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Formation of melanocyte spheroids on the chitosan-coated surface

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Abstract

The search for biocompatible materials that can maintain function of melanocytes as the cellular patch is a feasible alternative for use in the autologous melanocyte transplantation for vitiligo. In this study, we demonstrated that the surface of chitosan-coated polystyrene wells supported the growth and phenotype expression of melanocytes. Depending on the seeding density and culture time, melanocytes were monolayered or spheroidal in morphology. At seeding densities above 10×10^3 cells/cm^2, human melanocytes started to aggregate on the surface of chitosan after 2 days in culture. These aggregates grew into compact melanocyte spheroids on day 3 and more melanocyte spheroids were observed when a higher seeding density was used. Cells remained viable in the spheroids and grew into dendritic melanocytes when they were reinoculated on polystyrene wells. Conversely, the time for the formation of melanocyte spheroids needed a longer period at lower seeding density. For example, melanocytes at as low as 1.25×10^3 cells/cm^2 did not aggregate until the 20th day of culture. In order to interpret the phenomenon further, we proposed the formation of melanocyte spheroids on the chitosan is mediated by a balance between two competing forces: the interactions of cell–chitosan and cell–cell.

Keywords: Melanocytes; Spheroids; Chitosan; Cell density

1. Introduction

Human melanocytes, cells of neural crest origins, reside in the basal epidermis in physiological condition [1,2]. They produce melanin in melanosomes and transfer melanosomes to keratinocytes through their dendrites. The sizes and distribution of melanosomes in keratinocytes determine the skin color. Melanocytes are featured by melanin production and dendrites in culture conditions [3].

Vitiligo is a common disfiguring depigmented disorder resulting from destruction of functional melanocytes in the epidermis [4,5]. Non-invasive treatment includes topical steroids and phototherapy including psoralen plus UVA (PUVA), narrow-band UVB and low-energy laser irradiation [4–6]. In recalcitrant cases, autologous melanocyte transplantation has been used [4,7–9]. For treatment of extensive lesions, it is necessary to expand the number of melanocytes in vitro before transplantation. Then the melanocyte suspension is applied on a dermabraded vitiliginous area during transplantation process [4,8,9]. However, success rates for repigmentation of acral vitiliginous areas appear to be low using melanocyte suspensions [4]. It is suggested that acral skin is less capable of maintaining transplanted melanocytes, compared with skin of other part of the body [4]. One therapeutic strategy to improve the outcome of melanocyte suspension is to culture melanocytes on the biocompatible materials to form a cellular patch for transplantation. Chitosan is a deacetylated product of chitin that is a plentiful polysaccharide found in nature and is safe for the human body [10,11]. Little is known about the utility of chitosan in culturing human melanocytes in the development of tissue engineering. In this study, we demonstrated that the surface of chitosan-coated polystyrene

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wells maintains growth and phenotype expression of melanocytes. Further, melanocytes grew into three-dimensional muticellular spheroids when they were seeded on the chitosan at appropriate densities and culture time.

It has been shown that the survival, proliferation and function of cells can vary when the density, morphology, intercelluar organization of cells are changed [12–14]. Hepatocytes survive better in toxic environment when they form multicullar spheroids [15]. Compared with monolayer culture, the viability, levels of albumin production and detoxificatory function of hepatocytes are enhanced when they are cultured into multicellular aggregates [12,16–19]. Neuron precursor cells can only maintain its long-term proliferative potential and stem cell characters when they are cultured into neurospheres [20]. In this work, melanocyte spheroid showed a spherical structure with a smooth surface and individual melanocytes in the spheroid could not be delineated. Cells remained viable in the spheroid and grew into dendritic melanocytes when they were reinoculated on the polystyrene wells. In order to interpret the phenomenon further, we proposed the formation of melanocyte spheroids on the chitosan is mediated by a balance between two competing forces: the interactions of cell–chitosan and cell–cell.

2. Materials and methods

2.1. Preparation of culture plates 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 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 and assays for cell attachment and proliferation

Human melanocytes were cultured as previously described with modification [21,22]. Melanocytes were provided by cell bank of Department of Dermatology, National Taiwan University Hospital. The study protocol was approved by an Institutional Review Board. Briefly, melanocytes were isolated from fresh foreskin of healthy Chinese men aged from 20 to 35 years who underwent circumcision. The specimens were incubated in 0.2% protease (Sigma, USA) solution at 4°C for 1 day. The epidermal sheets were gently manipulated with forceps to dissociate the epidermal cells and to yield a cell suspension. The cells were seeded into a flask with a modified melanocyte medium (MMM) consisting of Ham's F12 nutrient mixture (Gibco-BRL Life Technologies) supplemented with 50 μg/ml of gentamicin (Gibco-BRL Life Technologies), 20 ng/ml of recombinant human basic fibroblast growth factor (bFGF) (PeproTech, USA), 20 μg/ml of isobutylmethylxanthine (IBMX) (Sigma, USA), 10 μg/ml of cholera toxin (Sigma, USA), and 20% fetal calf serum (Hyclone, USA). The flask was incubated at 37°C in an atmosphere of 95% air/5% CO_2. Geneticin (Sigma, USA) was added (100 μg/ml) for 3 days to eliminate keratinocytes and fibroblasts. After reaching confluence (mean interval between seeding and confluence was 2 weeks), the melanocytes were detached by means of trypsin-EDTA solution (Gibco-BRL Life Technologies), centrifuged, diluted, and seeded for subculture. After 17 to 22 days of 1 to 2 subcultures, the number of melanocytes increased 50- to 100-fold, with a doubling time of 2.6–3.7 days. The cells were then detached by trypsinization and spun down before the following experiments.

