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Exploratory descriptive study of the support tissue in keloids

By

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Submitted in partial fulfilment of the requirements for the degree of

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Department of Anatomy

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Co-supervisor: **Ms H Taute**

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Faculty of Health Sciences

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Abstract

Keloids are benign hyper-proliferative growths of fibrous tissue, where increased fibroblast activity results in abnormal collagen deposition. Scientific literature related to the morphological features of keloids especially at an ultrastructural level is outdated. Therefore the aim of this study was to reassess present knowledge of the ultrastructural features of keloids and possibly through this process identify new cellular therapeutic targets.

The research was conducted on normal (control) and keloid human skin samples collected from consenting patients undergoing keloid removal and skin transplantation surgeries at the Steve Biko Academic Hospital. The tissue structure of normal/control skin and keloids as well as mast cell and collagen distribution were evaluated using histological techniques. Transmission electron microscopy techniques were undertaken in order to investigate morphological and ultrastructural features of cells of the epidermis and dermis. A further detailed analysis of the ultrastructure of keloid fibroblasts and mast cells was undertaken. The findings of this study have lead to a new hypothesis related to keloid formation.

Increased fibroblast activity, intracellular collagen production and fibroblast and mast cell interactions were seen in keloid tissue. Changes in the morphology of keratinocytes and melanocytes were observed, where the cytoplasmic processes of both cells were shorter and cells were packed closer together in keloids. Keloid tissue appeared to be in a hyperproliferative state similar to that of the granulation phase of wound healing. Increased amounts of collagen were found in the extracellular matrix (ECM) of keloid tissue. This is the first study in which the abnormal accumulation of insoluble collagen fibrils was observed in the cytoplasm. Degranulation of mast cells had occurred and these cells were found in close association with fibroblasts. In some instances phagocytosis of collagen by mast cells was also observed.

These observations have led to the hypothesis that transforming growth factor β (TGF-β) derived from mast cells, inhibits keratinocyte proliferation and stimulates increased collagen production through increased expression of lysyl oxidase (LOX) by fibroblasts. Intracellular insoluble collagen formation then occurs due to the rapid, intracellular removal of the C terminal pro-peptide sequence by C-proteinase which initiates the cascade of insoluble collagen fibre formation within the fibroblast. Normally this process occurs only within the ECM in response to the increasing mass of collagen and in an attempt to establish normal tissue homeostasis the mast cells engulf the bundles of collagen fibres. Increased stress on the epidermal layer causes increased keratinocyte proliferation, which results in further growth factor mediated replication of fibroblasts. This creates an endless cycle of collagen synthesis, mast cell degranulation and mast cell mediated collagen phagocytosis, physical stress on the epidermal layer and subsequent growth factor release and fibroblast activation, collagen synthesis and subsequent crowding of keratinocytes and melanocytes.

In conclusion, this study identified keloid formation as a defect of procollagen synthesis and processing. Phagocytosis of collagen by mast cells indicates that accumulation of these cells may be a secondary effect to excessive collagen synthesis. In addition, the release of interleukins, mediators and growth factors may further stimulate collagen fibril formation with the imbalance toward increased synthesis. This study also identified and confirmed the findings of other studies that procollagen C-proteinase is an important therapeutic target.

Declaration

I, Sandra Arbi declare that this thesis entitled,

"Exploratory descriptive study of the support tissue in keloids"

Which I herewith submit to the University of Pretoria for the degree Master of Science in Anatomy with specialization in Human Cell Biology and Histology, is my own original work and has never been submitted before for any academic award to any other tertiary institution for any degree.

_____ _____

S Arbi **Date**

Department of Anatomy, Faculty of Health Sciences,

University of Pretoria

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South Africa

Ethical Clearance

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.

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Faculty of Health Sciences Research Ethics Committee

4/09/2014

Approval Certificate
New Application

Ethics Reference No.: 336/2014

Title: Exploratory descriptive study of the support tissue in keloids

Dear Prof Megan Bester

The **New Application** as supported by documents specified in your cover letter for your research received on the 4/08/2014, was approved, by the Faculty of Health Sciences Research Ethics Committee on the 27/08/2014.

Please note the following about your ethics approval:
- Ethics Approval is valid for 2 years.
- Please remember to use your protocol number (**336/2014**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

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(In the name of God, the most Beneficent, the most Merciful)

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Articles published and conferences attended

Articles:

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Conference:

Research poster presented at University of Pretoria Faculty Day, 2014.

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= 10 µm.

List of tables

List of abbreviations, symbols and chemical formulae

α	Alpha
β	Beta
δ	Delta
γ	Gamma
ε	Epsilon
%	Percentage
µm	Micrometer
ºC	Degrees celcius

A

ATP	Adenosine triphosphate
ATPase	Adenosine triphosphate

B

Bcl	B-cell lymphoma

C

$C_6H_3N_3O_7$	Picric acid
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
COPII	Coat protein II
CTGF	Connective tissue growth factor
CUL3-KLHL	Ubiquitin ligase

D

DAMPs	Damage associated molecular pattern molecules
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DOPA	Dihydroxyphenylanine

E

ER	Endoplasmic reticulum
EMC	Extracellular matrix

F

Fc	Fragment crystallisable
FcεRI	Fragment crystallisable epsilon receptor I
$FeCl_3$	Iron (III) chloride
FGF	Fibroblast growth factor

G

GAG	glucoseaminoglycan
g	Gram

H

H&E	Haematoxylin and eosin
H_2O	Water
HCl	Hydrochloric acid
HIF	Hypoxia inducible factor
HLA	Human leukocyte antigen

I

IF	Interferon
IFN	Interferon
IgE	Immunoglobulin E
IL	Interleukin

K

$KAl(SO_4)_2$	Potassium aluminium sulphate
KGF	Keratinocyte growth factor
kDa	Kilodalton
kg	Kilogram

L

L	Litres
LC-MC	Liquid chromatography- Mass spectrometry
LM	Light microscopy
LOX	Lysyl oxidase

M

M	Molar
MC1-R	Melanocortin-1 receptor
MCDP	Mast cell degranulation peptide
MCT	Mast cell containing tryptase
MCTC	Mast cell containing tryptase and chymase
ml	Millilitres
MMP	Matrixmetalloproteinase
mRNA	Messenger ribonucleic acid
MSH	Melanocyte stimulating hormone
mTOR	Mammalian target of Rapamycin

N

NaCl	Sodium chloride
NaH_2PO_4	Sodium phosphate
NaOH	Sodium hydroxide
$-NH_2$	Amide
$NaIO_3$	Sodium Iodate
nm	Nanometer

O

-OH	Hydroxide

P

PAR-2	Protease activated receptor- 2
PBS	Phosphate buffered solution
PCP	Procollagen C- proteinase
PDGF	Platelet derived growth factor

PGE_2	Prostaglandin E_2
PGF	Placental growth factor
pH	Potential hydrogen
PI3K	Phosphoionositide 3- kinase
PR	Picrosirius red

R

RER	Rough endoplasmic reticulum
ROS	Reactive oxygen species

S

SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sFRP2	Secreted frizzled- related protein- 2
SMA	Smooth muscle actin
SMAD	Similar to Mothers Against/Mothers Against Decapentaplegic

T

TEM	Transmission electron microscopy
TGF-β	Transforming growth factor β
TNF	Tumour necrosis factor
TYR1	Tyrosinase 1

U

UV	Ultraviolet

V

VEGF	Vascular endothelial growth factor
vs	*Versus*

Chapter 1: Introduction

Keloids present a common and serious condition among a large number of the South African population, providing discomfort both physically and psychologically; due to aesthetic changes, which in turn leads to a poorer quality of life for the individuals affected. Keloids are still a relatively new field of research, and even though various studies have been conducted on the treatments and causes of keloids, little is known about the underlying mechanisms and cellular changes which take place in the tissue of keloid patients.

Keloids in South Africa have been recorded as early as 1954 by Allan and Keen (Allan and Keen, 1954), in a study where the researchers tried to investigate the effect of radiation therapy on post-surgical sites following keloid removal. It was reported that the South African population is highly susceptible to keloid development with a high rate of occurrence. The authors suggested possible causes to be genetic factors, post-operative and injury care, or the lack of availability of efficient treatments.

Research and the resultant development of wound healing products and strategies have been developed for the optimal healing of injured skin. Nevertheless, for disorders such as keloids wound healing products and treatment strategies do not always effectively treat and/or prevent the reoccurrence of keloids. Further knowledge about the mechanisms involved in keloid development is needed to enable future research on possible treatments and therapies.

The focus of this study was to investigate the cellular changes that occur in keloid skin compared to normal skin. This included the histological and transmission electron microscopy analysis in order to investigate morphological and ultrastructural differences in skin-associated cells and support tissue.

Chapter 2: Literature Review

The skin covers the external surface of the body and is its largest organ, both in its surface area and weight. In adults it covers an area of two square meters and can weigh between 4.5 to 5.0 kg. It ranges in thickness depending on the area location, and together with its accessory structures; hair, nails, glands and muscles makes up the integumentary system of the body. Its main role is to protect the body from changes in the external environment, and to maintain a constant body temperature which is essential for the correct enzyme and general organ function of the entire body. The skin is easily exposed to injury, infection and disease, but several biochemical and cellular mechanisms are able to prevent damage and disease (Tortora and Derreckson, 2008).

Keloids are a pathological feature of the skin that affect the epidermal surface as well as the dermis of the skin. To understand and possibly identify new cellular targets of treatment it is necessary to understand normal skin and keloid structure and function. Therefore the aim of this literature review is to reassess present knowledge regarding the structure, physiology and cellular features of normal skin. In addition, wound healing as well as the cellular and biochemical processes involved in keloid formation will be discussed. Present theories of keloid formation and treatment options will also be considered.

2.1 STRUCTURE, PHYSIOLOGICAL FUNCTION AND THE CELLS OF THE SKIN

The skin is divided into two main layers, the epidermis and the dermis. The outer epidermis is the thinner layer, composed of keratinized stratified squamous epithelium, consisting of four main types of cells; melanocytes, keratinocytes, Langerhans' and Merkel cells. The majority (90%) of these cells are the keratinocytes. Throughout the epidermis keratinocytes are found in different stages of growth and differentiation and this ensures the rapid and constant replacement of cells that are shed during daily living. The dermis is below the epidermis and consists of connective tissue, nerves and blood vessels. Fibroblasts present in the dermis are the proliferative cells of this layer (Tortora and Derreckson, 2008) which release paracrine factors that regulate the growth and differentiation of other cells such as keratinocytes endothelial cells and immune cells. A schematic diagram of the basic tissue and cellular structure of human skin is shown in Figure 2.1. Figure 2.2 is a typical histological section of human skin also showing the main tissue and cellular components of skin.

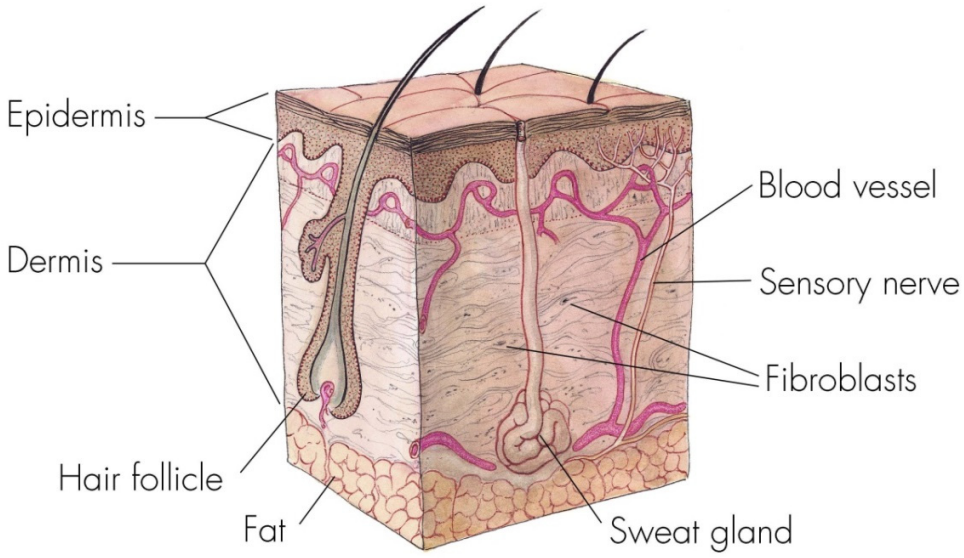


Figure 2.1: Schematic diagram of a cross section of the skin showing the dermis, epidermis and skin appendages. (http://publications.nigms.nih.gov/biobeat/10-07-21/10-07-21-2.jpg).

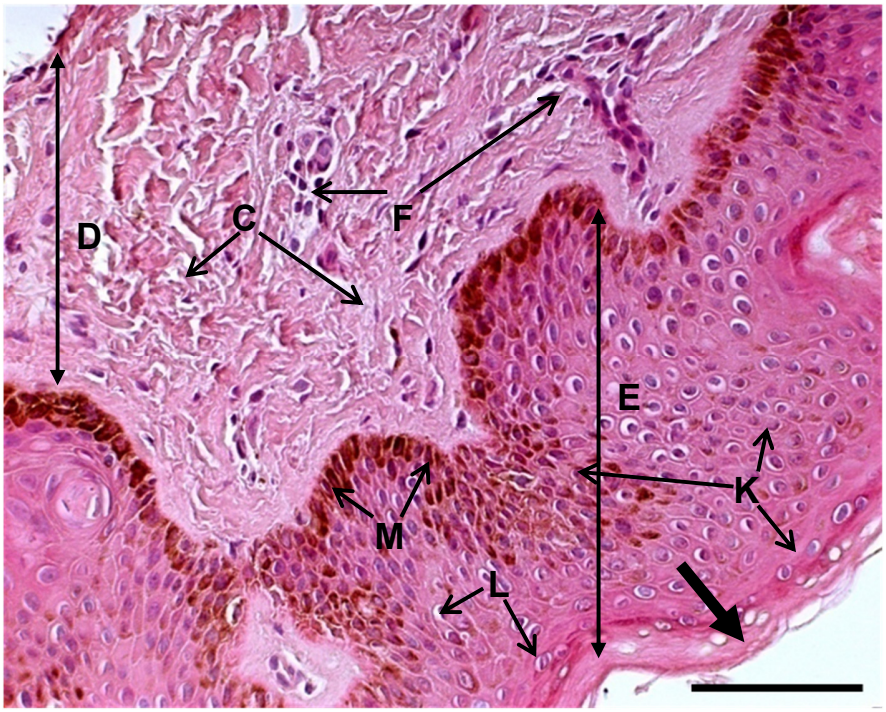


Figure 2.2: Histological section of normal human skin showing the epidermis (E) and dermis (D), the presence of collagen (C), keratinocytes (K), melanocytes (M), Langerhans' cell (L), fibroblasts (F) and the keratinised layer of epidermis (arrow). Bar = 10 µm.

2.1.1 Structure of the of the epidermis

The epidermis consists of stratified squamous epithelium with four layers of cells in thin skin and five layers in thick skin. The first innermost layer is the *stratum basale* which contains the proliferating or mitotically active keratinocytes which serve as the stem cells of the epidermis from which new keratinocytes grow. These cells contain some melanin in their cytoplasm which is transferred to these cells by the surrounding melanocytes. With growth the keratinocytes move to the next layer the *stratum spinosum.* This layer consists of spinous, larger keratinocytes, containing short processes which form between adjacent cells. These processes can be clearly seen with a light microscope as the cells shrink slightly during sample preparation. The next layer is the *stratum granulosum* which contains large flattened cells with intensely staining cellular granules rich in histidine and cysteine which are precursor molecules of the protein flaggerin, that later forms the keratin filaments in keratinised cells of the uppermost layer. The s*tratum granulosum* can consist of up to three cell layers, and is easily seen in histological sections. *Stratum lucidium* is the next layer and is found only in thick skin. It is believed to be a subdivision of the *stratum corneum*. It stains poorly in histological sections and contains highly keratinized cells devoid of nuclei and cytoplasmic organelles. The final and uppermost layer in contact with the external environment is the *stratum corneum*. It contains flattened and desiccated keratinocytes. These cells are the most differentiated cells of the epithelium and are coated with a layer of lipids which provide a water barrier. The thickness of the layer varies according to its location in the body, and it can become thicker when exposed to unusual amounts of friction. Found throughout the epidermis are melanocytes, keratinocytes, Langerhans' cells and Merkel cells (Tortora and Derreckson, 2008).

2.1.2 Structure of the dermis

The dermis is found directly below, and is attached to the epidermis. Attachment between the two layers is enhanced by increasing the areas of contact by papillae or ridges associated with the dermal epidermal border, projecting into the dermis. Areas of skin where there is a lot of mechanical stress such as the palmar surface of the hand and the plantar surface of the foot have a thicker epithelial layer. The dermal papillae in such areas are longer and more closely spaced to increase the strength of attachment to the dermis.

The dermis is mainly made up of connective tissue, including collagen and elastic fibres. The dermis consists of two layers, the papillary and the reticular layers. The papillary layer is more

superficial and contains loose connective tissue. The collagen in this layer is thinner and consists mostly of type I and type III collagen while the elastic fibres are thread- like and form an irregular pattern. The papillary dermis also contains nerve fibre endings and capillary blood vessels. In contrast the reticular layer contains thicker and irregular type I collagen bundles with fewer cells than the papillary layer. The thickness of the papillary layer varies and the elastic fibres in this layer are coarser and form regular lines of tension in the skin. Blood vessels, lymphatic vessels and nerves are also found in the reticular dermis. Layers of adipose tissue, smooth muscle or striated muscle can be found directly below the reticular layer. The cells found in this layer include the keratinocytes, fibroblasts, melanocytes, Langerhans' cells and Merkel cells, as well as mast cells (Tortora and Derreckson, 2008)

2.1.3 Physiological functions of the skin

There are six main functions of the skin and all contribute equally to normal body function. The first is thermoregulation to keep the body temperature constant. The skin achieves this by producing sweat at its surface in response to external environmental heat or internally produced heat, generated by exercise and metabolism. When sweat production is increased it cools down the skin surface as it evaporates. In the case of a low environmental temperature, sweat production is decreased and heat production is conserved, the blood vessels at the surface constrict, reducing the flow of blood and therefore conserving heat energy. The skin also serves as a blood reservoir as the dermis has many blood vessels which carry 8 - 10% of the total blood in a resting adult. Protection is the third function; the keratinised layer of cells in the epidermis protects the underlying tissues from microbes, water and toxic chemicals as well as abrasions and heat. Lipids that are released by lamellar granules make the surface waterproof, thereby preventing moisture from reaching tissues as well as leaving them through evaporation (Tortora and Derreckson, 2008). Sebum naturally contains bactericidal chemicals which kill bacteria and the acidic pH of the skin retards their growth if attached. Epidermal Langerhans' cells alert the immune system of any microbial invaders which pass the first two barriers and macrophages in the dermis are activated to engulf the invading organisms. Another function is cutaneous sensation and this includes pressure, vibration, tickling, and temperature changes on the surface of the skin as well as pain. Pain is extremely important as this is associated with tissue damage. Tactile disks in the epidermis, corpuscles of touch in the dermis and hair root plexuses at the surface of the skin are utilised in the transmission of these sensations. The fifth function is excretion of water, salts, ammonia and urea through sweat. An adult will lose

between 200 ml and 400 ml of water through the evaporation of sweat every day without exercise. These amounts may increase depending on the amount of physical activity. Water soluble substances can be absorbed through the skin, as well as fat soluble vitamins, some drugs, oxygen and carbon dioxide. Toxins can also be absorbed and these include organic solvents, heavy metals such as arsenic as well as plant toxins. Synthesis of vitamin D is the sixth function of the skin and involves the activation of a precursor vitamin D molecule in the skin's surface by ultraviolet light (Tortora and Derreckson, 2008). Enzymes in the liver and kidney then modify this molecule to produce calcitrol from a precursor during a multistep process. In short, 7- dehydrocholesterol is converted into cholecalciferol, which is hydroxylated by liver enzymes into calcifediol. Calcifediol is then hydroxylated into the active ligand, or active form of vitamin D, calcitrol (Gottfried *et al*., 2006).

2.1.4 Cellular components of the skin

2.1.4.1 Keratinocytes

Keratinocytes are the predominant cell type of the dermis and originates in the *stratum basale.* Characteristic to keratinocytes is the production of keratin which forms the intermediate filaments which make up 85% of the fully differentiated cell. Histologically the mature cytoplasm of keratinocytes appears basophilic with granules. Ultrastructurally these cells have free ribosomes, small Golgi apparatus, rough and smooth endoplasmic reticulum and mitochondria. Keratinocytes are cuboidal or columnar in shape, have a diameter of 6 - 10 µm, with a basophilic cytoplasm and a large nucleus.

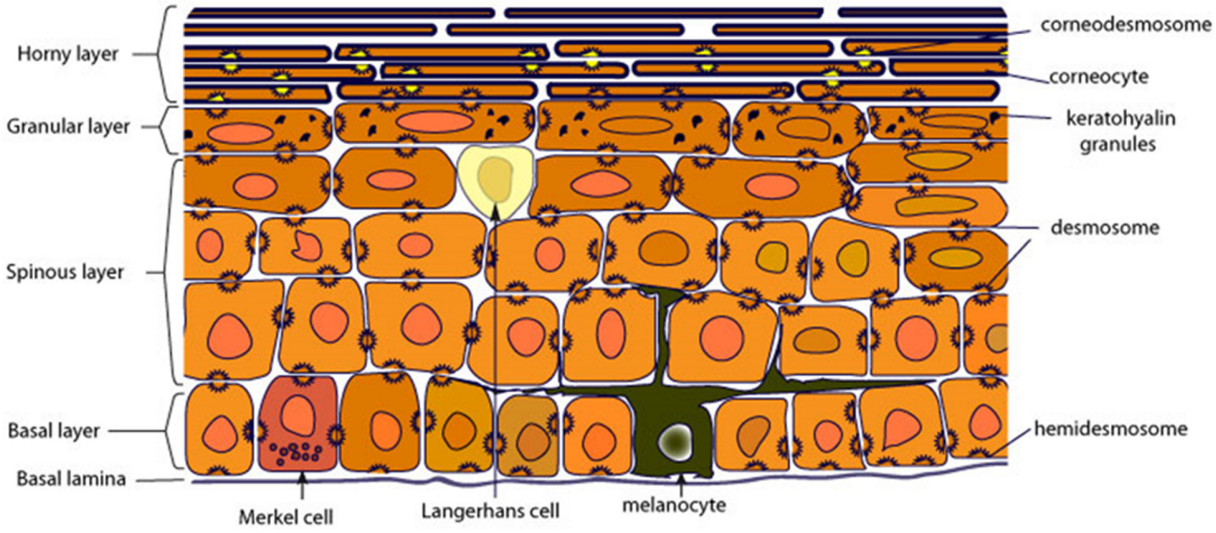


Figure 2.3: Schematic diagram of keratinocyte layers in the epidermis. (http://biologiedelapeau.fr/spip.php?article3&lang=fr)

In the basal cell layer keratinocytes are attached to the basement membrane by hemidesmosomes (Figure 2.3). When viewed with an electron microscope (EM) melanosomes are in a perinuclear distribution. Loose tonofilaments and electron dense cytoplasmic bundles of intermediate tonofilaments which contain keratin polypeptides insert into the attachment plaques of desmosomes and this contributes to the mechanical resistance of keratinocytes (Kanitakis, 2002). Between keratinocytes and adjacent cells are intercellular junctions with gap and adherence junctions (Figure 2.3) being the most characteristic.

In the spinous layer, S*tratum spinosum,* which is above the basal layer (Figure 2.3) the keratinocytes are larger (10 – 15 µm) and are polygonal in shape with a vesicular nucleus. These keratinocytes contain coarse bundles of tonofilaments which are concentrated at the periphery of the cytoplasm and are also found in the projections that end in the desmosomal junctions which connect the keratinocytes and do not pass from one keratinocyte to another. The spaces between the keratinocytes can contain fine processes of melanocytes and Langerhans' cells.

In contrast the keratinocytes in the granular layer (Figure 2.3) are flattened and lay parallel to the surface of the skin, are 25 µm in diameter containing keratohyaline granules and highly basophilic polygonal grains of proflaggrin protein and keratin. In the lamellar bodies, also known as keratinosomes, are keratohyaline granules which are involved in desquamation and formation of a lipidic pericellular coat which acts as an impermeable barrier.

The outer layer, the horny layer or the *stratum corneum* (Figure 2.3) is made up of highly flattened keratinocytes with no nuclei or cytoplasmic organelles. Instead the cells have a dense filamentous keratin matrix and thick cornified envelope of cross linked proteins (Young and Heath, 2007; Kanitakis, 2002).

Besides a protective role keratinocytes also have a secretory role and affect surrounding cells, but also other areas by modulating the immune system. Fibroblast behaviour is believed to be modulated by keratinocytes as was reviewed by Werner *et al*. (2007).

2.1.4.2 Melanocytes

Melanocytes are derived from the embryological neural crest and migrate to enter the developing epidermis and are later found scattered around the basal cells in the *stratum basale.* The functional association of these cells is established during migration, and a set ratio of melanocyte cells to fibroblast cells is created and maintained throughout life. This ratio is between 1:4 and 1:10 depending on the area of the body, and it is constant in all races (Kanitakis, 2002). Variation in skin colour is dependent on the amount of melanin produced. In adults undifferentiated melanocytes remain around the follicular bulge, an area around the hair follicle, and differentiation is regulated by the Pax 3 gene. Only following differentiation do the melanocytes move to the surrounding areas, where the melanocytes produce and distribute melanin to the surrounding keratinocytes in order to protect the cells from the damaging effects of ultraviolet (UV) radiation (Kanitakis, 2002).

Melanosomes are the cellular sites for the synthesis, storage and transport of melanin pigment in melanocytes. Fate and function of melanocytes varies according to the cell type and these include the mammalian skin melanocytes, chordial melanocytes and retinal pigment epithelial cells in the eye. The function of mammalian skin or epidermal melanocytes is to supply the neighbouring keratinocytes with melanosomes and therefore pigment. Synthesis of melanin is stimulated by UV irradiation which generates deoxyribonucleic acid (DNA) photoproducts and leads to the release of various autocrine and paracrine factors, most important being α-melanocyte stimulating hormone or propiomelanocortin which is secreted by keratinocytes. It activates melanocortin1 receptor in the plasma membrane of skin melanocytes and results in the cyclic adenosine monophosphate (cAMP)-dependant signalling and stimulation of expression of microphthalmia-associated transcription factor (MITF) which regulates melanocyte function and melanogenesis. Its function is affected by different genes that are involved in

melanocyte survival, motility, differentiation and apoptosis (Raposo and Marks, 2007; Harper *et al*., 2008; Theos *et al*., 2006; Valencia *et al*., 2007).

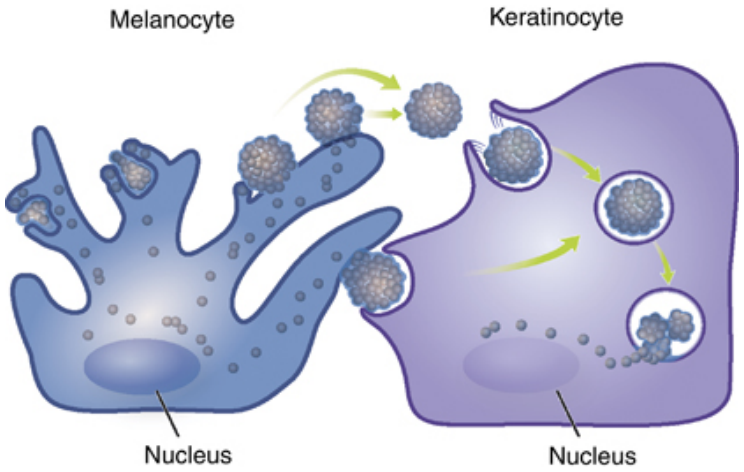


Figure 2.4: Schematic diagram of melanosome transfer. Melanosomes are packed in globules enclosed by the melanocytes plasma membrane, released into the extracellular space from the melanocyte dendrites and phagocytosed by keratinocytes and dispersed around the nucleus (Ando *et al*., 2012).

In the process of melanosome maturation, four distinctive stages can be identified. In stage one, pre-melanosomes which are non-pigmented granules originate from the endosomal system. During stage two, the melanosomes acquire their characteristic internal structure and here the trans-membrane proteins tyrosinase and tyrosinase-1 (TYR1) initiate pigment synthesis and further maturation. This leads to melanin pigment being deposited within the striations. At stage three, tyrosine is converted to dihydroxyphenylalanine (DOPA) which is then polymerised into melanin (Kanitakis, 2002; Rapso and Marks, 2007; Harper *et al*., 2008; Theos *et al*., 2006). Stage four is characterised by fully melanised melanosomes. The melanosomes pigment is then able to bind to a protein and be transported along cytoplasmic processes to be transferred through phagocytosis into the cytoplasm of basal *stratum spinosum* keratinocytes as shown in Figure 2.4 (Hearing, 2005; Nascimento, 2003; Seabra, 2004; Ross and Paulina, 2004; Ando *et al*., 2012)

In light microscopy, the dark colour of melanin can be used for the identification of these cell types in histological sections. If the melanin content of melanocytes is low, it can be detected histologically using ammoniacal silver nitrate, a DOPA histoenzymatic reaction or immunocytochemistry with antibodies for melanocyte specific antigens (Kanitakis, 2002).

Ultrastructurally melanocytes appear to have an electron-lucent cytoplasm with loose intermediate filaments (vimetin) and melanosomes at various maturation stages (Young and Heath, 2006).

2.1.4.3 Langerhans' cells and other immune cells

Langerhans' cells are round, dendritic appearing cells which originate from the lymphoid progenitor cells. They are the antigen presenting cells of the epidermis. Together with mast cells and macrophages these cells support the immunological and inflammatory processes in the skin (Harper *et al*., 2008; Theos *et al*., 2006). Langerhans' cells are able to take up endogenous antigens that are associated with skin infections. These antigens are processed and then presented to T-cells. Langerhans' cells are present in all layers of the epidermis and are easily seen in the *stratum spinosum* as the cytoplasm is pale staining and extends between the keratinocytes. Specific antigens used to identify these cells using immunocytochemistry are the CD4a antigens and antigens associated with the Bibeck granules. Histoenzymatic ATPase can also be used to identify these cells (Harper *et al*., 2008). Ultrastructurally, the cytoplasm of these cells contains Birbeck granules which are rod like structures with regular cross striations that distend into vesicles. The role of Bibeck granules is not well understood (Shevchuk *et al*., 2014). Most studies theorise that Bibeck granules are involved in receptor-mediated endocytosis (Valladeau *et al*., 2000) and participate in the antigenic function of Langerhans' cells (McDermott *et al*., 2002).

2.1.4.4 Mast cells

Human mast cells arise from the $CD34^{+}/CD117^{+}$ pluripotent progenitor cells in the bone marrow (Skaper *et al*., 2012). The morphology and phenotype of mast cells is characteristically that of a 20 µm ovoid or irregularly elongated cell with an ovoid nucleus. They are also seen to contain metachromatic cytoplasmic granules caused by heparin and chondroitin sulphates. Crystalline granules are seen when observing the mast cell with EM, but the granules become amorphous

after activation of the cell (Hsu *et al*., 2009; Metz *et al*., 2008). Mast cells are identified ultrastructurally by a monolobed nucleus, narrow elongated folds in the cell membrane and the cytoplasmic granules. Mast cell cytoplasm also contains Golgi apparatus, mitochondria and ribosomes, intermediate filaments and lipid bodies (Dvorak, 2005). Mast cells in humans are classified according to locations in the body and these are mucosal, serosal or brain. Mast cells can also be classified according to their granule content. Another distinguishing factor of mast cells is whether the cell contains tryptase alone, or tryptase and chymase, in which case it is referred to as a mast cell containing tryptase (MCT) or a mast cell containing tryptase and chymase (MCTC) (Stone *et al*., 2010). Tryptase is a major protease of human mast cells, and it stimulates collagen synthesis, as was seen in cell cultures of human lung fibroblasts. Tryptase increases cell numbers, DNA synthesis as well as collagen synthesis in fibroblasts. The ability of tryptase to stimulate collagen production is consistent with it having a role in fibrosis, and it is seen to specifically stimulate the production of type I collagen (Cairns and Wells, 1997).

Mast cells have the ability to change their phenotype depending on their environment (Pang *et al*., 1996; Shanas *et al.,* 1998). MCTs are predominantly found in connective tissue and are the predominant mast cell type. Tryptase specific staining is a common way to identify all mast cells (Stone *et al*., 2010).

The main function of mast cells in the human body is to respond to signals of adaptive and innate immunity, releasing inflammatory mediators. The release of these mediators can be both immediate and delayed. The presence of mast cells in tissues is mostly associated with pathogenesis, hypersensitivity and fibrosis. In the skin, large numbers of mast cells are seen in areas of wound healing (Noli and Miolo, 2001; Wulff and Wilgus, 2013)

As essential components of inflammation (Prussin and Metcalfe, 2003), mast cells trigger hypersensitivity reactions through antigen specific immunoglobulin E (IgE) production and the binding of IgE to fragment crystallisable epsilon receptor I (FcεRI) receptors on mast cells. Delayed and immediate release of inflammatory mediators occurs as a response to the binding of IgE to the FcεRI receptors. Mast cell mediators are found in the granules of mast cells and are released during inflammation (Stone *et al*., 2010). The release of these mediators is then followed by the synthesis of lipid mediators and then the synthesis of cytokines and chemokines (Galli and Tsai, 2012; Metcalfe, 2008). The ability of mast cells to immediately release their mediators, leading to the release of other substances over time, indicates a catalytical function for mast cells, i.e. amplifying and prolonging cellular and molecular responses.

Mast cell mediator release occurs through the degranulation of the mast cells. Degranulation is affected by IgE, as mentioned above, and a few other factors such as compound 48/80, opiates, neuropeptides in skin (Barke and Hough, 1993; Banks *et al*., 1990), anaesthesiology drugs (Marone *et al*., 1993) as well as asthma factors such as stress that can cause mast cell degranulation linking mast cells to nerve function (Bienenstock *et al*., 1991). Bee sting venom contains a peptide called mast cell degranulation peptide (MCDP) which stimulates degranulation and release of the granular contents of mast cells (Chen and Larviere, 2010).

Human dermal mast cells express messenger ribonucleic acid (mRNA) for epithelial and connective tissue growth factors, which are fibroblast growth factors (FGF)-2, 7, 10 and heparin binding- epidermal growth factor (HB-EGF)-like growth factor. Mast cells are also able to induce the secretion of FGF-2 and -7 from human dermal fibroblasts, with the FGF-7 secretion being mediated by histamine (Artuc *et al*., 2002). Histamine, tryptase, chymase, vascular endothelial growth factor (VEGF) and tumour necrosis factor (TNF)-α when released by mast cells during the start of wound healing will affect bleeding and subsequent coagulation and acute inflammation (Artuc *et al*.,1999). Histamine and heparin initiate events that have been associated with hypertrophic scarring and keloid development. Elevated histamine levels were seen to increase collagen production and heparin increases vascularisation in a developing keloid. Due to the presence and increased serum IgE associated with keloids it has been hypothesised that keloid formation is associated with mast cell hypersensitivity (Mouhari-Toure *et al*., 2012). An increased number of MCT seen in older scars suggests that the tissue is exposed to all of the factors that mast cells release throughout the scar remodelling process (Artuc *et al.,* 1999).

2.1.5 Fibroblasts and associated fibres

The over production of collagen by fibroblasts is a hallmark of keloid pathology. Fibroblasts, responsible for collagen production, are mostly present in the dermis. Histologically, fibroblasts appear as elongated cells with condensed nuclei and are arranged parallel to the direction of collagen fibres. The cytoplasm is reduced in volume with long cytoplasmic processes that connect with other fibroblasts. At an ultrastructural level fibroblasts appear to be large cells with a moderately condensed nuclei, however fibroblasts are surrounded by collagen and elastin fibrils, and have a small quantity of cytoplasm, most of which is filled with rough endoplasmic

reticulum (RER). Golgi apparatus and a few mitochondria are also present (Young and Heath, 2006).

2.1.5.1 Myofibroblasts

Myofibroblasts are differentiated fibroblasts that appear temporarily in granulation tissue during wound healing and can also be found in fibrotic lesions (Grinell 1994; Eddy, 1988). Myofibroblasts cause wound contracture during the final stages of wound healing and scar formation and can be detected in the tissue three days after wounding and will reach highest numbers during the period of contraction. These cells may continue to be detected in scars long after the process of wound healing has ceased (Majno *et al*., 1971). The ultrastructure of a myofibroblast is described as an intermediate between a fibroblast and a muscle cell (Ryan *et al*., 1974; Gabbiani 1976; Gabbiani 1994). RER, mitochondria and Golgi apparatus are classic fibroblast features, with the addition of bundles of cytoplasmic filaments which have the appearance and antigenic properties of myofilaments found in muscles. The presence of α-smooth muscle actin (α-SMA) is a characteristic by which myofibroblasts can be identified using immunocytochemistry (Gailit *et al*., 2001). In EM myofibroblasts are identified by the presence of a fibronexus, a fibronectin fibril near the lamina. It is differentiated from the lamina by increased staining density compared to the lamina and a deviation of the fibronexus from the cell surface into the matrix. The fibronexus is rigid and straighter looking than the lamina and has an internal structure that consists of fine longitudinal filaments (Eyden, 2001).

The majority of fibres produced by fibroblasts are interstitial collagen, mainly type I and type III collagen which makes up about 98% of the dried dermis mass. The function of these collagen fibres is to provide mechanical strength to the skin. Collagen fibres have a diameter of 100 nm and in longitudinal section have cross striations. These fibres are arranged in bundles which are loose in the papillary dermis and are more densely arranged in the reticular dermis. Type IV collagen is also found in the dermis and is located in the basement membranes, blood vessels, muscle and nerves. Type IV collagen is found in the anchoring fibres of the dermal-epidermal junction (Kanitakis, 2002).

Elastic fibres are also produced by fibroblasts and in the skin are responsible for the ability of the skin to retract. The fibres are thin in the papillary dermis, and become thicker in the reticular dermis. The arrangement to the elastic fibres is scattered to horizontal. Elastic fibres are composed of elastin and are surrounded by microfibers of fibrillin (Kanitakis, 2002).

2.1.5.2 Elastin

Elastin is an important structural, rubber like protein that is arranged as fibres and/or discontinuous sheets and is found in the extracellular matrix (ECM) of most connective tissues, particularly of the skin, lung and blood vessels, where it allows stretching and elastic recoil. Elastin is synthesised by fibroblasts as tropoelastin which undergoes polymerisation in the extracellular tissues. To deposit elastin in the form of fibres requires the presence of microfibrils of the structural glycoprotein fibrillin which then becomes incorporated around and within the elastic fibres (Young and Heath, 2000). Proelastin is a soluble precursor of elastin and the presence of many cross links makes mature elastin insoluble. Cross links form between the lysine and alanine groups. Cross links that are found in collagen are also present in elastin (Stryer, 1981).

2.1.6 Ground substance

Proteoglycans form the ground substance of connective tissue and are composed of polysaccharide chains. Seven types with different repeating disaccharide and protein units have been identified. One of the units is usually a uranic acid and the other an amino sugar forming glycosaminoglycan (GAG). The predominant GAG found in loose connective tissue is hyaluronic acid. The proteoglycans in the ground substance determine the viscoelastic properties of connective tissues. Ground substance is seen between the cells as an amorphous transparent material with semi-gel like properties. Tissue fluid is loosely associated with ground substance, forming the medium for the passage of molecules through supporting tissues and for the exchange of metabolites with the circulatory system (Stryer, 1981; Young and Heath, 2000).

2.1.7 Adipose

Adipose tissue or fat is considered an organ with metabolic functions which forms 15 – 20 % of weight in a normal adult person, providing a large energy reserve (Geneser, 1986). Adipose tissue is found in the connective tissue under the skin of the abdomen, buttocks, axilla and thigh. Gender differences in adipose thickness and distribution result in different shapes in males and females. Inside the body adipose tissue is found in the greater omentum, mesentery and retroperitoneal space and is abundant around the kidneys. Adipose tissue is a good insulator and reduces heat loss through the skin and it supports and protects internal organs

(Tortora and Derreckson, 2008). Lipid is most suited to energy storage as the bound chemical energy per weight or volume unit is larger than that of carbohydrate or protein (Geneser, 1986).

Yellow or white adipose tissue makes up the main part of body fat. It is also known as unilocular adipose tissue as the cells contain only a single lipid droplet. Brown adipose tissue is scarcer and occurs only in certain regions. It is known as multilocular adipose tissue as the cells contain many small lipid droplets (Geneser, 1986). In adults, adipose tissue is white and in foetuses and infants it is primarily brown. The colour variation is as a result of the many pigmented mitochondria in brown adipose tissue. The mitochondria participate in cellular respiration, and therefore brown adipose helps to maintain body heat in newborns (Tortora and Derreckson, 2008) while white adipose tissue functions in energy storage, insulation and cushioning of vital organs.

Adipose is a loose connective tissue in which adipocytes and fibroblast derived cells, are specialized for the storage of triglycerides. The cells fill up with a single large triglyceride droplet which forces the cytoplasm and nucleus to the periphery of the cell (Tortora and Derreckson, 2008). The nuclei of adipocytes are oval to flat with finely granular chromatin and have no nucleoli. Mitochondria and small Golgi apparatus are visible with EM and an agranular endoplasmic reticulum (ER) in the cytoplasm near the lipid droplet. Unilocular fat cells can be more than 100 µm in diameter and form a layer called the *panniculus adiposus* or hyperdermis in the connective tissue under the skin. The spherical cells distort each other when they occur in groups, resulting in polyhedral shaped cells. In histologic examinations the cells are seen as collapsed cells, as the lipid dissolves during sample preparation (Geneser, 1986).

In summary, the skin is a complex organ that contains many cell types, each that interact with each other through autocrine and paracrine mechanisms. Keloids are associated with an overproduction of collagen and therefore its structural features, cellular biosynthesis and degradation will be discussed in greater detail.

2.1.8 Collagen

Collagen is the main fibre found in most supporting tissues and is the most abundant protein in the human body. It provides tensile strength to the tissues and is secreted into the ECM in the form of tropocollagen, a helical structure consisting of three α- chains with a length of 300 nm

and a diameter of 1.5 nm. The tropocollagen molecules are polymerised in the ECM to form collagen fibres (Young and Heath, 2006).

There are various types of collagen. At least 28 different types exist in vertebrates and these are named from I to XXVIII (Myllyharju and Kivirikko, 2001). The collagen family also consists of collagen-like proteins that are involved in immunity and neurotransmission, such as C1q and mannan binding protein, and cholinesterase respectively (Myllyharju and Kivirikko, 2001; Hulmes, 1992). Fibril forming collagens are intermediate in length and range in diameter from 12 – 500 nm depending on the stage of development and tissue (Bella *et al*., 2006). As the focus of this study is skin, collagen types found in the support tissue in skin will be discussed in greater detail.

Type I collagen is found in fibrous supporting tissue, the dermis of the skin, tendons, ligaments and bone. It may be arranged loosely or densely depending on the mechanical support required by the tissue. Type II collagen is found in hyaline cartilage and consists of fine fibres dispersed in the ground substance (Young and Heath, 2000). This type of collagen acts as a shock absorber in the joints and vertebrae (Diegelmann, 2001).

Type III collagen found in the reticulum, has affinity for silver salts and is present in highly cellular tissue such as the liver, bone marrow and lymphoid organs (Young and Heath, 2000).

Adult skin contains about 80% type I and 20% type III collagen. Newborns have a high percentage of type III collagen in their tissues, making it flexible and supple. Flexibility of blood vessels is due to the presence of type III collagen, and its expression is increased during the initial stages of wound healing (Diegelmann, 2001).

Type IV collagen form anchoring fibrils that link to the basement membrane and basal lamina by interacting with the non-collagenous components of the basement membranes to form a filtering meshwork which functions as a filtration system. It is found in the blood vessels, kidney and skin where it controls the movement of oxygen, filters blood and separates the epidermis from the dermis, respectively (Young and Heath, 2006; Diegelmann, 2001).

Type V collagen is found in all tissues and is associated with types I and III. It is found around many cells and functions as scaffolding. The largest amount of collagen type V is found in the intestine which has a larger amount of type V collagen than any of the other tissues (Diegelmann, 2001).

2.2 CELLULAR BIOSYNTHESIS OF COLLAGEN

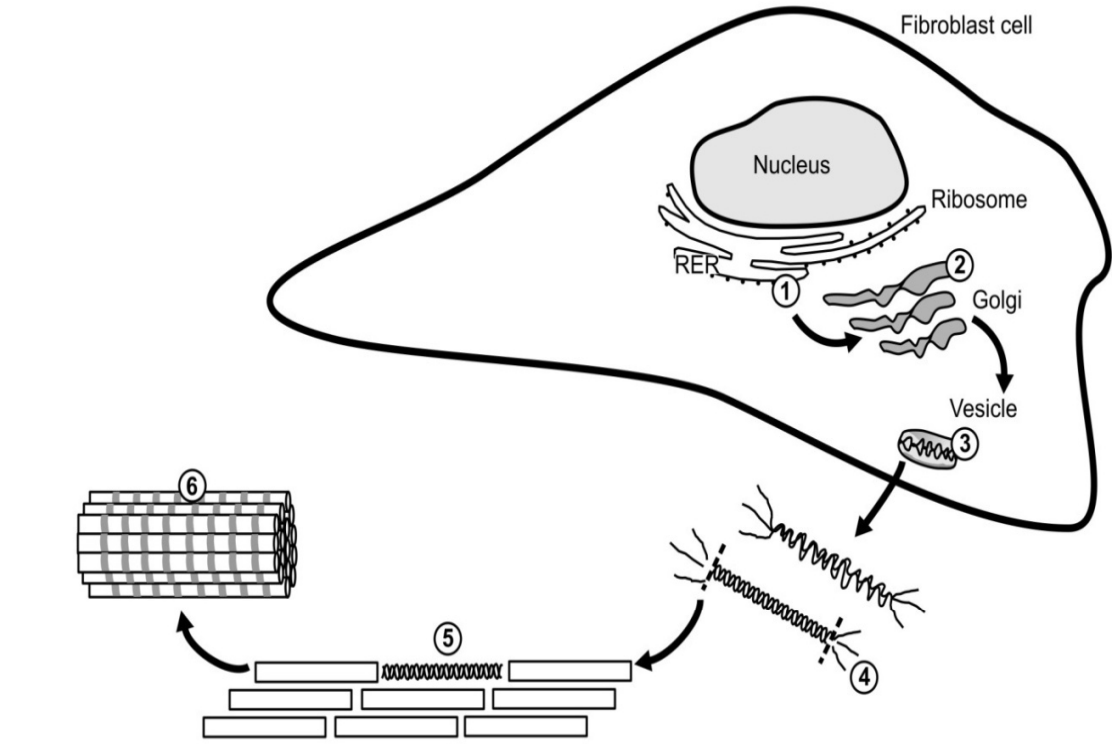
2.2.1 Intracellular

The process of intracellular collagen formation is shown in figure 2.5. Each collagen type is encoded by a specific gene and these genes are found on different chromosomes. mRNA is transcribed for each collagen type and then undergoes various steps in mRNA processing. The final mRNA attaches to the site of protein synthesis and translation occurs on the RER where pro-collagen is produced during the translation step (Diegelmann, 2001) (Figure 2.5; Step 1). Pro-collagen contains extension proteins on each side called amino and carboxy pro-collagen extension pro-peptides. These peptide sequences increase the solubility of the molecule which allows easy movement in the cell as it undergoes further post translational modifications which take place in the Golgi apparatus of the endoplasmic reticulum (ER) (Kivirikko and Risteli, 1976).

Procollagen molecules are assembled within the RER from the three constituent polypeptide (α) chains. Each chain has a repeating Gyl-X-Y sequence in which Gly are glycyl residues on every third position, X position is proline and the Y position is occupied by 4-hydroxyproline (Bella *et al*., 2006; Lamande and Bateman, 1999). The Gly-X-Y domain contains around 1000 residues and is uninterrupted in most collagens (Kadler *et al*., 2007).

For the pro-collagen to be formed into collagen a few steps occur. Firstly, to form the pro-collagen molecule, three of the pro-collagen chains need to be wound together. This is achieved through the formation of disulphide bonds at the amino terminals of the pro-collagen chains. Then three pro-collagen chains align and wrap around each other to form a single structure. This process is called registration and it takes place in the RER. The three chains are held together by hydrogen bonds (Bella *et al*., 2006).

Chain assembly depends on collagen type and can either be identical as in type III collagen which has three α1(III) chains, or different as in type I collagen with two α1(I) chains and one α2(I) chains. As described earlier pro-collagen has non triple helical domains at their N- and C-termini, known as collagen propeptides (Kadler *et al*., 2007). The C-propeptide is believed to determine chain selection as well as direct chain association during intracellular assembly of the procollagen molecules (Hulmes, 2002) causing the chain to associate into groups of three. Nucleation and folding of the triple helical regions from the C- to the N- terminus follows (Lees and Bulleid, 1994; Engel and Prockop, 1991). This folding involves the formation of intra- and interchain disulphide bonds (Lees and Bulleid, 1994).



① Procollagen synthesis
Hydroxylation of proline + lysine
Initial glycosylation + formation of triple helices
} In ribosomes of RER
② Terminal glycosylation in Golgi apparatus
③ Packaging into secretory vesicles and procollagen exocytosis
④ Non-helical prodomain cleaved
⑤ Assembly of procollagen ➡ formation of collagen fibrils (near cell surface)
⑥ Cross-linking of fibrils ➡ formation of collagen fiber

Figure 2.5: Process of collagen formation inside a fibroblast, exocytosis and its assembly and cross-linking in the extracellular matrix.

Hydroxylation follows registration (Figure 2.5, Step 1), and selected proline and lysine amino acids are turned into hydroxyproline and hydroxylysine with the help of hydroxylases, and

vitamin C and iron as cofactors. Hydroxyproline is necessary to form strong collagen molecules and its absence results in a molecule that is unable to form proper helical structures. These structures are weak and easily destroyed, which leads to poor wound healing and scurvy which is associated with a deficiency of vitamin C.

Glycosylation of some of the hydroxylysine residues occurs with the addition of glucose or galactose (Figure 2.5, Step 2). The glycosylation enzyme activity is high in the very young and decreases with age. The procollagen molecule is extremely soluble so that it can be transported easily within the cell in transport vesicles to the cell surface where it is then secreted into the extracellular space (Diegelmann, 2001) (Figure 2.5, Step 3). Steps 4 – 6 (Figure 2.5) will be discussed in section 2.2.3.

