GENE EXPRESSION PROFILE OF PRIMARY FIBROBLASTS FROM KELOID AND NORMAL SKIN TREATED WITH PALM OIL TOCOTRIENOL RICH FRACTION (TRF)

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by

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TABLE OF CONTENTS

		Page
Acknowledgements		**ii**
Table of Contents		**iv**
List of Tables		**x**
List of Figures		**xii**
List of Abbreviations		**xvi**
List of Symbols		**xx**
Abstrak		**xxi**
Abstract		**xxiv**
CHAPTER 1- INTRODUCTION		
1.1	Human Skin	**1**
	1.1.1 Structure of the human skin	**1**
	1.1.2 Functions of the human skin	**5**
1.2	Wound healing	**6**
	1.2.1 Mechanism and genes involved in wound healing	**6**
	1.2.2 The role of cytokines and growth factors in wound healing	**12**
1.3	Abnormal scarring	**16**
	1.3.1 Keloids	**16**
	1.3.2 Gene expression and pathogenesis of keloids	**20**
	1.3.3 Treatment of keloids	**26**
1.4	Vitamin E	**27**
	1.4.1 Palm oil tocotrienol	**31**

1.5 Primary cell culture **35**

1.5.1 Fibroblasts culture **36**

1.6 Screening of regulated genes in keloids formation using microarray technique **37**

1.7 Problem statement **42**

1.8 Objective of the study **44**

1.8.1 General objective **44**

1.8.2 Specific objectives **44**

CHAPTER 2 – MATERIALS AND METHODS

2.1 Sample collection and flowchart of the study **45**

2.2 Primary fibroblast cultures **48**

2.2.1 Reagents for primary fibroblast cultures **48**

2.2.2 Preparation of Dispase **50**

2.2.3 Preparation of Collagenase Type I **50**

2.2.4 Preparation of complete culture medium **50**

2.2.5 Protocol for primary fibroblast cultures **51**

2.2.6 Protocol for epidermal keratinocyte culture **56**

2.2.7 Assessment of cell viability and cell counts **56**

2.2.8 Maintenance of fibroblast cultures **57**

2.2.9 Subculturing **57**

2.2.10 Frozen cell stocks **58**

2.2.11 Cell recovery **59**

2.3 Verification of human dermal fibroblasts **60**

2.3.1 Reagents for verification of human dermal fibroblasts **60**

2.3.2 Protocol of verification of human dermal fibroblasts **61**

2.4 Growth characteristic of normal dermal and keloid fibroblasts **63**

2.5 MTT assay **64**

2.5.1 Preparation of MTT **64**

2.5.2 Protocol for MTT assay **64**

2.6 Fibroblast cells treatment **68**

2.6.1 Palm oil TRF preparation **68**

2.6.2 Protocol for palm oil TRF treated fibroblast cells **70**

2.7 RNA extraction **71**

2.7.1 Reagents for RNA extraction **71**

2.7.2 Protocol for RNA extraction **71**

2.7.3 Determination of RNA concentration and purity **73**

2.7.4 Quantitative measurement **73**

2.7.5 Determination of the RNA quality **74**

2.8 Electrophoresis **75**

2.8.1 Preparation of 1X LB Buffer solution **75**

2.8.2 Staining material **75**

2.8.3 Loading buffer **75**

2.8.4 RNA marker/ladder **76**

2.8.5 Preparation of agarose gel **76**

2.8.6 Protocol for agarose gel electrophoresis **76**

2.9 Whole genome gene expression profile **77**

2.9.1 cRNA amplification **77**

2.9.2 Reagents for cRNA amplification **77**

2.9.3 Protocol for cRNA amplification **78**

2.9.4 Reagents for hybridization **84**

2.9.5 Protocol for hybridization **84**

2.9.6 Reagents for washing and staining **85**

2.9.7 Protocol for washing and staining **85**

2.9.8 Scanning **86**

2.9.9 Analysis of microarray gene expression **86**

2.9.9.1 Genome Studio software **86**

2.9.9.2 GeneSpring (GX) software **87**

2.9.9.3 Database for Annotation, Visualization and Integrated Discovery (DAVID) software **88**

2.10 cDNA synthesis **90**

2.10.1 Reagents for cDNA synthesis **90**

2.10.2 Protocol for cDNA synthesis **90**

2.11 Gene expression of selected genes using Real-Time PCR **91**

2.11.1 Determination and standardization of cDNA concentration **91**

2.11.2 Serial dilution of cDNA **91**

2.11.3 Program setup for real-time PCR system **92**

2.11.4 Reagents for real-time PCR system **92**

2.11.5 Preparation of master mix for the amplification of *GAPDH* gene **93**

2.11.6 Preparation of master mix for the amplification of targeted genes (*TGFβ1, TGFβ2, TGFβ3, COL3A1, FN1, TAGLN* and *IGFBP5*) **100**

2.11.7 Relative quantification analysis **104**

2.12 Statistical analysis **105**

CHAPTER 3 – RESULTS

3.1 Primary dermal fibroblast cultures **106**

3.2 Verification of primary human dermal fibroblasts using HSP47 and FSP antibodies **108**

3.3 Growth characteristic of normal dermal and keloid fibroblasts **112**

3.4 Effect of palm oil tocotrienol rich fraction (TRF) on human skin fibroblast *in vitro* **115**

3.4.1 Effect of palm oil TRF on normal dermal fibroblasts **115**

3.4.2 Effect of palm oil TRF on keloid fibroblasts **121**

3.5 Whole genome gene expression profile using microarray **125**

3.5.1 Gene expression profile of keloid fibroblasts using microarray **128**

3.5.2 Gene expression profile of palm oil TRF treated keloid fibroblasts using microarray **132**

3.5.3 Pathways related to keloid formation **143**

3.6 Gene expression of selected genes using Real-Time PCR **146**

3.6.1 Amplification of reference gene (*GAPDH*) and targeted genes (*TGFβ1, TGFβ2, TGFβ3, COL3A1, FN1, TAGLN* and *IGFBP5*) **147**

3.6.2 Melting curve analysis **147**

3.6.3 Standard curve **148**

3.6.4 Expression of *TGFβ1, TGFβ2, TGFβ3, COL3A1, FN1, TAGLN* and *IGFBP5* genes in untreated keloid fibroblasts **157**

3.6.5 Effect of palm oil TRF treatment on *TGFβ1, TGFβ2, TGFβ3, COL3A1, FN1*, *TAGLN* and *IGFBP5* expression in keloid fibroblasts **158**

CHAPTER 4 – DISCUSSION

4.1 Primary dermal fibroblasts culture **164**

4.2 Verification of primary human dermal fibroblasts **167**

4.3 Growth characteristic of normal dermal and keloid fibroblasts **171**

4.4 Effect of palm oil TRF on human skin fibroblast *in vitro* **174**

4.4.1 Cytotoxicity effect of palm oil TRF on normal dermal fibroblasts **174**

4.4.2 Suppressive effect of palm oil TRF on keloid fibroblasts **178**

4.5 Whole genome gene expression profile using microarray **182**

4.5.1 Gene expression profile of keloid fibroblasts using microarray **184**

4.5.2 Gene expression profile of palm oil TRF treated keloid fibroblast **186**

4.6 Pathways related to keloid formation **191**

4.7 Expression of *TGFβ1, TGFβ2, TGFβ3, COL3A1, FN1, TAGLN* and *IGFBP5* genes in keloid fibroblasts using Real-Time PCR **195**

4.7.1 Real-time PCR for microarray data validation **198**

4.8 Limitation of the study **209**

4.9 Recommendation for future research **210**

CHAPTER 5 – CONCLUSION **212**

REFERENCES **217**

APPENDICES

LIST OF PUBLICATIONS AND PRESENTATIONS

LIST OF TABLES

		Page
Table 1.1	Differences between keloids and hypertrophic scars.	19
Table 1.2	List of common genes reported to be up or down regulated in keloids by microarray studies.	24
Table 2.1	Composition of tocotrienols and tocopherols in Gold Tri. E 70.	69
Table 2.2	Reagents mixture for first strand cDNA synthesis.	80
Table 2.3	Reagents mixture for second strand cDNA synthesis.	81
Table 2.4	Reagents mixture for cRNA synthesis.	83
Table 2.5	Primer sequence used for the amplification of *GAPDH*, TGF*β1, TGFβ2*, *TGFβ3*, *COL3A1*, *FNI*, *TAGLN* and *IGFBP5* genes.	94
Table 2.6 (a)	Final concentration and volume of reagents used for the amplification of *GAPDH* gene. 100 ng of cDNA was used as a template.	96
Table 2.6 (b)	Final concentration and volume of reagents used for the amplification of *GAPDH* gene. 1000 ng of cDNA was used as a template.	97
Table 2.7(a)	Experimental Real-time PCR protocol for the amplification of *GAPDH* gene for cDNA concentration of 100 ng was used as a template.	98
Table 2.7(b)	Experimental Real-time PCR protocol for the amplification of *GAPDH* gene for cDNA concentration of 1000 ng was used as a template.	99
Table 2.8	Final concentration and volume of reagents used for the amplification of targeted genes.	101
Table 2.9	Experimental Real-time PCR protocol for the amplification of targeted genes.	102
Table 2.10	Concentration of cDNA, number of cycles and annealing temperature of each targeted genes.	103