For determination of appropriate seeding densities that would yield melanocyte spheroids, 1 ml MMM of melanocyte suspension containing the cell number of 5×10^3, 10×10^3, 20×10^3, 40×10^3, and 80×10^3, respectively, was added to each chitosan-coated well (2 cm^2 per well) and maintained at 37°C in an atmosphere of 95% air/5% CO_2. Special attention was taken to make sure that melanocytes were seeded as single cells.

For the measurement of cell adhesion, cells were washed with PBS to remove nonadherent cells, and the number of attached cell was counted using a Neubauer counting-chamber under microscope 4 h after cell seeding. On the other hand, cell proliferation was determined by MTT assays [23] at the indicated times because counting of cells in the spheroid involving mechanical dissociation has a lower accuracy than automated quantitation. 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 enzyme-linked immunosorbent assay (ELISA) plate reader (ELx800, BIOTEK) at 570 nm.

2.3. Cell morphology and histology

For morphological observation, the cells adhering to the well were photographed by digital camera coupled to an inverse phase contrast microscope. Besides, scanning electron micrographs were also taken as follows. The cells adhering to the well were washed with PBS and then fixed with 2.5% glutaraldehyde in PBS for 1 h at 4°C. After thorough washing with PBS, the cells were dehydrated by graded ethanol changes and then critical point dried. The specimens were then gold sputtered in vacuum and examined by using a scanning electron microscope (SEM).

Melanocyte spheroids formed on the chitosan-coated surface after 5 days in culture were used for histological examination. Briefly, complete spheroids were fixed in 4% buffered formalin for 1 h and made into paraffin blocks. The specimen was then sectioned into 5–7 μm in thickness and stained with haematoxylin and eosin for optical microscopic examination.

2.4. Quantification of cell number and viability in melanocyte spheroids

After culture for 5 days, seven larger melanocyte spheroids with similar diameters were removed from chitosan-coated surfaces using a pipette carefully without disturbing the melanocyte spheroids in three separate experiments. The diameters of the selected melanocyte spheroids were determined by photographs before removing spheroids from the well. The melanocyte spheroids were then suspended into single cells by means of trypsin-EDTA solution and mechanical agitation. Single cells were reseeded on polystyrene plate and cell numbers were calculated through direct counting under microscope. The viability of the cells was determined by trypan blue exclusion test [24].

3. Results

3.1. The density-dependent formation of melanocyte spheroids on the chitosan-coated surfaces

For the first experiment, melanocytes were cultured on the chitosan-coated surfaces for 5 days to study cell behavior at different seeding densities. Fig. 1 shows all melanocytes having a dendritic morphology on the chitosan-coated surfaces at different seeding densities after 1 day of culture. At very lower seeding densities (2.5×10^3 and 5×10^3 cells/cm^2), melanocytes were scattered discretely without intercellular contact (Figs. 1(a) and (b)). At higher seeding densities (10×10^3, 20×10^3, and 40×10^3 cells/cm^2), intercellular contact could be observed (Figs. 1(c)–(e)). Melanocytes cultured on the control wells without chitosan coating exhibited the same trend. Figs. 1(e) and (f) show similar dendritic melanocytes cultured on wells with and without chitosan coating at an initial density of 40×10^3 cells/cm^2.

After culture in chitosan-coated wells for 2 days, melanocytes at the seeding density of 2.5×10^3 cells/cm^2 still remained discrete and dendritic (Fig. 2(a)). However, intercellular contact was observed at the seeding density of 5×10^3 cells/cm^2 (Fig. 2(b)). Interestingly, melanocytes at seeding densities of 10×10^3, 20×10^3, and 40×10^3 cells/cm^2 started to aggregate (Figs. 2(c)–(e)). Such a trend to aggregate was more obvious at higher seeding densities. After 3 days of culture, melanocyte aggregates at seeding densities of 20×10^3 and 40×10^3 cells/cm^2 grew into compact black round spheroids in chitosan-coated wells (Figs. 3(d) and (e)). More melanocyte spheroids were observed in the more densely seeded wells. Between spheroids, dendritic melanocytes were still present on the chitosan-coated surface. For the cases of 2.5×10^3 and 5×10^3 cells/cm^2, no spheroid was observed on day 3 (Figs. 3(a) and (b)). Though initial aggregates could be observed on day 2 with a seeding density of 10×10^3 cells/cm^2 (Fig. 2(c)), aggregates became less obvious on day 3 (Fig. 3(c)). It is noted that melanocyte aggregate occurring on day 2 at seeding densities of 10×10^3 cells/cm^2 was not observed always in the repeated experiments. Therefore, this phenomenon may be attributed to the uneven cell distribution locally. On the other hand, even in the control group with the highest seeding density of 40×10^3 cells/cm^2, no melanocyte aggregates or spheroids were observed on days 2 and 3 (Figs. 2(f) and 3(f)). Actually, no aggregation was observed in the control wells at various seeding densities (not shown here).

