Assessment of Melanogenesis in a Pigmented Human Tissue-Cultured Skin Equivalent

Nadja Nicole Zöller, Matthias Hofmann, Manuel Butting, Igor Hrgovic, Jürgen Bereiter-Hahn, August Bernd, Roland Kaufmann, Stefan Kippenberger, Eva Valesky

Abstract

Background: Organotypic tissue-cultured skin equivalents are used for a broad range of applications either as possible substitute for animal tests or for transplantation in patient-centered care. Aims: In this study, we implemented melanocytes in a tissue-cultured full-thickness skin equivalent, consisting of epidermis and dermis. The versatility of this skin-like model with respect to pigmentation and morphological criteria was tested.

Materials and Methods: Pigmented skin equivalents were morphologically characterized, and melanogenesis was evaluated after treatment with kojic acid – a tyrosinase inhibitor and forskolin – a well-known activator of the cyclic adenosine 3,5-monophosphate pathway. Pigmentation was measured either by determination of the extinction at 400 nm after melanin extraction with KOH correlated to a melanin standard curve or by reflectance colorimetric analysis, monitoring reflectance of 660 nm and 880 nm emitting diodes. Results: The morphological analysis revealed characteristic epidermal stratification with melanocytes located at the basal layer. Stimulation with forskolin increased the pigmentation, whereas treatment with kojic acid caused bleaching. Conclusion: The present study demonstrates that the herein-introduced organotypic tissue-cultured skin equivalent is comparable to the normal human skin and its versatility in tests regarding skin pigmentation. Therefore, this model might help understand diseases with dysfunctional pigmentation such as melasma, vitiligo, and postinflammatory hyperpigmentation.

Key Words: Forskolin, kojic acid, melanocytes, pigmentation, tissue engineering

Introduction

Skin pigmentation is a prominent aspect of the human complexion. The major source contributing to brownish skin color is melanin which is produced by melanocytes located in the stratum basale of the epidermis. Melanin is transported through the melanocytic dendrites to 35–40 keratinocytes that are connected to each individual melanocyte. These epidermal melanin units are crucial for pigment distribution within the epidermis. Even minor defects leading to an uneven pigmentation can lead to social stigmatization. Apart from the pigmentation disorders, changes in the skin color can be socially motivated, fitting a certain beauty ideal. For screening of possible pigmentation-altering drugs, monolayer cultures of melanocytes are commonly used. The necessity to consider the interaction between different cell species during any kind of treatment is well recognized, particularly when the transfer and the distribution of melanin is considered. Rheinwald and Green provided the basis for the development of epidermal equivalents or skin equivalents that could be used for more complex analysis. The aim of this study was the development of a pigmented organotypic tissue-cultured skin equivalent (TCSE) and verifying the functionality of the pigmentary system by treatment with known effectors of pigmentation. The pigmentation status of the TCSEs was evaluated using two different quantitative methods, namely, melanin extraction and remission photometry.

Materials and Methods

Cell isolation and tissue-cultured skin equivalent generation

Normal human fibroblasts, keratinocytes, and melanocytes were isolated from infantile foreskins of...
donors with varying pigmentation status (Fitzpatrick scale II–V). The isolated cells were seeded at a density of $2 \times 10^6$ cells/ml in appropriate culture media (keratinocytes: DermaLife K, melanocytes: DermaLife M [CellSystems, Troisdorf, Germany] and fibroblasts: Dulbecco’s modified eagle's medium [Gibco, Karlsruhe, Germany]) as described. Fibroblasts were seeded at a cell density of $4 \times 10^6$ cells/cm$^2$, into bovine collagen type I scaffolds (Henkel, Düsseldorf, Germany), and were cultivated in the aforementioned medium for 14 days. Thereafter, primary melanocytes and keratinocytes were seeded on top of the scaffolds at the same cell density ($4 \times 10^6$ cells/cm$^2$). The equivalents were then further propagated in the specified keratinocyte medium under submersed conditions for 7 days. Epidermal differentiation was induced by transferring the models to the air–liquid interface. All studies were conducted according to the Declaration of Helsinki principles and in agreement with the institutional review board.

