EFFECT OF TUALANG HONEY TREATMENT ON HUMAN KELOID AND NORMAL FIBROBLAST AND ITS RELATIONSHIP WITH TGF-β EXPRESSION

By

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TABLE OF CONTENTS

Page

CHAPTER 2 – LITERATURE REVIEW

Page

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES

LIST OF PLATES

Page

LIST OF ABBREVIATIONS

AAS	Atomic Adsorption Spectrometry
αvβ6	alpha v beta 6
amu	atomic mass unit
BMs	Basement membranes
c-Abl	c-Abelson
cells/cm^2	cells per centimeter square
cells/mL	cells per mililitre
cm^2	centimeter square
cNHDF	commercial Normal Human Dermal Fibroblasts
CO_2	Carbon dioxide
DK-SFM	Defined Keratinocyte Serum Free Media
D-MEM	Dulbecco's Minimal Eagle Medium
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
D-PBS	Dulbecco's Phosphate Buffered Saline
DPPH	1,1-diphenyl-2-picrylhydrazyl
ECM	Extracellular matrix
EGF	epidermal growth factor
ELISA	Enzyme-linked Imunosorbent Assay
EMT	epithelial-mesenchymal transition
FAMA	Federal Agriculture Marketing Authority
FBS	Fetal Bovine Serum

FSP	fibroblast specific protein
GC-MS	Gas Chromatography-Mass Spectrometry
g	gram
g	gravity
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HOS	Human osteosarcoma
HRP	Horseradish peroxidase
hrs	hours
HSP-47	heat shock protein-47
HUSM	Hospital Universiti Sains Malaysia
IHC	Immunohistochemistry
IL-1	interleukin-1
INDEX	inside needle dynamic extraction
JNK	Jun N-terminal kinase
L	Litre
MEOH	Methanol
µg/mL	microgram per mililitre
µL	microlitre
µm	micrometer
mg	miligram
min	minute
mm^2	milimeter square
mL	mililitre
MS-nose	Mass Spectrometry-nose

MSE-nose	Mass Spectometry Electronic-nose
MTT	3-(4, 5-dimethyl-2 thiozolyl)-2, 5-diphenyl-2H-tertrazolium bromide
MTS	3-(4, 5-dimethylthiazol-2-yi)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tertrazolium bromide, inner salt
NaOH	sodium hydroxide
Na_2SO_4	Disodiumsulphate
NIST	National Institute of Standards and Technology
nm	nanometer
OD	Optical Density
OSCC	Oral squamous cell carcinomas
P_0	Passage zero
P_1	Passage one
P_2	Passage two
PDGF	platelet derived growth-factor
PI3K	Phosphoinositide 3 kinase
PMS	phenazine methosulfate
pNHDF	primary Normal Human Dermal Fibroblast
pNHEK	primary Normal Human Epidermal Keratinocyte
pKHDF	primary Keloid Human Dermal Fibroblast
psi	pound per square inch
R-SMADs	receptor-regulated SMADs
RT-PCR	Real-time Polymerase Chain Reaction
sd	standard deviation
SHS	static head space

SMAD	SMA *(Caenorrhabditis elegans)* and MAD *(Drosophila melanogaster)*
SPME	solid phase micro-extraction
TAK1	TGF-β-activated kinase 1
TGF-β	Transforming Growth Factor-Beta
TIC	Total Ion Chromatogram
TNF	tumor necrosis factor-alpha
VEGF	vascular endothelial growth factor
zNOSE™	piezo-electric nose

LIST OF SYMBOLS

^{o}C	degree celcius
$^{o}C/min$	degree celcius per minute
$\geq$	equal to or more than
%	percent

KESAN RAWATAN MADU TUALANG KE ATAS FIBROBLAS KELOID DAN NORMAL MANUSIA DAN PERKAITANNYA DENGAN PENGEKSPRESAN TGF-β

ABSTRAK

Pembentukan parut keloid pada kulit manusia berlaku selepas trauma, pembedahan atau disebabkan kulit terbakar. Keloid terbentuk melangkaui kawasan yang terluka dan ciri ini membezakan keloid daripada parut hipertrofik. Teknik pembuangan keloid dilakukan disebabkan ketidakselesaan pertumbuhan keloid terutamanya pada kawasan yang terdedah. Walaubagaimanapun, pertumbuhan keloid biasanya berulang selepas pembedahan. Madu Tualang adalah madu tempatan yang didapati daripada pokok Tualang yang lebih dikenali dengan nama saintifiknya *Koompassia excelsa* yang boleh dijumpai di hutan hujan tropika Asia Tenggara. Madu Tualang adalah madu multiflora yang dihasilkan oleh *Apis dorsata* atau lebah Asia yang membuat sarang di atas pokok Tualang. Pemahaman terhadap rawatan keloid menggunakan madu masih terhad dan belum mendapat kata sepakat. Oleh itu, penyelidikan asas perlu dilakukan. Objektif-objektif penyelidikan ini adalah untuk menentukan keserasian madu Tualang kepada sel secara *in vitro*, untuk menentukan komposisi kimia madu Tualang, untuk mengkaji kesan komponen kimia madu Tualang tertentu pada proliferasi fibroblas secara *in vitro* dan melihat kesan ekspresi faktor pertumbuhan (TGF-β) terhadap keloid selepas dirawat dengan madu Tualang secara *in vitro*. Kultur fibroblas keloid dan normal dirawat dengan kepekatan madu Tualang yang berbeza dengan nisbah 1:2 bermula daripada 12.5% sehingga 0.10% dan ketoksikan sel diuji dengan ujian-ujian MTT dan