Table 3.1	Mean of viable cells for nHDF and KF for 4 days of cultures.	114
Table 3.2	Viable cells of nHDF for 24 h, 48 h and 72 h of incubation with palm oil TRF tested using MTT assay and analyzed using Kruskal-Wallis test.	118
Table 3.3	Viable cells of KF for 24 h, 48 h and 72 h of incubation with palm oil TRF tested using MTT assay and analyzed using Kruskal-Wallis test.	124
Table 3.4	Gene list of differentially expressed genes in untreated KF and nHDF analyzed by GeneSpring GX11 software.	130
Table 3.5	Differentially expressed of selected genes in palm oil TRF treated KF and untreated KF.	134
Table 3.6	Summary of differentially expressed genes with their functions.	140
Table 3.7	Relative expression ratio of *TGFβ1, TGFβ2, TGFβ3, COL3A1, FN1, TAGLN* and *IGFBP5* in untreated KF versus nHDF and palm oil TRF treated KF versus untreated KF as determined by REST© software.	159
Table 3.8	Summary of the relative expression of 7 genes selected from microarray data.	163
Table 4.1	List of Cdks and cyclin	193

LIST OF FIGURES

		Page
Figure 1.1	Schematic diagram of the skin.	3
Figure 1.2	Schematic diagram for the four phases of wound healing process.	8
Figure 1.3	The appearance of keloid from patients of this study before surgery. (a) Keloid on shoulder (b) Keloid on earlobe.	18
Figure 1.4	Structure of vitamin E compounds. (a) Tocopherols (b) Tocotrienols.	30
Figure 2.1	Flowchart of the study	47
Figure 2.2	Skin samples in DKSFM transport medium with a sample description form.	49
Figure 2.3	Washing procedures using PBS and 70 % ethanol to remove adipose tissue and excesses blood.	53
Figure 2.4	Skin fragments in dispase for overnight incubation at 4 ºC.	54
Figure 2.5	Epidermal and dermal layer separation.	55
Figure 2.6	Illustration of the MTT test performed in 96 wells culture plate.	67
Figure 3.1	Primary dermal fibroblast cells. (a) Rounded and floating cells in suspension during the first day of culture (b) Fibroblast cells at day 3 of culture (c) Fibroblast cells at day 5 of culture.	107
Figure 3.2	Immunocytochemistry staining of HSP47 antibody. (a) pNHDF culture positively stained with HSP47 antibody (b) cNHDF cell line positively stained with HSP47 antibody (c) NHEK showed negative reaction to HSP47 antibody and no colour produced after staining (d) Fibroblast cells without HSP47 antibody staining served as an antibody control.	109

Figure 3.3	Immunocytochemical staining of FSP antibody. (a) pNHDF culture positively stained with FSP antibody (b) cNHDF cell line positively stained with FSP antibody (c) NHEK showed negative reaction to FSP antibody and no colour produced after staining (d) Fibroblasts cell without FSP antibody staining served as an antibody control.	111
Figure 3.4	Growth pattern of nHDF and KF cultures. Mean of viable cells of nHDF and KF for 4 days of cultures was counted using trypan blue dye. (n =15).	113
Figure 3.5	Effect of palm oil TRF on nHDF cultures. nHDF were incubated with various concentrations of palm oil TRF for 24 h, 48 h and 72 h in humidified incubator at 37 °C with 5% of CO_2. Cell viability was measured using MTT assay. (n = 15).	116
Figure 3.6	Determination of IC_{50} value for nHDF at different incubation period.	120
Figure 3.7	Effect of palm oil TRF on KF cultures. KF were incubated with various concentrations of palm oil TRF for 24 h, 48 h and 72 h in humidified incubator at 37 °C with 5% of CO_2. Cell viability was measured using MTT assay. (n = 15).	122
Figure 3.8	PCA plot to view the separations between groups of replicates. (a) Untreated KF and nHDF (b) Palm oil TRF treated KF and untreated KF.	127
Figure 3.9	Heatmap of untreated KF and nHDF.	129
Figure 3.10	Heatmap of palm oil TRF treated KF and untreated KF.	133
Figure 3.11	ECM-receptor interaction pathway.	144
Figure 3.12	Cell cycle pathway.	145
Figure 3.13	Amplification and melting curves of *GAPDH* gene. (a) Amplification of *GAPDH* gene of untreated and treated samples (b) Melting peak of *GAPDH* amplification shown the amplicons were fully melted at 88 ºC.	149

Figure 3.14	Amplification and melting curves of *TGFβ1* gene. (a) Amplification of *TGFβ1* gene of untreated and treated samples (b) Melting peak of *TGFβ1* amplification shown the amplicons were fully melted at 91 ºC.	150
Figure 3.15	Amplification and melting curves of *TGFβ2* gene. (a) Amplification of *TGFβ2* gene of untreated and treated samples (b) Melting peak of *TGFβ2* amplification shown the amplicons were fully melted at 85 ºC.	151
Figure 3.16	Amplification and melting curves of *TGFβ3* gene. (a) Amplification of *TGFβ3* gene of untreated and treated samples (b) Melting peak of *TGFβ3* amplification shown the amplicons were fully melted at 90 ºC.	152
Figure 3.17	Amplification and melting curves of *COL3A1* gene. (a) Amplification of *COL3A1* gene of untreated and treated samples. Single last curve is the excess primers in negative control sample (non-template sample) (b) Melting peak of *COL3A1* amplification shown the amplicons were fully melted at 88.5 ºC.	153
Figure 3.18	Amplification and melting curves of *FN1* gene. (a) Amplification of *FN1* gene of untreated and treated samples. Single last curve is the excess primers in negative control sample (non-template sample) (b) Melting peak of *FN1* amplification shown the amplicons were fully melted at 86 ºC.	154
Figure 3.19	Amplification and melting curves of *TAGLN* gene. (a) Amplification of *TAGLN* gene of untreated and treated samples (b) Melting peak of *TAGLN* amplification shown the amplicons were fully melted at 91 ºC. A single peak with a lower melting temperature is the excess primer in negative control sample.	155
Figure 3.20	Amplification and melting curves of *IGFBP5* gene. (a) Amplification of *IGFBP5* gene of untreated and treated samples. Single last curve is the excess primers in negative control sample (non-template sample) (b) Melting peak of *IGFBP5* amplification shown the amplicons were fully melted at 91 ºC	156

Figure 3.21	Relative expression level of *TGFβ1, TGFβ2, TGFβ3, COL3A1*, *FN1*, *TAGLN* and *IGFBP5* in untreated KF compared to nHDF as determined by RT-PCR. The relative expression level of each targeted genes along with the relative expression level of reference gene (*GAPDH*) was calculated based on Cp values using the REST© software. *Significant (p <0.05) (n=3)	160
Figure 3.22	Relative expression level of *TGFβ1, TGFβ2, TGFβ3, COL3A1*, *FN1*, *TAGLN* and *IGFBP5* in KF after treated with palm oil TRF for 72 h as determined by RT-PCR. The relative expression level of each targeted genes along with the relative expression level of reference gene (*GAPDH*) was calculated based on Cp values using the REST© software. *Significant (p <0.05) (n=3)	161

