Immunohistochemical and Ultrastructural Evaluation of the Pathology and Aetiopathogenesis of Keloid Formation

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Shamin Bux
Student no: 863865882
University of KwaZulu-Natal, Durban

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Supervisor
PROF ANIL MADAREE

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ABSTRACT

Introduction
Keloids are formed by the excessive production of scar tissue, which extends beyond the margins of the original injury, often resulting in lesions of grotesque dimensions.
Keloids present a major dilemma to surgeons because of the high recurrence rate with recurrent growth often larger than the original keloid. The high recurrence rate and the poor response of keloids to therapy present a great challenge to surgeons. The numerous therapeutic regimens demonstrate that to date there is no single therapy that is absolutely successful. Therefore, it is necessary to comprehensively establish the pathology of keloids and to determine the aetiopathogenesis of the lesion in order to eventually provide unfailing specific effective treatment and to better understand the mechanisms regulating fibrosis in various fibroproliferative diseases.

Aim
To evaluate the pathology and aetiopathogenesis of keloid formation.

Methods
The research protocol for the study was approved by the Nelson R Mandela Faculty of Medicine Ethics Committee. Informed consent was obtained before the biopsies were taken. Keloid and non-lesional skin biopsies were obtained from thirty two patients who had multiple lesions in various locations, bringing the total number of keloids and apparently normal skin biopsies processed and examined to fifty eight. The biopsied specimens were processed for paraffin wax embedment and routine haematoxylin and eosin, differential and immunocytochemical staining. Sections were scrupulously examined using the Olympus BH-2 microscope; features pertinent to the study were photographed with the Olympus DP 10 microscope digital camera system. The stored images were studied, using the Camedia graphics processing programme.

Results
The results of the study showed that keloids comprise many distinct regions categorized as: the zone of hyalinising collagen bundles, fine fibrous areas, areas of inflammation, zone of dense regular connective tissue, nodular fibrous area and area of angiogenesis. Fibroblastic phenotypes present ranged from spindle, fibrohistiocytic, epitheloid, elongated flattened condensed fibroblastic cells to few wavy, fuzzy, polygonal and atrophic cell types. Immunocytochemically these cells were vimentin-positive and actin- and desmin-negative. Few myofibroblastic phenotypes were also identified and these were vimentin- and alpha smooth muscle actin-positive and desmin-negative. The fibroblastic and myofibroblastic phenotypes were in proliferative or degenerative stages and pathological features exhibited were the presence of vesicular, degenerate or calcified nuclei; nuclear and plasma membrane damage; cytoplasmic and nucleoplasmic clearing; atrophy, pyknosis and swelling.
Severe, moderate to mild paravascular inflammation was observed around the microvessels of the sub-papillary plexus and within the keloid. There was compression and occlusion of small blood vessels, coagulation necrosis and dissolution of mural cells of small blood vessels and small peripheral nerves. Also present in keloids were oedematous areas, disorganised and hyalinised connective tissue fibres and increased numbers of degranulated and degranulating mast cells. Elastic fibres in keloids were minimal or absent whereas at the border of keloids there was an increase.

Discussion
Degenerate, occluded and compressed microvessels were a widespread pathological feature in keloids. This resulted in impaired vascular supply to each of the keloid regions which impacted directly on the pathology of keloids where degeneration and necrosis, manifesting the lack of nutrients and oxygen to tissue, were found throughout the keloid. The vascular supply was

impaired because of the chronic inflammatory destruction of the microvessels and the elevated stress within keloids. Factors contributing to increased intrinsic stress were: 1) the lack of elastic fibres in keloids which decreased the elastic limit, leading to effects of excessive deformational force which were compression and stiffening of tissue; 2) the high tension skin covering keloid prone areas had low stretch and a low elastic modulus; 3). protruding hard connective tissue such as bony prominences or cartilage into the dermis of keloid prone skin; 4) contractile forces exerted by wound healing fibroblastic cells; and 5) external forces. Compression and occlusion of blood vessels induced ischaemic and reperfusion tissue injury. During the reperfusion phase blood rich in growth factors returned to tissue stimulating tissue growth. Tissue growth was also promoted by elevated internal stress which stimulated increasing levels of gene expression, collagen synthesis and mitotic activity. All these growth promoting effects resulted in keloid formation.

DECLARATION

I, Shamin Bux declare that:
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S. Bux
Department of Physiology,
School of Laboratory Medicine and Medical sciences
Nelson R Mandela School of Medicine
University of KwaZulu-Natal South Africa
7 February 2013

TO UVIR MADAREE

A PERFECT, DEVOTED, RESPONSIBLE, COMMITTED, LOVING AND TRULY GREAT SON;

A DOTING, PLAYFUL, PROTECTIVE, HUMOROUS, SMILING, CHEERFUL AND ANGELIC BROTHER;

A GENTLE, ADORING AND CARING GRANDSON;

A WARM, KIND, CONSIDERATE, HELPFUL AND HAPPY NEPHEW;

AN AWESOME, AMAZING, AFFECTIONATE, FAITHFUL AND GENEROUS COUSIN;

AN OBEDIENT, RESPECTFUL, DUTIFUL, SCHOLARLY AND VERY SUCCESSFUL STUDENT;

A LOYAL, THOUGHTFUL AND TRUSTWORTHY FRIEND;

A SELFLESS, SYMPATHETIC, COMPASSIONATE AND GRACIOUS GIVER TO THE POOR AND NEEDY;

A HELPER, SUPPORTER AND CAREGIVER OF THE AGED AND……..

YOUR LOVE FOR ALL WAS UNCONDITIONAL AND FULL OF DEPTH, CONSISTENT WITH GODLY LOVE

"THE LORD DIRECTS THE STEPS OF THE GODLY. HE DELIGHTS IN EVERY DETAIL OF THEIR LIVES"

YOU IMPRESSED GOD SO GREATLY BY BEING YOU, THAT HE WANTED YOU TO BE AT HIS SIDE FOREVER

FROM A DISTANCE WE SEND YOU, OUR SHINING STAR, A BIG HUG AND TONS OF LOVE

To Mikael, Akiel, Nikiel and Sohan

Sons, you will outgrow our laps, but never our hearts

Our prayer is that you will grow into the men you are meant to be: wonderful human beings, kind hearted gentlemen, loving and caring individuals who

- do the right thing even if it is the hard thing,
- seek God first in all that you do,
- stand firm in the face of temptation

We wish you a life full of love, peace, joy, happiness, confidence, success, balance, and contentment.

Always accept who you are and be proud of yourself. Do not expect perfection at all times, allow yourself to be human. Peace will not be far behind.

We wish you joy. It can be found in the simplest things: the wind blowing through the trees, a child's smile, dogs playing. If you learn how to look, you will find it.

We wish you happiness. Focus on what you enjoy. Listen to your heart. Make choices that will result in positives, not in negatives. Happiness will follow.

We wish you confidence. Know that are a capable, responsible person. Do not be afraid to make mistakes, this is how you will learn. You will build confidence.

We wish you success. Work hard, even when it seems it's not making a difference. We promise you it is and success will follow.

We wish you balance. Know what is important, and put these at the top of your list. Remember to respect yourself and others. Balance the personal and professional. This is a must.

We wish you contentment. Know that where you are is exactly where you are supposed to be. Life changes so fast. There is nothing wrong with striving for better, but don't forget to enjoy the now. Look around and be proud of your accomplishments.

We wish all of this and so much more for you. You are our heart, our life, our greatest accomplishments.

We love you.

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ACRONYMS AND ABBREVIATIONS

αSMA	Alpha smooth muscle actin
AgNORs	Silver-stained nucleolar organizer regions
CTGF	Connective tissue growth factor
Cu	Copper
CXGR2	CXC chemokine receptor two
DDR1	Discoidin domain receptor 1
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
G6PDH	Glucose-6-phosphate dehydrogenase
H_2O_2	Hydrogen peroxide
HCB	Hyalinising collagen bundles
HSP47	Heat shock protein 47
Hsp70-1A	Heat shock 70 kDa protein 1A
Hsp70-1L	Heat shock 70 kDa protein 1L
Hsp70-9B	Heat shock 70 kDa protein 9B
IFN- α	Interferon-alpha
IFN-β	Interferon- beta
IFN-γ	Interferon-gamma
IgA	Immunoglobulin A
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor-1 receptor
IGFBP	Insulin-like growth factor binding protein
IGF-I	Insulin-like growth factor -1
IGF-IR	Insulin-like growth factor-1 receptor
IgG	Immunoglobulin G
IgM	Immunoglobulin M

IL-1	Interleukin-1
IL1R	Interleukin 1 receptor
IL-6	Interleukin-6
IL-6aR	Interleukin-6 a receptor protein
IL-7	Interleukin-7
IL-8	Interleukin-8
LFA-1+	Lymphocyte function antigen-1+
MAPK	Mitogen-activated protein kinases
MHC	Major histocompatibility complex
MIF	Macrophage inhibiting factor
MME	Membrane metalloendopeptidase
MMP	Matrix metalloproteinase
MMP-2	Matrix metalloproteinase-2
MMP-3	Matrix metalloproteinase-3
MMP-9	Matrix metalloproteinase-9
Mn	Manganese
mRNA	Messenger RNA
MSH	Melanocyte stimulating hormone
NF1B	Nucleus factor of transcription1B
NGF-β	Nerve growth factor β
PAI-1	Plasminogen activator inhibitor type-1
PBMC	Peripheral blood mononuclear cell
PDGF	Platelet derived growth factor
RNOS	Reactive oxygen/nitrogen species
ROS	Reactive oxygen species
Se	Selenium
TGF-β	Transforming growth factor-beta
TGF-β1	Transforming growth factor-beta-1
TGF-β2	Transforming growth factor-beta-2

TNC	Tenascin C
TNF-α	Tumour necrosis factor-alpha
TNF-β	Tumour necrosis factor-beta
VEGF	Vascular endothelial growth factor
Zn	Zinc

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CHAPTER 1

INTRODUCTION

1.1 Introductory note

The lack of understanding of the pathogenesis of keloids and related disorders involving an accumulation of extracellular matrix components, leading to dermal fibrotic lesions, has hampered progress towards finding effective therapy for their prevention and treatment. Keloids represent a prototype of excessive scar tissue formation during an abnormal wound healing process. Keloids present a major dilemma to dermatologists and surgeons because of the high recurrence rate with recurrent growth often larger than the original keloid. Recurrent rates have been estimated to be between 45-100 % (1), (2). A reduction in recurrence rate resulted with the use of precise surgical techniques and adjuvant therapies (3). However, to date, there is no guaranteed effective treatment, despite the large number of treatment regimens available. To a great extent, this is due to the lack of understanding of the pathogenesis of these dermal fibrotic disorders.

1.2 Definition

Keloids can be defined as dermal fibroproliferative lesions that develop as a consequence of a dysregulated response to cutaneous wounding, resulting in disfiguring scars that often progress to grotesque dimensions. Keloid formation is associated with the involvement of trauma, surgery or other forms of wounding; however there are few reports on the occurrence of spontaneous keloids in the absence of injury (4), (5). In patients with keloids, after the initial injury, the wound heals, apparently normally. Three months or more, thereafter,

keloids start to form and continue growing to variable dimensions. The initial stages of the wound healing process appear to progress well, suggesting that the coagulation, inflammatory and epithelialisation stages of the wound healing process proceeded normally and that the abnormalities leading to keloid formation occur during the granulation and remodeling phases of wound healing.

1.3 Normal wound healing

It is important to understand the sequence of normal wound healing stages before assessing the pathogenesis of keloids. In the normal wound healing process the main stages comprise the coagulation, inflammatory, matrix formation, angiogenesis, fibroplasia, epithelialisation and remodelling stages. The granulation tissue stage encompasses the phases of angiogenesis and fibroplasia. In the first 24 hours of the normal wound healing process, polymorphonuclear monocytes migrate to the wound site where they digest damaged tissue and bacteria, fragment in the next 48 hours, releasing factors chemotactic for macrophages, which continue to accumulate over a 72-hour period. Fibroblasts start accumulating from about the 4th day, and this coincides with an accumulation of ground substance commencing the matrix production stage. Initially the matrix is rich in hyaluronate which, appears to be essential for the subsequent production of collagen. The hyaluronate is progressively degraded and the young fibroblasts proliferate and vigorously begin collagen production and proteoglycan synthesis.

1.3.1 Granulation phase of normal wound healing

During the granulation phase there is proliferation of fibroblasts, capillaries (angiogenesis) and epithelial cells. Macrophages and their production of interleukin-I (IL-I) play an important part in stimulating these processes by increasing intracellular growth factors (PDGF and somatomedins) and by promoting protein synthesis. Endothelial buds from small blood vessels

surrounding the wound vascularise the healing tissue. By the end of the 1st week and extending into the 2nd week there is extensive collagen production by granulation tissue fibroblasts; this provides the wound matrix with tensile strength. The young dividing fibroblastic cells of granulation tissue acquire properties of increased motility and contractility and transform into myofibroblasts. At first granulation tissue contains rapidly increasing amounts of type III collagen (increased 4 to 5 times). Wound contraction occurs as a result of the contractile function of myofibroblasts in the granulation tissue. As the tissue begins to shrink type I collagen becomes the abundant type; this change coincides with the disappearance of myofibroblasts and their replacement with fibrocytes. This takes place 4 weeks after the wound was initiated. Myofibroblasts undergo apoptosis and thereafter scar tissue formation takes place.

1.3.2 Remodelling phase and scar maturation in normal wound healing

After the deposition of the early extracellular wound matrix, the matrix starts to remodel its collagen framework and proteoglycan filler substance to provide skin and scar tissue with tensile strength. During this process, hyaluronan is replaced by the sulphated proteoglycans decorin, biglycan, and versican, which contain dermatan-sulphate and chondroitine-sulphate side chains (6). These sulphated proteoglycans contribute to tissue resilience (7) and are produced by mature scar fibroblasts (8). Chondroitine-sulphate side chains are released in the earlier phases of wound healing followed by the release of dermatan-sulphate side chains contained in decorin first and by biglycan later (9, 10).

The remodeling process could continue for months or years after injury, during which phase scars become progressively softer and flatter and the collagen fibers become thicker, denser and more orderly arranged. The number of capillaries, small blood vessels and fibroblasts present in older scars is greatly reduced. With remodeling of scar tissue there is a gain in wound tensile strength. The strength of an early wound is based on interactions between fibrin,

fibronectin, collagen, glycosaminoglycans, and fibroblasts (11) whereas the gradual gain during the later stages is achieved by new collagen deposition, collagen remodeling, and the formation of larger collagen bundles with altered intermolecular cross-links (7)**.**

1.4 Hypertrophic scars in comparison with keloids

Hypertrophic scars are raised collagenous pathological scar tissue growths occurring in certain individuals, when the biochemical process of wound repair is abnormal and excessive scar tissue production is stimulated. In assessing the clinical characteristics, hypertrophic scars are similar to keloids, appearing as raised masses of excessive scar tissue, which are often painful, pruritic and occasionally form strictures. Hypertrophic scars usually appear within 4 weeks after trauma, whereas the onset of keloids is generally at 3 months and can be delayed up to several years after trauma (12), (13). They differ from keloids, in that they do not grow beyond the confines of the original injury as keloids do, they grow rapidly within the first 6 months and then gradually regress to flat scars after 12-18 months but keloids persevere with no signs of regression. Keloids have a higher tendency to recur following excision (45-100%), whereas in hypertrophic scars, recurrence after excision is uncommon (10%). Regarding differences in general structure, keloids are characterized by the presence of haphazardly arranged thick, hyalinized collagen bundles with relatively few fibroblasts whereas in hypertrophic scars collagen fibres are orientated parallel to the long axis of the epidermis or they form nodular structures together with large numbers of fibroblasts (14) .

Hypertrophic scars and keloids are unique to humans and they do not develop in animals; even attempts to grow these tumours in animal models have, by and large, been ineffective (15), (16). This exclusivity is partially explained by the predilection for keloids to preferentially occur in areas with a high concentration of sebaceous glands and humans are the only mammals with true sebaceous glands (17).

1.5 Immunological differences and similarities between hypertrophic scars and keloids

Some immunological differences and similarities found between hypertrophic scars and keloids are as follows:

- Keloid tissue showed positivity for alpha smooth muscle actin (α-SMA) expressing myofibroblasts in 33.3% of keloid scars, while the collagen nodules of hypertrophic scars contained no α-SMA expressing myofibroblasts, although, they were cellular
- The presence of antinuclear antibodies from different immunoglobulin classes against fibroblasts, epithelial, and endothelial cells, in keloids and the absence of these antibodies in hypertrophic scars (18)
- The immune-cell infiltrate in both hypertrophic scars and keloids was composed of CD3+, CD45RO+, CD4+, human lymphocyte antigen (HLA)-DR+, and lymphocyte function antigen (LFA)-1+ T lymphocytes strictly associated with CD1a+/CD36+, HLA-DR+ and intercellular adhesion molecule (ICAM)-1+ dendritic cells (19). Also, this study supported the involvement of a cell-mediated, major histocompatibility complex (MHC)-class II-restricted immune response in the pathogenesis of keloids and hypertrophic scars (19)
- The pronounced staining for PCNA in keloid fibroblasts was greater than that displayed by hypertrophic scar or normal dermal fibroblasts (20). Also, these authors reported a significantly higher mean density of fibroblasts in both hypertrophic scars and keloids when compared with their normal dermis
- The CXC chemokine, MGSA/GRO alpha and its receptor CXCR2 were found to be present in some myofibroblasts and lymphocytes in the nodular areas of

keloids, while strong immunopositivity for CXCR2 was observed in endothelial cells in keloids. No staining for these antigens was found in hypertrophic scars, normal dermis or cultured keloid fibroblasts (21, 22)

- Teofoli et al reported that pathologic scarring in keloids and hypertrophic scars results from enhanced proliferation of fibroblasts (shown by Ki67 positive immunostaining) and an alteration of the apoptotic programme triggered by protooncogene c-jun and c-fos overexpression (23)
- Hypertrophic scar fibroblasts have an enhanced ability to contract the fibrin matrix gel while keloid fibroblasts have an enhanced ability to contract the three-dimensional collagen-I lattice (24)
- Studies ascertaining the levels of degrading proteases in excessive scar tissue showed that in keloid and hypertrophic scars matrix metalloproteinase-9 (MMP-9) was very low or undetectable, while matrix metalloproteinase-2 (MMP-2) was high when compared with normal skin (25). Matrix metalloproteinase-9 is involved in early tissue repair and MMP-2 is involved in the prolonged tissue remodeling stage. This indicates that anomalies of the remodeling stage of the wound healing process are implicated in the pathogenesis of keloids and hypertrophic scars

1.6 Gross characteristics and prevalence of keloids

Keloids present the highest incidence in the second decade, commonly occuring in the 10 to 30-year age group, and show the same prevalence in male and female sexes (13), (26), (27). These lesions occur in individuals of all races, but have a 15-fold propensity to form in patients with darker skin (13), (28). African, Negroid, Asian and Spanish people have a 4.5 to

16 percent greater predisposition to develop keloids than fair-skinned individuals (13), (29). Other racial groups reported to be at greater risk of forming keloids are African Americans and Asians in the United States when compared with white Americans (30), (31) (32). The frequency of occurrence in the Afro-American population was reported to be 1/30 while for the whole American population the incidence was 1/625 (33). Using random sampling methods, it has been estimated that the prevalence of keloid formation ranges from 6% to 16% in African populations (34). The association of higher melanin content of skin with keloid formation is supported by the finding that the occurrence of keloids had not been reported in albinos of any race group (34). Albinism is an inherited condition present at birth, characterized by a lack of the dark pigment melanin, which helps protect the skin from the sun's ultraviolet radiation.

1.7 Keloid-prone vs. keloid-protected areas

There is a predilection for keloids to form on the upper torso as single or multiple lesions. The main areas where these pathological scars form are the head (earlobes and cheeks), neck, upper arms and presternal, back and deltoid areas. The reason for this susceptibility of skin in these regions is not known. The weak expression of the myofibroblast phenotype in keloid-protected areas and the enhanced apoptosis of cultured palmar fibroblasts (also a keloid protected area), suggest that keloid formation in keloid-prone areas is promoted by the presence of a larger number of myofibroblasts and the diminished levels of apoptosis (35).

Additional work comparing keloid-proclivity skin with that of keloid-protected skin should provide more evidence in this regard. Some areas where keloids were rarely or never located are the eyelids, genitalia, palms, soles, cornea and mucous membranes (36), (37), (38), (39), (29), (40), (28). In some of these structures such as the eyelids and genitalia the “tension” hypothesis might be applicable. According to the “tension” hypothesis, keloid formation often occurs after

high tension skin injury, e.g. above big joints and in the chest area. Also, this hypothesis explains why keloids seldom develop in skin with minimal tension as in old people, eyelids and genitalia, but it fails to explain why the ear lobes, where skin tension is minimal, are prone to keloid formation (41).

The recurrence rate of lesions differed at the various anatomical sites of keloid formation: in the thorax it was as high as 49%, whereas in the head and neck areas the rate was the lowest at 2% (42). More recently, it was found that the recurrence rate for surgical excision and post-operative irradiation was 70% at a mean follow up of 19 months (2). The type of injury that initiated keloid formation also influences the prognosis of recurrence: e.g., post-burn keloids showed a poorer success rate for complete abolition, without recurrence after removal than those forming after surgery or mechanical trauma (42).

1.8 Some abnormal extracellular matrix quantities and sites of location in keloids

In patients with keloids, after the initial injury, the wound heals, apparently normally. Three months or more, thereafter, keloids start to form and continue growing to variable dimensions. As the initial stages of the wound healing process appear to have progressed well in keloids, it is assumed that the coagulation, inflammatory and epithelialisation stages proceeded normally. Granulation and remodelling stage abnormalities are indicated in keloids by persisting increased amounts of type III collagen whereas during normal wound repair, collagen type III decreases from 60 percent at 1 week after wounding, to 28 percent in the mature scar (43). Quantitative studies on the proteoglycan content of keloids showed that chondroitine-sulphate levels remain high (14-fold); this is associated with the persisting high amounts of versican, which contains 12 to 15 chondroitine-sulphate side chains per molecule. It is mainly found in the nodular areas of excessive scars where proliferating fibroblasts that overproduce collagen are

present (44-46). The high proteoglycan content was associated with perivascular T-lymphocyte infiltrates (47).

The amount of biglycan remains elevated in hyperplastic scar tissue whereas the amount of decorin becomes reduced compared with normal dermis and mature normal scars (48-50). The small amount of decorin is mainly found in the deep dermis and a narrow zone under the epidermis and decorin is reduced or absent in the nodular areas of excessive scars. In normal scars on the other hand, decorin shows intense staining throughout the whole dermis (51, 52). In contrast to decorin, biglycan expression is markedly up regulated in excessive scar tissue, especially in the nodular areas (53). In normal uninjured skin, it is found focally in the epidermis and in a narrow continuous band beneath the basal membrane of the epidermis (54).

1.9 Ultrastructure of keloids

Histological features generally exhibited by keloids were: (a) flattening of rete ridges and dermal papillae, (b) Hyperkeratosis, hypergranulosis and spongiosis of the epidermis, (c) basal cell vacuolar change in the epidermis (d) scarring of papillary dermis, (d) absence of scar tissue in the papillary dermis, (e) presence of hyalinised collagen bundles, (f) absence of conspicuous vertically oriented blood vessels, (g) presence of disordered arrangements of fibrous tissue fascicles, (h) presence of the growing edge as a band of lesional tissue below the apparently normal epidermis and papillary dermis, (i) telangiectasia in the papillary dermis, (j) layer of horizontal cellular connective tissue in the upper reticular dermis, (k) conspicuous layers of bands of dense regular connective tissue, (l) aggregating blood vessels below the epidermis, and (m) perivascular moderate chronic inflammatory infiltrate, and (n) scattered mast cells (55-57).

Ultrastructurally, fibroblasts in keloid showed a well-developed rough endoplasmic reticulum and the main protein produced was observed as banded collagen fibrils which were organized into thick fibers that were separated from the membrane surface of the fibroblast by a diffuse amorphous substance surrounding the surface of the cell (58). The chemical composition of this pericellular structure is unidentified and it was reported that in tight skin mice, the pericellular material vanished 3 weeks post injury (59). In keloids, this pericellular material persisted for as long as 2 years, but was not found in hypertrophic scars or normal dermis (58). Other ultrastructural features of keloid fibroblasts are described in the section 1.10 below under "immunohistological studies on keloid tissue".

1.10 Immunohistological studies on keloid tissue

1.10.1 α-SMA expressing myofibroblasts

Generally, immunohistochemical studies on keloidal tissue showed variable α-SMA positivity in fibroblastic cells fluctuating from absence of α-SMA staining to 45% cases showing positivity, and elevated levels with 70% positivity to most cases in another study. These α-SMA positive fibroblasts were identified as myofibroblasts and were differentiated from smooth muscle cells by the absence of desmin immunostaining (58).

Myofibroblasts, differ from normal fibroblasts by their characteristic cytoplasmic bundles of microfilaments and other ultrastructural features including nuclear indentations and cell-to-cell or cell-to-stromal connections. Enhanced synthetic and secretory function in myofibroblasts was indicated by extensive rough endoplasmic reticulum, conspicuous Golgi bodies and the presence of intracellular collagen within invaginating tubular membranes (60). Many myofibroblasts exhibited a close relationship with mast cells through filopodial processes and some mast cells were degranulated and their released granules were scattered

in oedematous and granular extracellular matrix (60). Myofibroblasts in the vicinity contained dilated rough endoplasmic reticulum and many vacuoles. The close relationship and interaction between mast cells and myofibroblasts prompted researchers to embrace mast cells and their products as important participants in the aetiopathogenesis of keloid formation (60).

Myofibroblasts appear briefly in granulation tissue during normal wound healing, but appear to be present permanently in keloids and other fibrotic lesions. Thus myofibroblasts are considered to play an important role in pathogenesis of keloid. Dispersed diffuse S-100 immunostaining was reported in keloids, indicating the presence of scattered Langerhans cells and regenerating nerve fibres (58).

The variable expression of α-SMA reported in keloid fibroblasts indicates that this feature is not reliable in distinguishing different types of scars and that keloid fibroblasts do not all originate from the same clone. Morphological and biochemical studies by Knapp et al. revealed many phenotypic variances of keloid lesional cells (61).

1.10.2 Altered response of keloid fibroblasts to chemokines

Keloid fibroblasts responded differently to various chemokines and enzymes when compared with normal dermal fibroblasts. Macrophage inhibiting factor (MIF) activated cPLA2 to a greater extent in keloid than in normal fibroblasts, resulting in an increased supply of arachidonic acid. Macrophage inhibiting factor promotes fibroblast proliferation and fibrosis. This process is also promoted by decreased activation of another enzyme, COX-2, in keloid fibroblasts, resulting in decreased products of COX-2 enzyme, one of which is prostaglandin PGE2, which has an inhibitory effect on cell proliferation and collagen synthesis. In addition to diminished levels, PGE2 activity is further reduced by the decreased expression of PGE2 receptors on the plasma membrane of keloid fibroblasts (62). Decreased PGE2 activity

abrogates its inhibitory effects on cell proliferation and collagen synthesis in keloid fibroblasts, promoting lesional growth.

1.10.3 Difference in activity of keloid fibroblasts based on location

The activity of keloid fibroblasts also differed according to central or peripheral sites of location. In the centre of the keloid, mesenchymal tissue and scattered fibroblasts prevail, while in the peripheral area the fibroblasts were dynamic, rapidly proliferating and had a reduced apoptotic rate which was half that of normal fibroblasts. Immunohistochemical studies showed increased concentrations of inhibitors for proteolytic enzymes in the apoptotic pathway in the peripheral area of keloid in comparison to the central region. Proteolytic enzymes have a catalytic function in proteolysis, breaking long chain molecules of proteins into smaller fragments. Inhibition of proteolytic enzymes, therefore, facilitates accumulation of scar tissue and promotion of keloidal growth. Contrary to growth promoting effects in the peripheral area, the cell death rate was elevated in the central keloid area by fibroblasts which showed increased expression of ADAMs gene for apoptosis (63, 64).