**Altering tissue-cultured skin equivalent pigmentation status**

To modulate pigmentation, TCSEs cultivated at the air–liquid interface were treated for 14 days with medium supplemented with 40 $\mu$M forskolin (Sigma-Aldrich, Schnelldorf, Germany) to induce melanogenesis or with 250 $\mu$M kojic acid (Sigma-Aldrich) to inhibit tyrosinase, the key enzyme in melanogenesis. TCSEs were exposed to fresh medium every other day. The experiments were repeated with different donors.

**Histological analysis**

Epidermal architecture was investigated by staining sections with hematoxylin/eosin (Millipore, Schwalbach, Germany), HMB-45 (Linaris, Wertheim-Bettingen, Germany), and anti-human Ki-67 (clone MIB-1, 1:50; DakoCytomation, Hamburg, Germany) according to the manufacturers’ protocols. Photographs were taken with a digital camera (Sony Cyber-shot 3.3, Sony, Cologne, Germany) connected to a Zeiss Axioskop (Zeiss, Oberkochen, Germany).

**Evaluation of the pigmentation status**

Shifts in pigmentation of TCSEs after 14 days in presence of forskolin or kojic acid or without treatment were evaluated by reflectance colorimetric analysis. Light reflection of the TCSEs using 660nm and 880nm emitting diodes was measured with the SPA 99 (CK electronic, Cologne, Germany; non-invasive). Additionally the relative melanin content after KOH extraction and extinction measurement at 400nm against a melanin standard curve was determined as described.

**Presentation of data and statistical analysis**

All data were presented as mean values ± standard deviation. Statistical significance of the data was evaluated by the Wilcoxon-Mann–Whitney U-test (BIAS, Frankfurt, Germany). Each set of data was related to the referring untreated control. Differences were considered statistically significant at $P<0.05$.

**Results**

**Morphological criteria of the TCSE**

The morphological and immunohistochemical properties of normal human skin [Figure 1a, c and e] and the herein-presented TCSE [Figure 1b, d and f] are comparable. The epidermal part was fully differentiated featuring all characteristic epidermal cell layers and selected differentiation- and proliferation-specific markers at physiological locations and allowed apart from the systemic treatment also topical application of substances. In both tissues proliferative, Ki-67 positive cells [Figure 1c and d] could only be identified in the stratum basale. Immunohistochemical staining against Pmel17/gp100 using HMB45 [Figure 1e and f] revealed that melanocytes were exclusively found in the stratum basale of human skin and TCSEs. Melanocytes were evenly scattered in the stratum basale of the TCSE, and melanin was clearly identifiable in the different epidermal layers [Figure 1f, yellow arrows].

**Evaluation of the pigmentation status of the TCSEs**

The pigmentation status was on the one hand by total melanin extraction and on the other hand spectrophotometrically determined. Pigmentation in kojic acid-treated TCSEs was 11% (melanin extraction, dotted bars) to 20% (spectrophotometric, striped bars) lower than the respective untreated controls, whereas a treatment with 40 $\mu$M forskolin increased the melanin content by 20% (melanin extraction) to 50% (spectrophotometric) [Figure 2].

**Discussion**

Skin equivalents are popular in cosmetic and pharmaceutical research, but are also used in the treatment of skin defects. Supplementation with externally added growth factors could be limited due to the synergistic relation (e.g., internutrition) between fibroblasts, keratinocytes, and melanocytes. In recent years, investigations showed that fibroblasts and fibroblast-secreted growth factors were important modulators for melanogenesis; therefore, skin equivalents featuring physiological dermal structures fitted closer to the in vivo situation than epidermal equivalents. The herein-introduced model consisted of dermal and epidermal cells cultured in a collagen matrix. The comparability concerning morphological and immunohistochemical properties of normal human skin and the herein-presented TCSE was not limited to the differentiation status as previously shown. The exclusive localization in the stratum
basale as well as the evenly scattering of the seeded melanocytes in a ratio that was very similar to bona fide skin could be assumed to be caused by the cell-specific ability of self-assembly of complex tissues. Furthermore, we showed the presence of functional epidermal melanin units, by detection of melanocytic dendrites that originated in the stratum basale reaching the upper epidermal layers. Normal human interfollicular skin harbors melanocytes producing melanin appearing as brownish and sometimes reddish complexion. This complexion was clearly visible not only macroscopically but also in the hematoxylin-eosin and immunohistochemical stains. Validation of our pigmented TCSEs after exposure to well-described pigmentation-influencing agents revealed that already visible distinction between the hyper- and depigmentation-inducing agents was possible. To substantiate these observations spectrophotometry is widely used in human studies when noninvasive approaches are required—and total melanin extraction is applicable for in vitro studies or punch biopsies. Comparison of both measuring methods showed comparable results and therefore substantiated the applicability of our TCSE for pigmentation analysis. In contrast to other pigmented skin equivalents, our TCSE was already able to detect pigmentation changes after treatment with 250 µM kojic acid, whereas in literature and in the application notes of commercial skin equivalent distributors, concentrations of 250 µM to 140 mM were used poststimulation, e.g., by α-MSH.