LDH. Kandungan volatil madu Tualang dikenalpasti dengan menggunakan lima pelarut organik yang berbeza dan dianalisis dengan kaedah GC-MS. Tiga puluh lima jenis kandungan volatil dikenalpasti daripada pengekstrakan madu Tualang. Madu Tualang yang diekstrak dengan metanol digunakan untuk diuji pada sel fibroblas dan kesan sel proliferatif diuji dengan ujian MTS. Ekspresi TGF-β pada keloid yang tidak dirawat dan dirawat dengan madu Tualang diuji dengan TGF-β Immunoesei. Hasil kajian menunjukkan madu Tualang pada kepekatan yang tinggi (12.5%) adalah toksik terhadap sel fibroblas tetapi ketoksikan madu Tualang terhadap kulit fibroblas manusia semakin menurun selepas pencairan. Madu Tualang yang diekstrak dengan metanol telah menunjukkan kebolehannya untuk merencatkan proliferasi sel. Didapati asid lemak berkemungkinan menyumbang kepada kesan antiproliferatif ini. Ekspresi TGF-β1 dan TGF-β2 pada fibroblas keloid yang dirawat menurun secara signifikan berbanding fibroblas keloid yang tidak dirawat. Ini telah membawa kepada penemuan baru di mana madu berupaya merencatkan sintesis kolagen dan seterusnya menurunkan tahap TGF-β dalam keloid. Penemuan-penemuan ini berpotensi membantu untuk penyelidikan terhadap model haiwan secara *in vivo* juga untuk kajian dari segi mekanisme molekular pada masa akan datang.

EFFECT OF TUALANG HONEY TREATMENT ON HUMAN KELOID AND NORMAL FIBROBLAST AND ITS RELATIONSHIP WITH TGF-β EXPRESSION

ABSTRACT

Keloid scar develops on the human skin after trauma, surgery or burn. Keloid grows beyond the boundaries of the wound site and is distinguishable from hypertrophic scar. Due to discomfort caused by keloid growth especially at exposed area, keloid excision might be needed. However, recurrence is common after surgical incision. Tualang honey is a local honey which is found on Tualang tree known as *Koompassia excelsa* found in Southeast Asia rainforests. Tualang honey is a multifloral honey which is produced by *Apis dorsata* or Asian rock bees that nested on the Tualang tree. There is no consensus and limited understanding in the keloid treatment using honey and therefore fundamental research is needed. The objectives of this study were to determine the degree of cytocompatibility of Tualang honey *in vitro*, to determine the chemical composition profile of Tualang honey, to investigate the effect of defined chemical components of Tualang honey on the proliferation of human skin fibroblasts *in vitro* and the effect on the expression of transforming growth factor-beta (TGF-β) in keloids after being treated with Tualang honey. The cultured keloid and normal fibroblasts were treated with several concentrations of Tualang honey ranging between 12.5% and 0.10% with 1:2 dilution followed by cytotoxicity testing using MTT and LDH assays. Volatile compounds of Tualang honey were identified based on five different organic solvents using Gas Chromatography-Mass Spectrometry (GC-MS) technique. Thirty five volatile

compounds have been identified from the extracts of Tualang honey. Methanol extract of Tualang honey was used to treat fibroblasts and the cell proliferative effect was determined by MTS assay. Expression of TGF-βs in untreated and honey treated keloid fibroblasts were determined using TGF-β Immunoassay. This study revealed that higher concentrations (12.5%) of Tualang honey was cytotoxic to fibroblasts cells but its toxicity to human fibroblasts reduced following dilution of the honey. Methanol extract of Tualang honey inhibited cell proliferation suggesting that its fatty acid contents may possess antiproliferative effect. TGF-β1 and TGF-β2 expressions in Tualang honey-treated keloid fibroblasts were lower compared with the untreated keloid fibroblasts. This finding indicated that honey has the ability to reduce collagen synthesis in keloid and simultaneously decreased TGF-βs level in keloid represent a new discovery in this field. The above findings have potential to be investigated in animal model *in vivo* as well as for studies at the molecular mechanism levels.

CHAPTER 1 - RESEARCH BACKGROUND

1.1 Skin Anatomy

Human skin is made up of three main layers which are epidermis, dermis and subcutaneous layer (Figure 1.1). The largest organ in human is skin. Skin consists of many types of cells and structures. There are several functions of skin including protecting the body from environment, for example, sun exposure, against infection, avoiding excessive loss of water from the body by evaporation, protecting organs and tissues from mechanical damage, regulates temperature, excretion of excess salts, water and urea from the body (Naficy, 2000).

Epidermis is the superficial layer of the skin. Epidermis is thicker on palms of hands and soles of feet compared to face and eyelids which have thinner epidermis (Wildcrafted's natural skin care newsletter, 2004). Epidermis is made from cells called keratinocytes. Dermis is thicker than epidermis which consists of connective tissue. Fibroblasts can be found in dermal layer. Dermal layer contains sense organs such as touch, pressure, pain and temperature. Subcutaneous layer lies below the dermis which consists of loose connective tissue and fat. It acts as a protective cushion as well as for temperature regulation.

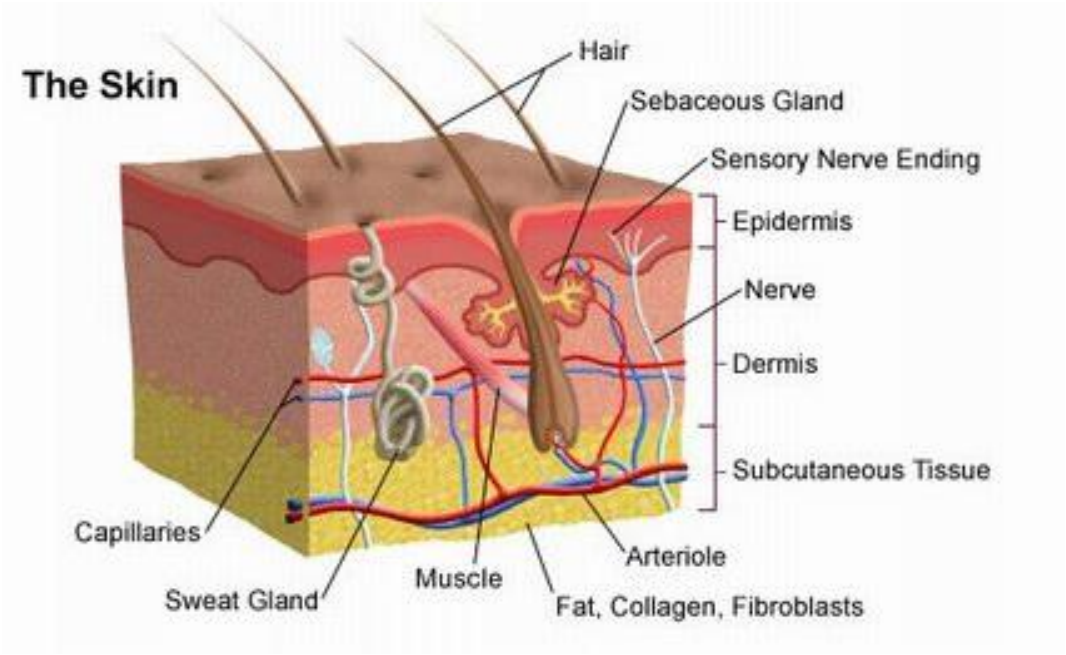

Figure 1.1. Anatomy of human skin which consists of three main layers; epidermis, dermis and subcutaneous tissue. (Adapted from: Naficy, 2000)