1.11Epithelial/mesenchymal interactions play a role in the pathogenesis of keloid formation

In keloids the main perpetrator responsible for the promotion of lesional growth by overproduction of collagen and other extracellular matrix components, has conventionally been the keloid fibroblast. More recent research, however, has shown that epithelial–mesenchymal interactions play an important role in influencing keloid fibroblast division and activity through paracrine effects of their secretory products. It has been established that keloid keratinocytes have the following effects on both normal and keloid fibroblasts:

- They modulate growth and proliferation of both normal and keloid fibroblasts

- They stimulate collagen production by both normal and keloid fibroblasts
- They can influence normal fibroblasts to overproduce collagen comparable to the action of keloid fibroblasts
- They use the cytokine insulin-like growth factor to attain their mitogenic effect on fibroblasts
- In addition to stimulating enhanced proliferation, the keratinocytes overlying the keloid lesion also decrease the rate of apoptosis in the underlying fibroblasts (65).

Contradictory to the above stimulatory action of keratinocytes on the growth and proliferation of normal and keloid fibroblasts, previous investigations showed that the absence of an epidermis, thus the absence of keratinocytes, was associated with excessive ECM production by dermal fibroblasts (66). These researchers also reported that the normal epidermis may yield signals that suppress scar formation.

1.11.1 Genomics of keloid keratinocytes

Some alterations at the genomic level found in keloid keratinocytes in vitro, were a decreased expression of cell adhesion genes, intermediate filament genes and a set of transcription factor genes, all of which are crucial for normal epidermal morphology. Genes exhibiting elevated expression were genes associated with wound healing, cellular motility, and blood vessel development. VEGF was also immunolocalized in the epidermis of keloids and normal skin and was intensely expressed by keratinocytes during wound healing and in vitro (66). The immunolocalization of VEGF in the nucleus of normal skin keratinocytes suggested nuclear sequestration prior to routing into the cytoplasm and next into the ECM. This was demonstrated by enhanced positive VEGF staining in the extracellular space in keloids (65).

Other characteristics displayed by keloid keratinocytes were higher migration rates than normal keratinocytes in vitro and reduced expression of desmosomal proteins in vivo.

These abnormalities suggest that keloid keratinocytes have a more influential role in keloid scarring than previously recognized (67).

However, the apparently normal appearance of the keloid papillary dermis which lies immediately below the epidermis, favours the view that the epidermis does not play a role in promoting keloid formation. A drawback of studies showing that epithelial–mesenchymal interactions influence keloid fibroblast division and activity was that they were in vitro studies which may exclude important anti-proliferation and other factors released sequentially in the in vivo wound healing process (68).

1.12 Immunohematological factors implicated in the pathogenesis of keloids

Immunohistochemical investigations on the immune-cell infiltrate in the exaggerated inflammatory response of keloids showed large numbers of activated immune cells, consisting of CD3+, CD4+, CD 45R0. The significantly higher $CD4^+$:$CD8^+$ (Th:Ts) ratios and increased levels of complement factors C3 and C4 , reported to be present, were proposed to contribute to keloid scarring. Other immunohaematological factors involved were the presence of particular human leukocyte antigen subtypes, a cell-mediated MHC-class II-restricted immune response and increased numbers of mast cells in keloid tissue. Also, an association between human leukocyte antigen B14, 21, BW35, DR5, and DQW3 and group A blood type was found in patients with keloids (69). Further, it was found that patients who develop keloids have a disproportionately high incidence of allergic conditions and elevated levels of serum immunoglobulin E. Multiple reports have found patterns in the serum complement, immunoglobulin G (IgG), and immunoglobulin M (IgM) levels in patients with keloids, suggesting a systemic immune state genetically predisposed to keloid formation (70). In keloids deposits of IgG were demonstrated to be present among collagen fibres and the presence of immunoglobulin A (IgA) and IgM was also observed in the lesion (71-73).

The presence of antinuclear antibodies from different immunoglobulin classes directed against fibroblasts, epithelial, and endothelial cells reinforce the theory of immunologic involvement in the pathogenesis of keloid (18), (74). These antinuclear antibodies were reported to stimulate proliferation of keloid fibroblasts and their production of collagen; they appear in the proliferative phase of the fibroblasts, thereafter, the antibody concentration plummets (72). The presence of antibodies directed against one's own fibroblasts in keloids has led to the classification of keloids as a connective tissue autoimmune disease which is comparable to systemic scleroderma(72)

The influence of T-lymphocytes on the induction of collagen formation by fibroblasts through reductions in concentrations of interferon alpha (IFN-α), interferon gamma (IFN-γ), and tumour necrosis factor alpha (TNF-α) lend further support for the implication of the immune system in the aetiology of keloid formation (75), (76), (77). The significance of the induction and implication of such a complex, yet confined, immune reaction in keloid lesions, have not been established. However, the stimulation of such a multitude of immune factors indicates that a host of potent factors are involved in the pathogenesis of keloid formation. The influence of immunologic mediators was reported to be more prevalent in the early stages of keloid development, with "early" being defined as less than two years of age and "late" as more than two years (18)**.**

1.13 **Immune reaction to sebum as a predisposing factor to keloid formation**

The immune reaction to sebum theory stated that dermal injury exposes the pilosebaceous unit to the systemic circulation, initiating a cell-mediated immune response in persons who retain T lymphocytes sensitive to sebum. Subsequent release of cytokines, including various interleukins and transforming growth factor beta (TGF-β), stimulate chemotaxis of mast cells

and production of collagen by fibroblasts. As the keloid expands, further pilosebaceous units on the advancing border are disrupted, leading to further propagation (78). Keloids preferentially occur in areas with a high concentration of sebaceous glands, including the chest wall, shoulder, and pubic area, while rarely occurring in areas relatively devoid of sebaceous glands, such as the palms and soles. Because humans are the only mammals with true sebaceous glands, the sebum reaction hypothesis partly explains why keloids only occur in humans (17).

1.14 Other immunohistological antigens present in keloids

Other immunohistological studies on keloidal tissue reported the presence of heat shock 70 kDa protein 9B (Hsp70-9B or mortalin), dnaK-type molecular chaperone, heat shock 70 kDa protein 1L (Hsp70-1L), and dihydropyrimidinase-like 2 protein. Proteins that were upregulated in keloids include crocalbin-like protein, calumenin, vimentin, tropomyosin 1, and heat shock 70 kDa protein 1A (Hsp70-1A) (79).

1.15 Genetic elements implicated in the pathogenesis of keloid formation

1.15.1 Keloid prone individuals show a genetic predisposition to keloid formation

Individuals with an inherited tendency to form keloids and those expressing atypical genetic features during dermal wound healing are at high risk of keloid formation. Although there are no clearly defined genetic loci indicating risk for keloids, there is strong support for the genetic susceptibility to keloid formation.

1.15.2 Predominance in Black ethnic groups

A genetic predisposition to keloid formation is supported by the finding that keloids are 5-15 times more predominant in ethnic groups with darker pigmented skin than Whites (80). It is estimated that 15-20% of Blacks (African-American, African-Caribbean and Africans), Hispanics and Oriental individuals are inflicted with keloidal growths (80). These darker skinned ethnic groups were reported to be 15 times more likely to develop keloids and this predilection was attributed to the enhanced action of melanocyte stimulating hormone (MSH) in the highly pigmented skin. The following findings support this assumption:

- Highly pigmented individuals of various races are more prone to keloid formation
- Melanocyte numbers are high at the main locations of keloid formation
- During puberty and pregnancy increased hormone production by the pituitary gland stimulates melanocyte proliferation and synthetic activity. This increased activity is associated with increased pigmentation and the incidence of keloids is higher during this period.
- The positive response of keloids to treatment with steroids is thought to be associated with the inhibition of MSH production. This assumption is supported by association between hypopigmentation, steroid injection and MSH suppression (58).

1.15.3 Familial inheritance

The existence of keloids within families and in twins suggests that genetic elements contribute to keloid formation. A report effectively illustrating the inherited familial propensity to form keloids, presented the cases of 3 European Caucasian brothers who developed keloids as teenagers following surgery, ear piercing and chicken pox scarring in the eldest to the youngest respectively (81).

Another study showed that 96 of the 341 family members displayed keloid formation; 36 were male and 60 were female and the age of infliction ranged from early childhood to late adulthood (82). The severity of keloids in the affected members varied from small earlobe keloids to very severe large keloids affecting different areas of the body (82). In twins keloid formation was found in 4 sets of identical twins and occurred simultaneously in a pair of twins at their vaccination sites (40, 82). The modes of inheritance in families and twins were found to be autosomal dominant or recessive, but there is greater evidence for the autosomal dominant mode. This type of inheritance in keloid patients displayed incomplete penetrance and genetic expression (83). In addition, familial inheritance studies have reported that aggressive keloid disease and multiple-site keloid development was associated with a positive family history and black African ethnic origin (84).

Other factors favouring a genetic predisposition in keloid formation are the higher rate of keloid development in people with type A blood and the following tissue compatibility antigens: HLA B14, Bw16, B21, Bw35, DR5, DRB1*, DQA1, DQB1 [20-23]. Keloids are often concomitant with many common genetic diseases of connective tissue including scleroderma, progeria, Ehlers-Danlos syndrome or Rubinstein-Tabi syndrome [24].

1.15.4 Genetic polymorphisms in keloid tissue

In analysing genetic polymorphisms in keloid tissue, chromosome loci that positively correlated with keloid formation were: 2q23, 7p11 and 14q22-q23 (72, 80, 85). Comparative analysis of gene expression in fibroblasts of keloids and reactive scars revealed differences in 500 genes of various metabolic and cellular pathways. Genes that were abundantly expressed in keloid fibroblasts were genes for insulin-like growth factor binding protein (IGFBP) and connective tissue growth factor (CTGF) while genes that exhibited the most distinct differences were genes

for nerve growth factor β (NGF-β), cyclin D2 , receptor genes for thrombin and thrombin-like growth factor (F2R and F2RL2 respectively) as well as for TGF-β receptor I (72, 85).

Many genes encoding second messengers in intracellular pathways regulating cell growth and differentiation were also excessively expressed in keloids. These included dishevelled-associated activator of morphogenesis 1 – activator from the Wnt protein family (DAAM1), JAG-1, encoding second messengers of the Notch signalling pathway as well as nucleus factor of transcription 1B (NF1B) (72, 85). Excessive expression of genes encoding extracellular matrix components were COL1A1 gene encoding type I collagen, ELN gene encoding elastin and genes encoding collagen types: V, VI, X, XV and XVI and also periostin, thrombospondin 4, lumican, mimecan, versican, syndecan-2 and decorin (72, 85). Another gene that showed excessive expression in keloid fibroblasts was the gene for 311 protein but, its function is not fully understood. However, it is thought to be an oncogene as neoplasms of the central nervous system (astrocytomas) show an excessive expression of this protein (72).

1.15.5 Keloid fibroblasts show reduced expression of many genes

There is diminished expression of many genes in keloid fibroblasts mainly, genes for inhibitors in intracellular signalling pathway of the Wnt protein family (DKK1, DKK3 and SFRP1 and 2), and for inhibitors in the cyclin signalling pathway (CDKN1C, CDKN3) (72). Another group of genes showing decreased expression in keloids included: interleukin 1 receptor (IL1R) gene and interleukin 1 controlled genes, encoding chemokines and their ligands (CXCL1, CXCL12 and CXCL14) (72). Also, there was reduced expression of genes encoding interleukin-7 (IL-7) and interleukin-8 (IL-8). Exceedingly diminished expression was reported for genes encoding metalloproteinases (extracellular matrix proteins degrading enzymes); these included matrix metalloproteinase 3 (MMP3) and membrane metalloendopeptidase (MME).

Other genes exhibiting significantly decreased expression were the receptor II encoding gene for TGF-β type 2 and a family of HOX genes (72). Genes of this family regulate precise differentiation of germ layer cells during the early stage of embryogenesis and many of these genes regulate fibroblast proliferation in adults. Research shows that specific "silencing" of some of these genes is associated with the development of adenocarcinoma in the lung (72).

1.16 Wound closure techniques may predispose to keloid formation

The technique of wound closure used by surgeons may also contribute to keloid formation. These techniques include:

- Placement of scar along lines of relaxed skin tension and natural lines are less prone to develop keloids (86).
- Wounds closed under tension are more likely to develop keloids (86).
- The use of highly reactive suture material increases the risk of keloid formation (87).

1.17 Other predisposing factors

Other predisposing factors implicated with the formation of keloids include foreign body reaction (87), viral infection (88) and degraded collagen acting as a catalyst for scar hyperplasia (86). A hormonal influence was suggested as well, because keloids often appear at puberty, (40) resolve after menopause, and enlarge during pregnancy (26). In addition, active keloids show a higher level of testosterone-binding receptors (89).

1.18 Proposed aetiopathogenic mechanisms of keloid formation

The aetiopathogenesis of keloids is unknown and although there are many hypotheses on the topic, the exact mechanism by which these benign fibrous growths form, is not implicit. Numerous factors are implicated in the aetiology and pathogenesis of excessive scar formation.

1.18.1 Growth factor implication in the aetiopathogenesis of keloids

Aberrant wound healing in keloids and hypertrophic scars may be caused to some extent by abnormal growth factor activity. A major aetiological factor in the pathogenesis of keloids is the production of increased or decreased amounts of the various growth factors causing prolonged or abnormal cell signalling mechanisms.

1.18.1.1 Transforming growth factor-beta

One of the most important and well-researched cytokines implicated in the pathogenesis of excessive scar formation is TGF-β. This cytokine has a pleiotropic role that influences cell growth, differentiation, matrix production and apoptosis (90). Transforming growth factor-β has an important regulatory function in tissue repair where, its prolonged production and action leads to the growth of fibrous tissue (91). At the site of injury TGF-β stimulates the production of new ECM proteins such as collagens, fibronectin and proteoglycans; and inhibits the synthesis of proteases, but stimulates the production of protease inhibitors. Transforming growth factor-β also increases the expression of cell surface integrin to enable cell-matrix interaction and matrix assembly (92). The effects of TGF-β are enhanced by the autocrine induction of its production (93).

Transforming growth factor β is secreted by many cell types, including fibroblasts, lymphocytes, platelets,and activated macrophages. It has 3 isoforms: TGF-β1, TGF-β2, and TGF-β3 which have superimposing functions (94). Transforming growth factors β1 and β2 are overproduced by keloid fibroblasts compared with foetal and normal skin fibroblasts (95). TGF-β1 and TGF-β2 are reported to be profibrotic, whereas, TGF-β3 is reported to have anti-fibrotic functions. When treated with exogenous TGF-β1, keloid fibroblasts produced 12 times more collagen than normal fibroblasts and 4 times more than hypertrophic scar

fibroblasts(95). Transforming growth factor-β1 promoted fibroblast proliferation by blocking fas-mediated apoptosis, preventing the activation of caspases-3, -8, and –9, creating an obstruction in the apoptotic pathway( ). This anti-apoptotic outcome of TGF-β1 was not demonstrated in hypertrophic scars or normal fibroblasts (96). In addition to increasing the proliferation of keloid fibroblasts TGF-β1 also stimulated the synthesis of extracellular matrix components such as elastin, fibronectin, and types 1 and 3 collagen (97), (98), (99) (100). Another effect of TGF-β1 was the enhancement of contractile activity in keloid fibroblasts ( ) and the increased synthetic activity of myofibroblasts, resulting in the production of exuberant stromal tissue (102). Transforming growth factor-β2 specifically activated fibroblasts to synthesize and secrete collagen and the TGF-β2 levels were elevated in both keloids and hypertrophic scars ( ). In in vitro experiments the increased synthetic and contractile activity of keloid fibroblasts; was reversed by the addition of TGF-β2 antibody (104).

The binding of TGF-β to proteoglycans close to the cell surface or in the matrix was proposed to be the signal that terminated its production after the repair process was complete (94). Recent research describes that the mechanism of action of TGF-β1 is by constant activation or reduced inhibition of signalling pathways involving intracellular effectors such as mitogen-activated protein kinases (MAPKs) and Sma- and Mad- related proteins (Smads), leading to a persistent autocrine-positive feedback loop affecting an overproduction of extra cellular matrix proteins and ensuing fibrosis (105).

Thus fibroblasts stimulated by TGF-β are the main perpetrators of scar formation. In foetal wounds which heal without scarring, messenger RNA expression of acidic and basic fibroblast growth factors and TGF-β_1 was decreased in fibroblasts compared with adult fibroblasts (95). Despite strong evidence supporting the increased production of TGF-β1 in

cultured keloid fibroblasts compared with cultured normal skin fibroblasts, in the in vivo situation, the plasma of keloid patients did not reflect this and no statistically significant difference was found in the plasma levels of TGF-β1 between keloid and control groups (106).

The key role of TGF-β in stimulating fibroblast proliferation and synthetic activity has attracted many research groups to investigate the modulation of TGF-β signalling mediators or the use of TGF-β inhibitors as a means of treating keloids (105). Ashcroft et al, (107) reported that normal skin and scar fibroblasts treated with conditioned media from keloid fibroblasts showed higher expression of fibrosis- linked molecular markers (including TGF-β) and increased synthetic and mitogenic activity. They indicated that keloid fibroblasts alter the proliferation rate and cellular activities of surrounding normal fibroblasts by their secretions through paracrine effects, promoting lesional growth.

It was reported that keloid fibroblasts have an uncontrolled or different response to TGF-β signalling pathways and demonstrate higher collagen I expression compared to normal fibroblasts (107). TGFβ-1 also stimulated the production of fibronectin (FN), α-smooth muscle actin-(SMA), plasminogen activator inhibitor 1 (PAI-1), connective tissue growth factor (CTGF) and TGFβ. Ashcroft et al (107) attributed the fervent keloid fibroblastic response to autocrine, paracrine, genetic and epigenetic effects as well as to the paracrine effects of secretions of inflammatory cells and re-epithelialising keratinocytes.

Exogenous TGF β1 upregulates the expression of platelet derived growth factor (PDGF) α receptor in keloid-derived fibroblast cells but not in normal dermal fibroblasts. (105). It has also been shown that TGF β stimulates the expression of vascular endothelial growth factor (VEGF) in keloid fibroblasts (105).

TGF-β moderates the expression of matrix metalloproteinase (MMPs), which can cleave all extra cellular matrix and basement membrane constituents. Expression of MMP-1 (interstitial collagenase) and MMP-2 (gelatinase-A) in keloids is increased and is associated with enhanced migration and invasion by lesional fibroblasts (58).

1.18.1.2 Platelet derived growth factor and insulin-like growth factor

The increased quantities of profibrotic cytokines such as platelet derived growth factor (PDGF), TGF-β, and insulin-like growth factor-1 (IGF-1), produced during the exaggerated inflammatory phase and often observed in excessive scarring, were proposed as aetiological factors contributing to keloid formation (108). These authors also reported that the excess of platelets produced during the amplified inflammatory phase in keloids, were a source of the fibrosis-promoting cytokines, TGF-β and IGF-1. Many IGF-binding and IGF-related proteins were reported to be over-expressed in cultured keloid fibroblasts although IGF-1 effects on keloid fibroblasts were shown to be non-stimulatory. It was suggested that the action of IGF-1 was by augmenting paracrine TGFβ- scar forming effects through the increased expression of collagen I, fibronectin and PAI-1 by these fibroblasts (107).

1.18.1.3 Role of interferon, tumour necrosis factor, interleukins and other cytokines in the aetiopathogenesis of keloids

The deficiency of interferon-alpha (IFN- α), which has a down-regulatory effect on collagen synthesis, (75), (109) and reduced levels of interferon-gamma (IFN-γ) (110) which stimulates collagenase activity (109), (111) were other suggested causal factors implicated in the

aetiopathogenesis of keloids. A study on the bloods from keloid patients showed increased amounts of tumour necrosis factor-alpha (TNF-α), beta-interferon (IFN-β), and markedly reduced levels of IFN-α, IFN-γ and tumour necrosis factor-beta (TNF-β). The net effects of these cytokines (at the increased or decreased concentrations), were reported to be related to elevated quantities of collagen, glycosaminoglycans and decreased collagenase levels in keloidal specimens (75), (109)

One of the most basic inflammatory cytokines, IL-6, was proposed to participate in the pathogenesis of keloids. Apart from promoting the inflammatory processes, it also actively stimulated proliferation of fibroblasts. In normal dermal fibroblasts IL-6 favours secretion of metalloproteinases, whereas in keloid fibroblasts, this process does not occur. Metalloproteinases are endopeptidases that cleave a wide range of extracellular, bioactive substrates, and regulate the activity of these extracellular proteins (112). Some of the main non-catabolic functions of MMPs include the release of growth factors from the cell membrane or ECM, cleavage of growth factor receptors from the cell surface, shedding of cell adhesion molecules, and activation of other MMPs (113). Experiments where exogenous IL-6 was administered to cultured fibroblasts showed that interleukin 6 increased the expression of type I collagen and adding an inhibitor for IL-6 to the culture abrogated this result (114). Immunohistochemical studies on keloid fibroblasts showed an increase in the expression of interleukin-6 a receptor protein (IL-6aR) and other proteins viz., JAK1, STAT3, RAF, ELK and gp130, which are second messengers in the signalling pathway of this cytokine ( ). The increased expression of IL-6 genes in keloid fibroblasts when compared with control fibroblasts, and the higher levels of IL-6 in peripheral blood mononuclear cell (PBMC) -fractions of black patients with keloids, supported the proposition that IL-6 may play a role in pathogenesis of keloid (75), (115). Other cytokines such as epidermal growth factor (EGF) and connective tissue growth

factor (CTGF) were indirectly associated with the pathogenesis of keloids after activation by TGF-β (116), (117).

1.18.2 Essential fatty acid deficiency, nitric oxide and p53 gene mutations involved in the pathogenesis of keloids

An essential fatty acid deficiency of aracidonic acid precursors (linolenic acid, gamma linolenic acid and dihomo-γ-linolenic acid) was established in keloid lesions (118). The large numbers of proliferating fibroblasts and inflammatory cells produced during keloid formation required proportionally larger quantities of arachidonic acid precursors for cell membrane synthesis. This excessive requirement resulted in a deficiency of the fatty acid which promoted keloid formation. The deficiency of arachidonic acid precursors was exacerbated by the lower intake of calcium and copper and the higher intake of magnesium; these abnormalities in micronutrient intake resulted in aberrant essential fatty acid metabolism (119).

Other aetiological factors involved in the pathogenesis of keloids were nitric oxide and mutated p53 genes. Increased levels of nitric acid which stimulates synthesis of collagen by fibroblasts involved in excessive scar tissue formation (120) and focal mutations in the tumour suppressing p53 gene were identified in all patients with keloids (121).

1.18.3 Characteristics of keloid fibroblasts and their impact on the pathogenesis of keloids

Crucial to the formation of keloids and hypertrophic scars is a modification of the fibroblast phenotype. When compared with normal fibroblasts, keloid fibroblasts showed increased growth and proliferation characteristics; alterations of nuclear and cytoplasmic features; altered metabolic activities and apoptosis; increased numbers of growth-factor receptors which permit a more rapid response to growth factors like TGF- β; etc. These

changes up regulate propagation and synthetic activity of these atypical cells from the commencement of wound healing.

1.18.3.1 Origin of keloidal fibroblasts

The various distinctive characteristics displayed by keloid-fibroblasts indicate that they are phenotypically different from normal dermal fibroblasts**.** It was suggested that keloidal fibroblasts were derived from the unsuspecting pericyte **(122)**, which is different, in many respects, to dermal fibroblasts. In time this view was altered and favoured the outlook that resident fibroblasts, endothelial cells or undifferentiated cells from the growing tips of microvessels were precursor cells of keloid fibroblasts (122).

1.18.3.2 Myofibroblasts in keloid tissue

The view that keloid-fibroblasts are essentially myofibroblasts does not appear to be well supported. Although there is consensus on the presence of myofibroblasts in granulating wounds and hypertrophic scars, there are contradictory reports about their occurrence in keloidal specimens: some studies found that the nodules containing myofibroblasts, described in hypertrophic scars, are rarely found in keloids or that myofibroblasts are generally absent in keloids (123, 124). Matsuoka et al described the presence of rare fibroblasts displaying convoluted nuclei and tangled mictofilaments, but failed to demonstrate muscle-restricted actins or cell-to-cell junctions, resulting in their conclusion that true myofibroblasts are not a component of keloid lesions (125). A confusing issue was the non-detection of myofibroblasts in vivo yet there was a predilection for keloid fibroblasts to express alpha smooth muscle actin (α-SMA) in vitro (126). The loss of α-SMA upon matrix contraction and its absence in the subsequent stage when matrix gel relaxation occurs and tension is low, may partially explain the controversy about the presence of myofibroblasts in keloids.

Other studies reported the definite presence of myofibroblasts in keloids (126, 127). Many of these α-SMA positive cells were found to be spindle-shaped, suggesting that these cells experienced outside tension. The actin isoforms equipped myofibroblasts to perform their contractile and retractile functions that they perform in the normal wound healing process, marking the transformation of granulation tissue into scar tissue. Thereafter, myofibroblasts containing α-SMA disappear, probably as a result of apoptosis. In contrast, myofibroblasts persist in excessive scarring and in fibrotic conditions (128). A causal link between myofibroblasts and other forms of fibrosis has also been suggested (129).

Many of the characteristics exhibited by keloid fibroblast phenotypes are directly related to the promotion of keloidal growth.

1.18.3.3 Growth and proliferation characteristics of keloid fibroblasts contributing to keloid growth

Some altered growth and proliferation characteristics of keloid fibroblasts that may contribute to keloid growth are listed below:

- The lower inherent threshold for entry into S phase of the cell cycle and the high mitotic rate of keloid fibroblasts (130) (131).
- The reduced population doubling times of certain keloid fibroblasts and their decreased rate of apoptosis (132).
- The significantly higher mean density of fibroblasts in keloids when compared with their normal counterparts (20).
- The increased commitment of keloid fibroblasts to DNA synthesis in response to wounding in vitro (133).

- The presence of incessantly proliferating cells at the dermal-keloid interface and the increased number of proliferating cells in the basal layer of the epidermis and dermis in keloids (134).

1.18.3.4 Nuclear alterations in keloid fibroblasts and their impact on the pathogenesis of keloids

Some nuclear alterations implicated in the pathogenesis of keloids are as follows:

- Focal mutations in the p53 tumour suppressor gene in keloidal tissue (135) predispose to increased cell proliferation and decreased cell death in keloids.
- Elevated numbers of silver-stained nucleolar organizer regions (AgNORs) which indicate increased cellular activity and proliferation in keloid fibroblasts (136).
- Increased concentrations and expression of the apoptosis-reducing protooncogenes c-jun and c-fos in keloid fibroblasts promoted longevity of these cells
- Increased fibronectin gene transcription (three-fold increase) in keloid fibroblasts led to a four-fold increase in fibronectin biosynthesis (137).

1.18.3.6 Altered expression of various proteins in keloid fibroblasts and their impact on the pathogenesis of keloids

The cytoplasmic alterations in keloid fibroblasts that may be implicated in the pathogenesis of keloids are outlined below:

- Overexpression of plasminogen activator inhibitor type-1 (PAI-1) mRNA by keloidal fibroblasts (138) which decreased or inhibited fibrinolytic activity (32). A

study investigating the effects of PAI-1 on pulmonary fibroblasts showed that it inhibits apoptosis, stimulates fibroblast proliferation and differentiation into myofibroblasts and it promotes collagen synthesis by these fibroblasts. The mechanism of activation of these effects was via the Ca^{2+}, ERK and AKT signalling pathway (139).

- Keloid fibroblasts overexpressed insulin-like growth factor-1 receptor (IGF-IR) which mediates the growth-stimulating and metabolic regulatory functions of insulin-like growth factor -1 (IGF)-I (140). Other receptors that keloid fibroblasts showed increased expression for were epidermal growth factor receptor (EGFR) and discoidin domain receptor 1 (DDR1); also greater phosphorylation of these molecules was exhibited (141). The discoidin domain receptor1, is activated by triple-helical collagen (142) and it is implicated in the pathogenesis of fibrotic diseases, cancer, atherosclerosis and arthritis (143). Epidermal growth factor receptors are tyrosine kinase receptors that function in cell signaling, cell proliferation and differentiation (144). The increased expression of activated EGFR during tumour formation is thought to control the proliferative process (145).