LIST OF ABBREVIATIONS

Ca^{2+}	Calcium
cDNA	Complementary Deoxyribonucleic acid
cells/ml	Cells per mililitre
cNHDF	Commercial Normal Human Dermal Fibroblast
CO_2	Carbon dioxide
COL3A1	Collagen type III alpha 1
CP	Crossing point
cRNA	Complementary Ribonucleic acid
Ct	Cycle threshold
Cy3	Cyanine 3
DAB	3, 3-diaminobenzidine
DAVID	Database for Annotation, Visualization and Integrated Discovery
ddH_2O	Deionized distilled water
DEPC	Diethylpyrocarbonate
DKSFM	Defined Keratinocyte Serum Free Medium
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
E	PCR efficiency
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FBS	Fetal Bovine Serum
FGF	Fibroblast growth factor
FNI	Fibronectin
FSP	Fibroblasts Surface Protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GX	Gene expression
h	Hour

HCl	Hydrochloride acid
HM-CoA	3-hydroxy-3-methyl glutaryl CoA reductase enzyme
HRP	Horseradish peroxidase
HSP47	Heat-shock protein 47
HUSM	Hospital Universiti Sains Malaysia
IC_{50}	50% inhibitory concentration
IFN-γ	Interferon gamma
IGF	Insulin-like growth factor
IGFBP5	Insulin-like growth factor-binding protein 5
IHC	Immunohistochemistry
IL-1	Interleukins l
IL-10	Interleukins l0
IL-1α	Interleukins l alpha
IL-1β	Interleukins l beta
IL-4	Interleukins 4
IL-6	Interleukins 6
IL-8	Interleukins 8
IVT	In vitro transcription
KF	Keloid fibroblast
KGF	Keratinocyte growth factor
L	Litre
LB	Lithium boric acid buffer
LDL	Low density lipoprotein
m^2	Square meters
mg/ml	Miligram per mililitre
Mg^{2+}	Magnesium
$MgCl_2$	Magnesium chloride
ml	Mililitre
mm	Milimetre
mM	Milimolar
MMP-2	Matrix metalloproteinase 2

MMP-9	Matrix metalloproteinase 9
MMPs	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid Micromolar
MTT	3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
ng	Nanogram
ng/µl	Nanogram per microlitre
nHDF	Normal Human Dermal Fibroblast
nm	Nanometer
OD	Optical density
P_0	Passage zero
P_1	Passage one
P_2	Passage two
PAI-1	Plasminogen activator inhibitor 1
PBS	Phosphate Buffered Saline
PCA	Principle Component Analysis
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived growth factor
pNHDF	Primary Normal Human Dermal Fibroblast
pNHEK	Primary Normal Human Epidermal Keratinocyte
qRT-PCR	Quantitative real-time polymerase chain reaction
R	Ratio
REST©	Relative Expression Software Tool
RLT	RNeasy Lysis Buffer
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Revolution per minute
RT-PCR	Real-time Polymerase Chain Reaction
RW1	RNeasy Wash Buffer
SD	Standard deviation
SE	Standard error

sec	Second
SPSS	Statistical Package for the Social Sciences
T25	$25cm^2$ cell culture flask
T75	$75cm^2$ cell culture flask
TAGLN	Transgelin
TBS	Tris Buffered Saline
TGF-β	Transforming growth factor-beta
TGF-β1	Transforming growth factor-beta 1
TGF-β2	Transforming growth factor-beta 2
TGF-β3	Transforming growth factor-beta 3
TIMP-1	Tissue inhibitor of metalloproteinase 1
TNF-α	Tumor necrosis alpha
t-PA	Tissue-plasminogen activator
TRF	Tocotrienol Rich Fraction
µg	Microgram
µg/ml	Microgram per mililitre
µl	Microlitre
µM	Micromolar
µm	Micrometer
u-PA	Urokinase-plasminogen activator
UV	Ultraviolet
V	Voltage
VEGF	Vascular endothelial growth factor
β-ME	β-mercaptoethanol
ΔCP	Crossing point different

LIST OF SYMBOLS

I	One
III	Three
IV	Four
V	Five
VII	Seven
α	Alpha
β	Beta
γ	Gamma
δ	Delta
%	Percent
<	Less than
ºC	Degree celcius
ºC/sec	Degree celcius per second

PROFIL PENGEKSPRESAN GEN DARI FIBROBLAS UTAMA KELOID DAN KULIT NORMAL YANG DIRAWAT MENGGUNAKAN PECAHAN TOKOTRIENOL (TRF) MINYAK KELAPA SAWIT

ABSTRAK

Keloid adalah penyakit kulit fibroproliferatif yang terbentuk disebabkan oleh tindak balas luka yang tidak normal pada individu tertentu. Perubahan terhadap faktor pertumbuhan dan sitokin dalam proses penyembuhan yang tidak normal membentuk keadaan yang tidak seimbang antara sintesis dan degradasi kolagen dan komponen ekstraselular matriks yang lain. Pelbagai rawatan telah dicuba untuk keloid, tetapi secara klinikal, kebanyakan terapi masih tidak memuaskan. Kepentingan saintifik tokotrienol telah meningkat kerana sifatnya sebagai antioksida dan sifat berkaitan pelindung. Walau bagaimanapun, terdapat kekurangan bukti saintifik mengenai keberkesanan tokotrienol dalam pengurusan keloid dan parut. Oleh itu, kajian ini telah dijalankan untuk menyiasat kesan-kesan berfaedah pecahan tokotrienol (TRF) minyak kelapa sawit pada fibroblas keloid. Keloid dan kulit normal dikumpulkan sebagai sampel dalam kajian ini. Fibroblas manusia daripada dermis normal dan keloid yang utama telah berjaya dikultur. Penanda antibodi protein kejutan haba 47 (HSP47) dan protein permukaan fibroblas (FSP) telah digunakan untuk pengesahan fibroblas manusia daripada dermis normal yang utama (pNHDF). pNHDF bewarna positif selepas pengeraman dengan antibodi HSP47 dan FSP. Untuk membezakan ciri-ciri pertumbuhan antara fibroblas manusia daripada dermis normal (nHDF) dan fibroblas daripada keloid (KF), sel hidup nHDF dan KF dinilai menggunakan pewarna biru tripan. Kedua-duanya menunjukkan kadar

pertumbuhan linear tetapi KF yang menunjukkan kadar pertumbuhan yang lebih cepat berbanding nHDF. Peratusan sel hidup nHDF dan KF telah diuji dengan *3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide* (MTT) untuk menilai kesan toksik dan menindas TRF. Peratusan sel hidup nHDF meningkat secara signifikan terhadap masa apabila dirawat dengan kepekatan TRF antara 0.16 µg/ml hingga 19.5 µg/ml. Keputusan yang bertentangan dilihat apabila nHDF dirawat dengan kepekatan 39 µg/ml dan 78 µg/ml. Peratusan sel hidup KF menurun terhadap masa apabila dirawat dengan semua kepekatan kecuali kepekatan 19.5 µg/ml. Pada kepekatan 19.5 µg/ml, peratusan sel hidup KF meningkat terhadap masa pengeraman. Kepekatan sebanyak 39 µg/ml secara signifikan menindas pertumbuhan sel-sel KF. Walau bagaimanapun, kepekatan TRF tertinggi (78 µg/ml) didapati menjadi toksik kepada kedua-dua nHDF dan KF. Teknik mikroatur menggunakan beadchip Illumina *HumanRef-8 Expression* telah digunakan untuk mengenalpasti pengekspresan gen dalam KF terhadap tindak balas kepada TRF. Terdapat 906 gen ditemui secara signifikan (p <0.05) berbeza dalam pengekspresan dengan perubahan sekurang-kurangnya dua kali ganda dalam KF yang dirawat berbanding KF yang tidak dirawat. Pengekspresan 524 gen meningkat dan pengekspresan 382 gen menurun dalam KF yang dirawat. Pengekspresan *transforming growth factor beta I* (*TGFβ1), transforming growth factor beta II (TGFβ2), transforming growth factor beta III (TGFβ3), collagen type III alpha I (COL3A1), fibronectin (FN1), transgelin (TAGLN)* dan *insulin-like growth factor-binding protein 5 (IGFBP5)* telah disahkan menggunakan *real-time PCR*. Menariknya, TRF didapati menurunkan pengekspresan gen tertentu yang biasanya diekspreskan tinggi dalam KF seperti gen yang terlibat dalam laluan interaksi ECM-reseptor dan kitaran sel, komponen ekstraselular matriks, sitokin yang mendorong fibrosis, pertumbuhan sel dan

penyembuhan luka. TRF didapati telah mengubah pengekspresan gen *TGFβ2, COL3A1, FN1*, *TAGLN* dan *IGFBP5* yang tidak normal dalam KF. Hasil kajian mendapati bahawa TRF mempunyai kesan bersifat menindas ke atas KF. TRF mempunyai kesan kesihatan yang signifikan melalui kesan anti-fibrogenik yang sangat baik ke atas KF berdasarkan keupayaan untuk mendorong degradasi ekstraselular matriks dan menghalang pembentukan kolagen dan komponen ekstraselular matriks lain yang berlebihan. Keputusan ini telah menghasilkan penemuan yang berharga untuk tujuan kosmetik kerana berdasarkan profil keselamatan, TRF menjanjikan strategi terapi untuk merawat parut keloid atau untuk mencegah pembentukannya.

