

Development and characterisation of human skin equivalents and their potential application as a burn wound model

Topping G • Malda J • Dawson R • Upton Z

Abstract

Wound healing is a complex physiological process; hence a reproducible *in vitro* model of skin provides a valuable tool to further understand the biology of dermal wound repair and to investigate techniques to improve wound healing. Human skin equivalent models (HSEs) have been proposed to serve as an *in vitro* model for these purposes; however, there is currently no readily available HSE model in Australia.

In this study, we describe the production of a HSE obtained by seeding human keratinocytes onto a de-epidermised dermis, (DED) which was then submerged in medium for 3 days and subsequently cultured at the air-liquid interface for up to 20 days. The model was characterised morphologically and biochemically over the 20 days of culture at the air-liquid interface and showed histological features similar to those observed in an *in vivo* epidermis. Immunohistochemistry of the epidermal markers keratin 6, keratin 14, keratins 1/10/11 and the basement membrane marker collagen type IV, revealed typical differentiation.

The HSE was then examined for its potential as a burn wound healing model. Burn wounds were created in the model and the re-epithelialisation of the wounds was followed for 6 days by keratinocyte morphology; metabolic activity was analysed every 2 days. Keratinocytes began to migrate into the wound bed after 2 days and continued to migrate for the next 4 days, suggesting that the HSEs generated may be of great value for studies of the wound healing process and for the evaluation of new therapies.

Topping G, Malda J, Dawson R & Upton Z. *Development and characterisation of human skin equivalents and their potential application as a burn wound model. Primary Intention* 2006; 14(1):14-21.

Gemma Topping

Jos Malda

Rebecca Dawson

Zee Upton*

Tissue Repair and Regeneration Program
Institute of Health and Biomedical Innovation and
Science Research Centre
Queensland University of Technology
GPO Box 2434
Brisbane QLD 4001
Tel: (07) 3864 2342
Fax: (07) 3864 1534
E-mail: z.upton@qut.edu.au

* Corresponding author

Introduction

Skin, the largest organ in the body, performs many important roles including protection against physical and chemical insults, regulation of temperature and fluid loss, and immunity and sensorial roles. Once damaged, skin is unable to perform these functions and numerous complications such as infection or fluid loss can occur¹. To restore the protective barrier function of skin, the body needs to close the wound as quickly as possible.

Models are essential to aid further understanding of the complex process of wound healing². In particular, the use of *in vitro* three-dimensional skin models – human skin equivalents (HSEs) – hold promise for the study of wound healing and evaluation of potential therapeutic agents. At the same time, these models also have potential in terms

of minimising the number of animals required for testing. This issue that is becoming increasingly important since it is widely recognised that preclinical studies in porcine models, rather than small animals, are, in the main, most successful in terms of facilitating the translation of laboratory findings to a successful wound healing therapeutic³.

In view of this, the development of a reproducible three-dimensional HSE, suitable for optimisation of therapies and wound healing treatments *in vitro* prior to porcine and clinical studies, holds benefits from not only economic, but also regulatory and ethical perspectives.

In Australia currently no HSE is readily obtainable. Although some HSEs, such as EpiSkin^{® i}, EpiDerm^{® ii} and SkinEthic^{® iii}, have been made commercially available, transport logistics, quarantine issues and affordability make them almost impossible to obtain for research laboratories and small to medium enterprises⁴. There is also the risk of discontinuation of these commercial models, which in turn may place any experimental work, such as validation or continuation studies, into jeopardy. For example, the discontinuation of the Skin2[®] model due to the bankruptcy of the manufacturer, Advanced Tissue Sciences, in 2002 has created some challenges for a number of laboratories⁴.

These problems have driven various institutions to develop in-house models. Therefore, the aim of this study was to establish a HSE model and to characterise the model using histological and immunohistochemical techniques. In addition, we investigated the potential of the HSE model as a system for evaluating burn wound healing.

