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Construction of pigmented skin equivalent and its application to the study of congenital disorders of pigmentation

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ORIGINAL ARTICLE

Construction of pigmented skin equivalent and its application to the study of congenital disorders of pigmentation*

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Abstract
We have constructed a pigmented skin equivalent and used it to study the hyperpigmentation seen in café-au-lait macules to elucidate whether the pigmented skin equivalent could be used as a model of congenital hyperpigmentary disorders. When we used fibroblasts derived from café-au-lait macules of neurofibromatosis type 1, the amount of pigment was significantly greater than in models using cells derived from normal skin. Quantities of pigment were not seen when keratinocytes derived from solitary café-au-lait macules were used, a possible reason being that keratinocytes on the skin equivalent are in a proliferating condition and are not well-differentiated enough to act on other cells. Our results suggested that our pigmented skin equivalent is useful for the study of congenital hyperpigmentary disorders, although insufficient differentiation of keratinocytes might be a disadvantage.

Key Words: *Pigmented skin equivalent, café-au-lait macule, hepatocyte growth factor, stem cell factor, endothelin-1, melanocyte*

Introduction

Pigmented skin equivalent has been used for the study of the ultra-violet-induced pigmentation [1–3], growth of keratinocytes and melanocytes, and their interactions [4,5], the effects of chemicals on the growth of melanocytes [6], and the mechanism of transfer of melanosomes [7]. However, there has been no report to our knowledge on the use of pigmented skin equivalent for congenital hyperpigmentary disorders of skin.

The café-au-lait macules are one of the congenital hyperpigmentary disorders and are generally divided into two groups: multiple (which are an indication of cutaneous sign in patients with syndromic diseases such as neurofibromatosis type-1 (NF1) and Albright syndrome), and solitary macules that develop in normal people and are not associated with syndromic diseases [8]. We have reported previously that the epidermal hyperpigmentation in multiple macules of NF1 (Recklinghausen's disease) were associated with increased secretion of stem cell factor and hepatocyte growth factor by dermal fibroblasts [9] whereas the hyperpigmentation in solitary café-au-lait macules was associated with increased secretion of endothelin-1 by epidermal keratinocytes [10]. In this study, we constructed the pigmented skin equivalent as a model of café-au-lait macules, and studied the differences in the amount of pigment between the café-au-lait models and the normal skin model, and consider its applicability for the study of congenital hyperpigmentary disorders.

Materials and methods

Cell isolation and cell cultures

Specimens of normal skin were obtained from the patients during plastic surgery. Skin specimens of

*An outline of this study was reported at the 13th Research Council Meeting of Japan Society of Plastic and Reconstructive Surgery (October 21–22, 2004).

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solitary café-au-lait macules were obtained from the lower leg of an 11-year-old patient, and that of Recklinghausen macules were obtained from the chest of a 4-year-old patient with neurofibromatosis type 1 who fulfilled the diagnostic criteria according to the National Institutes of Health Consensus Development Conference Statement [11]. Informed consent was obtained from all patients before operation.

Human keratinocytes were isolated using the method reported previously [9]. The specimens were washed three times in phosphate buffered saline (PBS) and finely shredded with scissors, then incubated with 0.25% trypsin and 0.02% EDTA in PBS for 16–24 hours at 4°C. The epithelium was separated from the dermis with forceps, and keratinocytes were isolated from the subepithelial side. These were grown in a modified serum-free keratinocyte growth medium (Kyokuto Seiyaku, Tokyo), which consisted of MCDB153 with high concentrations of amino acids, transferrin (final concentration 10 μg/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), phosphorylethanolamine (14.1 μg/ml), and bovine pituitary extract (40 μg/ml). The final concentration of calcium ions in the medium was 0.03 mmol. The serum-free melanocyte growth medium was bought from the Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan), which consisted of MCDB153 supplemented with basic fibroblast growth factor (final concentration 0.5 ng/ml), bovine pituitary extract (150 μg/ml), phorbol-12-myristate 13-acetate (10 ng/ml), and cholera toxin (10 ng/ml). Human melanocytes were isolated by the subculture of the primary keratinocyte culture; the first passage of keratinocyte cultures were trypsinised and seeded in melanocyte growth factor seven days after the primary culture, which selectively eliminated keratinocytes [12]. Human fibroblasts were isolated to be explanted after they had been separated from the epithelium, and grown in fibroblast growth medium, which consists of Dulbecco's modified Eagle's medium, 0.6 mg/ml glutamine, and 10% fetal calf serum.