1.2 Definition of Scar

Scar formation is the result of natural healing process which occurs after wounds, trauma, burns, surgical incision, vaccinations or disease (Gauglitz *et al*., 2011; Edriss and Mueak, 2005). In order to close a wound, skin repairs itself from normal to scar tissue and thus prevents infection (Edriss and Mueak, 2005). There are several types of scar. Among common ones are keloid and hypertrophic scars (Figure 1.2). These two scars can be distinguished by its size and shape. Keloid scars are larger in size and extend beyond the margins of the original wound compared to hypertrophic scars which remains within the wound border (Jagadeesan and Bayat, 2007).

Formation of scars can be characterized by an overproduction of extracellular matrix particularly collagen (Chin *et al*., 2004). Normal skin contains collagen bundles that are arranged parallel to the skin surface. However keloid consists of dense mass and randomly organized collagen and fibroblasts (Jagadeesan and Bayat, 2007). Fibroblast is a type of cell that synthesizes the extracellular matrix and a protein called collagen.

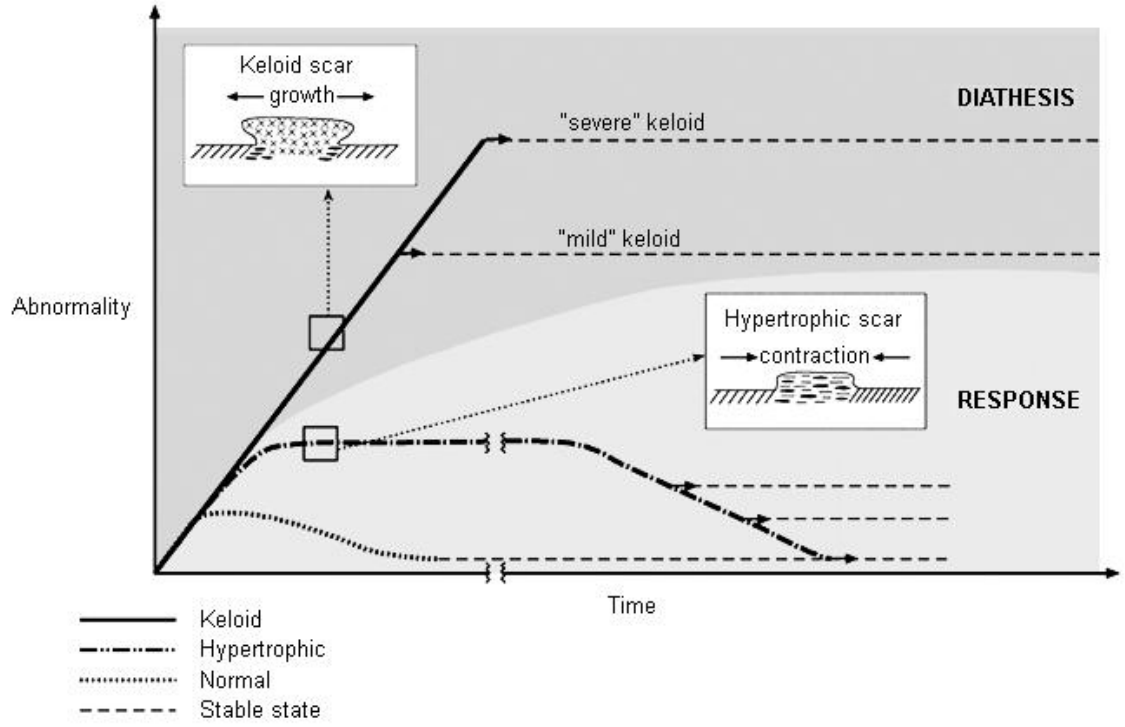

Figure 1.2. Keloid grows faster than hypertrophic scar whereas it proliferates to higher level of abnormality within short time frame and finally stabilized. Hypertrophic scar involves contraction or contractures but this condition does not occur in keloid.

(Adapted from: BMJ Evidence Center, 2011)

1.3 Expression of Growth Factors

High expression of certain growth factors or cytokines commonly associates with excessive accumulation of collagen in keloid scar. One of the important cytokine involved in scar formation is transforming growth factor beta (TGF-β) (Tuan and Nichter, 1998). TGF-β is a group of cytokines which is also called as 'The TGF-β superfamily'. It regulates epithelial cell growth and differentiation (Chin *et al*., 2004) and is found expressed by all cells (Chin *et al*, 2004). It can act either as a stimulator or inhibitor in cellular replication and control production of extracellular matrices (Streuli *et al*., 1993).

There are several types of TGF-β where the most common ones are TGF-β1, TGF-β2 and TGF-β3. TGF-β1 and TGF-β2 are involved in promoting fibrosis and formation of scar while TGF-β3 can either induce or reduce scarring (Chin *et al.*, 2004). From Figure 1.3, it shows that the formation of scar occurs due to the induction of TGF-β1 by connective tissue growth factor (CTGF). In this study, TGF-β1 and TGF-β2 expression from keloid were determined *in vitro*. Several treatments for keloid excision have been described including surgical, nonsurgical and combined modality treatment. Keloid treated with simple excision tends to recur and the use of laser is expensive (Kokoska and Prendiville, 2007). In this study, cultured keloid fibroblasts were treated with Tualang honey to study the ability of honey in reducing keloid scar.