Materials and methods

Collection of material

Skin was collected from consenting patients undergoing breast or abdomen reductions at the St Andrews and Wesley Hospitals, Brisbane, Australia. Ethics approval was obtained from both the hospitals and Queensland University of Technology, Brisbane, Australia. Skin was collected into sterile jars in Antibiotic/Antimycotic^{iv}. The samples were stored at 4°C for no longer than 12 hours before processing.

Isolation and culture of keratinocytes

Keratinocytes were isolated from donor skin as previously described⁵ with the following modifications. After the epidermis was removed from the dermis with forceps, a scalpel was used to gently scrape the keratinocytes from the dermis. The freshly isolated keratinocytes were then

expanded in culture on a feeder layer of lethally irradiated 3T3 fibroblasts as described previously in Greens media⁶. Keratinocytes were expanded for one passage (P1) for 7 days with the medium replaced every 3-4 days.

Preparation of dermal equivalent (de-epidermised dermis)

The dermal equivalent was prepared as previously described⁷ with slight modifications. Briefly, the skin was incubated in 1M sodium chloride at 37°C for 12 hours. The epidermis was removed using forceps, leaving behind the de-cellularised de-epidermised dermis (DED).

Preparation of skin equivalent and composite culture

The DED pieces were trimmed to approximately 1.4cm² and placed in a 24-well culture plate with papillary side up. Sterile stainless steel rings^v with a silicone washer base and a diameter of 7mm were placed on top of the DED⁸. Keratinocytes (P1) suspended at a concentration of 1x10⁵ cells/cm² in 200µL were added to each ring and the DEDs were incubated at 37°C and 5% CO₂. After 3 days the rings were removed and the composites (dermis plus cells) were elevated to the air-liquid interface by transferring the composites to a stainless steel grid in a 6-well culture plate (Figure 1). Cultures were maintained for 20 days at 37°C and 5% CO₂ and the medium was replaced every 5 days.

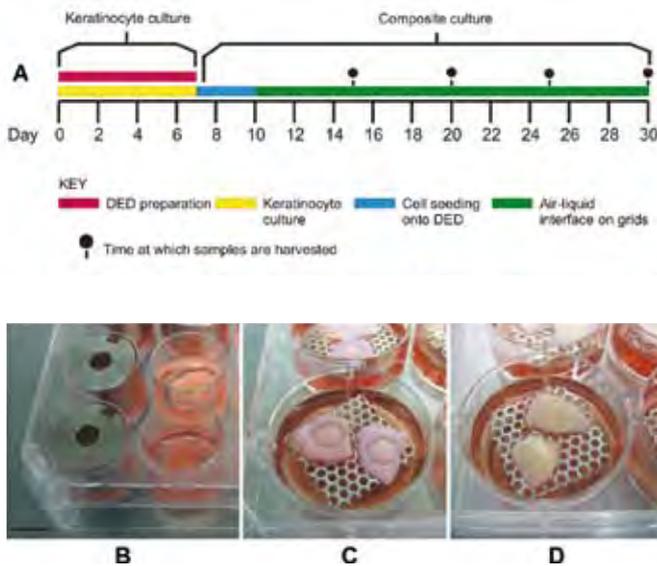
For the time-course studies, triplicate samples were harvested every 5 days. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide conversion (MTT) assay⁹ was performed on one of the samples for each time point. This involved submerging the samples in 6mL of 0.5mg/mL MTT dye^{vi} and incubating the samples at 37°C for 120 minutes. After this time, metabolically active cells could be visualised by a purple colour and were photographed. After this photography, the MTT-stained sample and the other two samples were fixed and embedded in paraffin using standard procedures for hematoxylin and eosin (H/E) histological analysis. In addition, DED samples without any seeded keratinocytes and normal donor skin samples functioned as controls.