- Increase in both types I and III procollagen mRNA, with higher levels of the type I. This resulted in greater ratios of type I to III procollagen mRNA (146, 147).

- A twenty-fold upregulation of collagen types I and III mRNA in keloid tissues as well as a 16-fold increase of its molecular chaperone, HSP47 (147).

- Elevated levels of TGF-β1 and TGF-β2 proteins in keloid fibroblast cultures (148).

- Enhanced expression of the extracellular matrix glycoprotein, tenascin C (TNC), by keloidal fibroblasts (149). Increased TNC expression is thought to promote TGF-β effects and fibrosis post injury (150).

- Elevation of alpha smooth muscle actin (αSMA) in keloid fibroblasts (151) and the presence of the myofibroblast phenotype were proposed to be causal factors associated with interstitial fibrosis (129).

- The unceasing production of high levels of collagen, fibronectin, elastin, and proteoglycans by keloid-derived fibroblasts (129), (152), (98), (153).

- Elevated levels of intracellular and extracellular fibronectin, increase in fibronectin mRNA, increased biosynthesis of fibronectin and high steady state levels of the glycoprotein in keloid fibroblasts, which also exhibited a coordinate increased expression of the fibronectin receptor (152). It was shown that fibronectin promoted proliferation of breast cancer cells in response to adhesion-mediated signals associated with the altered binding of integrins to fibronectin with different conformational changes. These alterations were caused during fibronectin interaction with various extracellular matrix components such as hyaluronic acid, collagen and proteoglycan (154).

- A decrease in the density of hyaluronic acid receptors on keloid fibroblasts when compared with the presence of enhanced numbers of this receptor in fibroblasts of hypertrophic scars (155). A reduction of hyaluronic acid receptors equate to low concentrations of hyaluronan applied to keloid fibroblasts; when this application involved stem cells the proliferative activity was enhanced (156).

- Increased expression of proteins of proliferation-enhancing protooncogenes Bcl-2, c-jun and c-fos in fibroblast-like cells (23).

1.18.3.7 Altered apoptosis in keloid fibroblasts and its impact on the pathogenesis of keloids

The following alterations of the apoptotic mechanism in keloid fibroblasts were implicated in the pathogenesis of keloids:

- Alteration of apoptosis in keloid fibroblasts (38), (40). The under expression of eight out of 64 of apoptosis-related genes was found in keloid fibroblasts. These changes alter physiological mechanisms controlling apoptosis and thus prolong fibroblast existence and the production of extracellular matrix (ECM) elements.
- The increased expression of proliferation-enhancing proto-oncogenes Bcl-2, c-jun, and c-fos, the lack of expression of the p53 tumor suppressor gene and the decreased expression of other pro-apoptosis-associated genes, (23)
- Alterations of the apoptotic mechanism in keloid fibroblasts led to their resistance to apoptotic cell death (157). Also keloid fibroblasts were found to be resilient to Fas-mediated apoptosis (96).

1.18.3.8 Altered properties of keloid fibroblasts dissimilar to normal dermal fibroblasts impacting on the pathogenesis of keloids

Some differential properties exhibited by keloid fibroblasts that may promote keloid growth are outlined below:

- The elevated activity of glucose-6-phosphate dehydrogenase (G6PDH) under saturated oxygen conditions (158); this increased expression of G6PDH was shown to augment resistance to hydrogen peroxide (H_2O_2) -induced cell death (159).

- The reduced requirement for serum growth factors (116). Tumour cells in general have a different reaction to growth regulatory factors and have been grown in serum-free media (160).

- The failure of hydrocortisone to down-regulate collagen and elastin synthesis in keloid fibroblasts, while in normal dermal fibroblasts this function was down-regulated (98).
- The production of highly cross-linked collagen that provides more stability contributing to their long-lasting qualities and accumulation (149).

1.18.3.9 Keloid fibroblasts produce extracellular matrix components different to normal skin:

Keloid fibroblasts produce extracellular matrix components that are different to that of normal skin. Some anomalous differences found in the extracellular matrix components of keloid when compared with normal skin are:

- The high proliferation rate of fibroblasts that have a prolonged high rate of collagen synthesis, and their production of high levels of fibronectin, elastin, and proteoglycans (129), (152), (98), (153).
- Presence of collagen that is highly cross-linked (up to 6-fold increase in content of major cross-links), facilitating their accumulation (161).
- The increase of both type I and III procollagen mRNAs in keloid, resulted in raised ratios of type I to III procollagen mRNA (162) and in an increase in type I collagen (163) and a 10% increase of collagen type III in keloids (164).
- Increased content of glycosaminoglycans in keloid tissue (165).
- Elevated levels of intracellular and extracellular fibronectin (152).

- Marked increase of two small proteoglycans, decorin and biglycan (53).
- Low or undetectable levels of matrix metalloproteinase-9 (MMP-9) and high levels of matrix metalloproteinase-2 (MMP-2), which are proteins of the matrix metalloproteinase (MMP) family which are involved in the breakdown of type IV collagen (25).
- Diminished levels of hyaluronan as well as alterations in the distribution of this glycoprotein in the epidermis overlying keloids (166).
- Trace element irregularities such as a marked increase in manganese (Mn) and decreased levels of zinc (Zn), copper (Cu), and selenium (Se) (167). These trace elements scavenge free radicals and are co-factors of important enzymes such as superoxide dismutase, catalase and glutathione peroxidase. The antioxidant effects protect tissues from injury by oxidative stress caused by accumulated reactive oxygen species (ROS) and reactive oxygen/nitrogen species (RNOS) (168).

1.19 Synopsis of a critical review of previous investigations on the pathogenesis of keloids

A critical analysis of previous investigations on keloids pertaining to the lack of understanding of the pathogenesis has revealed some important aspects that have been neglected. In this respect investigations in the following areas were sparse or absent:

- Factors stimulating immunological reactions in keloids
- Clarification on the persistent involvement of myofibroblasts in keloid formation
- Factors causing blood vessel occlusion
- The distribution of collagen types I and III in the lesions
- Studies investigating the entire keloid
- In depth *in vivo* morphological studies
- *In vivo* immunocytochemical studies

- A holistic approach integrating the various aetiological factors with previous work done and formulating hypotheses on the aetiopathogenesis of keloid formation

Despite the above inadequacies, each of the investigations contributes in some way, to create the "big picture" of the pathogenesis of keloids.

1.20 Theories on the aetiopathogenesis of keloid formation

In reviewing the literature and from assessing the results of pilot studies the likely postulates on the aetiopathogenesis of keloid formation include:

- Keloids form when a defect occurs during one or more of the steps in the granulation stage of the wound healing processes
- Fibroblasts of different phenotypes are implicated in the pathogenesis of keloids
- Programmed cell death does not occur in all fibroblast phenotypes, resulting in the continual existence of fibroblast populations unable to undergo apoptosis. The above fibroblasts produce collagen at a rapid rate and this is exacerbated by their prolonged existence because of a defect in their apoptotic apparatus. The above fibroblasts also overproduce some of the other extracellular matrix components like proteoglycans, glycoproteins etc., and fail to produce others.
- A certain phenotype of keloid fibroblasts is foetal in nature i.e. this group of fibroblasts is in the developmental stages and therefore, they are regarded as foreign. Recognition as foreign stimulates immunological mechanisms. Immunological mechanisms lead to autoimmune complications.
- These fibroblasts are regulated by various mediators such as cytokines, growth factors, neuropeptides and they express the appropriate receptors for these cytokines and neuropeptides.

- The effects of the various mediators and regulatory proteins on the different fibroblast phenotypes of the granulation phase of the wound healing process. The inherent properties of these fibroblasts and the immunological mechanisms stimulated are interlinked and implicated in the pathogenesis keloid formation.

1.21 Criteria to be investigated to test the above theories on the aetiopathogenesis of keloid formation

In order to assess the above theories the following criteria need to be investigated and established or resolved:

- Stages of wound healing recognisable in keloidal tissue
- The different fibroblast phenotypes present in keloid lesions
- Cell death in keloids
- The significance of hyalinised collagen bundles in keloids
- Why is there a predilection for keloids to occur in the upper torso?
- Why does chronic inflammation persist in keloids?
- Extracellular matrix components that are overproduced as well as the fibroblast phenotype responsible for this
- Which extracellular matrix components are absent and why
- What cell types or cellular elements stimulate immunological reactions
- Is there any evidence that keloid fibroblasts or other elements initiate an autoimmune type reaction?

1.22 Additional tools required for the holistic evaluation of keloid aetiopathogenesis

Evaluation of the aetiopathogenesis of keloid formation requires a sound understanding of the morphology of connective tissue elements in normal skin, normal scar tissue and normal wound healing stages. Even at these levels there are many unanswered questions. For example, do normal dermal components such as mast cells, dendritic cells and melanophages have a role to play in the wound healing process in skin; are reticular cells implicated in the immune system of the skin and if so, what is their function and their cell of origin; why does one often observe non-specific inflammation in normal dermis: what are the implications? In the normal wound healing process, are granulation tissue fibroblasts derived from the same cell of origin as the normal dermal fibroblast; is wound contraction the main role of the myofibroblast; what are the control mechanisms of the remodelling process; even after epthelialisation why is there often an exaggerated and prolonged inflammatory reaction? Studies investigating the above problems will positively contribute towards understanding the molecular mechanisms of pathological scar formation.

In order to assess the aetiopathogenesis of keloid formation each of the stages of the granulation phase of the wound healing process, i.e., the matrix production, angiogenic and fibroplastic stage, have to be identified and evaluated in keloid scar tissue. The evaluation process should be integrated with the results of previous investigations on keloids revolving around fibroblastic growth, metabolic activities, synthesis of extracellular matrix components, response to different cytokines and forms of treatment etc. Contradictory results, especially with regard to the fibroblast phenotypes, some extracellular matrix components, and growth and functional characteristics of fibroblasts, should be clarified. It is important that comprehensive in vivo investigations are incorporated. A large proportion of previous molecular investigations were based on *in vitro* studies where the intricate molecular and cellular relations and the intact keloid environment are not conserved. Also, excluded from the in vitro system are:

- Dermal/epidermal interactions
- Mesenchyme and connective tissue formation
- Generation of various cutaneous tissue elements
- Skin immune system
- Inflammatory processes and reactions
- Induction of hypoxic conditions
- Effects of mechanical forces, etc.

These factors are implicated in keloid formation and are fundamental to understanding the pathogenesis of keloids.

1.23 Rationale leading to hypotheses tested and publications

From appraising the literature apparent gaps were identified in the broad overview of keloid aetiopathology; hypotheses were then formulated within these gaps around pertinent related aspects and focussed into different studies for investigation. The logical approach to ultrastructural morphological investigations of keloid formation was to comprehensively interpret the light microscopical features and complement this with prospective immunocytochemical and electronmicroscopical studies. It is imperative that all recognisable features are deciphered and explained before postulating the pathological processes involved. Piecing together the various parts of the study should eventually result in valuable information on the pathogenesis of keloid formation

1.23.1 Paper 1: Keloids show regional distribution of proliferative and degenerative connective tissue elements

In reviewing the literature on keloid morphology, there are many contradictory reports. These contradictions range from keloids exhibiting a hyperproliferation of dermal fibroblasts (87) to

few fibroblasts (169); from the presence of a higher number of blood vessels in keloids when compared with normal skin (170), to the presence of a reduced vascular component (171). Other conflicting reports on keloid morphology were about the patency of vessels: when compared to normal skin and normal scars, it was reported that in keloids, the blood vessels were dilated and longer (170), while other reports showed an increase in the number of occluded microvessels (172). The absence of distinct nodules in keloids and their conspicuous presence in hypertrophic scars was a major histological difference between the two types of excessive scar tissue formation reported by Ehrlich et al (123) and this was contradicted by Luo et al (63) who reported that a hallmark feature of keloids was the presence of "collagen nodules". Another contradictory report on collagen arrangement informed that "discrete bundle formation was virtually absent" in keloids. Also, there are conflicting reports on the collagen content of keloids: Clore et al (173) report the quantity of types I and III collagen in keloids to be the same as in normal dermis, whereas other researchers showed elevated levels to be present in keloids (174). The variable expression of α-SMA expression reported in keloid scars suggested that this feature could not reliably help in distinguishing the two types of scars and implies that the pathogenesis of keloid scars is multifactorial, repudiating the clonal theory of origin (57).

Hypothesis: Thorough in depth ultrastructural examination of the entire keloid lesion and subsequent establishment of baseline morphological characteristics of keloids should explain many of the past and present contradictions, regarding morphological features. Also such a study was essential for future ultrastructural research investigating the pathogenesis of keloid formation.

This resulted in the publication of the following original article:

- **Keloids show regional distribution of proliferative and degenerative connective tissue elements.**

1.23.2 Paper 2: Does the paucity of elastic fibres contribute to the process of keloidogenesis?

During excessive wound healing, as in keloids, in addition to the constant production and progressive accumulation of scar tissue, traction force is continuously generated by migrating fibroblasts. Excessive, prolonged traction forces deform the extracellular matrix up to their elastic limit; beyond this limit the friction force that the tissue can withstand is exceeded resulting in movement of the matrix (175). Stress applied beyond the elastic limit, results in plastic changes that occur at a rate greater than the tissue can tolerate causing injury and permanent deformation; this is called plasticity stress (175). Beyond the plasticity level, tissue cannot tolerate more stress and it ruptures (175). Could the disfiguring dermal deformations in keloids be a manifestation of gradually accumulated effects of stress from many lesser loads exerted by continuous traction force generated by wound healing fibroblastic cells, especially during the granulation, angiogenic and tissue remodeling stages? Supportive evidence for this includes: the formation of keloids months to years post injury, after apparent successful healing and the use of pressure therapy in the effective treatment of keloids (176). Pressure therapy compensates for the elastic insufficiency of the affected dermal tissue and reduces scar tissue formation by inducing localised hypoxia and subsequent fibroblastic degeneration (176). However, preexisting hypoxia was confirmed in keloids where the accumulation of hypoxia-inducible factor-1α protein was found (177). The effectiveness of pressure therapy treatment of keloids may, therefore, be by exacerbation of the existing hypoxic state. This corroborates an association between stresses (force applied), strain (deformation when stress is applied), elastic insufficiency, hypoxia and keloid formation; thus reinforcing the necessity to investigate the role of traction forces in the pathogenesis of keloids.

Hypothesis: Keloids are growths formed by excessive wound healing where traction force generated by migrating fibroblasts deforms the extracellular matrix up to their elastic limit.

Beyond this plastic changes occur and cause permanent deformation. As permanent deformation of tissue occurs in keloids and as elastic recoil is the principal function responsible for recovery of tissue deformed by traction forces, we hypothesise that, in keloids, connective tissue elasticity is decreased, reducing the elastic limit and resulting in augmented tissue deformation which progresses to keloid formation.

Morphological assessment of the elastic fiber content of keloids resulted in the publication of the following original article:

- **Does the paucity of elastic fibres contribute to the process of keloidogenesis?**

1.23.3 Paper 3: Involvement of upper torso stress amplification, tissue compression and distortion in the pathogenesis of keloids

There is a definite tendency for keloids to form in the upper torso of the body, the main areas being the head (earlobes and mandibular ridge), neck, back, presternal and deltoid areas (178). The reasons for the propensity of skin in these regions to form keloids may be related to the inherent characteristics of skin and the effects of intrinsic and extrinsic factors on it.

Hypotheses: The predilection for keloids to form in skin covering the upper torso of the body is related to elevated dermal stress caused by:

1. stress-amplification at the soft and hard supportive connective tissue interface
2. inherent skin characteristics such as skin thickness, the bundled arrangement of collagen in the reticular dermis, fat content, the existent high tension, the low elastic modulus and low stretch ability
3. contractile forces generated by wound healing fibroblastic cells
4. action of external forces

Augmented internal stress causes dermal distortion and compression resulting in increased loading on cells; this triggers anabolic effects, with amplification of gene expression, collagen

synthesis and mitosis. Microvessels that become compressed and occluded cause ischemia and reperfusion injury; reperfusing blood is rich in growth factors which stimulate growth when released into the extracellular matrix of the healing tissue. Brainstorming and substantiating the possible reasons that support these hypotheses resulted in the publication of the following original article:

- **Involvement of upper torso stress amplification, tissue compression and distortion in the pathogenesis of keloids**

1.23.4 Paper 4: The significance of hyalinized tissue in the pathogenesis of keloids

Hyalinised collagen bundles (HCB) are pathognomonic of keloids where they are a characteristic and diagnostic feature (179).

Hypothesis: The hyalinization process is crucial to the understanding of the pathogenesis of keloids and possible reasons for the occurrence are hypothesised below:

- To provide a protective role by sequestrating cytokines and proteases produced by mast cells
- To prevent autoimmune attack by providing a safely isolated region where injured cells, that might not have gained prior tolerance, can demise without the danger of exposure to immune reactivity. These cells include degenerative and necrotic myofibroblastic cells and other fibroblastic phenotypes.

Investigations into these hypotheses resulted in the preparation and submission of the following original article:

- **The significance of hyalinized tissue in the pathogenesis of keloids**

1.24 Expected outcome

In this study it is envisaged that verification or elucidation of the above hypotheses, using a holistic approach where all areas of the keloid are examined in routine wax sections stained with haematoxylin and eosin and various differential and immunocytochemical stains. Light microscopy is the gold standard for the examination of pathological tissue and the “maintenance and reinforcement of good quality microscopy remains a priority” not only at the health center level, but also at the research level (180). Light microscopy should therefore, be the main tool of examination of specimens and this should be complemented by electron microscopy. Integration of critically, comprehensively and accurately interpreted results, with pertinent information from previous investigations, should permit the formulation of novel concepts that would contribute to a better understanding of the pathology and aetiopathogenesis of keloid formation. It is anticipated that prospective microscopical investigations arising from the morphological work covered thus far, will direct biochemists and molecular biologists ultimately, to the invention of efficient, unfailing treatment.

CHAPTER 2

PUBLICATIONS AND SUBMITTED PAPER

2.1 Keloids show regional distribution of proliferative and degenerative connective tissue elements.

2. 2 Does the paucity of elastic fibres contribute to the process of keloidogenesis?

2.3 Involvement of upper torso stress amplification, tissue compression and distortion in the pathogenesis of keloids

2.4 The significance of hyalinized tissue in the pathogenesis of keloids

2.5 Abstracts in refereed proceedings

2.5.1. Lack of elastic fibres in the pathogenesis of keloids.

2.5.2. Keloids show proliferative and degenerative connective tissue element

2.5.3. The significance of hyalinised collagen bundles in keloids

2.5.4. Mast cell involvement in keloid formation.

2.5.5. Characterisation of keloidal fibroblasts.

2.5.6. Ultrastructural difference between keloid and normal skin

2.5.7. Amyloid profile of keloids at the epidermal edge of the lesion.

2.5.8. Alterations in the papillary dermis of keloid lesions.

2.5.9. A morphological study of the dermis of normal scar

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Keloids Show Regional Distribution of Proliferative and Degenerate Connective Tissue Elements

Shamin Bux[a] Anil Madaree[b]

[a]Department of Physiology, Faculty of Health Sciences, and [b]Department of Plastic Surgery, Faculty of Clinical Sciences, Nelson R. Mandela School of Medicine, University of Kwazulu-Natal, Durban, South Africa

Key Words
Degeneration · Hyalinization · Keloids · Proliferation · Vascularization · Wound healing

Abstract
Keloids, formed by the deposition of excessive scar tissue, are characterised by the presence of thick hyalinised collagen bundles. Contradictory reports about keloid morphology include: hyperproliferation of dermal fibroblasts versus few fibroblasts; rich as opposed to poor vascularisation; dilated against occluded microvessels; distinct collagen nodules versus their absence, and elevated levels of types I and III collagen as opposed to no change when compared with normal dermis. This study attempted to clarify the controversies concerning keloid morphology by examining entire keloids and establishing baseline histological characteristics. Keloidal specimens from 32 patients were processed and comprehensively examined using light microscopy. The results of the study showed that keloids comprise many distinct regions, categorized as the zone of hyalinising collagen bundles, fine fibrous areas, area of inflammation, zone of dense regular connective tissue, nodular fibrous area and area of angiogenesis. The microvascular supply to each of these regions was impaired and features of degeneration and necrosis of keloid fibroblastic cells and microvessels were ubiquitous. Impairment of the healing stage of chronic inflammation, inefficient healing by fibrosis, multiple and exaggerated phases of vascular and fibrous granulation and remodelling stages manifest in keloid formation. The uneven distribution of cells may be due to the generation of traction forces by keloid cells. These forces also modify DNA and protein synthesis, leading to an overproduction of extracellular matrix components. This study provides a structured basis for future ultrastructural and immunocytochemical research of keloids and other fibroproliferative disorders.

Abbreviations used in this paper

CT	connective tissue
DRCT	zone of dense regular connective tissue
HCB	hyalinising collagen bundles
TGF-β	transforming growth factor beta

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Shamin Bux
Department of Physiology, Faculty of Health Sciences
Nelson R. Mandela School of Medicine, University of Kwazulu-Natal
Private Bag 7, Congella, 4013 Durban (South Africa)
Tel. +27 31 260 4362, Fax +27 31 260 4455, E-Mail Bux@nu.ac.za

Introduction

Keloids are formed by the excessive production of scar tissue, which extends beyond the margins of the original injury, often resulting in lesions of grotesque dimensions. The overproduction of collagen by proliferating fibroblasts is thought to be a major contributing factor [Cobbold, 2001]. Macroscopically, keloids exhibit a nodular, often lobulated, firm, non-encapsulated mass that may be pedunculated or sessile. Keloids seldom extend deep to the dermis and are devoid of hair and other adnexa. They are usually asymptomatic, but can be tender or pruritic. Described histological features of keloids include haphazard deposition of thick hyalinised eosinophilic collagen fibres in a mucinous extracellular matrix and collagen nodules consisting of dense masses of collagen and fibroblasts [Jagadeesan and Bayat, 2007]. However, there are many contradictory reports in the literature with regard to the morphology of keloids. While some authors confirm that keloids exhibit a hyperproliferation of dermal fibroblasts [Slemp and Kirschner, 2006], other authors [Blackburn and Cosman, 1966] report that keloids showed few fibroblasts. Also, there are contradictory data on the vascularization of keloids. Some studies report a higher number of blood vessels in keloids when compared with normal skin [Amadeu et al., 2003], while other studies showed that keloids have a reduced vascular component [Ueda et al., 2004]. Other conflicting reports on keloid morphology were about the patency of the vessels in these lesions: when compared to normal skin and normal scars, it was reported by some researchers that in keloids, the blood vessels were dilated and longer [Amadeu et al., 2003], while others [Kischer and Shetlar, 1974] showed an increase in the number of occluded microvessels. The absence of distinct nodules in keloids and their conspicuous presence in hypertrophic scars was a major histological difference between the 2 types of excessive scar tissue formation reported by Ehrlich et al. [1994]. This was contradicted by Luo et al. [2001], who reported that in keloids, a hallmark feature was the presence of 'collagen nodules'. Conflicting reports concerning the collagen content of keloids exist, where Clore et al. [1979] report the quantity of types I and III collagen to be the same as in normal dermis, whereas elevated levels of these fibres in keloids were shown by Uitto et al. [1985]. In view of the controversy that exists with regard to keloid structure, it is important to establish baseline morphological characteristics of these lesions so that the data can be used for the following: (1) to explain past contradictions; (2) as a reference for the histopathological features of keloids; (3) to form the basis for future ultrastructural research investigating the pathogenesis of keloid formation, and (4) to stimulate detailed study of the histopathology of fibroproliferative disorders. As, to date, there is no comprehensive study embracing the array of histopathological features of keloids, we undertook a microscopy study to ascertain the morphological characteristics of these lesions.

Materials and Methods

The research protocol for the study was approved by the Nelson R. Mandela Faculty of Medicine Ethics Committee. Informed consent was obtained from the patients before the biopsies were taken. Keloid biopsies were obtained from 32 patients undergoing surgery for the removal of these lesions. These patients were confirmed to have no other underlying medical afflictions. Many of the patients had multiple lesions in various locations, bringing the total number of keloids processed and examined to 58. The age of the keloids was recorded when this information was available. Among the 18 patients where this was known, the keloid age ranged from 5 months to 6 years. Patient details are recorded in table 1.

Immediately after procurement, the biopsied specimens were placed in 10% formalin saline (4% formaldehyde) and processed for paraffin wax vacuum embedding using conventional methods. We ensured that the entire keloid was processed and sectioned. The large keloids were cut up into smaller blocks for optimal penetration by processing agents and so that the cut specimens would fit into the embedding moulds. Multiple sections were cut of each specimen at a thickness of 3–5 μm for routine hematoxylin and eosin staining. Sections were also stained with Giemsa for the identification of mast cells. The stained keloid sections were scrupulously examined using the Olympus BH-2 microscope; features pertinent to the study were photographed with the Olympus DP 10 microscope digital camera system. The stored images were studied, using the Camedia graphics processing programme (Olympus). Independent processing and assessment of the specimens was carried out by the department of anatomical pathology where qualified pathologists confirmed the diagnosis of keloids.

Results

Keloids were characterised by a thickened reticular dermis that showed varying patterns of arrangement of connective tissue elements demarcated within regions which were not always clearly defined and often merged into each other through transitional areas. The regions or zones identified were categorised and sub-grouped as indicated in table 2.

Zone of Hyalinising Collagen Bundles

The region of hyalinising collagen bundles (HCB) was usually located below the sub-papillary plexus and con-

Table 1. Patient details

Patient No.	Race	Sex	Age years	Diagnosis and location	Age of scar
1	C	F	7	Keloid, suprapubic area	6 months
2	A	M	21	Keloid, left ear lobe	2 years
3	A	F	15	Keloid, right ear lobe	2 years
4	A	F	18	Keloids, left and right ear lobes	5 months
5	A	F	16	Keloids, left and right ear lobes	1 year
6	A	F	26	Keloids, left and right ear lobes	2 months
7	A	F	16	Keloids, left and right ear lobes	2 years
8	A	F	27	Keloids, multiple (neck, back, presternal, chest)	2 years
9	A	F	29	Keloid, suprapubic area	3 years
10	A	F	33	Keloids, left and right ear lobes	2 years
11	A	F	18	Keloids, left breast, neck	1 year
12	A	F	21	Keloids, left and right ear lobes	NA
13	A	F	23	Keloid, left ear lobe	NA
14	A	F	18	Keloids, bilateral ear lobes	NA
15	A	F	26	Keloids, left ear lobe, shoulder	NA
16	A	F	22	Keloid, left ear lobe	NA
17	A	M	24	Keloids, left and right ear lobes	NA
18	A	M	49	Keloid, left ear	NA
19	A	F	17	Keloid, right ear	NA
20	A	F	27	Keloids, left and right ear lobes	4 years
21	A	M	24	Keloid, left ear lobe	2 years
22	A	F	26	Keloids, suprapubic area	NA
23	A	F	18	Keloid, left ear lobe	NA
24	A	F	26	Keloid, left ear lobe	5 years
25	A	M	45	Keloid, back of head.	2 years
26	A	M	26	Keloid, left ear lobe, posterior	2–3 years
27	A	F	18	Keloids, bilateral ear lobes	3 years
28	A	M	28	Neck (below right ear), submandibular area	NA
29	C	F	5	Neck (below chin)	NA
30	A	M	36	Keloids, face and RHS of left mandible	NA
31	A	M	24	Keloids, left and right ear lobes	6 years
32	A	F	24	Keloid, right ear lobe	NA

C = Caucasian; A = African; RHS = right-hand side; NA = not available.

tained many HCB, each of which was composed of thick, dense, eosinophilic aggregates of collagen fibres and other extracellular matrix components. These bundles ranged from being elongated tubular bundles, bundles displaying a whorled pattern, to small, spherical or irregular bundles. Smaller HCB were usually less compact and they often appeared to merge into each other and become compacted into the larger bundles. Individual bundles were separated by fibrous cellular or fibromyxoid stroma, wide extracellular spaces, or intermediate combinations of these (fig. 1a–c). Generally, the fine fibrous stroma between HCB contained fibroblastic and epithelioid cells with vesicular nuclei and prominent nucleoli (fig. 1d). Fine fibrous areas with slightly enlarged extracellular spaces contained cells that were plump and round to spherical with condensed hyperchromatic nuclei (fig. 1a, b). Wide extracellular spaces around HCB were associated with stroma showing scant fibres and elongated, flattened, curved fibroblastic cells that often lined HCB, sometimes in a palisading pattern. Wavy forms were usually found within the wide extracellular spaces (fig. 1c). Fibromyxoid stroma around HCB contained cells that were epithelioid to spherical and condensed (fig. 1d).

