UNIVERSITI PUTRA MALAYSIA

MORPHOLOGICAL CHANGES AND EXPRESSION OF PROTEIN MARKERS DURING REMODELING OF TISSUE - ENGINEERED SKIN

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MORPHOLOGICAL CHANGES AND EXPRESSION OF PROTEIN MARKERS DURING REMODELING OF TISSUE - ENGINEERED SKIN

By

NORHAYATI MOHD MONZAI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

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This thesis is especially dedicated to:

My family: to my mother, Sa'ayah Binti Haji Bejo and my father, Mohd. Monzai Haji Alias who are infinitely precious to me, thank you……..

My husband: Abang, Wan Ali Wan Ishak and my son 'Si Comel Umar' for his forbearance concerning all things during my study & I might have done instead, , I love you…..

My friends, who have filled my life with joyous and balances between sadness and happiness

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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March 2009

Chairman : Professor Dr Fauziah Othman, Ph.D.

Faculty : Faculty of Medicine and Health Sciences

The study was carried out to evaluate the skin remodelling and skin development after bilayered fibrin – fibroblast / fibrin – keratinocytes skin equivalent (B FF/FK SE) and fibrin without seeded cell (FWC) were transplanted into eight weeks old athymic mice. During skin remodelling, the structural, ultrastructural features and protein expression were investigated. The keratinocytes and fibroblasts were isolated and cultured until propagated. The B FF/FK SE was produced by incoporated the keratinocytes and fibroblasts with human fibrin. Then, constructed skin was harvested after 1, 7, and 14 days *in vitro* and 30 and 60 days *in vivo* for electron microscopy analysis and immunolabelling. Grossly, the wound margin transplanted with B FF/FK SE appeared constantly pink whereas with FWC, necrotic zone appeared yellowish. Light microscopy revealed that B FF/FK SE has good skin remodelling capacity with 6-12 cells thick after 60 days post-transplantation whereas FWC was only

3-4 cells thick. The mean number of keratinocytes and fibroblast was pursued by using Duncan test and one way ANOVA which showed that B FF/FK SE was capable for regenerating the dermal-epidermal layer in a shorter period of time compared to FWC ($p \leq 0.05$). Further studies were done using the structural features of B FF/FK SE and FWC *in vitro* and *in vivo*. Scanning electron microscopy (SEM) revealed that keratinocytes and fibroblasts in B FF/FK SE showed an excellent adherence in fibrin matrix and changes in their morphology after 1 to 14 days *in vitro.* It ranges from rounded to elongated and stellate shape, whereas, for FWC, no cells were detected. Stratified layer with sloughed off stratum corneum was seen developing after B FF/FK SE and FWC were transplanted onto athymic mice. Transmission electron microscopy (TEM) showed that the ultrastructural features during epidermal differentiation and regeneration as well as basement membrane formation were well developed after B FF/FK SE and FWC transplanted onto athymic mice. The presence of keratinocyte clusters which migrated superiorly and fibroblast clusters which migrated anteriorly at fibrin matrix mimicked a bilayered skin tissue whereas in contrast FWC showed no cell migration. However, both B FF/FK SE and FWC after implantation, showed the formation of columnar stratum basale, stratum spinosum, stratum granulosum with keratohyaline granule and stratum corneum suggesting epidermal differentiation and regeneration might have occurred. Development of basement membrane in B FF/FK SE with cell junction components such as hemidesmosome, lamina lucida, lamina densa and

anchoring fibril network was established which was similar to native human skin. Furthermore, dermal organisation of B FF/FK SE showed a similarity to native human skin which has a compact basket weave pattern arrangement of collagen bundles whereas FWC showed a loose arrangement. Confocal microscopy revealed that immunolabelling of desmoglein 3 and plakophilin 1 at stratified layer, type IV collagen, integrin α6 and type VII collagen at basement membrane zone and type I collagen at dermal margin were present after 60 days B FF/FK SE post-transplantation which was similar to native human skin. In contrast, such observation was not detected for FWC. In conclusion, the B FF/FK SE showed the better skin regeneration similar to native human skin and required a shorter period of time during wound healing without any contraction.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PERUBAHAN MORFOLOGI DAN EKSPRESSI PENANDA PROTEIN SEMASA BINAAN SEMULA TISU KULIT MELALUI KAEDAH KEJUTERUTERAAN TISU

