), endothelins, growth factors (basic fibroblast growth factor, stem cell factor, hepatocyte growth factor and nerve growth factor) (Eisinger and Marko 1982; Halaban et al. 1987; Imokawa et al. 1998; Yada et al. 1991; Peacocke et al. 1988). 5-fluorouracil (5-FU) is utilized to isolate pure populations of melanocytes free from keratinocytes (Tsuji and Karasek 1983). Isolation of melanocytes from human skin explants is usually performed in a two-step protocol, Dispase incubation to separate the dermal and epidermal layers, followed by Trypsin treatment to release the epidermal cells (Tang et al. 2014a, b). Although Trypsin is utilized for a variety of cell culture applications, it is a porcine or bovine pancreas-derived enzyme. Animal-derived Trypsin may contain toxic substances and induce cellular damages. For clinical use, the good manufacturing

practice (GMP) guidelines do not recommend reagents that are derived from animal proteins because they can be a source of zoonosis diseases. Therefore most of the techniques described in the literature contain a limiting factor for clinical application.

This work describes a new method for isolation of melanocytes, based on one-step enzyme incubation using the TrypLE™, an animal origin-free and recombinant enzyme derived from microbial fermentation. TrypLE™ is an alternative source of Trypsin and can be used to dissociate various mammalian cell types (Manira et al. 2014). We reported the number of cells isolated from different limited sizes of skin tissue and characterized the cell population. In addition, we compared our novel procedure with an established technique (Godwin et al. 2014), studying the functionality of the extracted melanocytes.

Materials and methods

Collection of skin biopsies

Skin samples were obtained from discarded skin following abdominoplasty with informed donor consent (five patients, 43 ± 17 years, skin types II–IV). The hypodermis layer was removed. Subsequently, skin biopsies of 3 mm in diameter were realized and put for 10 min in phosphate-buffered saline (PBS) containing 100 IU/ml penicillin and 100 µg/ml streptomycin (PS) (all from Pan-Biotech, Dutscher, Brumath, France). In total, 45 biopsies were collected per skin and 6 distinct groups were realized according to the number of biopsies (5–10).

Dissociation of epidermis from dermis and separation of epidermal cells

Method 1 Simple enzymatic digestion (TrypLE™) (Fig. 1). Biopsies were incubated in TrypLE™ enzyme 1X (Table 1, GIBCO® TrypLE™ Select™ CTS™, ThermoFisher Scientific, Villebon-sur-Yvette, France) at room temperature overnight (17 h). During the treatment, the epidermal layer was easily separated from the dermis. After incubation, all dermis were discarded, then melanocyte culture medium was added and epidermal cells were disaggregated mechanically by repeated pipetting up and down. Mixtures were filtered through 70 µm cell

Fig. 1 Schematic diagram for the isolation of melanocytes from small amount of human skin, via the TrypLE™ method. The initial seeding density for initiating the primary culture is $2\text{--}4 \times 10^4$ cells per 25 cm^2 tissue culture flask. Generally, one such flask is set up from an initial yield of $4\text{--}10 \times 10^5$ cells isolated from about $42\text{--}70\text{ mm}^2$ piece of skin

Table 1 Composition of the GIBCO® TrypLE™ Select™ CTS™, ThermoFisher Scientific

Components	Molecular weight	Concentration (mg/l)	mM
Potassium chloride (KCL)	75	200	2.67
Potassium phosphate monobasic (KH ₂ PO ₄)	136	200	1.47
Sodium chloride (NaCL)	58	8000	137.93
Sodium phosphate dibasic (Na ₂ HPO ₄ ·7H ₂ O)	268	2160	8.06
EDTA	416	457.6	1.1
rProtease (controlled fermentation process)	Confidential		

strainers, and centrifuged for 5 min at 1100 rpm. Cell pellets were resuspended in melanocyte medium which consists of M254 culture medium (ThermoFisher Scientific) supplemented with human melanocytes growth supplement (HMGS, Life Technologies, ThermoFisher Scientific), 100 IU/ml penicillin and 100 µg/ml streptomycin. Epidermal cells were counted, placed into 25 cm² tissue culture flasks at a cell density of $2\text{--}4 \times 10^4$ cells/cm², and kept at 37 °C in a humidified atmosphere with 5% CO₂.