Immunohistochemistry

For immunohistochemical analysis, paraffin sections were cut and deparaffinised in ethanol and xylene. After incubation with primary antibodies for keratin 6 (1:1), keratin 14 (1:20), keratins 1/10/11 [1:400]^{vii}, and collagen type IV^{viii}, sections were stained using a DAKO Envision kit^{ix} as described by

Figure 1. Photographs of the HSE model at various stages of development and descriptive timeline of events in the construction of the model.

- Timeline of the model construction.
- Steel rings are used to seed keratinocytes on top of the centre of the DED in a 24-well culture dish. Scale bar: 5 mm
- Grids are used to bring the HSE to the air-liquid interface after the rings are removed.
- Model after 10 days culture at the air-liquid interface.



the supplier, with a minor modification in that PBS was used instead of Tris-buffered saline. All sections were counterstained with hematoxylin and examined by light microscopy.

Burn protocol

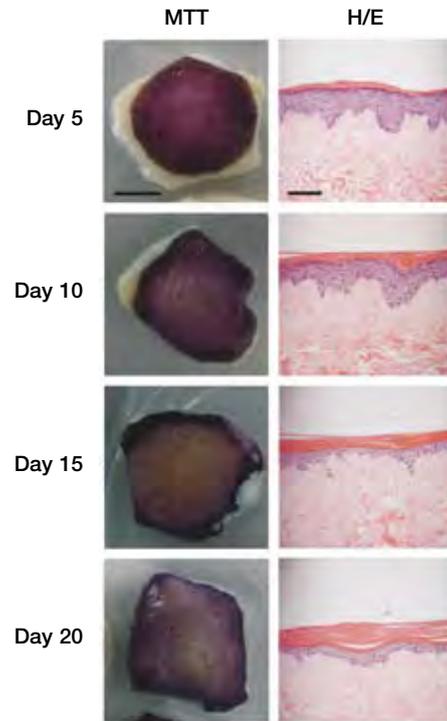
Several burn protocols were evaluated in pilot studies prior to using a method that involved a stainless steel rod with a 0.32cm² base, heated with an open flame for 3 seconds then placed on top of the model for 3 seconds. The healing of the burn in the HSE was followed via an MTT assay performed at Days 0, 2, 4 and 6 post-injury as well as through histological analysis.

Results

Morphological and MTT analysis of HSE models

Over a period of 20 days, the number of metabolically active cells in the HSE models, as ascertained by MTT and histological analysis, appeared to decrease and the stratum corneum increased in thickness (Figure 2). The MTT assay

Figure 2. MTT and histological analyses of the HSE over 20 days. Migration of metabolically active keratinocytes was visualised with the MTT assay; while H/E sections demonstrate the morphology of the stratified epidermis for both models at 5, 10, 15 and 20 days after being lifted to the air-liquid interface. Scale bar in MTT assay: 5 mm. Scale bar in histology: 100 µm.

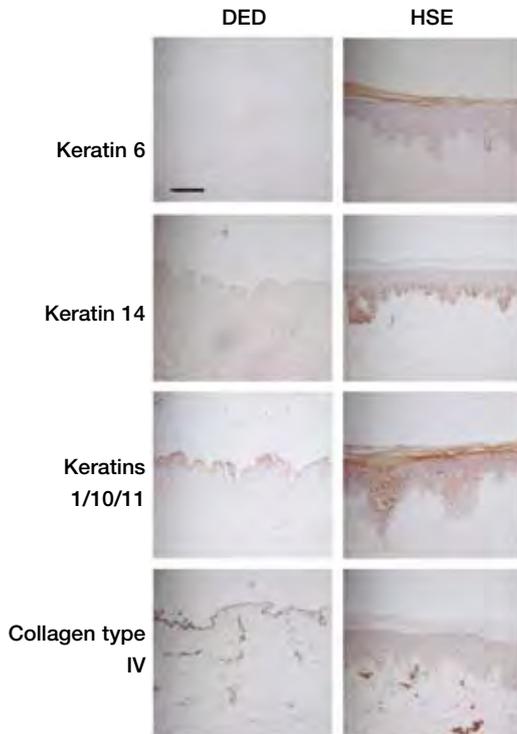


also confirmed that the keratinocytes migrated from the centre seeding region to the edge of the DED over this period. Morphological analysis of the HSE revealed a fully stratified epidermis characteristic of normal human skin at Days 5-15. All cell layers appeared to be present, including stratum basale, stratum spinosum, stratum granulosum and a stratum corneum with a basket weave appearance similar to that found normally observed *in vivo* in skin.