Hyalinising collagen bundles that were found as small single bundles or as small groups of 2–5 bundles were categorized as early HCB. These HCB were usually surrounded by fibromyxoid stroma containing spindle and

Table 2. Regions identified in keloids

No.	Regions	Sub-regions
1	Zone of hyalinising collagen bundles	a. Isolated b. Aggregated
2	Fine fibrous areas	a. Fine fibrous cellular areas b. Fine fibrous less cellular areas c. Wavy fine fibrous areas d. Fibrous tubular areas
3	Area of inflammation	a. Severe inflammation b. Moderate inflammation c. Mild inflammation
4	Zone of dense regular connective tissue	a. Eosinophilic b. Pale
5	Nodular fibrous area	a. Nodular fine fibrous cellular areas b. Nodular fine fibrous tubular areas
6	Area of angiogenesis	

epithelioid cells with condensed or large hyperchromatic nuclei (fig. 1e).

Features of degeneration and necrosis were prominent in the HCB region: atrophic and degenerate cells and coagulation necrosis were observed here. Mast cells were closely associated with degenerate cells and made contact with them through cellular processes (fig. 1e, f). The vascular supply of the HCB area was generally poor to avascular. Although blood vessels were found in the HCB area in the form of microvessels, mainly venules, they were usually compressed, showed occluded lumen or they were patent but displayed degenerate to necrotic mural cells with hyperchromatic nuclei and very eosinophilic cytoplasm (fig. 1e).

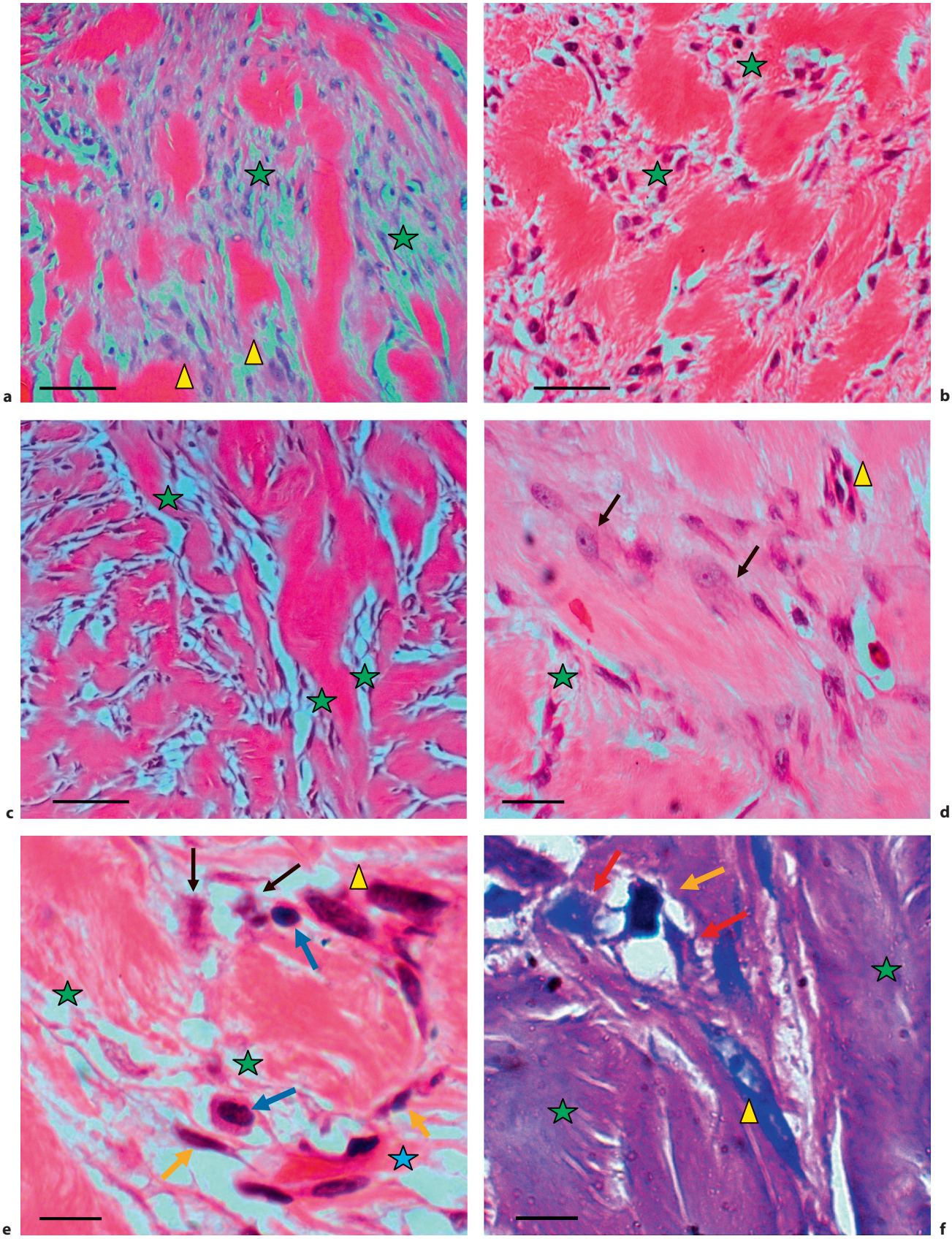
Fine Fibrous Areas

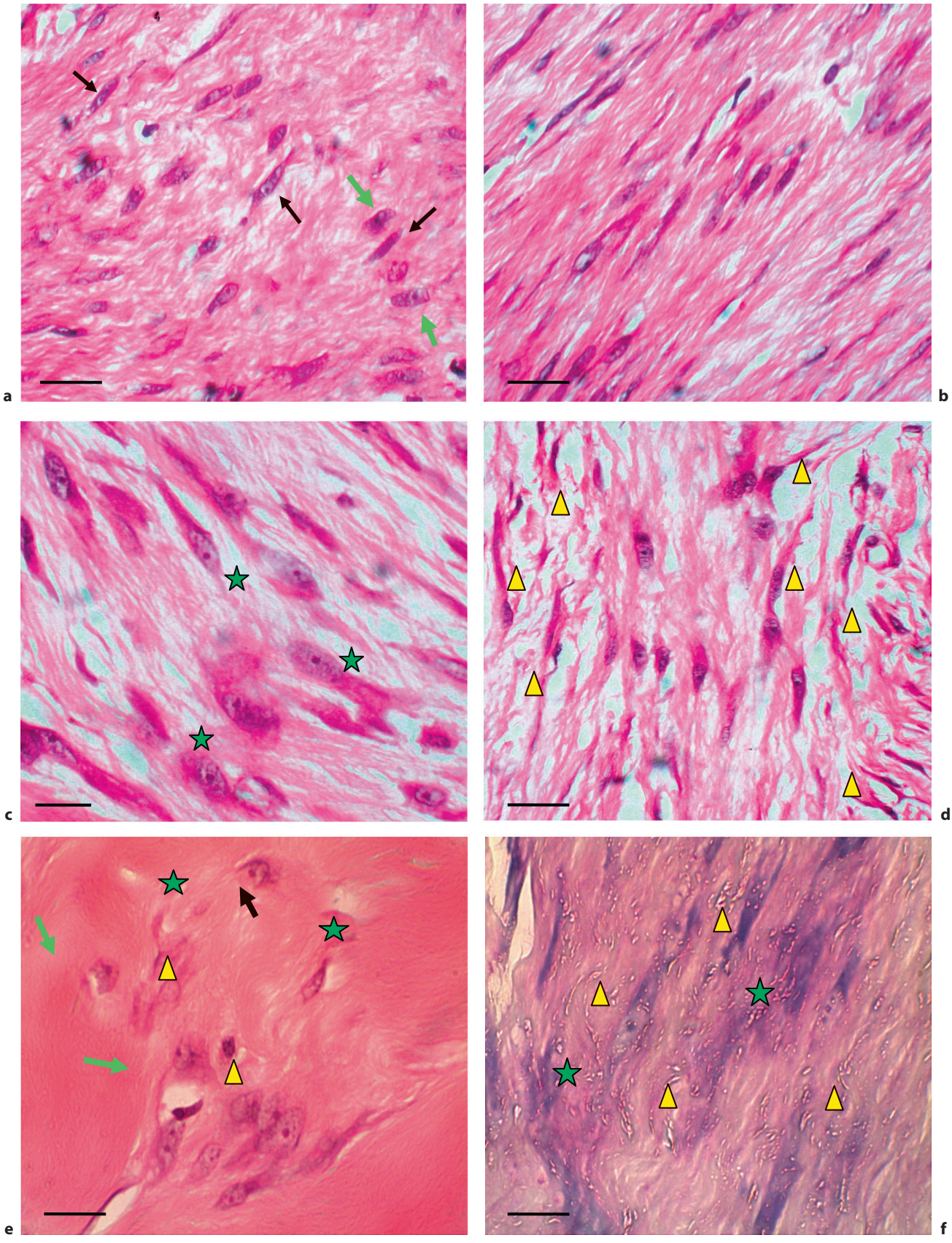
Fine fibrous areas in keloids were divided into the following types:

- fine fibrous cellular areas with many fibroblastic cells and fine fibres;
- fine fibrous less cellular areas with fewer cells and fine fibres;
- wavy fine fibrous areas where cells and fibres showed a wavy configuration;
- fibrous tubular areas where clumps of fine fibres or tubules were attached to a main tubular stalk.

Fine fibrous cellular areas contained increased numbers of cells strewn among loosely arranged collagen fibres and other extracellular matrix constituents. These areas showed diverse cell and fibre morphology, which included: spherical to rounded cells with vesicular nuclei and prominent nucleoli in a stroma of short curvy fibres (fig. 2a); elongated flattened condensed fibroblastic cells longitudinally aligned in the direction of long strands of collagen fibres (fig. 2b); epithelioid cells with plump nuclei, prominent nucleoli and condensed eosinophilic cytoplasm in fine fibrous to myxoid stroma (fig. 2c) and oedematous areas where there was widening of extracellular spaces adjacent to elongated fibroblastic cells with long processes (fig. 2d). The fine fibrous cellular areas were located around HCB areas, between collagen bundles in the HCB region, around perivascular chronic inflammatory areas and below the papillary dermis. Features of degeneration and necrosis were prominent in fine fibrous areas, and these included: membrane damage, swollen nuclei, cytoplasmic and nucleoplasmic lysis, fragmented tubular structures and dissolution of the extracellular matrix adjacent to degenerate cells (fig. 2e, f). Occasionally, degranulating or degranulated mast cells were present within wide intercellular spaces, in the vicinity of degenerate aggregates of epithelioid cells (fig. 3a). Occasional foci of mildly oedematous tissue were scattered within fine fibrous cellular areas; here cells were flattened, elongated, condensed and pyknotic (fig. 3b). Few wavy cells were present within enlarged extracellular spaces and fuzzy, polygonal and atrophic cells were scattered in the area (fig. 3b).

Fig. 1. Photomicrographs of HCB area. **a** Epithelioid (triangles) and fibroblastic cells (stars) in fibrous cellular stroma. Scale bar = 100 μm. HE. **b** Wide extracellular spaces, spherical cells with condensed nuclei (stars). Scale bar = 100 μm. HE. **c** Curved and wavy fibroblastic cells (stars) in wide extracellular spaces. Scale bar = 100 μm. HE. **d** Epithelioid cells with vesicular nuclei and prominent nucleoli (arrows) in fine fibrous stroma and condensed epithelioid and spindle cells in fibro-myxoid stroma (star). Note venule with degenerate mural cells and endothelium with hyperchromatic nuclei and eosinophilic cytoplasm (triangle). Scale bar = 20 μm. HE. **e** High-power photomicrograph of fibromyxoid stroma around early HCB showing epithelioid cells with large hyperchromatic nuclei (triangle), coagulation necrosis (black arrows), cellular atrophy (green stars), spindle cells (yellow arrows), mast cells (blue arrows) and capillary occluded with fibrinoid material (blue star). Scale bar = 20 μm. HE. **f** Mast cell (yellow arrow) in contact with degenerate cells (red arrows) through cellular processes. Note adjacent HCB (stars) and intact spindle cell (triangle). Scale bar = 15 μm. Giemsa.





In less cellular fibrous areas cell necrosis and degeneration of epithelioid cells was pronounced and early accumulation of eosinophilic collagen into small bundles was observed in their vicinity (fig. 3c, d). Some degenerate cells with large nuclei and prominent nucleoli were in palisade (fig. 3c). Microvessels present in fine fibrous less cellular areas were mainly capillaries and post-capillary collecting venules and they displayed degenerate or necrotic endothelium and pericytes; mural cell necrosis was occasionally associated with small calcified deposits (fig. 3e, f).

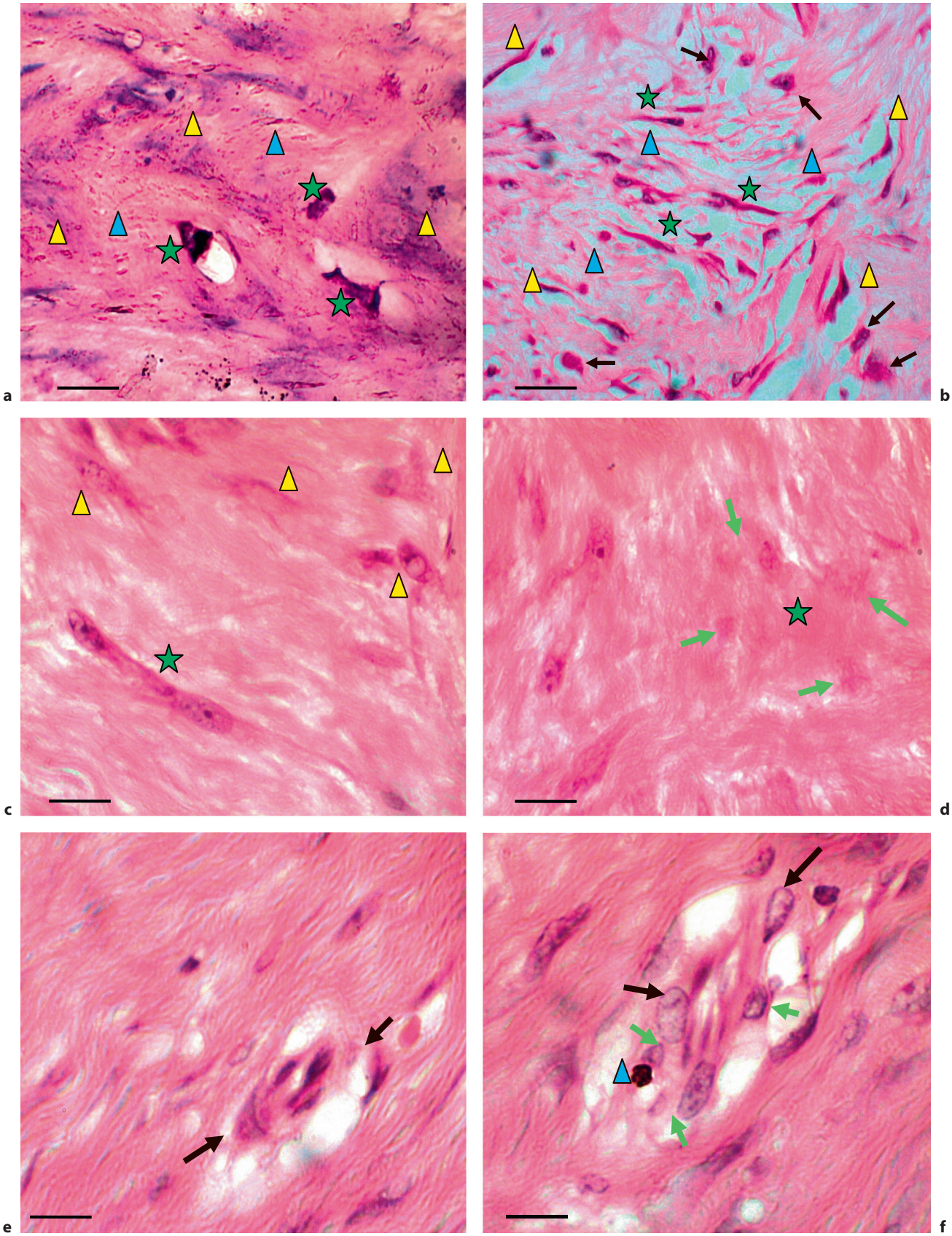
Wavy fibrous areas showed a wavy arrangement of cells and the extracellular matrix. The wavy cells were elongated to very elongated and flattened with bipolar basophilic cellular processes; other cells in this area were spindle-shaped (fig. 4a). The spindle cells contained degenerate or calcified nuclei and often had a fuzzy appearance. Generally, cell degeneration and necrosis were common features and necrotic remnants of cells were strewn in the area (fig. 4a). In some wavy fibrous areas wide extracellular spaces were present between parallel aggregates of eosinophilic collagen fibres, which showed a regular wavy arrangement (fig. 4b). Here calcification of nuclei of spindle and wavy cells was marked. Mast cells were occasionally found in close proximity to degenerate elongated wavy cells and they were located within wide extracellular spaces (fig. 4c).

Fibrous tubular areas comprised refractile, eosinophilic tubular structures that aggregated and attached to stalks; some stalks displayed protruding fibres. These structures were arranged in an assortment of patterns that included: slender stalks from which fringes of fibres of varying lengths emanated (fig. 4d); stalks to which aggregates of intertwined tubules were attached (fig. 4e); stalks attached to triangular aggregates of tubules that entwine into a single stalk (fig. 4f), and stalks enwrapped by winding tubules (fig. 5a). Cells found in fibrous tubular areas were condensed spindle cells and fuzzy epithelioid cells, often found in small aggregates (fig. 5b). Epithelioid cells contained vesicular nuclei with prominent nucleoli and generally were necrotic; their basophilic nuclear remnants were scattered in the extracellular matrix. These cells were embedded in myxoid stroma which had a basophilic tinge and was less eosinophilic and non-refractile when compared with the stroma associated with pyknotic spindle cells (fig. 5b). In some areas it appeared as if degenerate and necrotic cells were drawn into entwining tubular structures and enwrapped by them (fig. 5c). Mast cells were scattered in the vicinity of the degenerate cells enshrouded by tufts of fibres; the mast cells here displayed rounded, elongated and degranulating forms (fig. 5d). Fibrous tubular areas were poorly vascularised; few degenerate microvessels, often showing stages of development, were found here. Occasional differentiated venules were present; they exhibited necrotic or degenerate pericytes and endothelial cells; these endothelial cells frequently contained large calcified nuclei (fig. 5e, f).

Areas of Inflammation

Severe, moderate to mild paravascular inflammation (fig. 6a) was observed around the microvessels of the subpapillary plexus at the papillary dermis/keloid border and around hair follicles and sebaceous glands at the lateral keloid/non-lesional dermal border. It was noted that the 'younger' (<1 year) keloids showed increased severity of inflammation when compared with the older keloids (>2 years). Blood vessels associated with the inflammatory response were mainly venules and they generally displayed compressed lumen, degenerate and calcified endothelium and degenerate to necrotic mural cells embedded in eosinophilic material (fig. 6b). Mural cells outside the eosinophilic material were usually grossly swollen, vacuolated and necrotic (fig. 6b). Other features of degeneration and necrosis observed in the area were nuclear and plasma membrane damage, cytoplasmic and nucleoplasmic clearing and varying stages of cell atrophy (fig. 6c). Many lymphocytes, plasma cells and epithelioid cells were present in enlarged extracellular spaces, some of which contained myxoid material (fig. 6c). Inflammatory cells frequently exhibited calcified nuclei. Endothelial cells of inflamed vessels showed features of proliferation, pyknosis, swelling and necrosis (fig. 6c). Fine tubular structures and thin cellular processes were found around the wide extracellular spaces in areas of inflam-

Fig. 2. Photomicrograph of fine fibrous areas. **a** Spindle (black arrows) and spherical cells (green arrows) in stroma of short curvy fibres. Scale bar = 20 μm. HE. **b** Elongated flattened fibroblastic cells aligned parallel to collagen fibres. Scale bar = 20 μm. HE. **c** Epithelioid cells with large nuclei, prominent nucleoli and eosinophilic cytoplasm (stars). Scale bar = 20 μm. HE. **d** Wide extracellular spaces adjacent to elongated fibroblastic cells with long processes (triangles). Scale bar = 20 μm. HE. **e** Necrotic epithelioid cells showing plasma and nuclear membrane damage (black arrow), cytoplasmic and nucleoplasmic lysis (green arrows) atrophy (stars) and necrosis (triangles). Scale bar = 20 μm. HE. **f** Fragmented tubular structures (triangles) and necrosis of elongated fibroblastic cells (stars). Scale bar = 20 μm. Giemsa.



mation where features of necrosis were less marked (fig. 6d). Away from the sub-papillary plexus and deeper into the keloid, paravascular inflammation was conspicuously decreased or absent. General histopathological features associated with decreased paravascular inflammation were calcification of nuclei of inflammatory cells, and demarcation of the inflamed vessel by eosinophilic refractile tubular structures, while vessels that were not inflamed displayed deposition of eosinophilic substance in the wall and in adjacent less dense connective tissue that often showed some regular pattern of arrangement (fig. 6e, f). Also, uninflamed vessels in keloids showed better progress with regard to stage of differentiation, albeit they were not fully differentiated.

Zone of Dense Regular Connective Tissue

The zone of dense regular connective tissue (DRCT) comprised parallel bundles of collagen of varying thickness and length with scattered fibroblastic cells between the bundles. These bundles were in straight or wavy array and they were eosinophilic or pale-staining with eosin (fig. 7a, b).

When compared with the pale DRCT which showed a looser arrangement of fibres, in the eosinophilic type the fibres were more compactly arranged (fig. 7a, b). Fibroblastic cells associated with DRCT were spindle-shaped, elongated, slender and condensed and they frequently exhibited a fuzzy appearance; fuzzy cells were usually degenerate or necrotic (fig. 7c). In the wavy DRCT, fibroblastic cells often displayed a wavy contour and were elongated or spindle-shaped, slender and condensed (fig. 7b, d). It was noted that condensed spindle cells were occasionally closely associated with mast cells (fig. 7d). Blood vessels were scattered between the bundles in the DRCT, and many of these vessels were severely compressed, mildly inflamed and were surrounded by wide extracellular spaces (fig. 7e). Plasma cells were predominant in the paravascular inflammatory infiltrate (fig. 7f) in the region of DRCT. Mural cell degeneration associated with plasma cells was observed in inflamed microvessels, even when a paucity of plasma cells was present (fig. 8a). Wide extracellular spaces were often present between eosinophilic collagen bundles in the zone of DRCT with both straight and wavy configurations, and microvessels adjacent to these spaces suffered severe compression (fig. 8b, c).

Nodular Fibrous Areas

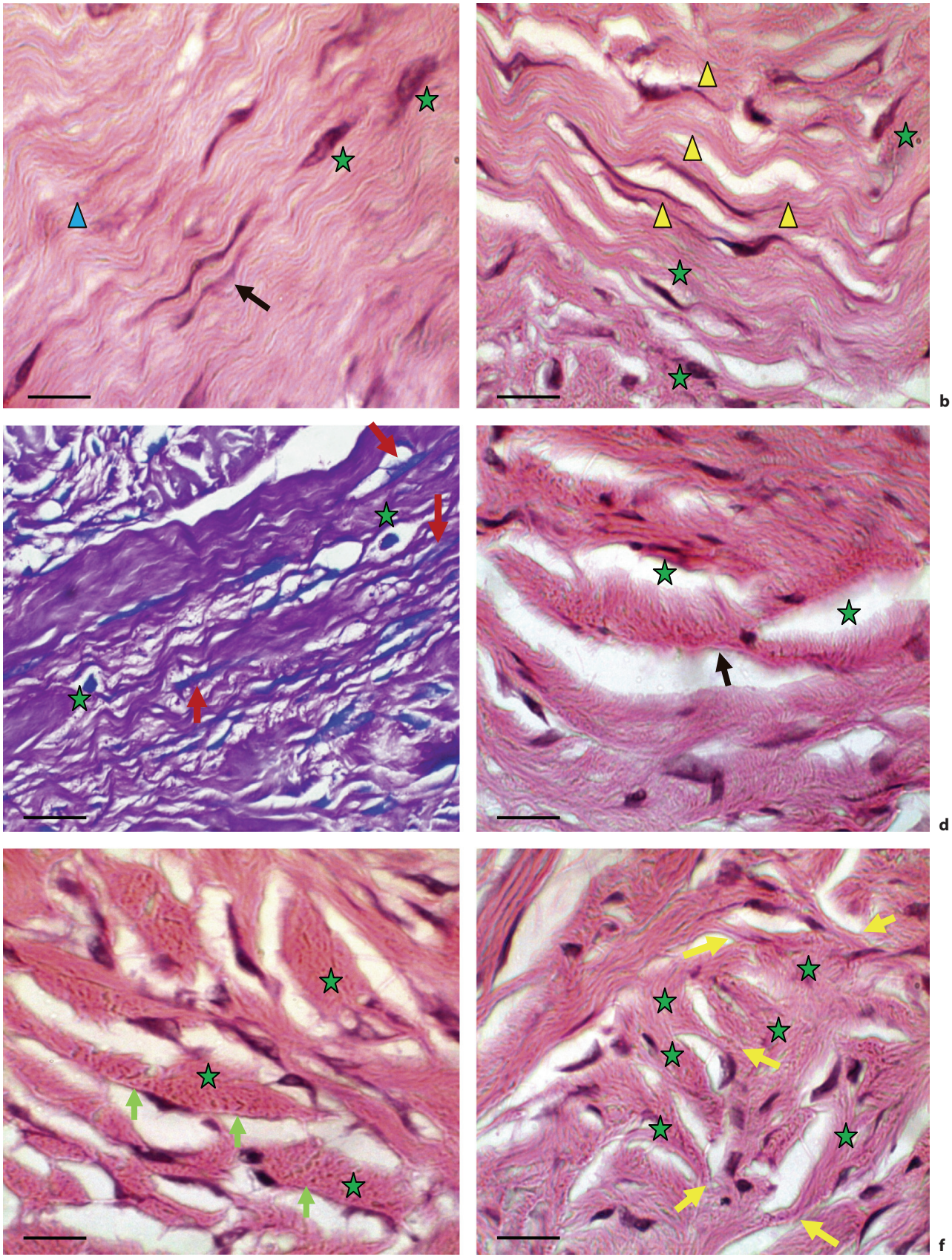
Nodular fibrous areas contained fine fibrous connective tissue arranged in a whorled pattern or as tufts of fine fibres branching from long stems of connective tissue (CT) or as fibrous tufts associated with fibroblasts (fig. 8d). The stems and branches of CT in the nodule showed similar arrangement to that in fibrous tubular

Fig. 3. a, **b** Fine fibrous cellular area. **a** Degranulated mast cells within wide extracellular spaces (stars) surrounded by necrotic epithelioid cells (yellow triangles) and tubular structures (blue triangles). Scale bar = 20 μm. Giemsa. **b** Wide extracellular spaces, associated condensed elongated cells (stars), wavy cells in enlarged extracellular spaces (yellow triangles), fuzzy polygonal (arrows) and atrophic cells (blue triangles). Scale bar = 20 μm. HE. **c–f** Less cellular fibrous area. **c** Necrotic epithelioid cells (triangles) and degenerate palisading cells (star) with large nuclei and prominent nucleoli. Scale bar = 20 μm. HE. **d** Marked cell necrosis (arrows) associated with early accumulation of eosinophilic collagen into small bundles (star). Scale bar = 20 μm. HE. **e** Capillary with necrotic pericytes (arrows). Scale bar = 20 μm. HE. **f** Venule with degenerate (black arrows) and necrotic (green arrows) pericytes. Note small calcified deposit (blue triangle) associated with necrotic pericyte. Scale bar = 20 μm. HE.

(For figures 4 and 5 see next pages.)