2.2.2 Transport and secretion

After synthesis and post translational modifications the procollagen is packaged into transport vesicles for delivery to the Golgi apparatus and then secreted into the ECM. The coat protein II (COPII) is a protein cage assembled on the ER membranes and assists in the transport of soluble secretory proteins and small transmembrane proteins to the Golgi apparatus. It coordinates the collection of secretory cargo with the formation of transport vesicles (Stephens, 2012; Zanetti *et al*., 2011; Townley *et al*., 2008; Barlowe *et al*., 1994).

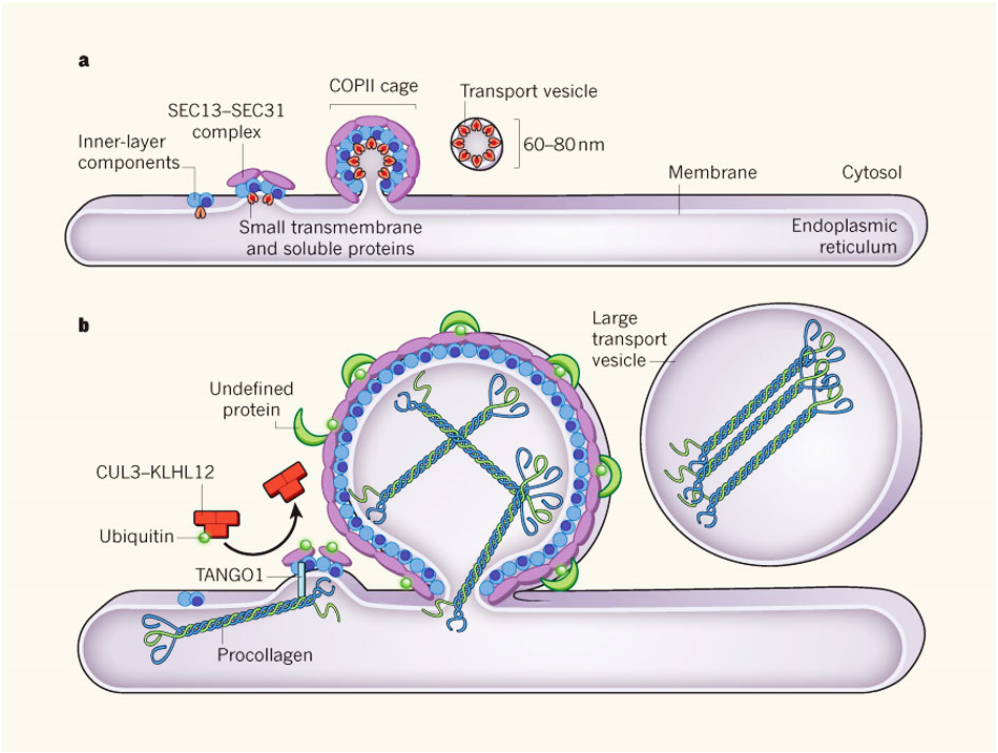


Figure 2.6: Schematic representation of COPII cage formation and subsequent transport vesicle formation on the ER (Stephens, 2012).

The assembly of these vesicles on the ER (Figure 2.6) is initiated by Sec12, a transmembrane protein within the ER membrane. Proteins that form the inner layer of the COPII coat associate first and then Sec12 serves as a catalyst in the recruitment of a complex known as Sec23/24 which captures cargo proteins into the budding vesicles.

The assembly of the COPII continues (Figure 2.6) with recruitment of another protein complex Sec13/31 which forms the outer layer or structural cage around the bud (Stephens, 2012; Sato and Nakano, 2005). Sec13 is required for transport vesicle formation on the ER (Salama *et al*., 1993) and for the maintenance of structural stability of Sec31 (Townley *et al*., 2008) while Sec31 is required for the ER to Golgi transport (Salama *et al*., 1997; Tang *et al*., 2000).

Membrane deformation occurs and a vesicle of 60 – 80 nm is formed from the bud (Figure 2.6) (Stephens *et al*., 2012). Because procollagen synthesised in the ER is 300 nm in size (Stephens, 2012) and transport vesicles of the ER are smaller than 100 nm (Zanetti *et al*., 2011) one of the COPII proteins, Sec31 is modified by ubiquitination. Here a smaller protein ubiquitin

is attached to the Sec31 protein in the Sec13/31 complex by the enzyme ubiquitin ligase (CUL3-KLHL12) (Jin *et al*., 2012).

The efficient assembly of the COPII coat, transport of the vesicles and secretion of the ECM is dependent on sufficient levels of Sec13/31. Suppression of Sec13 inhibits collagen secretion and deposition, and causes the ER to be distended with the accumulation of budding vesicles on the membrane (Townley *et al*., 2008). The distended ER and vesicle buds are seen in fibroblasts of patients with cranio-lenticulo-sutural dysplasia (Boyadjiev *et al*., 2006; Fromme *et al*., 2007). Collagen accumulation within the fibroblasts was observed in this disease. Cells depleted of Sec13 showed a decreased extracellular collagen deposition and intracellular accumulation of procollagen was observed (Townley *et al*., 2008).

2.2.3 Extracellular fibril formation

Following or during secretion into the ECM, pro-collagen proteases (N- and C- terminals) remove the extension peptides (Figure 2.5, Step 4). This causes the procollagen molecules to be assembled into fibrils and fibres (Kadler *et al*., 1996).

Processing and associated removal of the N- and C- collagen propeptides occurs proteolytically by specific N- and C- proteinases (Colige *et al*., 2005; Greenspan, 2005). This results in the soluble pro-collagens becoming mature, insoluble collagen molecules which then assemble into fibrils (Kadler *et al*., 1996). C-propeptide cleavage is required for fibrillogenesis (Greenspan, 2005) and specifically the presence of the C-terminal is responsible for solubility of the procollagen molecule, preventing fibril formation.

Under normal physiological conditions all fibrillar collagens are secreted into the ECM as soluble procollagen (Kadler *et al*., 2007). Procollagen I can be cleaved to a collagen molecule inside the cell and intracellular collagen fibrils were observed in protrusions of the plasma membrane, named fibripositors (Canty and Kadler, 2005).

The N-propeptide influences the shape and diameter of the fibril and does not prevent fibril formation (Brown and Timpl, 1995). N-proteinase cleaves types I, II and III at the junction of the N-propeptide triple helix and the telopeptide (Cal *et al*., 2001; Prockop *et al*., 1998). Telopeptides are the short non triple helical extensions of the polypeptide (Prockop and Fertala, 1998). If the cleavage process is delayed this leads to the accumulation of partially processed procollagen called pN-collagen which maintains the N-propeptides but lacks the C-propeptides

(Moradi- Améli *et al*., 1994; Rousseau *et al*., 1996). The presence of pN- collagen at 20% or less resulted in the formation of smaller diameter and cylindrical shaped collagen fibrils. At larger percentages the fibrils became increasingly lobular and at 75% formed stellate and branched fibrils in cross section, similar to fibrils seen for dermatosparaxis in animals where the skin is hyperextensible and fragile, which is similar to the Ehlers-Danlos syndrome in humans (Colige *et al*., 1999).

It is believed that these peptides then re-enter the cell and regulate collagen production by a feedback mechanism. Once the collagen is formed, the procollagen molecules align to form fibrils near the cell surface (Figure 2.5, Step 5) which then join to form collagen fibres in the ECM (Figure 2.5, Step 6). This step is called cross-link formation and lysyl oxidases serve as enzymes for this process. Intra and intermolecular links are formed to stabilise the final fibre and provides its strength.

2.2.4 Lysyl oxidase

Lysyl oxidase (LOX) plays an important role in collagen production and its cross linking with elastin in extracellular fibril assembly. It was first discovered in 1968 by Pinnell and Martin (Pinnell and Martin, 1968).

LOX is synthesised as a 46 kDa preproenzyme and undergoes signal peptide cleavage and N-glycosylation to yield a 50 kDa N-glycosylated proenzyme. The proenzyme is then secreted into the ECM where it is proteolytically cleaved to a 32 kDa mature enzyme by a metalloproteinase that is also secreted by fibroblasts (Smith-Mungo and Kagan, 1998; Trackman *et al*., 1992). The mature enzyme functions as a catalyst in the cross linking of collagen and elastin. Its expression is regulated by transforming growth factor beta (TGF-β), platelet derived growth factor (PDGF), angiotensin II, retinoic acid, FGF, altered serum conditions, and sheer stress (Smith-Mungo and Kagan, 1998).

LOX functions by initiating covalent cross linking between and within the molecular units of elastin and collagen by oxidising peptidyl lysine in the two molecules to peptidyl α-amminodipic-δ-semialdehyde. This peptidyl aldehyde can then be condensed with neighbouring amino groups or other peptidyl aldehydes to form covalent cross linkages seen in fibrillar collagen and elastin (Kagan, 1986). LOX may directly or indirectly affect the nuclear components of cells, as

LOX dependant alterations in chromatin structure were noted (Mello *et al*., 1995) as well as the presence of LOX in the nuclei of fibroblasts (Li *et al*., 1997).

Copper serves as a co-factor in the catalytic activity of LOX and subsequent collagen production (Gacheru *et al*., 1990). Decreased levels of LOX are seen in diseases involving copper metabolism and elevated levels of LOX are present in fibrotic conditions (Smith-Mungo *et al*., 1998).

TGF-β is known to activate the synthesis of collagen (Massague *et al*., 1994; Lawrence *et al*., 1994). The growth factor was seen to promote the expression of LOX in fibroblasts of human embryos (Roy *et al*., 1996). TGF-β1 was seen to cause a 6 - 7 fold increase in LOX mRNA synthesis. The increased synthesis is prevented in the absence of Prostaglandin E_2 (PGE_2) (Roy *et al*., 1996).

Studies have found that LOX may function within the cell as it has been localised within fibroblasts and other collagen producing cells as well as in some non-fibroblastic cells. It was not stated whether the intracellular proteins were proenzymes or mature forms of the enzyme as molecular weight of the protein was not determined (Wakasaki and Ooshima, 1990).

If the normal collagen in the tissue is injured it will be replaced by scar collagen, or collagen that is formed during wound healing. This collagen is always weaker, and scar collagen is remodelled by the body in order to attempt to achieve the ultrastructure that was present before the injury by collagen degradation and re-synthesis. Any condition that interferes with protein synthesis will have an adverse effect on collagen synthesis causing the rate of collagen degradation to be greater than collagen synthesis leading to a weak wound site. In some cases the synthesis of collagen is increased, leading to conditions such as hypertrophic scars and keloids.

The presence of different collagen types in fibres such as type I and III seen in skin is suggested to be a mechanism for diameter control through heterotypic collagen interactions (Birk 2001; Blaschke *et al*., 2000; Marchant *et al*., 1996). The N-propeptide remains in type III collagen and this may prevent the incorporation of the molecule into the centre of the fibril and force the N-terminal to the outside of the fibril which prevents further accretion and limits fibre diameter (Birk, 2001).

Maintenance of fibre diameters in connective tissues is important to structure as well as identification of the tissue. Tissue specific suprafibrillar architectures seen in polarised

microscopy are also important in identification. Fibrils of the skin have a complex three dimensional wave. The suprafibrilar architectures are characterised by distances of several micrometers compared to 0.3 µm length of individual collagen molecules (Giraud-Guille, 1996).

Early collagen fibre formation occurs in channels at the cell surface of fibroblasts (Birk and Trelstad, 1984). It has been suggested that this can begin intracellularly within the Golgi transport vesicles during embryogenesis (Canty *et al*., 2004). Fibrils form within the intermediate stage that includes nucleation and unilateral elongation of short primary fibrils which later fuse, resulting in diameter enlargement and bidirectional growth (Birk *et al*., 1996; Birk *et al*., 1995; Kadler *et al*., 2000).

Type V collagen plays a role in the initial fibril formation during collagen assembly, and it may be one of the earliest molecules assembled in the fibre as type V collagen is found deep within the collagen fibre (Birk *et al*., 1988). Under normal conditions type V collagen fibrils are assembled at the cell surface and form fibre intermediates which then enlarge by fibril fusion (Wenstrup *et al*., 2004).

2.3 COLLAGEN DEGRADATION

The breakdown of collagen is important in development and homeostasis in the wound healing process, and it can also contribute to various pathologies. There are a few collagenolytic enzymes in mammals such as alloproteinases, cathepsin K and neutrophil elastase (Fields, 2013). Matrix metalloproteinases (MMPs), cysteine proteinases and serine proteinases are able to degrade collagen *in vivo* (Sabeh *et al*., 2004).

Proteases that catalyse the hydrolysis of the collagen triple helix alter the mechanical and biochemical properties of interstitial collagen (Fields, 2013).

There are four pathways of collagen degradation and two main pathways are present in the ECM of skin. The first is a collagenase independent intracellular route which is of major importance in normal collagen turnover. Normal collagen turnover takes place intracellularly in lysosomes of fibroblasts after phagocytosis of collagen fibrils. Collagen fibrils are recognised by membrane bound integrin receptors on the surface of a fibroblast. The collagen fibril is then partially enclosed by a fibroblast. Partial digestion of the fibril and its surrounding non-collagenous proteins by gelatinase A, also known as matrix metalloproteinase 2 (MMP- 2) then takes place through hydrolysis (Jain *et al*., 2014; Madsen *et al*., 2011). MMP's belong to a family

of zinc containing enzymes (endopeptidases) that degrade cell components such as collagens, proteoglycans, laminin, elastin and fibronectin (Jain *et al*., 2014).

Phagocytosis/ endocytosis of the hydrolysed fibril fragments then takes place on mesenchymal cells, mediated by urokinase plasminogen activator receptor associated protein (uPARAP) or Endo180, a protein involved in the turnover of the ECM. On macrophages phagocytosis is mediated by the mannose receptor protein (Madsen *et al*., 2011). The mannose receptor protein has been identified to be responsible for the internalisation of collagen for intracellular degradation during fibrosis, cancer invasion and bone development (Jürgensen *et al*., 2014).

The fibril is then segregated within a membrane bound body known as a phagolysosome. A lysosome containing digestive enzymes is then able to fuse with the phagolysosome to form a digestive lysosome where the final digestion of the collagen fibril takes place by cysteine proteinases, cathepsin B and L. This process is modulated by growth factors and cytokines; TGF-β and interleukin (IL)-1α. TGF-β increases collagen fibril phagocytosis and IL-1α inhibits the phagocytic process (Jain *et al*., 2014).

The second main collagen degradation process is the mediated extracellular route. This route is important during the remodelling of tissue during wound healing and it involves large amounts of collagen and is often seen during inflammation. During this process MMPs are secreted as well as collagenase and gelatinase by fibroblasts and other cells (Jain *et al*., 2014). Extracellular MMP hydrolysis of collagen occurs and is followed by gelatinolytic MMPs that laterally diffuse on the extracellular collagen and find “tails” from the cleaved sites and denature the triple helix and further proteolyse the α-chains (Atkinson *et al*., 2001; Rosenblum *et al*, 2010).

Other collagen degradation routes include phagocytosis mediated by α1β2 integrin which leads to the lysosomal degradation by cathepsins (Arora *et al*., 2000). Collagen can also be degraded intracellularly by autophagy mediated lysosomal processes which may be another form of collagen degradation (Kim *et al*., 2012). Cathepsin K collagenolysis is another process which occurs in osteoclasts during mediated bone resorption (Costa *et al*., 2011).

Keloids are believed to form as a result of a defect in the normal wound healing process. To understand how such a defect in wound healing can arise normal wound healing will be discussed in greater detail.

2.4 NORMAL WOUND HEALING

Normal wound healing involves soluble mediators, blood cells, the ECM and parenchymal cells. There are three phases to wound healing; inflammation, epithelialisation and re-epithelialisation, with the final goal of any wound healing process being rapid wound closure and minimal scarring (Singer and Clark, 1990).

2.4.1 Stage 1: Inflammation

Inflammation occurs after tissue injury due to the disruption of blood vessels resulting in the spillage of blood cells and other constituents. The disruption of the blood environment causes the body to respond by attempting to re-establish homeostasis and provide a clear area for the movement and formation of new cells. This is possible due to the presence of platelets which form the hemostatic plug, as well as the secretion of PDGF which is a mediator in wound healing, attracting and activating macrophages and fibroblasts. Neutrophils are chemotactically attracted to the wound site where they remove foreign particles, bacteria and cell debris through phagocytosis. Damage associated molecular pattern molecules (DAMPs) are nuclear or cystolic proteins release by damaged cells into the ECM where they contribute to the induction of inflammation through recruitment of inflammatory cells (Adib-Conquy and Cavallion, 2007). Monocytes infiltrate the wound space in a response to fragments of ECM proteins, TGF-β, and other chemo-attractants. Reactive oxygen species (ROS) are also present in the ECM. Monocytes then differentiate into macrophages that release growth factors that result in the formation of granulation tissue. Macrophages also bind to proteins in the ECM and stimulate the phagocytosis of microorganisms and other fragments. Protein binding changes these macrophages into reparative or inflammatory macrophages that release various cytokines and growth factors (Singer and Clark, 1990). This is described in Figure 2.7 and Figure 2.8.

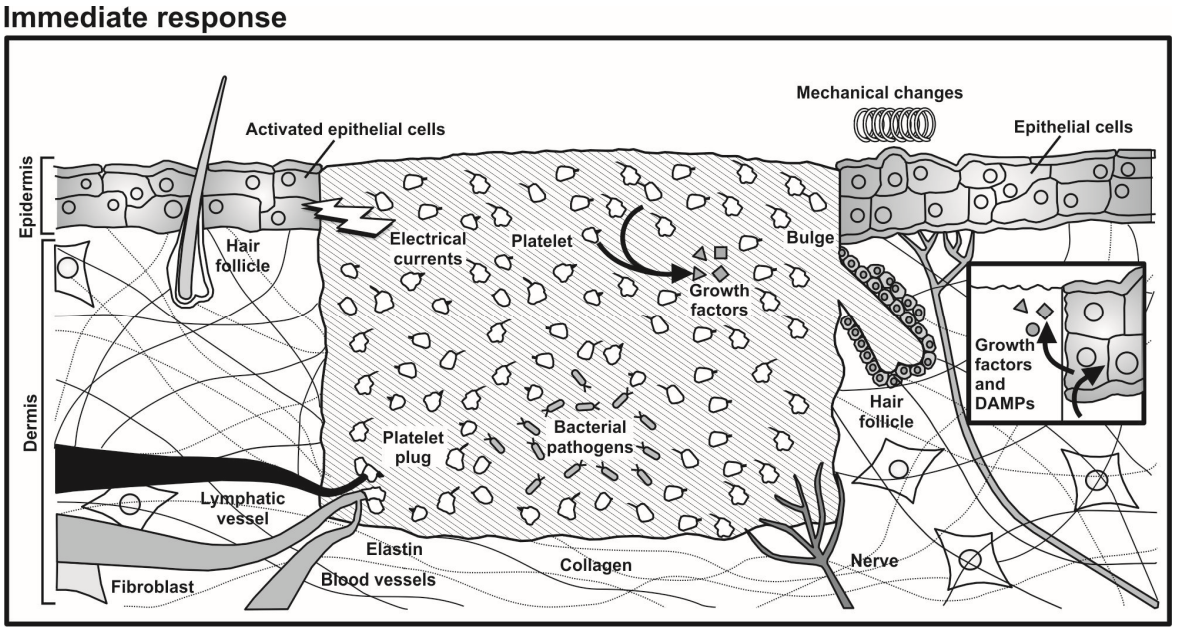


Figure 2.7: Schematic diagram of the inflammatory stage of wound healing showing the presence of the platelet plug and the other factors involved including the release of growth factors such as PDGF. DAMPs are also present (Adapted from Shaw and Martin, 2009).

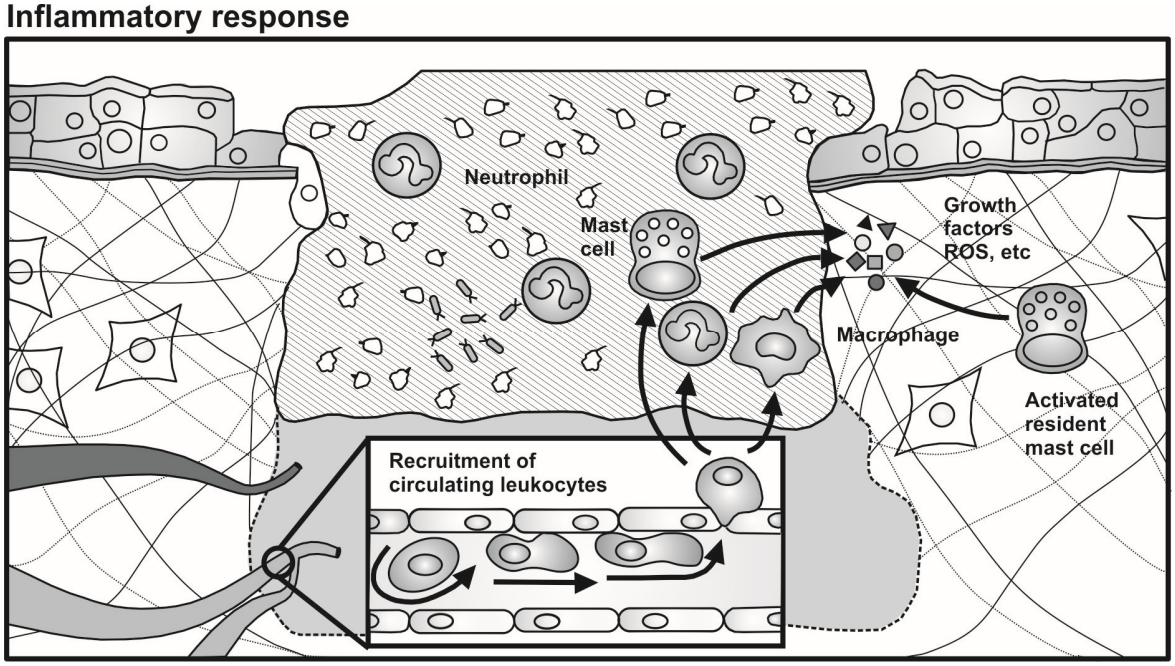


Figure 2.8: Schematic diagram of the second stage of the inflammatory stage of wound healing showing the role of the immune cells and associated products such as growth factors and ROS in this process. (Adapted from Shaw and Martin, 2009).

2.4.2 Stage 2: Epithelialisation

This occurs within hours after an injury where clotted blood and damaged cells are removed from the wound site and this allows the migration of new cells into the wound space. Cytoplasmic filaments are formed that promote cell migration. Epithelial and dermal cells become detached from one another, allowing epithelial cells to move laterally into the wound site. Epidermal cells produce collagenase which also assists with cell migration. Plasminogen activator activates plasmin and together with collagenase degrades collagen and ECM proteins. After one to two days, cells at the wound margins begin to proliferate and this is believed to be activated by the absence of neighbouring cells at the site of the injury, or by the increase in release of local growth factors (Singer and Clark, 1990).

Fibroblasts are responsible for the synthesis and remodelling of the ECM and a variety of enzymes such as cathepsin K, alloproteinases, neutrophil elastase and collagenase are responsible for this process (Fields, 2013). Fibroblasts migrate into the wound site and start with the formation of the ECM. Over time the ECM is gradually replaced with a collagenous matrix. Once enough collagen has been deposited in the wound, the fibroblasts stop producing collagen, and the fibroblast tissue undergoes apoptosis and form a scar. The process of epithelialisation is shown in Figure 2.9. Blood vessels are also formed during the epithelialisation process and this is also associated with the presence of fibroblasts in the wound space. A correct and uninterrupted sequence of the events will lead to an even closure of the wound space (Singer and Clark, 1990).

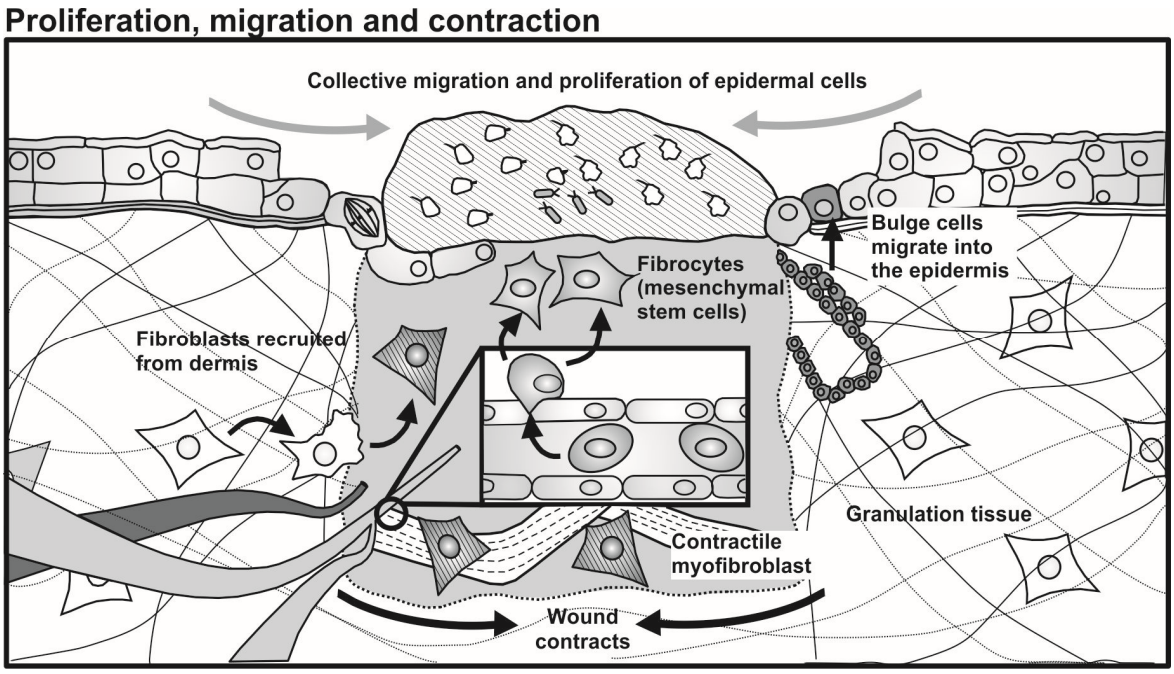


Figure 2.9: Schematic diagram of the process of the migration and proliferation of epidermal cells and the recruitment of fibroblasts from the dermis for matrix synthesis (stage 2) followed by wound contraction (stage 3). (Adapted from Shaw and Martin, 2009).

2.4.3 Stage 3: Re-epithelialisation, remodeling and wound contracture

Re-epithelialisation (Figure 2.9) forms part of the above mentioned process, and occurs within a few days after initial injury, until the wound is completely closed. Following re-epithelialisation wound contraction and scar maturation occurs. This results from the re-organisation of the ECM, its destruction and replacement by collagen. Collagen remodelling then occurs which is the degradation of excess collagen at a constant rate, controlled by proteolytic enzymes called matrix metalloproteins, secreted by macrophages, epidermal and endothelial cells. The contraction of the wound by myofibroblasts and collagen remodelling causes larger collagen bundles to form, and the wound to gain some of its original tensile strength. This process occurs fast in the first three weeks after injury and then slows down with time (Singer and Clark, 1990). Figure 2.10 shows the final outcome of the wound healing process, with a reduction of the inflammatory process by apoptosis of immune cells and myofibroblasts to end scar contracture (Shaw and Martin, 2009).

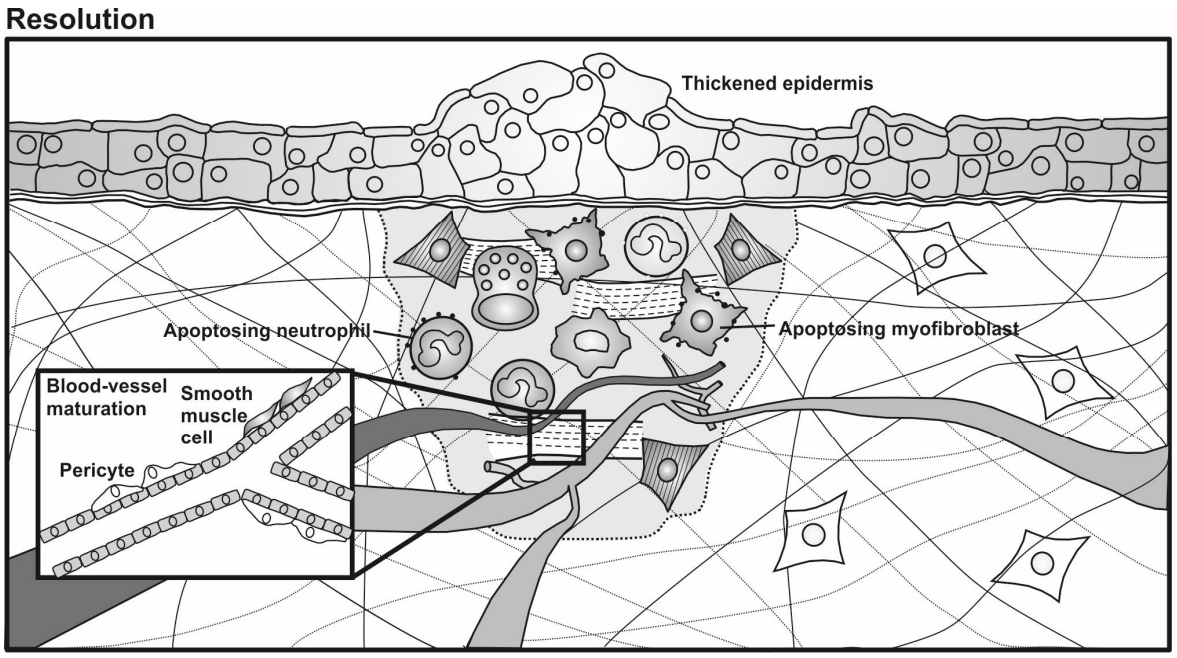


Figure 2.10: Schematic diagram of scar formation showing final wound closure with a thickened epidermis, the formation of blood vessels and reduction of the inflammatory process with apoptosing neutrophils and myofibroblasts **(**Adapted from Shaw and Martin, 2009).

A timeline representing the stages of normal wound healing described above is shown in Figure 2.11. It can be observed that the wound healing events are not independently occurring processes, but instead overlap and therefore have variable timing (Czbryt, 2012).

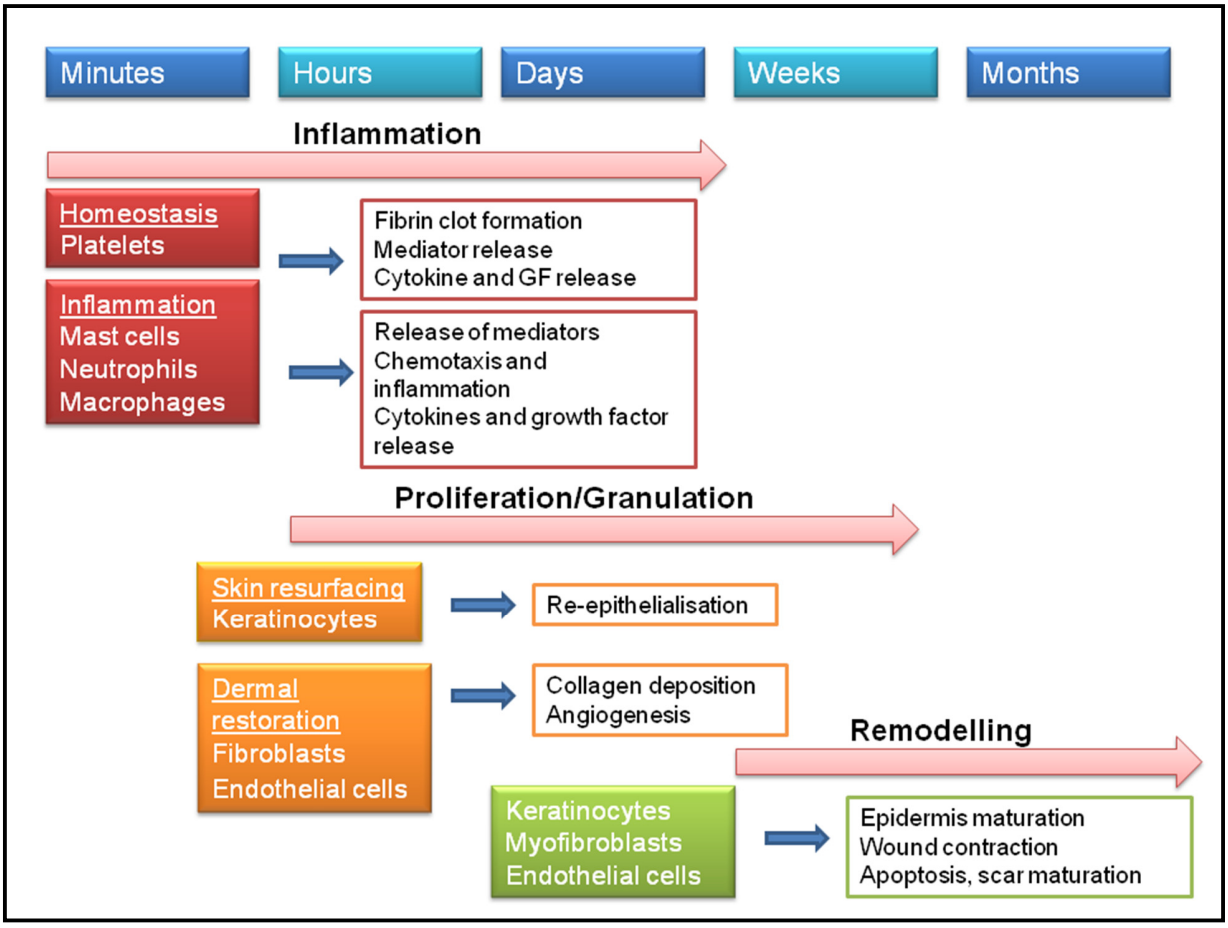


Figure 2.11: A timeline of the wound healing process, showing major cells and their effects on the various stages. (Adapted from Lin *et al.*, 2007).

2.5 ABNORMAL WOUND HEALING

Wound healing can be disrupted because of physiological and biochemical defects, a reduced supply of oxygen to the wound space, impaired granulocytic function and chemotaxis, resulting in poor signalling to the necessary cells to invade the wound space, and therefore in a decreased rate of secretion of the various cytokines and growth factors necessary for proper cell proliferation. Decreased or increased synthesis of collagen is the main reason for poor wound closure which results in uneven scarring and in the formation of keloids (Young and Heath, 2006; Tuan *et al.*, 1996; Al-Attar *et al.*, 2006).

Genetic predisposition and age are contributing factors to the inability to heal correctly, specifically in fibroproliferative disorders (Gosain and DiPietro, 2004; Keylock *et al.*, 2008; Swift

et al., 2001; Swift *et al*., 1999). There can also be secondary effects such as the constant auto-induction of growth factors, a defective apoptotic pathway and tissue hypoxia (Clark *et al*., 2009, Kischer *et al*.,1982b; Ladin *et al*., 1998; Schmid *et al*., 1998). Factors involved in wound healing are described in detail by Guo and DiPietro (2010), and factors most relating to this study are discussed below.

2.5.1 Growth factors

Correct wound healing is a result of a finely balanced interplay between autocrine and paracrine growth factors such as placental growth factor (PGF), VEGF, TGF, and connective tissue growth factor (CTGF) (Singer and Clark, 1990). Biopsies of fibroproliferative regions show elevated levels of growth factors and their receptors (Peltonen *et al*., 1991, Schmid *et al*., 1998). Fibroblasts removed from fibroproliferative regions showed increased sensitivity to growth factors *in vitro,* as well as increased collagen deposition (Bettirger *et al*., 1996; Colwell *et al*., 2005; Fujiwara *et al*., 2005; Haisa *et al*., 1994). TGF-β has been strongly implicated in the formation of abnormal scarring (Chalmers, 2011).This may be a contributing factor to the increased amount of connective tissue found in keloids.

2.5.2 Apoptosis

With normal wound healing distinct differences between the proliferative and remodelling phases of tissue repair can be identified. The formation of granulation tissue; its contraction, dehydration of the matrix and loss of cellularity through apoptosis are clearly defined as separate stages (Darby *et al*., 2002; Desmoulière *et al*.,1995; Gabbiani *et al*.,1971). Fibroblasts in a normal wound environment would undergo apoptosis in the wound remodelling phase, leading to a loss in tissue cellularity (Desmoulière *et al*., 1995). However, in fibroproliferative disorders the tissue remains in the proliferative stage (Clark *et al*., 2009; Kischer *et al*., 1982b; Ladin *et al*., 1998; Schmid *et al*., 1998). Keloids are characterized by an active proliferative phase and a remodelling phase with excessive deposition of collagen, particularly type I collagen (Aarabi *et al*., 2007; Bayat *et al*., 2003; Craig, 1975; Rudolph, 1987). It has been suggested that keloid fibroblasts *in vitro* show focal deregulation of p53 expression and up regulation of B- cell lymphoma (Bcl)-2 genes. Mutations in the p53 gene could decrease the rate of apoptosis. Decreased apoptosis has been reported in keloids when compared to normal skin

(Nirodi *et al*., 2000; Tanaka *et al*., 2004; Saed *et al*., 1998). Bcl-2 is another anti-apoptotic gene and its deregulation occurs in conditions associated with apoptotic resistance (Funayama *et al*., 2003). These factors lead to a combination of increased cell proliferation and decreased cell death through apoptosis (Ladin *et al*., 1998).

2.5.3 Tissue hypoxia

Vascular function is impaired during wound healing and this may result in a reduced oxygen supply to the wound site as a result of damage to the blood vessels upon injury. Often the influx of proliferative cells and immune cells during inflammation to the area will cause a decreased oxygen pressure to the area (Remensnyder and Majno, 1968). Good angiogenesis is therefore essential during wound healing (Knighton *et al*., 1981). Regulation of oxygen is also essential in other pathophysiological conditions, such as rheumatoid arthritis; age associated muscular degeneration and tumour growth (Folkman, 2006). Chronic or prolonged hypoxia delays wound healing (Bishop, 2008; Rodriguez *et al*., 2008) as do age (Swift *et al*., 1999; Gosain and DiPietro, 2004) and age associated changes in hormones (Gilliver *et al*., 2007), stress (Godbout and Glaser, 2006; Boyapati and Wang, 2007) and immune disregulation (Swift *et al*., 2001; Edwards and Harding, 2004 ; Menke *et al*., 2007). Because keloids are highly vascular scars and there is no connection between keloids and avascularisation, the shortage of oxygen in keloids is thought to be associated with perivascular contraction by myofibroblasts and microvascular occlusion leading to tissue hypoxia which is an important factor in tissue scarring (Kischer *et al*., 1982b).

Hypoxia may drive fibrogenesis through a direct increase of collagen gene products that are directly involved in regulating ECM production. Hypoxia increases the expression of collagen I and decreases the expression of MMP-2 (Orphanides *et al*., 1997). It was also seen to increase the expression of CTGF in kidneys (Higgins *et al*., 2004) and VEGF expression is regulated (Sánchez -Elsner *et al*., 2001). A hypoxic environment has been linked to the formation of granulation tissue (Sørensen *et al*., 2014).

Gene expression based on oxygen sensitivity can occur from hypoxia inducing and non-hypoxia inducing factors like TGF-β/SMAD3 signalling pathway as is shown through the regulation of VEGF (Sánchez- Elsner *et al*., 2001). Hypoxia increases “gene similar to Mothers Against/gene Mothers Against Decapentaplegic” (SMAD)3 mRNA levels, which promotes the thrombospondin dependant release of latent TGF-β2 and therefore the activation of TFG-β signalling (Zhang *et*

al., 2003). This suggests that hypoxia may affect TGF-β signalling as increased TGF-β1 levels were observed in a response to hypoxia (Orphanides *et al*., 1997; Norman *et al*., 2000) and are associated with the production of collagen by fibroblasts (Falanga *et al*., 2002; Saed *et al*., 1999). In fibrotic disease the expression of TGF-β1 strongly correlates with tissue fibrosis and with the composition and deposition of the ECM. TGF-β1 regulates gene expression through the regulation of SMAD transcription. Renal epithelial cells were seen to increase and remodel the ECM when stimulated with TGF-β (Chen *et al*., 2003; Yu *et al*., 2003).Hypoxia also enhances endothelial-mesenchymal transition (EMT) through hypoxia inducible factor (HIF)-1, activation and increases the production of ECM components (Higgins *et al*., 2007).

The inhibition of LOX had the same effect as the inactivation of HIF-1α *in vivo* during fibrogenesis. This suggests that the LOX genes play a role in endothelial mesenchymal interactions and fibrosis (Higgins *et al*., 2007). LOX were identified as downstream targets for HIF (Higgins *et al*., 2008). LOX-2 is capable of promoting EMT (Peinado *et al*., 2005) which was shown to induce renal fibrosis (Bauter *et al*., 2006) sometimes occurring through the EMT cell transition into myofibroblasts (Kalluri and Nelson, 2003; Liu, 2004).

Higgins *et al*. (2008), suggest that HIF-1 mediates the increase in LOX gene expression which in turn promotes fibrosis by activating the cells in the ECM, transitioning the cells into a fibroblast phenotype. This was based on the HIF-1 induction of LOX seen in cancer cell migration in cancer (Erler *et al*., 2006; Schietke *et al*., 2007).

HIF-1 is therefore identified as oxygen regulated transcription factor which increases the expression of LOX and epithelial-mesenchymal transition, promoting fibrogenesis.

2.6 ABNORMAL WOUND HEALING: KELOIDS

2.6.1 Clinical features of keloids

A fine line scar is the expected outcome of a well healed wound and undisturbed wound healing in adults (Seifert and Mrowietz, 2009). The formation of a keloid mass in the surface of the skin results from an interruption of a part of the wound healing process, or from prolonged wound healing.

Keloids were first identified by Baron Jean-Louis Albert in 1908 (Cosman *at al*., 1961). Keloids are classified as benign hyper-proliferative growths of fibrous tissue that do not regress on their

own and continue to grow with time. Keloids are most commonly found on the chest, shoulders, upper back and earlobes, occurring after inflammation of the skin such as acne or severe burn trauma. Keloids are also caused by direct invasive injury as in the cases of surgery and body piercing. Table 2.1 (below) lists the body regions most associated with keloids as well as the injuries causing keloids in these regions. Keloids are not found on the palms or on the soles, a proposed reason for this is due to a lack of sebum secretion, or lower melanocyte concentration in these areas (Young and Heath, 2006; Singer and Clark, 1990; Al-Attar *et al*., 2006). Some studies have shown a positive family history of keloids and of keloid scars in multiple sites. In such a study Bayat *et al.* (2005), suggested that in depth genetic studies will be required to identify the genetic factors that lead to keloid formation. There have been cases recorded of spontaneous development of keloids, but this spontaneous occurrence of keloids can be the result of minor, overlooked trauma (Pitche, 2006), although two cases were reported where keloids did not form from any trauma, but were possibly associated with a genetic mutation of collagen type IV, as both patients had Bethlem myopathy (Collins *et al*., 2012). This type of myopathy is caused by a type IV collagen mutation where contractures develop on multiple joints, along with general muscle weakness and wasting (Jöbsis *et al*., 1996).

Table 2.1: Body regions and type of injury that leads to keloid formation

Region	Type of injury
Trunk (Abdomen, flanks, back)	Laceration Burns
Chest (Breast and sternum)	Laceration Acne and chickenpox Burns, surgery and bee stings
Upper limb (Deltoid, upper and lower regions)	Vaccination Chickenpox and laceration
Ear	Piercing Laceration, burns and surgery
Head and neck	Laceration Burns, acne, warts and surgery
Scalp	Acne Burns, chickenpox, bee stings, warts and laceration
Lower limbs (Buttocks, feet and pubic mound)	Acne Burns, chickenpox, bee stings, warts and laceration

2.6.2 Histology of keloids

A feature of keloids is microvascular occlusion resulting in an increased hypoxic environment. Keratinocytes then produce increased levels of growth factors which, via a paracrine mechanism results in an overproduction of collagen by fibroblasts. The growth factors involved are FGF, CTGF, TGF-β1, -β2, and VEGF (Ehrlich *et al*., 1994; Buchanan *et al*., 1999).

There have been different opinions on the structure of the epidermal layer of keloids. Amadeu *et al*. (2003), suggest that the epidermis is thicker in keloids, while other researchers have reported that the epidermal layer is the same as that of normal skin (Young and Heath, 2006; Tuan *et al*., 1996). In contrast, all agree that the dermis of a keloid is visibly thicker than that of normal skin and has a higher density of mesenchymal cells. Collagen associated with fibroblasts found in the dermis is thicker and more abundant, closely arranged and often irregular (Amadeu *et al*., 2003; Young and Heath, 2006; Tuan *et al*., 1996, Singer and Clark, 1990). An abundant deposition of the ECM is seen in keloids (Neissen *et al*., 1999). Irregular collagen and its increased deposition are associated with an abnormal collagen metabolism. Elastic fibres are often absent in keloids (Abergel *et al*., 1985; Clore *et al*., 1979; Uitto, 1986).

Keloids are seen to grow beyond the borders of the original scar over normal appearing epidermis and clearly hyalinised collagen is a distinguishing histological feature (Robles and Berg, 2007; Al-Attar *et al*., 2006). Keloids have a rich vasculature which makes them histologically different from normal scars which do not have such an extensive vascular system (Rockwell *et al*., 1989; Lee *et al*., 2004).

Keloids differ from normal skin by their fibroblasts overproducing type I procollagen, and increased levels of growth factors; VEGF, TGF- β1 and -β2, as well as by the decreased rate of apoptosis. The overproduction of collagen by fibroblasts is seen in chronic inflammation and is associated with immune cells, particularly mast cells (Noli and Miolo, 2001; Abel and Vliagoftis, 2008). Studies done specifically on the proliferation and degeneration of tissue in keloids observed that promitogenic and prosecretory mechanisms are involved in the formation of keloids (Appleton *et al*., 1996).

A hypothesis put forth by Kischer and co-workers in 1990, stated that progressive cell degeneration and possibly apoptosis occurred during the transition from granulation phase to mature scar in keloids and other scars (Kischer *et al.*, 1990). It was demonstrated by Appleton *et al.* (1996), that proliferation, apoptosis and necrosis occur simultaneously in keloids and that

these processes are independent of each other and occur at specific times during the transition (Appleton *et al.*, 1996).

During the maturation of a keloid, apoptosis and necrosis result in selective removal of certain cellular populations that result in avascular fibrotic collagen. On the other hand, the proliferation of fibroblasts in the keloid dermis causes collagen deposition and fibrosis (Appleton *et al.*, 1996).

2.6.3 Theories of keloid formation

Various theories have been proposed for keloid formation. These include ECM and growth factor differences that result in an uneven distribution of cells necessary for the correct epithelialisation processes to take place.

Abnormal regulation of collagen is another theory, where the deposition of collagen in the wound site is increased, or continuously produced, so that the collagenous tissue either continues to proliferate, or forms very thick bands. The tension hypothesis states that keloids will form in areas of high skin tension such as joints, and not form in areas with less tension, as the uneven distribution of forces causes the wound to shift and have poor alignment, causing excessive scarring, and irregularly oriented collagen bundles (Figure 2.12). Mechanical forces in wound healing influence gene expression as well as growth factor and inflammatory mediator synthesis. Forces at a cellular level include shear, tension, osmosis, compression and gravity. The role of the different mechanical forces on the skin and cells during wound healing is described in detail by Agha *et al*. (2011). Since mechanical forces have known effects on the efficiency of wound healing it is expected that they would play a role in keloid formation. The theory also suggests that surgical incisions made along the tension lines in the skin will have a lesser chance of keloid formation as compared to incisions made across the tension lines (Akaishi *et al*., 2008).

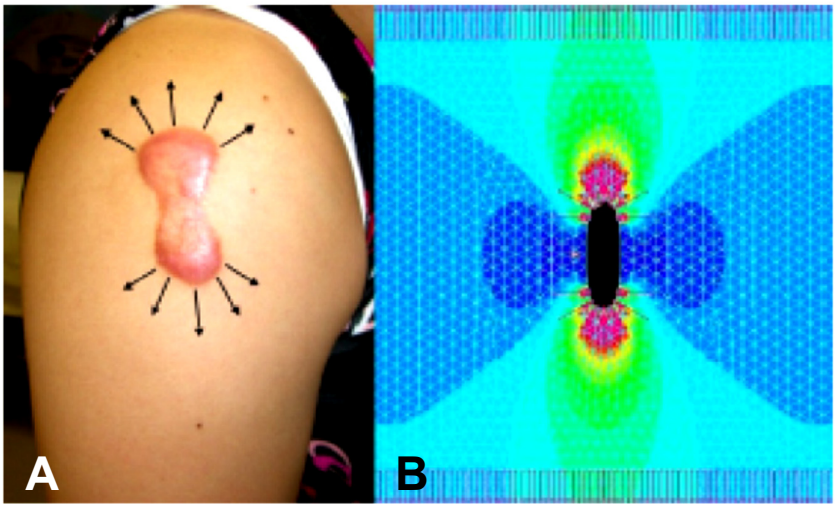


Figure 2.12: A) Keloid growth pattern due to the presence of tension lines across on the shoulder of the patient supporting the tension hypothesis. Areas of higher tension (top and bottom) are seen to have a larger area of development of the keloid as compared to that in the middle where there is less tension. B) Computer simulated skin tension around the keloid. High tension from longitudinal stretching creates high forces (shown in red). In A this area of high tension is inflamed and raised, showing a correlation between tension and keloid formation (Akaishi *et al*., 2008).

Genetic immune dysfunction is also suggested as a theory, where there is an abnormal immune response to dermal injury that may lead to keloid formation. This theory is supported by findings of keloids within families, and particularly in darker skinned individuals, suggesting a genetic relation through an immune phenotype. Some studies show an association with blood groups, however a study done by Mouhari-Toure *et al*. (2012), showed that there was no significant association between blood groups and the presence of keloids in the patients examined (Mouhari-Toure *et al*., 2012).

The sebum reaction hypothesis involves the association of keloids with the immune response. This may be as the result of skin sensitivity to sebum, and may occur as a result of destruction of sebum glands in the skin after injury. This is commonly seen in acne related keloids, where the presence of an excess of sebum in the skin, and tissue injury due to inflammation may cause a hypersensitivity reaction to the sebum. As the keloid grows, it continues destroying the sebum glands, contributing to the inflammation process all over again (Al-Attar *et al*., 2006).