On day 5, no melanocyte spheroids were observed in the chitosan-coated wells with seeding densities of 2.5×10^3, 5×10^3 and 10×10^3 cells/cm^2 (Figs. 4(a)–(c)) and in the control wells with seeding density of 40×10^3 cells/cm^2 (Fig. 4(f)). On the other hand, melanocyte spheroids were still present in chitosan-coated wells with seeding densities of 20×10^3 and 40×10^3 cells/cm^2 (Figs. 4(d) and (e)). Under the inverse phase contrast microscope, the diameters of the spheroids ranged from 50 to 250 μm. These data suggest higher seeding density promotes formation of melanocyte spheroids.

3.2. Cell adhesion

After 4 h of incubation, number of cells ($21.9\pm3.3\times10^3$ cells/cm^2) attached to the chitosan-coated surface is slightly less than that ($24.2\pm4.7\times10^3$ cells/cm^2) attached to the uncoated surface (seeding density was

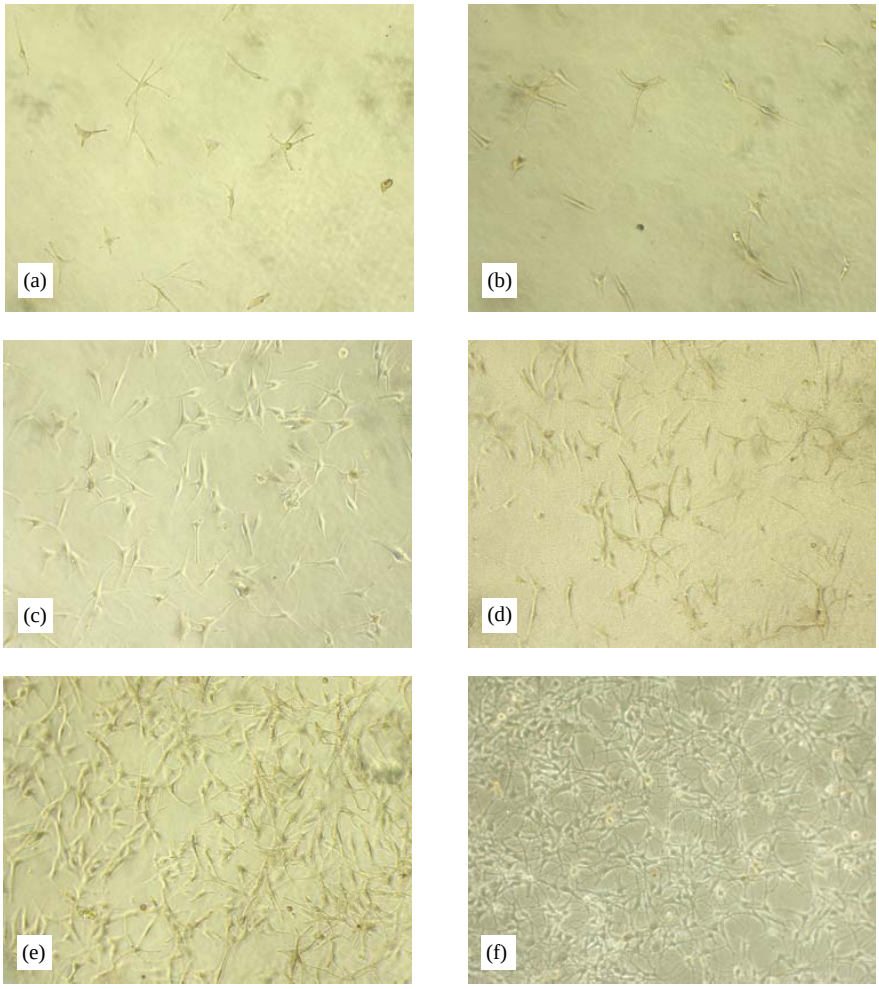


Fig. 1. Cell morphology after 1 day in culture. Melanocytes form dendrites on chitosan-coated surfaces. Almost no intercellular contact can be observed at the seeding densities of 2.5×10^3 (a) and 5×10^3 (b) cells/cm^2. More intercellular contact can be observed at higher seeding densities of 10×10^3 (c), 20×10^3 (d) and 40×10^3 (e) cells/cm^2. Cells in the control group are also dendritic ((f), seeding density 40×10^3 cells/cm^2). (Phase contrast, $\times$ 100).

30×10^3/cm^2). Optical microscope observation further confirmed that melanocyte showed a poorer attachment to the chitosan-coated surface than to the uncoated surface (Fig. 5). The majority of melanocytes maintained a round morphology on the chitosan-coated surface while most cells on the uncoated surface already form many dendrites.