Fig. 4. a–c Wavy fine fibrous area. **a** Elongated flattened wavy cells (arrow), degenerate spindle cells (stars) and necrotic remnants (triangle). Scale bar = 20 μm. HE. **b** Wide extracellular spaces between parallel aggregates of eosinophilic collagen fibres, spindle (stars) and wavy (triangles) cells with calcified nuclei. Scale bar = 20 μm. HE. **c** Mast cells (stars) surrounded by wide spaces in close proximity to degenerate elongated wavy cells (arrows). Scale bar = 20 μm. Giemsa. **d–f** Fibrous tubular area. **d** Tubular stalk (arrow) from which fibrous fringes emanate (stars). Scale bar = 20 μm. HE. **e** Stalks (arrows) to which aggregates of intertwined tubules (stars) attach. Scale bar = 20 μm. HE. **f** Triangular aggregates of tubules (stars) that entwine to form stalks (arrows). Scale bar = 20 μm. HE.

Fig. 5. Fibrous tubular area. **a** Stalks enwrapped by tubules (arrows). Scale bar = 30 μm. HE. **b** Pyknotic spindle cells (triangle) and aggregates of necrotic fuzzy epithelioid cells embedded in myxoid stroma with a basophilic tinge (green stars). Note myxoid stroma is less eosinophilic and non-refractile when compared with adjacent stroma associated with pyknotic spindle cells (blue stars). Scale bar = 20 μm. HE. **c** Pyknotic spindle cells (stars) drawn into entwining eosinophilic tubules (triangles) and enwrapped necrotic spindle cells (arrow). Scale bar = 20 μm. HE. **d** Rounded (star), elongated (yellow arrow) and degranulated (red arrow) mast cells close to degenerate cells (orange arrows) enshrouded by tufts of fibres (triangles). Scale bar = 20 μm. Giemsa. **e** Degenerate developing microvessels (star). Scale bar = 20 μm. HE. **f** Venule (triangle) exhibiting endothelial cells with large calcified nuclei (arrows) and degenerate pericytes (stars). Scale bar = 20 μm. HE.



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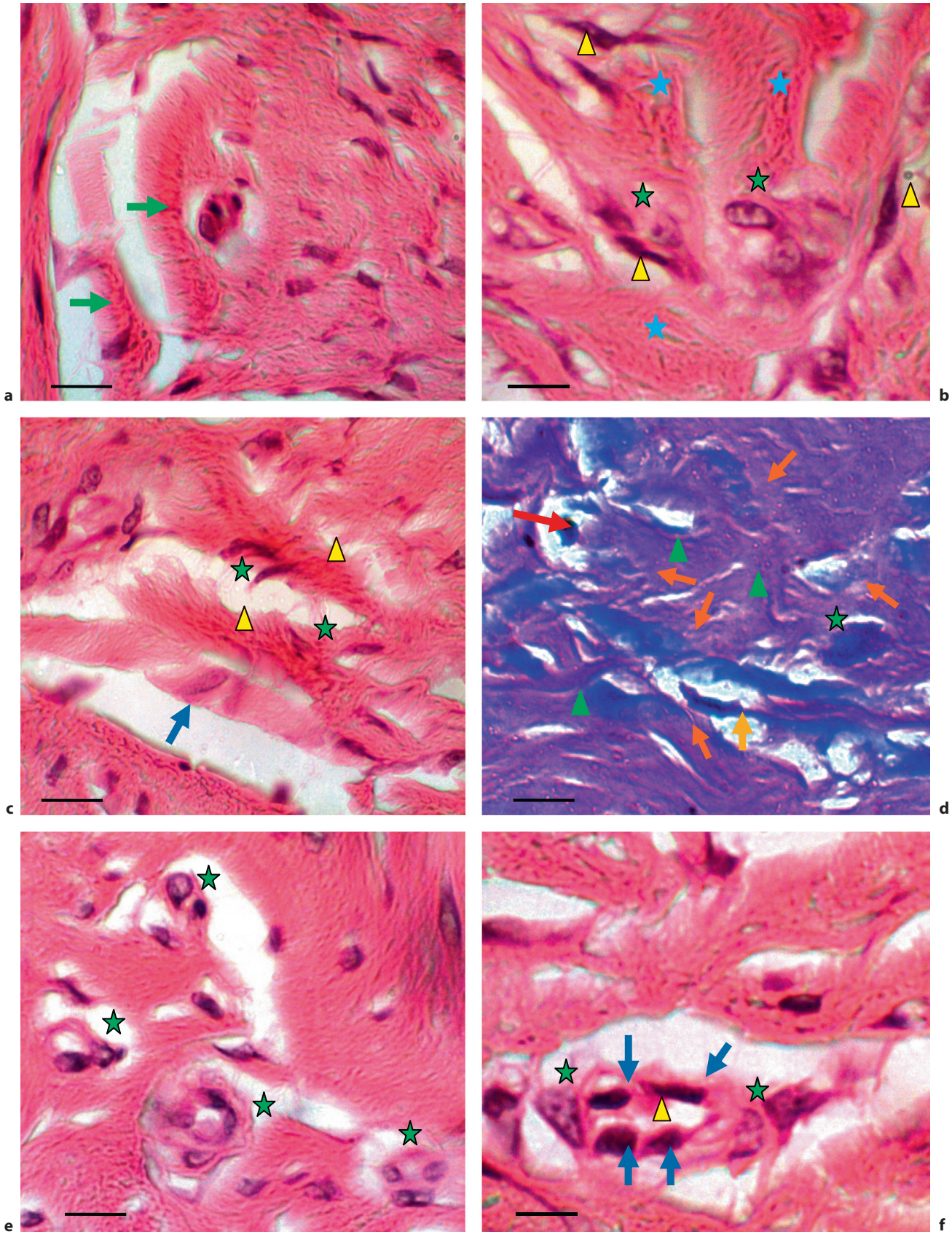

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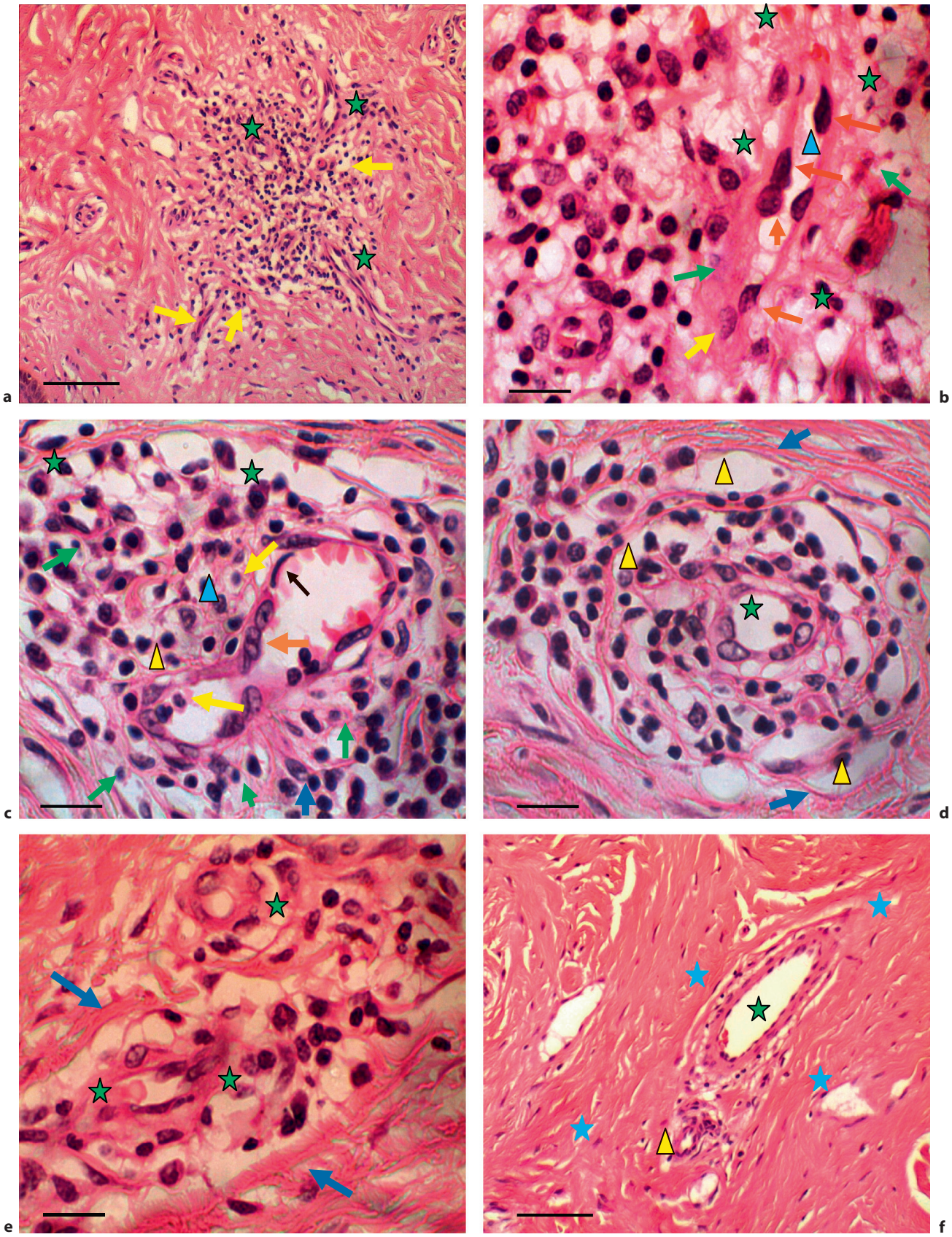


areas; sometimes the CT exhibited a wavy pattern where the small wavy bundles were separated by wide extracellular spaces (fig. 8e). Small blood vessels with thickened eosinophilic hyalinised walls and small or obliterated lumen were present in wide extracellular spaces located within or outside the nodules (fig. 8d–f). These vessels generally displayed degenerate or necrotic mural cells. The cells within the nodules were plump fibroblastic cells and they often showed space-forming characteristics; these cells were mostly degenerate. Cells at the edge of or around the nodules were spindle-shaped (fig. 9a). Some nodular fibrous areas contained early HCB interspersed in fine fibrous connective tissue (fig. 9b); their formation appeared to be by curling of bands of CT demarcated by wide extracellular spaces containing compressed blood vessels (fig. 9c). Areas of paravascular inflammation around compressed microvessels were occasionally present adjacent to nodules (fig. 9d) close to the keloid-papillary dermis border and some of the inflammatory cells appeared to infiltrate the adjacent nodular area. The inflammatory infiltrate contained multinucleate giant cells and lymphocytes amassed in myxoid material; some lymphocytic cells with eosinophilic cytoplasm were in palisade (fig. 9e). Hyalinisation of occluded blood vessels and adjacent bundles of collagen and associated widening of extracellular spaces were occasionally observed around nodular areas (fig. 9f).

Areas of Angiogenesis

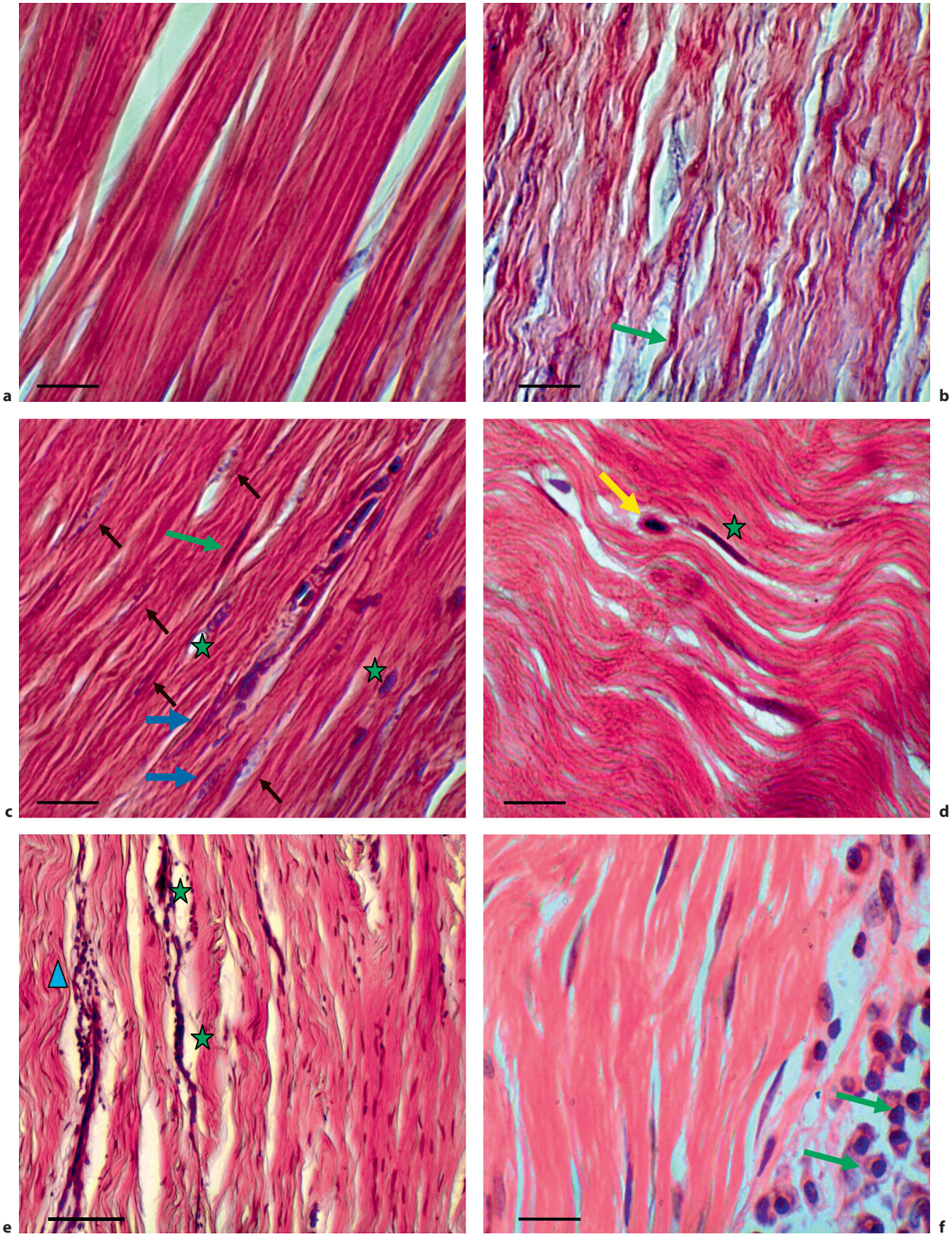
Areas of angiogenesis displayed endothelial buds and progressive stages of differentiation of small blood vessels and small neurovascular bundles (fig. 10a). These areas were located in the keloid, in close proximity to the borders between keloid and non-lesional skin and between keloid and the papillary dermis. Areas of angiogenesis were usually located below inflamed vessels of the subpapillary plexus and they were smaller and less frequently observed in keloids that were more than 2 years old. Many morphological abnormalities were observed in areas of neovascularisation and these included: compression of small blood vessels, disorganisation and hyalinisation of connective tissue fibres around compressed and degenerate vessels (fig. 10b), coagulation necrosis and dissolution of mural cells of small blood vessels and of small peripheral nerves (fig. 10c). Hyalinisation of small aggregates of fibres was evident in close proximity to developing neurovascular bundles (fig. 10c). Deeper into the keloid these bundles showed degeneration and loss of

Fig. 6. Photomicrographs of area of inflammation. **a** Moderate inflammation around microvessels (stars) of the sub-papillary plexus. Note compressed vessels (arrows). Scale bar = 100 μm. HE. **b** Inflamed venule (blue triangle) showing degenerate (orange arrows) and calcified (red arrows) endothelial cells and degenerate (yellow arrow) to necrotic (green arrows) mural cells embedded in eosinophilic material. Outside this, mural cells are swollen and vacuolated (stars). Scale bar = 20 μm. HE. **c** Cytoplasmic (yellow arrow) and nucleoplasmic (blue arrow) lysis, atrophic cells (green arrow), lymphocytes (yellow triangle), plasma cells (stars), epithelioid cells (blue triangle), proliferative (orange arrow) and pyknotic (black arrow) endothelial cells. Scale bar = 20 μm. HE. **d** Inflamed microvessel (star) with swollen and necrotic endothelial cells and wide extracellular spaces (triangles) surrounded by tubular structures and thin cellular processes (arrows). Scale bar = 20 μm. HE. **e** Mild paravenular inflammation in keloid. Note mural cell necrosis (stars) and demarcation of areas of inflammation by eosinophilic refractile tubular structures (blue arrows). Scale bar = 20 μm. HE. **f** Absence of paravascular inflammation within keloid and presence of eosinophilic material in wall of arteriole (green star), venule (triangle) and some adjacent collagen bundles (blue stars). Scale bar = 100 μm. HE.

(For figures 7 and 8 see next pages.)

Fig. 7. Photomicrographs of zone of DRCT. **a** Parallel eosinophilic bundles of collagen. Scale bar = 20 μm. HE. **b** Pale DRCT with a looser arrangement of fibres and wavy configuration. **c** Fibroblastic cells that were spindle-shaped (stars), elongated (blue arrows), slender and condensed (green arrow), or fuzzy and degenerate or necrotic (black arrows). Scale bar = 20 μm. HE. **b**, **d** Wavy DRCT with wavy cells (green arrow) and condensed spindle cell (star) closely associated with mast cells (yellow arrow). Scale bars = 20 μm. HE. **e** Compressed (stars) and mildly inflamed (triangle) microvessels between bundles of DRCT separated by wide extracellular spaces. Scale bar = 100 μm. HE. **f** Plasma cells (arrows) in the inflammatory infiltrate in areas with DRCT. Scale bar = 20 μm. HE.

Fig. 8. a–c Photomicrographs of zone of DRCT. **a** Mural cell degeneration (stars) associated with few plasma cells (arrows) around mildly inflamed venules. Scale bar = 20 μm. HE. **b**, **c** Wide extracellular spaces (triangles) between eosinophilic collagen bundles with straight or wavy configurations. Note compressed microvessels (stars). Scale bars = 100 μm. HE. **d–f** Photomicrographs of nodular fibrous areas. **d** Whorled arrangement of fine fibrous tissue and intra-nodular microvessels with thickened eosinophilic walls and small or obliterated lumen present in wide extracellular spaces (stars). Scale bar = 100 μm. HE. **e** Tufts of fine fibres (black arrows) branching from long stems of CT (green arrow) or associated with fibroblasts (blue arrows). Wavy arrangement of branching CT separated by wide extracellular spaces (stars). **e**, **f** Small blood vessels with thickened eosinophilic hyalinised walls (yellow arrows) and small or obliterated lumen (orange arrows) present in wide extracellular spaces located outside the nodules. Scale bars = 100 μm. HE.



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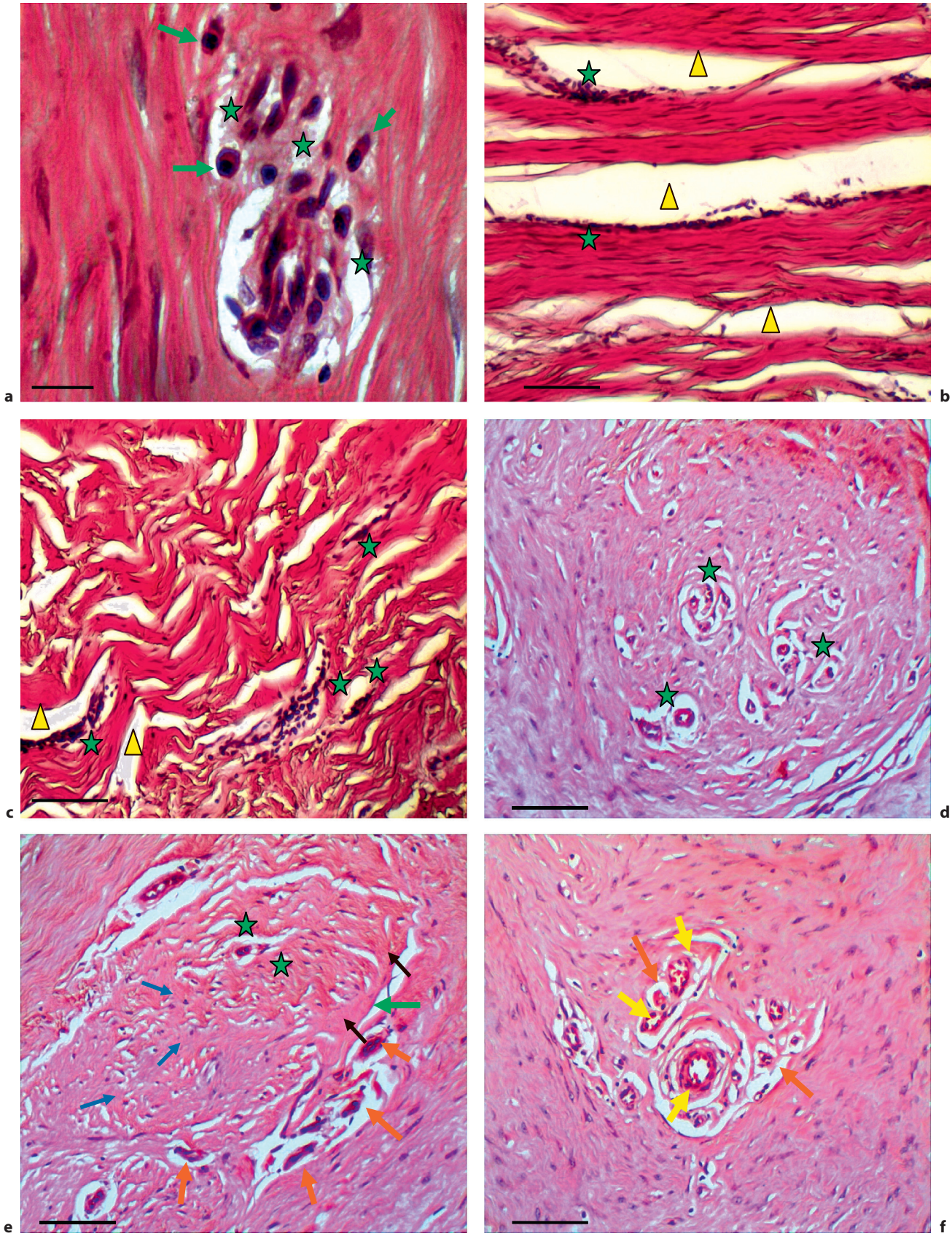

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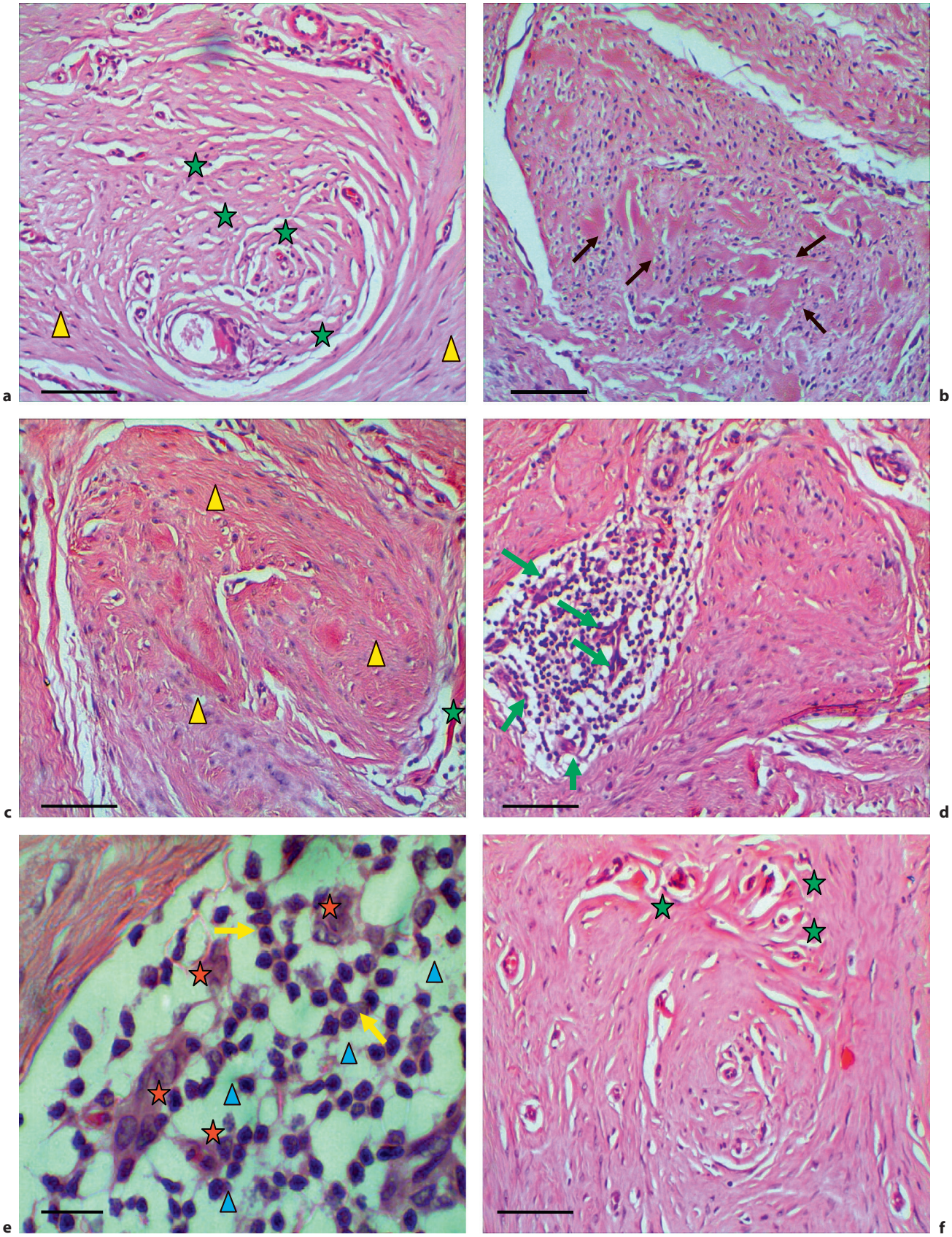


small peripheral nerves, hyalinisation and necrosis of mural cells and change in morphology of the surrounding CT to a fine fibrous cellular type (fig. 10d). Occasional areas of angiogenesis were isolated within nodules of sparse CT with enlarged extracellular spaces (fig. 10e); some intranodular vessels progressed to differentiated arterioles (fig. 10f). It was noted that blood vessels isolated within nodules displayed less severe pathological changes than developing vessels not contained within nodules, where inflammation was evident and necrosis augmented. Inflammation around developing blood vessels was usually mild and the infiltrate contained occasional mast cells and few lymphocytes, of which some were plasma cells. Inflammatory cells here displayed calcified nuclei and eosinophilic cytoplasm (fig. 11a, b). Some developing blood vessels displayed mural hyalinisation and this appeared to preserve the tissue, albeit the cells were degenerate. In comparison, blood vessel walls that were unhyalinised exhibited lytic change of mural cells (fig. 11c, d). Occasionally, calcified debris associated with cellular remnants was found in wide extracellular spaces adjacent to blood vessels with degenerate and necrotic mural cells (fig. 11e, f). The connective tissue around degenerate blood vessels in areas of angiogenesis displayed oedematous change due to the leaky vessels (fig. 11b–f).

Discussion

The results of this study showed that keloids comprise an assortment of proliferative connective tissue elements which displayed a regional pattern of arrangement, and that the microvascular supply to each of these regions was impaired. Even in the areas of angiogenesis the vascular supply was inefficient, as very few differentiated functional blood vessels were found here. The features of degeneration and necrosis identified in all regions of the keloid were, presumably, a manifestation of the lack of nutrients and oxygen to these cells. Why was the vascular supply to keloid regions impaired? Inflammation of the microvessels of the sub-papillary plexus and the ensuing damage and destruction of these vessels was the apparent reason. The inciting factor of the inflammation appears to originate in the vicinity of the sub-papillary plexus located between the unaffected papillary dermis and the keloidal lesion. The selective adverse effect on the reticular dermis while the adjacent papillary dermis is unaffected is an enigma. Sub-papillary vascular injury in keloids was associated with significant paravascular tissue damage, chronic inflammation and fibrosis, which are the hallmark features of chronic inflammation.