An additional theory is that in keloids there is an association between keloid formation and melanin production (Devika and Arockiamary, 2011; Dubato-Brown, 1990; Ramakrishnan *et al*., 1974) and this is based on the finding that there is a higher incidence of keloids in blacks, Hispanics and Asians with a 4.5 - 16% incidence rate (Al-Attar *et al*., 2006; Oluwasanmi, 1974;

Omo-Dare, 1975). There is also an increased incidence in women and an association with puberty and pregnancy which suggests a link between changes in hormonal levels and this is based on the observation of McGrouther *et al*. (1994), which found elevated androgen levels in keloid tissues.

2.6.4 Keloid genetics

Keloid genetics and recent advances in the field have been reviewed by Halmi *et al*. (2012). Familial aggregation, occurrence in identical twins, Mendelian modes of inheritance, expression studies and the high prevalence of keloids among different ancestral groups provides evidence of genetic factors in keloid formation.

Autosomal dominant inheritance pattern is the predominant mode of inheritance. Involvement of more than one gene is likely to be involved. Keloids are 5 - 15% more prevalent in black populations than in white (LeFlore, 1980) and an estimate 15 - 20% of Black, Hispanic and Orientals suffer from keloids (Tuan and Nichter, 1998). Aggressive keloid formation was noticed in familial keloids with black African ethnic origin (Bayat *et al*., 2003).

Non overlapping associated genes and genomic regions have been identified in studies of people of different ethnicities. A complex inheritance model is proposed with multiple genetic factor contributions together with environmental factors which may trigger the condition in genetically susceptible individuals (Halmi *et al*., 2012).

Familial heritage and prevalence in twins indicates that in some families there is a genetic susceptibility for keloids (Shih and Bayat, 2010; Maneros *et al*., 2001; Ramakrishnan *et al*., 1974). Family history is also linked with multiple site keloid development, and this indicates a genotype-phenotype association (Bayat *et al*., 2005). Different trends of keloid formation were observed in different families and are believed to reflect different mutations of the same keloid gene, locus heterogeneity or random variations (Clark *et al*., 2009).

Family history is associated with more severe forms and multiple sites of keloid formation (Bayat *et al.,* 2005). In cases where minor trauma was the cause of keloids, there was a history of familial keloids but a statistical association could not be found to be associated between history of fibrotic disorders and development of keloids (Bayat *et al*., 2005). Shih and Bayat, (2010), have proposed different inheritance models for keloid scarring based on literature from previous

studies on clinical incidences in familial keloids as well as various population and case studies (Shih and Bayat, 2010) and these results are presented, summarized in Table 2.2

Table 2.2: Inheritance models of keloid formation

Regular autosomal dominant (Bloom, 1956)
Autosomal dominant with incomplete penetrance and variable expression in familial keloid studies (Chen *et al*., 2006; Clark *et al*., 2009; Maneros *et al*., 2001)
Cross linked recessive in family case studies with multiple keloids (Omo- Dare, 1975)
Autosomal recessive in a selected population (Omo- Dare, 1975)

Increased familial aggregation, higher prevalence in certain races, parallelism in identical twins and alteration in gene expression identifies keloids as a genetic disease. However no single gene mutation has been identified. As shown in Table 2.2, genetic factors are usually found in certain families or selected populations and in addition environmental, physiological and environmental factors may contribute to keloid formation or increase penetrance.

Keloid fibroblast cells also have altered patterns of DNA methylation and histone acetylation (Russell *et al*., 2010) indicating a possible epigenetic change in gene expression.

2.6.5 Growth factors and keloid formation

SMAD proteins act as transcription factors and together with TGF-β families have been suggested as keloid genetic markers. SMAD has previously been reported to be involved in fibrotic disorders. Studies done on Chinese populations suggested that SMAD is a possible gene involved in keloid pathogenesis and a susceptibility locus on chromosome 18q21.1 (Yan *et al*., 2007).

Over recent years and with progressing research, the understanding of molecular mechanisms has led to the development of recombinant TGF-β3, anti-TGF-β1, interleukin (IL-10) and mannose 6-phosphate antibodies that inhibit the activation of TGF-β1 and TGF-β2. Keloids are also seen to be associated with Rubinstein-Taybi and Goeminne syndrome, supporting the genetic background for keloids (Goeminne, 1968; Hendrix and Greer, 1996). Rubinstein-Taybi

syndrome is known to be caused by mutations of genes encoding transcriptional co-activators cAMP Response Element Binding (CREB) protein and EP300 which also activate the SMAD/TGF-β signalling pathway, which has been suggested to be involved in the development of keloids (Warner *et al*., 2004). Other studies suggest that the human leukocyte antigen (HLA) system can be associated with the development of keloids and could explain the ethnic differences in the prevalence of keloids (Lu *et al*., 2008).

2.6.6 Immunology of keloid formation

Immunohistochemistry (IHC) studies have shown the presence of large numbers of lymphocytes and macrophages in keloids. Most of the immune cells present are CD3 T-lymphocytes and CD68 macrophages. B-lymphocytes are also seen in keloids; however T-lymphocytes are more abundant. Both B- and T-lymphocytes are observed in higher numbers in keloids than in normal skin (Castagnoli *et al*., 1997). T-cells are known to be associated with an excess accumulation of dermal collagen (Wallace *at al*., 1994) and an increased activity of macrophages is known to play an important role in wound healing (Kumar *et al*., 2007). Shaker *et al.* (2011), observed macrophages in contact with fibroblasts and lymphocytes in keloids.

Immunologically, an increase in IgG in keloid lesions was seen by Rossi and Bozzi, (1989), and IgA and IgM can be detected in keloids in higher levels than in normal skin (Kazeem, 1988; Kischer *et al*., 1983). The numbers of white blood cells; macrophages, Langerhans' cells and mast cells are increased as well. Mast cells may contribute to elevated HIF-1α and VEGF in keloids (Eishi *et al*., 2003; Zhang *et al*., 2006).

2.6.7 Cell biology of keloid formation

The synthesis and remodelling of the ECM, which is the most essential part of wound healing is controlled by various important factors (Ehrlich *et al*., 1994; Buchana *et al.,* 1999). TGF-β is chemotactic for fibroblasts, keratinocytes and inflammatory cells and stimulates collagen I production by fibroblasts. CTGF is profibrotic and increases the deposition of collagen fibres and other ECM components, but has no effect on epidermal and inflammatory cells. Both factors are increased in wound healing. VEGF is a glycoprotein produced by fibroblasts, keratinocytes and macrophages (Buchana *et al*., 1999). Increased production of VEGF is stimulated by hypoxia (Shweik *et al*., 1992). VEGF regulates angiogenesis and vasculogenesis

and is seen to increase the rate of wound healing and is associated with reduced scar or keloid formation. Large keloids usually have a good blood supply which is secondary to keloid formation and is rather a result of an increased oxygen requirement.

FGFs are cytokines involved in the regulation of cell proliferation, differentiation and migration in the wound and the up-regulation of FGF is associated with an increase in collagen production and consequent scarring and keloid formation (Buchana *et al*., 1999).

The major component of green tea extract (-)-epigallocatechin-3-gallate was seen to inhibit the production of type I collagen by blocking the PI-3/Akt/mTOR signalling pathway responsible for type I collagen production (Zhang *et al*., 2006).

It has also been suggested that lymphocyte migration often drives the fibrotic process, as the proliferation of fibroblasts was seen to occur only in the area of dense lymphocyte population (Appleton *et al*., 1996). This fibrotic process is believed to be under the influence of an endogenous antigen for which keratin and sebum are believed to be the effectors (Molwem, 1951; Yagi *et al*., 1979).

Enlarged fibroblasts were found in keloids and this is due to the increased number of RER and extensive Golgi complexes in the cells (Dyer and Enna, 1975). In some fibroblasts nuclear folds have been identified as well as the presence of cytoplasmic filaments. Ehrlich *et al*. (1994), could not show the presence of cytoplasmic microfilaments in the keloid fibroblasts; however the presence of a well-developed RER was confirmed. In another study by Kischer *et al*. (1982a) microfilaments in keloid fibroblasts were identified but only around the small vessels in the tissue. Pathological fibrosis in internal organs is also characterised by an over deposition of collagen as well as by increased presence of myofibroblasts (Buchanan *et al*, 1999). The role of these cells in keloids remains unknown and there is possibly a common mechanism of development where keloid formation is a localised mechanism while pathological fibrosis is a systemic disorder (Buchanan *et al*., 1999). A study by Matsuoka *et al.* (1988), also found that myofibroblasts were not present in keloid tissue although fibroblast activity and density was increased.

When dermal fibroblasts are co-cultured with keloid keratinocytes the proliferation of the dermal fibroblasts is greatly increased, suggesting that soluble factors secreted by the keratinocytes have an effect on fibroblast growth and therefore suggest a possible influence of keratinocytes on fibroblast growth in a keloid (Matsouka *et al*., 1988).

In another study keloid fibroblasts were co-cultured with either normal or keloid keratinocytes. Keloid fibroblasts in a co-culture with the keloid keratinocytes grew faster (Funayama *et al.,* 2003).

The ability of keratinocytes to promote proliferation and inhibit apoptosis of fibroblasts has also been investigated (Funayama *et al.,* 2003). The proliferation of normal fibroblasts was increased when co-cultured with keloid keratinocytes. In these co-cultures, the keloid fibroblasts were more resistant to apoptosis than normal keratinocytes following a four day stress starvation period.

Other studies have shown that keloid keratinocytes contribute to increased fibroblast proliferation as well as increased collagen production (Lim *et al*., 2002, Lim *et al.,* 2001). These authors suggested that a prolonged proliferation and a short remodelling state in keloids is due to the presence of hyperproliferative and apoptosis resistant fibroblasts, which is further enhanced by the presence of keloid keratinocytes that mediate increased collagen production.

During keloid formation there is an increase in fibroblasts that are in the active growth phase. These fibroblasts overproduce type I collagen while the type III collagen production is unchanged. Levels of soluble collagen were found to be increased, confirming an increase in collagen production. Wound fibroblasts differentiate into myofibroblasts that cause wound contraction during scar formation and this increased rate of differentiation and/or activity would also contribute to scar formation (Desmoulière *et al*, 2005). Myofibroblasts are a part of normal wound repair and these cells undergo apoptosis once the process is complete. However, if apoptosis does not occur, hypertrophic scars develop (Ehrlich *et al*, 1994). Myofibroblasts are rarely found in keloids and can be distinguished from fibroblasts due to the presence of bundles of cytoplasmic microfilaments (Ehrlich *et al*, 1994). Myofibroblasts also have a prominent RER and Golgi apparatus in the cells, reflecting their secretory activity, as does the frequent presence of intracellular collagen within tubular membranes (Lee and Vijagasingam, 1995). Myofibroblasts can be identified by immunohistochemical staining for fibronectin and vimetin (Eyden, 2003).

Cell culture studies have focussed on collagen, chondroitin and fibronectin levels and mechanisms of formation in fibroblasts (Babu *et al*., 1989; Ikeda, 2009; Abergel *et al*., 1985; Kischer *et al*., 1989; Kischer and Hendrix, 1983) and growth factors released by fibroblasts (Okazaki *et al*., 2003; Ashcroft *et al*., 2013). Although increased numbers of fibroblasts were seen in keloid tissue (Ryan *et al.,* 1974) the findings of several studies is contradictory as some

studies found that there was no differences in the fibroblast growth characteristics compared to normal tissue (Diegelmann *et al*., 1979; Russell and Witt, 1976) whereas other studies have shown differences in growth parameters in keloid fibroblasts which results in an overproduction of collagen (Abergel *et al*., 1987; Diegelmann *et al*.,1979; Uitto *et al*., 1985). Recent studies have focused on the effects of drugs on keloid fibroblast collagen secretion (Yi *et al.,* 2014) and potential sources of abnormal keloid fibroblasts (Iqbal *et al.,* 2012), as well as their gene expression (Russell *et al*., 2010).

One of the theories on keloid formation is related to the overproduction of fibronectin which, during the early stages of wound healing is responsible for the formation of granulation tissue (Diegelmann *et al*, 1979; Grinell, 1984; Grinell *et al*., 1981; Kischer *et al*., 1981; Kurkinen *et al*., 1980). This increased formation of fibronectin in turn causes abnormal fibroblast function which is associated with increased collagen production. The theory of overproduction of fibronectin is further supported by other studies done on fetal and embryonic wound healing which showed that scar-less wound healing occurred in the absence of fibronectin (Mak *et al*., 2009; Linder *et al*., 1978).

Increased amounts of fibroblast growth factors are found in keloid tissue and these include TGF-β (Xia *et al*., 2006; Chin *et al*., 2001), FGFs, CTGF and PDGF which are seen in wound healing with increased scar formation and are associated with keloids. CTGF and PDGF are both profibrotic factors greatly linked to keloid development (Xia *et al*., 2006). VEGF increases the rate of wound healing and reduces scarring, however levels are increased in keloids due to increased vasculature in some larger keloid masses (Babu *et al.,* 1989). Keloid fibroblasts also have an increased number of receptors for the factors as compared to normal fibroblasts. Responses to these factors would increase cellular recruitment causing an increased synthesis of collagen, proteoglycans and other ECM components. Molecular studies have shown that keloids contain more of these components than both normal and hypertrophic scars (Meenakshi *et al*., 2005).

2.6.8 Treatment of keloids

Keloids are difficult to treat and have been described as particularly resistant to medical management and sometimes too aggressive for surgery (Rudolph 1987). The most effective therapy for keloids is the identification of keloid susceptible individuals and prevention of the

formation of a keloid mass. Difficulty arises as patients susceptible to keloid formation are usually only identified once a keloid has formed.

General guidelines that apply to handling the skin of those susceptible to forming keloids during surgery are; to avoid mid chest incisions, to prevent infection at post-operative sites, to maintain normal tension in surgical wound closure and to ensure that skin excision and incisions are in the same direction as skin tension lines (Kelly, 2004).

2.6.8.1 Surgical removal of keloids

Surgical excision is used for larger keloids but there is a 50% to 80% possibility of reoccurrence without post-operative treatment measures (Darzi *et al*., 1992). Therefore primary excision is most commonly followed by intralesional corticosteroid injections. Other adjunctive therapies include silicone gel sheeting, imiquimoid cream or interferon injections. Larger keloids may require more complex treatment. Triamcinolone acetonide is injected into the wound site after suture removal and continued for some time. The treatment however can cause hypopigmentation for 6 months or more. Pressure garments and silicone gel sheeting are important co therapies and are applied two weeks after suture removal.

Debulking or shaving of the lesion to the level of the surrounding clinically normal skin is needed for large keloids where primary excision is not possible. This procedure causes the post-operative site to become hyperpigmented and the texture does not match the surrounding normal skin.

Surgical excision alone shows poor results and varying recurrence rates (Al-Attar *et al*., 2006). Excision stimulates additional collagen synthesis which results in rapid re-growth and a much larger keloid than the excised one. Both subtotal and complete excisions are performed in the surgical removal of keloids (Tang, 1992; Cosman and Wolff, 1972). Sutures are removed as soon as possible and intradermal subcuticular closure is preferred to avoid suture marks that develop into keloids (Anate, 1990). Braided sutures are avoided to limit the inflammatory reaction in the area (Niessen *et al*., 1997). Wounds can also be closed with flap advancement, autographs and allografts (Orgill *et al*., 1999; reviewed by Al-Attar *et al*., 2006).

Cryosurgery is performed with nitrogen to freeze keloid tissue causing cell and microvascular damage. Anoxia results in tissue necrosis, sloughing and tissue flattening (Rusciani, 1993). The nitrogen is applied for 15 - 20 seconds as anything above that time can result in

hypopigmentation as a consequence of melanocyte destruction. Eight to ten treatments are needed to obtain complete flattening and this occurs in half of the treated patients (Kelly, 2004). This treatment also improves the organisation of keloid collagen bundles (Har-Shai *et al*., 2003). Cryosurgery used with intralesional steroids can give an 84% success in treatment (Cielley and Barin, 1979). Cryosurgery patients experience pain and slow healing and often do not return to complete the treatment and hypopigmentation or depigmentation from the treatment can last for many years (Al-Attar *et al*., 2006).

2.6.8.2 Nonsurgical therapies

2.6.8.2.1 Steroid injections

This is one of the most frequently and commonly used treatments. Steroids induce ultrastructural changes in collagen synthesis through fibroblast glucocorticoids receptors. Normal and keloid fibroblast proliferation and collagen production is inhibited while collagenase production is increased (Ketchum *et al*., 1966; McCoy *et al*., 1980; Cruz and Korchin, 1994; Kauh *et al*., 1997, Gadson *et al*., 1984). The use of triamcinolone is the most effective way to treat keloids and its efficacy exceeds 80% (Ketchum *et al*., 1966; Ketchum *et al*., 1971; Griffith *et al*., 1970). Triamcinolone is administered into the papillary dermis where collagenase is produced. The corticosteroid inhibits α2-macroglobulin a collagenase inhibitor, collagenase accumulates and collagen degradation occurs (McCoy *et al*., 1980). These injections are painful and require multiple needle pricks. Additional medication for pain relief such as lidocane with epinephrine is administered around the lesion prior to using intralesional triamcinolone. Use of silicone gel sheeting in conjunction with intralesional injections of triamcinolone is more effective than using these therapies alone (Kelly, 2004).

2.6.8.2.2 Interferon therapy

Interferons (IF) are cytokines secreted by T-helper lymphocytes and have an antifibrotic effect (Harrop *et al*., 1995; Berman and Duncan, 1989; Tredget *et al*., 1997; Tredget *et al*., 1998). IF-α and –δ inhibit type I and type II collagen synthesis by causing a reduction in cellular mRNA (Jimnez *et al*., 1984). IF is injected into the skin surrounding the post-operative site immediately after surgery and injections are continued for one or two weeks. IF treatment has an 18.5%

recurrence rate if used after keloid excision versus that of 51% with excision alone and 58% recurrence if using excision combined with interleukin therapy (Berman and Flores, 1998).

2.6.8.2.3 5-flourouracil therapy (5-FU)

Small keloids can be treated successfully with 5-FU (Fitzpatrick, 1999). 5-FU is an antimetabolite that inhibits fibroblast growth (Fitzpatrick, 1999; Uppal *et al*., 2001; Manuskiatti and Fitzpatrick, 2002). When combined with triamcinolone better results are obtained. This treatment is very painful and often leads to noncompliance by the patient (Kelly, 2004).

2.6.8.2.4 Imiquimod therapy

Imiquimod 5% cream induces the local production of interferons at site of application. Imiquimod treatment is started immediately after surgery and continued daily for 8 weeks (Berman and Kaufman, 2000). Mild irritation can occur with the use of this cream and hyperpigmentation was recorded in 50% of patients. Large wounds require a four to six week postoperative delay of use of treatment to avoid causing the surgical site to splay or dehisce upon early application (Kelly, 2006).

2.6.8.2.5 Radiation therapy

Radiation therapy can be used alone, but this is not always effective as there is a high rate of reoccurrence (Borok *et al*., 1988). Using large doses of radiation for a more effective treatment is not ideal as the development of squamous cell carcinoma can occur 15 - 30 years after treatment (Hoffman, 1982). It can be combined with surgery to prevent the reoccurrence of keloid and radiation as a single therapy is effective in reducing pruritus and tenderness. Radiation therapy is most effective given two weeks after excision or in early keloids as this is when the number of proliferative fibroblasts is higher (Norris, 1995). Radiation damages the fibroblasts directly; increasing the rate of apoptosis and reverting to the normal balance between apoptotic and proliferative cells, as well as affecting the keloid collagen structure (Luo *et al*., 2001; Borok *et al*., 1988). Pre and postoperative radiation has not been found to be more effective than only postoperative radiation. Iridium 192 interstitial irradiation after surgery has been shown to have a low rate of 21% reoccurrence in 783 keloids evaluated (Escarmant *et al*.,

1993). High dose rate brachytherapy gives better results than external beam radiation both in terms of low recurrence and better cosmetic results. This therapy combined with surgical excision appears as an effective and safe way to treat keloids (Guix *et al*., 2001).

A benefit of radiation therapy is that starting the therapy early after surgery will not affect wound adhesion (Ogawa *et al*., 2003; Ragoowansi *et al*., 2003). There has been no association between radiation therapy for keloids and cancer in large clinical trials (Ogawa *et al*., 2003; Cosman and Wolff, 1972; Borok *et al*., 1988; Klumpar *et al*., 1994; Ragoowansi *et al*., 2003; Kovalic and Perez; 1989). The more frequent use of radiation therapy is suggested in cases where there is no consequent exposure to visceral structures (Al-Attar *et al*., 2006).

2.6.8.2.6 Pressure therapy and ligatures

Pressure garments are used to treat keloids postoperatively or after applying a potent steroid treatment or flurandrenolide tape. Tape with pressure dressings enables a reduction in thickness and size of the keloid through the reduction of intralesional mast cells and decreasing histamine production (Kelly, 2004). Pressure seems to decrease α-macroglobulins whose role is to inhibit the breakdown of collagen by collagenase. This results in an increased breakdown of collagen by collagenase. Pressure therapy could also decrease scar hydration causing mast cell stabilisation and a decrease in neovascularisation and ECM production (Baur *et al*., 1976) or hypoxia that leads to the degeneration of fibroblasts and collagen (Kelly, 2004). Elastic compression bandages, elastic adhesive bandages, pressure earrings and compression wraps can also be used to apply pressure to keloids. This is a long term treatment and patient compliance decreases with the duration of the treatment.

The practical use of pressure therapy is limited to ear lobes. Pressure therapy after surgical excision gives success rates of over 80% (Brent, 1978; Mercer and Studd, 1983; Linares *et al*., 1993; Lawrence, 1996; Russell *et al*., 2001). Pressure therapy is thought to work by altering wound tension and pressure induced ischemia that promotes collagen degradation and modulates fibroblast activity (Kischer *et al*., 1975).

Ligatures can be used in cases where surgery is contraindicated or refused by the patient. A suture is tied around the base of the keloid and replaced every two weeks. The sutures gradually cut off the keloid causing it to fall off. Pain medication can be given for a few days after the ligature is applied (Kelly, 2004).

2.6.8.2.7 Lasers

The argon laser was the first laser to be used in keloid therapy and was successful in early keloids. Pruritus and other symptoms can improve with the use of argon laser. The carbon dioxide laser had a variable rate of reoccurrence ranging between 40 - 90% even when used with additional therapies such as intralesional corticosteroids. It is mainly used for debulking of larger keloids so that they can be treated with other modalities (Abergel *et al*., 1984).

Collagen metabolism was seen to be selectively inhibited by neodymium:yttrium-aluminium-garnet (Nd:YAG) laser, without affecting fibroblast viability or DNA replication (Abergel *et al*., 1984). Softening, size reduction and normalisation of colour was seen at a three year follow up of two patients treated with this laser. Due to the small number of patients these results cannot be extrapolated to represent a larger population. Another study reported improvement in 16 out of 17 treated patients but no follow up was discussed (Sherman and Rosenfield, 1988). Using a pulse dye laser with intralesional triamcinolone increased the effectiveness of this laser treatment which was previously used to treat sternostomy scars (Alster and Williams, 1995).

The flash lamp-pumped pulsed dye laser causes selective thermolysis of haemoglobin molecules which results in microvascular damage and coagulative necrosis and finally tissue hypoxia. The laser is also thought to cause the dissociation of collagen bundles (Alster and Williams, 1995; Dierickx *et al*., 1995). With this laser melanin is a competing chromophore and is therefore not as effective in darker skinned individuals who are at a higher risk for keloids (Al-Attar *et al*., 2006).

2.6.8.2.8 Silicone gel sheeting

Silicone gel is commonly used as prophylaxis to abnormal scarring in elective incisions (Quinn, 1987, Ahn *et al*., 1991; Clugston *et al*., 1995). It results in more rapid healing and can be used with carbon dioxide laser to prevent the recurrence of keloids (Ehrlich *et al*., 1994; Gold, 1994). Silicone gel sheeting works through a combination of hydration and occlusion (Kelly, 2004) by the sheet serving as an impermeable membrane which keeps the skin hydrated and functioning as the *stratum corneum* (Chang *et al*., 1995; Sawada and Sone, 1992). The effects of silicone are not affected by external parameters such as temperature and pressure (Quinn, 1987; Ahn *et al*., 1991; Fulton, 1995).

Silicone is said to be able to down regulate TGF-β2, but non silicone gel dressings have shown similar results, and are more effective on younger keloids. As it is a painless treatment, it is used in the treatment of keloids in younger children. The gels take 6 to 12 months of therapy to achieve the best results but patients usually discontinue the treatments due to the duration of the treatments. The gel sheets are worn for 22 to 23 hours a day and taken off to aerate the wound site. Silicone gels alone are not as effective in keloid treatment as using the gel sheeting. Polyurethane dressings also known as Curad can be worn for 20 to 22 hours a day to soften keloid and some regression is observed after 8 weeks of treatment. Using polyurethane with compression increases the treatment effectiveness (Kelly, 2004).

As mentioned above, no single treatment therapy is always successful in the treatment of keloids. For this reason two treatment therapies are usually combined, and the most common ones are mentioned below as a summary in Table 2.3, together with their efficacy rates and most common adverse effects.

Table 2.3: Commonly used combination therapies in keloid treatment

Therapy	Effect	Side Effects	Additional Notes
Surgery and steroids	80% (Ketchum *et al*., 1971; Griffith *et al*., 1970)	Atrophy and pigment change which resolve on their own	
Surgery and 5-FU	Cure rates exceed those of surgical excision alone	Rare skin irritation (Uppal *et al*., 2001)	Injections are painful
Surgery and silicone gel sheeting	Exceeding 80% (Ahn *et al*.,1991; Sproat *et al*., 1992; Gold, 1994)	Minor skin irritation and maceration	Patient compliance is a problem
Surgery and compression earrings	Exceeding 80%	Minimal adverse effects (Brent, 1978; Pierce, 1986)	Preferred for earlobe keloids
Surgery and radiation	65 - 99%	Minimal adverse effects, skin pigmentation changes (Ragoowansi et *al*., 2003; Borok *et al*., 1988; Klumpar *et al*., 1994)	Not to be used in paediatric populations and pregnant women
CO_2 and steroids	Cure rates comparable to scalpel excision and steroid injection (Norris, 1995)	Decrease of postoperative pain (Norris, 1991; Henderson *et al*., 1984)	Costly

2.6.8.3 Other therapies

Flurandrenolide tape applied to the keloid for 12 - 20 hours a day usually causes the keloid to soften and flatten as well as eliminate pruritus. Long term use of this treatment may cause cutaneous atrophy. Clobestasol ointment or gel used twice a day has a similar effect and can reduce pain and tenderness but long term use also causes atrophy and hypopigmentation of the treated area. Various other topical treatments for keloids exist. Tracolimus is said to mute the gli-1 oncogene expressed in keloids, methotrexate is known to prevent most reoccurrences of keloids (Kim *et al*., 2001). This treatment is given orally prior to keloid removal surgery and continued until the site is healed. Pentoxifylline is thought to improve circulation to the post-operative area and eliminate fibroblast growth factors.

Other tried therapies include retinoids which have been tried experimentally but have not been clinically accepted as a treatment for keloids. Vitamin A and its derived retinoids, administered topically and intra-lesionaly are known to enhance new wound healing and scar regression

(Sporn, 1986) and were seen to improve the appearance of keloid scars when taken orally (Russo and Laguens, 1985). Retinoids enhance epidermal proliferation while inhibiting fibroblast proliferation and shift the wound healing process towards normal in keloid tissue (Jassen de Limpens, 1983; Prutkin, 1971; Christophers and Lagner, 1974). Clinical trials have shown response to topical retinoid applied to keloids (Jassen de Limpens, 1980; Daly and Weston; 1986) and data from *in vitro* studies suggests that retinoids can modulate collagen production through modulating fibroblast growth (Cruz and Korchin, 1994; Christophers and Lagner, 1974; Nelson and Balian, 1984; Abergel *et al*., 1985). Retinoids also suppress sebum production (Zouboulis *et al*., 2014; Stewart *et al*., 1984) which could play a role in keloid control. Adverse effects of retinoid use include skin irritation, photosensitivity and atrophy in some cases (Jassen de Limpens, 1980; Daly and Weston., 1986).

Calcium channel blockers can be used in keloid therapy (Palamaras and Kyriakis, 2005). Phenylalkylamine calcium channel receptor antagonist injected intra-lesionaly after keloid excision has shown promising results in clinical trials (Viera *et al*., 2010; Copcu *et al*., 2004). Verapamil was also a tried treatment (D'Andrea *et al*., 2002; Lawrence, 1996,). The mechanism of action involves the inhibition of calcium dependant reactions involved in ECM production and enhancement of its degradation by increasing procollagenase synthesis (Lee and Ping, 1990; Lee *et al*., 1994; Doong *et al*., 1996).

Histamine antagonists specific to the H1 subtype receptor relieve some of the pruritus and discomfort associated with keloids and can also modulate keloid size (Topol *et al*., 1981; Cohen *et al*., 1973; Bairy *et al*., 1991). Pruritus is thought to be associated with the histamine release during mast cell degranulation (Cosman and Wolff, 1972) and the histamine could also contribute to the increase in collagen synthesis in keloids (Sandberg, 1962; Topol, 1981).

Zinc ointments inhibit LOX but have had limited success in the treatment of keloids (Soderberg *et al*., 1982). Inhibitors of LOX; penicillamine and β-aminopropionitrile interfere with collagen cross linking making the collagen weaker and more susceptible to collagenases. The LOX inhibitors are given orally and in combination with colchicines which increases collagenase activity, inhibits collagen synthesis and microtubular disruption. There are no documented adverse effects with the use of this therapy (Peacock, 1981).

Cyclosporine (Duncan *et al*., 1991), D-penicillamine (Schorn *et al*., 1979) and Relaxin (Unemori *et al*., 1990) have been tried but with little therapeutic success or having more risk than benefit.

Long wavelength ultraviolet A may help prevent keloid reoccurrence after excision by inhibiting mast cells. A flavanol, Quercitin, has been found to inhibit the proliferation and contraction of scar derived fibroblasts. Prostaglandin E_2 softens tissue and can restore normal wound healing. The use of a strong bleaching agent has been suggested as a potential therapy as keloids are not seen in albinos and have regressed when vitiligo develops in the keloid area. Gene therapy is another new potential therapy (Kelly, 2004).

Oils rich in Omega-3 fatty acids such as Shea butter and boa constrictor oil used traditionally by West African patients for keloid treatment have been shown to be an effective treatment. Using fibroblast cell cultures and Boa constrictor oil was more effective then triamcinolone in keloid treatment (Oliatan *et al*., 2011).

TGF-β has been identified as a growth factor most central to keloid pathology (Al-Attar *et al*., 2006). TGF-β1 and -β2 promote fibrotic scarring in animal models and TFG-β3 has the opposite effect of enhancing physiological healing (Shah *et al*., 1994; Shah *et al*., 1995; Tyrone *et al*., 2000). Investigations into TFG-β3 agonists and TGF-β1 and -β2 antagonists can have therapeutic potential (Al-Attar *et al*., 2006).

Studies on rat models showed an improvement in wound healing and scar formation quality by involvement of polyphenols in green tea (Kapoor *et al*, 2004). Collagenase activity and collagen formation was suppressed with the use of green tea (Zhang *et al*., 2006; Nakamuta *et al*., 2005).

Recent studies have shown botulin toxin type A (BoNT-A) to be effective in the treatment of keloids. Its use as prophylaxis prior to surgery in order to reduce muscle tension in the area surrounding the wound and improve the appearance of postsurgical scars is accepted, but intralesional use of BoNT-A in the treatment of keloids is still uncertain (Gauglitz, 2013), even though clinical trials have shown it to be effective (Zhao and Miaobo, 2009). Optical profiling of keloids treated with BoNT-A has shown no changes compared to the controls and no effects of BoNT-A on TGF-β were found *in vitro* (Gauglitz *et al*., 2012), while other researchers found down regulation of TGF-β, MMP-1, CTGF, PDGF and VEGF genes in keloid fibroblasts after treatment with BoNT-A (Xiaoxue *et al*., 2014).

There is a need to develop better therapies to improve the quality of life of keloid patients especially the cosmetic implications, physical impairment, discomfort as well as decreased quality of life (Seifert and Mrowietz, 2009; Bock *et al*., 2006). Effective treatment options for

keloids are limited and a re-evaluation of basic tissue and cellular features may provide new insight into possible new treatment strategies.

2.7 AIM AND OBJECTIVES

The aim of this study is to investigate the distribution, localisation and ultrastructure of keratinocytes, melanocytes, collagen, fibroblasts and mast cells, as well as the physical interactions between each cell type in keloid tissue compared to normal skin tissue using microscopic techniques.

The objectives are:

1. To evaluate the histological changes of keloid tissue related to collagen morphology, mast cells, fibroblasts and melanocyte distribution using light microscopy.
2. To evaluate the morphology and distribution of collagen, mast cells, fibroblasts and melanocytes in keloid tissue using transmission electron microscopy.
3. To describe the interactions between melanocytes, fibroblasts and mast cells using transmission electron microscopy.
4. Identify specific differences related to collagen fibre formation and cell-cell interactions between normal/control and keloid skin

It is hypothesized that there is a significant cellular difference histologically in keloid tissue when compared to normal, with prominent changes in keratinocytes, fibroblasts and mast cells as well as collagen synthesis and fibre formation.

Chapter 3: Materials and Methods

3.1 SAMPLE COLLECTION

Keloid skin samples were obtained from a plastic surgeon with ethical approval and clearance; under protocol number 336/2014 as were normal skin (excess left over skin following skin transplantation) which were used as a control. For this study three control and eight keloid samples were randomly selected and were used in this study.

3.2 RESEARCH PROCEDURE

All research was conducted in the research facilities of the Department of Anatomy of the Health Sciences Faculty and the Unit for Microscopy and Microanalysis, University of Pretoria.

3.2.1 Reagents, Equipment and disposable plastic ware

Sodium hydroxide (NaOH), sodium phosphate (NaH_2PO_4), sodium chloride (NaCl), fuchsin dye powder (CI NR 42510), iron (III) chloride ($FeCl_3.6H_2O$), picric acid ($C_6H_3N_3O_7$), potassium aluminium sulphate ($KAl(SO_4)_2.12H_2O$), sodium iodate ($NaIO_3$) and Entellan mounting medium were of analytical quality and were obtained from Merck Chemicals, Modderfontein South Africa (SA). Sirius red dye powder (Direct red 80) was obtained from the Sigma-Aldrich Company, Atlasville, SA. Fuchsin (CI NR 42510) was obtained from Riedel-de Haën A-G Germany. Methyl orange powder (CI NR 2226), Eosin yellow water soluble (standard stain), Lead citrate, and thymol were obtained from British Drug House (BDH) Chemicals LTD, Poole England.

3.2.1.1 Equipment:

Microtome Leica RM2255 supplied by the Scientific Laboratory Equipment Company (LASEC), Cape Town, S.A. JEOL transmission electron microscope (TEM) (JEM 2100F). Nikon Optiphod transmitted light microscope.

3.3 LIGHT MICROSCOPY

Light microscopy is a tool used most widely in histology and is used to distinguish the fine structure of cells and tissues. The specimens need to be thin for the light beams to pass through. There is no level of useful contrast in unstained specimen sections, for this reason various stains are applied to the specimens after paraffin sectioning in order to provide contrast or to stain for specific components. Microscopes magnify images to a level at which the retina can resolve the information that is otherwise below the limit of resolution. Resolution is dependent upon the light source wavelength, specimen thickness, quality of fixation and staining intensity (Ross *et al*., 2003).

Fixation is a process which preserves the structural integrity of specimens that are to be used in microscopic observation by stopping the cell metabolism. Formaldehyde is commonly used as a fixative and it preserves the general structure of the tissue through reactions with amino acid groups of proteins (Ross *et al*., 2003). Once fixed the specimens are washed and then dehydrated in increasing concentrations of ethanol and cleared in xylene after which the sections are infiltrated with paraffin wax and embedded in wax blocks to allow easy sectioning.

Sectioning of tissue embedded in wax is done on a microtome using a steel knife and sections are cut 3 – 5 µm thick depending on the structures investigated (Ross *et al*., 2003). Once cut the sections are mounted on glass slides, de-waxed and rehydrated according to the stain that is to follow.

All methodologies for tissue processing and staining was optimized and these optimized methods for human normal and keloid skin are presented in Section 3.3.2.

3.3.1 Tissue processing

Tissue prepared for paraffin wax embedding was fixed overnight in 4% formaldehyde and 0.1 M phosphate buffered solution (PBS) (0.2 M Na_2HPO_4, 0.2 M $NaH_2PO_4 \cdot H_2O$, 0.15 M NaCl, pH = 7.4). The following day the samples were washed with PBS buffer three times for 30 minutes each and then dehydrated by an increasing series of ethanol concentrations (50% for 30 minutes, 70% for 1 hour, 90% for one hour, twice in 100% for one hour, and 100% ethanol overnight. The tissue was cleared of ethanol by placing the samples in 50% xylene in ethanol for 30 minutes, then 100% xylene for 2 hours. The tissue was then infiltrated with paraffin wax of increasing purity (30%, 70% and 100%) for one to two hours at 60ºC. The samples were

embedded in paraffin wax in grids and moulds, cooled at 4°C. Sections of 3 – 5 µm were cut using a Leica RM2255 microtome and a disposable steel blade. After mounting on glass slides, the sections were deparaffinised by placing the slides in 100% xylene two times for 5 minutes each. Rehydration was achieved by placing the slides in 100% ethanol for two minutes, also two times, and then in 90% and 70% ethanol for 1 minute each and finally in distilled water for 1 minute. Staining methods were done according to the specific requirements for visualisation of tissue components followed. After the slides were stained they were rinsed and dipped in 70%, 90%, 100% ethanol, then a xylene dip and mounted in resin with a coverslip.

3.3.2 Tissue Staining

3.3.2.1 Staining theory

In order to provide contrast and allow for differentiation of tissue components, sections to be viewed under a light microscope need to be stained.

3.3.2.1.1 Haematoxylin and Eosin

Haematoxylin and Eosin staining was undertaken to investigate general tissue structure within the keloid and to differentiate between the cells in the epidermis and dermis as well as to show the orientation and thickness of collagen fibres in the dermis. In this stain aluminium acts as a mordant so that haematoxylin can effectively bind to the negatively charged components of tissue such as DNA, RNA and other negatively charged proteins. Sodium iodate is used to oxidise the haematoxylin to haematein which, when bound to aluminium, is positively charged enabling it to bind to the negatively charged molecules. The oxidation of haematoxylin continues after formulation is complete and for this reason the haematoxylin dye is stronger after a period of time.

Decreased staining occurs at a decreased pH, as the application of acidic solution will break the bond between haematein and aluminium or aluminium and tissue. Using acetic acid solution of water or 70% ethanol or hydrochloric acid in ethanol will remove (differentiate) non-specific background staining of cytoplasm or in some cases mucin. Differentiation steps can be applied so as to optimise the staining contrast. Buffered solutions of organic acids are used for this purpose and increase the clarity and definition of the cytoplasm. A blueing reagent is needed

with haematoxylin stain to buffer the solution and darken the reddish brown colour of haematoxylin to a purple blue and provide sufficient contrast with the eosin.

Eosin is a commonly used cytoplasmic stain and is an anionic dye that is attracted to protein groups which are positively charged, cationic amino groups. The amino groups on proteins become ionised when bound to a hydrogen ion and this charged group attracts the eosin ions to form bonds between the lysine residues of protein and the negatively charged auxochromic groups of eosin. Eosin staining gives several shades to the tissue and the staining quality is depressed at high pH since amino groups are now unionised and no longer attract and bind eosin (Cook, 2006).

The haematoxylin dye solution was prepared by dissolving 1 g of haematoxylin in 1000 ml of distilled water, final concentration 0.1%. To this solution, 0.2 g of sodium iodate and 50 g of potassium aluminium sulphate were added and dissolved. Then of 1 g citric acid and 50 g of chloral hydrate was also added and then dissolved. Aging of the stain is not required as the oxidative agent causes oxidation and will continue to do so over time.

To prepare the eosin stain, 2 g of yellowish eosin powder was dissolved in 200 ml of distilled water. Scott's buffer solution was prepared by dissolving 2 g of potassium bicarbonate and 20 g of magnesium sulphate in 100 ml of distilled water.

To stain the tissue the slides were de-waxed according to the procedure mentioned above, and rehydrated, then stained with haematoxylin for 10 minutes, Scott's buffer for 10 minutes and dipped in eosin, followed by distilled water for one minute. The sections were then dehydrated and mounted with a coverslip and xylene based mounting media.

3.3.2.1.2 Picrosirius Red

Direct red 80 or Sirius red is a poly azo dye and is used for the red staining of all types of collagen with yellow and green birefringence of fibres (Dapson *et al.*, 2011). Picrosirius red (PR) allows for evaluation of different polarizing colours of collagen. This stain enhances the normal birefringence of collagen fibres in tissue sections. Thin fibres exhibit green to yellow polarizing colours and thick fibres show yellow-orange to orange-red colours. Colour and intensity of birefringence are due to the difference in their pattern of physical aggregation and thickness of collagen fibres (Junqueira *et al.*, 1979). Thin fibres form a loose network interacting with ground

substance and this physical aggregation probably results in the weak green-yellow birefringence and variable green to yellow to orange and finally red birefringence (Velindala *et al*., 2014).

Green to yellow also indicates poorly packed collagen while an orange to red colour indicates tightly packed fibres (Hirshberg *et al*., 1999; Junqueira *et al*., 1999). Loosely packed thin fibrils of type III collagen have greenish to yellow colour (Perez Tamayo and Manfort, 1980). Yellow orange to reddish orange is type I collagen arranged in thick bundles (Velindala *et al*., 2014)

Sirius red is a strong cationic dye that stains collagen by reacting with its sulphuric acid and basic groups present in the collagen molecules. Collagen molecules, being rich in basic amino acids are able to react strongly with acidic dyes. Sirius red is an elongated dye molecule, which reacts with collagen and promotes the enhancement of its normal birefringence as the dye molecules are aligned parallel with the long axis of the collagen molecule (Montes and Junqueira., 1991). The reaction takes place between the sulphonic acid groups of the dye and the basic groups of collagen. PR staining increases sensitivity and resolution, as thin fibres not visible with normal microscopy becomes visible with the use of PR and polarised light (Junquiera *et al*., 1979).

In polarization microscopy a polarizer or a polarizing filter is located between the light source and the specimen. The ability of a crystalline or a paracrystalline structure to rotate the plane of polarized light is called birefringence, meaning double refraction (Ross *et al*., 2003). Crystalline and fibrous components of biological material possess a characteristic orientation of molecules and present with specific birefringence properties (Gesener, 1986).

To prepare PR stain, 0.5 g Sirius red dye was weighed out and dissolved in 500 ml of aqueous solution of picric acid. Acidified water was used for washing. The tissue was stained in hematoxylin prepared as described above for 8 minutes and rinsed in running tap water for 10 minutes. The PR solution was applied for one hour and then washed twice with acidified water. The tissue was then dehydrated three times in 100% ethanol and then cleared in xylene. The samples were visualised using a Nikon Optiphod transmitted light microscope.

3.3.2.1.3 Luna Stain

The Luna stain dyes elastic fibres purple, mast cells purple, and nuclei black with a yellow background. The stain consists of three different dyes;

Aldehyde Fuchsin (AF) dye was first introduced as a stain for elastic fibres, beta cells in the islets of pancreas, basophils and mucin (Gomori, 1950). AF stains tissue components which are rich in dissociable acidic groups (Buehner *et al*., 1978). Various authors have described different bonding structures with AF and these are reviewed by Puchtler *et al.* (1979), but the staining mechanisms of AF are diverse and difficult to explain on the basis of its conventional formula of a Schiff reaction.

In the periodic acid Schiff reaction; hexose rings of carbohydrates contain adjacent carbons, each of which has a hydroxyl (-OH) group. The hexosamines of glucosaminoglycans can contain adjacent carbons with an (-OH) group and another with a ($-NH_2$) group. Periodic acid cleaves the bond between the adjacent carbon atoms and forms aldehyde groups. These aldehyde groups react with Schiff reagent to give a purple/magenta colour (Ross *et al*., 2003). Paraldehyde depolymerises in the presence of HCl to form acetylaldehyde which condenses with free form amino groups of basic fuschin to form a Shiff base (Bangle, 1954)

Methyl orange is used as a negative or cationic counter stain to the positively charged collagen and other cations in the tissue and is used as staining is lighter than eosin.

Haematoxylin is used to stain the nuclei as described in Section 3.3.2.1.1. Iron haematoxylin is used in this stain and contains ferric salts, either ferric chloride or ferric aluminium sulphate. This stain is darker and more resistant to acidic counterstains. Ferric salts are oxidising agents as well as mordants and will accelerate the oxidation of haematoxylin to haematein.

For Luna staining an aldehyde fuchsin solution was prepared by dissolving 1 g basic fuchsin dissolved in 200 ml of 70% alcohol, 2 ml concentrated HCl and 2 ml paraldehyde which was left to stand for 2 - 3 days and filtered before use.

An iron haematoxylin stock solution was prepared by adding 1 g haematoxylin to 100 ml of 95% ethanol. A second stock solution of 4 ml ferric chloride in 95 ml distilled water with 1 ml concentrated HCl was also prepared. A working solution was made up by using equal parts of the two stock solutions.

The methyl orange stain was prepared by dissolving 0.25 g methyl orange in 100 ml of 95% ethanol, which was filtered before use.

For staining the slides were de-waxed and rehydrated to 95% ethanol and stained with AF for 30 minutes, after which they were rinsed 3 times in 95% ethanol. Haematoxylin staining was

done for 10 minutes and washed off in running tap water for 10 minutes and a 95% alcohol rinse. The methyl orange stain was applied for 10 minutes, and the slides were then dehydrated and mounted.

To achieve objectives 2 and 3, the following technique was used.

3.4 TRANSMISSION ELECTRON MICROSCOPY

Electron microscopy (EM) increases the detail at which subcellular structures can be studied. EM is used to study the ultrastructure of a cell, including the detailed structure of the cytoplasm and membrane that are not visible with the light microscope. EM allows visualisation of finer ultrastructural components of cells by using an interaction of electron beam and specimen to produce an image. Strong resolving power is dependent on specimen preparation and thickness. EM has high resolution but low penetrative power so correct specimen preparation is essential. The portions of the specimen that electrons pass through are seen as bright areas while those of dark are areas where electrons have been absorbed due to the density of the area or heavy metals added during preparation of the specimens (Ross *et al*., 2003).

Tissue preparation for EM requires finer methods than LM and the specimens need to be smaller in size and thinner. Glutaraldehyde is used in fixation to preserve proteins by cross linking them and osmium tetroxide reacts with phospholipids and gives electron density to cell and tissue structures and enhances the images formed on the TEM. Tissue for EM is sectioned with a diamond knife and once cut; the sections are floated out on water and picked up with copper mesh grids. The grids have 50 to 400 holes for the electron beam to pass through and onto the specimen to enable viewing (Ross *et al*., 2003).

Staining of TEM sections is needed to increase the contrast to enable easier viewing of cell structures. Tissue is soaked in solutions of heavy metal ions to enable the ions to bind to the tissue and increase its electron density and provide higher resolution to the images. Osmium tetroxide used in the fixation process binds to phospholipids of membrane giving them additional density. Uranyl acetate and lead citrate are commonly used to stain tissue after it has been mounted on grids (Ross *et al*., 2003).

In this study tissue samples were fixed in 2.5% Glutaraldehyde/Formaldehyde in 0.075 M phosphate buffer for 1 hour and rinsed three times in 0.075 M sodium potassium phosphate buffer (pH = 7.4) for 15 minutes before being placed in a secondary fixative, 1% osmium tetraoxide solution, for 1 hour. Following secondary fixation, the samples were rinsed again as

described above. The samples were dehydrated in 30%, 50%, 70%, 90% and three changes of 100% ethanol and were then embedded in Quetol resin. Ultra-thin sections (70 - 100 nm), cut with a diamond knife using an ultramicrotome, were then made and were contrasted with uranyl acetate for 15 minutes followed by 10 minutes of contrasting with lead citrate. Finally the samples were allowed to dry for a few minutes before examination with the JEOL Transmission Electron microscope (JEM 2100F).

Cells were identified according to the characteristics described in the literature review and presented in table 3.1:

Table 3.1: Morphological characteristics used to identify cell types

Cell	Morphological features
Keratinocytes	Desmosomes/ desmosomal junction, tonofilaments, keratinosomes
Fibroblasts	Spindle shaped cell, elongated nucleus
Myofibroblasts	Fibronexus
Mast cells	Cytoplasmic granules, villous cell membrane
Melanocytes	Cytoplasmic melanosomes, cellular processes extending into epidermis
Collagen	Cross striation in longitudinal section
Elastin	Regular contours, central amorphous matrix, fibrillin microfilaments

Chapter 4: Results and Discussion

Chapter 4A: Light Microscopy

Light microscopy with haematoxylin and eosin (H&E) staining was used in this study to investigate the general morphology of control and keloid tissue. Using this technique the structure of the dermis and epidermis, the epidermal-dermal junction, the rete ridges and the distribution of the collagen in the dermis was evaluated. With specific staining methods, the distribution and arrangement of collagen as well as the distribution of mast cells was investigated in greater detail.

4.1 EPIDERMIS AND DERMIS

The surface of normal skin has a loose keratin layer on the surface, below which is a thin epidermal layer followed by the dermal layer, consisting of an ECM made up of collagen, fibroblasts and various immune cells. The epidermal layer contains keratinised stratified squamous epithelial cells, melanocytes, Langerhans' and Merkel cells. The dermis is divided into the papillary and reticular dermal layers. The papillary dermis consists of fine collagen and thicker more compact fibres are found in the lower reticular dermis. The epidermis is joined to the dermis by a dermal-epidermal junction formed by basal epidermal cell membrane, the lamina lucida, the lamina densa and the fibroreticular lamina. The role of the dermal-epidermal junction is to bind the epidermis to the dermis, to determine the polarity of the basal keratinocytes, to serve as a selective barrier as well as to serve as a glycoprotein foundation for re-epithelialisation in wound healing. Bordering on this layer are the basal keratinocytes, melanocytes and Langerhans' cells. The dermal-epidermal border is irregular and contains rete ridges and the depth of these rete ridges depends on the thickness of the skin which correlates to shearing forces on the skin. This means that thicker skin will have more rete ridges than thinner skin. In Figure 4.1, the epidermal and dermal layers consisting of the reticular and papillary dermis can be identified. In the control skin samples used in this study the dermal layer is thin as the skin used was excess skin from skin transplants. The depth of the rete ridges is variable and they are either absent (Figure 4.1a), present (Figure 4.1b) or penetrate into the dermis (Figure 4.1c).