Method II Double-enzymatic digestion (Dispase and Trypsin). A control group was set up by incubating biopsies in 10 mg/ml Dispase II (GIBCO®, ThermoFisher Scientific) at 4 °C overnight (17 h). The epidermis was separated from the dermis using fine forceps. Isolated epidermal layers were put in 0.05% Trypsin/0.02% EDTA solution (Pan-Biotech, Dutscher) at 37 °C for 10 min.

After incubation, melanocyte culture medium was added and epidermal cells were disaggregated mechanically by repeated pipetting up and down. The following steps were as for TrypLE™ method.

Primary melanocyte cultures

Culture medium was firstly changed after 48 h, then every 3 days. When melanocytes reached 70% confluency, they were harvested by 3 min treatment at 37 °C with 0.05% Trypsin/0.02% EDTA solution. The cell suspension was neutralized with fetal bovine serum (Pan-Biotech, Dutscher) and centrifuged for 5 min at 1100 rpm. The cell pellet was resuspended in melanocyte medium. Viable cell counts were performed before seeding melanocytes at 16×10^3 cells/cm² into new culture flask for another round of incubation. Images of cell cultures were acquired using an Olympus IX50 inverted microscope.

Characterization of melanocytes by flow cytometry

Two and ten days after extraction, cells were fixed in 3% paraformaldehyde (PFA) in PBS for 10 min, then rinsed in PBS and permeabilized for 15 min in 0.1% Triton X-100 in PBS. Nonspecific binding sites were blocked at room temperature by a 10 min incubation in 1% glycine in PBS followed by a 1 h incubation in 3% bovine serum albumin (BSA) (Sigma-Aldrich, St.

Quentin-Fallavier, France) solution in PBS containing 10% normal goat serum (Sigma-Aldrich) and 0.1% Triton X100. Then cells were incubated at 4 °C overnight with polyclonal rabbit anti-human microphthalmia-associated transcription factor (MITF) at 1:1000 dilution, along with appropriate isotype control. They were rinsed twice and stained 1 h at room temperature with goat anti-rabbit antibody conjugated with fluorescein isothiocyanate (FITC) diluted 1:2000 (all antibodies from Sigma-Aldrich). Cells were centrifuged and suspended in 2 mM EDTA in PBS. They were analyzed on a Beckman Coulter FC 500 flow cytometer.

Viability assay

Melanocytes at passage 2 were seeded at a density of 6×10^4 cells/well in 12-well plates. After 1, 2, 3 and 4 days of culture, cells were harvested by Trypsinization and counted using trypan blue exclusion.

Measurement of melanin content

Melanocytes at passage 2 were plated at 400,000 cells on 25 cm² flask and grown for 5 days to 70% confluence. They were washed with PBS, collected by scraping and pelleted. Intracellular melanin was extracted by solubilizing frozen cell pellets in 1 N NaOH containing 10% DMSO for 2 h at 90 °C. The samples were then centrifuged at 1100 rpm for 10 min and the supernatants were transferred to fresh tubes. The melanin content in these supernatants was measured spectrophotometrically at an absorbance of 420 nm against a standard curve of known concentrations of synthetic melanin (0–50 µg/ml, Sigma-Aldrich).

The protein level in cell pellet was determined using the Bradford assay (Sigma-Aldrich). The intracellular melanin content was adjusted by the amount of protein in the same sample.

Immunocytochemistry

Melanocytes at passage 2 were grown in 8-well chamber slides (Nunc Lab-Tek Chamber Slides, Dutscher) at a density of 1×10^4 cells/well. After 24 h, cells were fixed in 3% PFA in PBS, then rinsed in PBS and permeabilized for 15 min in 0.1% Triton X-100 in PBS. Nonspecific binding sites were blocked

Fig. 2 Epidermal-dermal separation using TrypLE™ enzyme 1X (a) and Dispase II (b). After TrypLE™ incubation, epidermis (arrow) was completely separated from dermis, whereas after Dispase treatment, it was not perfectly detached

Fig. 3 Skin epithelial cells isolated from multiple 3-mm punch biopsies. Comparison of number of cells using TrypLE™ and Dispase/Trypsin methods. Cell counts were performed at the extraction day and the first passage (P1). Histograms are mean $\pm$ SE of five independent experiments with skin from different donors. Whatever the method used, there was no significant difference in cell number from 6 biopsies to 10 biopsies ($P > 0.05$)

at room temperature by a 10 min incubation in 1% glycine in PBS followed by a 1 h incubation in 3% BSA solution in PBS containing 10% normal goat serum and 0.1% Triton X100. Cells were incubated at 4 °C overnight with polyclonal rabbit anti-human MITF at 1:1000 dilution, then 1 h at room temperature with goat anti-rabbit antibody conjugated with rhodamine diluted 1:2000. They were double-stained with FITC-conjugated phalloidin for 15 min at room temperature (all antibodies from Sigma-Aldrich). Samples were mounted on glass slides with Fluoromount™ medium and observed under a Zeiss Axioskop 40 fluorescence microscope.