3.3. Cell proliferation

For determination of the MTT conversion of melanocyte spheroids on the chitosan-coated surface, a seeding density of 30×10^3/cm^2 was chosen because melanocytes could constantly develop spheroids on the chitosan-coated surface at the seeding density between 20×10^3 and 40×10^3 cells/cm^2. The time course of MTT conversion of melanocytes cultured on chitosan treated and untreated wells is depicted in Fig. 6. Increased formazan absorbance indicates that melanocytes were able to convert the MTT into a blue formazan product and continued to proliferate during the culture period, either on treated or untreated wells. Although control wells had higher accumulation of formazan compared with the chitosan-coated wells during the culture period, the shape of the MTT-conversion curves (i.e., cell proliferation rate) was similar between them. Therefore, although the cell proliferation rates were similar on chitosan coated and uncoated wells, less MTT conversion of the chitosan-coated wells during the culture period may be explained by the lower cell adhesion at 4 h.

3.4. SEM and histological examinations of melanocyte spheroids

SEM photographs showed that the structure of the round compact melanocyte spheroids on day 5 were spherical (Fig. 7(a)). Some cells on the surface of the

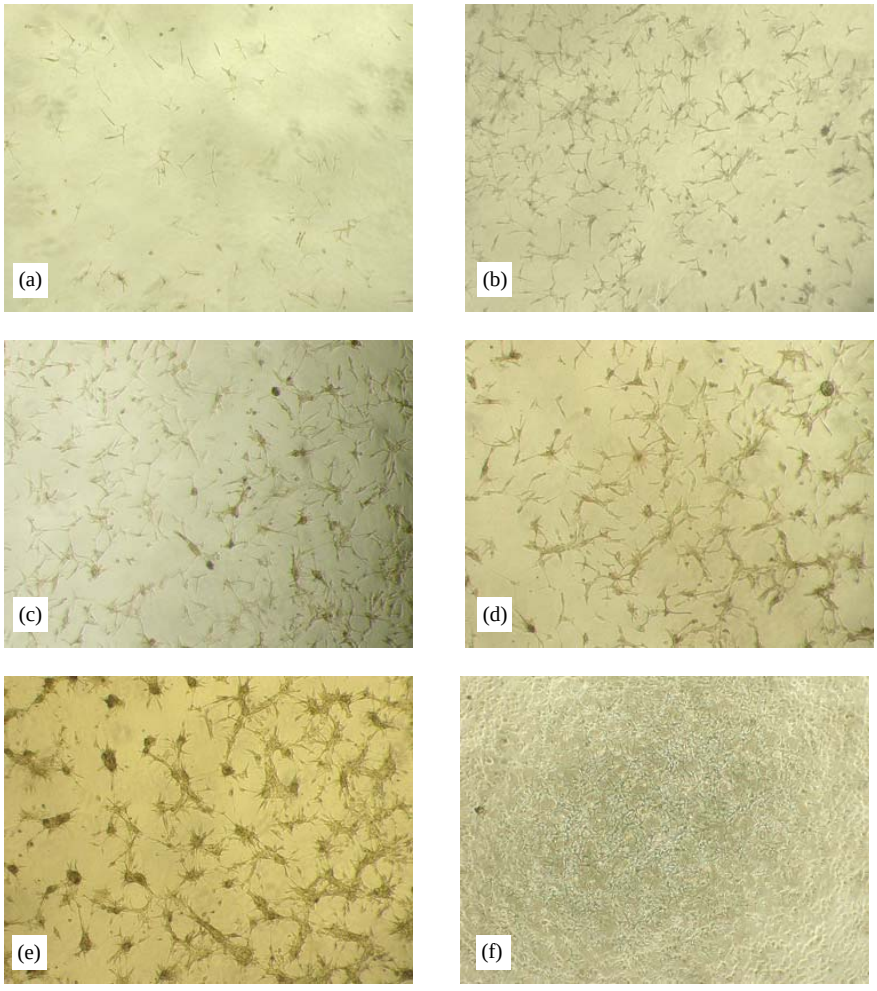


Fig. 2. Cell morphology after 2 days in culture. Melanocytes start to aggregate on chitosan-coated surfaces. Melanoctyes are still discrete at the seeding density of 2.5×10^3 cells/cm^2 (a). Intercellular contact can be observed at the seeding density of 5×10^3 cells/cm^2 (b). Cells start to aggregate at higher seeding densities of 10×10^3 (c), 20×10^3 (d) and 40×10^3 (e) cells/cm^2. No aggregation is observed in the control group ((f), seeding density 40×10^3 cells/cm^2). (Phase contrast, $\times$ 100).

spheroid showed a round contour but single cells cannot be clearly delineated. The cells in the control wells with the seeding density of 40×10^3 cells/cm^2 remained spread-out and dendritic (Fig. 7(b)). Though focal piling-up cells could be seen in the control wells, no spheroids were observed.