Conclusion

We showed morphological and physiological homologies of the herein-presented TCSE to normal human skin and the comparability of an invasive and a noninvasive method to quantitate pigmentation. The herein-introduced skin model allowed studying the interaction between different skin cell species under physiological conditions with particular regard to pigmentation disorder, such as, melasma, vitiligo, or postinflammatory hyperpigmentation. On the one hand, potential disease triggers, for example, of cytokines in case of postinflammatory hyperpigmentation, could be evaluated as a single active agent/component as well as in variable combinations, and on the other hand, therapeutic strategies could also be investigated with
such TCSEs. Furthermore, modifications of the expression of alleged target genes are a step to obtain an individualizable in vitro disease model. This TCSE shows potential to bridge the gap between the monolayer studies and in vivo studies. Besides basic research, this model also allows testing the efficacy of new agents in the aforementioned clinical conditions.

Acknowledgments
The authors would like to thank Katja Härle and Ellen Thiem for technical assistance. We are grateful to Wendy Stevens for comments and discussion. The study was supported by the Prof. Harry und Rosa Neumann-Stiftung and the Dr. Paul and Cilli Weill Stiftung.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

References
1. Weiner L, Fu W, Chirico WJ, Brissette JL. Skin as a living coloring book: How epithelial cells create patterns of pigmentation. Pigment Cell Melanoma Res 2016;27:1014-31.
2. Kippenberger S, Bernd A, Bereiter-Hahn J, Ramirez-Bosca A, Kaufmann R. The mechanism of melanocyte dendrite formation: The impact of differentiating keratinocytes. Pigment Cell Res 1998;11:34-7.
3. Nicolaidou E, Katsambas AD. Pigmentation disorders: Hyperpigmentation and hypopigmentation. Clin Dermatol 2014;32:66-72.
4. Rheinwald JG, Green H. Serial cultivation of strains human epidermal keratinocytes: the formation of keratinizing colonies form single cells. Cell 1975;6:331-43.
5. Zöller NN, Kippenberger S, Thaçi D, Mewes K, Spiegel M, Sättler A, Teißier MH, et al. Evaluation of beneficial and adverse effects of glucocorticoids on a newly developed full-thickness skin model. Toxicol In Vitro 2008;22:747-59.
6. Baqué M, Kasraee B. Discrimination between cutaneous pigmentation and erythema: Comparison of the skin colorimeters dermamatch and mexamer. Skin Res Technol 2014;20:218-27.
7. Bernd A, Ramirez-Bosca A, Kippenberger S, Martinez-Liarte JH, Holzmann H, Solano F, et al. Levels of dopachrome tautomerase in human melanocytes cultured in vitro. Melanoma Res 1994;4:287-91.
8. Schlotmann K, Kaeten M, Black AF, Damour O, Waldmann-Laue M, Förster T, et al. Cosmetic efficacy claims in vitro using a three-dimensional human skin model. Int J Cosmet Sci 2003;25:309-18.
9. Roguet R, Cohen C, Leclaire J, Tessonnneaud E, Gagne C, Leclaire J, et al. Use of a standardized reconstructed epidermis kit to assess in vitro the tolerance and the efficacy of cosmetics. Int J Cosmet Sci 2000;22:409-19.
10. Cario-André M, Briganti S, Picardo M, Nikaido O, Gall Y, Ginerst J, et al. Epidermal reconstructs: A new tool to study topical and systemic photoprotective molecules. J Photochem Photobiol B 2002;68:79-87.
11. Schoeppe S, Schäcke H, Bernd A, Zöller N, Asadullah K. Identification of novel in vitro test systems for the determination of glucocorticoid receptor ligand-induced skin atrophy. Skin Pharmacol Physiol 2010;23:139-51.