GENE EXPRESSION PROFILE OF PRIMARY FIBROBLASTS FROM KELOID AND NORMAL SKIN TREATED WITH PALM OIL TOCOTRIENOL RICH FRACTION (TRF)

ABSTRACT

Keloids are dermal fibroproliferative disease that developed due to abnormal wound response in predisposed individuals. The alteration of growth factors and cytokines in abnormal healing process formed an imbalanced condition between collagen and other extracellular matrix components synthesis and degradation. Various treatments for keloids have been tried but most of the therapeutic approaches remain clinically unsatisfactory. Tocotrienols have gained increasing scientific interest due to their antioxidant and related protective properties. However, there is lack of scientific evidence regarding the efficacy and therapies of tocotrienol in keloid and scar management. Thus, this study was carried out to investigate the effects of palm oil tocotrienol rich fraction (TRF) on human keloid fibroblasts. Keloids and normal skin were collected as samples in this study. Primary human dermal fibroblast from normal skin and keloid tissues were successfully cultured. Heat-shock protein 47 (HSP47) and fibroblast surface protein (FSP) antibody markers were used for primary normal human dermal fibroblast (pNHDF) verification. pNHDF was positively stained after incubation with HSP47 and FSP antibodies. To differentiate growth characteristic between nHDF and keloid fibroblast (KF), the cell viability for both cells were assessed using trypan blue dye. Both nHDF and KF exhibited linear growth pattern but KF grew faster compared to nHDF. The percentage of viable cells of nHDF and KF were assessed by 3-

(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay in order to evaluate cytotoxic and suppressive effects of TRF. The percentage of viable cells of nHDF significantly (p <0.05) increased in a time dependent manner when nHDF treated with concentrations of TRF at 0.16 µg/ml to 19.5 µg/ml. An opposite result was seen when nHDF were treated with TRF at concentrations of 39 µg/ml and 78 µg/ml. The percentage of viable cells of KF decreased in a time dependent manner when KF treated with all concentrations except at 19.5 µg/ml. At concentration of 19.5 µg/ml, the percentage of viable cells of KF increased over the incubation time. TRF at concentration of 39 µg/ml significantly (p <0.01) suppressed KF cells growth. However, TRF at highest concentration (78 µg/ml) was found to be cytotoxic to both nHDF and KF. Microarray gene expression using Illumina HumanRef-8 Expression BeadChips was used to identify the changes in gene expressions in KF in response to TRF. There were 906 genes found to be significantly (p <0.05) differentially expressed with at least a 2 fold change in treated KF compared to untreated KF. 524 genes were up-regulated and 382 genes were down-regulated in treated KF. The expression of *transforming growth factor-beta I* (*TGFβ1), transforming growth factor-beta II (TGFβ2), transforming growth factor-beta III (TGFβ3), collagen type III alpha I (COL3A1), fibronectin (FN1), transgelin (TAGLN)* and *insulin-like growth factor-binding protein 5* (*IGFBP5)* was validated using real-time PCR. Interestingly, TRF down regulates the expression of certain genes that are commonly highly expressed in KF such as genes that are involved in ECM-receptor interaction and cell cycle pathways, ECM components, fibrosis-inducing cytokines, cell growth and wound healing. TRF appeared to reverse the abnormal expression of *TGFβ2, COL3A1, FN1, TAGLN* and *IGFBP5* genes in KF. The findings of this study concluded that TRF has a suppressive effect on KF. These

indicated that TRF have a significant health promoting effects through its excellent anti-fibrogenic effect on KF based on its ability to induce ECM degradation and inhibit the overproduction of collagen and other ECM components. These results provide valuable finding for cosmetic reasons because considering its safety profile, TRF represents a promising therapeutic strategy to treat keloid scarring or to prevent its development.

CHAPTER 1

INTRODUCTION

1.1 Human Skin

1.1.1 Structure of the human skin

The integumentary system is one of the major human body systems that consist of the skin, hair, nails, subcutaneous tissue and assorted glands. It has multiple roles including in homeostasis, protection, temperature regulation, sensory reception, biochemical synthesis and absorption. All the body systems work in an interconnected way with each other to maintain the internal conditions of the body thus making it to function properly (Farabee, 2010).

Skin is an organ of the integumentary system made up of a layer of tissues that cover the underlying muscles and organs. It is the largest organ of the body, making up about 8% of the body weight with surface area about 1.6 m^2 (Igarashi *et al.*, 2005). Although structurally consistent throughout the body, the skin thickness is varies depending on the person's age, gender and area of the body. The variation of skin thickness due to variation in collagen and elastin levels. Women have less thick skin than men because

skin collagen decreased with age and was less in females at all ages. (Shuster *et al.*, 1975). This feature make it a unique organ compared to the others.

The complex structures of the skin are shown in Figure 1.1. The multi-layered structure of the skin is made up by the different cells, fibers and other components and each of them has different functions. The outermost layer of the skin is epidermis which serves as the physical and chemical barrier between the interior body and exterior environment. The middle layer is known as dermis which provides the structural support of the skin and the innermost layer is the subcutaneous tissue. Veins, capillaries and nerves form vast networks inside this structure and hairs stick out from the inside of skin. Numerous fine hair are scattered over the surface of skin (Igarashi *et al.*, 2005).