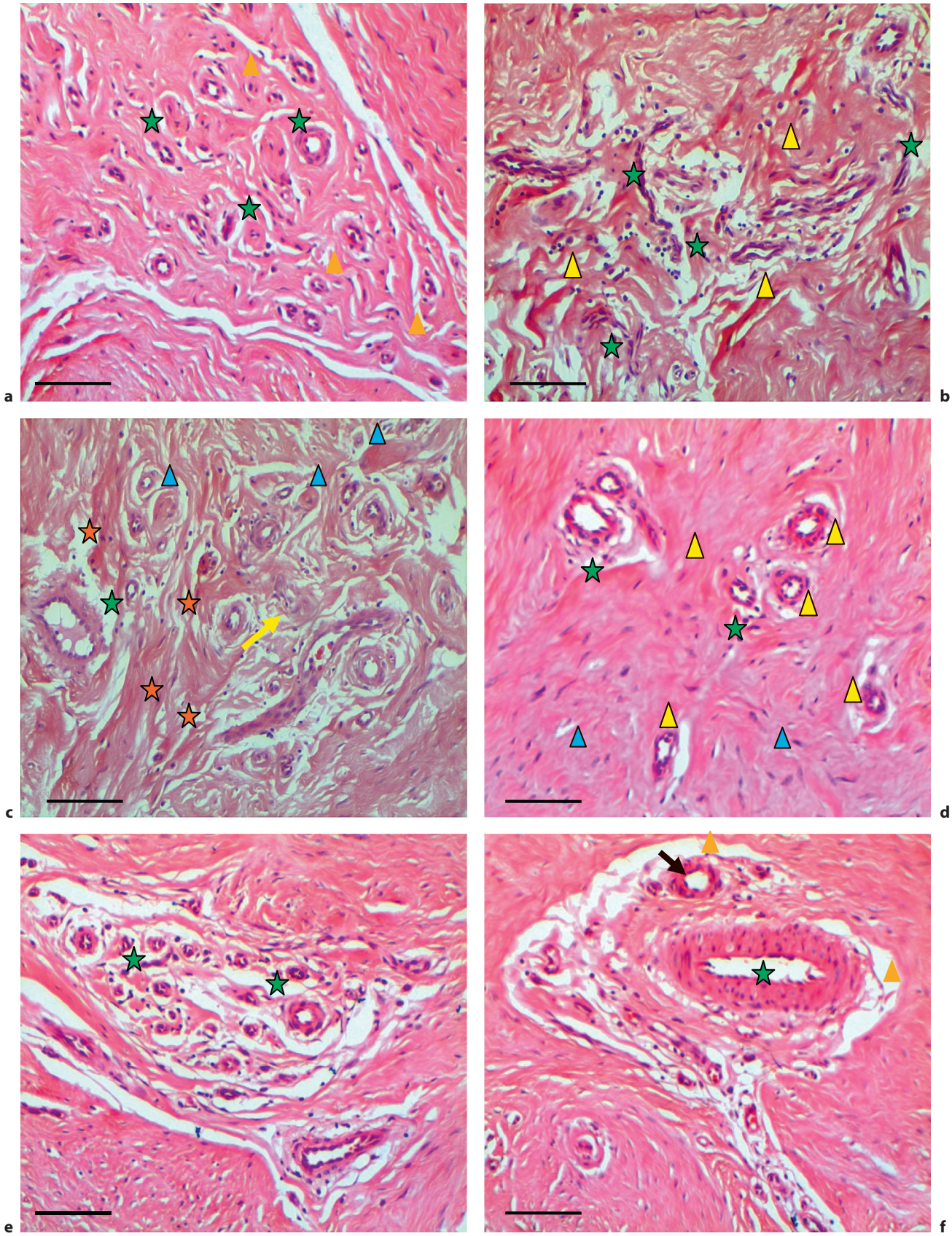
As acute inflammation was not observed in the keloid specimens examined and as multinucleate giant cells were present in areas of inflammation together with lymphocytes and plasma cells, the subset of chronic inflammation found in keloids would be specific chronic inflammation. However, as no infectious agent or foreign bodies were identified, it is uncertain as to whether the

Fig. 9. Photomicrographs of nodular fibrous areas. **a** Plump fibroblastic space-forming cells (stars) within the nodules and spindle cells (triangles) in the fibrous tissue around the nodules. Scale bar = 100 μm. HE. **b** Early HCB (arrows) interspersed in fine fibrous cellular tissue. Scale bar = 100 μm. HE. **c** Curling bands (triangles) of CT separated by wide extracellular space containing compressed blood vessels (star). Scale bar = 100 μm. HE. **d** Area of moderate inflammation around compressed blood vessels (arrows) adjacent to nodules. Scale bar = 100 μm. HE. **e** Multinucleate giant cells (stars), myxoid material (triangles) and palisading eosinophilic lymphocytes (arrows) in the inflammatory infiltrate. Scale bar = 20 μm. HE. **f** Para-nodular hyalinisation of occluded microvessels and adjacent bundles of collagen and associated widening of extracellular spaces (stars). Scale bar = 100 μm. HE.

(For figures 10 and 11 see next pages.)

Fig. 10. Photomicrograph of areas of angiogenesis. **a** Stages of differentiation of small blood vessels (triangles) and small neurovascular bundles (stars). **b** Compression of microvessels (stars), disorganisation and hyalinisation of connective tissue fibres (triangles). **c** Coagulation (green star) and dissolution (arrow) necrosis of mural cells of small blood vessels, degeneration of small peripheral nerves (triangles), and early hyalinisation of small aggregates of collagen fibres (orange stars). **d** Hyalinisation of walls of microvessels (yellow triangles), degeneration of peripheral nerves in neurovascular bundles (stars) and change in morphology of CT surrounding hyalinising blood vessels to a fine fibrous cellular type (blue triangles). **e**, **f** Enlarged extracellular spaces around developing (stars) and differentiating blood vessels (triangles). **f** Note advanced differentiation of arteriole (star) and venule (arrow) enclosed within nodules. **a–f** Scale bar = 100 μm. HE.

Fig. 11. Photomicrograph of areas of angiogenesis. **a** Mild inflammation (star). Scale bar = 100 μm. HE. **b** Scattered inflammatory cells with calcified nuclei and eosinophilic cytoplasm, including mast cells (arrows), plasma cell (star) and lymphocytes (triangles). Scale bar = 20 μm. HE. **c**, **d** High power of coagulation necrosis of mural cells in unhyalinised wall (stars) or partially unhyalinised wall (arrow) of blood vessels and preservation of cells in hyalinised wall of blood vessel (triangles). Note paravascular lymphocytic inflammation in **c**. Scale bars = 20 μm. HE. **e**, **f** Calcified deposits (arrows) in the vicinity of mural cell necrosis (star) and degeneration (triangle). Scale bars = 20 μm. HE.



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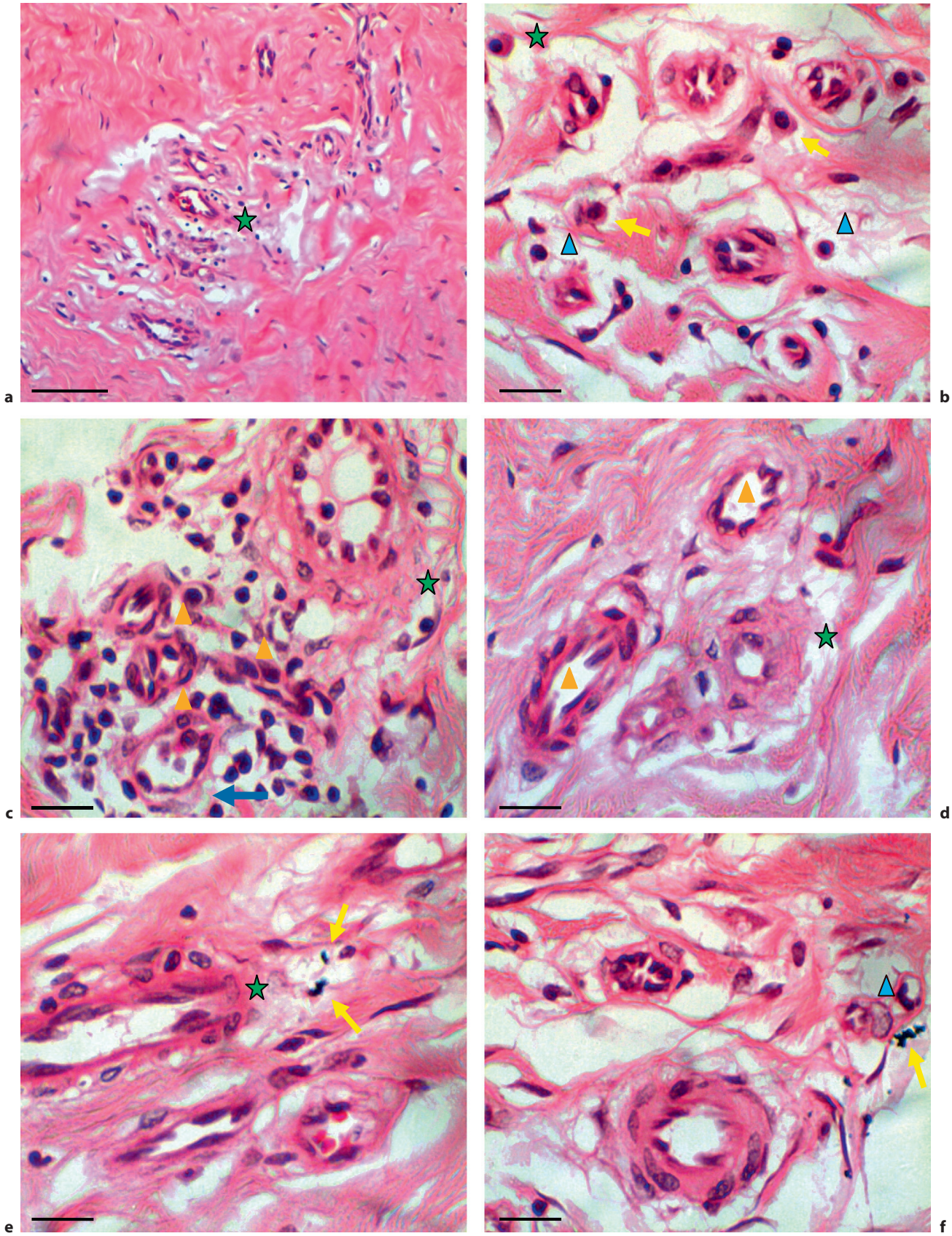

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mechanism involved in this type of inflammation is immune or non-immune. Specific chronic inflammation appears de novo in response to certain types of injurious agents and heals by fibrosis.

In keloids, fine fibrous cellular areas in the vicinity of chronic inflammation are morphologically similar to fibrous granulation tissue of normal wound healing. This must have progressed from earlier vascular granulation tissue, which in normal healing fills the site of active tissue damage and inflammation and is typified by proliferating vessels and fibroblasts. In keloids, areas of angiogenesis and fibrous cellular areas may represent exaggerated phases of the vascular granulation tissue stage of the healing process. This is in keeping with keloids being described as scarring that is the end result of an 'overexuberant' healing response. In normal wound healing, over time the vessels of vascular granulation tissue regress, collagen fibrous tissue matures and is remodelled in an orderly fashion to eventually form fibrous scar tissue. Remodelling of the mature collagen fibres of fibrous granulation tissue in keloids was evident in fine fibrous areas and wavy fine fibrous areas with a regular arrangement and in regions of DRCT. These areas of collagen remodelling in keloids showed increase in size and number when compared with normal wound healing.

From the above, we could expand the description of keloids to a form of excessive wound healing that occurs when the healing stage of chronic inflammation is impaired. Some important factors associated with impaired wound healing are poor blood supply, persisting tissue damage and sequestered dead tissue. All of these features were present in keloids. Microvessels in all keloid regions displayed damage, evident as endothelial and mural cell injury and constriction of the lumen. Also, tissue degeneration and necrosis were found in all keloid regions, which, most likely, was a consequence of the diminished blood supply resulting from poor lesional vascularisation and the impaired nature of these microvessels. Hypoxia might be a contributory factor causing cell injury and cell death in paravascular tissue in areas of inflammation and angiogenesis, while ischaemic loss of blood supply from impeded arteriolar flow would be the most likely apparent cause of cell death in all keloid regions, especially in the HCB, fine fibrous and nodular fibrous areas where the microvasculature appeared non-functional, resulting in a lack of blood supply and the subsequent degeneration and necrosis of tissue. The enlarged extracellular spaces present in these areas result, presumably, from the reduced venous drainage characteristic of ischaemia. Venules were the predominant microvessels showing constriction, occlusion and mural damage in keloids.

While cell death and tissue necrosis were pronounced in all keloid zones, the processes for removal of dead tissue were lacking. Macrophages, which were plentiful in the papillary dermis overlying keloids, were absent within the keloid. This resulted in the accumulation of large aggregates of dead tissue in all keloid regions, contributing to the bulky nature of the lesion. The decreased cell numbers in the less cellular fibrous regions where necrosis was pronounced appear to be a direct consequence of cell death and digestion, perhaps by autolytic enzymes.

Cellular proliferation was marked in fine fibrous cellular areas and in areas of angiogenesis. The paradox of proliferation of connective tissue cells and fibres in a hypoxic or ischaemic environment could be explained by results of in vitro studies which showed the reduced growth-factor requirement of keloid-derived fibroblasts [Russell et al., 1988]. Also, it was reported that the ATP content of keloids remained high for prolonged periods of time and that the pathway employed for the production of this ATP was anaerobic glycolysis [Ueda et al., 2004]. However, most of the fibroblast cells in the various keloid regions were degenerate or necrotic, implying that, in the long term, there is deprivation of nutrients, oxygen and other blood-borne essential factors; this is, presumably, a consequence of the ischaemic effects discussed earlier. In the less cellular fibrous regions, necrosis was pronounced and the decreased cell numbers here appear to occur as a direct result of cell death and autolysis. In addition to an increase in their numbers, keloid fibroblasts also overproduced extracellular matrix components, the main ones being collagen type I [Uzawa et al., 1998] and collagen type III [Naitoh et al., 2001] which was evident in all the fibrous regions. Collagen overproduction by keloid fibroblasts has been shown to be stimulated by transforming growth factors (TGF)-β1, -β2 and -β3 which are expressed in increased amounts in keloid fibroblasts [Robles and Berg, 2007; Murata et al., 1997]. The copious production of collagen in keloids was further augmented by the higher levels of TGF-β receptor 1 found in keloid fibroblasts [Sun et al., 2006], the proliferative state and prolonged survival of keloid fibroblasts, their lower rates of apoptosis and a down-regulation of apoptosis-related genes in these fibroblasts [Robles and Berg, 2007].

In normal wound healing the collagen is remodelled in an orderly pattern and appropriately orientated to withstand tensile forces exerted on the area of repair. In keloids, in addition to remodelling zones that are similar to that in normal scars, there are a range of orderly pat-

terns of arrangement of collagen in the zones of dense regular, less dense regular and wavy connective tissue as well as in nodular fibrous areas. These patterns of collagen arrangement, most likely, are formed in response to prolonged stresses exerted, presumably, to offer greater resistance to traction forces. These forces are known to be generated by cells during cell adhesion to the extracellular matrix and are important for wound healing, especially during extracellular matrix remodelling and angiogenesis [Kiehart et al., 2000; Sottile, 2004; Friedl et al., 2004]. When traction is sufficient to deform the extracellular matrix network, it results in net alignment of collagen fibres; cells that come in contact with these aligned fibres move with bidirectional bias along the axis of fibre alignment [Tranquillo and Murray, 1992]. This leads to an uneven distribution of cells; cell accumulation in cellular areas of keloids may have occurred as a result of this. Cell traction-generated forces also modify DNA and protein synthesis in cells and cell differentiation [Tranquillo and Murray, 1992]; effects of all these modifications are evident in keloid fibroblasts. These include upregulation of collagen type I [Uzawa et al., 1998]; collagen type III, heat shock protein 47 [Naitoh et al., 2001]; enhanced expression of IL-6 and its receptors in keloid fibroblasts, with a concomitant increase in collagen biosynthesis [Uitto, 2007]; up-regulation of 250 genes, including TGF-β1 and nerve growth factor and down-regulation of 152 genes [Chen et al., 2003]. The ability of keloid fibroblasts to generate traction forces was demonstrated in a study where these fibroblasts populated a collagen lattice and significantly increased lattice contraction when compared with lattices populated by normal fibroblasts [Mukhopadhyay et al., 2005].

The regional distribution of proliferative and degenerating connective tissue elements described in this study reinforces that scar tissue is dynamic and shows that keloids encompass not only the remodelling/scar maturation stage, which in the form of aligned bundles of collagen composes normal scar tissue, but also earlier wound healing stages of angiogenesis and inflammation. Persistent inflammation is known to continually stimulate TGF-β-induced matrix deposition by fibroblastic cells; this would contribute to both growth and heterogeneity (varying stages of the healing process) of keloid specimens [Border and Noble, 1994]. This is evident at both the microscopic (this study) and macroscopic levels [Bayat et al., 2004]. The heterogenous appearance of keloids may also be an outcome of the wide range in age of the lesions (5 months to 6 years). The dynamic processes ongoing in keloids, the persistent inflammation and wide range of keloid age, thus, could explain past controversies regarding keloid structure. These discrepancies could also be clarified as follows from this study, which outlines the basic regions morphologically identified in keloids. Researchers viewed specimens that showed the presence or enlarged versions of the regions that they reported on and the absence of other regions that would contradict their results. For example, with regard to the contradictory data on the vascularisation of keloids, studies that report a higher number of vessels in keloids [Amadeu et al., 2003] might have viewed areas of angiogenesis in specimens or these areas might have been enlarged here, while other studies that showed a reduced vascular component in keloids [Ueda et al., 2004] might have assessed biopsies where areas of angiogenesis were absent.

The regional distribution of proliferative and degenerating connective tissue elements described in this study broadly encompasses most of the morphological characteristics of keloids, and provides a baseline which permits categorizing of future results into each of these regions. From ongoing studies, it is anticipated that, using immunocytochemistry, patterns of antigen localization pertinent to certain regions will emerge. Such synchronization will contribute meaningfully towards working out aspects of the pathogenesis of keloid formation. This study provides a structured basis for future ultrastructural research of keloids, whereby investigations could focus on one region at a time, before reconstructing into the holistic result. As keloids are a prototype of fibroproliferative disorders, a similar regional approach could be used to investigate the pathogenesis of other disorders within this group of diseases.

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Does the paucity of elastic fibres contribute to the process of keloidogenesis?

Bux S [1], Madaree A [2].
1.Bsc, MMedSc, Department of Physiology, Faculty of Health Sciences, Nelson R Mandela School of Medicine, University of Kwazulu-Natal, Durban, South Africa.
2.FRCS {England},FCS (Plastics) (South Africa).Department of Plastic Surgery, Faculty of Clinical Sciences, Nelson R Mandela School of Medicine, University of Kwazulu-Natal, Durban, South Africa

Corresponding author and author to receive reprints: Shamin Bux (corresponding author)
Dept of Physiology, Faculty of Health Sciences, Nelson R Mandela School of Medicine, University of Kwazulu-Natal, Private Bag 7, Congella 4013, Durban, South Africa
Tel: 031-2604362 Fax: 031-2604455 e-mail: Bux@nu.ac.za

Key words: Elastic fibres; wound healing fibroblasts; deformation forces; histomorphometric analysis.Running title: Comparative histomorphometric analysis of elastic fibres in lesional and non-lesional skin of patients with keloids

List of abbreviations

AI	Area of inflammation	FFWA	Fine fibrous wavy areas
ATP	Adenosine tri-phosphate	Fig	Figure
CT	Connective tissue	FFT	Fine fibrous tubular
DRWA	Dense regular wavy areas	HCB	Hyalinising collagen bundles
DRWW	Dense regular wavy areas with	K/PD	Keloid/papillary dermis border
wide spaces		LAS	Leica Application Suite
ECM	Extracellular matrix	SEM	Standard error of mean
EVG	Elastic-Van Gieson	SPSS	Statistical Package for the
FFA	Fine fibrous areas	Social Sciences	
FFCA	Fine fibrous cellular areas	TGF-beta	Transforming growth factor

SUMMARY

Introduction: Keloids are a prototype of excessive wound healing. Keloid fibroblasts generate traction force, which deforms and realigns the collagen network during migration. Cells move bidirectionally along aligned fibres resulting in nonuniform cell distribution. This and the anisotropic collagen properties displayed in keloids, may be governed by traction force. Excessive traction force (>elastic limit), causes permanent deformations. As recovery from deformational forces is attained mainly by elastic recoil, we hypothesised that in keloids the elastic limit is decreased by reduced numbers of dermal elastic fibres, leading to permanent plastic deformation of dermal tissue by traction force.

Objective: To quantitate and compare the elastic fibre content of keloids and non-lesional skin

Methods: Sections of keloids and non-lesional skin from 32 patients were stained with elastic Van Gieson. The elastic fibre content was histomorphometrically quantified and the mean (± SEM) percentage area of elastic fibres in lesional and non-lesional skin was compared.

Results: Elastic fibres at the border of keloids were increased whereas internally they were minimal or absent. Statistical analysis (Wilcoxon signed ranks test) showed significant differences ($p < 0.05$) in elastic fibre content between non-lesional dermis and keloids.

Conclusions: The lack of elastic fibres in keloids decreases the elastic limit, leading to effects of excessive deformational force. These include compression and stiffening of tissue, increased mitogenesis and cell contractility, modified DNA and protein synthesis and increased collagen biosynthesis. The manifestation of these effects in keloids, supports the hypothesis that decreased elasticity in keloids promotes permanent dermal deformation by traction forces.

[Afr J Health Sci. 2011; 19:1-14]

Introduction

As keloids are a prototype of excessive wound healing, the lesional fibroblastic cells constantly exert traction forces to facilitate migration and extracellular matrix remodelling. During this process the collagen network may be realigned to provide contact guidance for 3D migration of cells along the axis of fibre alignment, resulting in bi-directional cell migration and non-uniform cell distribution. This and the anisotropic collagen properties displayed in the various keloid regions identified2 occurred, probably, to offer greater resistance to prolonged traction forces generated by migrating fibroblasts. Cell adhesion and migration are prominent in the angiogenesis and remodeling stages of wound healing [1, 2, 3]. During excessive wound healing, as in keloids, in addition to the constant production and progressive accumulation of scar tissue, traction force is continuously generated by migrating fibroblasts. Excessive, prolonged traction forces deform the extracellular matrix up to their elastic limit; beyond this limit the friction force that the tissue can withstand is exceeded [4] and the collagenous matrix is unable to resist cellular traction force, resulting in movement of the matrix [7]. Cell movement only occurs if the collagenous matrix can resist the traction force exerted by cells [5]. Stress applied beyond the elastic limit, results in plastic changes that occur at a rate greater than the tissue can tolerate causing injury and permanent deformation; this is called plasticity stress [4]. Beyond the plasticity level, tissue cannot tolerate more stress and it ruptures4. Could the disfiguring dermal deformations in keloids be a manifestation of gradually accumulated effects of stress from many lesser loads exerted by continuous traction force generated by wound healing fibroblastic cells, especially during granulation tissue formation, angiogenic and tissue remodeling stages? Supportive evidence for this includes: 1) formation of keloids months to years post injury, after apparent successful healing and 2) use of pressure therapy in the effective treatment of keloids [6]. Pressure therapy compensates for the elastic insufficiency of the affected dermal tissue and reduces scar tissue formation by inducing localised hypoxia and subsequent fibroblastic degeneration [6]. However, pre-existing hypoxia was confirmed in keloids where the accumulation of hypoxia-inducible factor-1α protein was found[7]. The effectiveness of pressure therapy treatment of keloids may, therefore, be by exacerbation of the existing hypoxic state. This corroborates an association between stress (force applied), strain (deformation when stress is applied), elastic insufficiency, hypoxia and keloid formation; thus reinforcing the necessity to investigate the role of traction forces in the pathogenesis of keloids. As elastic recoil is the principal function responsible for recovery of tissue deformed by traction forces, we hypothesise that, in keloids, connective tissue elasticity is decreased, reducing the elastic limit and resulting in augmented tissue deformation; progressive accumulation of deformed dermal tissue leads to keloid formation. To assess this, we (1) histologically assessed elastic fibre distribution in keloids, (2) histomorphometrically quantified and compared the mean (± SEM) percentage area of elastic fibres in lesional and non-lesional skin of patients with ear lobe keloids, (3) histomorphometrically quantified and compared the mean (± SEM) percentage area of collagen fibres in lesional and non-lesional skin of patients with ear lobe keloids and (4) compared the collagen:elastic fibre ratios of keloids, non-lesional skin and normal skin. This study is the first to show the distribution of elastic fibres in keloids and implicate abnormal elastic fibre distribution in the aetiopathogenesis of keloid formation.

Patients and Methods

The Ethical Committee of the Nelson R Mandela School of Medicine approved this study and all patients gave informed consent. The ethical policy is in compliance with the rules for human experimentation stated in the 1975 Declaration of Helsinki. Wedge biopsies were taken from lesional and non-lesional skin of thirty patients with keloids and no other underlying medical condition. Patient details are recorded in Table 1. The specimens were placed in 10% formalin saline (4% formaldehyde) immediately after procurement and were processed for paraffin wax embedment using conventional methods. Four and five micron sections of each specimen were stained with haematoxylin and eosin and elastic Van Gieson (EVG), respectively. Semiquantitative assessment of elastic fibre distribution in all sections of keloids and non-lesional skin biopsies was performed using the Olympus BH-2 microscope. From the 30 patients, a group of ten closely-matched patients with a common site of keloid formation and cause of initial injury (Table 1, blue rows) was selected for statistical analysis of the elastic fibre content in the keloid and adjacent normal skin. Close equivalence of patients minimized preexisting differences between the groups and maximized probability that differences found were associated with keloid formation. Patients chosen for the study were aged between 15 and 21 years, of a common race (African) and gender (females) who showed common cause of injury (ear-piercing) and site of keloid formation (ear lobes). The non-lesional skin biopsies were taken from the apparently normal area adjacent to the keloid.