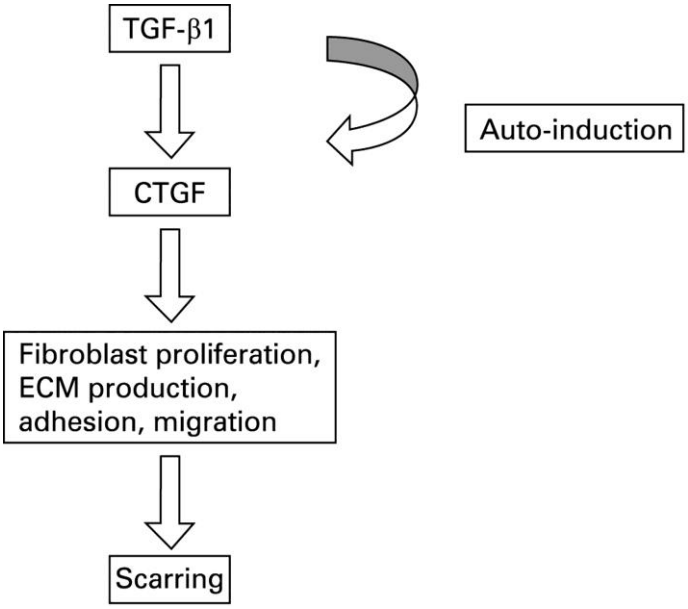

Figure 1.3. Induction of TGF-β1 through connective tissue growth factors (CTGF) causes increase of fibroblast proliferation and leads to scar formation. [Abbreviations: ECM, extracellular matrix].

(Adapted from: Klass *et al*., 2009)

1.4 Tualang Honey

For a long time, honey has been valued for its medicinal properties. Honey has been used to treat infections in various types of wounds including burns, diabetic ulcers, pressure ulcers, traumatic ulcers and Fournier's gangrene (Molan, 2001). The antiseptic and antibacterial properties of honey have recently been explained at a chemical level (Jeffrey and Carlos, 1996).

In Malaysia, one of the most common honey found is Tualang honey. The Tualang tree known as *Koompassia excelsa* is a majestic tree of the Southeast Asia rainforest best known for the disk-shaped honeycombs which hang from its horizontal branches. It is mostly found in lowland forests of Peninsular Malaysia, southern Thailand, northern Sumatra and Borneo, and can grow to heights greater than 85 meters. The trees are valued by the locals due to its honey. In fact, a standing Tualang tree is more valuable for its honey than if it were felled for its timber. The honey from the combs of this tree is known as Tualang honey and is produced by *Apis dorsata* or Asian rock bees (Tropical Rainforests, 2006).

This study focused on two major fields which are basic pharmacology study and *in vitro* study. In the field of pharmacology, a spectrometry technique was carried out to identify its volatile compositions which have never been elucidated before. Meanwhile *in vitro* technique was done to determine the viability of cells and expression of growth factors in fibroblast cells, exposed to the Tualang honey.

1.5 Hypothesis

Tualang honey is effective in treating keloid scar and is able to reduce expression of TGF-β in keloid.

1.6 General Objective

To study the mechanism of healing capability of Tualang honey on keloid scar formation.

1.7 Specific Objectives

- To determine the degree of cytocompatibility of the Tualang honey using an *in vitro* study model.
- To determine the composition profile of Tualang honey using GC-MS.
- To investigate the effect of methanolic extraction of Tualang honey on the proliferation of human skin fibroblasts *in vitro*.
- To determine the effect on the expression of transforming growth factor-beta (TGF-β) in keloid fibroblasts after treatment with Tualang honey using biochemical method.

CHAPTER 2 - LITERATURE REVIEW

2.1 Keloid Scar

2.1.1 Characteristic of Keloid

Keloid is a type of scar which extends beyond the boundaries of the original wound (Figure 2.1). It can spread to the surrounding skin by invasion. Clinical appearance of keloid is a raised growth and is usually related with pruritus and pain. Certain areas of the body such as sternum, deltoid (Kokoska and Prendiville, 2007) and the back (Edriss and Mueak, 2005) are susceptible to keloid formation. Some opinions conclude that motion and tension play a major role in the formation of keloid (Edriss and Mueak, 2005). Previous *in vivo* and *in vitro* studies have found that the increase of tension level has been shown to increase the production of fibroblasts and myofibroblasts (Edriss and Mueak, 2005).

Keloid scars commonly occur only in humans. Keloid healing remains unravelled since the pathogenesis of biochemical mechanisms of keloid is still unknown (Vincent *et al*., 2008). Development of keloid contains atypical fibroblasts and consists of overabundant of extracellular matrix components include collagen, fibronectin and certain proteoglycans. During the early stage of granulation tissue, fibronectin production is high and collagen deposition is low. When granulation tissue matures, collagen production becomes high while fibronection is low (Babu *et al*., 1992).

Collagen is a component of extracellular matrices which play various roles in mammals. There are 27 types of collagen that have been identified with specific α-chains which are encoded by more than 40 different genes (Ishida *et al*., 2006). Type I collagen

consists of two α1 chains and one α2 chain. Newly synthesized polypeptides of procollagen are cotranslationally transferred into the endoplasmic reticulum (ER). In order to ensure correct folding of collagen in the cell, several molecular chaperones are involved. These molecular chaperones include BiP/Grp78, Grp94, protein disulfide isomerase (PDI), prolyl 4-hydroxylase (P4H) and heat-shock protein of 47 kDa (HSP-47).

Increase of collagen mRNA expression, propyl-4 hydroxylase and fibronectin synthesis (Jagadeesan and Bayat, 2007) in keloid scar result in higher level of type III collagen deposition compared to normal (Kokoska and Prendiville, 2007; Jagadeesan and Bayat, 2007). Collagen deposition could result either from enhanced biosynthetic activity or reduced rate of degradation. This condition leads to an imbalance collagens in the dermis (Uitto and Kouba, 2000). Fibroblasts and myofibroblasts are responsible for the deposition of dense extracellular matrix which consists of collagen and glycosaminoglycans. Certain studies reported that level of fibronectin which is produced by fibroblasts has increased within keloids (Kokoska and Prendiville, 2007). Compared to normal fibroblasts, explanted keloid fibroblasts synthesize abnormal collagen deposition (Babu *et al*., 1992).