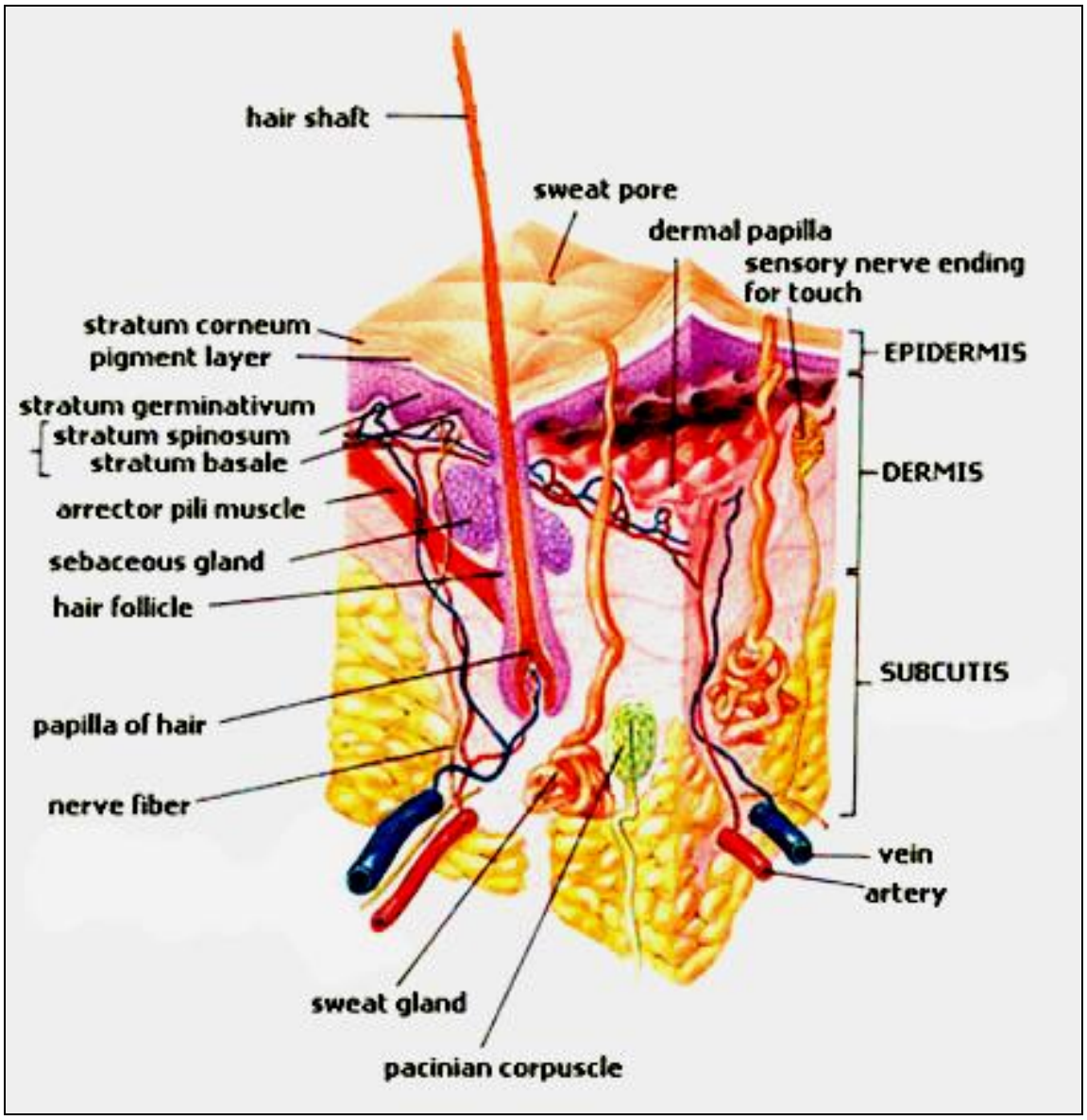

Figure 1.1: Schematic diagram of the skin. Complex structure of the skin that consists of many components to perform a wide variety of functions Adapted from (http://www.daviddarling.info/encyclopedia/S/skin.html).

The epidermis is a complex multiple layered membrane and the thickness varies over the whole body surface in different individuals. It is divided into five sublayers which from the bottom are stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum (Poirier, 2003). The epidermis contains no veins and capillary. Keratinocytes are the main type of cells which make up the epidermis. This cell produces keratin which contributes to the rigidity of the outermost layer of skin. It also protects the body from the external environment and maintains skin moisture. The other epidermal cells are melanocytes which produce the melanin pigment for the purpose of ultraviolet protection (Igarashi *et al.*, 2005).

The dermis is the layer beneath the epidermis. This layer is much thicker than the epidermis layer, ranging from 1 to 4 mm (Anderson and Parrish, 1982). Dermis consists of two layers which are papillary and reticular layer. It contains blood vessels, nerves, hair follicles, smooth muscle, glands and lymphatic tissue (Poirier, 2003). The dermis is made up of fibroblasts, which produce collagen, elastin and proteoglycans, together with mast cells and macrophages (Abdul Majid, 2007). Collagen fibers give the dermis strength and toughness while elastin maintains elasticity and flexibility of the dermis (Igarashi *et al.*, 2005). The other component such as proteoglycans provides viscosity and hydration.

Subcutis, also known as hypodermis is the third layer beneath the dermis. It is an elastic layer and contains a large amount of adipose tissue that functions as a shock absorber for blood vessels and nerve endings. The thickness of this layer ranged from 4 to 9 mm and

it differs between individuals and also depends on the body region (Igarashi *et al.*, 2005).

1.1.2 Functions of the human skin

The skin not only gives us our appearance and shape but it is an organ in direct contact with the environment. It is designed to adapt to stress and strain and protect the fragile systems within the body (Slemp and Kirschner, 2006). The primary function of the skin is as a protective barrier in preventing the internal tissues from physical trauma, ultraviolet (UV) radiation, temperature changes, chemical and pathogens (Butcher and White, 2005). Besides preventing harmful substances from entering the body, it also controls the loss of vital substances (Graham-Brown and Burns, 1998).

The other function of skin is as organ of sensation. It contains an extensive network of nerve cells that allow the body to detect any changes in the environment. There are separate receptors for heat, cold, touch and pain. These receptors allow the body to detect pain and changes in temperature, touch and pressure. Damage to these nerve cells results in a loss of sensation in the affected areas (Timmons, 2006).

Due to the rich capillary network and sweat glands, skin plays an important role in regulating heat loss from the body surface. It allows the body to respond to any changes in temperature by constricting or dilating the blood vessels (Abdul Majid, 2007).

Skin also functions as an organ of excretion. The waste products excreted by sweat contain water, urea and albumin. An oily substance known as sebum oil is excreted by the sebaceous glands, helping to lubricate and protect the skin (Timmons, 2006). Besides acting as a physical barrier, skin also plays an important immunological role. It contains all the elements of cellular immunity (Abdul Majid, 2007). It also synthesizes vitamin D which is required for calcium absorption. The skin needs to be maintained intact to allow the body to perform these vital functions. When it is injured, it rapidly repairs itself in order to maintain these external defense systems (Timmons, 2006).

1.2 Wound healing

1.2.1 Mechanism and genes involved in wound healing

The term ‘wound’ has been defined as a break in the epithelial integrity of the skin and accompanied by disruption of normal anatomical structure and function. Healing is defined as the complex and dynamic process that results in the restoration of anatomical continuity and function (Lazarus *et al.,* 1994). Wound healing is the body's natural process that involves the interaction of a complex cascade of cellular events that generates resurfacing, reconstitution and restoration of the tensile strength of wounded skin (Romo *et al.*, 2010).

Wound healing process involves changes in expression of specific genes at a specific phase of wound healing. However, an aberrant regulation of specific genes plays a key role in abnormal healing process (Shilo *et al.*, 2007). This process also involves a variety

of cells, cytokine mediators and extracellular matrix (ECM) components which need to interact continuosly towards tissue repair (MacKay and Miller, 2003). The normal healing response begins at the moment the tissue is injured. This process occurs in recognisable, usually progressively through the overlapping phases which are hemostasis, inflammatory, proliferation and remodeling phases (Figure 1.2) (Enoch *et al.*, 2006).

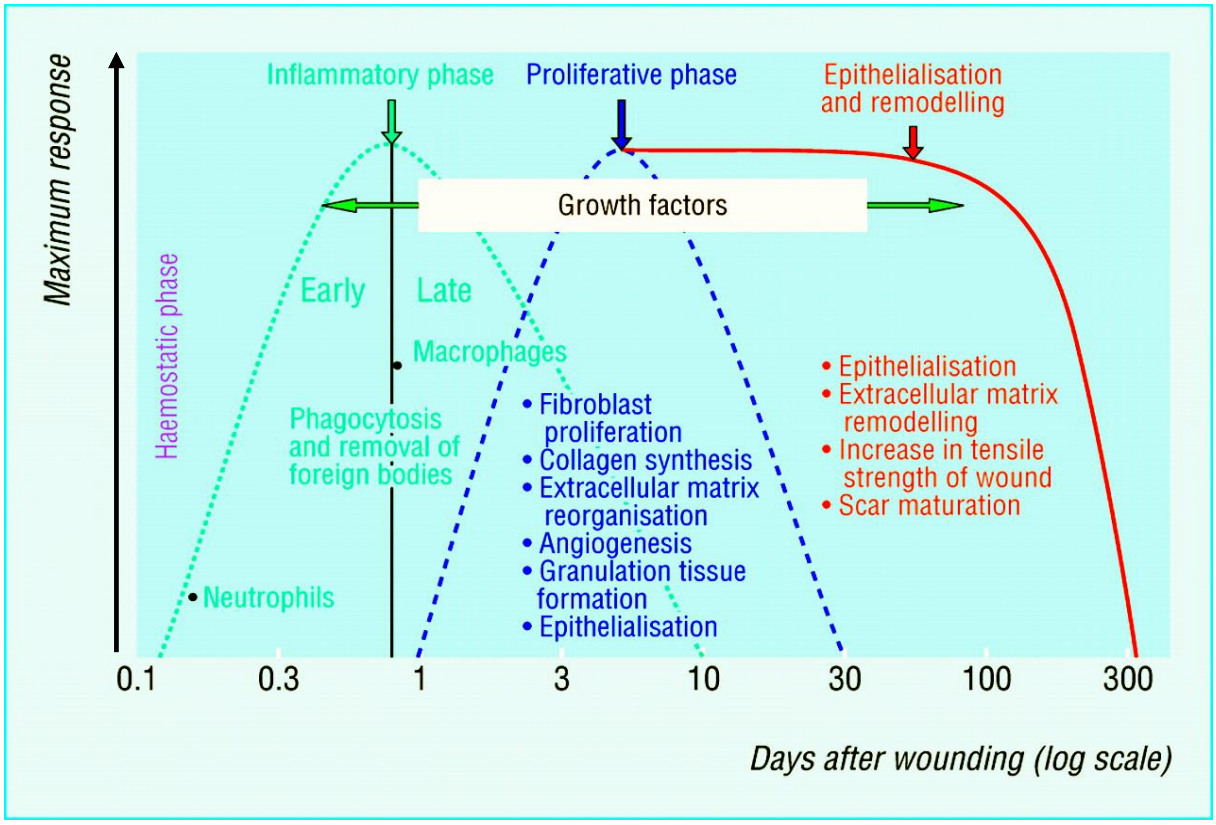

Figure 1.2: Schematic diagram for the four phases of wound healing process. Adapted from (Enoch *et al.*, 2006).