In the keloid tissue the same variations in the distribution of the rete ridges are found as seen in Figure 4.2c compared to 4.2f. In some instances the rete ridge is not as rounded as in normal control skin, i.e. they have a more blunted appearance (Figure 4.2f). The keloid has a well defined dermis, however it is difficult to distinguish between the papillary and reticular dermis. Furthermore the collagen arrangement is irregular and denser when compared to normal skin (Figure 4.1a).

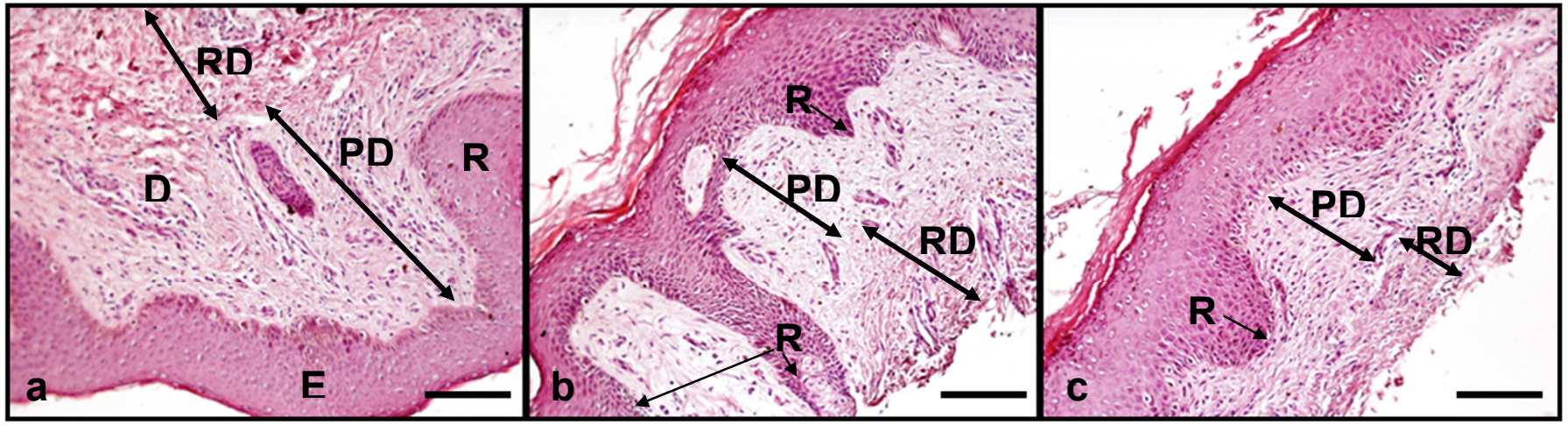


Figure 4.1: Control skin: Structure of the epidermis (E) and dermis (D) of different skin samples. Papillary dermis (PD), reticular dermis (RD) and rete ridges (R) are shown. H&E staining. Scale bars =20 µm.

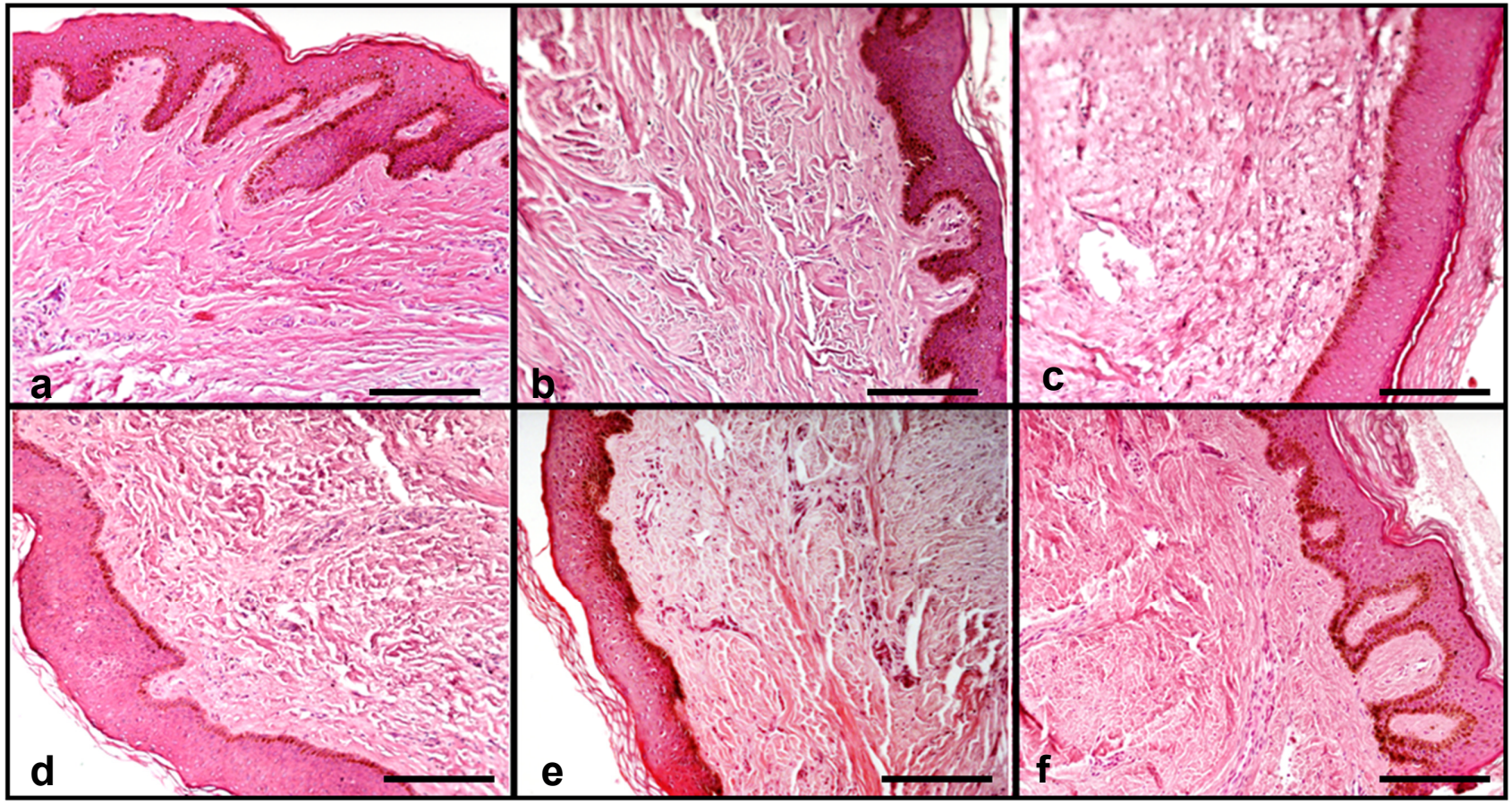


Figure 4.2: Keloid: Irregular structure of the keloid epidermis and dermis of different keloid samples, a - f each from a different patient. H&E staining. Scale bars = 25 µm.

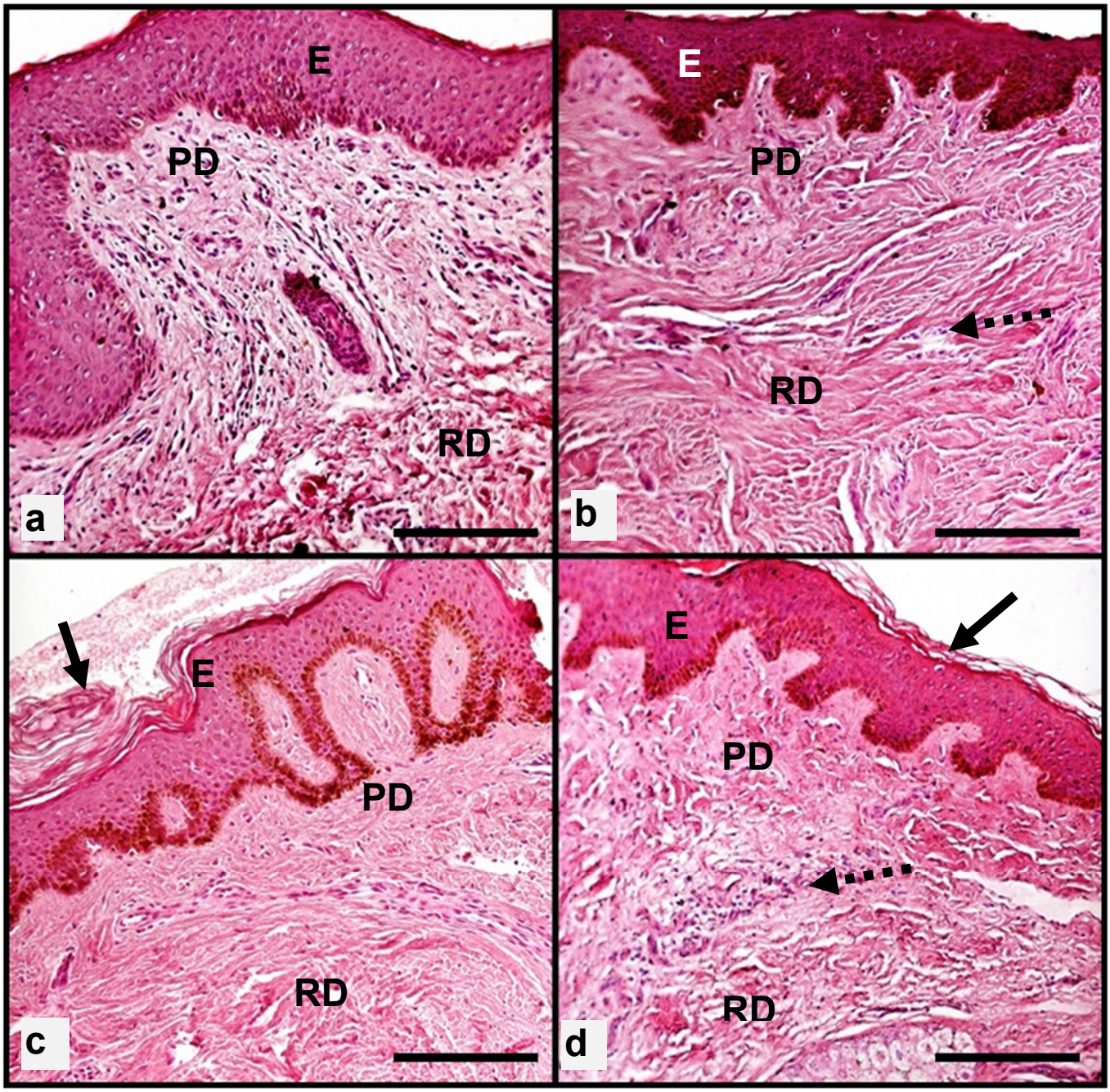


Figure 4.3: Normal, control (a) and **keloid** (b - d) skin showing the stratum corneum (arrows), epidermis (E), rete ridges as well as the papillary (PD) and reticular dermis (RD). Thickened and irregular arranged collagen is seen in keloid sections as well as fibroblast distribution (dashed arrow). H&E staining. Scale bars = 25 µm.

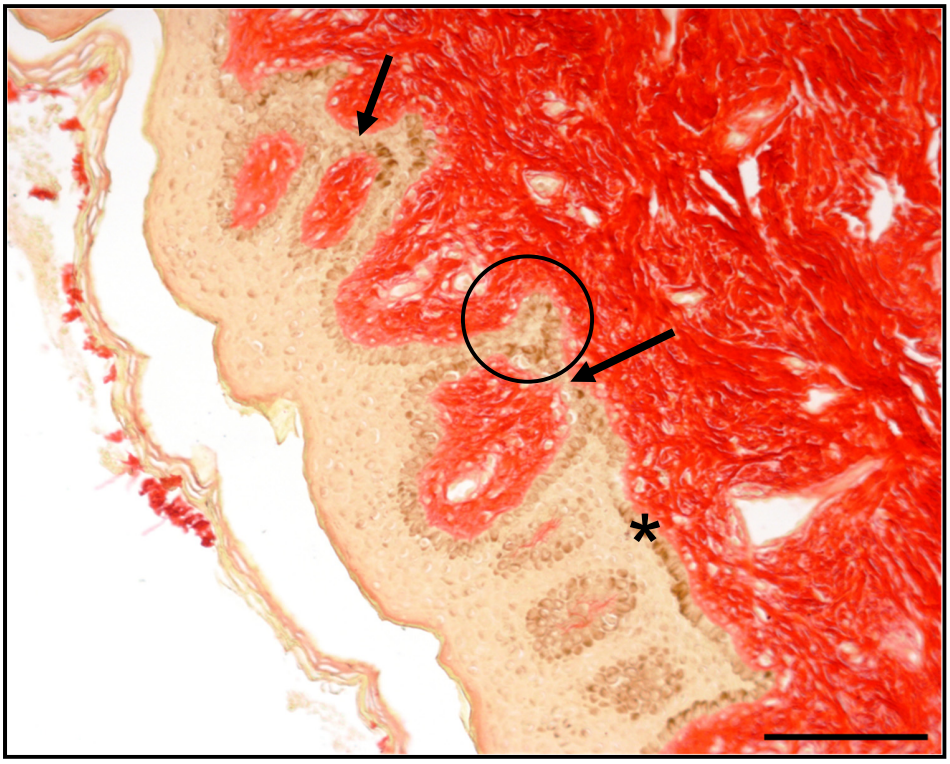


Figure 4.4: Keloid epidermis structure showing blunted epidermal ridges (star) with bulbous thickening (circle) and fusion of their lower portions (arrows) with thin suprapapillary epidermal plates. PR stain, brightfield light. Scale bar = 20 µm.

At a higher magnification (Figure 4.3) it can also be seen that the rete ridges of the epidermal-dermal junction are blunted as seen in Figure 4.3d. This may be due to the thickness and density of the collagen in the dermis below, that prevents the epidermal cells from moving into the dermis. The dermis of keloid skin has thick and dense collagen bundles that are arranged irregularly. Compared to the control where the distribution of fibroblasts is regular throughout the dermis, the distribution of fibroblasts in the dermis of keloid skin is variable with either a few (Figure 4.3b) or many (Figure 4.3d) fibroblasts present in the dermis (dashed arrows).

The formation of thin suprapapilary epidermal plates was seen on the keloid samples (Figure 4.4). The formation of suprapapillary epidermal plates was observed in psoriasis (D'Costa and Bhrambe, 2010) and in hereditary gingival fibromatosis which is characterised by gingival enlargement (Häkkinen and Csiszar, 2006). Formation of these plates can therefore be associated with diseases which cause tissue mass enlargement resulting in dermal irregularities. Elongated rete ridges seen in gingival fibromatosis were suggested to be caused by the increased proliferation of epidermal cells (Häkkinen and Csiszar, 2006). In the present

study the formation of suprapapillary epidermal plates and a blunting of the epidermal ridges instead of elongation, is proposed to be the result of dermal hyperproliferation. The increased forces of the dermal collagen on the dermis could reduce epidermal cell extention into the dermis.

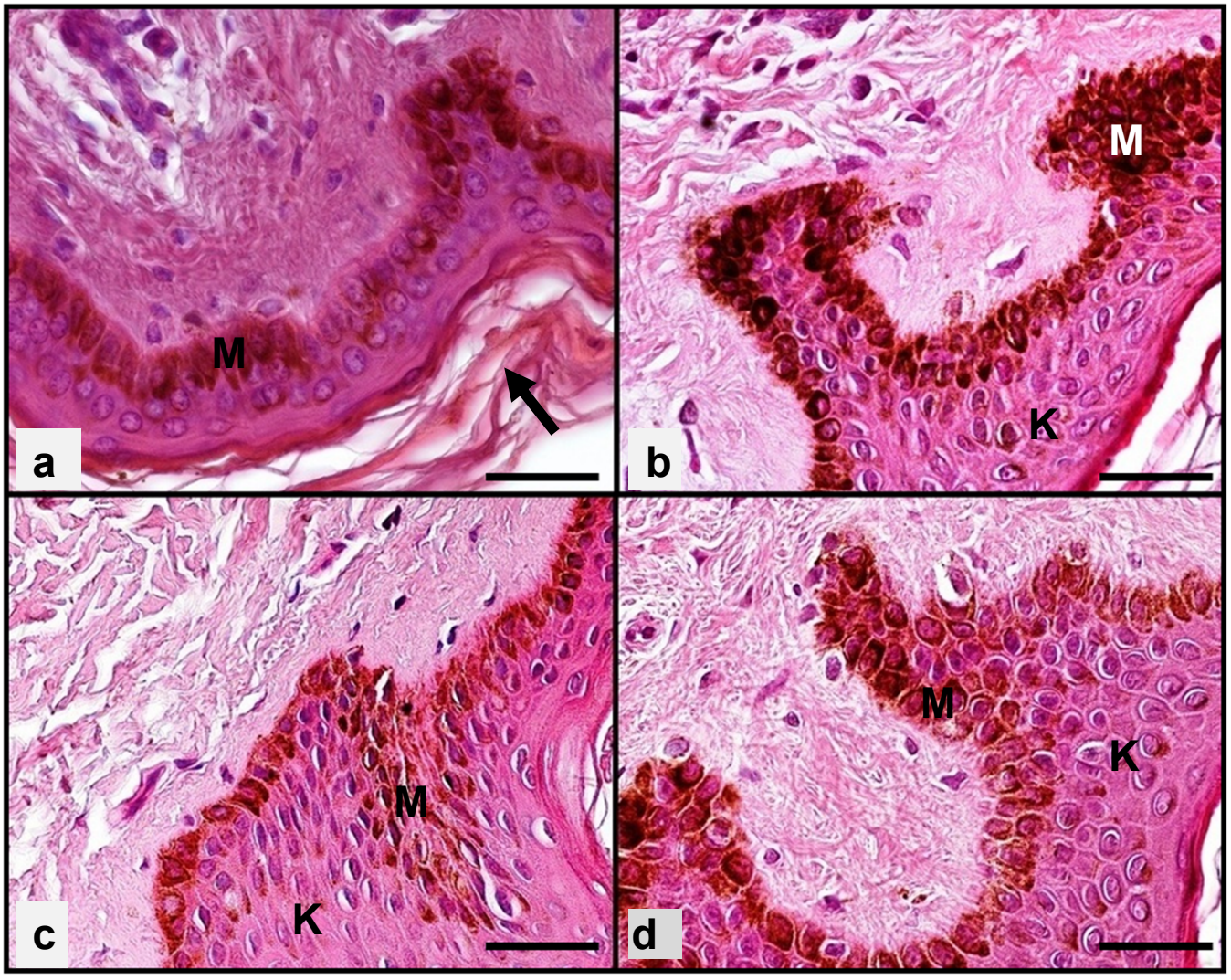


Figure 4.5: Normal, control (a, c) and **keloid** (b, d) epidermis showing the stratum corneum (arrow), as well as the distribution of the keratinocytes (K) and melanocytes (M). H&E staining. Scale bars = 5 µm.

Figures 4.5 a - d, are higher magnifications of the epidermis. Desquamation of keratin in the superficial layer of the skin, the stratum corneum, is shown in Figure 4.3 c and d as well as Figure 4.5 a and b. Desquamation is indicative of natural surface cell loss, where desquamated cells join together to form a membrane of dead cells (Gesener, 1986). Below the stratum corneum is the stratified layer of keratinocytes which at the epidermal-dermal junction are associated with melanocytes. The melanocytes are clearly visible in this region due to the presence of melanosomes containing melanin.

Ehrlich *et al*. (1994), showed the epidermal layer to be thicker in some keloids than in control tissue; however this was not a consistent finding. In the present study the epidermal layers of keloid skin appears to be thicker however this is not conclusive as the control skin was obtained from skin grafts which is generally thinner than normal skin and the number of samples used in this study are not sufficient for a statistically significant conclusion. In addition, the thickness of the epidermal layer is dependent on factors such as the area of the body, age of the patient as well as the size of the keloid.

4.2 COLLAGEN DISTRIBUTION IN THE DERMIS OF CONTROL AND KELOID SKIN

Normal skin has a distinct separate papillary dermis (Figure 4.3a) (label PD), with fine collagen fibres, and a reticular dermis (label RD) with denser arrangement of collagen. Collagen in normal skin is synthesised in fibroblasts and secreted into the ECM where it forms collagen bundles. In keloids collagen production is increased resulting in the formation of a keloid mass. Light microscopy with H&E was used in the present study along with a collagen specific PR stain in order to compare the structure and orientation of keloid collagen to normal skin (control).

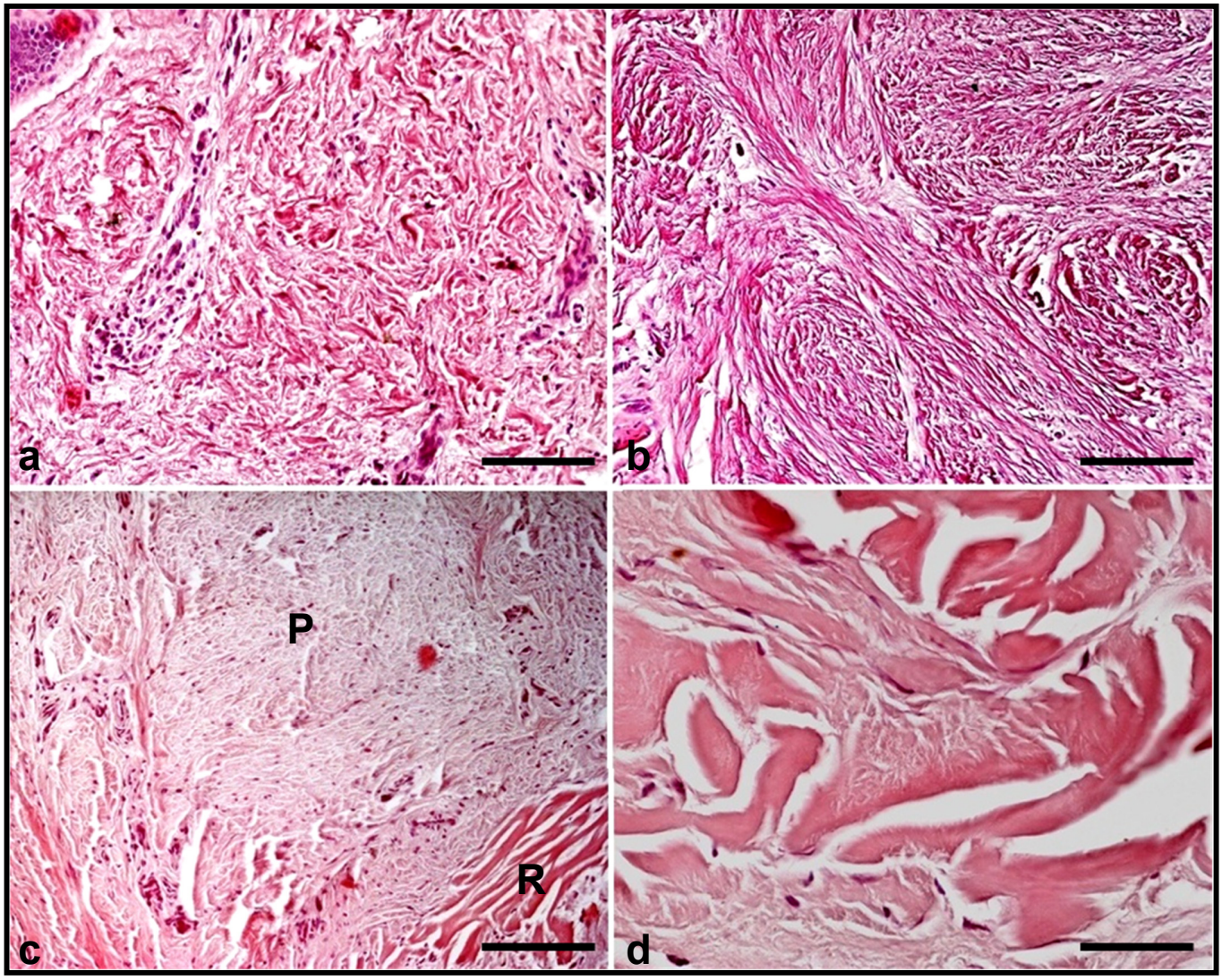


Figure 4.6: **Control** (a) and **keloid** (b, c, d) skin. In (a) the dermal collagen is fine and fibrillar compared to woven, thicker, irregularly arranged collagen of keloid tissue (b - d). In the keloid tissue the papillary dermis (P) collagen is fine, while reticular dermis collagen (R) is considerably thicker. (d) Increased magnification of reticular collagen in (c) showing large bundles of fibres. H&E staining. Scale bars (a, b, c) = 55 µm, scale bar (d) = 11 µm.

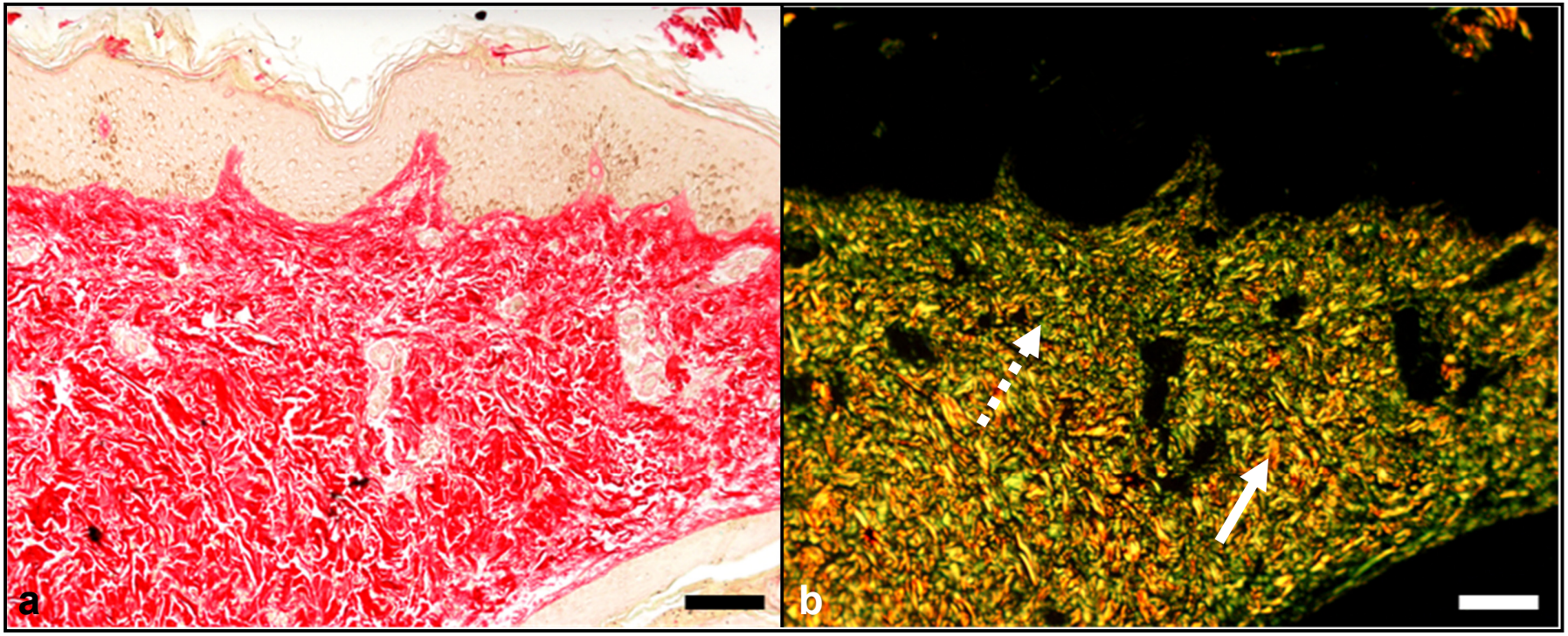


Figure 4.7: Control skin stained with PR and viewed with bright field (a) and polarised light (b). Birefringent collagen is visible in (b) Green-yellow type III collagen fibres (arrow) and orange, type I collagen fibres (dashed arrow) are indicated. PR staining. Scale bars = 30 µm.

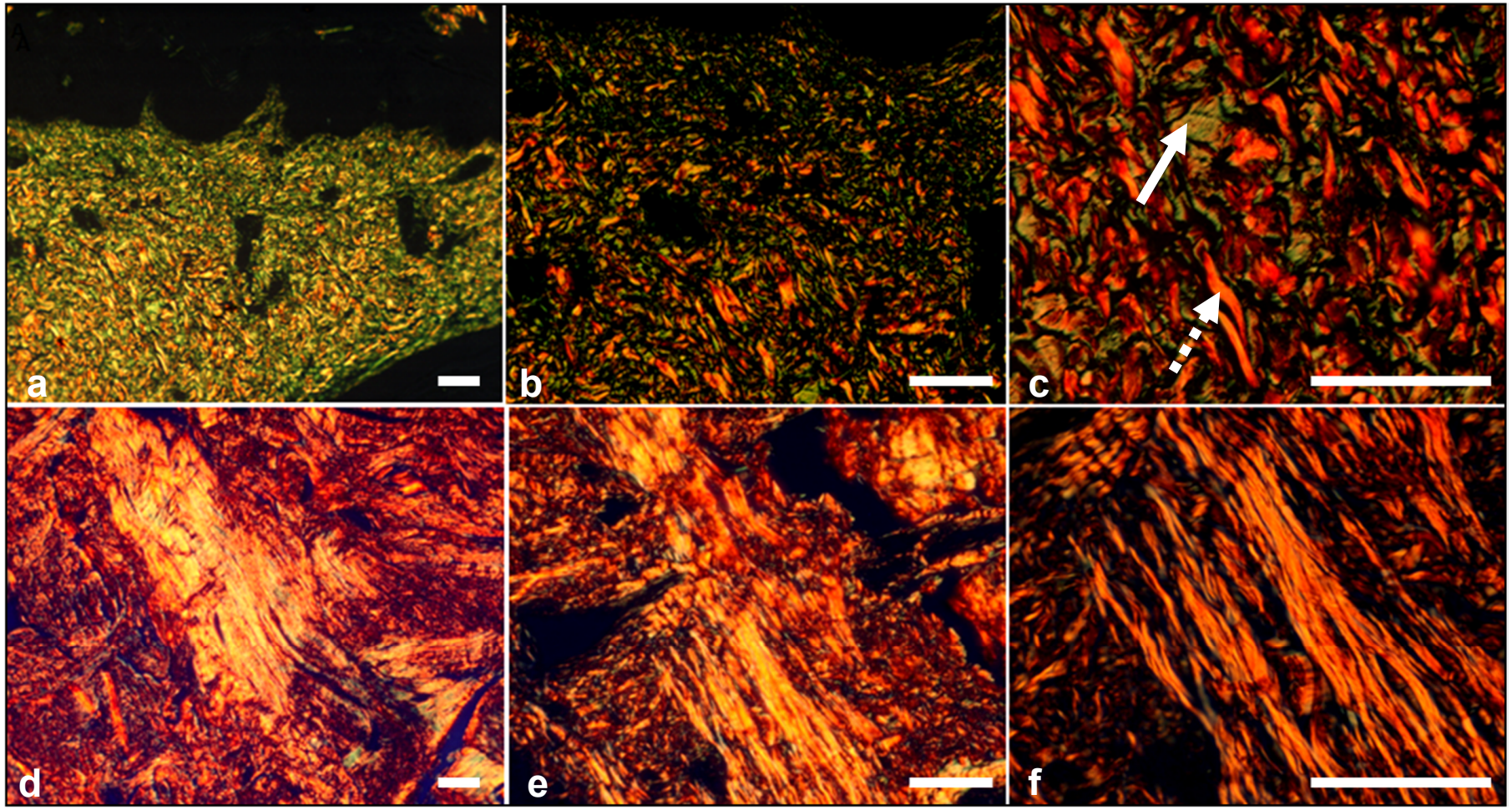


Figure 4.8: Picro Sirius staining of **control** (a - c) and **keloid** (d - f) keloid tissue, showing differences in the type, distribution and arrangement of collagen at increasing magnification. Collagen type III fibres (arrow) and type I fibres (dashed arrow) are indicated. PR staining. Scale bars = 10 µm.

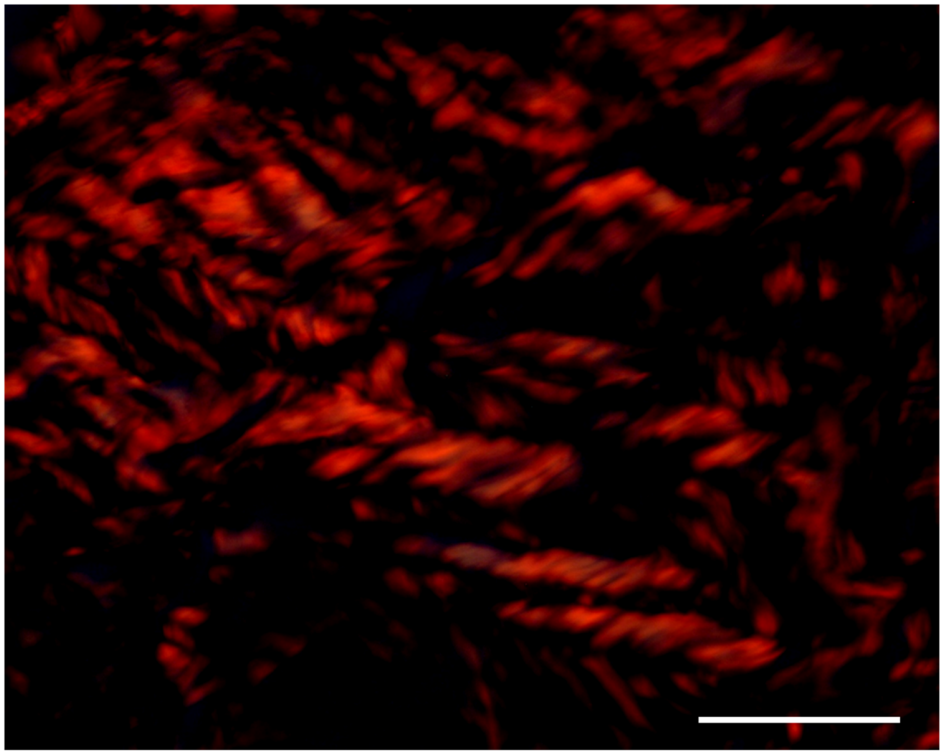


Figure 4.9a: **Keloid** bundle arrangement showing that collagen type I is the predominant type, arranged in thick bundles. PR staining. Scale bar = 5 µm.

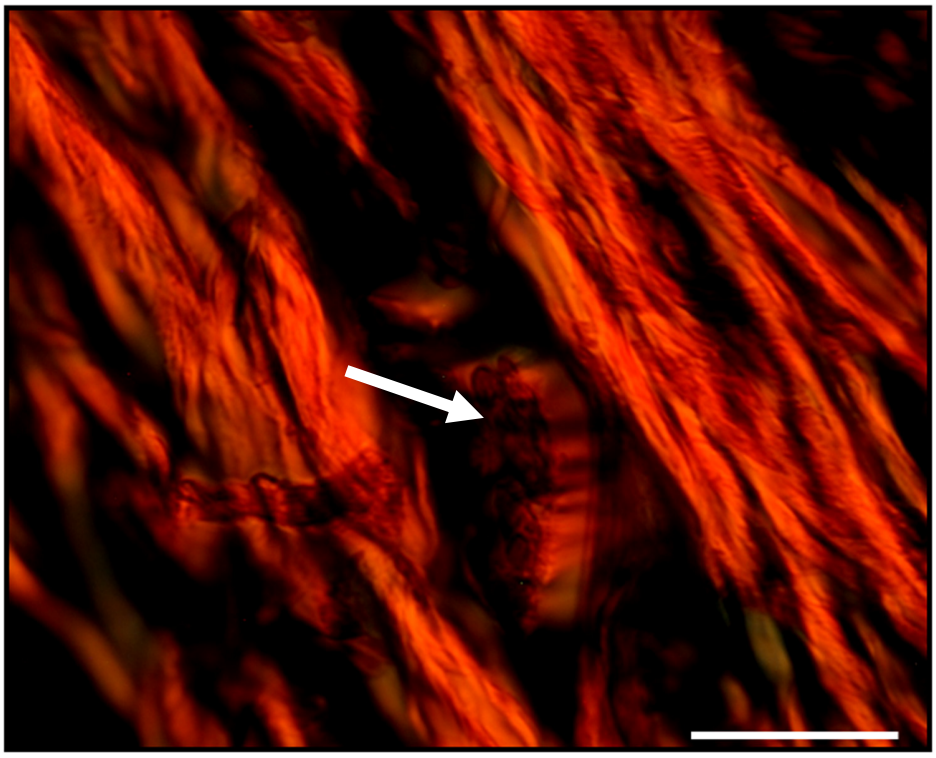


Figure 4.9b: **Keloid** bundle arrangement showing that collagen type I can also have an interwoven bundle arrangement. PR staining. Scale bar = 5 µm.

The dermal layer composition of control and keloid skin was evaluated and is shown in Figure 4.6. Keloid tissue (Figure 4.6b) has dense and thick collagen even in the area of the papillary dermis where finer collagen should be found. The papillary and reticular dermis are distinguishable in some the keloid sections (Figure 4.6c) with the reticular dermis collagen being a lot thicker when compared to control skin (figure 4.6a). In Figure 4.6d, no difference between the two layers of the dermis can be seen, instead the collagen is a mass of irregular fibres.

To better investigate the distribution of collagen in the dermal layers the tissue was stained with PR stain. PR is a collagen specific stain in which the orientation of collagen fibres and bundles can be evaluated. With bright field the PR stains specifically collagen in the dermal layer (Figure 4.4 and 4.7a). With polarised light, birefringent collagen is visible (Figure 4.7b). At higher magnifications the loose thinner type III collagen fibres appear green (arrow) and the denser, thicker type I collagen fibres appear orange or red (dashed arrow) (Figure 4.8c). Normal adult skin contains about 80% type I and 20% type III collagen (Liu *et al*., 2005; Diegelmann, 2001; Klein, 2001).

Velindala *et al*. (2014), used the PR stain to identify elastin in skin samples. Like collagen type III, elastin occurs in the ECM as fine fibres and with polarised light appears green in colour. As collagen type III is the lesser collagen type in skin and elastin also appears green with polarisation this may account for the amount of green staining seen in Figure 4.8 a - c.

Using this staining technique the pattern of collagen arrangement can also be evaluated. Control skin presented with an even interwoven distribution of green and orange fibres. Increased presence of collagen type I in keloids was found by Levame and Meyer, (1986), using immunocytochemistry. Shaker *et al*. (2011), also described the presence of predominantly collagen type I in keloids determined with the Herovici stain which differentiates between type I and type III collagen. Syed *et al*. (2011), reported the presence of active fibroblasts in the margins of the keloid mass responsible for the collagen production and increased deposition, as well as the formation of thick hyalinised collagen bundles in the growing margins of keloids. Keloid fibroblasts were reported by Uitto *et al*. (1985), to increase their synthesis of type I collagen, causing an over deposition of collagen in keloid and an imbalance between type I and III collagen.

In the present study at a higher magnification only type I collagen fibres are present (Figure 4.8 e and f). These fibres form thick bundles throughout the dermis. From Figure 4.2 and 4.6 collagen distribution in keloids is variable where in all keloid samples collagen type I is the

predominant type of collagen that can either be interwoven (Figure 4.9a) or arranged in thick bundles (Figure 4.9b). Excess collagen deposition can occur as a result of increased collagen production due to the up-regulation of growth factor synthesis, or an increased sensitivity of keloid fibroblasts to these growth factors, or a defective negative feedback (Kischer *et al*., 1982a). The sources of these growth factors are primarily keratinocytes and mast cells (Werner *et al*., 2007; Artuc *et al*., 2002; Noli and Miolo, 2001; Abel and Vliagoftis, 2008). Increased release of these growth factors is a result of increased cellular synthesis or increased cellular proliferation. This process will be described in greater detail in section 5.2.

4.3 CELLS OF THE EPIDERMAL LAYER

Cells of the epidermal layer, such as the keratinocytes, influence the growth of fibroblasts in keloid tissue via the release of growth factors such as IL-6 (Waelti *et al*., 1992; Smola *et al*., 1993; Boxman *et al*., 1996), keratinocyte derived IL-1 (Waelti *et al*., 1992; Maas-Szabowski and Fusenig, 1996; Maas-Szabowski *et al*., 1999), FGF, PDGF, TNF-α and TGF-β (Werner *et al*., 2007, Xia *et al*., 2004). Fibroblasts can influence the release of these growth factors in a double paracrine manner (Maas-Szabowski and Fusenig, 1996; Maas-Szabowski *et al*., 1999). Increased growth factor production and epidermal-dermal interactions are often associated with increased cellular proliferation and distribution (Phan *et al*., 2002; Lim *et al*., 2001) and this can indirectly lead to increased collagen synthesis by fibroblasts (Lim *et al*., 2002). Therefore the distribution and structure of these cells in the epidermal layer of keloid tissue was further investigated using light and transmission electron microscopy.

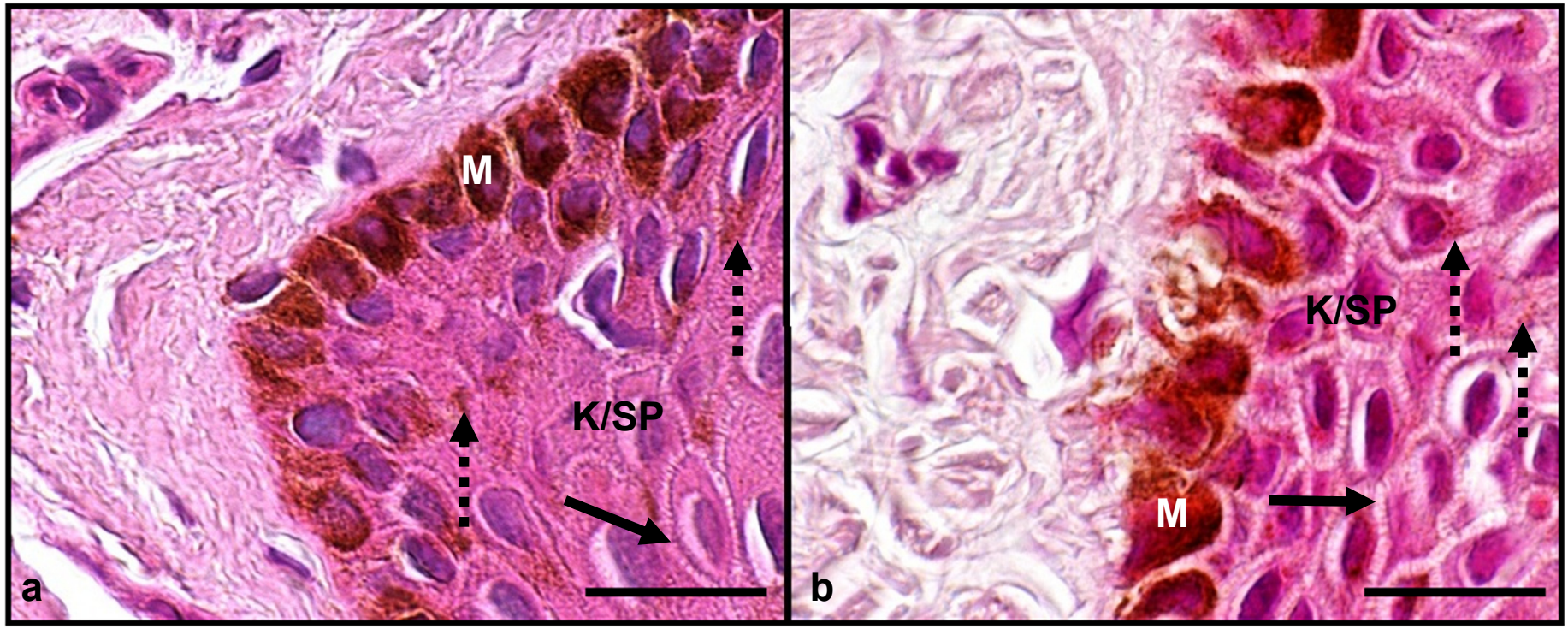


Figure 4.10: (a) **Control** and (b) **keloid** epidermis, showing the distribution of keratinocytes (K) in the *stratum spinosum* (SP) and melanocytes (M). In these sections keratinocyte cellular extensions of the *stratum spinosum* (arrows) are visible as well as the distribution of melanin in the keratinocytes (dashed arrow). H&E staining. Scale bars = 5 µm.

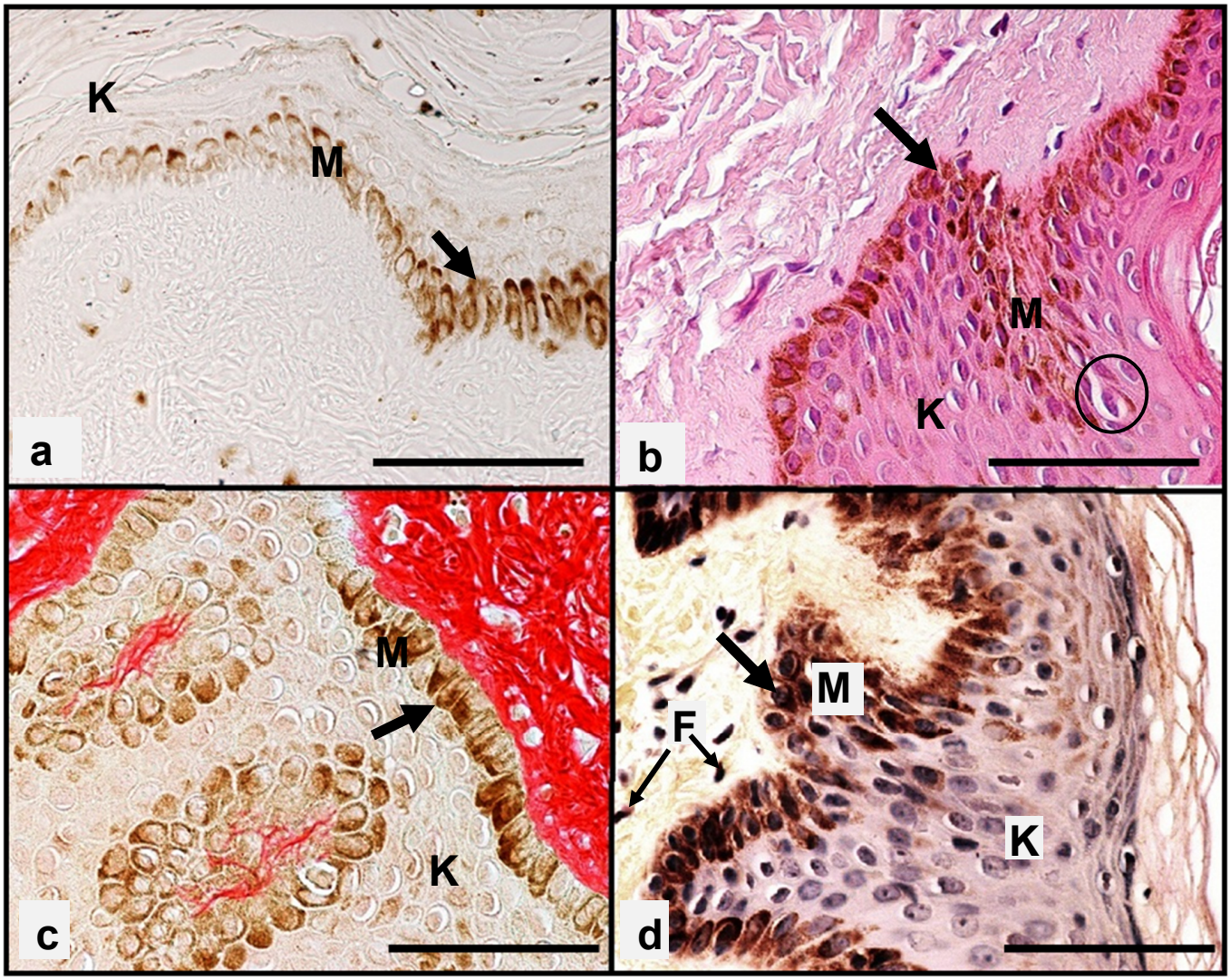


Figure 4.11: Keloid: Epidermal distribution of keratinocytes (K), melanocytes (M) and melanin containing keratinocytes, in (a) unstained tissue, showing the distribution of melanin (arrow), (b) H&E stained tissue showing keratinocyte distribution and the presence of a Langerhans' cell (circled), (c) PR with bright field showing the anterior distribution of melanin and (d) Luna stain that clearly shows nuclear morphology of the keratinocytes and fibroblasts (F). Scale bars = 20 µm.

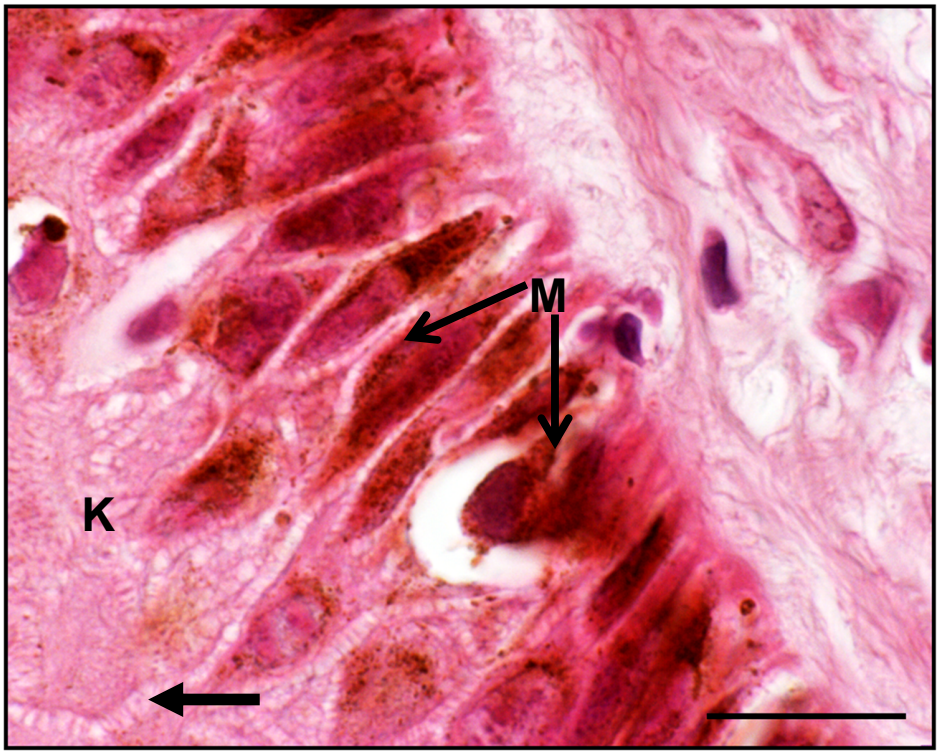


Figure 4.12a: Keloid epidermis showing melanocytes (M) with an elongated shape and keratinocytes (K) with the cellular projections between the cells (arrow). H&E staining. Scale bar = 5 µm.