Table 1: Patient details

Patient number	Race	Sex	Age	Diagnosis, location and size	Period after injury	Nature of initial injury and number of biopsies
1	C	M	31.5	Keloid. Presternal. 3.5 x 13 cm^2	4 years	Post thoracotomy. Bx, 2
2	A	F	19	Keloid, preauricalar, right. 7x11cm^2	2 years	Trauma. Bx,2
3	C		11.5	Keloid Suprapubic area. 2x4 cm^2	1year	Scar from surgery for undescended testis. Bx,2
4	A	M	21	Keloid. Left ear lobe. 2x2x2.5 cm^3	2 years	Ear piercing. Bx,1
5	A	F	15	Keloid. Right ear lobe	7 years	Ear piercing. Size NA. Bx,1
6	A	F	18	Keloids. Left and right ear lobes. 33x24x16 cm^3	Few months	Ear piercing. Bx,2
7	A	F	16	Keloids. Left and right ears lobes. Recurred after 1 year.R = 90x85x31 cm^3. L<R	1 year	Ear piercing. Bx,2
8	A	F	26	Keloids. Left and right ear lobes. 19x15X10 cm^3	2 months	Ear piercing. Bx,2
9	A	F	16	Keloid Left and right ear lobes 14x9x7 cm^3	1-2 years	Ear piercing. Bx,2
10	A	F	27	Multiple keloids. Recurred. Neck, back, presternal, chest. >18x3.5x1.1 cm^3	20 years	Plastic burns. Bx,8
11	A	F	29	Keloid. Suprapubic area. 2.1x3.5x1.1 cm^3	3 years	Ceasarian section scar. Bx,1
12	A	F	33	Keloids. Left and right ears lobes. Recurrent. 4.2x3.5x1.7 cm^3	2 years	Ear piercing. Bx,2
13	A	F	18	Keloids. Left breast, neck.	12 years	Neck, wire injury. Breast, abscess injury. Bx,2
14	A	F	21	Keloids. Left and right ear lobes. Recurrent	NA	Ear- piercing. Size NA. Bx,2
15	A	F	23	Keloid. Left ear lobe	NA	Ear-piercing. Size NA. Bx,1
16	A	F	18	Keloids. Bilateral ear lobes	NA	Ear piercing. Bx,2
17	A	F	26	Keloids. Left ear lobe, shoulder. Recurrent.	NA	Ear piercing. Shoulder, NA. Bx,2
18	A	F	22	Keloid. Left ear lobe. Recurrent	NA	Ear piercing. Bx,1

19	A	M	24	Keloids. Left and right ear lobes	NA	Ear piercing. Bx,2
20	A	M	49	Keloid. Left ear	NA	Stab wound. Bx,1
21	A	F	17	Keloid. Right ear	NA	Ear piercing. Bx,1
22	A	F	27	K. Left and right ears lobes. Recurrent for 3rd time	4 years	Ear piercing. Bx,2
23	A	M	24	Keloid. Left ear lobe.	2 years	Accident. Bx.1
24	A	F	18	Keloid. Left ear lobe	NA	Ear piercing. Bx,1
25	A	F	26	Keloid. Left ear lobe	5 years	Ear piercing. Bx,1
26	A	M	45	Keloid. Back of head. Occipital region. Prone to skin rash	2 years	Spontaneous. Bx,2
27	A	M	26	Keloid. Left ear lobe, posterior	2-3 years	Ear piercing. Bx,1
28	A	F	18	Keloids. Bilateral ear lobes	3 years	Ear piercing. Bx,2
29	A	M	28	Neck (below right ear), submandibular area	NA	NA. Bx,2
30	A	M	60	Keloid. Occipital area	NA	NA. Bx,2

C – Caucasian
A – African
M – Male
F – Female
Bx - Biopsy
RHS - Right hand side
NA - Not available

Morphometric and image analysis

The various regions composing the keloid were identified according to the classification of Bux and Madaree [8] and digital images of areas containing elastic fibres were captured using the EC 3 camera (Leica, Heerbrugg, Switzerland). Image analysis was performed using the Leica Application Suite (LAS), version 3.0. (Wetzlar, Germany). Prior to photography, the microscope was adjusted for optimal viewing by aligning for Kohler illumination and the system was calibrated using a 1000-µm stage micrometer. To exclude bias and repetition, the areas photographed were adjoining, non-overlapping fields of view showing the presence of elastic fibres; in non-lesional skin and each of the elastic fibre containing keloid regions. Acquired images were exported into Leica Qwin Pro (Wetzlar, Germany) for histomorphometrical evaluation using LAS version 3 image processing and analysis system and Leica Qwin Pro version 3.0, software (Wetzlar, Germany). The analysis included:

Non-lesional skin

1. Percentage area of elastic fibres in 100 fields of view (10 specimens in 10 fields of view at 40X)
2. Percentage area of collagen fibres in 100 fields of view (10 specimens in 10 fields of view at 40X)

Lesional skin

1. Percentage area of elastic fibres in 400 fields of view (4 areas X10 specimens in 10 fields of view at 40X)
2. Percentage area of collagen fibres in 400 fields of view (4 areas X10 specimens in 10 fields of view at 40X)

Also the mean % elastic and collagen fibre content of keloids and non-lesional were compared with that of normaldermis[9,10.]

Statistical analysis

All data were represented as means and standard errors (± SEM); these results are summarized in Table 2. Data obtained was entered in a computerized statistical analysis program, SPSS 15 for Windows, (SPSS, Chicago, IL) and comparative analysis of the mean percentage area of elastic and collagen fibres between non-lesional skin and each of the elastic fibre-containing keloid regions was performed using

Wilcoxon signed-rank non-parametric test. A p value of less than 0.05 was considered to be statistically significant.

Table 2: Specimen grouping details, mean and ± SEM of % area of elastic and collagen fibres in lesional and non-lesional skin of patients with keloids

Non-Lesional Skin (n = 10)							
Sp No	Region	No of Fields		Elastic Fibre % Area		Collagen Fibre % Area	
				Mean	±SEM	Mean	±SEM
1	Dermis	10		1.85	±0.078	71.89	±1.149
2	Dermis	10		10.64	±0.298	37.42	±0.919
3	Dermis	10		9.09	±0.196	43.05	±0.500
4	Dermis	10		4.2	±0.188	53.34	±0.718
5	Dermis	10		4.05	±0.191	59.2	±0.435
6	Dermis	10		3.52	±0.236	72.22	±0.687
7	Dermis	10		7.1	±0.314	57.99	±0.618
8	Dermis	10		9.09	±0.278	43.05	±0.375
9	Dermis	10		7.73	±0.227	55.81	±0.654
10	Dermis	10		8.92	±0.281	41.96	±0.593

Lesional Skin (n = 10)								
	Elastic Fibre % Area							
	FFT		DRWW		AI		K/PD	
Sp No	Mean	±SEM	Mean	±SEM	Mean	±SEM	Mean	±SEM
1	0.12	±0.012	0.39	±0.018	4.22	±0.1056	3.17	±0.10006
2	0.09	±0.007	0.09	±0.0114	5.02	±0.1013	4.52	±0.1483
3	0.46	±0.027	0.17	±0.0123	9.59	±0.1319	10.26	±0.1483
4	1.12	±0.137	0.42	±0.0149	2.4	±0.138	10.94	±0.2179
5	0.6	±0.052	0.26	±0.017	5.49	±0.1665	6.78	±0.1447
6	0.38	±0.027	0.22	±0.015	4.99	±0.1487	7.88	±0.0967
7	0.42	±0.028	0.18	±0.018	4.32	±0.1297	6.43	±0.148
8	0.45	±0.025	0.24	±0.014	7.01	±0.1137	7.98	±0.1191
9	0.19	±0,016	0.11	±0.019	4.80	±0.1307	5.55	±0.2095
10	0.67	±0.033	0.29	±0.014	5.21	±0.1378	8.97	±0.1294
	Collagen Fibre % Area							
	FFT		DRWW		AI		K/PD	
Sp No	Mean	±SEM	Mean	±SEM	Mean	±SEM	Mean	±SEM
1	70.01	±0.507	63.97	±1.09	6.72	±0.1414	55.3	±1.393
2	65.91	±0.561	33.41	±1.05	5.21	±0.1273	41.35	±0.941
3	76.36	±1.518	59.73	±0.945	23.25	±0.3099	53.24	±1.446
4	58.52	±1.437	46.19	±1.33	5.69	±0.1383	28.82	±0.901
5	60.33	±1.194	66.13	±1.73	20.56	±0.2078	31.04	±0.7148
6	90.62	±1.811	39.22	±0.527	5.63	±0.1404	46.98	±1.2407
7	64.17	±1.945	56.87	±1.357	6.43	±0.1643	30.33	±0.7008
8	64.39	±1.3576	48.07	±0.844	6.32	±0.1758	45.32	±1.1521
9	75.87	±1.635	58.99	±1.199	5.71	±0.1268	40.23	±0.7891
10	64.84	±1.652	49.6	±1,034	6.22	±0.1505	50.54	±0.705

Sp No – Specimen Number FFT –Fine fibrous tubular
AI – Area of inflammation K/PD – Keloid/papillary dermis border
DRWW – Dense regular wavy connective tissue with wide spaces

Results

Histological semiquantitative assessment of the elastic fibre content of non- lesional dermis and keloids showed that non-lesional skin contained the largest amount of elastic fibres with 66% showing a great increase in elastic fibres and 28% a moderate increase; this was followed by the keloid/papillary dermis border where there was a great increase in 28% of the specimens and a moderate increase in 47% (Table 3). Areas of inflammation located at the keloid/papillary dermis border showed moderate (19%) to scattered (25%) elastic fibres in 44% of the keloid specimens. Within keloids there was a paucity of elastic fibres; fibrous tubular regions showed the highest content (22% moderate and 66% few elastic fibres) followed by dense regular wavy areas (9% moderate and 53% few elastic fibres). Moderate to few elastic fibres were present around blood vessels in 69% of keloids and in nodular areas in 57% of the keloid specimens. Only 9% of keloid specimens showed the presence of elastic fibres in hyalinising collagen bundle regions; the distribution was sparse. Areas of angiogenesis displayed moderate numbers of elastic fibres in 15% and few in 41% of the keloid specimens.

With regard to the morphology of elastic fibres, at the papillary dermis/keloid border they were long and fairly thick, short and stubby, or thin and wispy; the long elastic fibres generally were oriented parallel to the long axis of the collagen fibre bundles (Fig 1a). Elastic fibres were increased in and around inflamed vessels of the sub papillary plexus at the papillary dermis/keloid border; elastic fibres were also present in the inflammatory infiltrate (Fig. 1b). It was remarkable that increased numbers of elastic fibres were located on the keloid side of the sub papillary plexus in 50% of the specimens, while on the papillary dermis side minimal amounts were found (Fig. 1b). At the papillary dermis/keloid border, some keloid zones, viz., fibrous tubular regions and dense regular wavy connective tissue areas, contained abundant elastic fibres (Fig. 1c, d) whereas matched areas within the keloid were devoid of elastic fibres or contained very few frail fibres (Fig. 1e, f). In non-lesional skin, the elastic fibres were irregularly arranged, less well-defined, clumped and paler staining when compared with those at the papillary dermis /keloid border (Fig. 2a); also the elastic fibres reoriented from parallel to perpendicular to the axis of collagen fibres and compacted to form thickened bands (Fig. 2b). This often occurred in close proximity to areas of tissue rupture or areas where collagen and elastic fibres appeared disheveled and degenerate (Fig. 2b). Within keloids there was a paucity of elastic fibres and this was restricted to certain keloid zones which included the fibrous tubular region and the dense regular wavy connective tissue areas (Fig. 1e, f); other zones such as fine fibrous cellular, and hyalinising collagen bundle areas were devoid of elastic fibres or showed occasional spots or blurred specs of positivity for elastin (Fig. 2c, d). Six % of the specimens displayed blurred patches of elastin staining within few hyalinizing collagen bundles (Fig. 2e). A consistent and conspicuous finding was the absence of elastic fibres in very cellular regions of the keloid, viz., fine fibrous cellular and wavy fine fibrous cellular areas (Fig. 2f).

Statistical analysis of ten ear lobe specimens using the Wilcoxon signed ranks test, showed statistically significant differences in the elastic fibre content between non-lesional dermis and: 1) fine fibrous tubular areas and 2) dense regular wavy areas in keloids (Table 4 and Fig. 4).

Table 4. Comparative statistical analysis of % elastic fibres in area of inflammation, fine fibrous tubular area, keloid/papillary dermis border area and dense regular wavy area with apparently normal area of keloids

	Elastic fibres in normal area of keloids versus Elastic fibres in keloids in area of Inflammation	Elastic fibres in normal area of keloids versus Elastic fibres in keloids in fine fibrous tubular	Elastic fibres in normal area of keloids versus Elastic fibres in keloids/papillary dermis border	Elastic fibres in normal area of keloids versus Elastic fibres in keloids in dense regular wavy wide
Z	-1.580(a)	-2.803(a)	-.764(b)	-2.803(a)
Asymp. Sig. (2-tailed)	.114	.005	.445	.005

a Based on negative ranks.
b Based on positive ranks.
c Wilcoxon Signed Ranks Test

Differences in collagen fibre content between non-lesional dermis and fine fibrous tubular areas in keloids as well as between non-lesional dermis and areas of inflammation were statistically significant (Table 5 and Fig. 5).

Table 5. Comparative statistical analysis of % collagen fibres in area of inflammation, fine fibrous tubular area, keloid/papillary dermis border area and dense regular wavy area with apparently normal area of keloids

	Collagen fibres in normal area of keloids - Collagen fibres in keloids in area of inflammation	Collagen fibres in normal area of keloids - Collagen fibres in keloids in fine fibrous tubular	Collagen fibres in normal area of keloids - Collagen fibres in keloid/papillary dermis border	Collagen fibres in normal area of keloids - Collagen fibres in keloids in dense regular wavy wide
Z	-2.803(a)	-2.599(b)	-1.784(a)	-.051(a)
Asymp. Sig. (2-tailed)	.005	.009	.074	.959

a Based on negative ranks.
b Based on positive ranks.
c Wilcoxon Signed Ranks Test

The elastic:collagen fibre ratios were calculated from the statistical data on % collagen and % elastic fibres in keloids and non-lesional dermis (Table 6). The elastic:collagen fibre ratios were as follows: 1) 1:1.73 in the areas of inflammation, 2) 1:154 in fine fibrous tubular areas, 3) 1:5.8 in the keloid/papillary dermis border area, 4) 1:220 in dense regular wavy areas and 5) 1:8.1 in the dermis of non-lesional skin. In normal dermis the elastic fibre content is 2-5% 9 and collagen 71.9% 10, giving a proportional ratio range of 1:14 to 1:36.

Table 6: Comparative analysis of mean % elastic and collagen fibre content of keloids, non-lesional and normal dermis

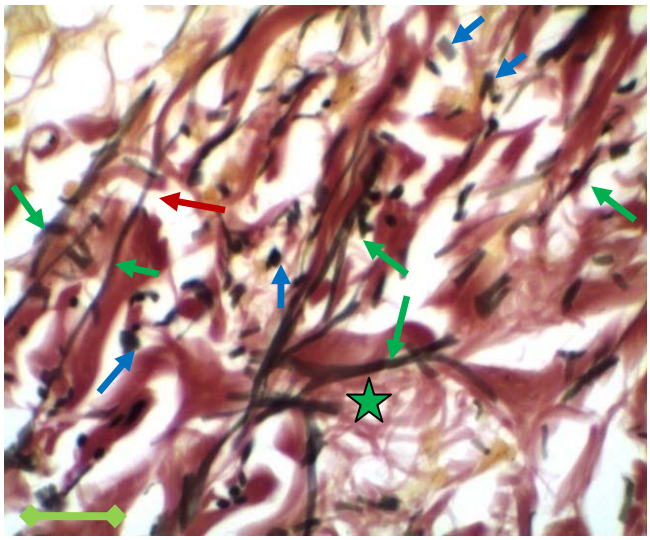
	Normal dermis	**Non-lesional dermis (100 fields)**	**Keloid/pap-illary dermis border (100 fields)**	**Areas of inflammation (100 fields)**	**Fibrous tubular areas (100 fields)**	**Dense regular wavy areas (100 fields)**
% elastic fibre content	2-5 (Gibson et al., 1965)	6.519	7.25	5.31	0.45	0.24
% collagen fibre content	71.9 (Neuman and Logan, 1950)	53.6	42.31	9.17	69.10	52.21
Proportional elastic fibre: collagen fibre ratio	1:14 to 1:36	1:8.2	1:5.8	1:1.73	1:154	1:220
Rating when compared with normal dermis % elastic fibre content		↑ ++	↑ ++	↑ +	↓ +++	↓ +++
Rating when compared with normal dermis %		↓ +	↓ ++	↓ +++	↓	↓ +

collagen fibre content						
Rating when compared with non-lesional dermis % elastic fibre content	↓ +		↑ +	↓ +	↓ +++	↓ +++
Rating when compared with non-lesional dermis % collagen fibre content	↑ ++		↓ ++	↓ +++	↑ ++	↓
Key: + mild, ++ moderate, +++ greatly ↑ Increase						
↓ Decrease						

When comparing the mean % elastic and collagen fibre content of normal dermis with that of lesional and non-lesional skin of patients with ear lobe keloids (Table 6), the following results were noteworthy:

1. The mean % elastic fibre content of non-lesional dermis and the keloid/papillary dermis border were moderately increased
2. The mean % elastic fibre content within keloids were greatly decreased
3. The mean % collagen content of non-lesional dermis and all keloid areas were decreased
4. All proportional elastic fibre:collagen fibre ratios were outside the range of normal skin, being low in the keloid border regions and very high within keloid

Figure 1(a,b)



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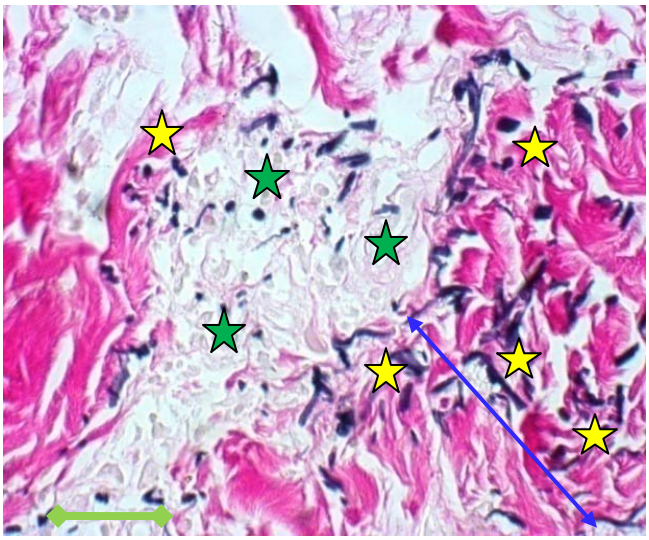

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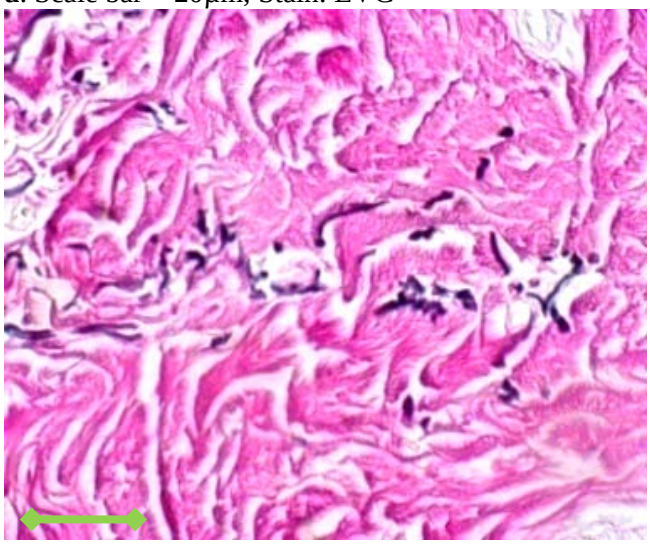

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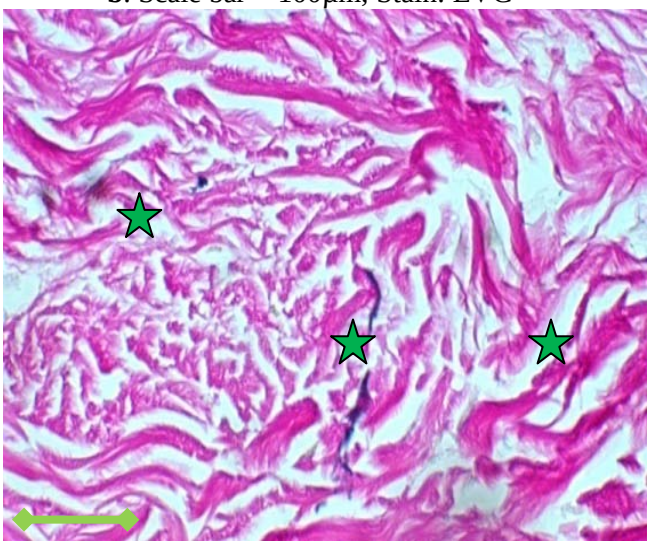

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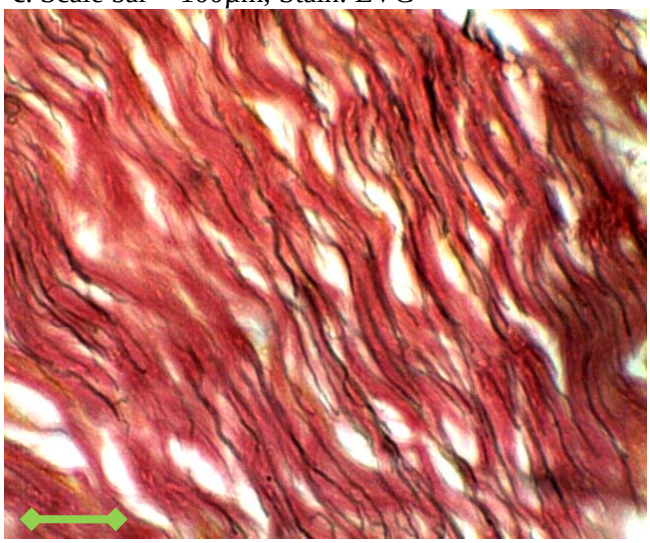

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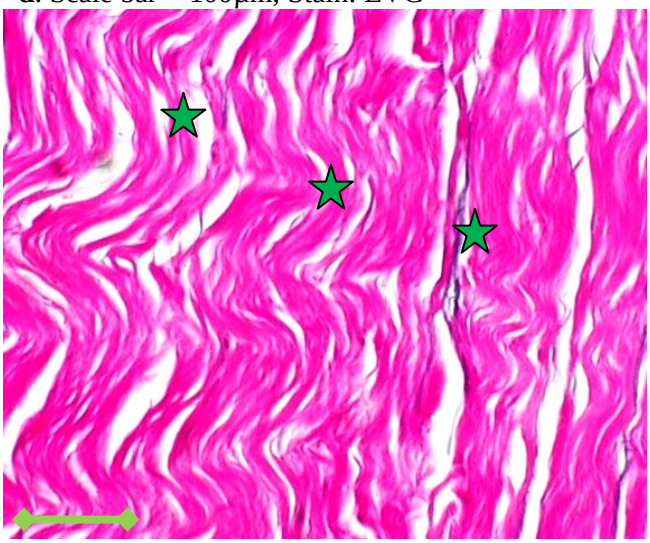
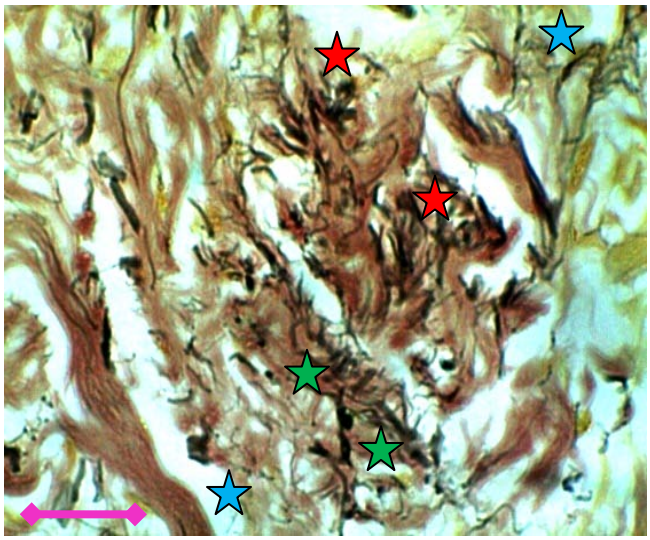


f. Scale bar = 100 µm; Stain: EVG

Figure 1. Photomicrograph of papillary dermis/keloid border showing: (a) long thick (green star), short stubby (blue arrows) and thin wispy (red arrow) elastic fibres. Note long elastic fibres oriented parallel to the long axis of collagen bundles (green arrows); (b) increase in elastic fibres adjacent to inflamed subpapillary plexus (yellow stars), few in area of inflammation (green stars) and increase in keloid side of the subpapillary plexus (violet line); (c,d,e,f) Fibrous tubular (c) and dense regular wavy (e) areas at the papillary dermis/keloid border showing abundant elastic fibres when compared with similar areas within keloids (d,f, respectively), with sparse, frail elastic fibres (green stars)**.**



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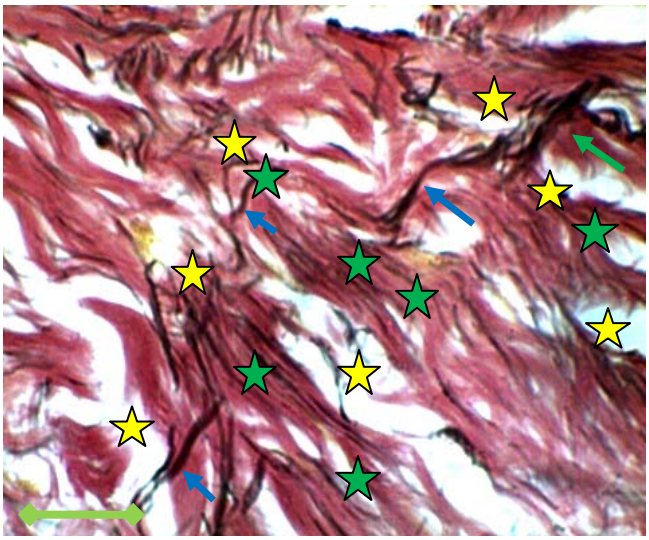

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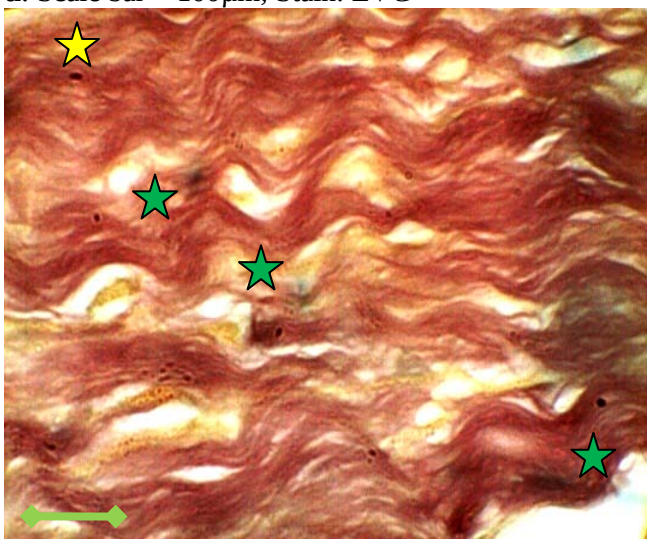

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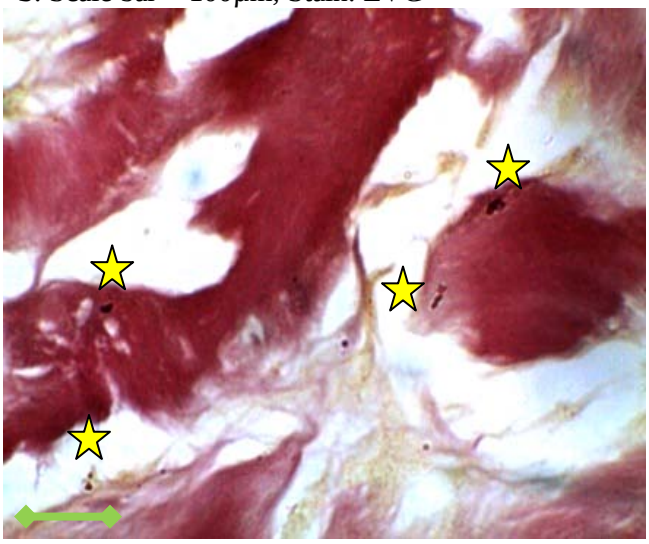

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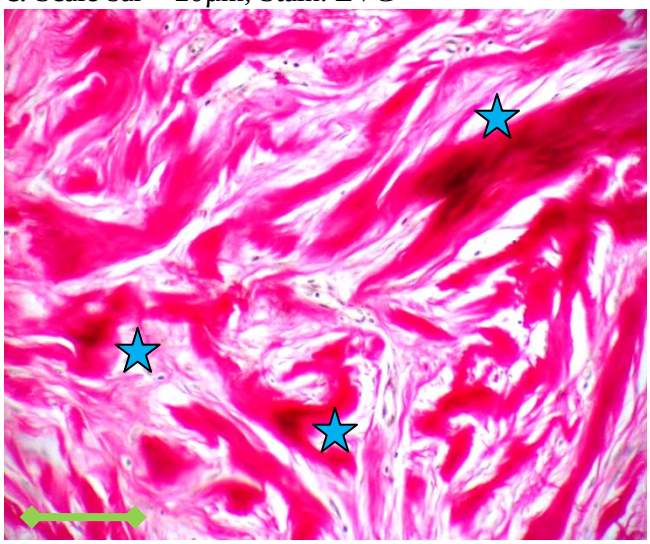

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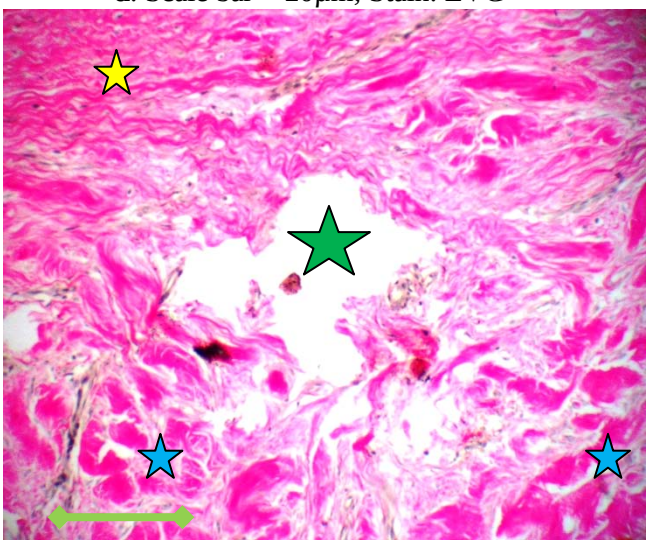

f. Scale bar = 100µm; Stain: EVG

Figure 2. Photomicrograph of (a) Increased elastic fibres in non-lesional skin showing irregular (green stars), clumped (red stars) and paler staining (blue stars) forms; (b) Elastic fibres reoriented from a parallel (green stars) to perpendicular arrangement (blue arrows) to the length of collagen fibres and aggregate to form bands (green arrow) in the vicinity of ruptured tissue and disheveled , degenerate collagen and elastic fibres (yellow stars); (c, d) Fine fibrous cellular, and hyalinising collagen bundle areas showing occasional specs (green stars) and spots (yellow stars), respectively; (e) Blurred patches of elastin staining within HCB (blue stars) and (f) Ruptured tissue (green star) between WFFA (yellow star) and HCB area (blue stars).