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Construction of the pigmented skin equivalent

Pigmented skin equivalents were constructed by modifying the method of organotypic cultures without melanocytes that was reported previously [13,14]. Keratinocytes and melanocytes were cultured three-dimensionally at the air-liquid interface on top of a dermal equivalent consisting of type I collagen and fibroblasts. The third passage of fibroblast cultures was used for experiments. Dermal equivalents were constructed by casting fibroblasts into pigskin type I collagen solution and pouring the cells into a 60 mm Petri dish, 10 ml/dish. The solution was allowed to gel and contract for seven days (Figure 1*a*). The final concentrations of collagen and fibroblasts were 1 mg/ml and 100 000 cell/ml, respectively. The preconfluent third passage of keratinocytic and melanocytic cultures were trypsinised and seeded at 50 000 cells/dish respectively, inside a glass ring (1.6 cm in diameter, area = 2 cm^2) on the surface of dermal equivalents. Twenty-four hours later, preconfluent third cultures of keratinocytes were seeded at 450 000 cells/ring. Pigmented skin equivalents were maintained in 60 mm tissue culture dishes supplemented with 10 ml of medium (a 1:1 mixture of keratinocyte growth medium and Dulbecco's modified Eagle's medium plus 10% fetal calf serum, in which the calcium ion concentration was adjusted to 1.8 mmol) (Figure 1*b*). From the fifth day, the medium was reduced to the level of the epithelial cell sheet, so that the epithelial cells were grown at the air-liquid interface. Every other day the medium was removed and replaced with fresh medium. The specimens of pigmented skin equivalents were harvested at three weeks.

Histological and immunohistochemical observation of pigmented skin equivalent

The specimens of pigmented skin equivalents were fixed in 4% paraformaldehyde and embedded in paraffin using standard techniques. Pigmented skin equivalents were mounted in blocks, cut into 4-μm vertical sections, and stained with haematoxylin and eosin. For immunohistochemical staining, mouse monoclonal antibodies to human tyrosinase (MAT-1) [15] were purchased from Pola R&D Laboratories (Yokohama, Japan). The sections were lightly counterstained with haematoxylin, mounted, and looked at with a light-microscope (Nikon Microphoto-FXA). In unstained control samples, phosphate-buffered saline was substituted for the primary antibody.

Measurement of pigments on the skin equivalents

Experiment 1: Models using cells derived from solitary café-au-lait macules compared with models using only cells derived from normal skin

The levels of melanin pigments were compared among the three groups (Table I); keratinocytes derived from solitary café-au-lait macules were cocultured with fibroblasts from normal skin, keratinocytes derived from normal skin were cocultured with fibroblasts from solitary café-au-lait macules, keratinocytes, and fibroblasts derived from normal skin. In all groups, melanocytes were obtained from normal skin. Normal melanocytes, keratinocytes, and fibroblasts were derived from a single parent.

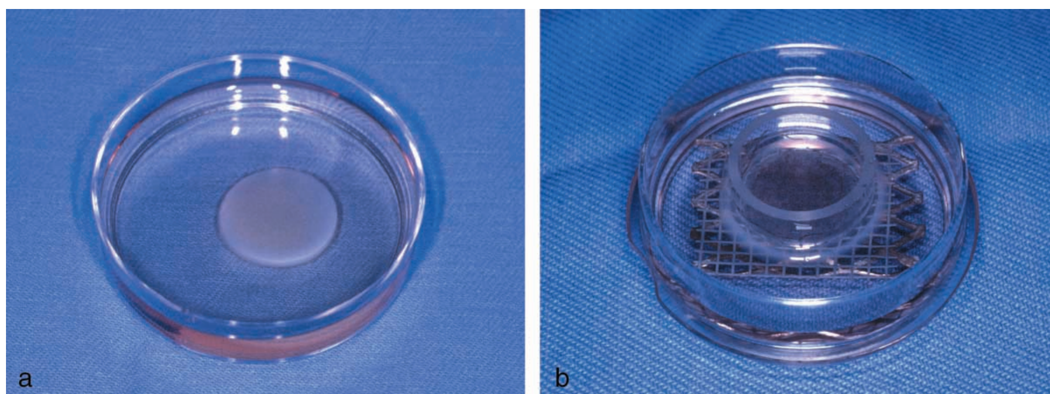

Figure 1. (*a*) Dermal equivalent cultured for seven days and contracted. The diameter of the dermal equivalent is about 2 cm. (*b*) Pigmented skin equivalent cultured for 21 days. The pigmentation can be seen on the top of dermal equivalent inside the ring.

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Experiment 2: Models using cells derived from Recklinghausen café-au-lait macules compared with models using only cells derived from normal skin

The levels of melanin pigments were compared among the three groups (Table II); keratinocytes derived from Recklinghausen café-au-lait macules were cocultured with fibroblasts from normal skin, keratinocytes derived from normal skin were cocultured with fibroblasts from Recklinghausen café-au-lait macules, keratinocytes and fibroblasts were derived from normal skin. In all groups, melanocytes were obtained form normal skin. Normal melanocytes, keratinocytes, and fibroblasts were derived from a single parent.