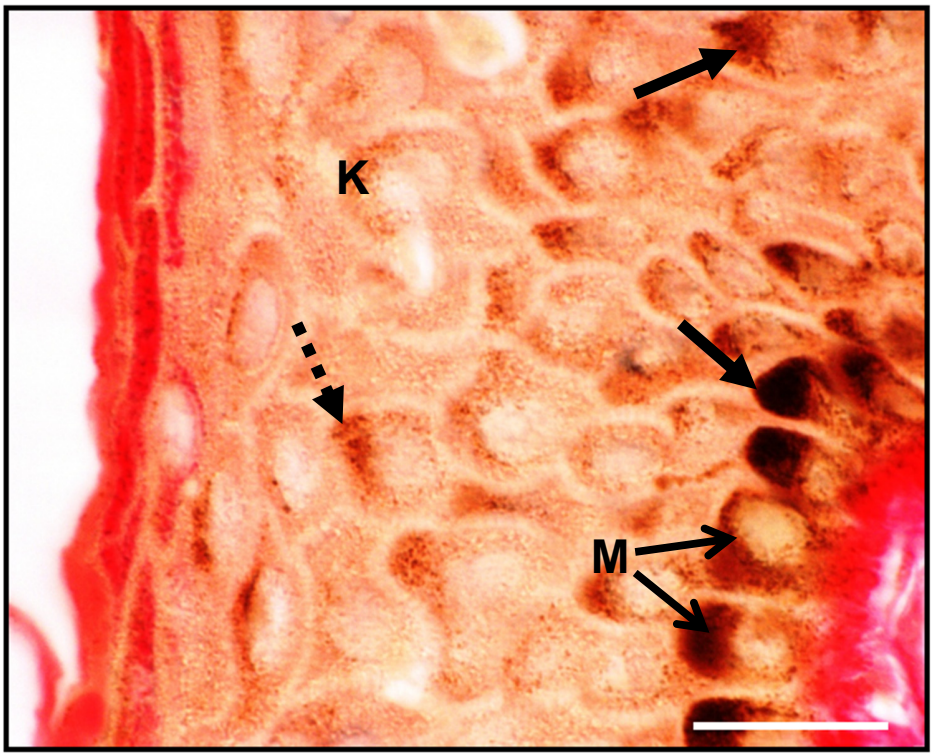


Figure 4.12b: Keloid epidermis showing melanin distribution (arrows) from melanocytes (M) to the surrounding keratinocytes (K). PR staining. Scale bar = 5 µm.

In Figure 4.10 the epidermis consists mainly of keratinocytes with the melanocytes occurring at the epidermal-dermal junction. Other cells found in the epidermal layer are Langerhans' and Merkel cells.

Melanocytes produce melanin, a dark brown pigment and are found in the *stratum basale* at the epithelial-dermal junction (Figure 4.5 a - d) and the ratio of melanocytes: keratinocytes is 1:10. These cells are round with long irregularly branched processes that extend between the *stratum basale* and *stratum spinosum*. The ends of the processes terminate as invaginations in the cells of the layers. Melanocytes contain few desmosomes and tonofilaments. The melanin is transferred to the cells of the *stratum basale* and *spinosum* through a process of cytocrine secretion. Therefore the melanocytes are the sites of melanin secretion while the keratinocytes are the sites of storage (Coetzee *et al*., 2003). As the *basale* keratinocytes divide and migrate to the upper layers melanin is transferred. This movement of melanin and subsequent distribution in the upper layers of the skin is shown in Figures 4.10a and 4.10b for control and keloid skin. Figure 4.12b clearly shows the high accumulation of melanin in the *stratum basale* and the gradual decrease in distribution of melanin from this layer towards the *stratum granulosum*. Accumulation of melanin in the keratinocytes is towards the anterior surface protecting the nuclei of keratinocytes from the damaging effects of UV radiation (Figure 4.12b, dashed arrow).

Langerhans' cells are also found in the epidermis (Figure 4.11b) and the morphology of these is similar to melanocytes in that Langerhans' cells also have cellular processes which extend between the cells of the epidermis. These cells lack desmosomes and tonofilaments and contain Birbeck granules (Coetzee *et al*., 2003). Langerhans' cells are antigen-presenting cells and play an important role in the immunity of the skin. Without immunocytochemistry staining for CD1a it is difficult to identify Langerhans' cells. The cell indicated in Figure 4.13 (circled) is possibly a Langerhans' cell. Merkel cells are derived from the neuronal crest (Mescher, 2010) and are found in the basal layer of keratinocytes at the dermal- epidermal junctions. Merkel cells are associated with free nerve endings and are pressure or touch receptors (Young and Heath, 2000) which are found in areas of high tactile sensitivity such as the fingertips (Mescher, 2010). Merkel cells were not identified in the tissue samples as they are difficult to distinguish from melanocytes and keratinocytes using light microscopy.

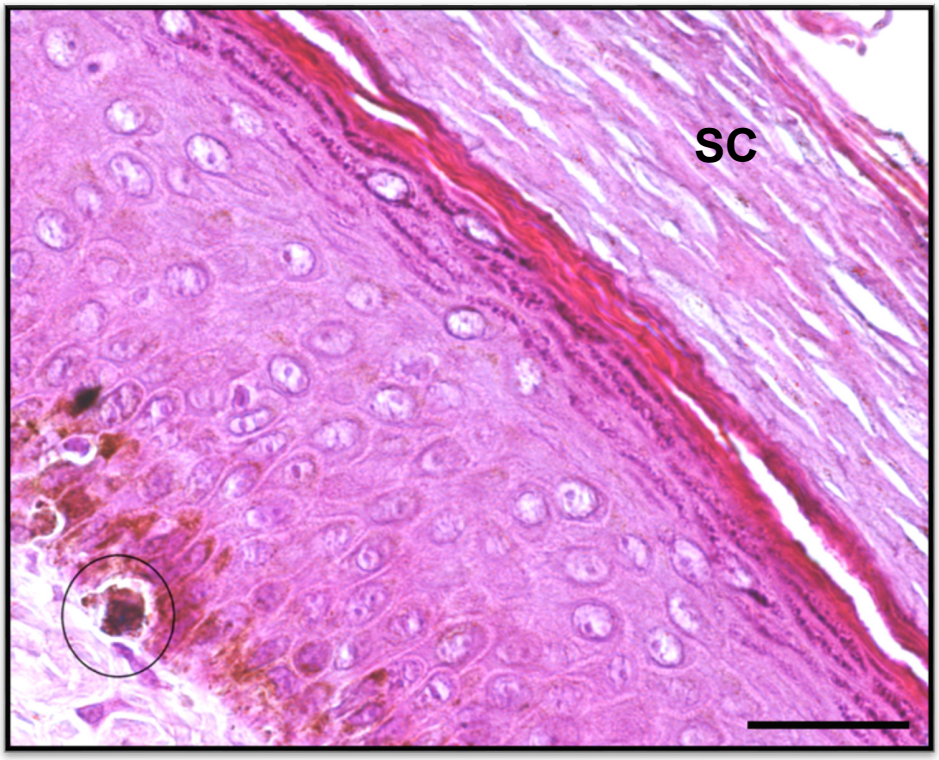


Figure 4.13: Keloid section showing possible Langerhans' cell at the dermis and epidermis junction (circled). A thick *stratum corneum* (SC) is visible H&E staining. Scale bar = 10µm.

4.4 CELLS OF THE DERMAL LAYER - FIBROBLASTS AND MAST CELLS

4.4.1 Fibroblasts

Fibroblasts are the most common connective tissue cells and are observed in histological sections as large flattened cells with tapering processes (Figure 4.14a). With H&E staining, in normal skin the cytoplasm is eosinophilic but usually only the flattened oval nucleus is visible as the cytoplasm is different to distinguish from the ECM (Figure 4.14a). With increased protein synthesis the cytoplasm of an active fibroblast becomes more basophilic due to increased biosynthesis of collagen and elastin (Gesener, 1986). With Luna staining (Figure 4.14b) the nuclei of the fibroblasts in the dermis are easier to identify against the yellow background of the ECM.

Fibroblasts are present in control and keloid skin (Figure 4.11d and 4.14 respectively). The distribution of the fibroblasts is dependent on the arrangement of collagen in the area. In normal skin, fibroblasts are found throughout the dermis. In some keloid sections only a few fibroblasts

are present especially in samples with very thick collagen. Appleton *et al.* (1996), described that keloids have a central area of increased fibroblast proliferation while fewer fibroblasts were found on the borders of the keloid.

PR staining is used to study the distribution of collagen in tissue. Interestingly in a keloid section either a pink or blue coloured spindle shaped structure (Figures 4.15a and 4.15b) was observed that resembles the shape of the nuclei of fibroblasts. An extensive search of scientific literature did not reveal an explanation for this observation. PR is an anionic dye and therefore cannot stain DNA. PR stain contains haematoxylin which stains the DNA and other basophilic components of the cell. Whether haemotoxylin has birefringent properties under polarised light is unknown. Nevertheless, indications are that these blue staining structures are fibroblasts contained within the keloid mass.

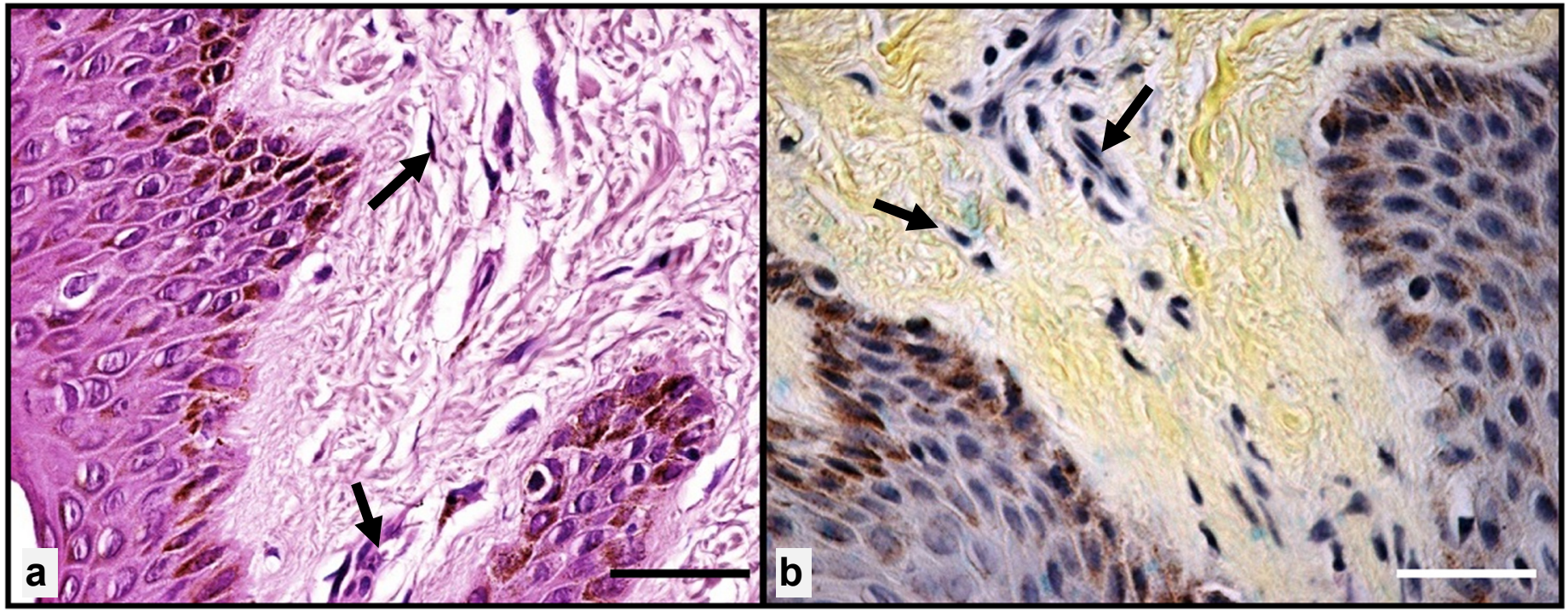


Figure 4.14: Control: Presence of fibroblasts in dermis (a) H&E stain and (b) Luna stain. Scale bars = 10 µm.

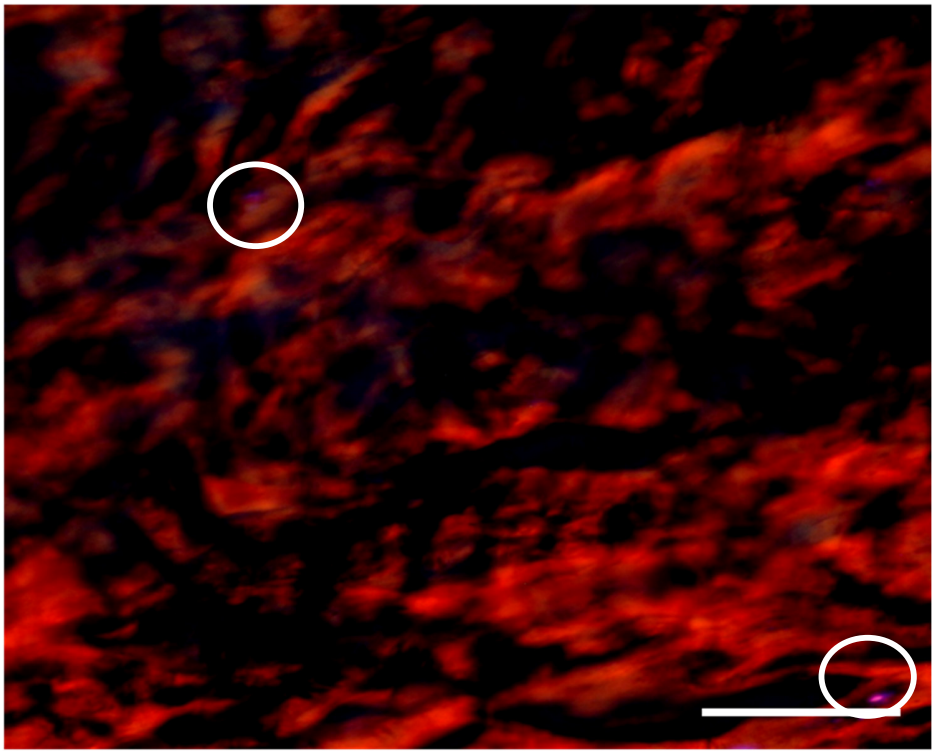


Figure 4.15a: Keloid cross section of the dermis stained with PR and visualised with polarised light showing the presence of pink (circle) staining spindle shaped structure (arrows). Scale bar = 2 µm.

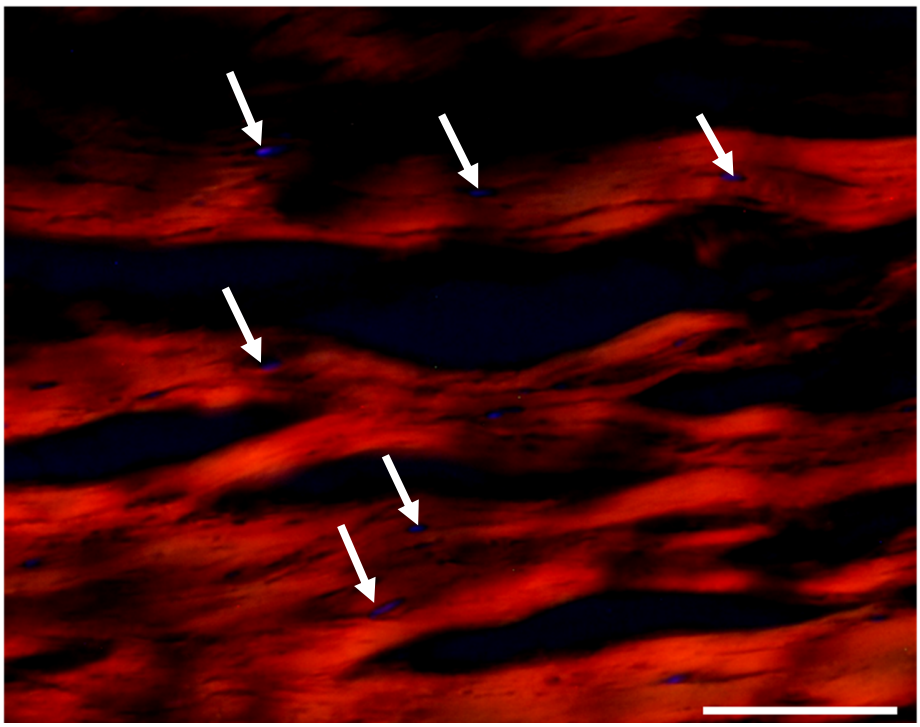


Figure 4.15b: Keloid: Cross section and longitudinal section of the dermis stained with PR and visualised with polarised light showing the presence of (a) pink (circle) and (b) blue staining spindle shaped structure (arrows). Scale bar = 2 µm.

4.4.2 Mast Cells

Mast cells have an immunological function in connective tissue of the skin and increased numbers of mast cells are observed in areas of inflammation and wound healing (Noli and Miolo, 2001; Wulff and Wilgus, 2013). Mast cells are large oval shaped cells with a small nucleus which is rarely seen as it is hidden by the large number of intercellular granules. Mast cell granules stain with basic dyes (Gesener, 1986) such as thionine, methylene blue, methylene violet, neutral red and toluidine blue (Young and Heath, 2000).

Luna stain dyes mast cells purple, nuclei black, and elastic fibers purple with a yellow background. No mast cells could be found in control skin (Figure 4.14b) while mast cells were found only in some of the keloid samples and were located in the dermis (Figure 4.16a). At a higher magnification the presence of histamine granules that are typical of mast cells can be seen (Figure 4.16c).

To better characterise the structure and activity of fibroblasts and mast cells, transmission electron microscopy analysis of this region of keloid tissue was further evaluated and these results are presented in sections 4.6.2. and 4.6.3.

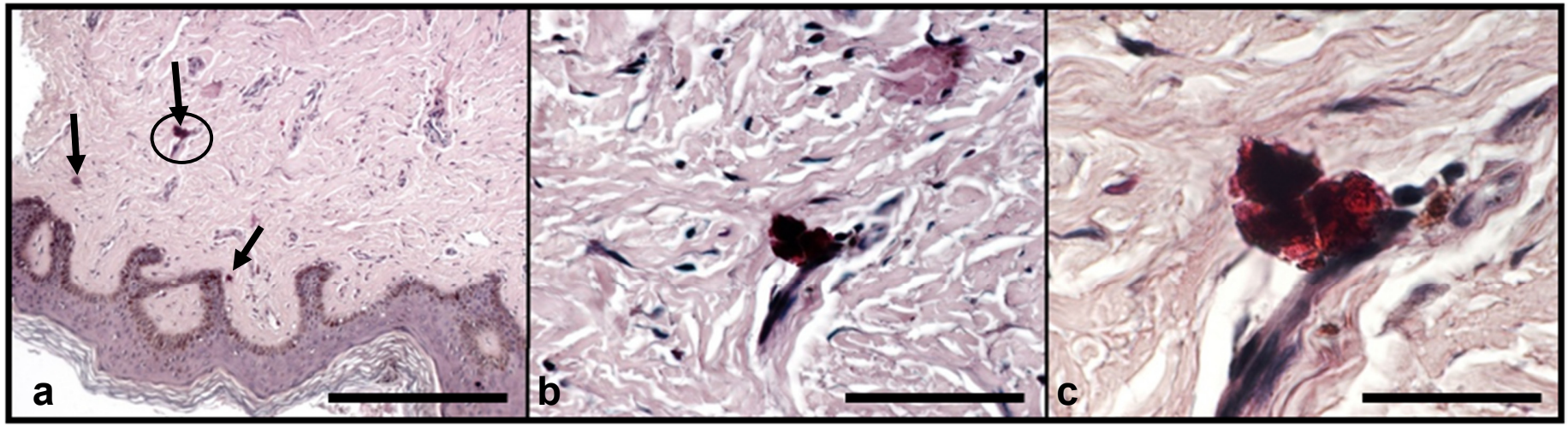


Figure 4.16: Keloid: (a, b, c) Mast cells (arrows) in increasing magnifications. (c) granular structure of the mast cell is visible. Scale bar (a) = 100 µm, scale bar (b) = 20 µm, scale bar (c) = 10 µm.

Chapter 4B: Transmission electron microscopy

The ultrastructure of normal, control and keloid skin was evaluated with transmission electron microscopy (TEM). This technique was used for the detailed evaluation of the structure of cells associated with the epidermis and dermis and included the evaluation of the structure and distribution of keratinocytes, melanocytes, collagen, fibroblasts and mast cells as well as the interactions of fibroblasts with mast cells. The cells described below were identified using the morphological characteristics of each cell type as shown in table 3.1.

4.5 EPIDERMIS

4.5.1 Keratinocytes

Keratinocytes are localised in the epidermal layers of the skin where they undergo differentiation and eventually form a surface layer of dead cells filled with the protein keratin which provides strength to the epidermis. The size, shape, quantity and the degree of differentiation is different in each layer. In the uppermost *stratum corneum* the remnants of keratinocytes, such as those seen in control skin are flattened and fused, appearing as smooth layers (Figure 4.13). The *stratum granulosum* underneath consists of granular keratinocytes containing keratohylin granules (Figure 4.18) as well as prominent tonofilaments, which are visible with TEM. The *stratum granulosum* could be observed only in keloid samples (Figure 4.18), as the epidermal layers of control skin were not distinguishable and this may be related to the source of the control skin.

The *stratum spinosum* is found beneath the *stratum granulosum* and contains keratinocyte cells with cytoplasmic cellular processes or spines, forming intracellular bridges, which are bound by desmosomes to the adjacent keratinocytes. This is seen in control skin in Figures 4.17 and 4.19 and in keloids in Figure 4.20. The keratinocytes in this layer are relatively large and polyhedral in shape. Cells in the *stratum spinosum* synthesise the protein keratin and it can be visualised as tonofilaments (Figures 4.17 and 4.19), formed by the intracellular aggregates of the protein cytokeratin. The tonofilaments can be found in the projections of the cytoplasm of the keratinocyte which terminate in desmosomal junctions that occur in the periphery of the cytoplasm and binds two keratinocytes with each other (Figures 4.17 and 4.19).

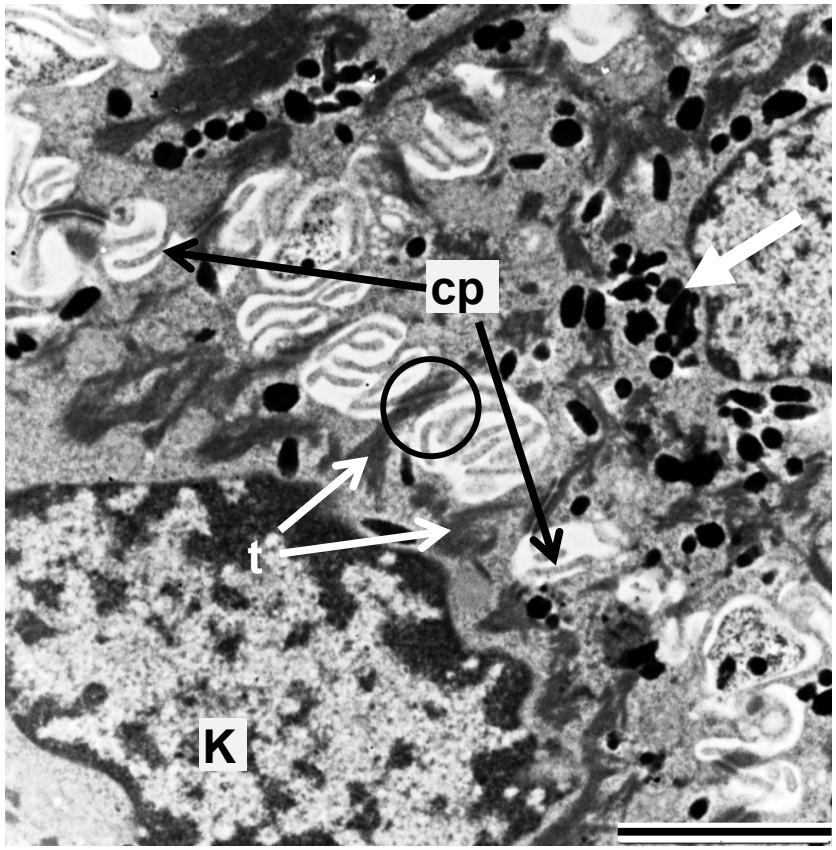


Figure 4.17: Control skin keratinocytes (K) in the stratum spinosum with cellular processes (cp), tonofilaments (t), melanosomes (arrow) and desmosomes (circled). Scale bar = 2 µm.

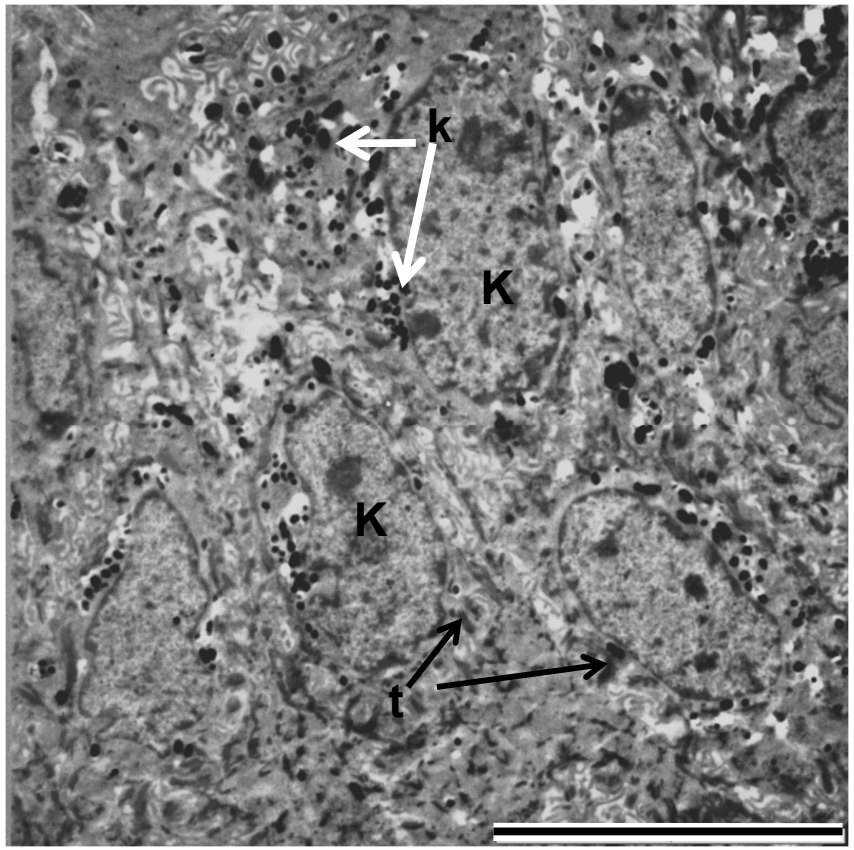


Figure 4.18: Keloid: Stratum granulosum showing keratohyalin granules (k), keratinocytes (K) and tonofilaments (t). Scale bar = 10 µm.

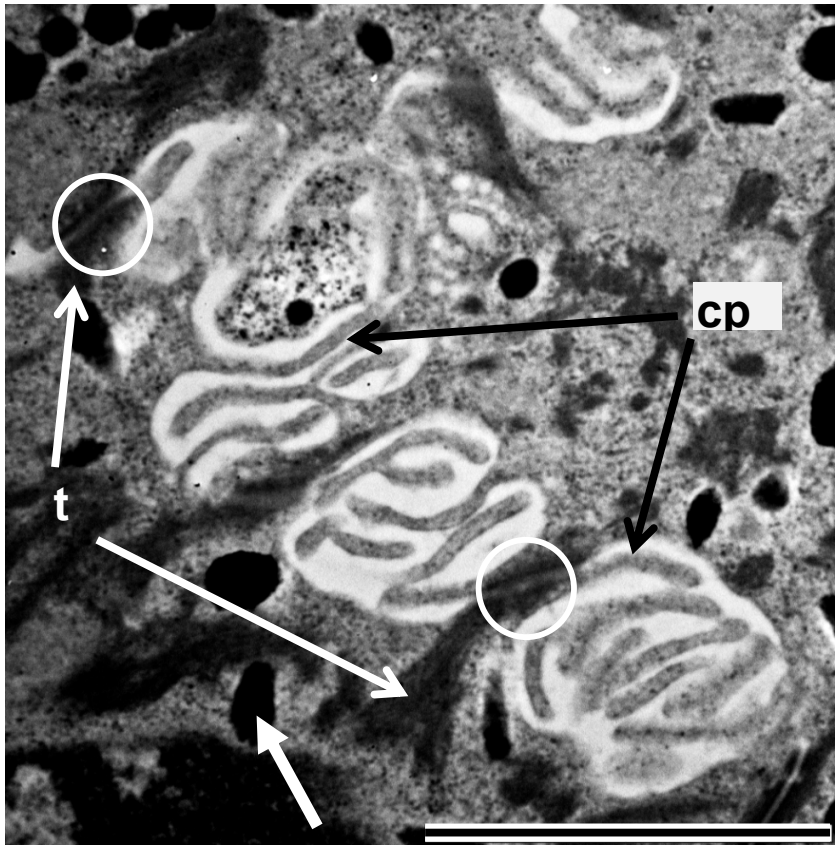


Figure 4.19: Control skin higher magnification of keratinocytes with cytoplasmic processes (cp), desmosomes (circled) melanosomes (arrow) and tonofilaments (t). Scale bar = 2 µm.

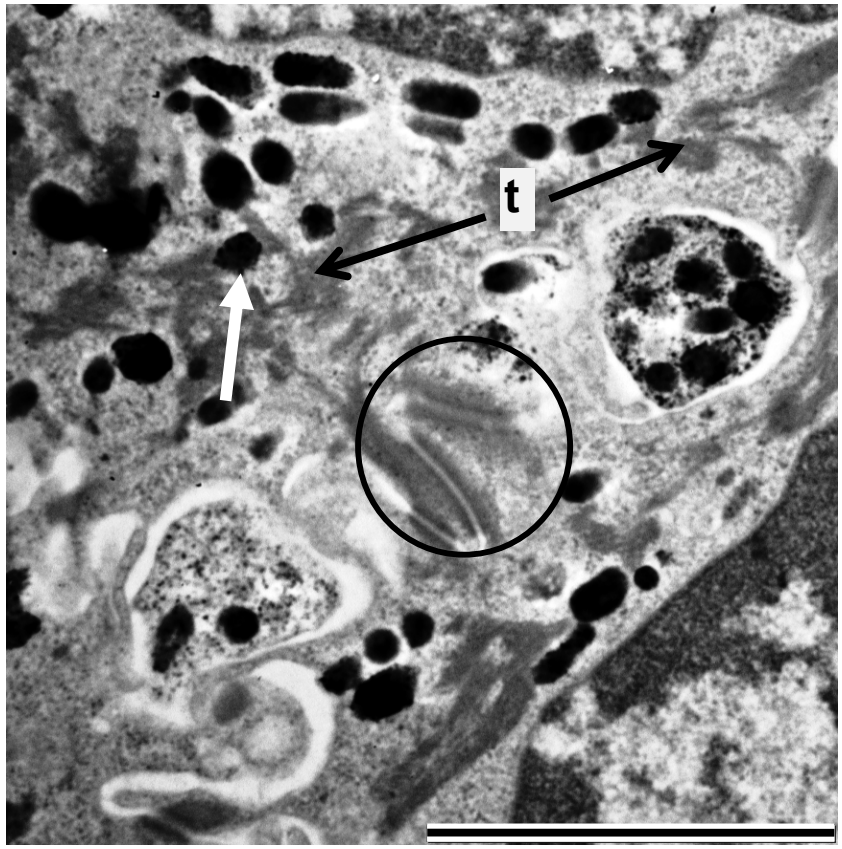


Figure 4.20: Keloid: Keratinocytes with desmosomes (circled), melanosomes (arrow) and tonofilaments (t). Scale bar = 2µm.

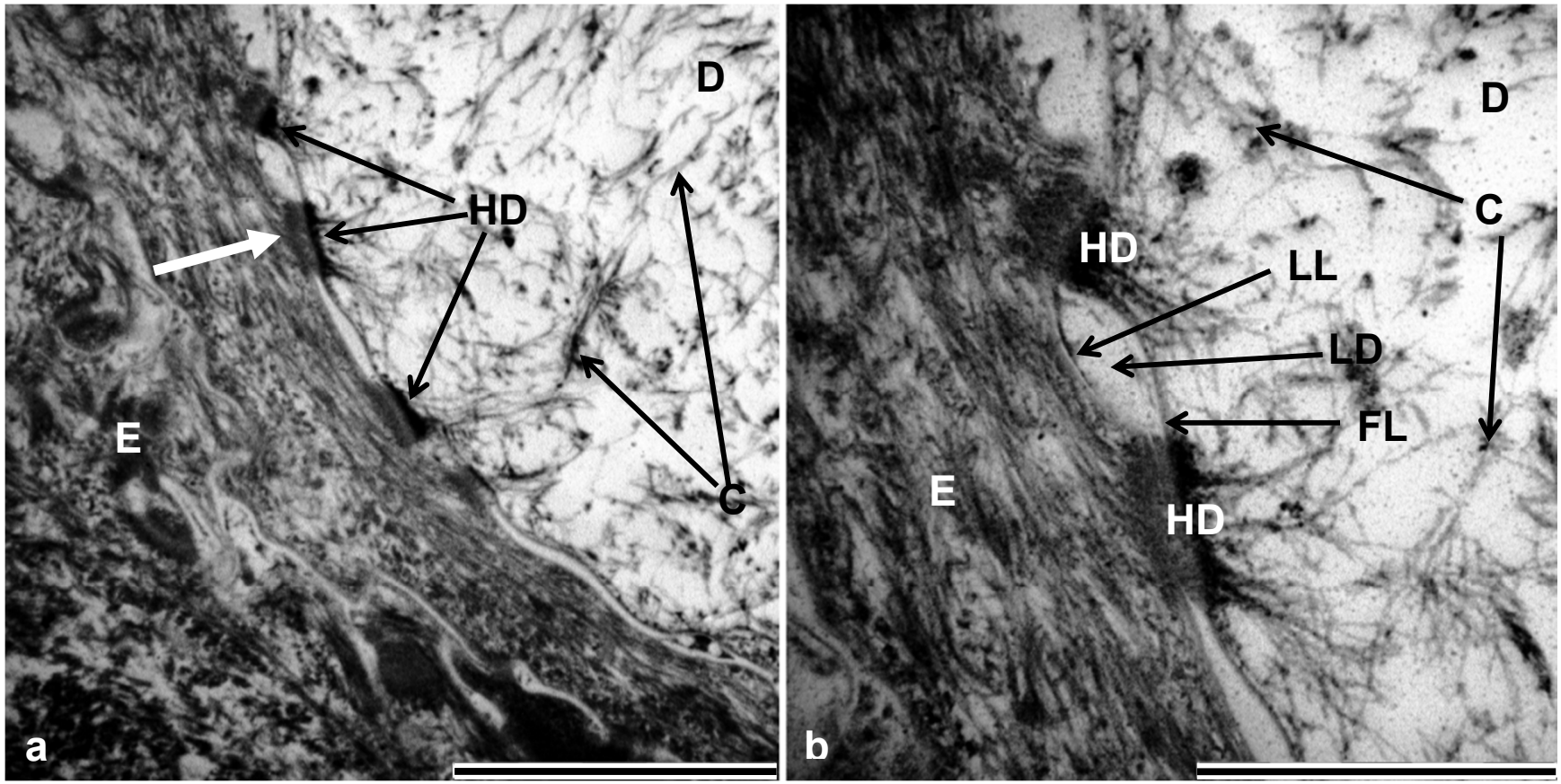


Figure 4.21: Control skin showing in (a) the basement membrane (arrow), hemidesmosomes (HD) between the epidermis (E) and dermis (D) and fine collagen (C). Figure (b) is a higher magnification of the basement membrane showing the three lamina layers; the lamina lucida (LL), lamina densa (LD) and fibroreticular lamina (FL). Scale bar (a) = 2 µm; scale bar (b) =1 µm.

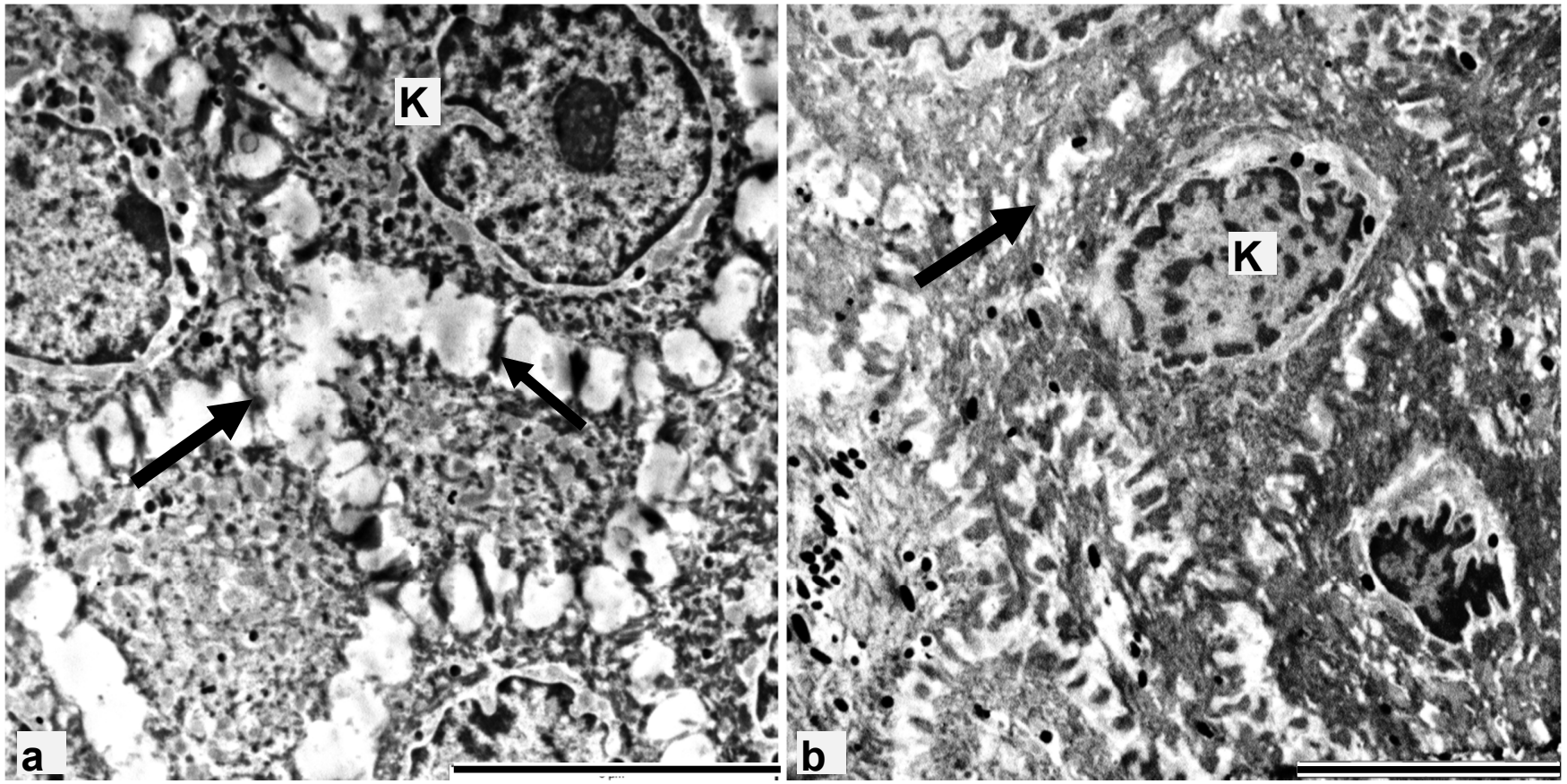


Figure 4.22: (a) Control and (b) keloid skin showing the contact (arrow) between keratinocytes (K). Scale bars = 5 µm.

The final layer before the dermis is the *stratum basale* which consists of highly irregular cells seen with TEM, which are bound to the basement membrane located just above the dermis by hemidesmosomes (Figures 4.21a). The dermo-epidermal basement membrane can be divided into three parts, the lamina lucida, the lamina densa and the fibroreticular lamina. The lamina lucida and densa consist of anchoring proteins found between the two layers as well as hemidesmosomes. The fibroreticular lamina contains proteins which connect the lamina densa to the collagen in the papillary dermis (Figure 4.21b).

The morphology of the keratinocytes in control and keloid tissue was compared (Figure 4.22a and b) and while control skin keratinocytes had the typical structure described for normal skin, keloid keratinocytes appeared to be more closely arranged with shortened cytoplasmic processes between cells (Figure 4.22b).

No studies have described the morphological differences between control, normal skin and keloid keratinocytes. This compaction of the keratinocytes in keloid tissue may be due to the presence of the collagen mass pushing against the epidermal-dermal junction. LM revealed that in the keloid tissue the rete ridges were more blunted and likewise the keratinocyte layer closest to the epidermal-dermal junction would also be compressed closer to each other.

Keratinocytes have been shown to influence the growth of other cells such as dermal fibroblasts. Normal fibroblasts were cultured together with keloid derived keratinocytes and these normal fibroblasts were shown to secrete collagen in the same manner as keloid fibroblasts (Lim *et al*., 2002). The authors hypothesized that keratinocyte-fibroblast interactions influence collagen production in a keloid, as increased soluble collagen type I and type III were observed. However, it has been noted by other authors that type III collagen remains unchanged in a keloid while only type I is increased (Ladin *et al*., 1995).

Interactions between keratinocytes and fibroblasts are known to occur during wound healing (Werner *et al*., 2007) and are evident in cell cultures where fibroblasts are used as a feeder cell layer for the growth of keratinocytes. Keratinocyte growth is dependent on the interaction between keratinocytes and fibroblasts. Substitution of the fibroblasts with growth supplemented medium is not as effective as the use of a feeder cell layer. This cell culture feature shows that keratinocytes influence fibroblasts to produce and secrete paracrine growth factors and cytokines. KGF or FGF-7 and IL-6 (Waelti *et al*., 1992; Smola *et al*., 1993) are some of the growth factors whose expression by fibroblasts is induced by keratinocyte-derived IL-1 (Maas-Szabowski *et al*., 1996). KGF-7 and FGF-7 are both growth factors that are expressed by

fibroblasts during wound healing. PDGF is expressed by epidermal cells that promote the growth of dermal cells. Some growth factors have been observed to have both autocrine and paracrine mechanisms of action, by controlling the expression of growth factors of the same cell type while also affecting the growth of other cell types (Werner and Grose, 2003). FGF-22 is expressed by keratinocytes (Nakatake *et al*., 2001; Beyer *et al*., 2003) and this growth factor also activates its own FGFR111b receptor (Zhang *et al*., 2006). An FGF binding protein is synthesised by epidermal cells and is upregulated during wound healing, where it binds to FGF-7, -10 and -22 and increases their activity at low concentrations (Beer *et al*., 2005). Proliferation of epidermal cells is stimulated by IL-6 and its expression is also increased in co-cultures of keratinocytes and fibroblasts (Grossman, 1989). Further evidence that epidermal-dermal interactions occur during wound healing is that wound healing of excisional wounds will not occur in IL-6 deficient mice (Gallucci *et al*., 2000; Lin *et al*., 2003). These are some examples of keratinocyte and fibroblast interaction during wound healing and a detailed discussion is given by Werner *et al*. (2007).

Keratinocyte-fibroblast interactions, such as epithelium with mesenchyme, occur in early fetal skin development and are responsible for the formation of limbs, skin and its appendages (Martin and Parkhurst, 2004). These interactions also occur in adulthood (Martin and Parkhurst, 2004) where they play a role in tumour development (Bhowmick *et al*., 2004; Mueller and Fusenig, 2004). It is known that keratinocytes have a secretory function and also plays a role in the modulation of the immune system (Lim *et al*., 2002; Boyce, 1994; Garner, 1998; Robles and Berg, 2007).

Several varied theories have been put forward about the role of keratinocytes in keloid formation. Increased expression of TGF-β1, -β2, TGF-β receptor 1 and SMAD2 was found in cell co-cultures of keloid keratinocytes and fibroblasts (Bock *et al*., 2005). Down regulation of apoptosis is suggested as a possible mechanism of keloid formation (Sayah *et al*., 1999) and normal and keloid fibroblasts co-cultured with keloid keratinocytes were more resistant to apoptosis than normal fibroblasts in co-culture. Levels of caspase-3, an important marker of apoptosis, was reduced in keloid keratinocyte and keloid fibroblast co-cultures. Both normal and keloid keratinocytes had a proliferative effect on normal fibroblasts. Bcl-2, an anti-apoptotic gene, was found to be up-regulated in keloid fibroblasts. Disregulation of the Bcl-2 gene occurs in conditions associated with apoptotic resistance (Funayama *et al*., 2003). These studies show that fibroblast growth is influenced by keratinocytes, and keratinocytes derived from keloids increase fibroblasts resistance to apoptosis.

Data from a study done by Lim *et al*. (2001), suggests that fibroblast proliferation in keloids is promoted by keratinocytes which are different to keratinocytes found in normal skin. TEM done by Tuan *et al*. (2002), showed that normal fibroblasts cultured with keloid keratinocytes produced more collagen fibrils than those fibroblasts cultured with normal skin keratinocytes. *In vivo* tissue matrix examination revealed structural similarities between the collagen fibrils formed in co-cultures and that found in keloid tissue. Autocrine and paracrine mechanisms are believed to occur where keratinocyte derived growth factors promote keratinocyte proliferation and the differentiation of myofibroblasts. This process of differentiation is due to the presence of pro-inflammatory and growth factors especially of a TGF-β which also has an anti-inflammatory function (Schmidt-Urlich and Paus, 2005; Tickle, 2006).

In this study the arrangement of the keratinocytes in the keloid tissue is disrupted. The cells are more closely packed together when compared to normal tissue and this may be due to the increased volume of dermal collagen that increases the pressure on the dermal layer. Besides the keratinocytes being closely packed together, this may also disrupt normal cellular processes and function as well as cell-cell interactions such as the interaction between keratinocytes and fibroblasts. Interestingly, studies that involve the isolation of keloid keratinocytes and subsequent cultivation in co-cultures with fibroblasts show that these keratinocytes are different from normal keratinocytes. This indicates that alterations to the keratinocyte genome have occurred. This effect seems to be localised to a specific site where the keloid has occurred and may be a result of later epigenetic events (Mann and Mann, 2013).

As will be shown later, there is an increase in the number of mast cells found in keloid tissue. Not only may the keratinocytes promote the growth of fibroblasts but they may also affect the function of these cells. Keratinocytes may cause an increase in the number of mast cells or activate mast cell degranulation through the production and release of anti-microbial peptides, endothelin-1 and cytokines (Metz and Mauer, 2009).

In normal tissue, cellular interactions between keratinocytes and fibroblasts are observed during mid and late stages of wound healing and this changes the wound environment from inflammatory to that of granulation tissue (Schmidt-Urlich and Paus, 2005; Tickle, 2006). In granulation tissue the number of apoptotic cells is increased and this process is responsible for the conversion of granulation tissue into scar tissue (Funayama *et al*., 2003). Keloid keratinocytes via increased expression of growth factors such as KGF-1 (Beer *et al.,* 2005),

effectively reduce the rate of apoptosis and as a consequence fibroblast proliferation is unaltered resultingin increased ECM formation.

4.5.2 Melanocytes

The difference in morphology, the absence of tonofilaments and the presence of melansomes at various stages of differentiation makes it easy to distinguish melanocytes from keratinocytes. Melanocytes are round cells with a large number of long cytoplasmic processes that extends from the cell body and run into spaces between the keratinocytes of the *stratum spinosum* (Figures 4.17 and 4.19 and 4.27) as well as into the collagen of the papillary dermis (Figures 4.23 and 4.24). Melanocytes are in contact with one another through cytoplasmic processes extending from the cell body (Figure 4.24). The long cytoplasmic processes were observed to vary between wavy (Figure 4.23) and straight (Figure 4.24).

Melanin containing granules of melanocytes are visible as dark staining oval shaped organelles (Figures 4.25 and 4.26). The pre-melanosomes which are the immature form of the melanosomes are striated. A membrane surrounding the organelles is visible. Striations are absent in mature melanosomes as the filaments that make up these striations are obscured by the high content of melanin present in the mature melanosomes (Figure 4.26). Melanocytes transfer their melanin to keratinocytes and a cap of melanin is formed in the keratinocytes directed towards the surface protecting the DNA present in the nucleus against UV radiation (Figure 4.26).

The exact mechanisms of the melanosome transfer to keratinocytes have not been elucidated. Three possible mechanisms have been established and they involve melanosome transfer through the fusion of melanocyte and keratinocyte membranes, the release of individual melanosomes from melanocytes and subsequent phagocytic uptake by keratinocytes and the partial phagocytosis of the melanosome containing dendrite tips of melanocytes by keratinocytes (Mottaz and Zelickson, 1967; Yamamoto and Bhawan, 1994; Seiberg, 2001; Scott *et al*., 2002; Van Den Bossche *et al*., 2006; Singh *et al*., 2008). Recently a new mechanism for melanosome transfer was reported by Ando *et al*. (2012). This method involves shedding vesicles which bud off from the plasma membrane and into the ECM, transferring intracellular components (Coccuci *et al*., 2009). In this case, melanosomes generated in melanocytes are concentrated and contained in shedding vesicles, which are trapped by microvilli and phagocytosed by keratinocytes (Figure 2.4). The phagocytosis is protease activated receptor-2

(PAR-2) dependent. The vesicles incorporated into the keratinocytes disappear as their membranes degrade, allowing for the movement and distribution of melanosomes around the keratinocyte nucleus (Ando *et al*., 2012).