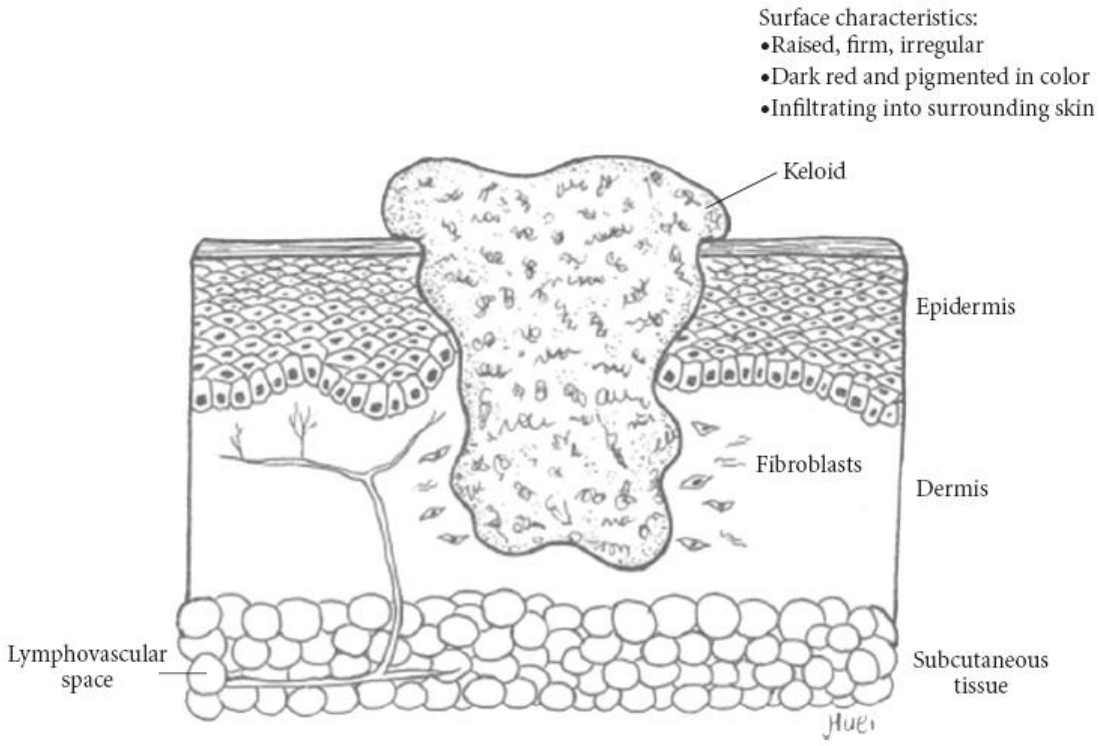

Figure 2.1. The characteristics of keloid mark its differences from hypertrophic scar. It is composed of disorganized type I and III collagens.

(Adapted from: Su *et al*., 2010)

2.1.2 Comparison between Keloid and Hypertrophic Scar

Keloid and hypertrophic scars are both benign proliferation of the dermis. Keloids occur genetically in approximately 10% of people with darkly pigmented skin. Even though keloid and hypertrophic scars look similar and are hard to differentiate because of an apparent lack of morphologic differences, they can be distinguished by their own special characteristics (Felice *et al*., 2009; Campaner *et al*., 2006). A keloid can be differentiated from a hypertrophic scar in which it grows beyond the boundaries of the original wound, continues to grow from time to time, is recurrent after surgical excision and does not regress spontaneously. However for hypertrophic scar, it remains within the boundaries of the original lesion, does not recur and often regresses spontaneously several months after the initial injury (Felice *et al*., 2009; Campaner *et al*., 2006). Similarly, formation of keloid and hypertrophic scar is related to collagen deposition. Collagen deposition is increased in keloid and hypertrophic scars due to uncontrolled production of extracellular matrix and reduced degradation of procollagen polypeptide (Ueda *et al*., 2004). Besides that, keloids are characterized by the presence of thick, hyalinized collagen bundles with mucinous ground substance and relatively few fibroblasts while hypertrophic scars are characterized by nodular structures with fine randomly organized collagen fibers (Campaner *et al*., 2006).

2.1.3 Mechanism of Formation of Keloid Scar

The specific mechanism of keloid formation is undetermined. One theory reported that allergic immunoglobulin E (IgE)-mediated response leading to a decreased percentage of mature cross-linked collagen and increased fraction of soluble collagen. A second theory stressed that either the deficiency of melanocyte-stimulating hormone (MSH) metabolism or excess accumulation of MSH such as during puberty and pregnancy (Edriss and Mueak, 2005) results in keloid formation. Recent study reported increased level of interleukin-6 (IL-6) expression and the role of insulin like growth factor-1 (IGF-1) and IGF-1 receptor increased the activity of keloid (Kokoska and Prendiville, 2007).

In the management of scars, one should know how to prevent scars and learn how to identify them immediately. In order to do this, a good understanding of wound healing and skin biomechanics is important (Santucci *et al*., 2001).

2.2 Phases of Wound Healing

Wound healing is a process that occurs after an injury which involves tissue repair and regeneration (Kumar *et al*., 2004; Adikwu and Enebeke, 2007). A wound is a break of the tissue continuity due to trauma or violence and if restoration of the wound site to normal condition occurred, the wound is considered as healed (Adikwu and Enebeke, 2007). Wound healing starts with inflammation and ends with scar formation (Kumar *et al.*, 2004). Three phases are involved in wound healing which are inflammatory, proliferation and remodeling (Table 2.1). In the inflammatory phase,

blood coagulation, hemostasis, infiltration of leukocytes and cytokine release occur (Figure 2.2). More immune response cells migrate to the healing wound. During the proliferative phase, formation of epithelium occurs in order to close the wound surface together with the growth of granulation tissue to fill the wound space within hrs (Li *et al.*, 2007; Adikwu and Enebeke, 2007). During this phase, cellular activity is dominant and the wound closes spontaneously by contraction with reepithelization occuring in early wound repair (Adikwu and Enebeke, 2007). There is also granulation tissue formation which involves fibroblasts proliferation, collagen deposition and development of new blood vessels (Figure 2.2).