Oleh

NORHAYATI BINTI MOHD MONZAI

Mac 2009

Pengerusi : Profesor Dr Fauziah Othman, PhD

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Kajian ini dilakukan untuk menilai pembinaan dan pembentukkan kulit selepas dwilapis fibrin-fibroblas/fibrin-keratinosit (B FF/FK SE) dan fibrin without seeded cell (FWC) ditransplan keatas 8 minggu tikus athymik. Ia dijalankan untuk mengkaji struktur mikroskopik, struktur ultra mikroskopik dan ekspresi protein semasa pembentukkan kulit. Keratinosit dan fibroblas telah diasingdan dikulturkan sehingga membiak. B FF/FK SE telah dihasilkan dengan mengambungkan keratinosit dan fibroblas bersama fibrin manusia. Selepas itu, konstruk kulit dituai setelah 1, 7 dan 14 hari *in vitro* dan 30 dan 60 hari *in vivo* untuk analisis mikroskopi elektron dan perlabelan immuno. Secara kasar, margin luka yang telah ditransplan dengan B FF/FK SE menunjukkan warna merah jambu yang konstan manakala FWC menunjukkan zon warna kekuningan nekrotik. Mikroskopi cahaya mendedahkan bahawa B FF/FK SE

selepas 60 hari mempunyai pembentukkan kapasiti kulit yang baik dengan 6 -12 ketebalan sel manakala FWC hanya mengandungi 3-4 ketebalan sel. Hitungan purata keratinosit dan fibroblast dijalankan dengan menggunakan ujian Duncan dan ANOVA 1 hala telah menunjukkan bahawa B FF/FK SE telah berupaya menghasilkan semula lapisan dermis-epidermis di dalam masa yang singkat berbanding dengan FWC ($p \leq 0.05$). Kajian seterusnya dijalankan dengan menggunakan ciri struktural B FF/FK SE dan FWC *in vitro* and *in vivo.* Dari analisis menggunakan mikroskop pengimbas elekron (SEM), keratinosit dan fibroblas yang terdapat pada B FF/FK SE menunjukkan pelekatan di matriks fibrin dan perubahan morfologi yang baik selepas 1 hingga 14 hari *in vitro* dari bentuk yang membulat kepada bentuk memanjang dan stellate manakala FWC adalah sebaliknya. Lapisan berstrata dengan penanggalan strata korneum telah terhasil selepas B FF/FK SE dan FWC ditransplan keatas tikus athymik. Seterusnya mikroskop transmissi elektron (TEM) telah digunakan untuk menilai struktur ultra semasa pembezaan dan regenerasi lapisan epidermis begitu juga lapisan membran bawah selepas B FF/FK SE ditransplan keatas tikus athymik. Kehadiran keratinosit yang bermigrasi ke bahagian superior dan kluster fibroblast ke bahagian anterior pada matriks fibrin seakan-akan menyerupai dwilapisan tisu kulit manakala berbeza dengan FWC iaitu tidak terdapat langsung sel yang bermigrasi. Walaubagaimanapun selepas ditransplan B FF/ FK SE dan FWC kedua-duanya menunjukkan pembentukkan strata basal yang kolumnar, strata spinosum, strata granulosum, bersama