The melanin pigments of each sample were photographed and each digital image was analysed with Image-Pro Plus (version 3.0, Media Cybernetics, Silver Spring, MA). The difference between an original image and its extracted background was estimated as the melanin pigments, and the area and intensity of each pigment were measured. The total amounts of melanin were compared using Bonferroni's multiple comparison tests.

Results

Histological and immunohistochemical observation of pigmented skin equivalents

Haematoxylin and eosin staining showed a stratified squamous epithelium containing melanocytes in the basal layer (Figure 2*a*). Immunohistochemical staining with MAT-1 showed staining of melanocytes in the basal layer (Figure 2*b*).

Table I. Cell sources and the numbers of each model used in experiment 1.

Keratinocyte	Fibroblast	No.
Solitary café-au-lait macules	Normal	6
Normal	Solitary café-au-lait macules	6
Normal	Normal	12

Measurement of pigments in the skin equivalents

Experiment 1: Solitary café-au-lait macules compared with normal skin

Harvested pigmented skin equivalents in three groups are seen in Figure 3*a*. The levels of pigments did not differ significantly among the three groups (Figure 3*b*).

Experiment 2: Recklinghausen café-au-lait macules compared with normal skin

Harvested pigmented skin equivalents in each group are seen in Figure 4*a*. The level of pigments in the group cocultured with fibroblasts derived from Recklinghausen café-au-lait macules was significantly higher than those in the other two groups (Figure 4*b*).

Discussion

The construction of pigmented skin equivalents was more difficult than that of the skin equivalent composed of only keratinocytes and fibroblasts. Melanocyte growth medium could not be used for their construction because it contains phorbol-12-myristate 13-acetate (10 ng/ml), which promotes the

Table II. Cell sources and the numbers of each model used in experiment 2.

Keratinocyte	Fibroblast	No.
Recklinghausen café-au-lait macules	Normal	6
Normal	Recklinghausen café-au-lait macules	6
Normal	Normal	12

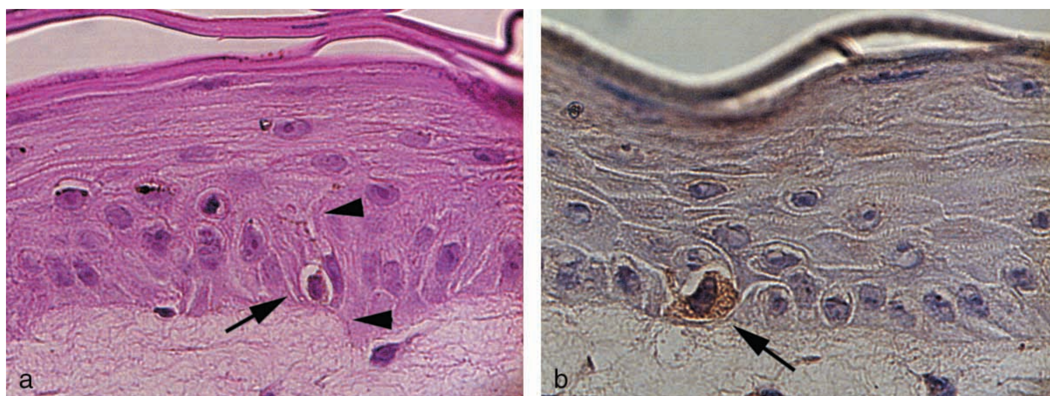

Figure 2. (*a*) The histological finding of the pigmented skin equivalent, haematoxylin and eosin staining (original magnification ×400). The melanocyte is located at the basal layer (arrow). The melanocytic dendrites extend into the basal and suprabasal layer (arrowhead). (*b*) The immunohistochemical finding of pigmented skin equivalent stained with MAT-1 and counterstained with haematoxylin (original magnification ×400). Melanocytes, identified by their dendritic appearance, are stained with MAT-1 (arrow).