The final remodeling phase is also known as delayed primary closure (Adikwu and Enebeke, 2007). It takes place when the new tissue is already well-formed and begins to restore tissue structural integrity (Li *et al.*, 2007). During this phase initial debridement of the wound for over a period of time followed by a formal closure of the wound by suturing or other mechanism are involved. This complex event includes extracellular matrix, cells, growth factors and cytokines (Adikwu and Enebeke, 2007). There are two most important factors in the process of scar tissue formation which are aggregation and proliferation of fibroblasts in the wound site; and the increase in the accumulation of other extracellular matrix components such as collagen (Kumar *et al.*, 2004). The major events that occur during this phase are the creation of a permeability barrier, the establishment of appropriate blood supply and reinforcement of injured dermal tissue (Li *et al.*, 2007). Many cytokines are involved during this phase including insulin like growth factor (IGF). Contraction results in smaller appearance of scar tissue (Adikwu and Enebeke, 2007).

Table 2.1. Wound healing involves three main phases: Inflammation, proliferation and remodeling. Wound healing is associated with immune cellular response; which involves presence of several important cells. [Abbreviations: PDGF, platelet derived growth-factor; TGF-β, transforming growth factor-beta; EGF, epidermal growth factor; VEGF, vascular endothelial growth factor; ECM, extracellular matrix; TNF-α, tumor necrosis factor-alpha; IL-1, interleukin-1].

PHASES	DAY	PRESENCE OF CELLS/ CHARACTERISTICS
Inflammation	0 – 6	• Number of platelets increase and release many growth factors including PDGF, TGF-β, EGF and VEGF. • Neutrophils and macrophages strongly increased between day 2 and 3.
Proliferation	1 – 14	• Reepithelialization starts a few hours after lesion. • Neutrophils increase to a maximum level at day 2 and slowly decreased at day 6. • Macrophages increase to a maximum level at day 3 and decrease slowly until day 13. • Fibroblasts cells increase, produce a new extracellular matrix for cell growth.
Remodeling	8 – 16	• An attempt to recover the normal tissue structure. • Most vessels, inflammatory cells (macrophages and lymphocytes) and fibroblasts disappear from the wound site through migration, apoptosis or other unknown cell death mechanism. • Fibroblasts are transformed into myofibroblasts and act as contractile tissue. • ECM reorganization, cytokines (TNF-α, IL-1 and TGF-β) released

(Adapted from: Mendonça and Coutinho-Netto, 2009)

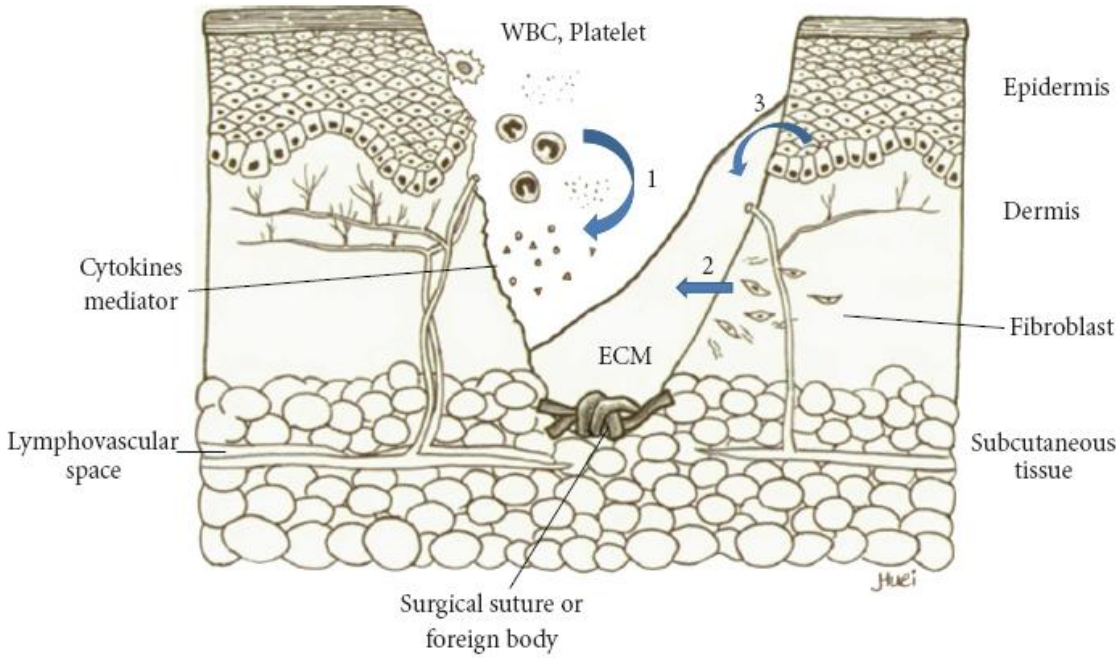

Figure 2.2. Normal process of wound healing. It represents: 1) The activation of coagulation cascade cause cytokines release 2) An abundance of fibroblasts and ECM accumulation 3) Immature type III collagen of early wound is modified into mature type I collagen in mature phase.

(Adapted from: Su *et al*., 2010)

2.3 Fibroblasts Proliferation

Fibroplasia is a process of formation of granulation tissue. It involves fibroblasts proliferation, migration into wound fibrin clot and production of new collagen and matrix proteins. Fibroblasts begin to proliferate at the wound edges. After several days, it starts to migrate into provisional matrix of the wound clot and form collagens, proteoglycans and elastin (Li *et al.*, 2007). Fibronectin and collagen provide a reservoir for cytokines and growth factors and thus contribute to granulation tissue formation.

Collagen is the most abundant protein in mammals. It plays various roles as a component of the extracellular matrices. Type I collagen is a typical fibril-forming collagen which is a major component of the extracellular matrix (ECM) whereas type IV collagen is a nonfibrillar network-forming collagen found in basement membranes (BMs) (Ishida *et al*., 2006). Collagen is crucial for various biological functions, and mutations of collagen are known to cause various collagen-related diseases including osteogenesis imperfecta and the Ehlers–Danlos syndrome (Myllyharju and Kivirikko, 2004).