12. Ali N, Hosseini M, Vainio S, Taieb A, Cario-André M, Rezvani HR, et al. Skin equivalents: Skin from reconstructions as models to study skin development and diseases. Br J Dermatol 2015;173:391-403.
13. Kaufmann R, Greiner D, Kippenberger S, Bernd A. Grafting of in vitro cultured melanocytes onto laser-ablated lesions in vitiligo. Acta Derm Venereol 1998;78:136-8.
14. Zöller N, Valesky E, Butting M, Hofmann M, Kippenberger S, Bereiter-Hahn J, et al. Clinical application of a tissue-cultured skin autograft: An alternative for the treatment of non-healing or slowly healing wounds? Dermatology 2014;229:190-8.
15. Golinski P, Menke H, Hofmann M, Valesky E, Butting M, Kippenberger S, et al. Development and characterization of an engraftable tissue-cultured skin autograft: Alternative treatment for severe electrical injuries. Cells Tissues Organs 2014;200:227-39.
16. Kondo S, Koosheh F, Sauder DN. Penetration of keratinocyte-derived cytokines into basement membrane. J Cell Physiol 1997;171:190-5.
17. Duval C, Cohen C, Chagnoleau C, Fliouret V, Bourreau E, Bernard F, et al. Key regulatory role of dermal fibroblasts in pigmentation as demonstrated using a reconstructed skin model: Impact of photo-aging. PLoS One 2014;9:e114182.
18. Kovacs D, Cardinali G, Aspite N, Cota C, Luzi F, Bélais B, et al. Role of fibroblast-derived growth factors in regulating hyperpigmentation of solar lentigo. Br J Dermatol 2010;163:1020-7.
19. Hachiya A, Sriwiriyanont P, Kaiho E, Kitahara T, Takeya M, Tsuboi R, et al. An in vivo mouse model of human skin substitute containing spontaneously sorted melanocytes demonstrates physiological changes after UVB irradiation. J Invest Dermatol 2005;125:364-72.
20. Duval C, Chagnoleau C, Pouradier F, Sextius P, Condom E, Bernard F, et al. Human skin model containing melanocytes: Essential role of keratinocyte growth factor for constitutive pigmentation-functional response to β- melanocyte stimulating hormone and forskolin. Tissue Eng Part C Methods 2012;18:947-57.
21. Yoon Td, Lei TC, Yamaguchi Y, Batzer J, Wolber R, Hearing VJ, et al. Reconstituted 3-dimensional human skin of various ethnic origins as an in vitro model for studies of pigmentation. Anal Biochem 2003;318:260-9.
22. Uter W, Benz M, Mayr A, Gefeller O, Pfahlberg R. Assessing skin pigmentation in epidemiological studies: The reliability of measurements under different conditions. Skin Res Technol 2013;19:100-6.
23. Watanabe F, Hashizume E, Chan GP, Kamimura A. Skin-whitening and skin-condition-improving effects of topical oxidized glutathione: A double-blind and placebo-controlled clinical trial in healthy women. Clin Cosmet Investig Dermatol 2014;7:267-74.
24. Ramirez-Bosca A, Bernd A, Werner R, Dold K, Holzmann H. Effect of the dose of ultraviolet radiation on the pigment formation by human melanocytes in vitro. Arch Dermatol Res 1992;284:358-62.
25. Kim SH, Ha YM, Moon KM, Choi YJ, Park YJ, Jeong H0, et al. Anti-melanogenic effect of (Z)-5-(2,4-dihydroxybenzylidene) thiazolidine-2,4-dione, a novel tyrosinase inhibitor. Arch Pharm Res 2013;36:1189-97.
26. Faig JJ, Moretti A, Joseph LB, Zhang Y, Nova MJ, Smith K, et al. Biodegradable kojic acid-based polymers: Controlled
delivery of bioactives for melanogenesis inhibition. Biomacromolecules 2017;18:363-73.

27. Lee JH, Lee ES, Bae IH, Hwang JA, Kim SH, Kim DY, et al. Antimelanogenic efficacy of melasolv (3,4,5-trimethoxycinnamate thymol ester) in melanocytes and three-dimensional human skin equivalent. Skin Pharmacol Physiol 2017;30:190-6.

28. Zhou J, Ren T, Li Y, Cheng A, Xie W, Xu L, et al. Oleylethanolamide inhibits α-melanocyte stimulating hormone-stimulated melanogenesis via ERK, Akt and CREB signaling pathways in B16 melanoma cells. Oncotarget 2017;8:56868-79.