Histological examination showed that the cells in the spheroids retained cytoplasm that contained large amount of melanin pigment, a unique feature of melanocytes (Fig. 7(c)), indicating that the cells still retained the melanocytic phenotype.

3.5. Viability of cells in the melanocyte spheroid

It is reasonable to assume that when cells aggregated into large spheroids, the viability of the central portion of the spheroid was impaired because the cells in the central portion of a large clump may decrease the nutrient uptake compared to separate cells due to diffusion limitation of nutrient. Therefore, we were interested in whether formation of spheroid influenced the viability of melanocytes. From three independent experiments, viability of melanocytes in the spheroid, determined by trypan blue exclusion, was higher than 95%, based on seven larger spheroids with the average diameter of 162 ± 22 μm and the average cell number of 909 ± 78. Furthermore, melanocytes in the spheroid were mechanically dissociated and replated as single cells in a new polystyrene culture plate. Fig. 8 showed they grew into a dendritic morphology after 1 day in culture.

4. Discussion

Generally, cells grow as a monolayer on the tissue culture plate in vitro. However, cells can also grow into a three-dimensional aggregate in the special

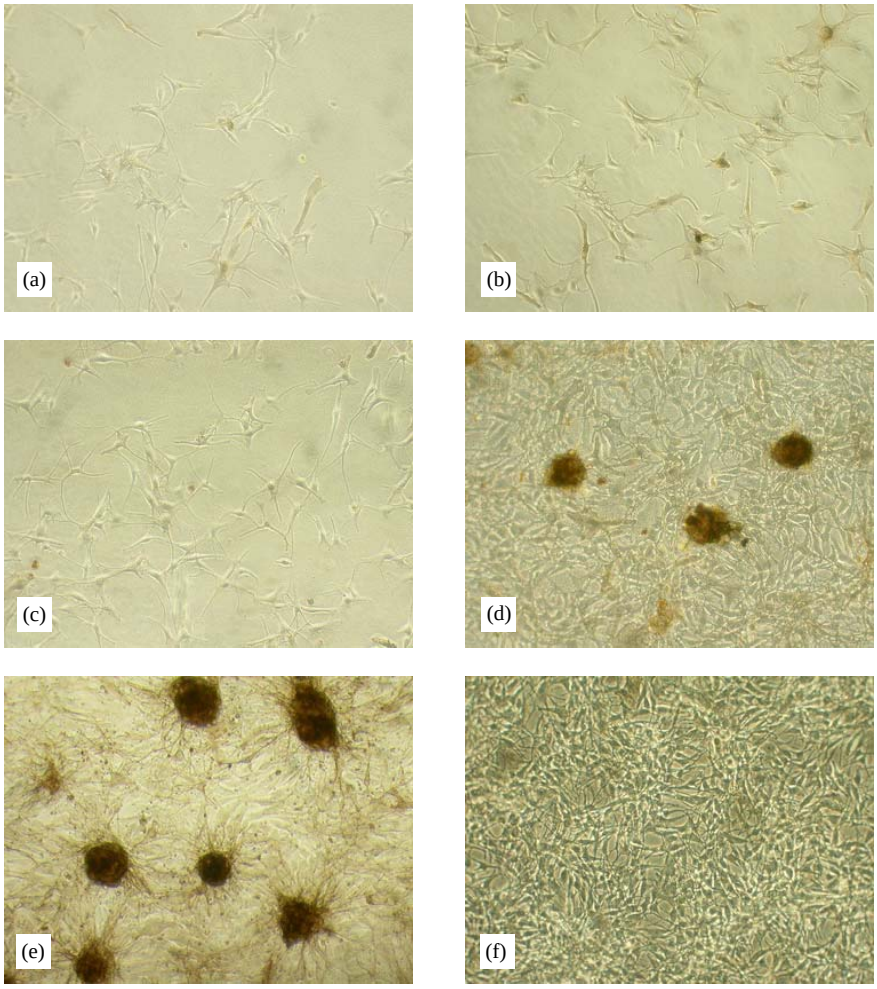


Fig. 3. Cell morphology after 3 days in culture. No melanocyte spheroids are observed at seeding densities of 2.5×10^3 (a) of 5×10^3 (b) and 10×10^3 (c) cells/cm^2. Compact melanocyte spheroids can be observed at the seeding densities of 20×10^3 (d) and 40×10^3 (e) cells/cm^2. No aggregation or melanocyte spheroid is observed in the control group ((f), seeding density 40×10^3 cells/cm^2). (Phase contrast, × 100).

environments, such as hepatocytes [19,25], neural stem cells [20], etc. It has been shown that the function, proliferation and viability of cells are related to the morphology and intercellular organization of cells [13,14,16,18,20,25,26]. In this report, we document the use of chitosan-coated surface in culturing human melanocytes. The development of melanocytes to be monolayered or spheroidal was in a seeding density-dependent manner on the chitosan-coated surface. Although the substrate can influence the attachment, growth and function of melanocytes [27–29], there has been no report using biocompatible biomaterials for melanocyte culture to examine its effect on the formation of melanocyte spheroids. We believe that this is the first time the dependence of formation of melanocyte spheroids on the seeding density was examined experimentally.