As the blood spills out, the platelets are attracted to the injury site and come into contact with exposed collagen and other elements of the ECM. Platelets are triggered to release the clotting factors as well as essential growth factors and cytokines (Diegelmann and Evans, 2004). This results in initial vasoconstriction that reduces blood flow through the damaged vessels. Generally, hemostasis occurs within minutes of the initial injury unless there are underlying clotting disorders (Timmons, 2006).

The expression of pro-inflammatory genes was increased at early phase of wounding. These genes involved in tumor necrosis factor (TNF) activation (TNF receptor associated factor 1 and 2) and interferon (IFN) activation. The expression of several macrophage markers (CD163, Fc-gamma receptor 1 (FcγRI), macrophage scavenger receptor 1 and major histocompatibility complex (MHC) class II α chain), T cells markers (RAB7L1, and RAB18), B cells (immunoglobulin heavy chain), neutrophils (cytochrome b-245), β2 integrin and CD18 were also increased (Kavita *et al.*, 2007).

In early inflammatory phase, the neutrophils enter the wound site and remove the foreign materials, bacteria and damaged tissue through phagocytosis. In the late inflammatory phase, monocytes are converted to macrophages and phagocytose the debris and pathogens (MacKay and Miller, 2003). Macrophages secrete the collagenase and elastase which break down injured tissue and release cytokines (Timmons, 2006). In addition, it also secretes a variety of chemotactic and growth factors that stimulate cell migration, proliferation and formation of the tissue matrix (MacKay and Miller, 2003). At the inflammatory phase of wound healing, platelet derived growth factor (PDGF) upregulates the expression of Syndecan-4 (SDC4) in fibroblasts. SDC4 interacts with

integrins in response to fibronectin and modulates cell adhesion, migration and proliferation (Szabad, 2010).

Fibroplasia, angiogenesis and epithelialization are three major processes in proliferative phase. Its duration dependent on the size of the wound. The formation of granulation tissue is a central event during this phase (Romo *et al.*, 2010). Progranulin promotes tissue granulation (He *et al.*, 2003). It activates extracellular kinases. During wound healing, progranulin are up-regulated in the dermis for at least 10 days following the wound. The expression of progranulin causes neutrophil infiltration and increases the accumulation of fibroblasts and blood vessels in the wound by promoting dermal fibroblast and endothelial cell proliferation and their migration to type I collagen (He and Bateman, 2003). Platelets and macrophages release the chemotactic and growth factors which stimulate the migration and activation of fibroblasts. Fibroblasts play an important role in this phase whereas it produces a variety of substances essential to wound repair, including collagen, fibronectin, glycosaminoglycans and proteoglycans (MacKay and Miller, 2003). The expression of methallothioneins was increased in metabolically active and proliferating cells of the dermis. The levels of methallothioneins were increased in the wound margin as mitotic activity was increased. It is thought to promote cell proliferation and re-epithelialization through its zinc and copper binding properties (Lansdown, 2002). During wound healing, fibroblasts express connective tissue growth factor (CCN2) which promotes matrix deposition, fibrobast proliferation and fibroblast adhesion (Kavita *et al.*, 2007).

New blood vessels are formed to deliver oxygen and nutrients to the healing tissues (Timmons, 2006). Signal transducer and activator of transcription 3 (Stat3) plays an important role in normal wound healing and response to injury. Activation of Stat3 resulting in upregulation of genes involved in cell invasion, chemotaxis, angiogenesis, blood coagulation and remodeling of extracellular matrix. These genes are chemoattractants family such as chemokine (C-C motif) ligand 2 (CCL2) and chemokine (C-X-C motif) ligand 2 (CXCL2), proteases, SERPIN families such as urokinase plasminogen activator (uPA) and its receptor uPAR, coagulation proteins such as fibrinogen, plasminogen activator inhibitor-1 (PAI-1) and thrombomodulin, genes involved in angiogenesis including endothelial PAS domain-containing protein 1 (EPAS1), adrenomedullin, angiopoietin-like protein 4 and vascular endothelial growth factor (VEGF) (Daniel *et al.*, 2005).

Cysteine protease and cathepsin S present during tissue repair to induce angiogenesis (Wang *et al.*, 2006). MMP-9 is a metalloproteinase which promotes angiogenesis indirectly by making VEGF more available to its receptor (Bergers *et al.*, 2000). Dermal wound repair models demonstrated that Neuropilin-1 (NRP1) is a co-receptor of VEGFR and is involved in the VEGF-mediated angiogenesis associated with dermal wound repair (Szabad, 2010). Integrin αv is important in angiogenesis, fibroblast migration and wound closure. Integrin αvβ5 is a receptor for vitronectin and integrin αvβ3 is a receptor for both fibronectin and vitronectin (Serini *et al.*, 2006). Fibroblast migration to an angiogenic factor CYN61 is mediated by integrin αvβ5. During normal wound healing, fibroblasts differentiate into myofibroblasts in granulation tissue where they play a role in wound contraction and scar formation (Lygoe *et al.*, 2004).

Remodeling is the final phase of wound healing process. The new collagen matrix then becomes cross-linked and organized during this phase to increase the tensile strength of wound. This process continues up to two years to be complete (MacKay and Miller, 2003).

The remodeling genes included type IV collagen and collagen modifying enzymes (procollagen C-endopeptidase enhancer and procollagen-lysine, 2-oxoglutarate 5-dioxygenase) and the integrin β5 (Kavita *et al.*, 2007). Extracellular S100A4 is involved with angiogenesis and the remodeling of extracellular matrix (Senolt *et al.*, 2006).

The successful completion of each step in the healing process is dependent on the interactions between stimulation and regulation of cytokine, growth factors, fibroblasts and other cells that populate the wound. However, for chronic wounds, the healing process can be prolonged by factors which may relate to the type of wound and other factors (Timmons, 2006).

1.2.2 The role of cytokines and growth factors in wound healing

The multiple steps of healing process involve a large number of regulatory molecules including cytokines and growth factors. These molecules exert a series of direct and indirect biological activities which might be important for wound healing. However, dysregulation in cytokine or growth factor expression alters the normal healing process and blocks the production of specific proinflammatory cytokines (Efron and Moldawer, 2004).

Cytokines are group of regulatory proteins that are released from various cell sources which binds to cell surface receptors to stimulate a cell response. They play an important role in wound healing process (Romo *et al.,* 2010). The different cytokines are expressed in wound site at different time periods during the healing process (Matsuoka and Grotendorst, 1989, Witte and Barbul, 1997). Tumor necrosis alpha (TNF-α) is released by macrophages to induce neutrophil recruitment and maturation (Rumalla and Borah, 2001, Feiken *et al.*, 1995, Kunkel *et al.*, 1989, Michie *et al.*, 1988). It increased vascular permeability and hemostasis and also induce collagen synthesis and degradation (Rumalla and Borah, 2001, Feiken *et al.*, 1995, Dinarello and Moldawer, 2001). The other cytokine essential in the healing process is interferon- gamma (IFN-γ) which is secreted by T lymphocytes. It induces polymorphonuclear leukocytes and macrophage activation and cytotoxicity. It also induces tissue remodeling and reduces wound contraction by increasing collagenase expression as well as by decreasing collagen production and crosslinking (Rumalla and Borah, 2001, Dinarello and Moldawer, 2001).

Interleukins l alpha and beta (IL-1α and IL-1β) have many similar functions with TNF-α. It activates neutrophils and promotes chemotaxis by inducing the endothelial cells to express proinflammatory cytokines (Loppnow *et al.*, 1998, Fong *et al.*, 1989). IL-1 is important in the healing process by increasing collagen synthesis as well as keratinocyte and fibroblast growth (Sauder *et al.*, 1990).