dengan granul keratohailin dan strata korneum telah dikenalpasti, ini menunjukkan perbezaan dan regenerasi epidermis telah berlaku. Pembentukkan lapisan bawah membran pada B FF/FK SE bersama komponen penting yang lain seperti hemidesmosome, lamina lucida, lamina densa dan rangkaian fibril sangkutan telah terbentuk seperti kulit manusia yang asal. Selain daripada itu organisasi bahagian dermis menunjukkan persamaan kulit manusia yang asal iaitu terdapat corak anyaman bakul yang padat pada berkas-berkas kolagen, manakala FWC menunjukkan susunan yang longgar. Mikroskop konfokal telah menunjukkan ekspressi protein desmoglein 3 dan plakophilin 1 di lapisan strata, kolagen IV, kolagen VII dan integrin α6 di lapisan bawah membran, kolagen I di margin dermis telah hadir selepas 60 hari pos-transplantasi sama seperti kulit manusia yang asal. Kesimpulannya, B FF/FK SE menunjukkan regenerasi kulit yang baik sama seperti kulit manusia yang asal dan memerlukan jangka masa yang pendek untuk penyembuhan luka tanpa pengecutan.

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Serdang, January 2009

Norhayati Mohd Monzai

I certify that an Examination Committee met on 6th March 2009 to conduct the final examination of Norhayati Mohd Monzai on his Master of Science thesis entitled 'Morphological Changes and Expression Of Protein Markers During Remodeling of Tissue - Engineered Skin' in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Putra Malaysia (Higher Degree) Regulations 1981. The committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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NORHAYATI MOHD MONZAI

Date:

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LIST OF ABBREVIATIONS

BDMA	Benzyldimethylamine
Cy3	Cyanine 3
DDSA	Dodecenyl succinic anhydride
DKSFM	Defined keratinocytes serum free medium
DPBS	Dulbecco's phosphate buffer saline
FCS	Fetal calf serum
FITC	Fluorescein isothiocynate
MNA	Methyl nadic anhydride
PBB	Patient based biomaterial
PBS	Phosphate buffered saline
OsO_4	Osmium tetroxide

CHAPTER 1

INTRODUCTION

Tissue and organ failure, resulting from various form of injuries traumatic, metabolic, inflammatory and other diseases, accounts for about half the total annual expenditure in world health care (Middelkoop *et al*., 2004). Various treatment modalities are employed to overcome the problems which include organ transplantation, surgical repair, plastic surgery, artificial prostheses, drug theraphy and the use of mechanical devices. However, organ and tissue damage cannot be repaired and healed by fibrous repair which result in permanent loss of functional tissue. In organ transplant, rejection may occur and frequent monitoring is needed. The presence of tissue engineering technology provides an alternative choice to solve this problem of tissue loss and it has been reported to be safe and side effects are minimal (Robert and Vacanti, 1993).

This technology is capable of producing adequate constructed skin from a small skin biopsy without any additional synthetic or extracellular matrix components from xenogeneic or allogeneic source. This approach can result in the formation of fully autologous skin substitute.

The term "tissue engineering" or "cell therapy" was defined as "the application of principles and methods of tissue engineering and life science toward

fundamental understanding of structure-function relationship in normal and pathological mammalian tissue or to improve tissue function" (Sachlos and Czernuszka, 2003).

This technology provides a suitable environment for cell proliferation and differentiation during tissue regeneration or tissue reconstruction processes (Moroni and Van Blitterswijk, 2006). This situation is an important factor to enable an increase in the self healing potential in diseases tissue of the patient. Treatment by this approach involves essential processes from cell recovery to tissue manipulation and grafting (Heinonen *et al*., 2005).

Stem cells have been used in tissue engineering technology because of it's potential to regenerate new tissue (Chapekar, 2000). However recent studies showed that the use of cells from specific organs such as keratinocytes or fibroblast isolated from skin also gave a positive result such as better cosmetic effects and quicker healing process without scar formation. In addition, usage of tissue engineering technology also gives a good impact in cartilage and bone defect restructuring, changed parts of the nervous system, liver, pancreas, blood vessels, striated muscle (Maniatopolous *et al*., 1988, Stark *et al*., 1999 and De Aza *et al*., 2003), heart valves (Vesely, 2005) and cardiovascular tissue affected by injuries (Mol *et al*., 2005).