It is reasonable to assume that high-density melanocytes form spheroids via migration after close intercellular contact can be reached. The effect of cell density on the behavior of cerebellar granule neurons has been reported in our previous study [13]. We showed that a positive correlation exists between cell density and cell survival through the growth factor secreted by neurons. However, such a mechanism cannot easily account for the behavior of melanocytes because cell density can regulate the formation of melanocyte spheroids but cannot improve cell proliferation. Based on the results of cell adhesion after 4 h of incubation, the chitosan-coated surface declined the attachment of melanocytes, indicating the chitosan possibly facilitated melanocyte migration. Therefore, we hypothesize a balance between two competing forces: the interactions of cell–substrate and cell–cell. When low density of melanocytes is seeded on the surface of chitosan, dominance of cell–substrate interaction results in cell attachment since the distance between cells is so large to cause relatively small cell–cell interaction. At this time, melanocyte aggregation is inhibited and cell behavior is similar to that on the polystyrene wells. Theoretically, enhancement of

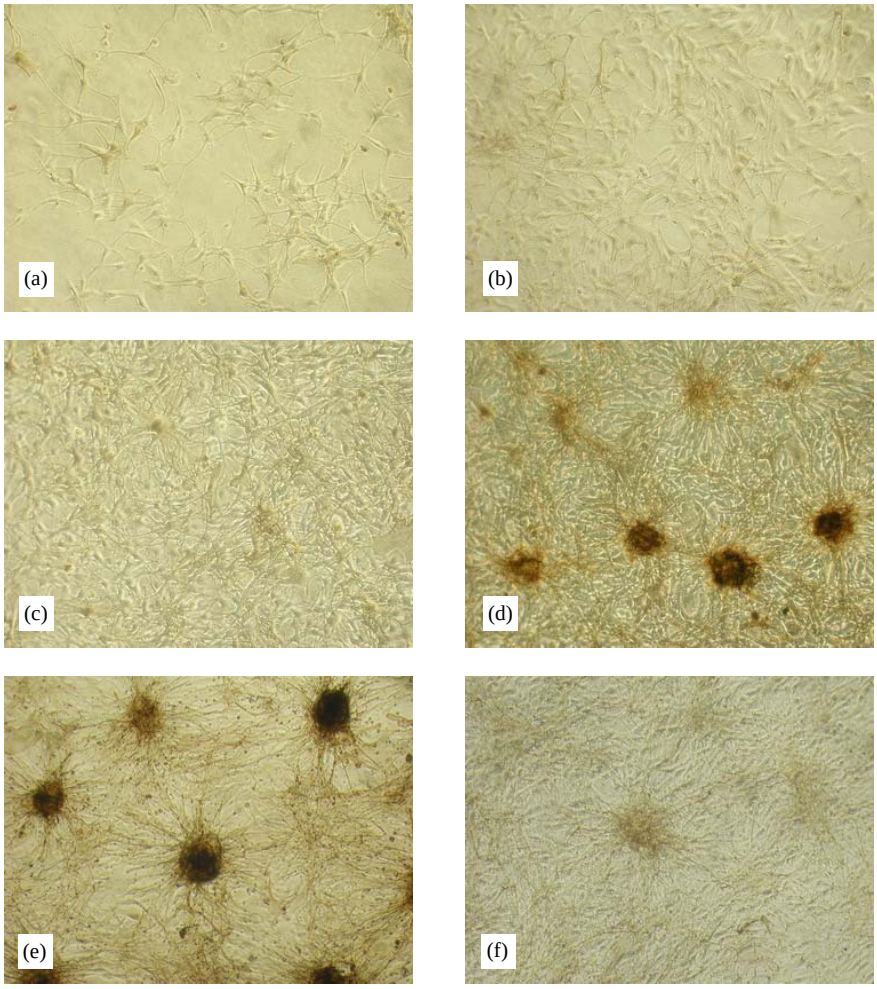


Fig. 4. Cell morphology after 5 days in culture. No melanocyte spheroids are observed at seeding densities of 2.5×10^3 (a), 5×10^3 (b) and 10×10^3 (c) cells/cm^2. Compact melanocyte spheroid can be observed at seeding densities of 20×10^3 (d) and 40×10^3 (e) cells/cm^2. No aggregation or melanocyte spheroid is observed in the control group ((f), seeding density 40×10^3 cells/cm^2). (Phase contrast, $\times$ 100).