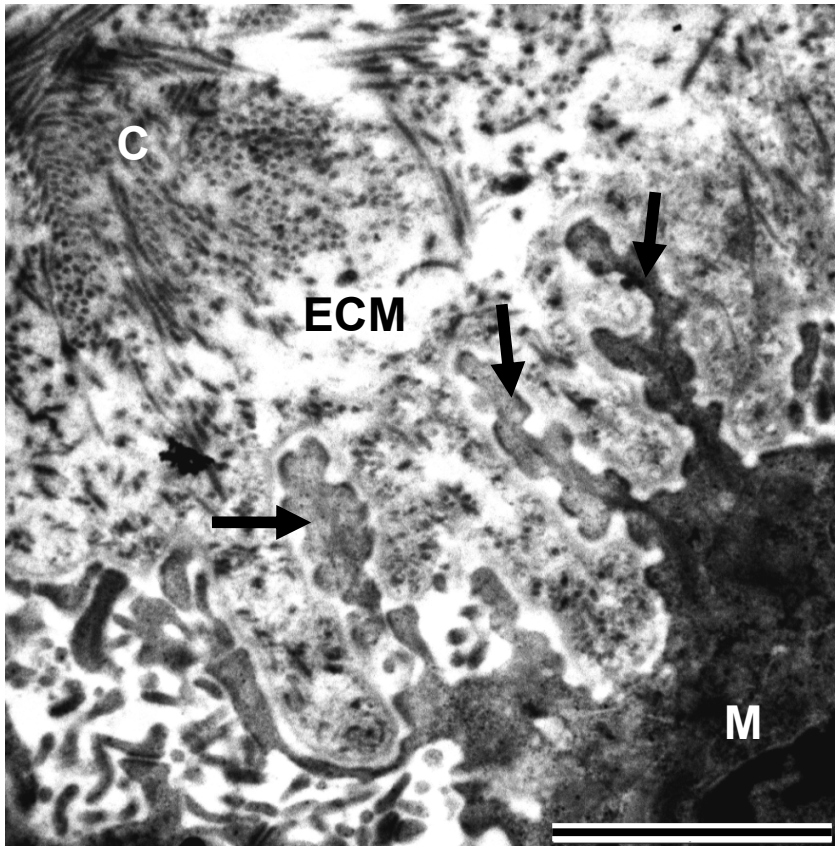


Figure 4.23: Control skin melanocyte (M) and its cytoplasmic processes (arrow) extending into the extracellular matrix (ECM). The regular arrangement of control skin collagen is present in the top left corner (C). Scale bar = 2 µm.

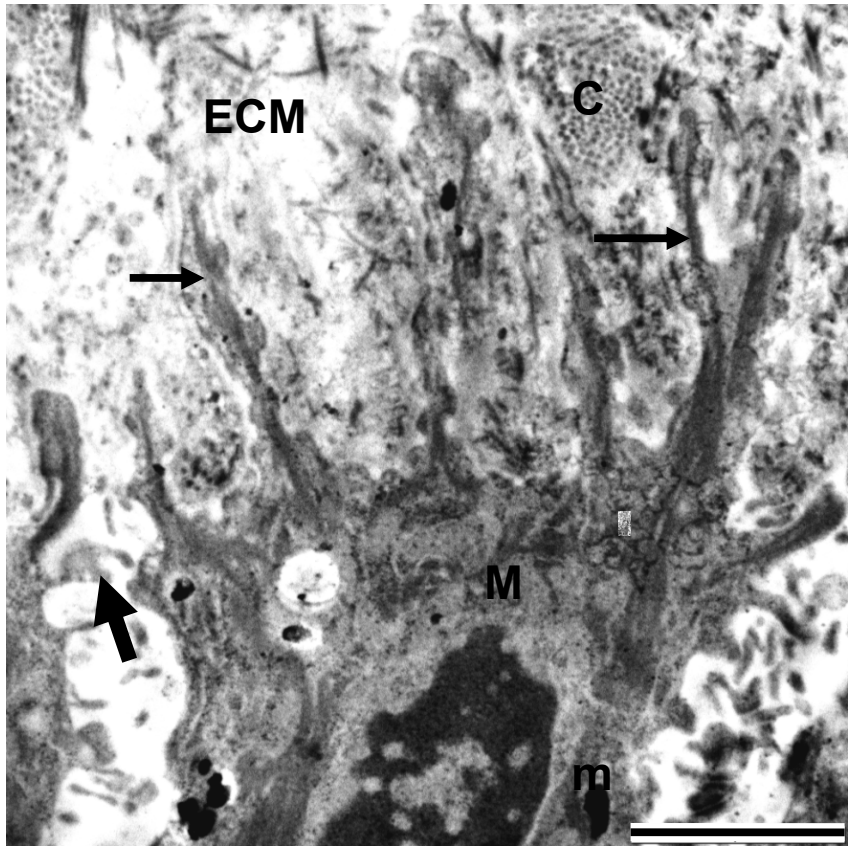


Figure 4.24: Control skin melanocyte (M) with various straight cell processes (thin arrow) and contact with the neighbouring cell (thick arrow). Collagen (C) is observed as a loose bundle in the ECM. Scale bar = 2 µm.

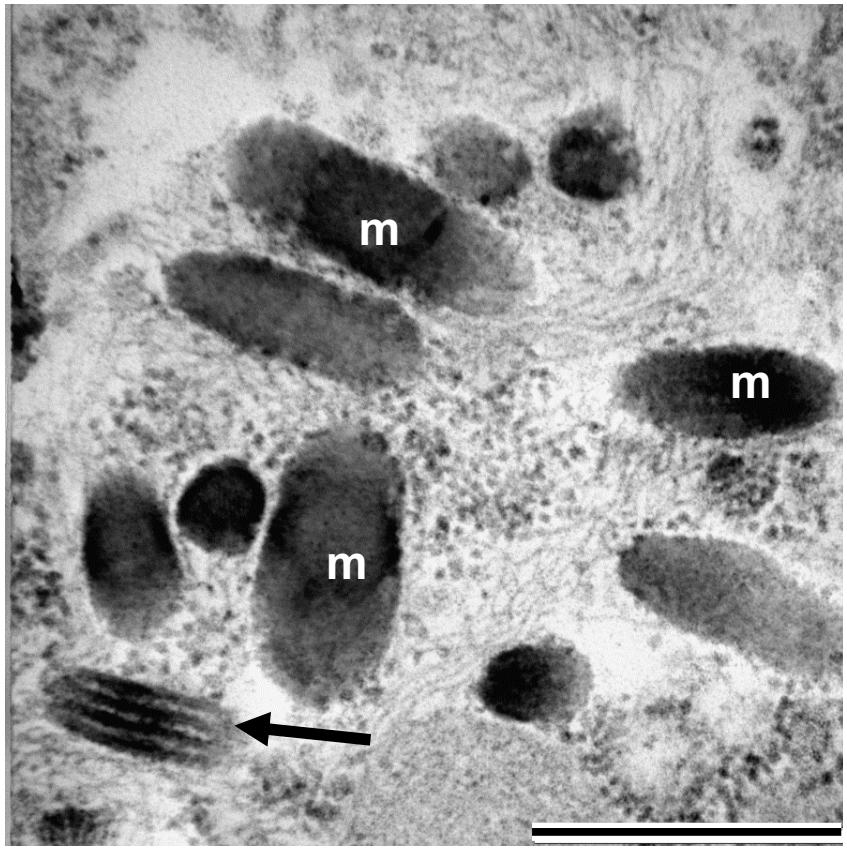


Figure 4.25: **Control** skin section that show premelanosomes which are striated (arrow) while mature melanosomes do not have striations (m). Scale bar = 500 nm.

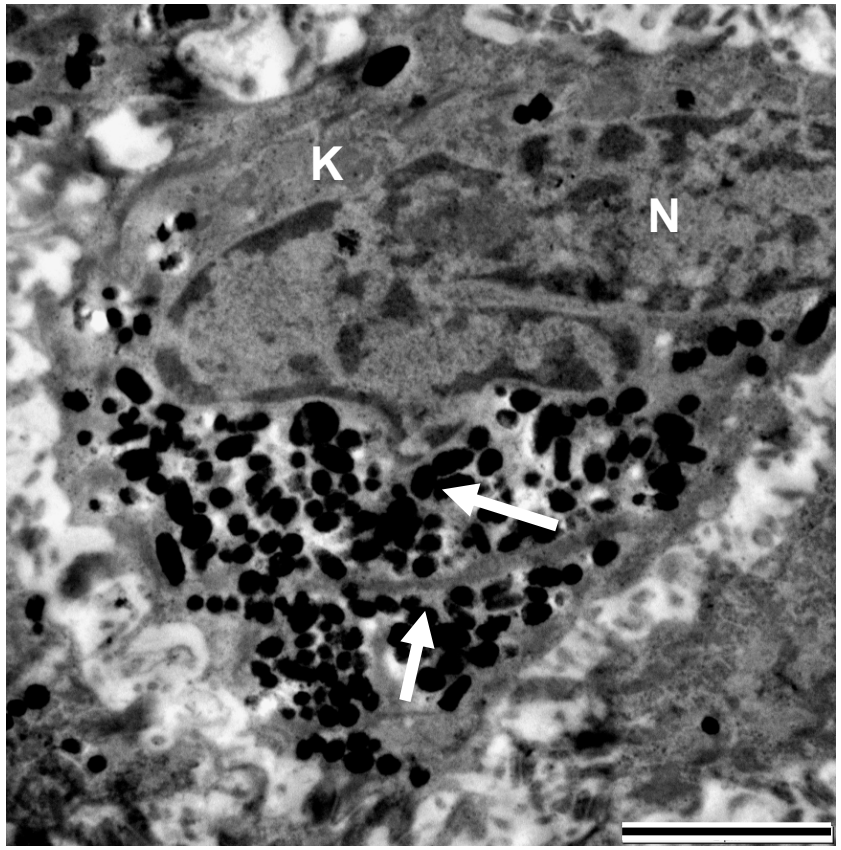


Figure 4.26: **Control** skin melanosomes (arrow) forming a melanin cap on a keratinocyte (K) nucleus (N). Scale bar = 2 µm.

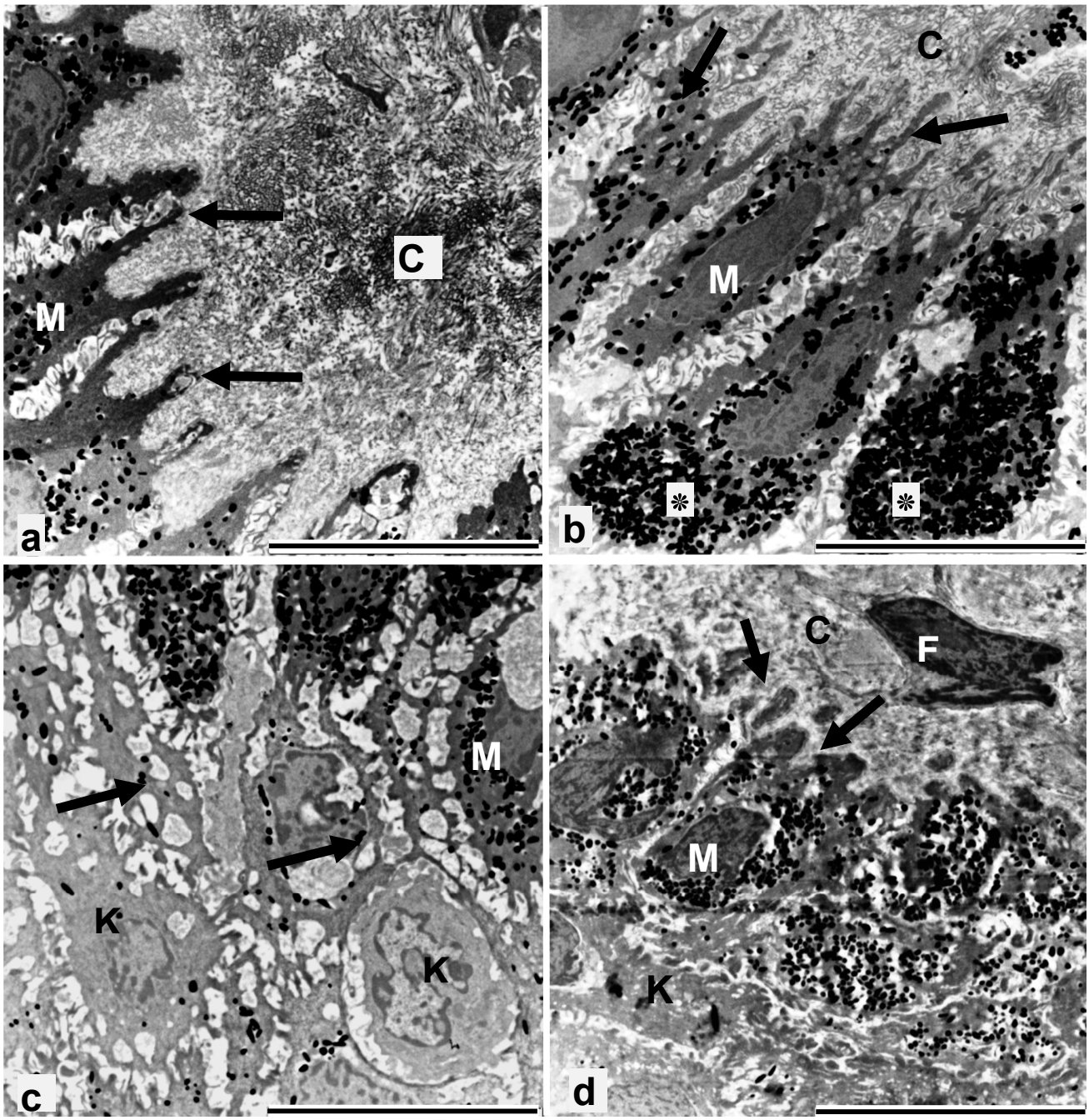


Figure 4.27: Keloid: (a) Melanocyte (M) in contact with collagen (C), the few cytoplasmic processes are shorter or restricted (arrows), (b) extension/enlargement of the melanocyte body in the epidermis (*) and shortened or restricted cytoplasmic processes (arrow) into the collagen (C), (c) melanocyte (M) in contact with keratinocytes (K) and transfer of melanin to the keratinocytes (arrows). (d) Very short or restricted cytoplasmic processes (arrows) extending into the collagen (C). A fibroblast (F) is visible in the collagen. (b and d) show differences in the morphology of the melanocytes in different samples. Scale bars = 10 µm.

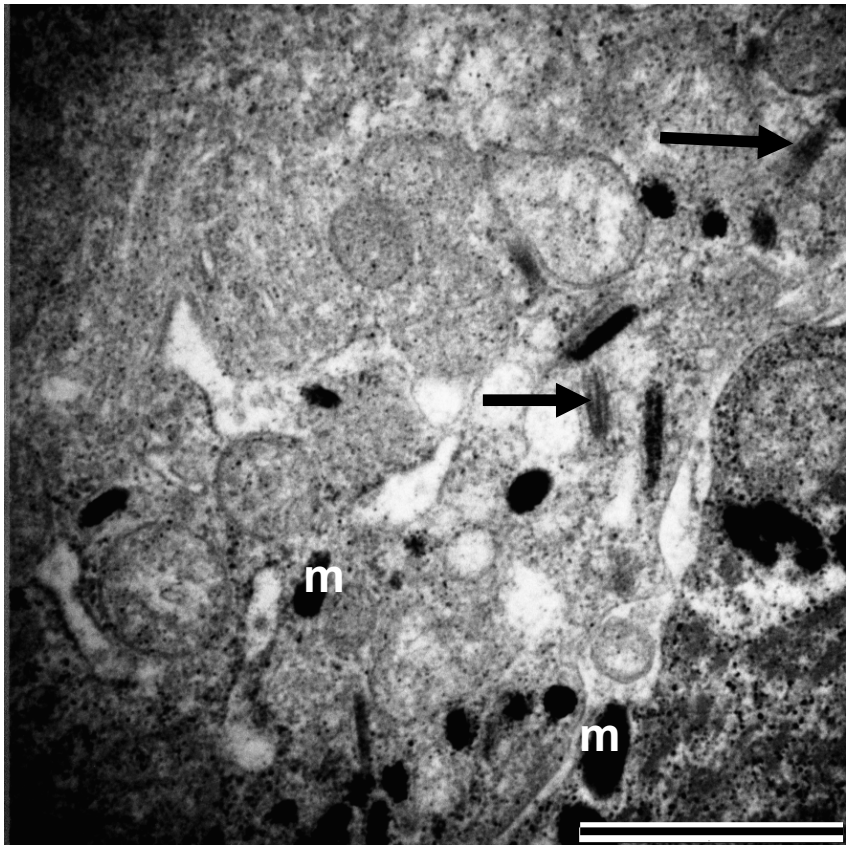


Figure 4.28: Keloid: Presence of pre-melanosomes (arrow) and mature melanosomes (m) in keloid skin. Scale bar = 1µm.

Differences were observed between control (Figures 4.23 and 4.24) and keloid melanocytes (Figure 4.27). The cytoplasmic processes of the melanocytes differed in that control melanocytes had processes extending into the dermis seen clearly in Figure 4.23, while those of keloid melanocytes appeared shorter and were fewer in number (Figures 4.27a and b) with smaller projections extending from the main processes (Figure 4.11a). Melanocytes in Figure 4.27d do not have any processes, only ridges at the dermal surface of the cell. It is observed that the cells in some instances project further into the epidermal layer, having a larger and more rounded cell body (Figure 4.27b) as if the collagen mass in the dermal layer has pushed the melanocytes towards the epidermis. The arrangement of keloid keratinocytes is irregular and the contact between keloid keratinocytes and melanocytes appears to be less organised (Figure 4.27c).

There were no structural differences observed between control and keloid melanosomes. There appeared to be no differences in the developmental stages of melanosomes in control (Figure

4.25 and 4.26) and keloid skin (Figure 4.28). In both tissue types, in some samples the different stages of melanosome development can be identified. This includes the presence of pre-melanosomes with striations (arrow), while the striations are not visible in the mature melanocyte due to the presence of melanin as can be seen in Figure 4.25 and 4.28.

A study undertaken by Snell in 1986, investigated guinea pig melanocyte morphology at different stages of wound healing. During wound healing the morphology of melanocytes varied. During the early stages of wound healing the cell bodies of melanocytes were either smaller than normal or very much larger. Melanocyte cell processes were observed to be either absent or very few in number, where the latter were either simple with very few branches and much shorter and stumpy when compared to these processes in normal skin. At nine days the melanocytes were similar to those of normal skin (Snell, 1968).

The melanocyte structure reported by Snell (1968), is similar to the structure of melanocytes found in this study, i.e. altered melanocyte body shape and dendritic processes that are reduced in size. Based on the stages of the wound healing process as described in detail in Chapter 2, the stage of wound healing in which this author found melanocytes with altered morphology corresponds with the granulation phase of wound healing. This information is consistent with the possibility that keloids are in a permanent granulation phase, based on the anti-apoptotic and pro-fibrotic factors and wound healing events described in the literature review. These will be discussed in greater detail in chapter 5.

Melanocytes may play a role in keloid formation. Skin pigmentation is determined by the activation of the melanocortin 1 receptor (MC1-R) (Lin and Fischer, 2007). The activation of this receptor in melanocytes increases the production of melanin, such that more black/brown than red/yellow melanin is formed. Agonists or ligands of this receptor include the alpha melanocyte stimulating hormone (α-MSH) and adrenocorticotropin (Muffley *et al*., 2011). The α-MSH was seen to reduce TGF-β1 induced synthesis of collagen types I and III in dermal fibroblasts (Bohm *et al*., 2004). In addition α-MSH has also been reported to reduce skin fibrosis in a mouse model for scleroderma (Kokot *et al*., 2009). The hormone downregulates inflammatory cytokines; IL-1, IL-4, IL-6, interferon (IFN)-□ and TNF-α (Brzoska *et al*., 2008). Research by Muffley *et al*. (2011), showed that the MC1-R receptor and its ligand is localised in the epidermal keratinocytes and dermal fibroblasts of acute burns and hypertrophic scars in humans. The presence of MC1-R and its ligand is significant as the ligand is reported to have anti-inflammatory properties and is able to limit or end the inflammatory response after injury to the

skin (Brzoska *et al*., 2008). The presence of the receptor ligand in keratinocytes suggests a role of MC1-R and the α-MSH in the proliferation of keratinocytes as well as their movement over the injured dermis (Gurtner *et al*., 2008). Accelerated epithelialisation of corneal wounds treated with an α-MSH derived tripeptide was observed (Bonfiglio *et al*., 2006). The presence of an epithelial layer is known to promote a more stable dermal environment during wound healing, hence the use of silicone gel sheeting in post surgical scar reduction (Kelly, 2004; Chang *et al*., 1995; Sawada and Sone, 1992). Fibroblast activity is affected indirectly when stable epithelium suppresses collagen synthesis induced by TGF-β *in vitro* and cutaneous fibrosis *in vivo* (Bohm *et al*., 2004). This indicates that keratinocytes, melanocytes and fibroblasts are essential for wound healing and each fulfils a specific role during each stage of wound healing.

MC1-R and its ligand α-MSH within the skin have several different effects in wound healing and these are anti-inflammatory, anti-fibrotic and pro-epithelialisation. It is widely reported that individuals of African and Asian descent keloids are more prevalent, however a higher level of MC1-R and α-MSH implies that wound healing would occur more efficiently. Alternatively in those patients where keloids do occur, these biochemical pathways and cellular interactions are disrupted or a totally different biochemical pathway may be involved.

4.6 DERMIS

The dermis is a thick layer of connective tissue to which the epidermis attaches to an irregular dermal-epidermal junction and continues into the subcutaneous tissue without a definite boundary, and this makes it hard to define the specific thickness of the layer. The dermis is composed of loose connective tissue in the uppermost, papillary dermis and thicker, denser connective tissue in the lower, reticular dermis. The reticular layer contains elastin and coarse collagen arranged in bundles forming fibres whose main direction is parallel to the surface of the skin. Cells of the dermal layer include fibroblasts and immune cells, mainly mast cells especially with inflammation (Geneser, 1986, Tortora and Derrickson, 2009).

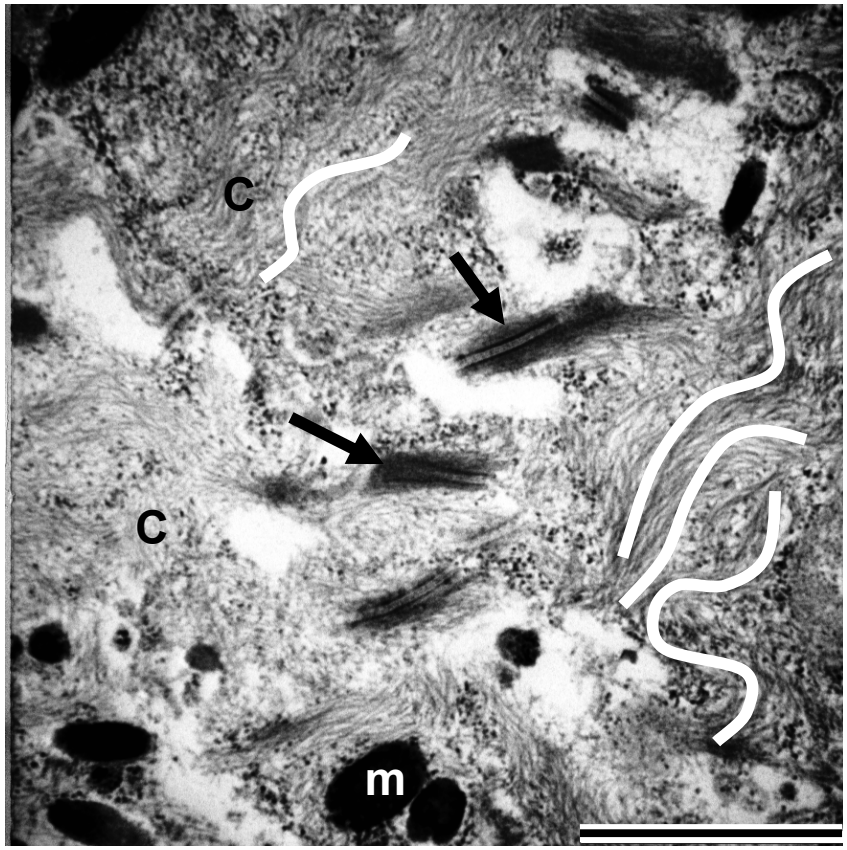


Figure 4.29: Control: Desmosomes (arrow) in the ECM near the epidermis,and melanosomes (m). Structure and orientation of collagen (C) in the ECM is visible. Scale bar = 1 µm.

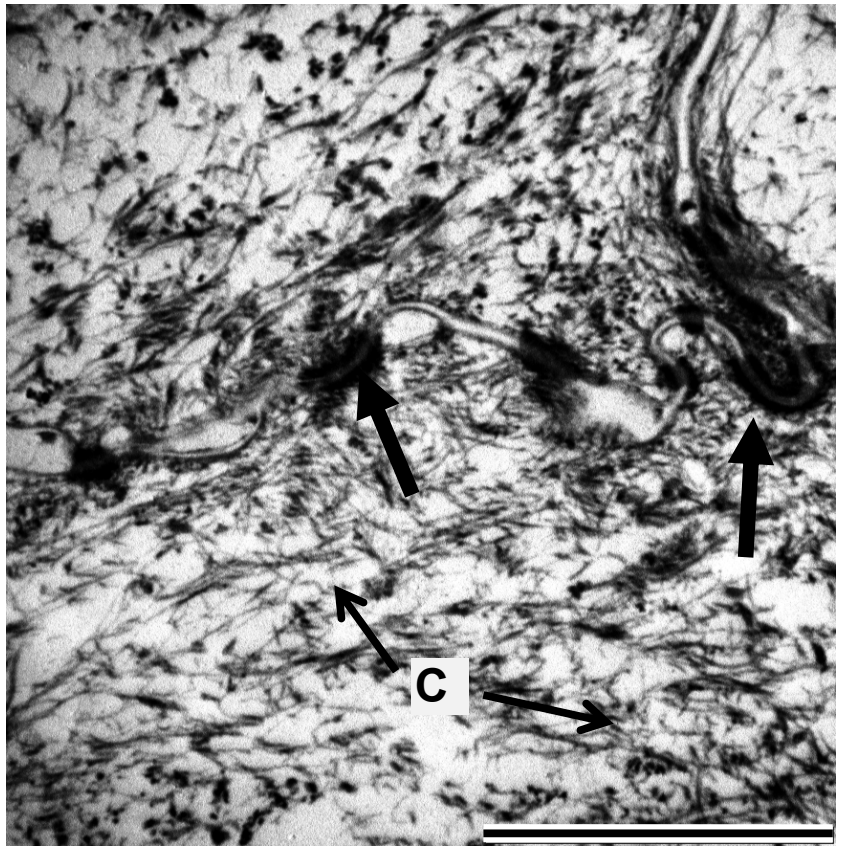


Figure 4.30: Control: Desmosomes (arrow) located deeper in the dermis Fine collagen (C) bands are observed in the ECM. Scale bar = 2 µm.

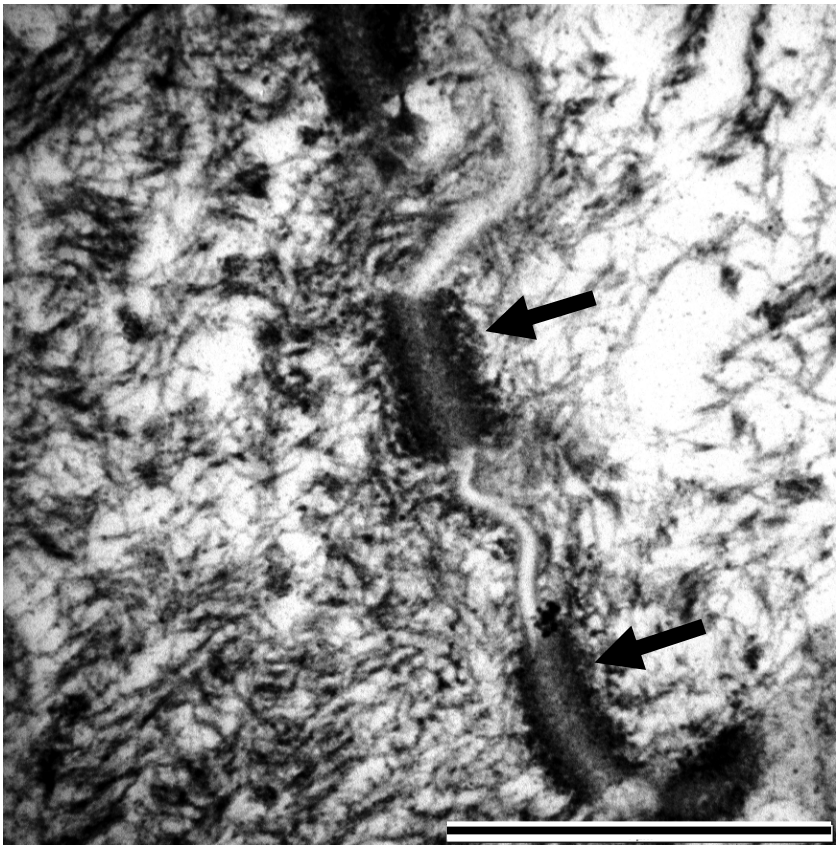


Figure 4.31: Control: A higher magnification of desmosomes (arrow). Collagen fibers are visible as fine fibrils with a loose arrangement of low density. Scale bar = 1 µm.

Hemidesmosomes and desmosomes provide structural integrity to surfaces under mechanical stress, and also serve to bind the epithelium into a single mass (Merscher, 2010; Young and Heath, 2000). Desmosomes are present at the dermal-epidermal junction and ensure that both layers are strongly attached to each other. The skin is often subjected to shearing forces and these structures ensure these two layers remain attached. Hemidesmosomes and desmosomes were observed in the basal layer of control skin (Figures 4.17, 4.19 - 4.21) and in the papillary dermis (Figure 4.29 - 4.31). Desmosomes could only be found in one control skin sample and were absent in keloid tissue. The lack of desmosomes in control skin may be related to the source of the skin as it was obtained from skin grafts and is often stretched. As a consequence the dermal layer is thin (Figure 4.1c) and the epidermal-dermal layer interactions are compromised. Desmosomes occur in areas of high tension or movement so it can be assumed that the hemidesmosome/ desmosome system at the epidermal-dermal junction is well developed. However, these structures could not be found in keloid tissue. This could be due to

the excessive amount of collagen in the dermis that either disrupts these interactions or masks the presence of these structures.

4.6.1 Collagen

Collagen is the most abundant protein in the human body and the main connective tissue fibre (Young and Heath, 2006). Skin collagen consists primarily of type I and type III collagen (Diegelmann, 2001). Collagen type III is the main type of collagen found in granulation tissue and with further wound healing is replaced with collagen type I which is re-orientated across the lines of tension with the formation of stable bonds. In granulation tissue collagen is randomly arranged (Figure 4.6b - d) while in normal tissue the collagen is arranged in parallel interwoven arrays (Figure 4.1 and Figure 4.3a).

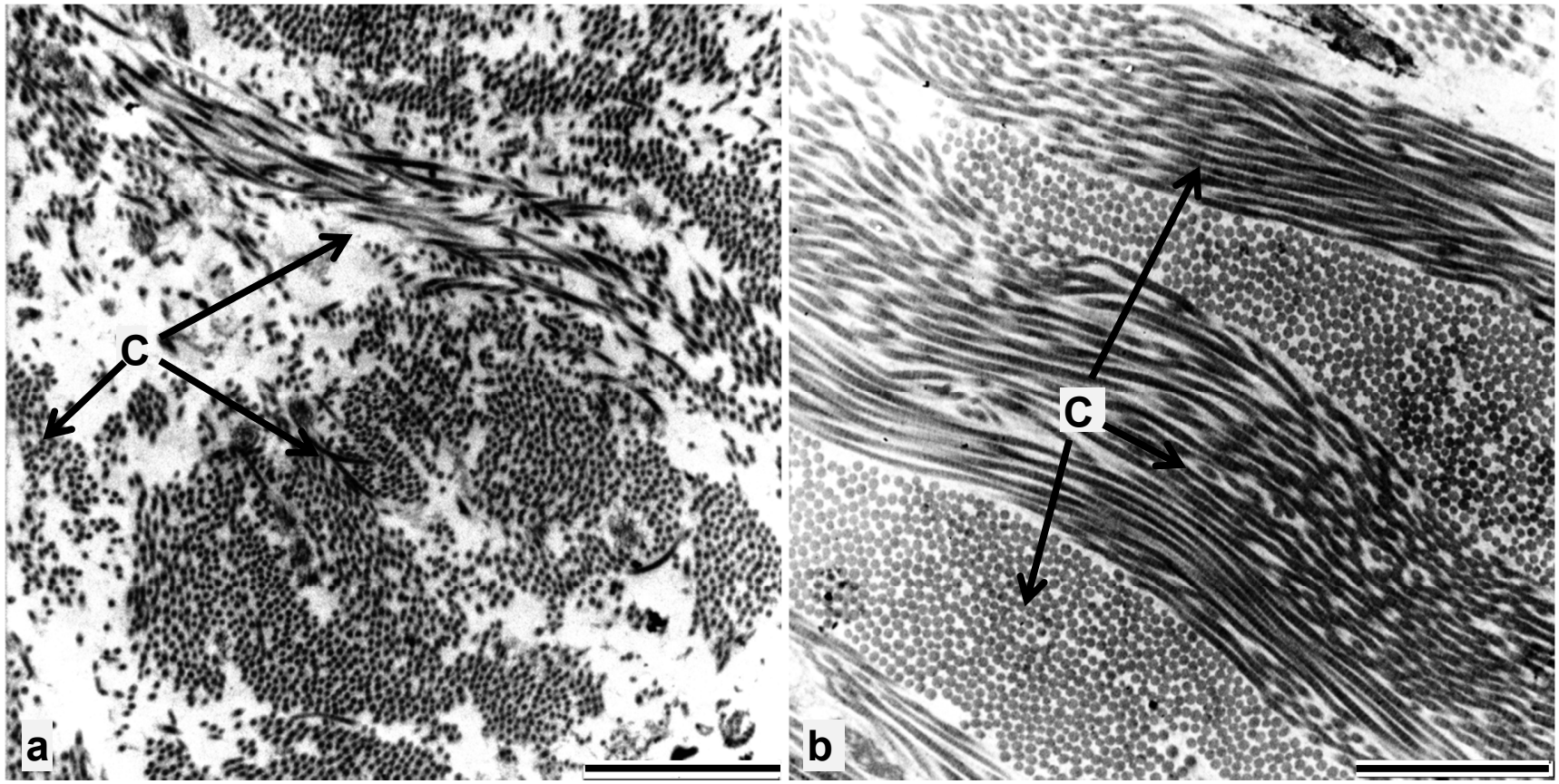


Figure 4.32: **Control** and **keloid**. Loose arrangement of collagen fibres (C) in control skin compared to (b) the denser and thicker collagen fibres (C) of keloid skin. Scale bar = 2 µm.

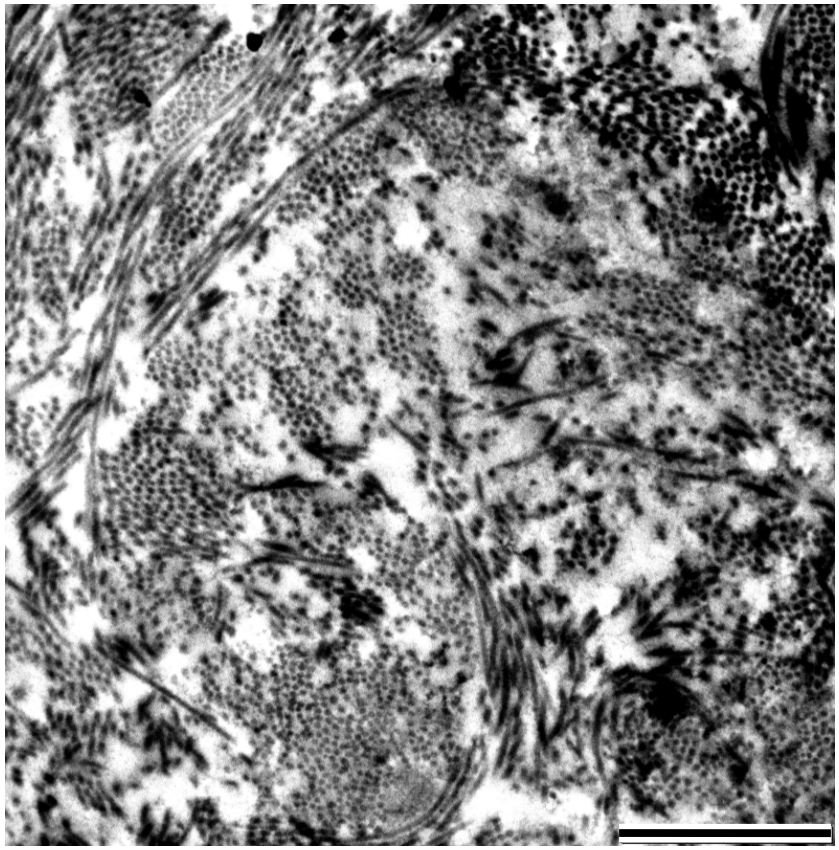


Figure 4.33 Control: Typical looser arrangement of control skin collagen. Scale bar = 2 µm.

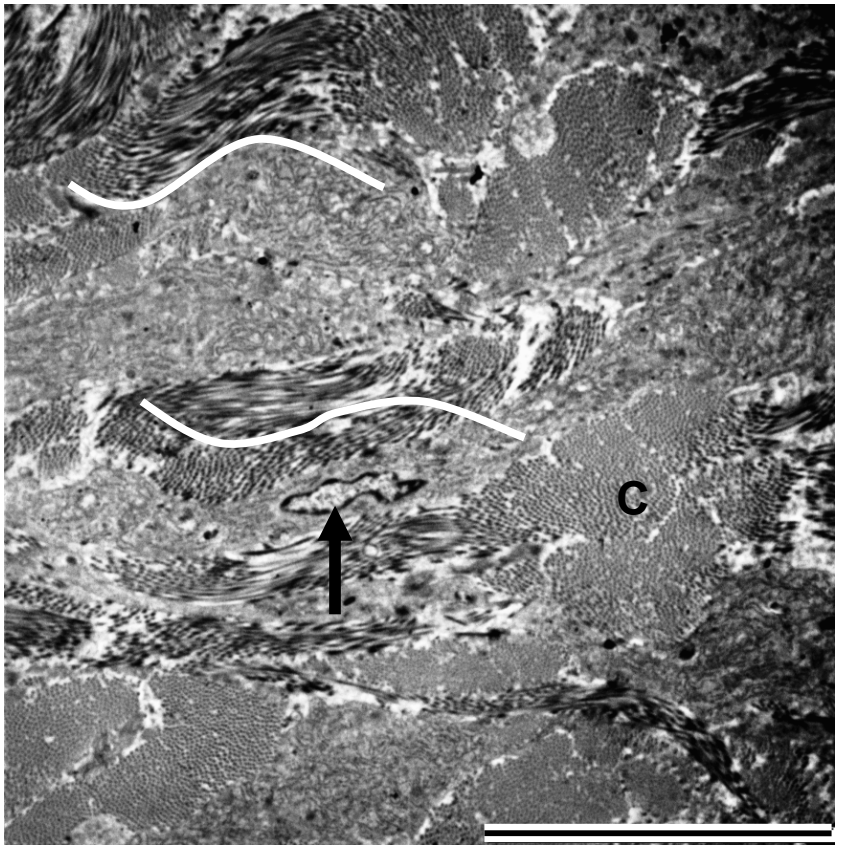


Figure 4.34 Keloid: Low magnification showing the dense arrangement and the wavy pattern (line) of the bands of collagen (C) fibres. A fibroblast (arrow) is visible in between the collagen fibres. Scale bar = 10 µm.

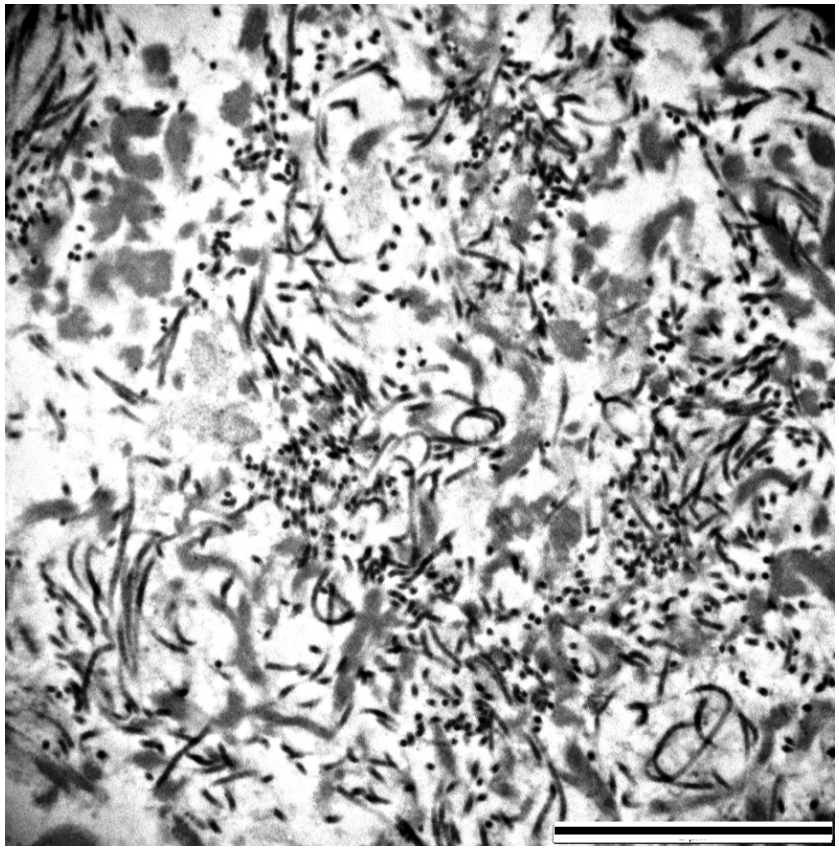


Figure 4.35: Scar tissue: Loose irregular arrangement of collagen in ECM. Scale bar = 2µm.

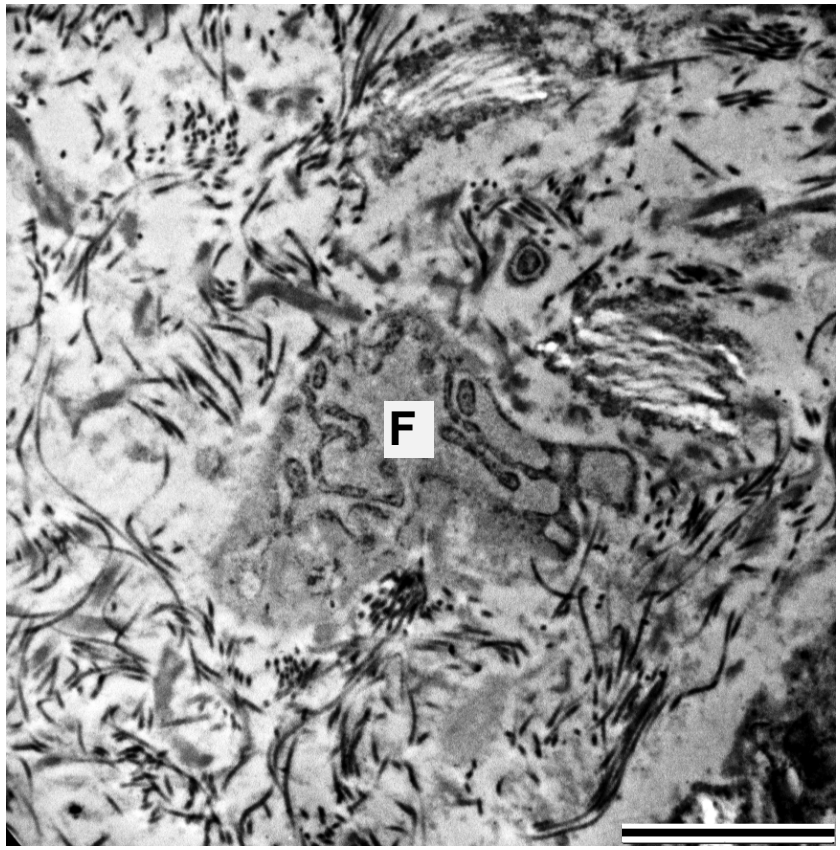


Figure 4.36: Scar tissue: Presence of a fibroblast (F) with intracellular features of increased protein synthesis (distended ER and increased number of ribosomes) is present in the ECM. Scale bar = 2µm.

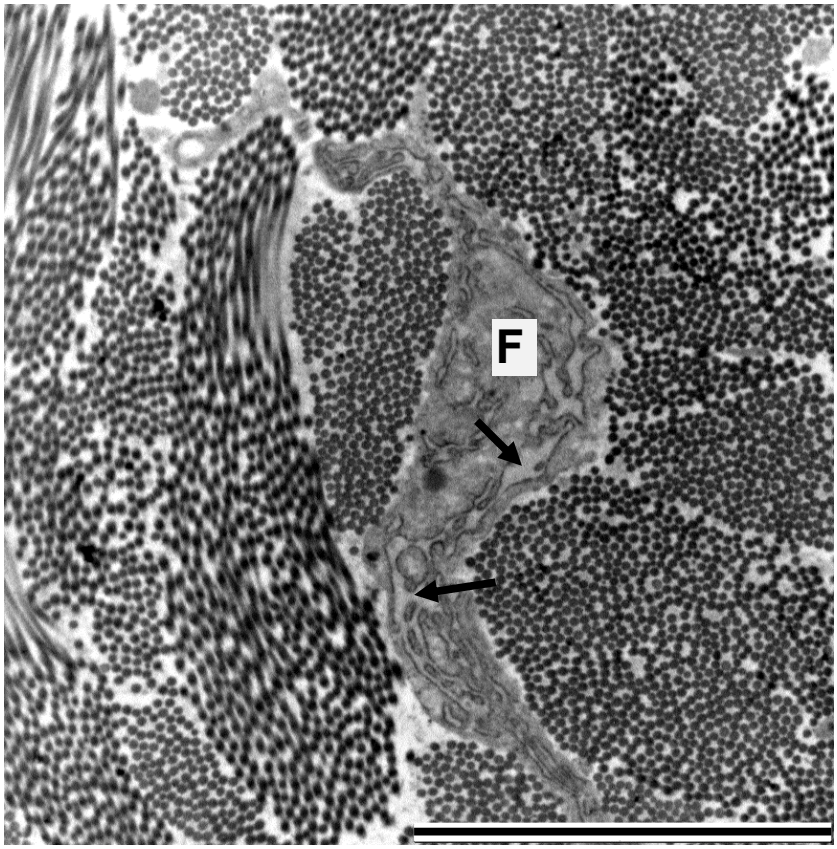


Figure 4.37: Fibroblast (F) in keloid tissue with the intracellular features of increased protein synthesis (distended ER and increased number of ribosomes) is present in the ECM (arrows). Scale bar = 5 µm.

The results obtained from the EM analysis of collagen confirmed the LM findings, that collagen fibers in keloid tissue are thicker (Figure 4.32b) and more densely arranged (Figure 4.34) compared to control skin (Figure 4.32a and Figure 4.33). EM at a low magnification clearly showed the arrangement of collagen in the dermis and its relation to the surrounding cells (Figure 4.34). Fibers were irregulary arranged in varied orientations close together in a woven pattern. The differences between the arrangement of the dermal collagen fibers of control skin, scar tissue and keloid were compared. Control skin (Figure 4.33) has a fine structure with collagen fibers evenly spaced throughout the ECM while in scar tissue (Figure 4.35) collagen fibres were sparse and loosely arranged. In contrast, keloid collagen was compact and densely arranged (Figure 4.32 b). For both scar and keloid tissue, several fibroblasts were present in the ECM. These fibroblasts had distended ER with many ribosomes indicating that these cells were actively synthesing protein such as collagen. However the amount, structure and arrangement of collagen fibers in the ECM differed considerably between scar (Figures 4.35 and 4.36) and

keloid tissue (Figures 4.34 and 4.37). Collagen was differenciated from elastin using the morphological characteristics discussed in the literature review, sections 2.1.5.2 and 2.1.8 and given in table 3.1. Scar tissue was used to show the ultrastructure of skin after normal wound healing. With scar formation and the keratinocyte mediated apoptosis of fibroblasts, scar tissue is essentially acellular. As mentioned in section 4.5.2 keloids are a tissue that is in a permant anti-apoptotic/proliferative or granulation state, with characteristic increased rates of fibroblast proliferation and collagen synthesis.

Blackburn and Cosman (1966), described keloids as having abnormally large collagen bundle complexes, these complexes were found to consist of numerous closely packed fibrils (Ehrich *et al*., 1994) due to increased synthesis of collagen type I and III (Syed *et al*., 2011). In normal skin the synthesis of collagen reaches its peak at six months after the initial injury and starts to decline over the next two to three years (Craig *et al*., 1975), however in keloids the synthesis of collagen is known to continue for several years (Diegelmann *et al*., 1979).

Typical of keloids is the presence of whorls of thickened collagen bundles with a normal appearing papillary and reticular dermis (Lee *et al*., 2004). The results of this study confirm the irregular and thickened appearance of collagen in keloids, but the papillary and reticular dermis did appear normal. In most instances the reticular dermis contained densely arranged collagen while in some instances collagen deposition was also increased in the papillary dermis.

Increased collagen gene-transcription, increased translation of collagen mRNA and/or decreased collagen degradation contributes to the formation of excessive amounts of collagen in keloids (Phan *et al*., 2002). The organisation, arrangement and degradation of collagen fibres are determined by a proteoglycan, biglycan, which is present in the ECM (Honda *et al*., 1986). Upregulation of biglycan is found in keloids and is associated with increased synthesis of collagen type I (Hunzelmann *et al*., 1996).

In addition to the over production of collagen type I and III in keloids (Ala-Kokko *et al*., 1987), abnormal crosslinking of this collagen occurs when compared to normal skin collagen (Di Cesare *et al*., 1990). This may contribute to the characteristic appearance of keloid collagen.