Immunohistochemical analysis

Immunohistochemical analysis was performed on the HSE after 10 days of culture at the air-liquid interface (Figure 3). All keratins were undetected in the DED control. Positive staining for keratin 6 was observed in the HSE model in the stratum corneum and suprabasal layers as well as very light intermittent staining in the basal layer. The expression of keratin 14 was observed in the basal cell layer and slight staining for keratin 1/10/11 was present in the suprabasal layers. The DED alone stained for collagen type IV along the

Figure 3. Immunohistochemical analysis of the DED and HSE model.
Expression of keratin 6, keratin 14, keratins 1/10/11 and collagen type IV in the DED control and the HSE model at day 10. Scale bar: 100 μ m.

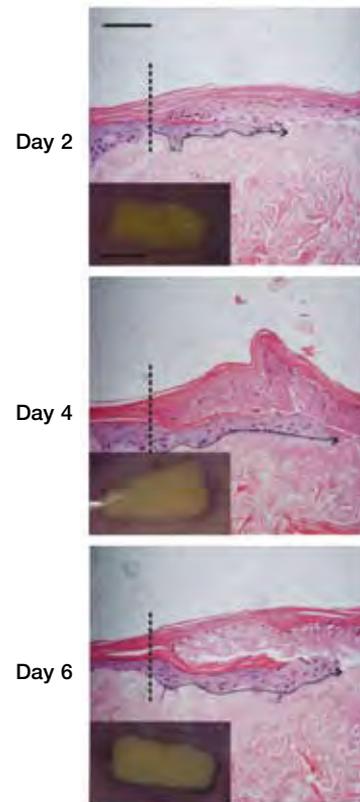


entire basement membrane and also sporadically throughout the dermis. The HSE model exhibited staining along the entire basement membrane, although this was less intense than staining in the DED control. Collagen type IV was detected throughout the dermis of the HSE as expected.

Evaluation of healing following burn

Immediately following burning, metabolically active keratinocytes, as determined by MTT assay, were evident in the HSE around a brown stain which represents the contact area of the burn (Figure 4). Histology revealed the death of tissue, as judged from the presence elongated and detached cells from the dermis, and a slightly compacted dermis in the area of the burn. From Day 2 onwards, keratinocytes began to migrate and proliferate into the wounded region and histological analysis revealed that a new thin stratum corneum was present underneath the damaged tissue. Over the next 4 days, the keratinocytes had migrated further into the wound and differentiated into a stratified epidermis.

Figure 4. MTT and histological analyses of healing of a burn on the HSE up to 6 days post-burn.
The HSE model was burnt after 8 days culture at the air-liquid interface. The dashed line shows the margin of the burn. To the right of the line is the area of the model that was burnt. The dotted arrow running beneath the basement membrane shows the migration of keratinocytes that are facilitating wound re-epithelialisation. Scale bar for histology: 100 μ m. The insets show MTT analysis of the burn area.



MTT analysis corroborated the enhanced activity, as indicated by the dark purple stain around the margin of the burn; this was especially evident at Day 6.

Discussion

The objective of this study was to develop and characterise, morphologically and biochemically, a HSE model and to evaluate its use in studying the healing of burn wounds. The HSE consisted of keratinocytes seeded on a DED, submerged for 3 days, and lifted to the air-liquid interface for up to 20 days. The DED was selected as the dermal scaffold in this study as it possesses significant advantages over other scaffolds¹⁰. In particular, while it is an acellular tissue matrix, it retains an intact basement membrane. This has

previously been shown to be important for the adherence of the epidermis to the dermis and for the differentiation of keratinocytes¹¹⁻¹⁴. Other dermal scaffolds, such as inert filters (SkinEthic®), and collagen matrices (EpiSkin®) do not have a basement membrane and, as such, are not ideal for use in dermal wound healing studies.