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growth of melanocytes but inhibits the growth and attachment of keratinocytes [12], whereas the growth and attachment of melanocytes requires keratinocytes in keratinocyte growth medium because it does not contain phorbol-12-myristate 13-acetate nor cholera toxin. Each melanocyte is normally associated with about 36 keratinocytes and together they make up the epidermal melanin unit [16]. However, melanocytes, if seeded with many keratinocytes, cannot attach closely to the dermal equivalent because melanocytes have less ability to attach than keratinocytes, so we first seeded keratinocytes and melanocytes at 50 000 cells/dish in keratinocyte growth medium, and seeded more keratinocytes at 450 000 cells/dish 24 hours later. Our pigmented skin equivalent was constructed in this way, and was stratified with dendritic melanocytes located in the basal keratinocyte layer. Pigmented skin equivalents have been used for the study of the pigmentation induced by ultra-violet light [1–3], for the growth of keratinocytes and melanocytes and their interactions [4,5], for the effects of chemicals on the growth of melanocytes [6], and to study the mechanism of melanosomes transfer [7]. However, there has been no report to our knowledge on their use for the study of congenital hyperpigmentary disorders. Several methods have been described for their construction; some reported the use of collagen sponge [17] or dermis with the epidermis removed [5] in place of collagen-gel, and a graft of epidermis [4,17] in place of the seeding of melanocytes and keratinocytes. The large advantage of our skin equivalent is that keratinocytes, melanocytes, and fibroblasts can be combined independently, and cells other than keratinocytes, melanocytes, and fibroblasts can be excluded. Many sets were constructed from a tiny piece of skin, so this skin equivalent is useful for control studies to elucidate the interaction among melanocytes, keratinocytes, and fibroblasts. A disadvantage of the present skin equivalent is that we might have altered the properties of the cells by culture or subculture, so the early passage cells were used to minimise their influence.

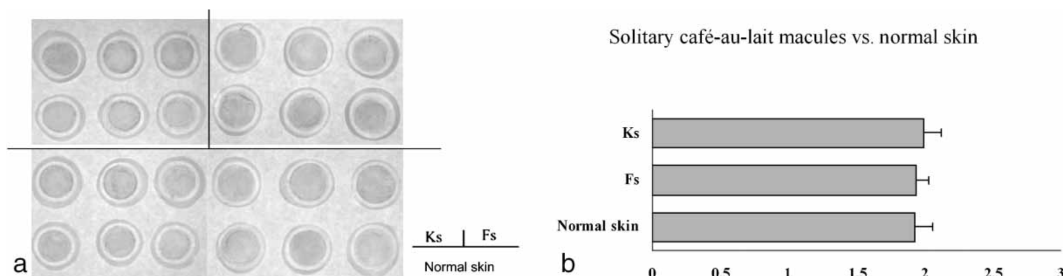

Figure 3. (*a*) Photographs showing the pigmented skin equivalents harvested in experiment 1. (*b*) Mean (SD) amount of pigments in pigmented skin equivalents after 21 days organotypic culture. There were no significant differences among the three groups. Ks = Keratinocytes derived from solitary café-au-lait macules were cocultured with fibroblasts from normal skin ($n = 6$). Fs = Fibroblasts derived from solitary café-au-lait macules were cocultured with keratinocytes from normal skin ($n = 6$). Normal skin = Keratinocytes and fibroblasts were derived from normal skin ($n = 12$).

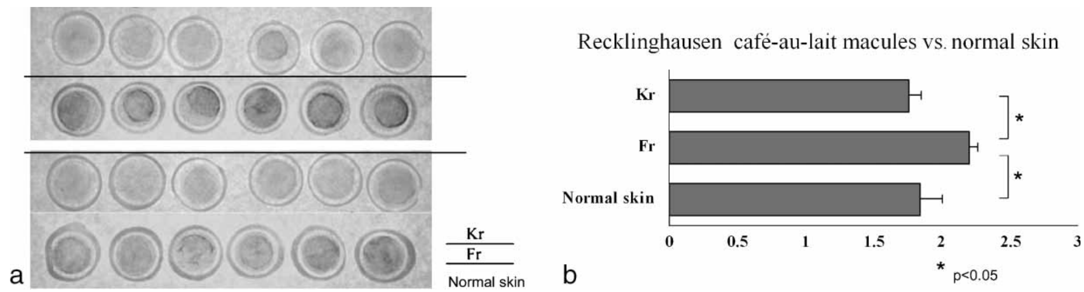

Figure 4. (*a*) Photographs showing the pigmented skin equivalents harvested in experiment 2. (*b*) Mean (SD) amount of pigments in pigmented skin equivalents after 21 days organotypic culture. There were significant differences between group Fr and the other two groups ($p < 0.05$). Kr = Keratinocytes derived from Recklinghausen café-au-lait macules were cocultured with fibroblasts from normal skin ($n = 6$). Fr = Fibroblasts derived from Recklinghausen café-au-lait macules were cocultured with keratinocytes from normal skin ($n = 6$). Normal skin = Keratinocytes and fibroblasts were derived from normal skin ($n = 12$).

Conclusion

The present pigmented skin equivalents are useful for the study of congenital hyperpigmentary disorders although insufficient differentiation of keratinocytes might be one of the disadvantages.

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