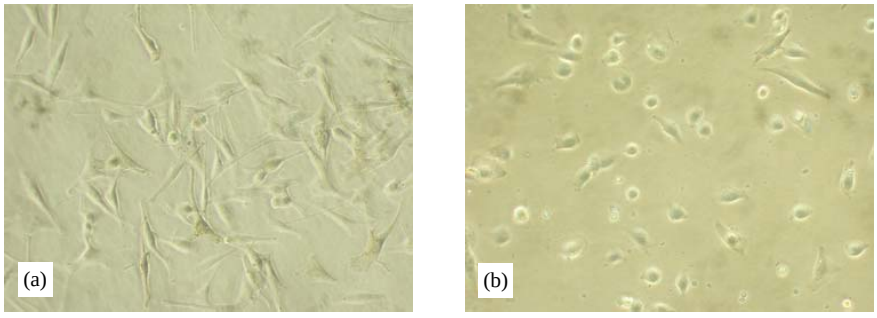


Fig. 5. Cell morphology 4 h after seeding (at the seeding density of 30×10^3/cm^2): (a) In the uncoated wells, cells with more than 2 dendrites can be observed. (b) The cells on chitosan-coated wells are mostly round and few cells have 1 or 2 dendrites. (Phase contrast, $\times$ 200).

cell–cell interaction can be obtained by decreasing the distance between cells. Hence, when the distance between cells decreases at high seeding density, the inhibition of cell migration by the chitosan is overcome by the enhanced cell–cell interaction, which allows cell aggregation and subsequent formation of spheroid. Melanocytes can proliferate continuously in the spheroids, resulting in gradual enlargement of melanocyte spheroids. Finally, intimate cell–cell contact within three-dimensional spheroids gives a different environment to further create a stronger cell–cell interaction. At the moment, the spheroid–chitosan interaction is greatly weakened because the contact area becomes very limited. Actually, melanocyte spheroids could be intactly detached by gently pipeting the culture medium during the culturing period. When dissociated cells are

reinoculated on the polystyrene wells, melanocytes grow as monolayered and dendritic cells.

With the behavior of melanocytes on the chitosan as an interpretive basis, it seemed possible that even low seeding density of melanocytes on the chitosan can accumulate into spheroids after culture for a longer period because lower seeding density after a long time of culture would yield a similar cell number to that of the higher seeding density, i.e., the space distance between the cells should be similar. To test this possibility, the time needed for the formation of melanocyte spheroids at all seeding densities in this study was measured and shown in Fig. 9. Clearly, the culture time for the formation of melanocyte spheroids increased in a seeding density-dependent manner when seeding density was less than 20×10^3 cells/cm^2. Although melanocyte spheroids were not observed at as low as 1.25×10^3 cells/cm^2 at the 20th day of culture, cells started to aggregate after this time. Therefore, provided that melanocytes survive on the chitosan longer enough, they finally will accumulate into spheroids.

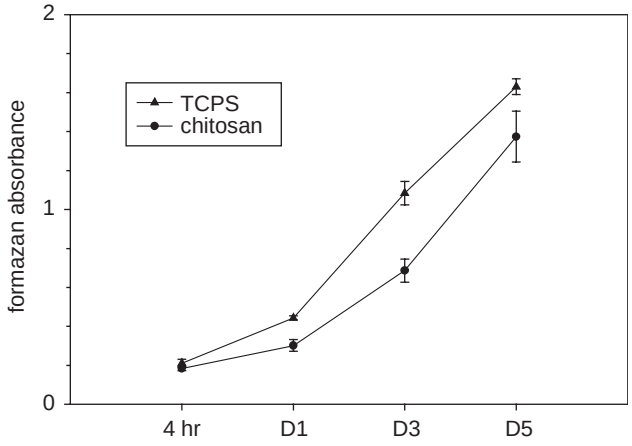
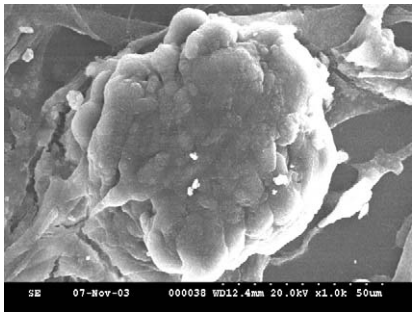
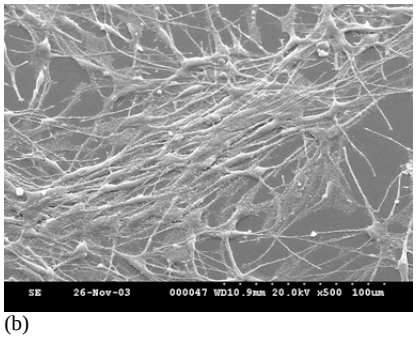


Fig. 6. MTT assays of melanocytes cultured on chitosan coated and uncoated wells at the seeding density of 30×10^3/cm^2.