For a HSE to be a valid skin model, it must correlate both morphologically and biochemically with normal *in vivo* skin¹⁵. As depicted in this paper, the keratinocytes in the HSE formed a fully differentiated epidermis with histological features similar to those observed *in vivo*. Thus the stratum basale, stratum spinosum, stratum granulosum and stratum corneum were evident after 5 days of culture at the air-liquid interface as assessed by histology. In addition, the morphology of the models is similar to other commercial and non-commercial models previously described in literature^{4, 16-20}.

Between 5 days and 10 days of culture at the air-liquid interface, the greatest number of viable cells and a complete stratified epidermis comparable to *in vivo* skin were evident in the model, as determined by histology. Over time, the stratum corneum increased in thickness and the number of metabolically active cells decreased. In view of these results, it was concluded that subsequent wound healing studies should take place 8 days after culture at the air-liquid interface. Interestingly, other studies have also demonstrated an increase of thickness of the stratum corneum over culture time^{21, 22}.

Immunohistochemical analysis of the HSE revealed the presence and localisation of the following biochemical markers – keratin 6, keratin 14 and keratins 1/10/11. While keratin 6 is a protein that is absent from normal skin *in vivo*, it is often associated with wound repair, hyperproliferation and abnormal differentiation^{20, 23, 24}; thus its presence in the HSE may be due to the presence of the proliferating keratinocytes. Indeed, keratin 6 has been found in other commercial skin models, albeit differences in localisation and intensity have been reported²³.

Keratins 1/10/11, on the other hand, are localised in all suprabasal layers of normal *in vivo* skin and serve as early markers of the terminal differentiation process²³; the HSE expressed keratins 1/10/11 as expected for skin *in vivo*. Proliferating basal cells are characterised by the presence of keratin 14²⁵ and the basal cell layer of the HSE model in this study also exhibited keratin 14. Thus, in general, the HSE exhibited biochemical markers similar to those found in skin

in vivo. As expected, the DED control showed no presence of epidermal keratins.

A previous study has shown that keratinocytes seeded onto a DED degrade the original basement membrane and regenerate a new one²⁶. To examine basement membrane degradation and formation in the models, the presence of collagen type IV, a major constituent of the basement membrane, was investigated. Immunohistochemical analysis of the DED control demonstrated that the original basement membrane was indeed intact before keratinocyte seeding, while the HSE model, at Day 10, exhibited minimal collagen IV along the basement membrane. In light of these results, we propose that, at Day 10, the keratinocytes in the HSE had degraded the original basement membrane and had begun to regenerate a new one.

HSEs may make it possible to study both the local events involved in wound healing as well as the effects of potential therapeutics. In an attempt to explore this, we investigated the potential of the HSE model as a burn wound healing research tool. After 8 days of culture at the air-liquid interface, the HSE was burnt using a metal rod heated with a flame and applied for 3 seconds. Histological analysis revealed a partial thickness burn with dead cells and debris in the contact area. However, metabolically active keratinocytes (as assessed by MTT assay) were present at the wound margin, and these presumably hold potential for the re-epithelialisation of the wound.

Indeed, this hypothesis was supported by further analysis of the burn wound healing model in which the healing of the HSE was evaluated immediately after the burn and every 2 days thereafter for up to 6 days post-injury. Histological analysis revealed that keratinocytes began to re-epithelialise the wound from Day 2 post-injury. Clearly, this demonstrates the potential of the HSE as a burn wound healing model, and raises the possibility that the model will also be useful for *in vitro* testing of new therapeutics.