Interleukin 8 (IL-8) has numerous functions including neutrophil activation, endothelial cell adhesion protein expression and keratinocyte maturation and margination (Liechty *et al.*, 1998a, Engelhardt *et al.*, 1998, Nanney *et al.*, 1995, Clark, 1993a). The other type

of cytokine frequently found in healing process is interleukin six (IL-6) which is secreted by fibroblasts, monocytes and polymorphonuclear cells to stimulate fibroblast proliferation (Mateo *et al.*, 1994, Saba *et al.*, 1996, Goodman and Stein, 1994, Pajulo *et al.*, 1999). In addition, interleukin four (IL-4) plays an important role in normal healing process by promoting fibroblast proliferation, proteoglycan synthesis and collagen production (Trautmann *et al.*, 2000, Postlethwaite *et al.*, 1992, Wegrowski *et al.*, 1995). Interleukin ten (IL-10) is an anti-inflammatory cytokine secreted by T lymphocytes which inhibits the production of proinflammatory cytokines such as TNF-α, IL-1 and IL-6 as well as preventing neutrophil and macrophage infiltration into the wound (Rumalla and Borah, 2001, de Waal Malefyt *et al.*, 1991, Sato *et al.*, 1999).

Healing process is also controlled by a variety of different growth factors. Growth factors are peptide mediators that act on target cells to influence cell proliferation and cellular activity, including migration and protein synthesis (Rumalla and Borah, 2001, Steed, 1997). It plays a major role in tissue repair. PDGF has been shown to be chemotactic for cells migrating into the wounded skin such as neutrophils, monocytes and fibroblasts. It enhances proliferation of fibroblasts and induces collagen and proteoglycan synthesis by these cells (Clark, 1993b, Heldin and Westermark, 1999). It also has been shown to enhance angiogenesis (Nakamura *et al.*, 1998).

Fibroblast growth factor (FGF) has over 20 isoforms which are secreted by macrophages and endothelial cells in the wound involved in angiogenesis and epithelialization. It has the ability to stimulate the fibroblast and keratinocyte proliferation and migration (Ono *et al.*, 1999, Detillieux *et al.*, 2003, Gerwins *et al.*, 2000, Mustoe *et al.*, 1991, Hebda *et*

al., 1990, Finetti and Farina, 1992, Pierce *et al.*, 1992). Keratinocyte growth factor (KGF) is part of FGF family (Xia *et al.*, 1999, Werner *et al.*, 1994b, Werner *et al.*, 1994a) and importantly involves in epidermal homeostasis by acting as an essential regulator for keratinocyte proliferation and maturation (Rumalla and Borah, 2001).

Epidermal growth factor (EGF) plays a role in epithelialization after being secreted by keratinocytes (Cohen *et al.*, 1995). It also increases collagenase secretion by fibroblast (Rumalla and Borah, 2001). VEGF is an angiogenic factor which is secreted by keratinocytes, macrophages and fibroblasts to induce angiogenesis in healing process (Taub *et al.*, 2000, Frank *et al.*, 1999, Shweiki *et al.*, 1992, Nissen *et al.*, 1998).

Literature reveals transforming growth factor-beta (TGF-β) to have important implications in all phases of healing process. In wound healing, it is mainly produced by the platelet, monocyte and fibroblast. Once the TGF-β binds to it receptor, the intracellular signaling pathway is activated and carried out by the Smads proteins (Faler *et al.*, 2006).

TGF-β plays a vital role in inflammatory phase of healing process by attracting inflammatory cells to the wound site and suppressing the production of proteases. In proliferative phase, this growth factor stimulates the fibroblast proliferation and migration. It also promotes angiogenesis and impacts epithelialization. TGF-β transforms fibroblasts into myofibroblasts for wound contraction in maturational phase (Faler *et al.*, 2006).

The beneficial effect of cytokines and growth factors in healing process are well determined. However, abnormal cytokines and growth factors expression can impair the normal healing process and associated with excessive scarring.

1.3 Abnormal scarring

1.3.1 Keloids

Healing process ends with the formation of a scar that is mainly composed of collagen which restores the functional integrity of the skin. Abnormal scars are formed as a result of alteration of wound healing process and may develop due to deep dermal injuries. The imbalance between synthesis and degradation of extracellular matrix components such as collagen, fibronectin and proteoglycan by fibroblasts lead to keloids and hypertrophic scars formation. This scarring not only causes pain, pruritus and contractures but it also affects the patient's quality of life both physically and psychologically (Gauglitz *et al.*, 2011a).

Keloids are skin abnormalities that are unique to humans. It is defined as benign dermal fibroproliferative tumours that occur due to abnormal wound response in predisposed individuals (Seifert and Mrowietz, 2009). The etiology of keloids remain unclear but there are several factors which may contribute to keloids formation such as surgery, tattoos, bites, vaccination, blunt trauma, burns and lobular piercing (Kakar *et al.*, 2006). It also may occur spontaneously (Meenakshi *et al.*, 2005a).

This scarring occurs in all races but it is more common in darker pigmented ethnicities compared to white people (Child *et al.*, 1999, Shaffer *et al.*, 2002). Higher incidences of this scarring has been reported in Asian, Black and Hispanic populations with value of 4.5 to 16% (Oluwasanmi, 1974). In contrast, only less than 1% of keloids occur in Caucasians (Bloom, 1956). Keloids can develop at any age but most of the cases reported are between 10 and 30 years old (Shaffer *et al.*, 2002).

Keloids more frequently occur on parts of the body with high concentrations of melanocytes such as chest, shoulders, neck, upper back, upper arms and earlobes (Figure 1.3) as firm nodules which are pruritic and painful (Bayat *et al.*, 2004). This scarring is rarely found on the soles of the feet and palms of the hand because the concentration of melanocytes is minimal (Kelly, 1988). It is variable in size from a few millimeters in diameter to larger areas (Eryilmaz and Uygur, 2010).

Differentiation between keloids and hypertrophic scar can be problematic. However, there are some clinical, histological and epidemiological differences can be used to distinguish both of these scarring. The clinical and histological differentiation of keloids and hypertrophic scars are shown in Table 1.1.

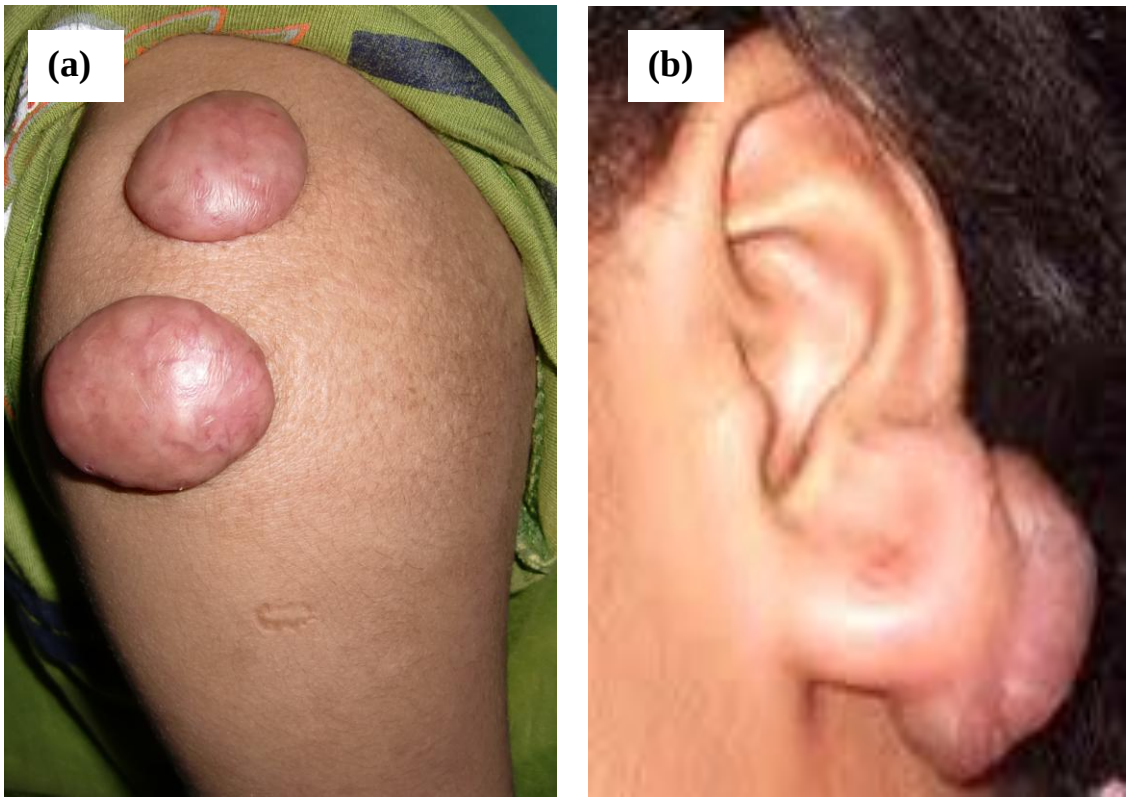

Figure 1.3: The appearance of keloid from patients before surgery. Keloid on (a) Shoulder, (b) Earlobe.