Statistical analysis

All values were expressed as mean $\pm$ SE. Statistical analyses between skin biopsy groups were performed using ANOVA with SigmaStat. Differences were considered significant if the P value was < 0.05 .

Results and discussion

Primary cultures of human melanocytes are appropriate models to study the pathomechanisms of pigmentation, the genetic disorders of melanocyte development or the efficacy of melanogenic compounds. Unfortunately, the clinical size and the anatomical location of melanocytic lesions often prevent a wide excision of skin and, thus make

Fig. 4 Morphology of cells extracted from 10 punch biopsies of skin, by TrypLE™ (a, c) and Dispase/Trypsin (b, c), at 2 days (a, b) and 10 days (c, d) after cell isolation. *Extracted cells attached to the culture flask within 2 days. After 10 days of*

incubation, melanocyte-like morphology (dendritic cells) was dominant with TrypLE™ method whereas melanocyte and keratinocyte-like morphologies (dendritic and polygonal cells) were observed with Dispase/Trypsin method. Bar 100 µm

difficult the establishment of melanocyte cultures. Many methods for extracting melanocytes are based on animal-derived enzyme. Here we have developed and validated a melanocyte extraction method being the simplest, the most effective and applicable to small skin samples.

Enzymatic dissociation of human skin tissue

Melanocytes were isolated from very limited size of human skin, equivalent of multiple 3-mm punch biopsies (not exceeding 70 mm² in total surface area). We used one-step enzyme incubation with TrypLE™,

an animal origin free and recombinant enzyme, for isolating epidermis from dermis and release epidermal cells. We compared our method with a standard dissociation protocol which is performed in two-step enzyme incubation. As shown in Fig. 2, the incubation in TrypLE™ resulted in a total separation of the epidermis from the dermis. The incubation with Dispase was less effective with a partial separation of the two skin layers on the outside edge of biopsies. Epidermal sheets were peeled from the dermis with forceps. Following TrypLE™ treatment, the intact epidermal layer was easily dissociated mechanically into a cell suspension. With the standard protocol, the

Fig. 5 MITF expression profiles of cells extracted from 10 punch biopsies of skin, by TrypLETM (a, c) and Dispase/Trypsin (b, c), at 2 days (a, b) and 10 days (c, d) after cell isolation. The percentage of cells expressing MITF (a marker of melanocytes)

is indicated in cytometry histograms. With TrypLETM method, 94–98% of cells were positive whereas with Dispase/Trypsin method, 53–57% of cells were positive

digestion of epidermis required a second enzyme incubation using Trypsin, supported by a mechanical action.

In addition, contrary to the traditional method, the advantage of our method is the absence of animal derived enzyme, a major condition for the production of advanced therapy medicinal products. Kormos et al. (2011) described the use of the Mel-mix medium supplemented with human serum for obtaining pure melanocyte cultures. Therefore, the combination of

different techniques offers valuable opportunity in cultured melanocytes-based transplantation therapies.

Growth pattern of extracted cells

As for Normand and Karasek (1995) who focused on keratinocytes, endothelial cells and fibroblasts, our method established melanocyte cultures from small amount of tissue. There was no significant difference in number of cell extracted among groups of 6–10 biopsies, and whatever the method used (Fig. 3).

Surface of skin tissue less than or equal to 5 biopsies (e.g., 35 mm²) did not allow survival and proliferation of extracted cells (data not shown). Extracted cells attached to the culture flask within 48 h. After 10 days of incubation, melanocyte-like morphology was dominant with TrypLETM method (Fig. 4). Many cells displayed dendrites. This was supported by flow cytometry analysis demonstrating that 98.5% of cells expressed MITF, a marker of melanocytes that acts as a master regulator of melanin production (Shi et al. 2016) (Fig. 5). Cells obtained from Dispase/Trypsin method exhibited dendritic and polygonal morphologies (Fig. 4). Analysis by flow cytometry indicated