Conclusion

The HSEs in this study are a simplified model of human skin. They lack both innervation and vascularisation, thus the inflammation and infiltration typical of *in situ* burns is absent²⁷. In addition, the HSE developed consisted only of keratinocytes. There are no melanocytes, Langerhans or Merkel cells, the three other epidermal cell types found *in vivo*, and no fibroblasts are present.

Nevertheless, while this model lacks certain qualities of *in vivo* skin, these cellular components may be able to be incorporated with further development and refinement of the HSE. Importantly, the results reported here demonstrate the successful development of a HSE model in Australia and indicate that this model holds particular promise as a potential model for *in vitro* burn wound healing. Moreover, this HSE is likely to prove to be a valuable tool in furthering our understanding of the biology of skin in general.

Acknowledgements

The authors would like to thank Mr Don Geyer (School of Life Sciences, Queensland University of Technology), the staff of the Faculty of Science Mechanical Workshop at the Queensland University of Technology and Mr David Topping for their technical assistance. In addition, the authors would like to thank Dr Anthony Kane and his patients for their kind donations of skin. The collagen type IV monoclonal antibody developed by Dr H Furthmayer was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa^x.

Manufacturers

- i L'Oreal, Paris, France.
- ii Mattek, Ashland, Massachusetts, USA.
- iii Skinethic, Paris, France.
- iv Invitrogen, Melbourne, VIC, Australia.
- v Aix Scientifics, Aachen, Germany.
- vi Sigma Aldrich.
- vii RDI Research Diagnostics, Massachusetts, USA.
- viii Developmental Studies Hydroma Bank, The University of Iowa, USA.
- ix Dakocytomation, Botany, NSW, Australia.
- x The University of Iowa, Dept of Biological Sciences, Iowa City, IA 52242.