Table 1.1: Differences between keloids and hypertrophic scars. Modified from (Ehrlich *et al.*, 1994)

Keloids	Hypertrophic scars
• Grow continuously and invasively beyond the original wound.	• Remain within the boundaries of the original wound.
• Do not regress spontaneously.	• Regress spontaneously.
• Appear within several months after initial scar and gradually proliferate indefinitely.	• Arise within 4 to 8 weeks and grow intensely for several months, then regress within one year.
• Commonly occur on the chest, shoulders, upper back, earlobes and rarely on the sole of the feet and palms of the hand.	• No predominant anatomical site.
• Pruritic and painful.	• Less pruritic and painful.
• Disorganized, larger and thicker collagen fibers with no nodules and myofibroblasts.	• Fine and well organized collagen fibers with nodules and myofibroblasts.

1.3.2 Gene expression and pathogenesis of keloids

The pathogenesis of keloids still remains unclear. Recently, most of the research has focused on the interaction between keloid fibroblasts and ECM components and abnormalities of keloid fibroblasts. In addition, several studies have been done to investigate the expression of cytokines, growth factors and their signaling pathways in keloids (Seifert and Mrowietz, 2009). Microarray studies provide a broad spectrum of differentially regulated genes that may play roles in the pathogenesis of disease. Literature reported that there was a difference in expression of a broad spectrum of wound-healing and fibrosis related genes between normal and keloid fibroblasts (Russell *et al.*, 1995, Smith *et al.*, 2008).

The formations of this abnormal scarring are due to genetic, cellular and molecular alteration in the orchestrated healing process (Seifert and Mrowietz, 2009). Imbalance between ECM synthesis and degradation lead to keloids formation. Keloids are characterized by the increasing of its cellularity. This characteristic increased the metabolic activity which is reflected by the increased activity of glycolytic enzymes and glycoproteins synthesis (Kakar *et al.*, 2006).

Tenascin C is a glycoprotein produced by fibroblasts or myocytes (Mackie *et al.*, 1998, Schalkwijk *et al.*, 1991). It expression increases in wound repair, inflammation and hyperproliferative skin diseases (Mackie *et al.*, 1998, Lightner, 1994, Dalkowski *et al.*, 1999). It is strongly expressed in granulation tissue and at wound edges in the dermis. However, it return to normal after wound contraction is completed (Mackie *et al.*, 1998).

The expression of tenascin C in keloid is increased and TGF-β1 and interleukin 1 β are reported to be potent inducers of tenascin C (Shrestha *et al.*, 1996, Pearson *et al.*, 1998, Chevalier *et al.*, 1996).

Type-I procollagen, fibronectin and proteoglycan are synthesized more in fibroblasts from keloids compared to normal dermal fibroblasts (Luo *et al.*, 2001, Rodland *et al.*, 1990). Plasmin and plasminogen activator is an important regulator involved in keloid formation. u-PA and tissue-plasminogen activator (t-PA) activate plasminogen to plasmin which acts as fibrinolytic and activates procollagenase (Declerk, 1993). Plasmin is involved in activating TGF-β from its latency form (Lyons *et al.*, 1990). Plasminogen PAI-1 and TIMP-1 are induced by TGF-β. This lead to decrease of plasmin and collagenase activity and thus resulting in diminished collagen degradation. The higher expression levels of PAI-1 in keloid fibroblasts leads to increase the accumulation of collagen and fibrin in this tissue (Tuan *et al.*, 2003, Tuan *et al.*, 1996).

According to Wu *et al*. (2004), the expression of VEGF is higher in keloid tissues and keloid fibroblasts compared to normal skin. VEGF stimulates the expression of PAI-1, in keloid fibroblasts (Wu *et al.*, 2004). VEGF may play an important role in keloid formation by altering ECM homeostasis and impaired degradation, thus causing excessive accumulation of ECM (Seifert and Mrowietz, 2009). Abundant deposition of collagen synthesis in keloid tissue is due to increased activity of prolyl hydroxylase enzyme (Murray, 1993, Tuan *et al.*, 2003).

Matrix metalloproteinases (MMPs) are involved in ECM degradation such as in breakdown of type I and III collagen and other types of collagen (Ghahary and Ghaffari, 2007). However, an imbalance in expression of MMPs has been implicated in dermal fibrosis (Ghahary *et al.,* 1996). The expression of MMP-2 is high while MMP-9 is low in hypertrophic scars and keloids (Neely *et al.,* 1999). MMP-2 has major effect on matrix remodeling in wound healing by degrading denatured collagen, while MMP-9 is involved in early wound repair by degrading native types IV and V collagen, elastin and fibronectin (Mauviel, 1993, Zhang *et al.,* 1998).

Although TGF-β plays an important role in normal healing process, it also has been implicated in a wide variety of fibrotic disorders. TGF-β1 and TGF-β2 are stimulator of collagen and proteoglycan synthesis (Szulgit *et al.,* 2002, Kose and Waseem, 2008). In contrast, TGF-β3 induced in the later stages of healing process and has been found to reduce connective tissue deposition (Bock *et al.,* 2005). A study from Lee *et al*. (1999) found the expression of TGF-β1 and TGF-β2 are increased in keloid fibroblasts compared to normal human skin fibroblasts while the expression of TGF-β3 did not differ (Lee *et al.,* 1999b).

IL-6 is reported to have an effect on inflammation and fibroblast proliferation in wound healing. However, the high levels of IL-6 have been found to play an important role in the pathogenesis of fibroproliferative diseases. A study from Hui *et al.* (2000) found the expression of the IL-6 gene is increased in fibroblasts isolated from keloids when compared with control fibroblasts (Hui *et al.,* 2000).

Gene profiling studies showed there was a significantly increased in expression of several IGF-binding protein such as IGFBP5, IGFBP7 and IGFBP2, jagged 1 (JAG1) and CTGF and decreased in expression of several inhibitors of Wnt such as DKK1, DKK3 and secreted frizzled-related protein 1 (SFRP1), MMP3 and dermatopontin (DPT) in keloid fibroblasts. The expression of the Wnt stimulatory molecules FZD4 and DAAM1 was increased in keloid fibroblasts (Smith *et al.*, 2008). Reduced expression of several inhibitors of Wnt signaling and increased of the Wnt stimulatory molecules in keloid fibroblasts indicates a role of this pathway in the pathogenesis of keloids (Sato, 2006).

Whole genome gene expression of keloid fibroblasts or tissues using microarray analysis have been conducted by several studies (Satish *et al.*, 2006, Seifert *et al.*, 2008, Smith *et al.*, 2008, Naitoh *et al.*, 2005, Hu *et al.*, 2006, Chen *et al.*, 2003, Na *et al.*, 2004). The list of differentially expressed genes founded by different studies is differed. Table 1.2 showed the list of most common genes reported to be dysregulated in keloids by various studies. It is likely that mutliple genes impart susceptibility to keloid development, with different genes contributing to keloid formation in different individuals.

Table 1.2: List of common genes reported to be up or down regulated in keloids by microarray studies.

Gene	Chen *et al.*	Na *et al.*	Naitoh *et al.*	Hu *et al.*	Satish *et al.*	Seifert *et al.*	Smith *et al.*
A2M	+			+			
ACAN			+			+	
ANXA1				+		+	
COL1A1		+	+		+	+	
COL1A2	+			+			
COL3A1	+						
COL4A2				+		+	
COL5A2	+		+	+			
COL15A1			+			+	
C5ORF13			+	+			
EGFR				-		-	
FN1	+		+		+	+	
HDGF	-			-			
HIF-1A				+		+	
IGFBP7				+		+	+
JAG1				+			+
POSTN			+	+			
VCAN			+			+	
KRT19				-			-