It has also been shown that hepatocytes only form multicellular spheroids when the cell–substrate adhesion is weakened [30]. However, the reasons for the weak adhesion of melanocytes on the chitosan are not clear at this stage. Taking the strength of cell–substrate adhesion into account, the chemical nature of the chitosan has a significant effect on the behavior of melanocytes. Gilchrest et al. [27] reported that surfaces coated with collagen and laminin did not improve attachment of melanocytes, which is similar to the responsiveness of melanocytes to chitosan in this study. Greenberg et al. [31] have demonstrated that fibronectin is strongly chemokinetic and chemoattractive for melanocyte precursors in vitro, suggesting a possible active role for this glycoprotein in the migration of melanocyte precursors. Cerdan et al. [32] also reported that glycoproteins regulated melanocyte behavior through sugar-specific



(a)



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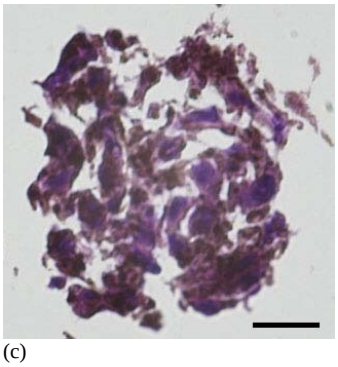

(c)

Fig. 7. Scanning electron micrographs and histology of melanocytes after 5 days in culture: (a) Cells on the surface of the melanocyte spheroid show a round contour, but single cells cannot be clearly delineated (chitosan-coated surface, seeding density 40×10^3 cells/cm^2). (b) The cells in the control group remained spread-out and dendritic. Though focal piling-up cells can be seen, no melanocyte spheroids are observed (polystyrene culture plate, seeding density 40×10^3 cells/cm^2). (c) The cells in the spheroid showed abundant melanin pigment in the cytoplasm (H&E staining, bar 20 μm).

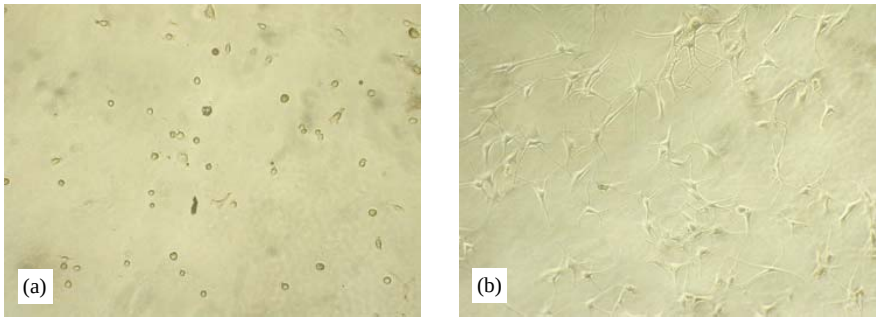

Fig. 8. Reinoculation of melanocyte spheroids on polystyrene plate: (a) Cells show a round morphology immediately after seeded on polystyrene plate. (b) Cells grow into dendritic melanocytes after 1 day in culture. (Phase contrast, × 100).

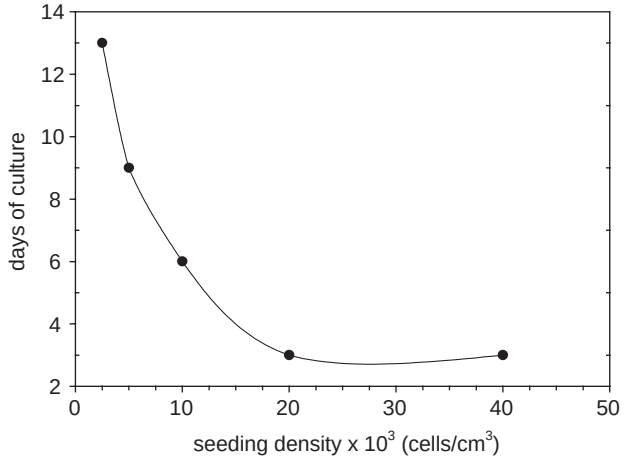

Fig. 9. The time needed for the formation of melanocyte spheroids on the chitosan at different seeding densities.

receptors expressed on melanocyte surface. Similarly, we cannot exclude the possibilities that chitosan, composed of chains of polysaccharide, might also regulate melanocyte behavior via sugar-specific receptors. On the other hand, the behavior of cells on biomaterials has been reported to be controlled by molecules adsorbed on the biomaterials, such as growth factors and adhesion molecules in the culture medium [33]. The different behavior of melanocytes on the chitosan and polystyrene may also be explained by the different adsorption abilities of the two materials. Finally, we cannot exclude the possibilities that chitosan may induce melanocytes to secrete soluble factors that promote melanocyte aggregation when a critical concentration of the soluble factors is reached at higher cell densities [34].

5. Conclusion

We succeeded in maintaining growth and phenotype expression of melanocytes on the chitosan. Depending on the seeding density and culture time, melanocytes were monolayered or spheroidal in morphology. At higher seeding density, melanocytes could aggregate on the surface of chitosan and grew into spheroids in a shorter culture period. Conversely, lower seeding density of melanocytes on the chitosan accumulated into spheroids after culture for a longer period because it would yield a similar cell number to that of the higher seeding density after a long time of culture. However, cells remained viable in the spheroids and grew into dendritic melanocytes when they were reinoculated on polystyrene wells. Therefore, two signals are required for melanocyte spheroid formation. Chitosan as the substrate serves as a first signal. The intercellular communication by way of contact seems to be a second signal. These in vitro results are very encouraging since such a three-dimensional culture system may serve as a cellular patch for future use in the treatment of vitiligo.

Acknowledgements

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