References

1. Moulin V, Auger F, Garrel D & Germain L. Role of wound healing myofibroblasts on re-epithelialization of human skin. *Burns* 2000; **26**:3-12.
2. Gottrup F, Agren MS & Karlsmark T. Models for use in wound healing research: a survey focusing on *in vitro* and *in vivo* adult soft tissue. *Wound Repair Regen* 2000; **8**:83-96.
3. Sullivan TP, Eaglstein WH, Davis SC & Mertz P. The pig as a model for human wound healing. *Wound Repair Regen* 2001; **9**:66-76.
4. Poumay Y, Dupont F, Marcoux S, Leclercq-Smekens M, Herin M & Coquette A. A simple reconstructed human epidermis: preparation of the culture model and utilization in *in vitro* studies. *Arch Dermatol Res* 2004; **296**:203-211.
5. Shaw A. *Epithelial Cell Culture: A Practical Approach* (AS, Ed). Oxford University Press, Oxford, 1996.
6. Chakrabarty K, Dawson R, Harris P, Layton C, Babu M, Gould L, Phillips J, Liegh I, Green C, Freedlander E & Macneil S. Development of autologous human dermal-epidermal composites based on sterilized human allodermis for clinical use. *Brit J Dermatol* 1999; **141**:811-823.
7. Huang Q, Dawson R, Pegg D, Kearney J & Macneil S. Use of peracetic acid to sterilize human donor skin for production of acellular dermal matrices for clinical use. *Wound Repair Regen* 2004; **12**:276-287.
8. Fischer E, Stingl A & Kirkpatrick C. Migration assay for endothelial cells in multiwells: application to studies on the effect of opioids. *J Immunol Methods* 1990; **128**:235-239.
9. Denizot F & Lang R. Rapid colorimetric assay for cell growth and survival: modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods* 1986; **89**:271-277.
10. Bhargava S, Chapple CR, Bullock AJ, Layton C & Macneil S. Tissue-engineered buccal mucosa for substitution urethroplasty. *BJU Int* 2003; **93**:807-811.
11. Breikreutz D, Stark HJ, Mirancea N, Tomakidi P, Steinbauer H & Fusenig NE. Integrin and basement membrane normalization in mouse grafts of human keratinocytes – implications for epidermal homeostasis. *Differentiation* 1997; **61**:195-209.
12. Smola H, Stark HJ, Thiekotter G, Mirancea N, Krieg TH & Fusenig NE. Dynamics of basement membrane formation by keratinocyte-fibroblast interactions in organotypic skin culture. *Exp Cell Res* 1998; **239**:399-410.
13. Smola H, Thiekotter G & Fusenig NE. Mutual induction of growth factor gene expression by epidermal-dermal cell interaction. *J Cell Biol* 1993; **122**:417-429.
14. Maas-Szabowski N, Szabowski A, Stark H J, Andrecht S, Kolbus A, Schorpp-Kistner M, Angel P & Fusenig NE. Organotypic cocultures with genetically modified mouse fibroblasts as a tool to dissect molecular mechanisms regulating keratinocyte growth and differentiation. *J Invest Dermatol* 2001; **116**:816-820.
15. Monteiro-Riviere NA, Inman AO, Snider TH, Blank JA & Hobson DW. Comparison of an *in vitro* skin model to normal human skin for dermatological research. *Microsc Res Techniq* 1997; **37**:172-179.
16. Rosdy M & Clauss LC. Terminal epidermal differentiation of human keratinocytes grown in chemically defined medium on inert filter substrates at the air-liquid interface. *J Invest Dermatol* 1990; **95**:409-414.
17. Netzlaff F, Lehr CM, Wertz P W & Schaefer UF. The human epidermis models EpiSkin, SkinEthic and EpiDerm: an evaluation of morphology and their suitability for testing phototoxicity, irritancy, corrosivity, and substance transport. *Eur J Pharm Biopharm* 2005; **60**:167-178.
18. Rehder J, Souto LR, Bernardino CM, Issa M, Puzzi MB. Model of human epidermis reconstructed *in vitro* with keratinocytes and melanocytes on dead de-epidermized human dermis. *Sao Paulo Med J* 2004; **122**:22-25.
19. Lee DY, Ahn HT, Cho KH. A new skin equivalent model: dermal substrate that combines de-epidermized dermis with fibroblast-populated collagen matrix. *J Dermatol Sci* 2000; **23**:132-137.
20. Boelsma E, Gibbs S, Faller C & Ponc M. Characterization and comparison of reconstructed skin models: morphological and immunohistochemical evaluation. *Acta Derm-Venereol* 2000; **80**:82-88.
21. Stoppie P, Borghgraef P, De Wever B, Geysen J & Borgers M. The epidermal architecture of an *in vitro* reconstructed human skin equivalent (Advanced Tissue Sciences Skin2 Models ZK 1300/2000). *Eur J Morphol* 1993; **31**:29-29.
22. Nolte CJ, Oleson MA, Bilbo PR, Parenteau NL. Development of a stratum corneum and barrier function in an organotypic skin culture. *Arch Dermatol Res* 1993; **285**:466-474.
23. Ponc M, Boelsma E, Gibbs S & Mommaas AM. Characterization of reconstructed skin models. *Skin Pharmacol Appl* 2002; **15**:4-17.
24. Takahashi K, Paladini RD & Coulombe PA. Cloning and characterization of multiple human genes and cDNAs encoding highly related type II keratin 6 isoforms. *J Biol Chem* 1995; **270**:18518-18592.
25. Leigh IM, Lane EB & Watt FM. *The Keratinocyte Handbook*. Press Syndicate of the University of Cambridge, Cambridge, 1994.
26. Altankov G, Hecht J & Dimoudis N. Serum-free cultured keratinocytes fail to organize fibronectin matrix and possess different distribution of beta-1 integrins. *Exp Dermatol* 2001; **10**:80-89.
27. Arturson G. Pathophysiology of the burn wound and pharmacological treatment. The Rudi Hermans Lecture, 1995. *Burns* 1996; **22**:255-274.