Fibronectin is a glycoprotein that is initially secreted for the enhancement of fibroblast activity. Fibronectin allows fibroblasts to bind to extracellular matrix and thus produce adherent base for cell migration. Both PDGF and TGF-β can stimulate fibroblast migration and up-regulate the expression of integrin receptors (Li *et al.*, 2007). On the other hand, fibrosis is a condition whereby abnormal and excessive accumulation of fibrous tissue, for example in the wound healing process following trauma or surgery. Fibrosis that occurs in the skin causes the formation of abnormal scars either

hypertrophic scars or keloids. This abnormal behavior can lead to other problems in tissue growth, function and movement (Campaner *et al*., 2006).

2.4 Transforming Growth Factor-βeta (TGF-β) Expression

2.4.1 Phases of TGF-β Secretion

TGF-β is known to play an important role in the pathogenesis of keloid and hypertrophic scarring via a distinct molecular mechanism (Chin *et al*., 2004). Cascade pathway takes place for tissue regeneration and repair process in fibrosis. It involves three main phases in which it begins with coagulation and inflammatory phase, formation of granulation tissue phase and ends with extracellular matrix deposition and termination of the response phase. Platelets and inflammatory cells released certain factors whereas these factors initiate the regeneration response (Frazier *et al.*, 1996). TGF-β was determined to be as one of the initiators in these processes (Wahl, 1992).

2.4.2 Stimulation of TGF-β in Relation to Other Growth Factors

TGF-β level is elevated in tissue during normal wound repair processes and skin fibrotic disorders processes (Frazier *et al.*, 1996). The biologically active TGF-β induces a variety of cell activities such as stimulation or inhibition of cell growth and differentiation depending on the cell type (Tang *et al.*, 1994). It is found in most parts of the body and is involved in the scarring process (Shah *et al.*, 1995). In addition, several actions of TGF-β have been found as a result of increased fibrotic tissue disorders. These include increased fibroblasts proliferation, increased synthesis of extracellular matrix

components such as fibronectin and type I collagen and decreased degradation of extracellular matrix due to stimulation of the synthesis of protease inhibitors (Frazier *et al.*, 1996).

Previous study has indicated that with the induction of platelet-derived growth factor (PDGF) genes, TGF-β can stimulate the growth of various fibroblastic cell types (Frazier *et al.*, 1996). Expression of TGF-β was found to occur during inflammatory phase whereas connective tissue growth factor (CTGF) was expressed during granulation tissue formation phase of tissue repair. These coordinate expressions help to functionally link both phases of this complex process (Frazier *et al.*, 1996). Overexpression of TGF-β has been reported in scleroderma lesions which suggested that there is an association between TGF-β and the increased in collagen deposition (Frazier *et al.*, 1996).

Activator protein-1 (AP-1) is known as a key regulatory protein in TGF-ß1-induced type I collagen synthesis in skin fibroblasts (Cho *et al.*, 2008). Formation of keloid is largely associated to fibronectin and collagen production. Fibronectin production is influenced by many regulatory factors and one of them is TGF-β. TGF-β plays a role in the extracellular matrix where it regulates the deposition of collagen, fibronectin, proteoglycans, tenascins, thrombopondin (Streuli *et al*., 1993; Ignotz *et al*., 1987) via transcriptional mechanism (Babu *et al*., 1992).

2.4.3 Inactive and Active Form of TGF-β

Synthesis and activation of TGF-β's family is a complex process. TGF-β is secreted from monocytes, lymphocytes and fibroblasts (Varga and Pasche, 2009) and is

synthesized in a latent form as large precursor molecules. When secreted, it is proteolytically cleaved from the precursor. Latent TGF-β is a large 390-412 amino acid precursor protein which is activated by proteolytic cleavage between amino acid into active TGF-β and Latency associated peptide (LAP) (Khalil N., 1999). The presence of TGF-β in tissue cannot be detected in a latent form (Frazier *et al.*, 1996). Even after cleavage it remains in a noncovalent association with the precursor sequence, bound to the latency-associated peptide (Campaner *et al.*, 2006; Tang *et al.*, 1994) by plasmin-dependent and plasmin-independent pathways (Bikfalvi, 1995). Most of the TGF-β in tissue and that is produced by cells in culture is in this latent form.

TGF-β–latency associated peptide complex is usually stored in the extracellular matrix but TGF-β in this complex is inactive. This latent TGF-β must be activated to obtain the active TGF-β (Frazier *et al.*, 1996) and thus TGF-β expression in culture cells can be detected. Activation of latent TGF-β involves catalyzes of the TGF-β by serine proteases, thrombospondin and cell-surface integrins (Varga and Pasche, 2009). In addition, it can also be activated through dissociation of the mature form from the TGF-β complex *in vitro* (Olofsson *et al.*, 1992). Once released from this complex, TGF-β1 is active and can bind to its cellular receptor.

From Figure 2.3, TGF-β depot is at the extracellular matrix whereas cytokine is sequestered in a latent form here. Latent TGF-β is catalyzed by alpha v beta 6 (αvβ6) integrin on epithelial cell membranes into active TGF-β. Active TGF-β then binds to serine/threonine kinase cell surface receptors that phosphorylate downstream SMAD2/3. SMAD pathway is an intracellular signaling pathway that TGF-β family member signal through (Wikipedia, 2011, Yu *et al.*, 2006). The term ‘SMAD’ is derived from a combination of homologue genes found in *Caenorrhabditis elegans*, SMA, and

Drosophila melanogaster, MAD (Yu *et al*., 2006). Phosphorylated SMAD2/3 form a complex with SMAD4 and accumulates within the nucleus resulting in transcription. Endogenous negative regulator includes SMAD7 inhibits TGF-β signaling by sequestering SMAD2 and SMAD3 inside the nucleus.