The choice of biomaterial is an important factor for tissue reconstruction process. Biomaterial must have biodegradable characteristic to ensure healing progresses in a right way (Palsson and Bhatia, 2004). There are many such biomaterials such as Type I collagen, fibrin, chitosan; usually used in tissue engineered skin and cartilage whereas polyDL-lacticco- glycolic acid (PLGA), Tricalcium phosphate / Hydroxy apatite (TCP/HA) in tissue engineered bone (Guerret *et al*., 2003, Guo *et al*., 2006, Wu *et al*., 2006, Hing *et al*., 1999 and Moore *et al*., 2001).

In Malaysia, Human Plasma Derivatives (HPD) or fibrin as a biomaterial was widely used to produce the skin construct or briefly called bilayered fibrin-fibroblast/fibrin-keratinocytes skin equivalent (B FF/FK SE). Fibrin was used as biomaterials to develop a fully autologous and 3- dimensional skin construct. Fibrin can provide a temporary scaffold that stimulate cell directs to the site of injury and create of a viable dermal compartment (Escamez *et al*., 2004; Casoli *et al*., 2004 and Guerret *et al*., 2003).

Bilayered fibrin-fibroblast/fibrin-keratinocytes skin equivalent (B FF/FK SE) is very different from Apligraf (Skin Graft) Human Skin Equivalent or Integra's Artificial Skin which is very popular in the US (Jones *et al*., 2002). The new approaches of skin equivalent consist of both fibroblast and keratinocytes. Both cells were simultaneously seeded inside the fibrin whereas Apligraf or Integra's

Artificial Skin used the neonatal fibroblasts / neonatal keratinocytes or either the fibroblasts or keratinocyte cells were seeded in one of side a silicone membrane (Burke *et al*., 1981 and Jones *et al*., 2002).

B FF/FK SE was applied to treat chronic wound and diabetic foot ulcer which showed a good healing process after transplantation with a layer of keratinocytes (epidermis) and fibroblast (dermis) (Mazlyzam *et al*., 2007). However, the determination of ultrastructural features and protein expression analysis during this process has not being conducted. Thus, this present study assesses the ultrastructural features and protein expression during skin remodeling.

1.1 Research hypothesis

$H_{Ø:}$ there is no similarity in term of morphology and protein markers of bilayered fibrin fibroblast/fibrin keratinocytes skin equivalent (B FF/FK SE) and native human skin

H_A : Bilayered fibrin fibroblast/fibrin keratinocytes skin equivalent (B FF/FK SE) have similar morphology and protein markers as native human skin

1.2 Objectives of the Study

The general objectives of this study are;

i) To construct bilayered fibrin fibroblast/fibrin keratinocytes skin equivalent (B FF/FK SE) *in vitro.*

ii) To transplant B FF / FK SE and fibrin without seeded cell (FWC) onto athymic mice *in vivo.*

The specific objectives are;

i) To observe the light microscopy of the wound healing, formation of epidermal-dermal layer of B FF/FK SE and FWC *in vitro* and *in vivo* in comparison to native human skin.

ii) To analyze the number of keratinocytes and fibroblast of B FF/FK SE and FWC *in vivo* in comparison to native human skin (NHS).

iii) To study the surface morphological development of B FF/FK SE and FWC *in vivo* in comparison to native human skin (NHS) using scanning electron microscopy.

iv) To study the ultrastructural features of B FF/FK SE and FWC *in vitro and in vivo* in comparison to NHS using transmission electron microscopy.

v) To study the protein expression during skin remodeling of B FF/FK SE and FWC at basement membrane zone, stratified and dermis layer

formation *in vitro and in vivo* in comparison to NHS by using immunolabelling technique.

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