Fig. 6 Number of viable melanocytes extracted by TrypLE and Dispase/Trypsin methods. Melanocytes at passage 2 were deposited in 12-well plates at a density of 6×10^4 cells/well. Cell numbers were measured after 1, 2, 3 and 4 days of culture. Data are expressed as the mean $\pm$ SE of 5 independent experiments carried out in duplicate. No difference was found ($P > 0.05$)

Fig. 7 Intracellular melanin content normalized to the amount of protein. Melanocytes at passage 2 were deposited on 25 cm² flask at a density of 4×10^5 cells/flask. Melanin and protein contents were measured after 5 days of culture. Data are expressed as the mean $\pm$ SE of 5 independent experiments carried out in duplicate. No difference was found between TrypLE and Dispase/Trypsin methods ($P > 0.05$)

that 57.3% of cells were positive for MITF (Fig. 5). These results supported that, in case of TrypLETM method, we obtained a relatively pure primary melanocyte culture. In contrast, primary cultures obtained from Dispase/Trypsin incubations were contaminated with keratinocytes, suggesting enzyme's activities less effective. Incubation for 17 h at room temperature to separate epidermis from dermis with TrypLETM method gave better results with less keratinocytes. TrypLETM incubation resulted in a total separation of the epidermis from the dermis, without mechanical help. The basement membrane was easily disrupted, resulting in the release of melanocytes from the basal layer of the epidermis. However in subsequent passages these clusters of keratinocytes disappeared, inducing the decrease of cell number between extraction day and end of passage 1. Extracted cells reached 70% confluency at around 14 days and they were subcultured at a 1:2 split ratio approximately every 10–14 days. As passaging can alter cell behavior and phenotype, we chose to culture melanocytes until passage 3. There was no difference in the cell yield of early-passage cells using both methods and from skin samples with more than 42 and less than 70 mm² of tissue (Fig. 3, data not illustrated for passage 2 and 3).

Functionality of melanocytes

Primary melanocytes in culture might be optimal to examine in vivo pathomechanisms, to assess biological effect of compounds or to treat pigmentary

Fig. 8 Immunostaining of cells extracted from 10 punch biopsies of skin, by TrypLETM (a) and Dispase/Trypsin (b), and cultured at passage 2. MITF (in red) was used as a

characteristic melanocyte marker while F actin (green) as a cell shape marker. *Cells extracted by both methods positively expressed MITF and F actin.* Bar 50 μ m. (Color figure online)

lesions. Previous work showed that non-enzymatic tissue incubation method for cell isolation preserves molecular markers (Tang et al. 2014b). Prolonged passages of cultured melanocytes tend to lose their pigmentation (Virador et al. 1999). Due to the limited proliferative capacity of primary melanocytes and the variability of phenotypes generated, it is necessary to limit melanocyte passage number. In the present work, we studied the functionality of melanocytes cultured at passage 2. We analyzed cell morphology and more precisely the dynamic cytoskeletal network by filamentous actin (F actin) labeling. Results showed no significant difference between each group of biopsies. Here only the data for 10 $\times$ 3-mm punch biopsies were represented. Numbers of viable cells over 4 days of culture were similar among TrypLETM and Dispase/Trypsin methods (Fig. 6). There was also no difference in the intracellular melanin content of melanocytes (Fig. 7). Immunocytochemical staining confirmed that cells extracted by both methods positively expressed MITF and F actin (Fig. 8). It was concluded that TrypLETM and Dispase/Trypsin methods are of similar efficacy for preserving cell biological characteristics. Melanocytes extracted with our protocol and cultured with classical conditions exhibited normal functionality with respect to dendritic morphology, pigment production and growth kinetic.

Conclusion

We reported in this study a novel and simple extraction method of human skin melanocytes based on the use of TrypLETM, a recombinant Trypsin. Compared to the well-established Dispase/Trypsin method, this protocol presented the advantage of providing pure populations of melanocyte before the first cell passaging. It appeared efficient, disrupting easily epidermis from dermis and releasing melanocytes with good yield and minimal damage. It preserved cell morphology and functional characteristics after subculture. The minimum size of skin sample that allows the extraction of functional cells for the establishment of primary cell cultures was 6 $\times$ 3-mm punch biopsies (e.g., 42 mm² or 6 $\times$ 3-mm punch biopsies). In addition, our procedure had the significant advantage to use an animal-free cell culture reagent, an ideal product for clinical studies and for the development of advanced cell therapy product indicated for the treatment of pigimentary disorders.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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