4.6.2 Fibroblasts and intracellular collagen

The ultrastructure of normal skin fibroblasts are spindle shaped cells with sparse granular ER and small Golgi apparatus. Fibroblasts with these characteristics are non-active fibroblasts sometimes termed fibrocytes. When stimulated, the amount of RER cisternae and Golgi apparatus in the fibroblasts increases with resulting increased synthesis of ECM components (Gesener, 1986). Abundant RER was also seen in the cytoplasm of keloid fibroblasts with TEM (Wang and Luo, 2013). Intracellular immature, pro-collagen is insoluble and is not visible with TEM. Collagen is produced by the fibroblasts and assembled on the RER as pro-collagen. Post translational modification and packaging takes place in the Golgi apparatus and ER, where budding ER membranes form transport vesicles. The collagen is then secreted into the ECM through a process of exocytosis. Within the extracellular environment collagen becomes insoluble once cross-linking has occurred and is visible with TEM (Diegelmann, 2001; Kadler *et al*., 2007). This is described in greater detail in section 2.2.

In fibroblasts with well-developed RER many ribosomes and collagen fibres are visible (Figure 4.38 a - d). Collagen fibres are visibly associated with the ER and Golgi apparatus (Figure 4.38d) and occur in vesicles as either single fibres (Figure 4.40) or as bundles (Figures 4.39 and 4.40) that are secreted into the ECM (Figure 4.40). An extensive literature search could not identify any other studies where the presence of intracellular insoluble collagen was described as a feature of keloids.

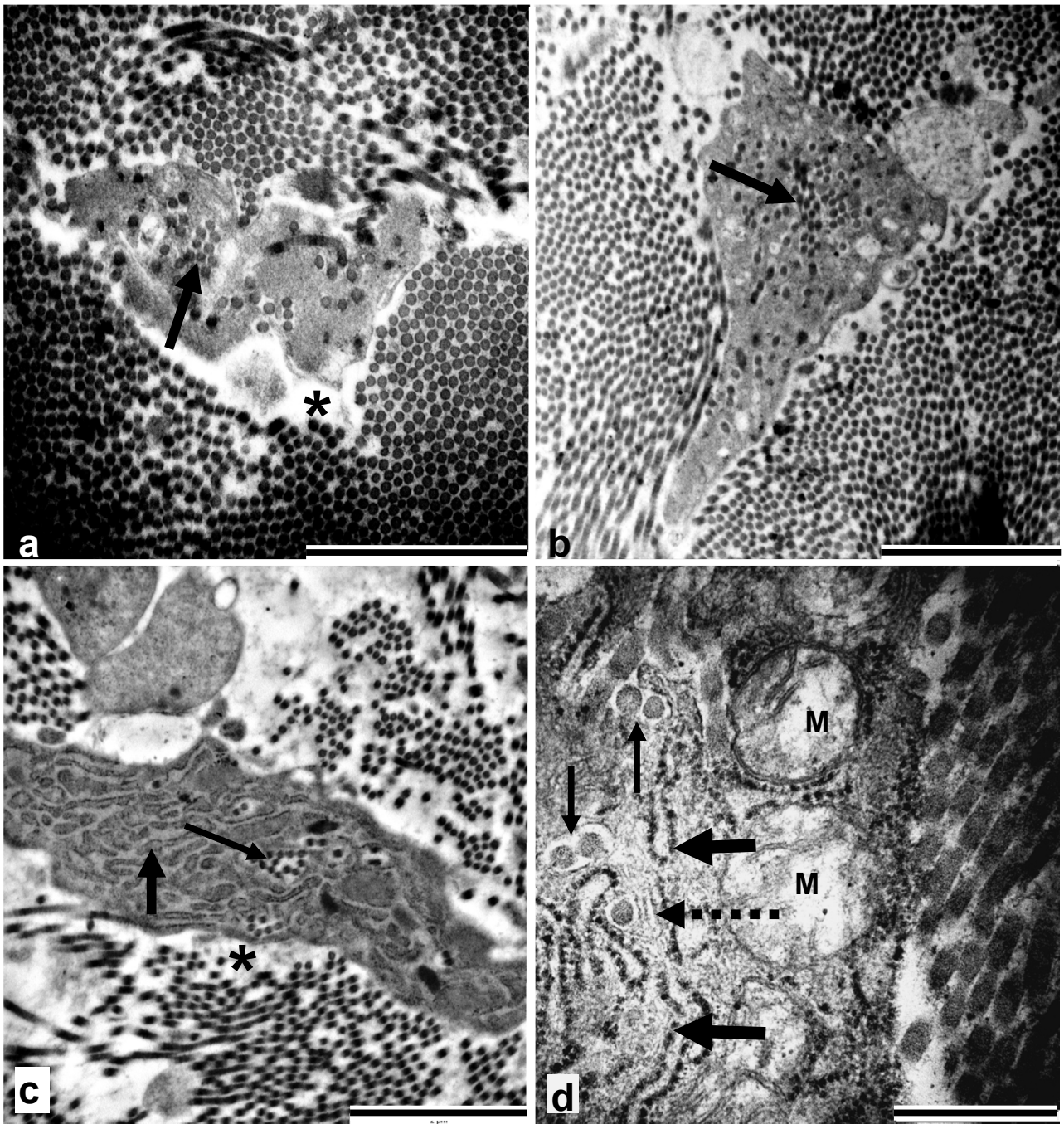


Figure 4.38: Keloid: (a - c) Fibroblasts with intracellular collagen present in vesicles (thin arrows). (a and c) A pericellular structure (*) is shown surrounding the fibroblasts. (c) Distended RER (bold arrow) is present, associated with active protein synthesis. In (d) a high magnification shows activation of fibroblasts indicated by abundant ribosomes (bold arrows). Fibroblast mitochondria (M) are visible in (d), as are vesicle bound collagen fibres (thin arrows) next to Golgi apparatus (dashed arrow). Scale bars (a, b, c,) = 2 µm, scale bar (d) = 500 nm.

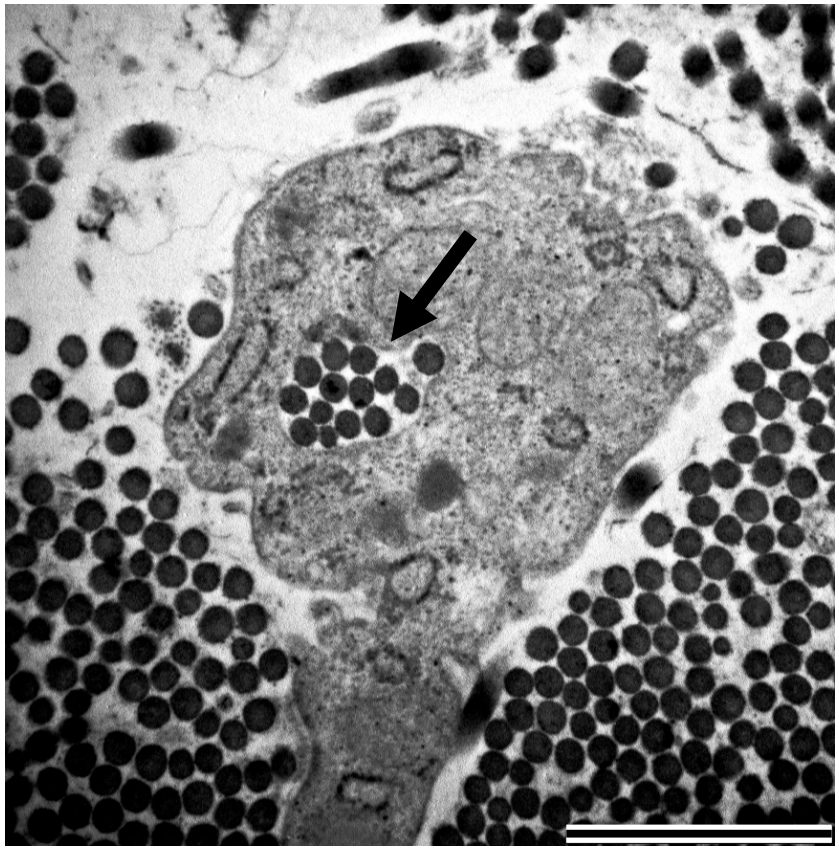


Figure 4.39: Keloid: Bundle of mature collagen fibres in a keloid fibroblast (arrow). Scale bar = 1 µm.

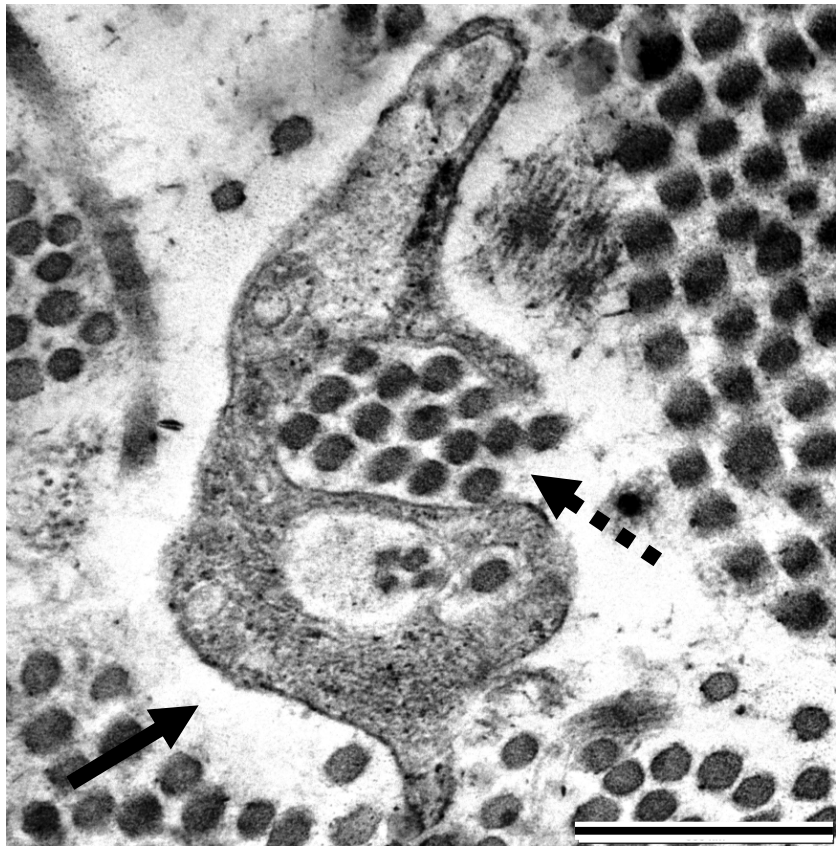


Figure 4.40: Keloid: Release of mature collagen from a keloid fibroblast into the ECM (dashed arrow). The pericellular structure is defined by an amorphous, collagen free area (arrow). Scale bar = 500 nm.

Myofibroblasts which are differentiated fibroblasts are found in granulation tissue and are reported to be present in keloid tissue (Santucci, 2001; Lee and Vijayasingam, 1995; Eddy *et al*., 1988; Schürch *et al*., 1992; Rannger-Brandle and Gabbiani, 1983; Grinell, 1994). Myofibroblasts have a contractile function and can be identified by the presence of cytoplasmic bundles of microfilaments, nuclear indentations and cell-to-cell stroma connections (Schürch *et al*., 1992). In all the EM samples evaluated in this study, no myofilaments could be identified in the cytoplasm of fibroblasts and this confirms the results of other studies where researchers have reported that myofibroblasts are absent in keloids (Köese and Waseem, 2008; Slemp and Kirschner, 2006; Ehrlich *et al*., 1994, Matsouka *et al*., 1988; Werner *et al*., 2007) or less differentiated (Eyden, 2003).

Mechanical tension (Hinz *et al*., 2001) and TFG-β (Desmoulière *et al*., 1993) activity are reported to be the main stimulus for fibroblasts to differentiate into myofibroblasts. Cell culture studies have reported the presence of myofibroblast characteristics in keloid fibroblast cultures, indicated by the presence of α-SMA positive cells as well as an increase in actin mRNA expression by keloid fibroblasts (Mukhopadhyay *et al*., 2007). Wound contraction is not a feature of keloids as the mass continues to grow over the original wound margins; therefore fibroblasts have probably not differentiated into myofibroblasts i.e. keloid tissue is trapped in the early granulation phase of wound healing. However the absence of myofibroblasts in these keloid tissue samples needs to be confirmed with IHC using myofibroblast specific antibodies such as α-SMA (Skalli *et al*., 1989; Skalli *et al*., 1986, Goodpaster *et al*., 2008). However this study does confirm the findings of previous studies that true myofibroblasts are either absent (Lee and Vijayasingam, 1995; Ehrlich *et al*., 1994) or poorly differentiated myofibroblasts (Eyden, 2003) as they contained features of increased ribosomes and distended RER seen in myofibroblasts (Eyden, 2003).

The presence of diffuse amorphous substance was noted by Ehrich *et al*. (1994), between the fibroblast and the surrounding keloid collagen, which separates the collagen fibres from the membrane surface of the fibroblast. This was also observed in some of the keloid samples used in this study and an example is seen in Figure 4.38a and c. This substance was described by Hembry *et al*. (1986), in research done on the wound healing of tight skin mice. The authors described the substance as a “pericellular structure” surrounding the fibroblast and noted that it disappeared in the mice at 3 weeks of wound healing. In keloids however, it was observed to remain for up to two years in samples investigated by Ehrlich *et al.* (1994). The authors suggested more research needed to be done on the matter as it could help to explain the

biological behaviour of keloid fibroblasts *in vivo*. Fibroblasts also synthesise elastin (Kanitakis, 2002).

4.6.3 Fibroblast and mast cell interaction

Interactions between cells in the skin take place through the release of cytokines and growth factors, where cells influence one another. These interactions and communications are increased during events such as cell injury and inflammation where a need for homeostasis exists in the cells' environment (Eming *et al*., 2007)

Various authors have reported the presence of mast cells in keloid tissue (Sansberg, 1962; Shaker *et al*., 2011; Smith *et al.*, 1987; Ehrich *et al*., 1994; Mueller, 2006; Zhang *et al.*, 2010; Beer *et al*., 1998; Rothe and Kerdel, 1991; Plack and Lewis, 1992). The number of mast cells is increased in chronic wounds, scars and keloids (Wulff and Wilgus, 2013) and increased angiogenesis in keloids was attributed to the presence of tryptase positive mast cells in tissue (Ammendola *et al*., 2013).

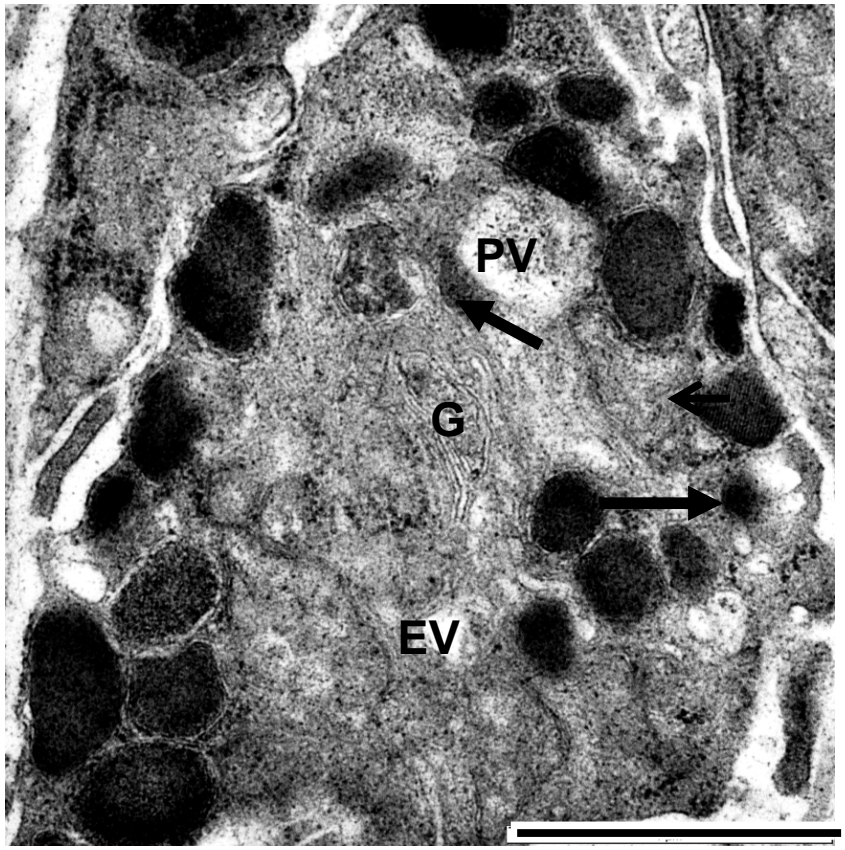


Figure 4.41: Keloid: Internal structure of a mast cell with high magnification showing mast cell granules (arrow), an empty vesicle (EV) and particle filled vesicle (PV). The Golgi apparatus is also indicated (G). Scale bar = 1 µm.

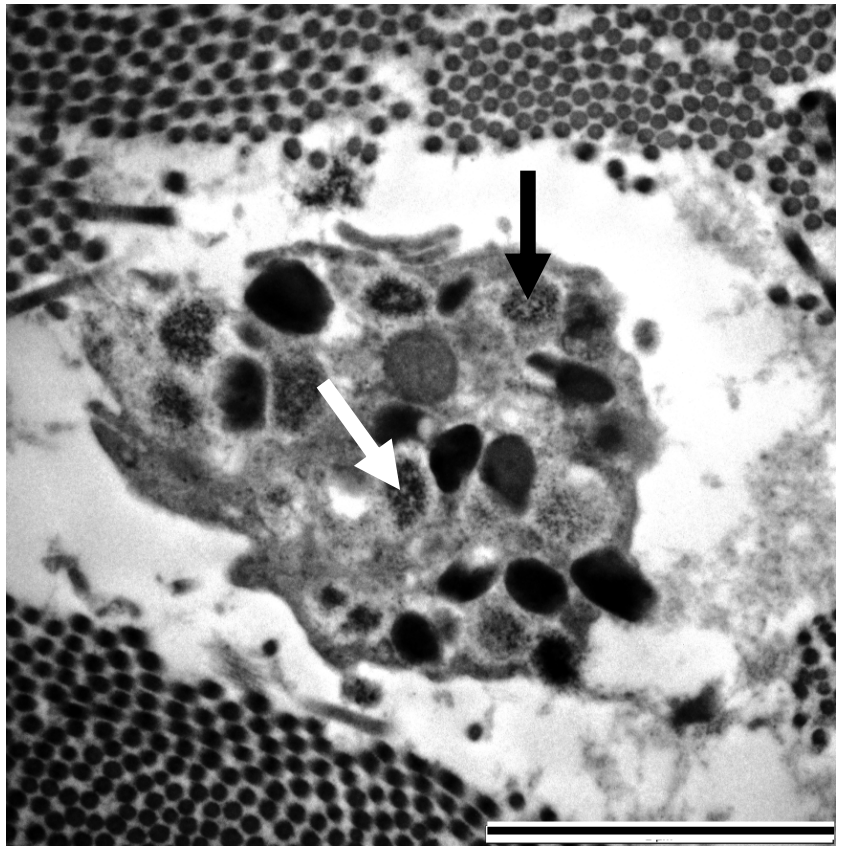


Figure 4.42: Keloid: Mast cell with visible degranulation of the vesicles (arrows). Scale bar = 2 µm.

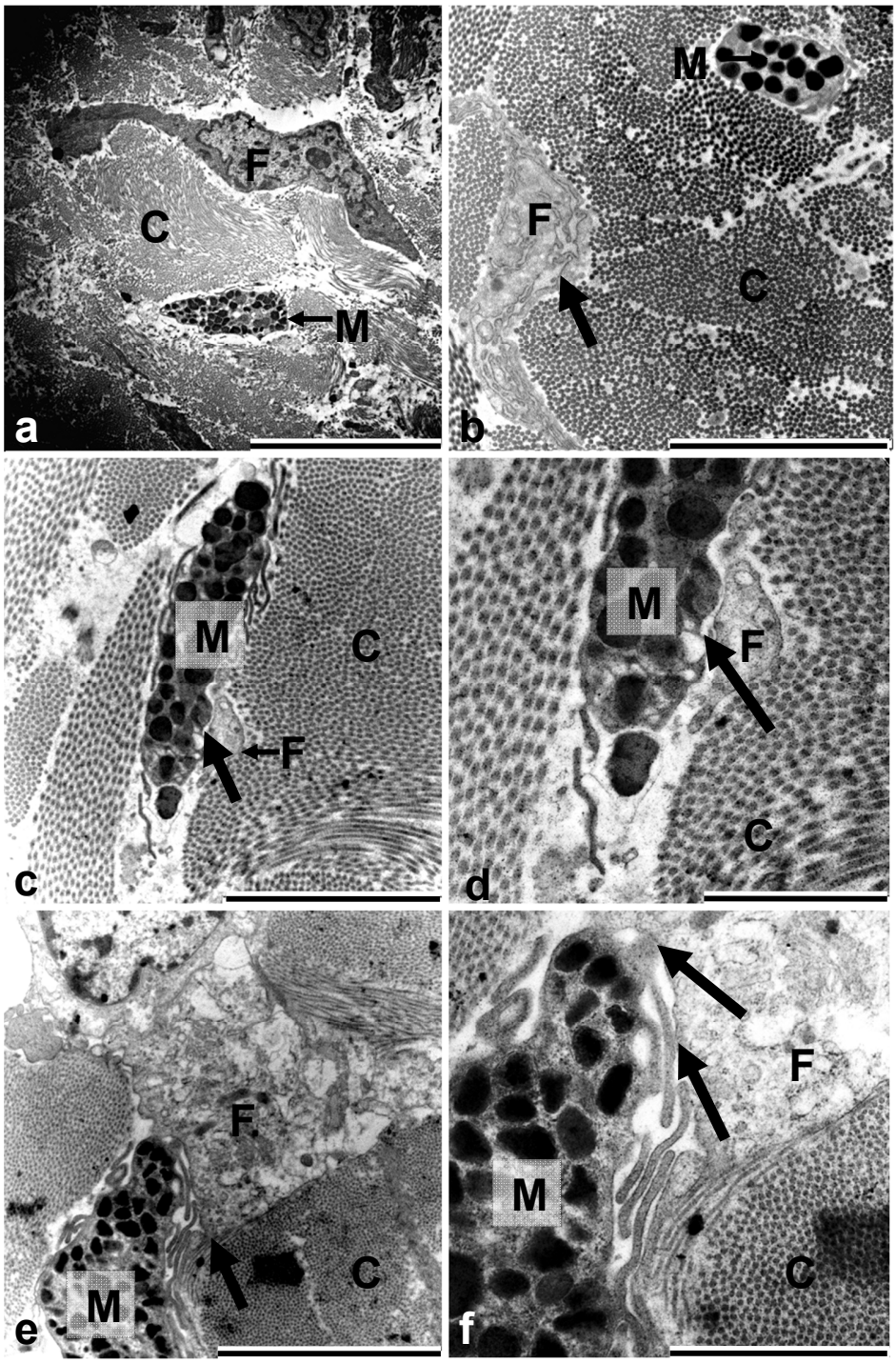


Figure 4.43: Keloid: (a) Close association of mast cel (M) and fibroblast (F). (b) Mast cell (M) and fibroblast (F) in close proximity to each other. Distended RER cisternae are visible in the fibroblast (arrow), indicating active protein synthesis. (c) Direct contact between a mast cell (M) and fibroblast (F) is shown (arrow). (d) A higer magnification of the contact in (c) (arrow). (e) Direct contact between mast cell (M) and fibroblast (F) (arrow). (f) A higher magnification if (e), showing areas where cell membranes of the two cells are in contact (arrows). Collagen (C). Scale bar (a) = 10 µm, scale bars (b, c, e) = 5 µm, scale bars (d, f,) = 2 µm.

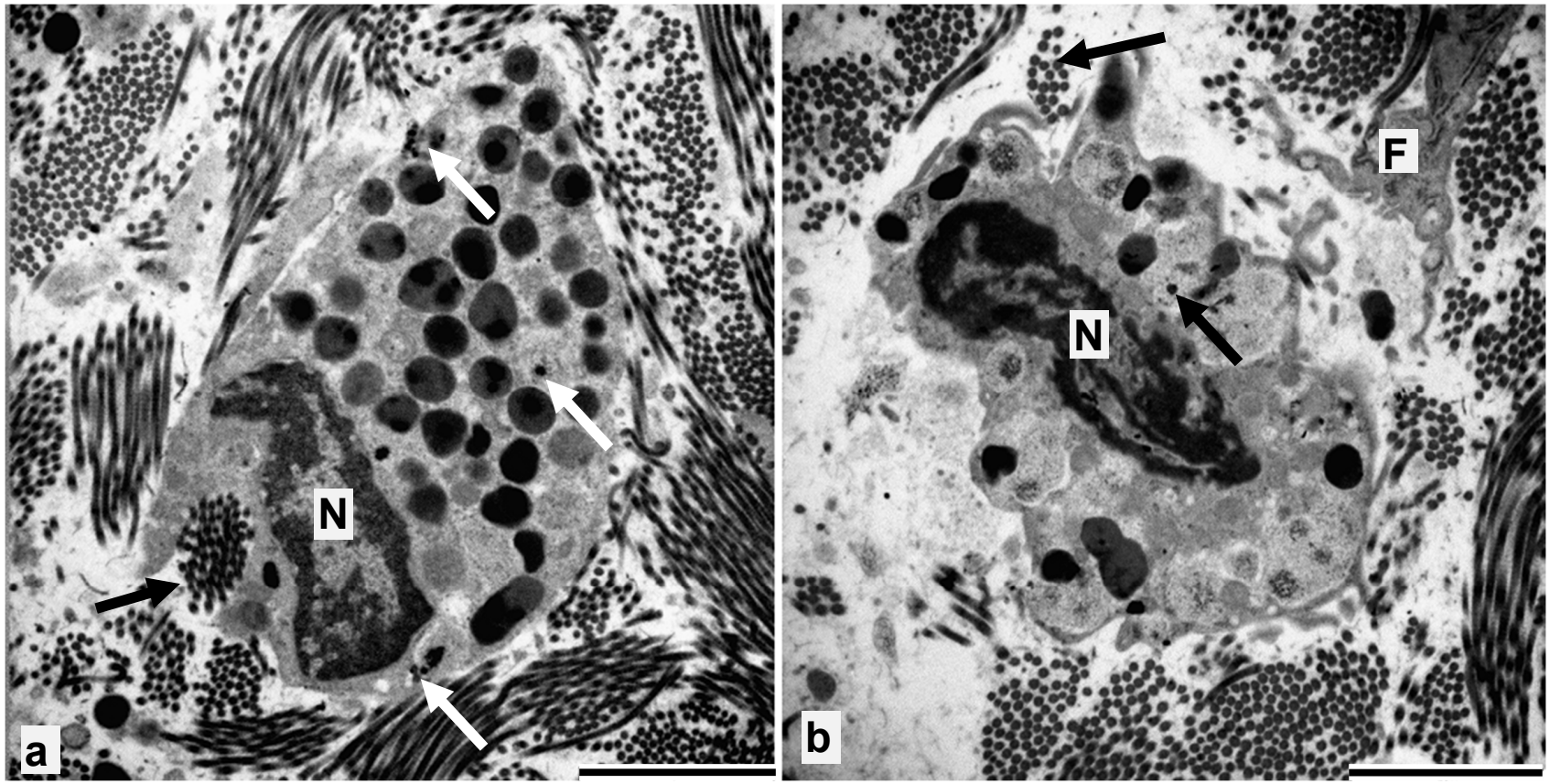


Figure 4.44: Keloid: (a) Mast cell engulfing a collagen bundle (black arrow) and (b) a mast cell preparing to engulf a collagen bundle (arrow). Intracellular collagen fibrils are seen in the mast cells (white arrows). An active fibroblast (F) is seen in (b). Nuclei (N). Scale bars = 2 µm.

Mast cells in keloid tissue have intact and empty vesicles that have undergone degranulation (Figure 4.41 and 4.42). Degranulation, associated with the breakdown of granules, a reduction of granule content and disruption of the cell membrane (Figure 4.42) indicates increased mast cell activity and the release of histamine and heparin (Dvorak, 2005).

In this study it was found that mast cells occur in close proximity to fibroblasts (Figure 4.43a and b). In some samples at a high magnification (Figure 4.43 c - f), mast cells are in direct contact with fibroblasts. Mast cells associated with these fibroblasts had degranulated vesicles in their cytoplasm. Direct contact between the two cell membranes is seen in Figure 4.43f. In many of these samples, the mast cells and fibroblast appear to be completely surrounded and entrapped in the dense collagen matrix (Figure 4.43 a and b).

Besides degranulation and cell-cell contact between mast cells and fibroblasts, mast cells were also engulfing bundles of collagen fibres as seen in Figure 4.44. Smaller groups of collagen fibres are present in the cytoplasm of the mast cell (Figure 4.44a). Another mast cell (Figure 4.44b) is seen with a collagen bundle isolated from the surrounding collagen and a slight splitting of the cell membrane (arrow), possibly prior to phagocytosis of the collagen bundle. Collagen fibres are also present in the cytoplasm of this mast cell.

Contact between mast cells and fibroblasts have been noticed by Shaker *et al*. (2011), and a close relationship between the two cell types was mentioned by Ozbilgin and Inan, (2003). Shaker *et al*. (2011), showed mast cell and fibroblast interactions in a LM study. Using TEM, the present study confirms that the interaction between mast cells and fibroblasts occurs (Figure 4.43).

Mast cells and fibroblasts can form gap junctions between cells which allow direct intercellular communication (Pistorio and Ehrlich, 2011; Shaker *et al*., 2011). The formation of gap junctions has been shown to stimulate fibroblast proliferation, myofibroblast differentiation and contraction (Au *et al*., 2007; Foley *et al*., 2011). Cell interactions termed “cell talk” were described by Lim *et al*. (2002), and these interactions are said to play a role in fibrogenesis of scars and other fibrotic lesions (Chizzolini, 2008; Chujo *et al*., 2009). Fibroblasts were found to produce mast cell growth factor which regulates mast cell survival and mast cells in turn affect the functioning of fibroblasts (Shaker *et al*., 2011).

Increased mast cell numbers during scarring have been observed in human samples and animal models (Wulff *et al*., 2012). Superficial wounds such as insect bites that do not go beyond the epidermis do not result in scarring. More traumatic injuries such as deep lacerations

and surgical incisions are deep seated skin wounds which require a longer wound healing period and often result in scar formation. Mast cell degranulation is associated with scarring. Wounds with fewer numbers of mast cells heal without scar formation. Examples of these are oral mucosal wounds as well as fetal skin wounds, which contain few mast cells that do not degranulate and after injury this leads to wound healing without scarring. More developed foetuses may present with fibrotic wounds which contain an increased number of mast cells that degranulate upon injury (Mak *et al*., 2009). Various animal models of mast cell function in tissue have been established and these are mentioned by Gurish and Austen, (2001). Studies on animal models have shown that preventing mast cell degranulation or blocking the activity of mast cell proteinase will effectively reduce scar tissue formation (Takato *et al*., 2011). Wound repair therefore depends on the type and size of the wound (Ansell *et al*., 2012; Greenhalgh, 2005). However, in the study by Bayat *et al.* (2003), the size of the wound did not influence the formation of keloids. Increased mast cell numbers and their mediators is seen in inflammatory and allergic conditions (Hallgren and Gurish, 2007; O'Sullivan *et al*., 2000; Brightling *et al*., 2002) and the prevalence of keloids in this population is higher. Histamine is stored in mast cells and released during degranulation in response to different stimuli (Hallgren and Gurish, 2007). Connective tissue mast cells have a histamine content that is greater than mucosal mast cells (Gurish and Austen, 2001). Histamine stimulates the production of pro-inflammatory mediators by keratinocytes (Giustizieri *et al*., 2004; Ishikawa *et al*., 2009; Kohdu *et al*., 2002) and stimulates keratinocyte proliferation (Wulff and Wilgus, 2013; Weller *et al*., 2006). Mast cell activation is a well-known feature of chronic inflammation where fibrosis occurs due to increased collagen production by fibroblasts (Rabiatti *et al*., 2000), and is also involved in tumour formation and angiogenesis (Wulff and Wligus, 2013; Tomita *et al*., 2001). Heparin is also present in mast cell granules and is released during degranulation. It is known to contribute to fibrosis by preventing the inactivation of FGFs (Yayon *et al*., 1991; Mueller *et al*., 1989; Saksela *et al*., 1988).

Previous research on mast cells and their effects on fibroblasts and collagen synthesis (Noli and Milo, 2001; Abel and Viagoftis, 2008; Cairns and Walls, 1997) has led to the theory that degranulation of mast cells in skin, results in the activation of fibroblasts and consequently increased collagen production.

This LM and TEM based study has revealed that keloid formation is a complex process that involves cellular, matrix, inflammatory and growth factors as well as defective collagen synthesis by fibroblasts. These effects will be discussed in greater detail in Section 5.2.

Chapter 5: Concluding Discussion

5.1 RATIONALE OF STUDY

Keloids are disfiguring and are often difficult to treat. Scientific literature related to the morphological features of keloids especially at an ultrastructural level is outdated. Therefore the purpose of this study was to reassess present knowledge of the ultrastructural features of keloids and possibly through this process identify new cellular therautic targets.

5.2 SUMMARY OF RESULTS

In this study the tissue and cellular structure of control, normal skin was compared to keloid tissue using LM and TEM in order to clarify the role of keratinocytes, melanocytes, fibroblasts and mast cells in keloid formation.

5.3 TISSUE STRUCTURE- LIGHT MICROSCOPY

Using LM the general morphology of control and keloid tissue was evaluated and this included the structure of the dermis and epidermis, the epidermal-dermal junction, the rete ridges and dermal distribution of the collagen. In keloid tissue variations in the distribution of the rete ridges were observed and morphologically were found to have a blunted appearance. Although keloids had a well-defined dermal layer, it was difficult to distinguish between the papillary and reticular dermis. Within the dermis the arrangement of collagen was irregular and denser when compared to control skin.

The distribution of collagen in normal and keloid tissue was further evaluated using a collagen specific stain. Control skin appeared to contain collagen type I and III arranged in an interwoven pattern. Collagen type I was found to be the predominant type of collagen in keloids, the distribution of which varied between samples and was either interwoven or had formed dense bands.

Cells of the epidermal layer such as the keratinocytes influence the growth of fibroblasts in keloid tissue via the release of growth factors such as IL-6 (Waelti *et al*., 1992; Smola *et al*., 1993; Boxman *et al*., 1996), keratinocyte derived IL-1 (Waelti *et al*., 1992; Maas-Szabowski and Fusenig, 1996; Maas-Szabowski *et al*., 1999), FGF, PDGF, TNF-α and TGF-β (Werner *et al*., 2007, Xia *et al*., 2004). Also present in the epidermal-dermal junction are melanocytes. With LM,

kertinocyte and melanocyte distribution appeared to be normal and Langerhans' and Merkel cells were difficult to identify.

Mast cells are associated with inflammation and wound healing. Mast cells were rarely found in control tissue but were found along the epidermal-dermal border and deeper in the dermis of keloid samples, and occurred in groups of three to five cells. Fibroblasts were present in the dermis of control and keloid skin samples. No differences in the distribution of fibroblasts were found in the samples evaluated.

5.4 CELLULAR AND MATRIX STRUCTURE – TRANSMISSION ELECTRON MICROSCOPY

The ultrastructure of normal, control and keloid skin was evaluated with TEM. This technique was used for the detailed evaluation of the structure of cells associated with the epidermis and dermis and included the evaluation of the structure and distribution of keratinocytes, melanocytes, fibroblasts and mast cells as well as the structure and distribution of collagen.

5.4.1 Keratinocytes

The morphology of the keratinocytes in control and keloid tissue was compared and while control skin keratinocytes had the typical structure described for normal skin, keloid keratinocytes appeared to be more closely arranged with shortened cytoplasmic processes between keratinocytes. This may be due to the collagen mass in keloid tissue pushing against the epidermal-dermal junction, this may also account for the blunted rete ridges observed with LM. Likewise, the cytoplasmic processes of melanocytes present in the epidermal-dermal junction were fewer, shorter and were in direct contact with the collagen in the dermal layer.

Hemidesmosomes are present at the dermal-epidermal junctions (Mescher, 2010), forming attachments between the basal layer and epidermal and dermal cells (Figure 4.21), as well as in the epidermis between the keratinocytes (Figures 4.17, 4.19 and 4.20). Desmosomes were also present in the dermis of control skin (Figures 4.29 - 4.31) where they were observed only in a single skin sample and both structures were absent in keloid tissue. The lack of desmosomes in control skin may be related to the source of the skin, as skin obtained from skin grafts is often stretched and as a consequence the dermal layer is thin and the epidermal-dermal layers interactions are compromised. The lack of these structures in keloids could be due to the

excessive amount of collagen in the dermis that either disrupts these interactions or masks the presence of these structures.

5.4.2 Melanocytes

In 1986, Snell reported that the morphology of melanocytes varied during wound healing. In the early stages of wound healing the cell bodies of melanocytes were either smaller than normal or very much larger. Melanocyte dendritic processes/cytoplasmic processes were observed to be either absent or very few in number. The cytoplasmic processes that were present were either simple with very few branches and much shorter and stumpy when compared to these processes in normal skin. Keloid melanocyte structure is similar to that found in the granulation phase of wound healing.

5.4.3 Fibroblasts

In a study involving wound healing in tight skin mice, developed based on the tension theory of keloid formation, Hembry *et al*., 1986, identified and described a collagen free pericellular region around fibroblasts. In these mice this region was found up till 3 weeks of wound healing. Ehrlich *et al*., 1994 described a similar feature to be present in keloids for up to two years (Ehrlich *et al*., 1994). In this study this pericellular region was also found to be present in keloids (Figure 4.38a, c and Figure 4.40) a feature associated with prolonged inflammation and granulation. The granulation state of keloids is further indicated in studies by the prolonged proliferative and short remodelling or apoptotic state (Lim *et al*., 2002; Lim *et al*., 2001) and excessive deposition of collagen in the remodelling phase due to p53 and Bcl-2 which reduces apoptosis and encourages proliferation respectively (Aarabi *et al*., 2007; Bayat *et al*., 2003; Craig, 1975; Rudolph; 1987). Fibronectin, responsible for the formation of granulation tissue (Diegelmann *et al*., 1979; Grinell, 1984; Kisher *et al*., 1981; Kurkinen *et al*., 1980), is increased in keloids and it leads to an abnormal fibroblast function and an increased collagen production. More so the absence of fibronectin leads to scarless wound healing (Mak *et al*., 2009; Linder, 1978). Based on this information available in literature and the general time line of wound healing, keloids can be descrbed as being in a permanent or at least extended granulation state with a great inflammatory component.

5.4.4 Fibroblast activity and collagen formation

Increased fibroblast activity was observed in some keloid samples with EM. The increased production and deposition of collagen in the ECM in keloids was seen in TEM, and is described by Shaker *et al*. (2011). Along with increased collagen production through fibroblast stimulation mast cells also affect collagen maturation and remodelling (Younan *et al*., 2011; Egozi *et al*., 2003; Iba *et al*., 2004). The presence of fully formed collagen fibres in the cytoplasm of fibroblasts in our results demonstrates abnormal production, as various stages of collagen formation. This is the first study that has observed this phenomenon in keloids and the intracellular presence of insoluble collagen fibrils identified by TEM has only been described for chick embryo tendon formation (Canty and Kadler, 2005).

5.4.5 Mast cells

In wound healing and fibrosis associated conditions, the number and the distribution of mast cells is increased. Various authors reported the increased presence of mast cells in keloid tissue (Shaker *et al*., 2011; Sansberg 1962; Smith *et al*., 1987; Ehrich *et al*., 1994; Mueller, 2006; Zhang *et al*., 2010, Beer *et al*., 1998; Rothe and Kerdel, 1991; Plack and Lewis, 1992). In keloid tissue an increase in mast cells was present and these cells were in various stages of degranulation indicating activation of mast cells and the release of inflammatory mediators from mast cell granules.

5.4.6 Fibroblast and mast cells

TEM analysis of the dermal region of keloid skin revealed close or direct association between mast cells and fibroblasts which implicates mast cell stimulation of fibroblasts via cytokines (Beer *et al*., 1998). Mast cells may influence cells in their immediate vicinity without direct contact, through the release of cytokines (Beer *et al*.,1998) and growth factors such as basic FGF and particularly TGF-β which is involved in dermal fibrosis through the expression of collagen genes in dermal fibroblasts (Zhang *et al*., 2010; Gruber, 2003). Direct contact of fibroblast and mast cells is possible through gap junctions and this can also influence collagen production (Hügle *et al*, 2012). Degranulation of mast cells present in keloid tissue results in the release of histamine, heparin and other factors (Dvorak, 2005).

The associations of mast cells with fibroblasts have previously been observed by Shaker *et al*. (2011). These associations are important in fibrotic conditions such as systemic sclerosis, chronic graft versus host disease and lung fibrosis, which is characterised by an overproduction of collagen by fibroblasts (Ruoss and Caughey, 1995; Atkins *et al*., 1985). In pulmonary fibrosis, mast cells were observed in partial degranulation and in close proximity to lung fibroblasts (Kawanomi *et al*., 1979; Heard *et al*., 1992). In a study on systemic sclerosis (Hugle *et al*., 2012), cell-to-cell contact between mast cells and fibroblasts was observed, but the authors were unable to determine whether the contact was a characteristic specific to systemic sclerosis or mast cell activation. Recently Foley and Ehrlich, (2014), have shown that fibroblasts co-cultured with mast cells transformed into myofibroblasts and caused an increase in collagen synthesis by fibroblasts. Both granulated and degranulated mast cells caused this occurrence, due to the formation of gap junctions between fibroblasts and mast cells. An inability to form gap junctions by mast cells led to fibroblasts being susceptible to hypertrophic scar therapy investigated in the study, whereas the co-culture of gap junction forming mast cells with fibroblasts caused fibroblasts to be resistant to the therapy (Foley and Ehrlich, 2014). The authors suggest that elimination of the ability of mast cells to form gap junctions would be useful in hypertrophic scar therapy, showing the importance of gap junctions in cellular communication.

These findings, together with the observations made in this study indicate that gap junction interaction between mast cells and fibroblast is a typical feature of fibrosis and also a causative agent for increased collagen production.

5.4.7 Hypothesis: Defective collagen synthesis and processing, leads to mast cell activation and collagen phagocytosis in keloids

Histologically significant cellular differences were found in keloid tissue when compared to normal/control, with prominent changes in keratinocytes, fibroblasts and mast cells as well as collagen synthesis and fibre formation. These findings have led to the hypothesis of a new theory regarding keloid formation.

As shown in Figure 5.1, normal collagen synthesis has an intracellular and an extracellular component. Intracellularly, collagen mRNA is synthesised in the nucleus, and transported to the RER (Engelmann, 2001) where the collagen is produced during translation and the steps of hydroxylation and initial glycosylation occur. The presence of the non-helical domains associated with each collagen, the propertied domains at the C- and N-terminals (Adler *et al*., 2007) play an important role in the collagen triple helical formation (Bella *et al*., 2006) (Figure

5.1, Step 1). The C-propertied determines chain selection and direct chain association during intracellular assembly of the precollege molecules (Hulme's, 2002) causing the chain to associate in a triple helical structure. The presence of these propertied sequences ensures that the large collagen molecule remains soluble. In the Golgi apparatus the collagen molecules undergo further post translational modifications (Kivirikko and Risteli, 1976) which involves further glycosylation steps (Figure 5.1, Step 2). The soluble pro-collagen is then transported via transport vesicles to the cell surface where it is then secreted into the extracellular space (Diegelmann, 2001) (Figure 5.1, Step 3).

Following or during secretion into the ECM (Figure 5.1, Step 4), processing and associated proteolytic removal of the N- and C- collagen propeptides occurs catalysed by specific N- and C- proteinases (Colige *et al*., 2005; Greenspan, 2005). The C-terminal ensures the intracellular solubility of the procollagen. Once removed, collagen becomes insoluble and fibrogenesis occurs (Greenspan, 2005). The N-propeptide influences the shape and diameter of the fibril and does not prevent fibril formation (Brown and Timpl, 1995).

The insoluble collagen then assembles into fibrils (Figure 5.1, Step 5), that are structurally stabilised by cross links (Figure 5.1, Step 6) (Kadler *et al*., 1996; Prockop and Hulmes, 1994) catalysed by lysyl oxidase (LOX). The intra- and intermolecular links that form stabilise and increase the tensile strength of the final collagen fibre. It is believed that these peptides then re-enter the cell and regulate collagen production by a feedback mechanism.

The presence of insoluble collagen fibrils within the Golgi apparatus and secretory vesicles (Figures 4.38 d and 4.40) implies that collagen processing in keloid fibroblasts is defective and this may be related to increased procollagen C-proteinase (PCP) activity which results in premature collagen fibril formation. As mentioned previously the C- terminal propeptide sequence ensures the solubility of intracellular collagen. Normally, this sequence is only removed once the procollagen is secreted into the extracellular environment (Figure 5.1; Step 6). Premature or early removal of this sequence results in decreased solubility and rapid crosslink formation by LOX. PCP also processes LOX to its active form, as a consequence of increased levels of PCP, LOX is also increased. Broder *et al*. (2013), have identified that metalloproteases α- and β-meprin are C- and N- procollagen proteinases. Both meprins are expressed by human dermal fibroblasts and remove the C- and N- propeptides from collagen type I and III. Expression of α- and β-meprin is increased in keloids (Kronenberg *et al*., 2010). Both Turtle *et al*. (2012), and Broder *et al*. (2013), have identified PCP and the meprins as possible therapeutic targets for fibrosis and possibly keloid formation. The cause of increased

levels of PCP and subsequently of LOX is unknown although increased LOX levels are associated with hypoxia, an important microenvironment factor in the development of fibrosis. As shown in Figures 4.34 and 4.37, fibroblasts that are overcrowded by collagen fibres can be assumed to be in a hypoxic environment.

TGF-β also stimulates the production LOX, an enzyme involved in the formation of cross links between collagen fibrils, resulting in collagen fibre formation. Direct contact of fibroblast and mast cells through gap junctions is also a possible means of association (Hügle *et al.,* 2012). Moyer *et al.* (2004), identified the presence of gap junction intercellular communication between mast cells and fibroblasts and that this interaction only occurred *in vitro* when cultured in a three dimensional environment. Cell-to-cell or direct contact between mast cells and fibroblasts *in vivo* was observed during TEM analysis (Figure 4.43). Associations of mast cells with fibroblasts have previously been identified as an important feature of fibrotic conditions such as systemic sclerosis, chronic graft vs. host disease and lung fibrosis characterised by an overproduction of collagen by fibroblasts in the area (Ozbilgin and Inan, 2003; Ruoss and Caughey, 1995; Atkins *et al*., 1985). In pulmonary fibrosis, mast cells were observed in partial degranulation and in close proximity to lung fibroblasts (Kawanomi *et al*., 1979; Heard *et al*., 1992). In a study on systemic sclerosis (Hügle *et al*., 2012), cell-to-cell contact between mast cells and fibroblasts was observed but the authors were unable to determine whether the contact was a characteristic specific to systemic sclerosis or due to mast cell activation. In this study, cell-to-cell contact between fibroblasts and mast cells was also observed. This observation and findings of other authors related to other types of fibrosis, leads one to the conclusion that this type of interaction is a common feature of fibrosis.

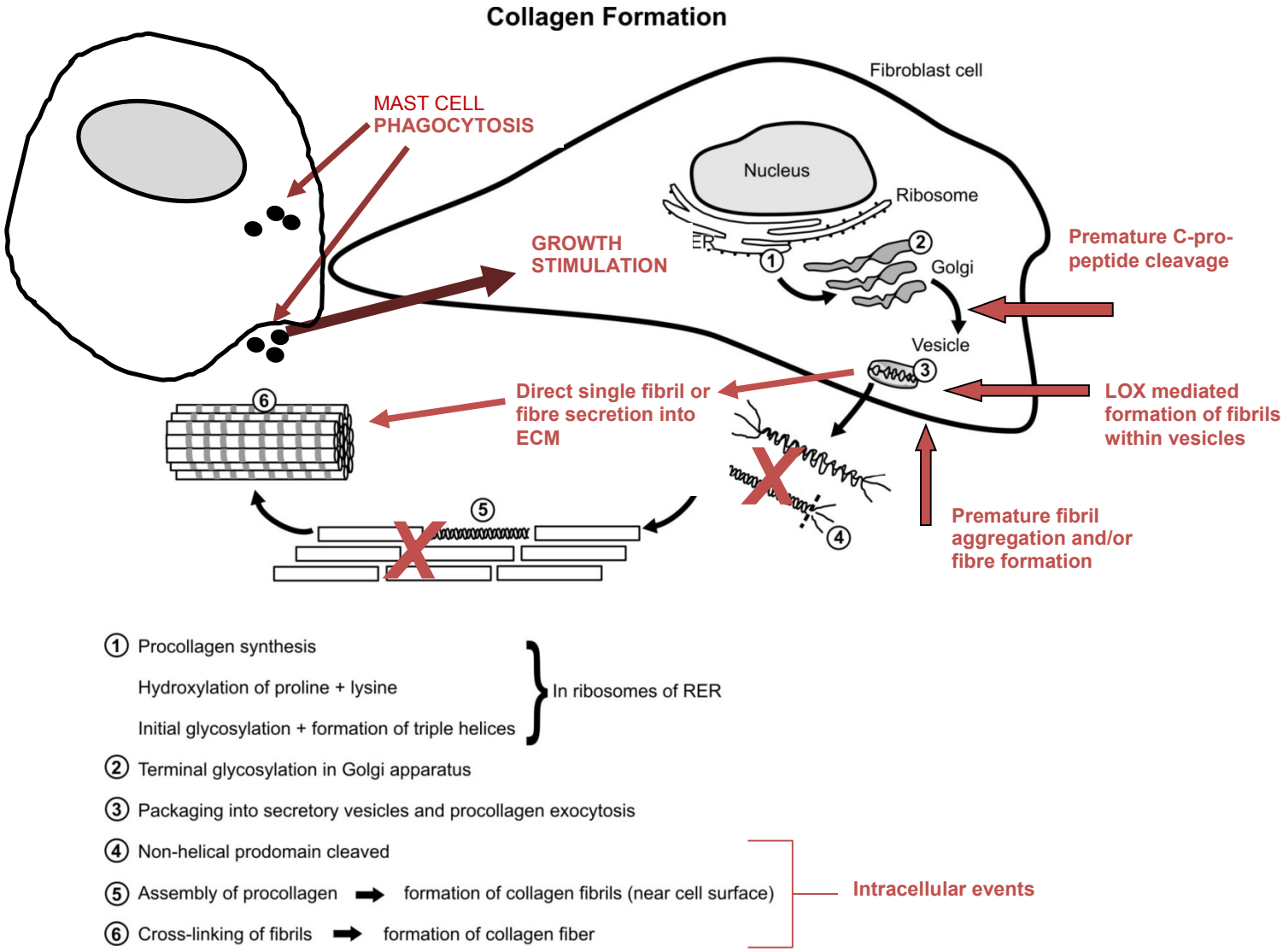


Figure 5.1: Schematic diagram of normal and abnormal keloid collagen processing.