TGF-β can also induce cellular responses, such as EMT (epithelial-mesenchymal transition), via SMAD-independent pathways involving the kinases JNK (Jun N-terminal kinase), p38, PI3K (Phosphoinositide 3 kinase), c-Abl (c-Abelson) and TAK1 (TGF-β-activated kinase 1). Degradation of the TGF-β is regulated by the uptake of the TGF-β receptor–ligand complex into caveolin-lined endosomes. Excessive TGF-β activity or intracellular SMAD signaling results in collagen overproduction and fibrosis (Varga and Pasche, 2009). TGF-β1 is liberated by extremes of pH and heat *in vitro* as well as by proteases such as furin and plasmin (Gleizes *et al*., 1997; Khalil, 1999).

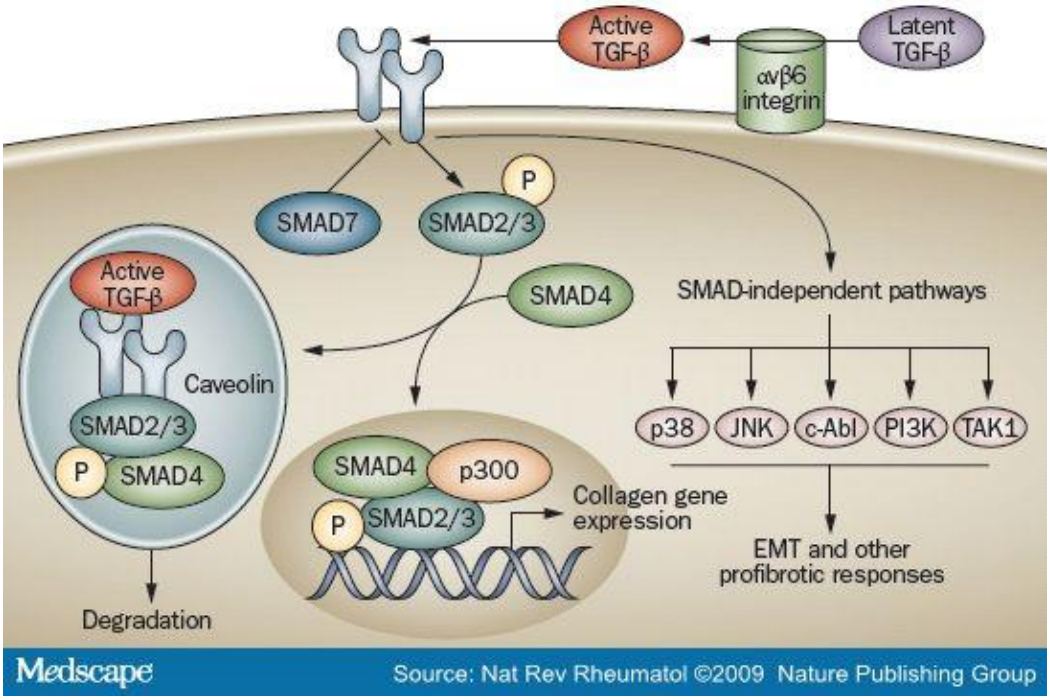

Figure 2.3. Mechanism of TGF-β signalling pathway involves several components in order to activate TGF-β, transcription and degradation process and profibrotic responses.

(Adapted from: Varga and Pasche, 2009)

2.5 Treatment of Keloid Scar

Effective treatment of fibrotic diseases is very limited. Despite the fact that there is a wide array of options such as corticosteroids, radiation, lasers, surgery and interferon, treatments are clinically challenging since recurrence of keloids after treatment is frequent, varying from 45 to 100% (Campaner *et al*., 2006). There are several ways in managing keloid. Prevention of the inflammation extension can be done. Anti-inflammatory agents such as intralesional corticosteroids which act by inhibiting fibroblast growth and decreasing alpha-2 macroglobulin levels and synthetic glucosteroids are commonly used in the treatment of keloid scars. Other than that, this inflammatory response can be regulated before, during or after inflammatory gene expression. Transcription factor such as nuclear factor-kB (NF-kB) is activated before gene expression while inducible enzyme such as cyclo-oxygenase (COX) is regulated after gene expression. Nonsteroid anti-inflammatory drugs (NSAIDs) inhibit either the NF-kB pathway or COX activity (Edriss and Mueak, 2005).

Another possible approach is inhibition of growth factor. TGF-β family especially TGF-β1 isoform is a key mediator of tissue fibrosis (Jagadeesan and Bayat, 2007). An animal model study suggested that treatment of fibrotic scarring can be done either by blocking the effects of TGF-β1 and TGF-β2 or by administration of TGF-β3 (Shah *et al.*, 1995). In an attempt to reduce scarring, anti- TGF-β1 antibody may help to treat keloid either by inhibition of collagen synthesis by blocking TGF-β1 or by degeneration of types 1 and 3 collagens (Jagadeesan and Bayat, 2007). A previous study by Shah *et al.* (1995) has found that the addition of neutralizing antibody to TGF-β1 and

TGF-β2 to wounded adult rodents helped in scar reduction. Suppression of SMAD3 signalling may also lead to inhibition of keloid fibroblasts (Phan *et al.*, 2005).

In addition, application of honey to scars may contribute to an alternative way of reducing a scar. A previous study has revealed the effectiveness of honey in wound healing for almost all types of wounds (Medhi *et al.*, 2008). Therefore, it is possible that honey can help to minimize scarring without any surgical interventions. Clinical observation has found that sucrose either with or without antiseptic supplementation was able to initiate granulation tissue formation (Knutson *et al.*, 1981). In addition, clinical studies have suggested that honey or sucrose may lower the incidence of keloid and hypertrophic scars (Kossi *et al.*, 2001, Knutson *et al.*, 1981). In keloid fibroblast culture, sucrose caused a clear decrease in proα1(I) collagen and a slight decrease in proα1(III) collagen mRNA level (Kossi *et al.*, 2001) while mannose may have value in the prevention or treatment of abnormal scarring since it decreases type I collagen gene expression and synthesis as well as decreases type I/III collagen ratio (Kossi *et al.*, 2004).

2.6 Honey and its Properties

2.6.1 Properties and Composition of Honey

Several beneficial properties of honey make honey a preferred choice for human. Honey has been used as a source of food since million of years ago by Paleolithic humans (Eaton and Eaton, 2000). Honey varies from one type to another in terms of geography, botany, floral origin, species of bees, age of honey and method of honey collection. Since ancient times, honey has been valued for its medicinal properties.