Lysyl-oxidase-like-2 (LOXL2) is increased in disease and its inhibition was found to result in a reduction of active fibroblasts, decreased production of growth factors and cytokines and a decrease in the TGF-β pathway signalling (Barry-Hamilton *et al*., 2010). The influence of LOXL2 on fibroblasts was observed to occur *in vivo* through the LOX function of collagen cross linking and resulting increases in the tension of the ECM (Wipff *et al*., 2007) which caused an activation of the TGF-β1 signalling (Wipff *et al*., 2007, Hinz *et al*., 2007). Several treatment options are available to prevent and/or treat keloids. These treatment strategies are not always successful.

Specific target enzymes within the biochemical pathway of collagen synthesis and fibre formation have been identified as therapeutic targets and these include inhibitors of LOX and PCP (Turtle *et al*., 2012; Broder and Becker-Pauly, 2013).

It is unknown whether the increased presence of mast cells is a consequence of overproduction of collagen i.e. an excess collagen in the ECM causes increased homing and/ or migration of mast cells into the dermis into regions of increased collagen production. Alternatively that increased presence of mast cells due to inflammation and the consequent release of cytokines or growth factors results in increased fibroblast proliferation. A recent study by Willenborg *et al.* (2014), has shown that genetic ablation of connective tissue type mast cells fails to prevent bleomycin induced lung fibrosis. Keloid fibroblasts have been isolated from keloid tissue and grown in cell culture; compared to normal cells, keloid fibroblasts have shown differences in growth properties and gene expression (Igota *et al*., 2013). Furthermore epigenetic changes have been implicated in the development of fibrosis in keloids (Mann, 2012). Recently Mastri *et al.* (2013), have identified secreted frizzled-related protein-2 (sFRP2) as an important mediator of fibrosis, as this protein enhances procollagen C proteinase activity and this leads to increased procollagen processing and deposition in cardiac fibrosis. Similarly sFRP2 could also have a similar role in keloid formation and this protein may, as for other types of fibrosis, be a specific target of therapeutic intervention (Mastri *et al*., 2013). Phagocytosis of collagen fibrils by mast cells (Figure 4.44a) may indicate that mast cells in keloid tissue play an important role in maintaining tissue homeostasis and turnover. The presence of collagen fibres in mast cells of keloids has also been reported by Ehrlich *et al*. (1994), and these authors hypothesised that this was related to an immunological dysfunction. However, recent scientific literature and the findings in the current study, leads us to believe that keloid formation is primarily due to a defect in collagen synthesis and increased mast cell accumulation is a consequence of collagen overproduction in the dermis.

To summarise increased fibroblast activity, intracellular collagen production and fibroblast and mast cell interactions were seen in keloid tissue. Keloid tissue is a hyperproliferative tissue, similar to that of the granulation phase of wound healing, where fibroblasts are in an increased metabolic state and secrete large amounts of collagen into the ECM. In addition an inflammatory reaction takes place in the dermis, where mast cells and fibroblasts influence each other into producing large amounts of collagen and pro-inflammatory mediators which in turn affect the collagen production in the fibroblasts. TGF-β derived from mast cells, inhibits keratinocyte proliferation and stimulates increased collagen production through increased

expression of LOX by fibroblasts. Intracellular insoluble collagen formation has occurred due to the rapid, intracellular removal of the C terminal propeptide sequence by C-proteinase which initiates the cascade of insoluble collagen fibre formation within the fibroblast. Normally this process occurs only within the ECM in response to the increasing mass of collagen and in an attempt to establish normal tissue homeostasis the mast cells engulf the bundles of collagen fibres. Increased stress on the epidermal layer causes increased keratinocyte proliferation, which results in further growth factor mediated replication of fibroblasts. This creates an endless cycle of collagen synthesis, mast cell degranulation and mast cell mediated collagen phagocytosis, physical stress on the epidermal layer and subsequent growth factor release and fibroblast activation and collagen synthesis (Figure 5.2).

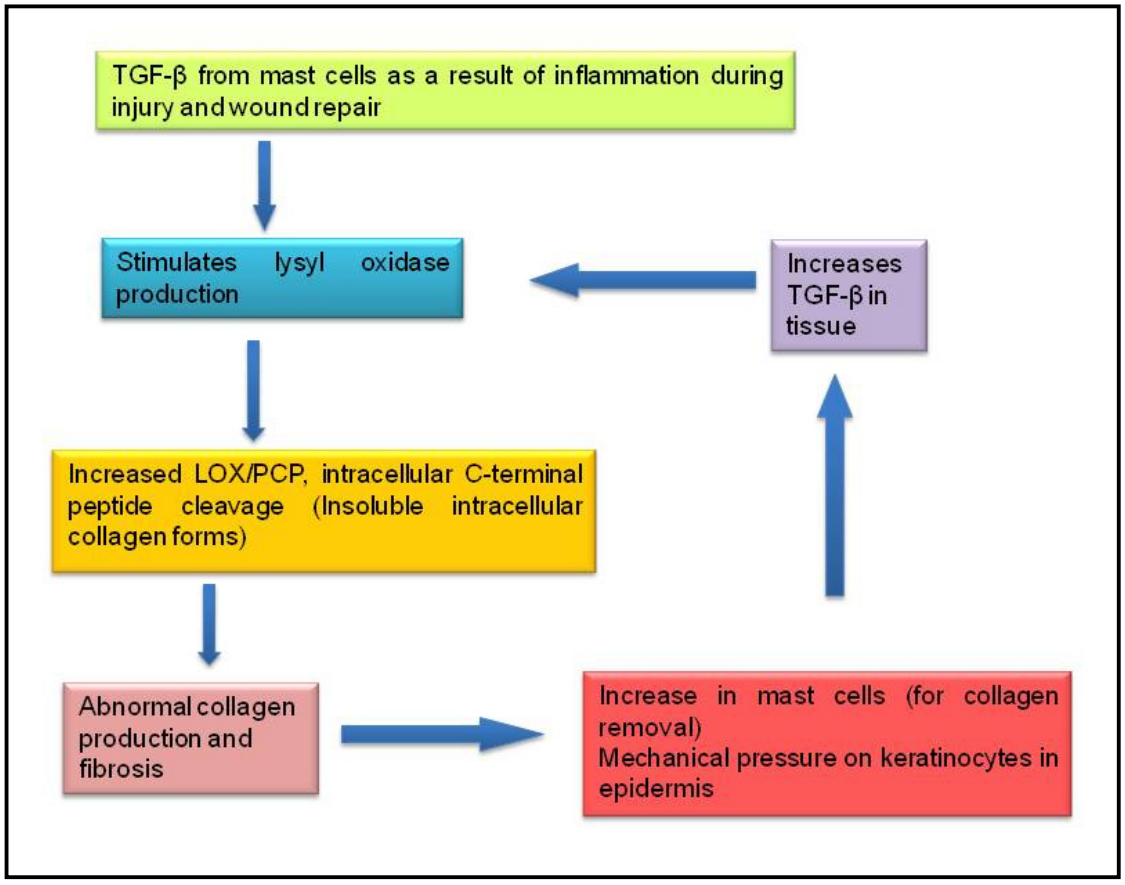


Figure 5.2: Flow diagram demonstrating a summary of the conclusion and effects of TFG-β, LOX and PCP on collagen production in keloids.

5.5 LIMITATIONS TO THE STUDY

5.5.1 The use of skin transplants as control

Control skin samples were obtained from patients who underwent skin transplantation. Procedures involved in preparation of skin for skin grafts include the use of tissue expansion to generate skin used as an epidermal graft. A balloon expander or hydro-gel (Bergé *et al*., 2001) is inserted under the epidermis and with time filled with saline solution, causing the epidermis to stretch and grow. This expanded flap of skin can then be used in scar therapy (Zhao *et al*., 2014). Ethical reasons permitted the use of this tissue and the study was therefore limited to using skin transplant skin which was not an ideal representation of normal human skin. The skin obtained as control was stretched which could have potentially led to the removal of some cells within the dermis. This led to difficulties in the comparison of the amounts of collagen and cell numbers as only half of the dermis of control skin was available in most samples. Some images used to show the epidermal layers of skin were from keloid skin as the control skin epidermis was thin and stretched out due to the collection procedure. Also the control skin and keloid were from different patients and it would be ideal to obtain samples from one patient to see if there are any differences. It is suggested for future studies that control skin be sourced as left- over tissue from cosmetic procedures that involve the removal of undisturbed whole skin, such as reduction mammoplasty and abdominoplasty.

5.5.2 Lack of Immunohistochemistry (IHC)

IHC staining on LM sections of keloids would more accurately identify mast cells, and differentiate between fibroblasts and myofibroblasts. IHC staining coupled with TEM would enable the detection of cytokines or any other factors released by keratinocytes or melanocytes *in vivo.* With regards to melanocytes; localisation of the MC1 R receptor in skin with low melanin content would give more insight into its relevance in keloids as there has been an association established between the receptor and its ligand in darkly pigmented skin and keloids. Looking at its presence in lighter pigmented keloid skin would focus on the inflammatory function without a melanin association.

We also suggest the investigation of the effects of AB0023 on keloids in place of tumours as done by Barry-Hamilton *et al*. (2010), using IHC with a LOXL2 polyclonal antibody. The study by these authors saw LOXL2 associated with α-SMA positive fibroblasts. We strongly suggest

future studies to investigate the presence of α-SMA to differentiate between fibroblasts and myofibroblasts in keloid tissue.

IHC to localise TGF-β in keloid fibroblasts and to further investigate its association with LOX should also be investigated as well as LOXL2, TGF-β and α-SMA.

5.5.3 Quantification

LM and TEM provide information regarding probable interactions. These techniques are qualitative rather than quantitative. To address this limitation IHC can be used to better identify cell types such as mast cells. IHC can also be used to determine if in keloid tissue LOX and/or PCP levels are increased.

5.6 FUTURE PERSPECTIVES

There is no specific mechanism identified for the formation of keloids and therefore no specific keloid treatment exists. The current standard treatments can be referred to as “broad spectrum” at a molecular level and further studies involving proteomics could contribute to finding a single or a more focused treatment. Previous studies have compared comparative proteomic analyses on keloids and hypertrophic scars (Ong *et al*., 2010) and on whole keloid tissue (Javad and Dey, 2012). Many proteins have been identified in keloid tissue and the suggested future perspective is to focus on the proteins/ enzymes implicated in collagen formation and wound healing such as LOX and PCP.

The presence of unknown proteins/enzymes can be further elucidated or verified using proteomics linked with liquid chromatography mass spectrometry (LC-MS). The results will then be compared to literature to identify proteins/ peptides that are known and those that are novel. The identified proteins/ peptides could be used to identify novel drug targets for the treatment of keloids.

The inability to compare non keloid skin regions of known keloid formers with normal skin using the method described above is a limiting factor in all keloid research. The comparison would give further insight into the molecular mechanisms and differences between normal and keloid forming individuals prior to actual keloid formation. However biopsies of normal skin, even with

patient consent for research or elective surgery purposes are ill advised and altogether avoided in keloid formers. This highlights the importance of animal models for keloid formation.

Formation of excessive granulation tissue is seen in horses and the condition is similar to that of keloids in humans (Theoret and Wilmink, 2013), posing athletic performance and discomfort problems to horses as well as various concerns to their owners (discussed by Sørensen *et al*., 2014). For this reason equine models for excessive granulation tissue have been established (Sørensen *et al*., 2014; Celeste *et al*., 2011; Deschene *et al*., 2011), and for the first time a potential animal model for keloids exists. Future research could utilise these models to study stages of wound healing with mediator expression leading to the formation of granulation tissue and excess collagen deposition, as well as the therapeutic potential of novel agents. A unique take on the use of an equine model is that benefits of research would serve both human and equine health as is not always the case with other animal studies.

In conclusion keloid formation is difficult to study and therefore a detailed morphological (as used in this study) and biochemical approach will help identify specific cellular and molecular targets for the development of new treatment strategies.

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Figure 2.1: http://publications.nigms.nih.gov/biobeat/10-07-21/10-07-21-2.jpg

Figure 2.3: http://biologiedelapeau.fr/spip.php?article3&lang=fr

Appendix A

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ORIGINAL ARTICLE

Premature Collagen Fibril Formation, Fibroblast-Mast Cell Interactions and Mast Cell-Mediated Phagocytosis of Collagen in Keloids

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ABSTRACT

Keloids are benign hyper-proliferative growths of fibrous tissue where increased fibroblast activity results in abnormal collagen deposition. Excessive inflammation is a characteristic feature of keloids, but little is known about the underlying ultrastructural features of keloids related to collagen processing, fibril and fiber formation, the interaction between fibroblasts and associated collagen fibers and mast cells. In this study, the ultrastructure of the dermis of keloid patients was evaluated using light and transmission electron microscopy techniques. Abnormal intracellular premature collagen fibril formation was observed. Phagocytosis of collagen fibrils by mast cells was a common ultrastructural feature of keloid tissue as was a close or direct association between fibroblasts and mast cells. Based on these findings and recent advances in knowledge related to collagen synthesis, fibril formation and processing, we hypothesize that keloid formation is primarily due to abnormal collagen synthesis where the consequent accumulation of collagen fibers causes increased mast cell recruitment and collagen phagocytosis. Subsequent release of mast cell-derived mediators then promotes further collagen synthesis. The observation of early formation in keloid tissue of premature insoluble collagen fibrils supports previous studies that enzymes such as procollagen C-proteinase are important early therapeutic targets.

Keywords: Collagen, fiber, fibroblast, intracellular, keloid, mast cell

Keloids are benign hyper-proliferative growths of fibrous tissue that may develop spontaneously or as a result of an abnormal healing after cutaneous injury. Keloids do not regress on its own and often continue to grow beyond the original wound margins, and the removal thereof often results in reoccurrence and difficulty in treatment [1–3]. The areas of the body most commonly associated with keloids include the chest, shoulders, upper back, back of the neck and earlobes [4]. Increased prevalence has been reported in African and Asian populations [5], whereas genetic factors, puberty, gender and pregnancy are associated with an increased probability of keloid formation [1,6,7]. Currently, there is no single effective treatment for keloids [1,8], and the identification of new therapeutic targets is an important focus of research.

Histologically, keloids are characterized by the presence of inflammatory cells such as mast cells and macrophages with a greatly expanded dermis being occupied by collagen fibers and fibroblasts [9]. Mast cells are present in the dermal and subcutaneous tissue layers of the skin and are an important cellular component of the innate immune system. Through a process of phagocytosis and reactive oxygen species production accompanied with degranulation, mast cells protect tissue against pathogens.

Increased numbers of dermal mast cells are seen in systemic fibrosis and other fibrotic disorders such as chronic graft versus host disease and lung fibrosis [10–12]. Due to the presence and increased serum IgE associated with keloids, keloid formation may likely be associated with mast cell hypersensitivity. Furthermore, patients with allergy symptoms were

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seen to have a greater number of keloids than patients without allergy, possibly linking keloids to mast cell activity [13,14].

Furthermore, in a recent study by Bakry et al., the authors investigated the possible role of hematopoietic stem cells in keloid pathogenesis by detecting these cells in keloid tissue by using immunohistochemistry. The authors used CD34 and c-KIT, antibodies specific for hematopoietic stem cell markers, and found an up regulation of these markers in keloid tissue compared with controls, indicating a possible role of hematopoietic stem cells in the formation of keloids [15].

Another important cell type involved in keloid formation is the dermal fibroblast, which is responsible for collagen production. Collagen synthesis and fibrogenesis is a complex process with distinctive intra- and extracellular phases. In keloids, overproduction of collagen only occurs at the wound site, whereas systemic sclerosis is associated with the progressive thickening and fibrosis of the skin. In this disorder, it was observed that mast cells directly activate fibroblasts *via* gap junctions resulting in increased collagen production [16]. Mast cells release several growth factors such as transforming growth factor (TGF)-β, fibroblast growth factor (FGF), cytokines as well as other factors such as angiogenesis factor and mitotic polypeptide that stimulate collagen formation by dermal fibroblasts resulting in the development of dermal fibrosis [17–19].

Likewise, after injury, mast cell infiltration is believed to promote keloid development through the stimulation of fibroblast activity. Mast cells and fibroblast may interact *via* gap junctions and release cytokines. It has been suggested that this interaction may cause fibroblasts to over produce collagen in the connective tissue of the skin [16,20].

Excessive inflammation and overproduction of collagen by fibroblasts is an accepted feature of keloids, but information related to the underlying ultrastructural features of keloids related to collagen processing, fibril and fiber formation, the interaction between fibroblasts and associated collagen fibers and mast cells is limited. Using light and transmission electron microscopy techniques, this study endeavors to provide more detailed information regarding these processes and cellular interactions in keloid tissue.

MATERIALS AND METHODS

Keloid and normal (excess skin from skin transplantation) skin samples were obtained from a plastic surgeon at Steve Biko Academic Hospital in Pretoria, South Africa. Ethical approval for the collection of skin was obtained from the research ethics committee at the University of Pretoria (protocol number 336/2014). Three control and eight keloid samples were used.

Light microscopy

Normal and keloid skin samples were fixed in 4% formaldehyde and dehydrated in a series of increasing ethanol concentrations, followed by infiltration and embedding in paraffin wax. Sections of 3–5 µm were prepared and stained with picrosirius red and viewed with polarized light to differentiate between collagen fiber types [21,22].

Transmission electron microscopy

The skin samples were fixed in 2.5% glutaraldehyde/formaldehyde in 0.075 M sodium phosphate buffer (pH = 7.4) and rinsed three times in the same buffer before it was placed in the secondary fixative, 1% osmium tetroxide solution for one hour. Following secondary fixation, the samples were rinsed again as described above. The samples were then dehydrated in 30%, 50%, 70%, 90% and three changes of 100% ethanol and were embedded in resin. Ultra-thin sections (70–100 nm) were cut with a diamond knife using an ultramicrotome. Samples were then contrasted with uranyl acetate and lead citrate, after which it was examined with a JEOL transmission electron microscope (TEM) (JEM 2100F, Tokyo, Japan).

RESULTS

To investigate the underlying ultrastructural features of keloids related to collagen processing, fibril and fiber formation, the interaction between fibroblasts and associated collagen fibers and mast cells, the general characteristics of collagen fiber arrangement in normal and keloid tissue was determined.

Figure 1(a–f) are normal (a–c) and keloid skin (d–f) viewed with polarized light, indicating the differences in fiber presence and thickness. Both type I collagen (orange red birefringence) (solid arrow in Figure 1c) and type III collagen (green–yellow birefringence) (dashed arrow in Figure 1c) were present in normal skin. Only type I collagen indicated by the presence of orange–red birefringence collagen fibers was found in keloid tissue (Figure 1f).

The ultrastructure of keloid tissue was then further evaluated using TEM. First, the general morphology and arrangement of fibers in keloid tissue was evaluated. Figure 2(a and b) are TEM micrographs of normal and keloid skin, respectively, showing a fibroblast and associated collagen fibers. The appearance of collagen in the extracellular matrix (ECM) of normal skin (Figure 2a) and keloid skin (Figure 2b)

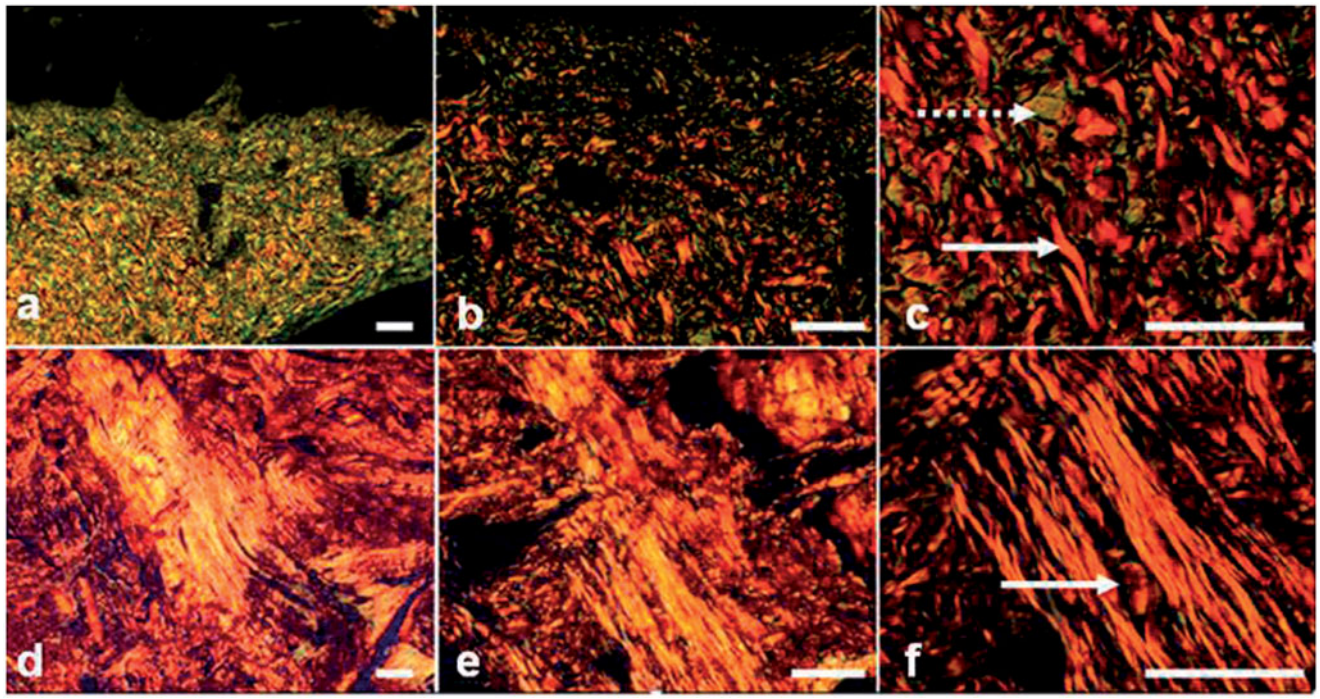

FIGURE 1. Increasing magnifications of normal Figure (a–c) and keloid Figure (d–f) tissue stained with PR and viewed with polarized light, showing differences in appearance and color distribution of the collagen fibers. White solid arrows indicate type 1 collagen fibers, and dashed arrow indicates type III collagen fibers (scale bar = 20 μm).

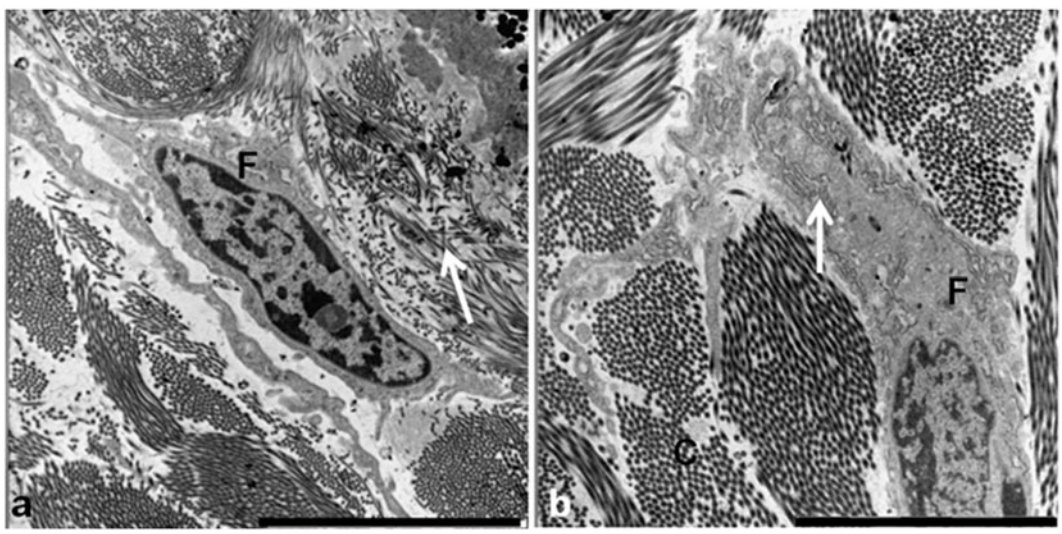

FIGURE 2. (a) TEM micrograph of normal skin indicating a fibroblast (F) with fibrillar collagen (arrow) and (b) keloid skin with thicker and non fibrillar collagen (C) and active protein synthesis due to visible rough endoplasmic reticulum in the cytoplasm (arrows) (scale bar = 5 μm).

differs. In normal skin, collagen fibers have a finer fibrillar structure, while keloid collagen fibers are larger and lack the fibrillar appearance of short individual collagen fibers. At a higher magnification, Figures 4 and 5 show the presence of intracellular collagen fibrils similar in size and shape as the fibers present in the ECM of keloid tissue.

Fibroblasts presenting with collagen in the cytoplasm also had extremely well developed rough endoplasmic reticulum (RER) with large numbers of ribosomes (Figure 3b, black arrow). This morphology is indicative of fibroblast activation and increased protein synthesis. Vesicles containing collagen are evident at a higher magnification in close proximity to Golgi apparatus (Figure 3b, white arrow).

The formation and release of collagen fibers are shown in the TEM micrographs in Figure 4(a–d). Figure 4(a) shows collagen formation and release as single (black arrow) and grouped (white arrow) fibrils. In Figure 4(b), various stages of collagen

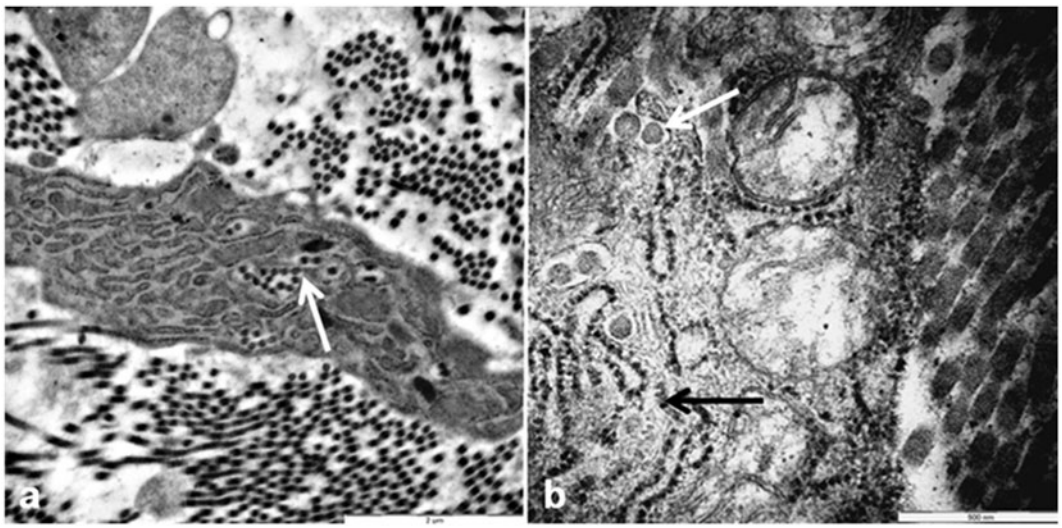

FIGURE 3. (a) TEM micrograph showing the presence of intracellular collagen fibers in a keloid fibroblast (white arrow); (b) higher magnification shows activation of fibroblasts indicated by abundant ribosomes (black arrow) as well as presence of collagen fibers in vesicles (white arrow) [scale bars (a) = 2 μm and (b) = 500 nm].

formation and maturation are indicated. These stages can be identified by the thickness of the collagen fibrils (white arrows in Figure 4b and d) and the extent of collagen fibril aggregation (Figure 4c and d). Figure 4(d) also clearly shows the fusion of vesicles containing collagen fibrils (white arrow) and the release of collagen fiber to the ECM (black arrow).

In Figure 5(a–f), the association of mast cells with fibroblasts are shown. In Figure 5(a and b), a mast cell (M) is in close proximity to a fibroblast (F) within a region of densely arranged and packed collagen fibers. In Figure 5(c–f), this association can be seen on higher magnification, where the mast cell and fibroblast are in direct cell-to-cell contact indicated by the arrows [Figure 5e (white arrow) and Figure 5f (black arrow)].

In Figure 6, a mast cell is engulfing a bundle of collagen fibers (black arrow). Another bundle of collagen fibers is seen in close proximity to the cell membrane of the mast cell. The presence of a slight indentation in the membrane (white arrow) around a collagen bundle indicates that this cell is preparing to engulf the collagen. Smaller groups of collagen fibers are present in the cytoplasm of the mast cell (thin white arrows). Degranulation of the mast cell is evident by the presence of lighter colored granules along with the normal granules as seen in this figure.

DISCUSSION

Type I collagen is found in fibrous supporting tissue, the dermis of the skin, tendons, ligaments and bone. It may be arranged loosely or densely depending on the mechanical support required by the tissue [6]. Type III collagen is found in the reticulum, and is present in highly cellular tissue such as the liver, bone marrow and lymphoid organs [6]. Normal adult skin contains about 80% type I and 20% type III collagen.

In this study, light microscopy with PR staining revealed the typical over deposition of type I collagen, which is a characteristic feature of keloid tissue (Figure 1d–f), as has been described by Shaker et al. [23]. TEM analysis revealed closely packed fibrils within densely arranged large fibers within the ECM. In contrast, in normal skin, the fibrils are more loosely arranged and fibrillar (Figure 2a and b). The presence of fully formed collagen fibrils and fiber aggregates in fibroblasts demonstrates premature fibril formation and subsequent processing. To our knowledge, this is the first study that has observed this phenomenon in keloids and the intracellular presence of insoluble collagen fibrils identified by TEM has only been described for chick embryo tendon formation [24].

Normal collagen synthesis has an intracellular and an extracellular component as shown in Figure 7. Intracellularly, collagen mRNA is synthesized in the nucleus and transported to the RER [25] where the collagen is produced. Early post-translational modification includes hydroxylation and glycosylation. The presence of the non-helical domains associated with each collagen, the propeptide domains at the C- and N-terminals [26] play an important role in the collagen triple helical formation [27] (Figure 7, step 1). The C-propeptide determines chain selection and direct chain association during intracellular assembly of the procollagen molecules [28]. The presence of these propeptide sequences ensures that the large procollagen molecule remains soluble. In the Golgi apparatus, the procollagen undergoes further post translational modifications, which involve further glycosylation steps (Figure 7, step 2). The soluble pro-collagen is then transported *via* transport vesicles to the cell

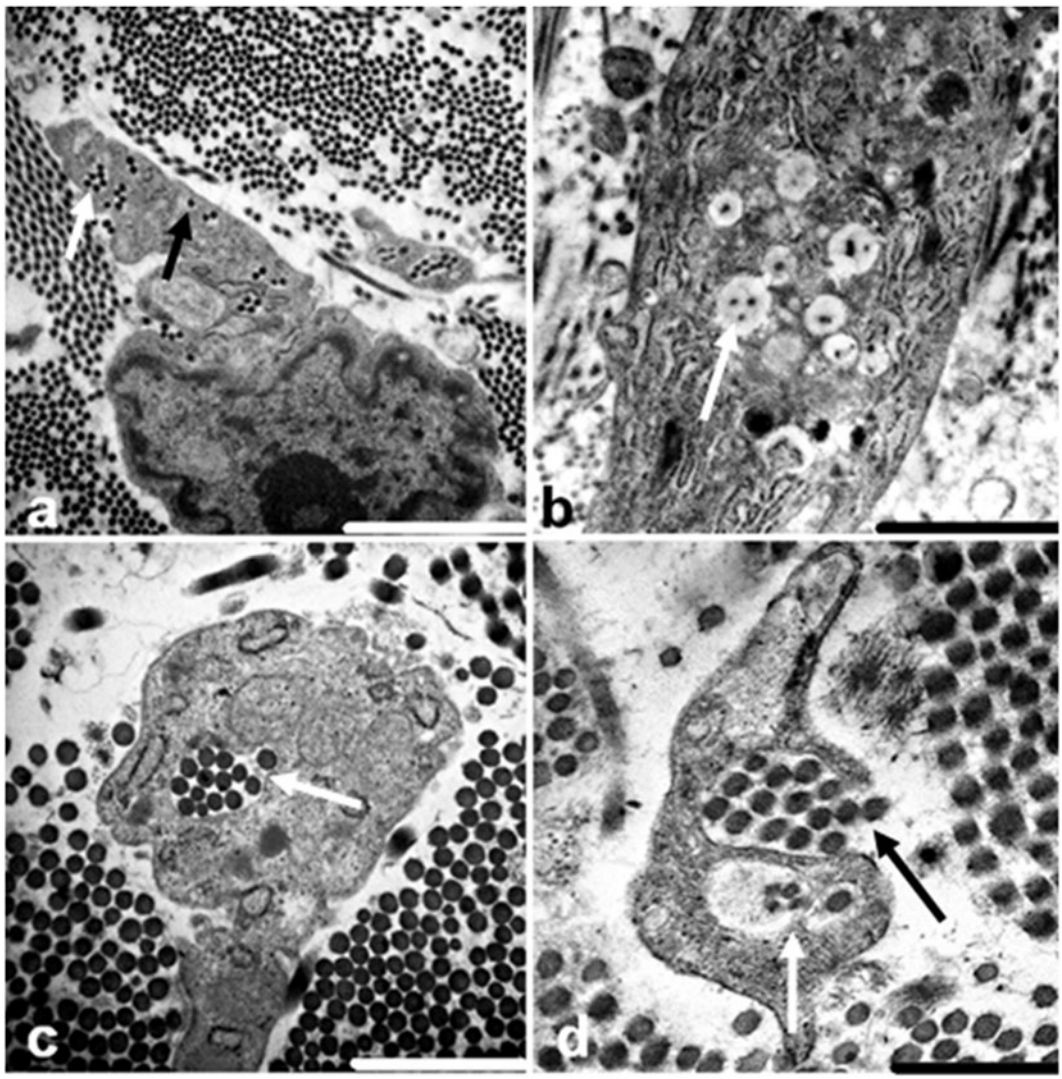

FIGURE 4. (a) Collagen formation and release as single (black arrow) and grouped (white arrow) fibrils (b) collagen formation and maturation identified by the presence of fibrils with a thinner diameter (arrow) (c) aggregated collagen fibrils forming an intracellular fiber (arrow) in fibroblast and (d) collagen vesicle fusion (white arrow) and release of collagen fiber to the extracellular matrix (black arrow) [scale bar (a and b) = 2 μm, (c) = 1 μm, (d) = 500 nm and (f) = 1 μm].

surface where it is then secreted into the extracellular space [25] (Figure 7, step 3).

Following or during secretion into the ECM (Figure 7, step 4), processing and associated proteolytic removal of the N- and C- collagen propeptides occurs, catalyzed by specific N- and C- proteinases (PCP) [29]. The C-terminal ensures the intracellular solubility, whereas the N-propeptide influences the shape and diameter of the fibril [30]. Once these sequences are removed, collagen becomes insoluble and fibrogenesis occurs [29].

The insoluble collagen which then assembles into fibrils (Figure 7, step 5), that are structurally stabilized by cross links (Figure 7, step 6) [26,31] are catalyzed by lysyl oxidase (LOX). Besides stabilization of the collagen structure, the intra- and intermolecular links increase the tensile strength of the final collagen fiber. It is believed that these peptides then re-enter the cell and regulate collagen production by a feedback mechanism [26].

The presence of insoluble collagen fibrils within the Golgi apparatus and secretory vesicles (Figures 3 and 4) implies collagen processing in keloid fibroblast is defective, and this may be related to increased PCP activity, which results in premature collagen fibril formation. As mentioned previously, the C-terminal propeptide sequence ensures the solubility of intracellular collagen. Normally, this sequence is only removed once the procollagen is secreted into the ECM (Figure 7, step 6). Premature or early removal of this sequence would result in decreased solubility and rapid crosslink formation by LOX. PCP also processes LOX to its active form; therefore as a consequence, increased levels of PCP also results in increased LOX levels and as a consequence rapid fibril formation. Broder et al. have identified that metalloproteases

α- and β-meprin are C- and N- procollagen proteinases [32]. Both meprins are expressed by human dermal fibroblasts and remove the C- and N-propeptides from collagen type I and III. Expression of α- and β-meprin is increased in keloids [32]. Both Turtle et al. and Broder et al. have identified PCP and the meprins as possible therapeutic targets for fibrosis and possibly keloid formation [32,33]. The cause of increased levels of PCP and subsequently of LOX is unknown although increased LOX levels are

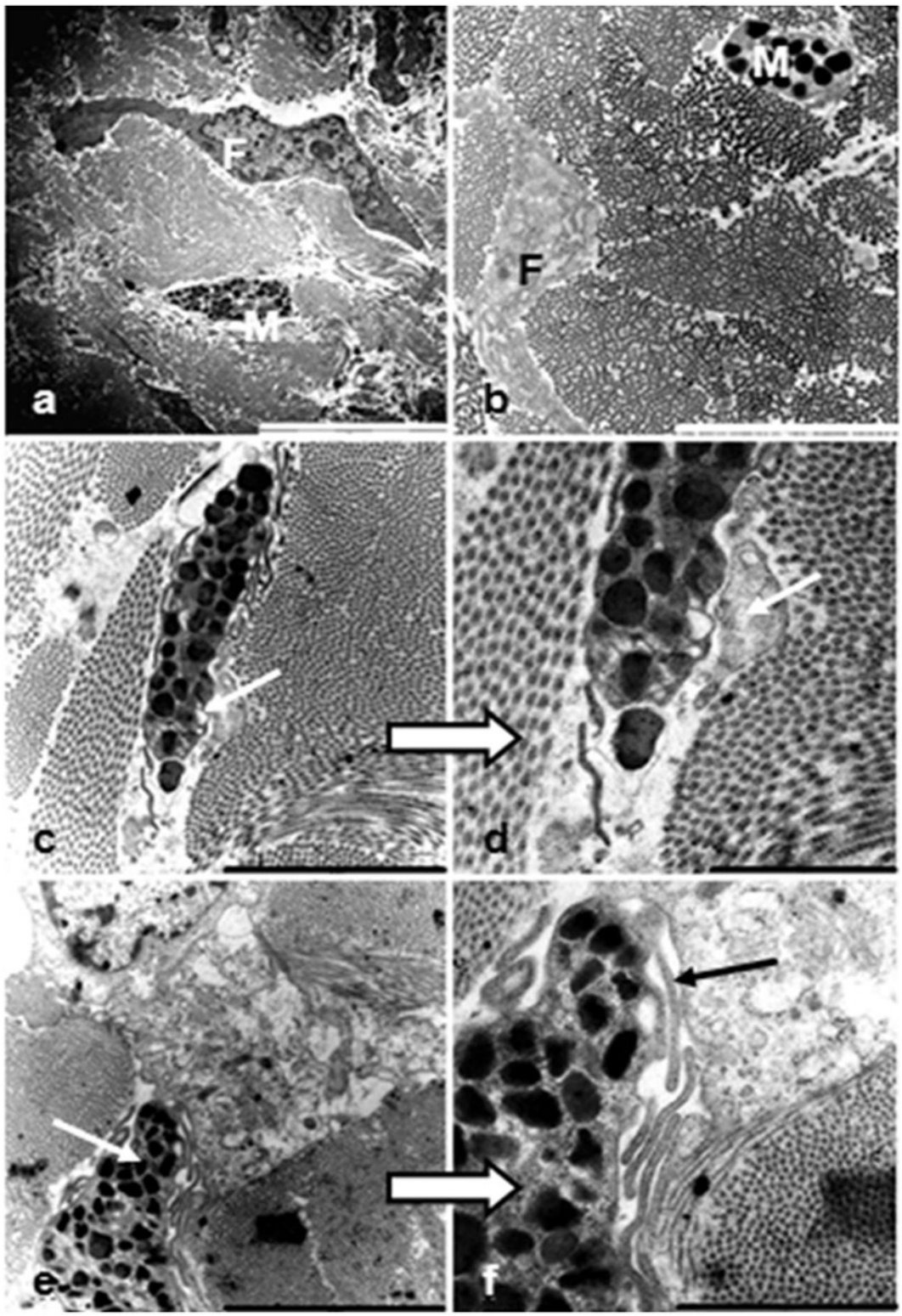

FIGURE 5. (a and b) Mast cell (M) and keloid fibroblast (F) in close proximity to each other in different keloid samples. (c and d) Mast cells and fibroblast cell-to-cell contact (white arrows), and (e and f) at higher magnification [scale bar (a) = 5 μm; (b) = 10 μm; (c) = 2 μm, (d and e) = 5 μm and (f) = 2 μm].

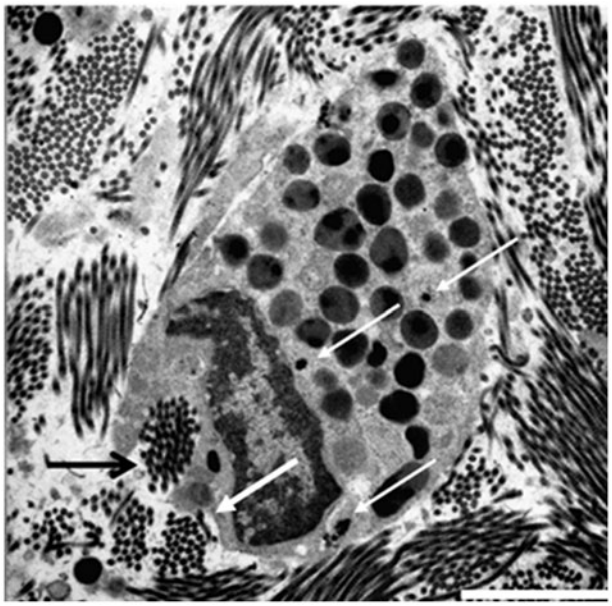

FIGURE 6. TEM micrograph of the internal structure of mast cell shows the phagocytosis of collagen (black arrow) and cytoplasmic indentation (thick white arrow) preparing for phagocytosis. Smaller groups of collagen fibers are seen (thin white arrows) (scale bar = 2 μm).

associated with hypoxia and the expression of hypoxia-inducible factor-1α, an important microenvironment factor in the development of fibrosis. A shown in Figures 2b and 5, fibroblasts, due to overcrowding by collagen fibers, can be assumed to be in a hypoxic environment, which would result in increased LOX levels possibly as a result of increased PCP.

In wound healing and fibrosis-associated conditions, the number and the distribution of mast cells is increased. Various authors reported the increased presence of mast cells in keloid tissue [13,18,19,34–38]. In this study, TEM analysis revealed the presence of mast cells in close proximity to fibroblasts (Figure 5a and b), which could indicate stimulation of fibroblasts *via* cytokines [19] and growth factors, such as basic FGF and TGF-β [18,39]. TGF-β also stimulates the production LOX. The presence of vacuoles and intact cell granules in a mast cell is characteristic of normal and non-active mast cells. Degranulation, as seen in Figure 6 (lighter stained granules), is indicative of mast cell activity and a release of histamine, heparin and other factors [40].

Direct contact between fibroblasts and mast cells through gap junctions is also a possible means of

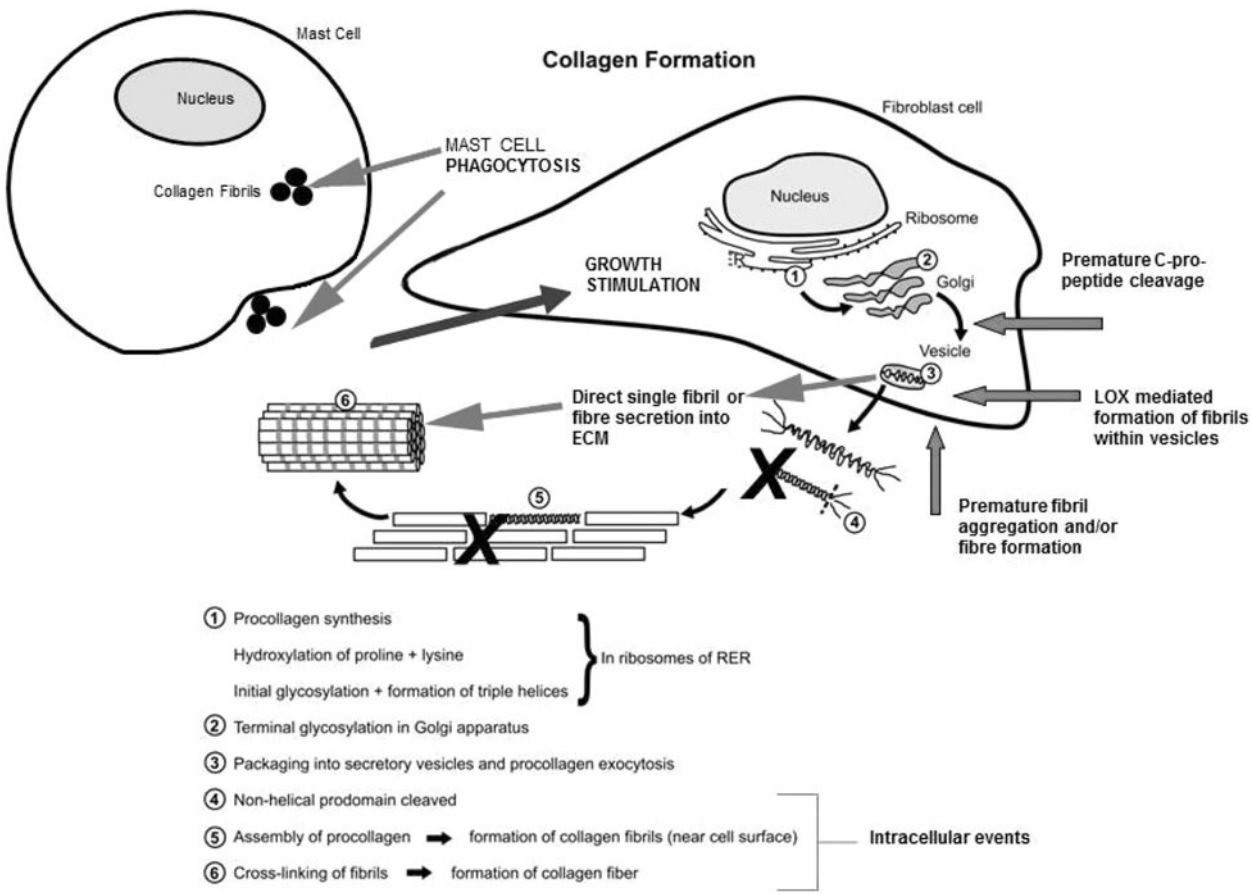

FIGURE 7. Schematic diagram of normal and abnormal keloid collagen processing.

association [16]. Moyer et al. identified the presence of gap junction intercellular communication between mast cells and fibroblasts and that this interaction only occurred in vitro when these cells were cultured in a three-dimensional environment [20]. Cell-to-cell or direct contact between mast cells and fibroblasts in vivo was observed during TEM analysis (Figure 5c–f). Associations of mast cells with fibroblasts have previously been identified as an important feature of fibrotic conditions such as systemic sclerosis, chronic graft versus host disease and lung fibrosis characterized by an overproduction of collagen by fibroblasts [12,41,42]. In pulmonary fibrosis, mast cells were observed in partial degranulation and in close proximity to lung fibroblasts [43,44]. A study on systemic sclerosis [16], cell-to-cell contact between mast cells and fibroblasts was observed, but the authors were unable to determine whether the contact was a characteristic specific to systemic sclerosis or due to mast cell activation. In this study, cell-to-cell contact between fibroblasts and mast cells was also observed, indicating, together with findings of other authors related to other types of fibrosis, this type of interaction is a common feature of fibrosis.

It is unknown whether the increased presence of mast cells is a consequence of overproduction of collagen, i.e. an excess collagen in the ECM causes increased homing and/or migration of mast cells into the dermis into areas of increased collagen production. Alternatively, the increased presence of mast cells due to inflammation and the consequent release of cytokines or growth factors results in increased fibroblast proliferation. A recent study by Willenborg et al. has shown that genetic ablation of connective tissue type mast cells fails to prevent bleomycin-induced lung fibrosis [45]. Keloid fibroblasts have been isolated from keloid tissue and grown in cell culture. Compared to normal cells, keloid fibroblasts have shown differences in growth properties and gene expression [46,47]. Furthermore, epigenetic changes have been implicated in the development of fibrosis in keloids [48]. Recently, Mastri et al. have identified Frizzled-related protein 2 (sFRP2) as an important mediator of fibrosis, as this protein enhances procollagen C proteinase activity and this leads to increased procollagen processing and deposition in cardiac fibrosis [49]. Similarly, sFRP2 could also have a similar role in keloid formation, and this protein may, as for other types of fibrosis, be a specific target of therapeutic intervention [49]. Smith et al. have reported that keloid fibroblasts growth in cell culture has more than a fivefold reduction in the expression of sFRP2 [47]. This, however, may be due to differences between an in vitro and an in vivo environment where in vivo environmental and cellular factors such as hypoxia and interactions with other cell types may alter gene expression.

Phagocytosis of collagen fibrils by mast cells (Figure 6) may indicate that mast cells in keloid tissue play an important role in maintaining tissue homeostasis and turnover. The presence of collagen fibers in mast cells of keloids has also been reported by Ehrlich et al., and these authors hypothesized that this was related to an immunological dysfunction [35]. In contrast, we believe, supported by recent scientific literature [32,33,50], that keloid formation is primary due to a defect in collagen synthesis and increased mast cell accumulation is a consequence of collagen overproduction or content in the dermis. As a consequence, in an endeavor to reduce the amount of collagen by phagocytosis, growth factors and mediators are released that further stimulate fibroblasts to produce more collagen.

CONCLUSION

In conclusion, this study clearly identifies keloid formation as a defect of procollagen synthesis and processing. Phagocytosis of collagen by mast cells indicates that accumulation of these cells may be a secondary effect to excessive collagen synthesis. In addition, the release of interleukins, mediators and growth factors may further stimulate collagen fibril formation with the imbalance toward increased synthesis. This study also identifies and confirms the findings of other studies that procollagen C-proteinase is an important therapeutic target [32,33].

DECLARATION OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of this article. The authors would like to thank the National Research Foundation for funding.

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