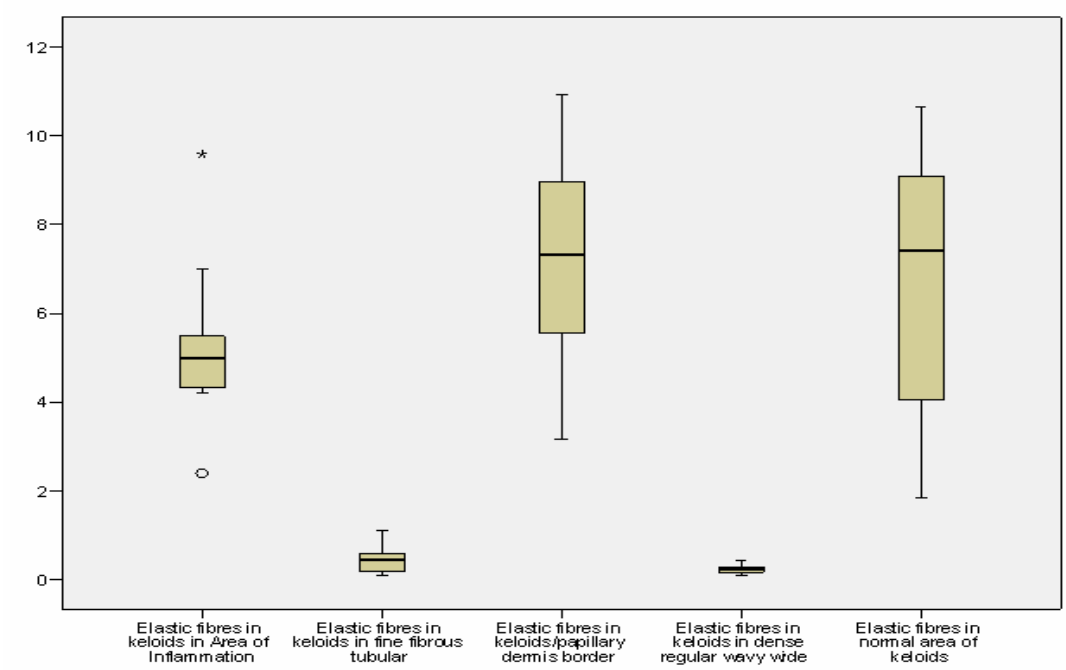

Figure 3. Elastic fibre content in area of inflammation, fine fibrous tubular area, keloid/papillary dermis border area, dense regular wavy area and apparently normal area of keloids.
Results are shown as mean percentage area of ten fields/specimen (n = 10). *p<0.05 compared with controls (normal area of keloids).

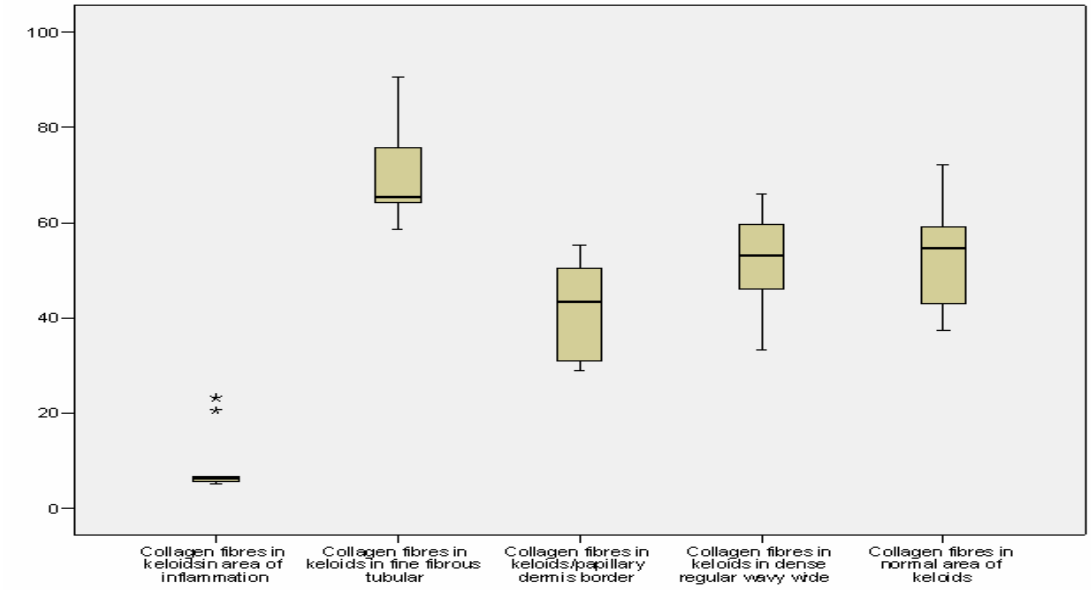

Figure 4. Collagen fibre content in area of inflammation, fine fibrous tubular area, keloid/papillary dermis border area, dense regular wavy area and apparently normal area of keloids. Results are shown as mean percentage area of ten fields/specimen (n = 10). **p<0.01 compared with controls (apparently normal area of keloids).

Discussion

Histomorphological and statistical analysis of the elastic fibre content in keloids showed that the highest concentrations were found at the keloid/papillary dermis border and non-lesional dermis. As the papillary dermis and non-lesional dermis adjacent to keloids are known to be normal with no evidence of scarring [11], it is anticipated that the elastic fibre content here is similar to that of normal unaffected skin where elastic fibres make up about 2-5% of the total volume of the dermis. In normal skin elastic fibres form an interconnecting network around collagen bundles to maintain normal skin tension by providing extensibility to accommodate deformation forces and elastic recoil to restore collagen to their original length after deformation9, provided that the strain among the collagen molecules is less than 3% when displacement of the collagen molecules is minimal [12]. Restoration of the collagen fibres to their original length also requires replacement of tissue fluid that was displaced during deformation; this permits associated collagen bonds to reorganize. In normal dermis fluid displaced into adjacent tissue compartments during deformation, is easily replenished by elastic recoil. In contrast, if elastic fibres are lacking, as in keloids, strain rapidly progress from the elastic to the plastic range, causing stiffening and permanent deformation of tissue and original tissue structure cannot be regained [4 9]. The higher than normal concentration of elastic fibres at the keloid/papillary dermis border and in non-lesional dermis may be a response to cope with traction forces exerted by keloid fibroblasts and resistance and shear forces wielded by firm inelastic keloids. The lower than normal concentration of collagen fibres quantified at the keloid/papillary dermis border and in non-lesional dermis was contrary to the proven finding that keloid fibroblasts overproduce collagen; this may be explained by gross tissue compression that occurs during permanent deformation. Lengthening and eventual rupture may also occur during permanent deformation. Connective tissue becomes progressively stiffer with lengthening and collagen fibres become aligned in the direction of the stretching; this is exhibited by keloids [13]. At a molecular level, in irreversibly deformed tissue, collagen cross-links are disrupted leading to ruptured collagen fibres, tissue failure and obstruction of the force-relaxation response [4] when stress cannot be absorbed and is transferred to adjacent areas. The compression of collagen fibres into thick bundles and elastic fibres into thick bands observed at the keloid border may be caused by stress transferred in this way. Areas of tissue failure with disheveled and degenerate collagen and elastic fibres may also be caused in this way.

The elastic:collagen fibre ratios of keloids and non-lesional skin are higher than that in normal skin; being 1.73 and 2.4 times higher in non-lesional dermis and the keloid/papillary dermis border respectively. This alters connective tissue biomechanics; the increase in collagen fibres may be a fibroblastic response to provide increased tensile strength to cope with stress transferred from the inflexible keloid. Non-lesional dermis and the keloid/papillary dermis border form the upper and lateral borders of the keloid and therefore, are the first areas around the keloid, to endure the consequences of transmitted compressive forces. Fibroblasts exposed to increased mechanical force showed stimulation of collagen and elastin synthesis [14]. The increased elastic fibre production at the upper and lateral keloid borders and the general increased collagen fibre production in keloids may thus be a consequence of cumulative forces: gravitational, displacement (exerted by rigid keloid tissue), traction (exerted by wound healing fibroblasts), shear, resistance and friction forces (wielded by regularly aligned and multi-directionally orientated dense collagen bundles). Shear, resistance and friction force may further be increased by movement of the matrix which occurs when keloid fibroblasts cannot resist traction forces [5]. In areas of inflammation the decreased elastic fibre:collagen fibre ratio was influenced by the high elastic fibre content. This was a consequence of the absence of elastase-producing neutrophils (infiltrate comprised lymphocytes and plasma cells [13]) and collagenase production mediated by inflammatory agents of wound healing fibroblasts [15].

In contrast with the large number of elastic fibres in the keloid/papillary dermis border and non-lesional dermis, the keloid proper exhibited a lack or absence of elastic fibres. Elastic fibres impart the properties of stretch and recoil to tissue, in their absence, the accumulative effect of deformation forces is compaction of fibres, a stiffer extracellular matrix, increased rigidity and alteration of shape and dimensions of the tissue. Increased rigidity alters matrix mechanics leading to activation of integrins, which promotes mitogenic signaling and cell contractility [16], further increasing matrix stiffness. This creates a positive feedback loop [17] that continually stimulates cell contractility, increasing stress, which, because of the lack of elasticity, progresses to plasticity stress and consequent permanent deformation. Evidence of stress reaching breaking point was found in ruptured tissue generally located below the papillary dermis/keloid border; this corresponded with the first part of the keloid with minimal elastic fibres, to encounter, additionally, the effects of atmospheric pressure (force of 1 kilogram per square centimeter). This and opposing upward

pressure rebound by the keloid, compress microvessels [2] leading to hypoxia and ischaemia.

The role of unrestrained stress in the pathogenesis of keloids is supported by innovative technical studies where custom-made silicon ear moulds were developed to apply homogeneous pressure to the ear [18]. There was a > 50% reduction in the size of keloids fitted with pressure devices made from Zimmer splints when assessed at one year 6. The success of pressure therapy was attributed to exacerbation of the previously existing hypoxic condition, reduction in number of myofibroblastic cells and reorientation of disorganized collagen to a parallel arrangement similar to that found in normal wound healing [19]. Pressure therapy also compensates for elastic insufficiency, as shown by the success of elastic bandages in the treatment of hypertrophic scars [22]. Zimmer splints abolish the need for elasticity by maintaining homogenous pressure.

This study is the first to confirm that elastic fibres are deficient in keloids. Reinstating elastic properties in keloids could gradually decrease the size of keloids, perhaps by conversion to the normal wound healing process. Treatment modalities for keloids should therefore include pressure therapy and research in the field of pressure therapy biomechanics, methodologies and devices should be continual.

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Involvement of upper torso stress amplification, tissue compression and distortion in the pathogenesis of keloids

Shamin Bux [a,*], Anil Madaree [b]

[a] Department of Physiology, Faculty of Health Sciences, Nelson R. Mandela School of Medicine, University of Kwazulu-Natal, Durban, South Africa
[b] Department of Plastic Surgery, Faculty of Clinical Sciences, Nelson R. Mandela School of Medicine, University of Kwazulu-Natal, Durban, South Africa

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ABSTRACT

Keloids are benign tumours composed of fibrous tissue produced during excessive tissue repair triggered by minor injury, trauma or surgical incision. Although it is recognized that keloids have a propensity to form in the upper torso of the body, the predisposing factors responsible for this have not been investigated. It is crucial that the aetiopathoical factors implicated in keloid formation be established to provide guidelines for well-informed more successful treatment. We compared keloid-prone and keloid-protected skin, identified pertinent morphological differences and explored how inherent structural characteristics and intrinsic factors may promote keloid formation. It was determined that keloid prone areas were covered with high tension skin that had low stretch and a low elastic modulus when compared with skin in keloid protected areas where the skin was lax with a high elastic modulus and low pre-stress level. Factors contributing to elevated internal stress in keloid susceptible skin were the protrusion of hard connective tissue such as bony prominences or cartilage into the dermis of skin as well as inherent skin characteristics such as the bundled arrangement of collagen in the reticular dermis, the existent high tension, the low elastic modulus, low stretch ability, contractile forces exerted by wound healing fibroblastic cells and external forces.

Stress promotes keloid formation by causing dermal distortion and compression which subsequently stimulate proliferation and enhanced protein synthesis in wound healing fibroblastic cells. The strain caused by stress also compresses and occludes microvessels causing ischaemic effects and reperfusion injury which stimulate growth when blood rich in growth factors returns to the tissue. The growth promoting effects of increased internal stress, primarily, and growth factors released by reperfusing blood, manifest in keloid formation. Other inherent skin characteristics promoting keloid growth during the late stages of wound healing in the upper torso are the thinner epidermis, the presence of vellus hairs, the absence of protective immunoglobulin A (IgA), and the thick fragile quality of upper torso skin. As it is not known why there is a predilection for keloids to form in the upper torso of the body, this hypothesis implicating and associating inherent morphological characteristics and elevated stress in the aetiopathogenesis of keloids is of potential value in terms of prevention, management and treatment of these enigmatic tumours.

Introduction

Keloids are dermal fibroproliferative lesions that develop as a consequence of a dysregulated response to cutaneous wounding, resulting in disfiguring scars that often progress to grotesque dimensions. They appear few months post injury and can be delayed up to several years after trauma [1,2]. There is a definite tendency for these keloidal growths composed of excessive scar tissue, to form in the upper torso of the body, the main areas being the head (earlobes and mandibular ridge), neck, back, presternal and deltoid areas [3]. The reasons for the propensity of skin in these regions to form keloids are not known. It is expected that inherent characteristics of skin and the effects of intrinsic and extrinsic factors on it, contribute to this susceptibility. We therefore, compared keloid-prone and keloid-protected skin, identified pertinent anatomical and morphological differences and explored how these structural features, when subjected to internal and external factors during the wound healing process, could contribute to the pathogenesis of keloid formation.

The hypothesis

Skin covering the upper torso of the body is susceptible to keloid formation post injury because of elevated dermal stress caused by:

* Corresponding author. Address: Department of Physiology, Faculty of Health Sciences, Nelson R. Mandela School of Medicine, University of Kwazulu-Natal, Private Bag 7, Congella 4013, Durban, South Africa. Tel.: +27 31 2604362; fax: +27 31 2604455.
E-mail address: Bux@ukzn.ac.za (S. Bux).

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(1) stress-amplification at the soft and hard supportive connective tissue interface;
(2) inherent skin characteristics such as skin thickness, the bundled arrangement of collagen in the reticular dermis, fat content, the existent high tension, the low elastic modulus and low stretch ability
(3) contractile forces generated by wound healing fibroblastic cells;
(4) action of external forces.

Augmented internal stress causes dermal distortion and compression resulting in increased loading on cells; this triggers anabolic effects, with amplification of gene expression, collagen synthesis and mitosis. Microvessels that become compressed and occluded cause ischaemia and reperfusion injury; reperfusing blood is rich in growth factors which stimulate growth when released into the extracellular matrix of the healing tissue.

Evaluation of the hypothesis

Thick skin and very thin skin are less susceptible to keloid formation

The upper torso of the body which is susceptible to keloid formation is covered with thin skin whereas areas of the body where keloids are rare or never develop are covered with either thick or very thin skin. Thick skin covered keloid-protected areas are the palms and soles, while thin skin covered regions that are keloid-protected or less prone to keloid formation include the eyelids, genitalia, hands, feet and areolae around the nipples of the breast [4]. The latter regions are covered with thin skin that has increased stretch or a high elastic modulus meaning that for the tissue to be distorted a large force has to be applied. Some inherent features contributing to this are the decreased fat content (eyelid, genetalia), increased smooth muscle content (arrector pili muscle in eyelid, areolae, genetalia), thick stratum corneum (palms and soles), etc. On the contrary, keloid prone areas are covered with skin that has a low stretch and a low elastic modulus. Features contributing to this are the increased fat content and thicker dermis with increased numbers of collagen bundles (deltoid, neck, mandible, chest etc.). Some reasons as to why skin categorized as thick or very thin are less susceptible to keloid formation are discussed below.

Inherent low prestress levels in the eyelids prevents keloid formation

The skin of the eyelids is the thinnest in the body (<1 mm), is highly folded and the dermal connective tissue is extremely loose and devoid of fat. The structure is well suited to permit the eyelid to perform its essential functions of blinking every 5–7 s to protect the eye from external debris and to spread tears over the cornea. The eyelid structure is optimally designed to balance external and internal forces. The eyelid crease and lax nature of eyelid skin indicates low prestress levels in the extracellular matrix which enable optimal storage, transmission and dissipation of tension imposed by external and internal forces.

Under normal physiological conditions the dermis of skin, except in lax skin, is under tension, even without the application of external forces. Tension in the dermis arises from mechanical, osmotic, hydrostatic, fluid, and electromechanical effects [5]. Application of external force will add to internal tissue stress. The extracellular matrix (ECM) manages stress by stretching of elastic and collagen fibres which store, transmit and dissipate the forces encountered. Collagen fibrils transfer tensile loads and elastic energy from the ECM to the dermal cell cytoskeleton via integrin–ECM interactions [5]. This leads to activation of the phosphorelay system that controls cell division, gene expression, and protein synthesis [5]. Through this process of mechanotransduction the metabolism of dermal cells is influenced by external and internal mechanical loading. At equilibrium all external forces acting on tissue equals zero. Alteration of this balance alters cellular metabolism: Increased stress stimulates an anabolic response, while decreased stress stimulates a catabolic response [6].

In the eyelid, skin laxity, crease and low prestress levels ensure that the mechanical loads imposed on the eyelid are tolerated and optimally employed and that the important functions of blinking and keeping the cornea moist to protect the eye from external debris, are maintained. Also, by preventing increased internal stress accumulation, these eyelid characteristics prevent over activation of the phosphorelay system and subsequent increased gene expression and protein synthesis by dermal fibroblasts, which are key events leading to keloid formation.

Loose arrangement of the dermal collagen fibres in eyelid skin promotes stress tolerance unlike hyalinized collagen bundles in keloids which increases internal strain

In the eyelids, the loose arrangement of the dermal collagen fibres immediately below the basal lamina of the epithelium, promotes stress tolerance by rapid dissipation and reduces the impact of shear, friction and compressive forces. In comparison, in keloid prone regions the skin is comparatively taut with elevated muscle and skin tension because of increased mechanical loading as well as less efficient transmission and distribution of this load. The elevated tension stimulates skin fibroblasts to produce increased amounts of collagen [7] which in the reticular dermis are arranged in dense bundles that are irregularly aligned and multi-directionally orientated. In keloids, where continual wound healing processes occur, cell adhesion and migration, are ongoing. During these process traction forces are generated, stimulating the fibroblastic cells to produce even more collagen, in response to the environmental demand to increase tensile strength [8,9]. Collagen types I and III are overproduced in keloids and they form fibrils with rigid and flexible domains; the flexible domains stretch and store elastic energy during tensile loading, while the rigid domains prevent collagen triple helical unfolding during mechanical load application [10]. In keloids collagen function is impaired by the hyalinization of collagen bundles. Hyalinisation decreases collagen stretch ability and load bearing capacity, resulting in inadequate transmission and distribution of mechanical loads. This further increases internal stress and exacerbates fibroblastic overproduction of collagen [7].

Absence of dermal fat in eyelid skin reduces internal tissue stress

The absence of fat in the dermis of eyelid skin and the sparse fat content of the hypodermis reduces the mass of the eyelid. The reduction of the number of adipocytes and thus their cell surface area, effectively reduces the collagen fibril-cell interface contact extent; this diminishes the action of internal forces at the point of contact as well as its associated anabolic effects [10]. The absence of fat in the dermis of eyelids reduces the mass of the eyelid which aids with combating the pulling down effects of gravity. Also, the reduced mass of the eyelids aids with combating the pulling down effects of gravity. The necessity to minimise the fat content in the eyelid is demonstrated in a microarray study on gene expression of corneal, skin and tendon fibroblasts subjected to linear shear stress and their subsequent down-regulation of some genes that are expressed at high levels in mature adipocytes [11]. Some of these genes are *Car3* (carbonic anhydrase 3) and *Lcn2* (lipocalin 2) [12]. Therefore, in essence, shear stress will influence differentiation of fibroblasts rather than adipocytes from mesenchymal precursor cells [13], in

order to cope with the increased stress: fibroblasts will produce collagen to provide greater tensile strength.

Protective role of immunoglobulin A (IgA) impedes keloid formation in eyelids

According to the keloid-triad hypothesis the 3 factors involved in the aetiopathogenesis of keloids, are infection, genetic factors and location of sutures in relation to body skin lines of tension and tension of sutures [14]. In the eyelid immunoglobulin A (IgA) protects the mucosal surfaces of the eyelid by preventing bacteria from adhering to the mucosa and by disposing of bacteria [15]. Immunoglobulin A is the major immunoglobulin in tears secreted by the lachrymal glands of the eyelid [15]. This is an important attribute in preventing keloid formation as infection is postulated to be one of the three factors involved in the aetiopathogenesis of keloids. In the keloid-triad hypothesis it was emphasized that the simultaneous interaction of the three factors viz., infection, genetic factors and location and tension of sutures was necessary for the growth of keloids. Excessive suture line tension increases shear and tensile stress in skin. This affects the reticular dermal layer the most because deformation of dense collagen bundles and displacement of tissue fluid, effectively, amplify shear and tensile stress. The main response of connective tissue cells to increased mechanical load is the production of the extracellular matrix, mainly collagen, which provides tensile strength to resist this force.

Thin layer of keratin makes thin skin more susceptible to keloid formation than thick skin

Skin that is prone to keloid formation is thin skin which differs from thick skin in that it has a thinner layer of keratin and contains hair follicles, sebaceous glands and arrector pili muscles. This thinner layer of keratin affords less protection and is less efficient in withstanding external forces acting on skin. Paravascular inflammation around microvessels of the sub-papillary plexus observed in keloids [16] may have been caused by agents that gained entry through the thinner epidermis. In keloids the papillary dermis which is located between the epidermis and the keloid which forms in the reticular dermis, is unaffected [16]. The discerning adverse effect on the reticular dermis while the adjacent papillary dermis is unaffected is most likely, related to the effects of stress increase caused by the presence of dense collagen bundles which individually have a very small area when compared with the papillary dermis containing loosely arranged fibres, making it a single large body. Quantitatively, stress is a measure of the average force per unit area of a surface within the body on which internal forces act. Therefore, forces acting on the numerous small dense bundles of collagen with small surface areas in the reticular dermis will amount to accumulated increased stress when compared with the large surface area of the papillary dermis. The internal forces acting on the dermis are a reaction to external forces applied on skin; these external forces are easily transmitted through the thin, less-resistant epidermis. In addition, internal forces are generated within the dermal ECM of keloids by contraction of wound healing fibroblasts and arrector pili muscles as well as by contracting muscles during body movement. These forces are transferred to the bundles of collagen, causing sliding and/or rubbing of adjacent collagen bundles. Initially, there is resistance to this sliding action caused by the frictional force between contacting collagen bundles and shear stress has to be applied to start the sliding or rubbing. Frictional force thus leads to shear force. The increased tension build-up between the multi-directionally orientated collagen bundles causes increased friction between converging collagen bundles in motion; this further increases stress levels. The situation is exacerbated in keloids because of poor transmission and distribution of the increased internal stress by hyalinized collagen bundles. Hyalinisation impairs collagen function by decreasing the stretch ability and load bearing capacity of the flexible and rigid domains, respectively, of collagen fibrils. The increased internal stress influences the metabolism of dermal fibroblastic cells, increasing gene expression and protein synthesis as well as cell division [5].

Keloid prone areas are covered with skin that has low stretch and a low elastic modulus

Skin covering keloid prone areas like the deltoid, neck, mandible, chest etc., is distorted by the application of small forces; features contributing to this are the increased fat content and thicker dermis with many small bundles of dense collagen. The tensile stress inflicted by the various forces on skin cause compression and stretching of skin, resulting in the occlusion of blood vessels and interstitial fluid-flow [17]. Shear force in skin, produced by frictional force combined with pressure has been reported to occlude skin blood flow [18,17]. In keloids Bux and Madaree [16] have shown extensive microvascular pathology and associated ischaemic effects in the lesions. Microvessels in all keloid regions displayed damage and were constricted or occluded [16]. Occluded microvessels were usually found within oedematous areas where interstitial fluid was trapped and flow was obstructed; these areas were surrounded by fibrous regions where the collagen fibres showed profuse unbundled or dense bundled arrangement [16]. The dense collagen bundles were orientated in a multi-directional, dense regular pattern. This arrangement may have been caused by the stretch effects of shear on collagen in response to increased internal tensile stress. In the process interstitial fluid was squeezed and entrapped in areas. These events led to the occlusion and constriction of vessels causing a diminished blood supply to tissue. These effects were evident in all keloid regions as patches of degenerate and necrotic tissue [16].

Elastic and collagen fibres maintain cell and tissue integrity during mechanical loading of skin

Effects of stretch caused by excessive external loading could be seen in keloids in areas of dense regular connective tissue where collagen fibres within bundles were straight and taut and cells were stretched and appeared pale, translucent, fuzzy and indistinct [16]. Generally, in tissue, at low strain levels (up to 0.3) forces of deformation are absorbed by the stretch of elastic fibres and collagen fibres offer little resistance to deformation. After the forces are removed the tissue returns to its elastic set point, i.e. elastic fibres will release the potential energy which it stored during stretch and the fibres recoil restoring tissue to its normal state. Bet strains of 0.3–0.6 the collagen fibres aid with offering resistance to deformation by stretching of the flexible regions within crosslinked collagen molecules [19]. The flexible domains reversibly store elastic energy during stretching; they become extended and thereafter stress is transferred to the rigid regions of collagen molecules. Thus, at larger strains the collagen fibres stiffen to ensure cell and tissue integrity [20] and prevent premature mechanical failure of collagen and elastic fibres in the dermis. In keloids rigidity of collagen fibres is indicative of the elevated internal strain experienced by the tissue, while the compact nature of these fibres is due to the compressive effects of pressure and shear in response to increased internal tensile stress. The stiffness and rigidity of collagen fibres in keloids present technical difficulty in tissue processing during cutting up of the tissue for wax embedment and during sectioning of blocks. Areas of tissue failure where high mechanical loading was experienced were evident in keloids as enlarged irregular spaces surrounded by ruptured fibres and other ECM elements [21]. Areas of visible rupture usually occurred at the borders of

fibrous regions showing dense regular realignment of collagen fibres [21] confirming that this region had previously experienced loading.

The lack of elastic fibres contributes to tissue deformation and growth in keloids

Skin is viscoelastic and responds to mechanical loading by proportional energy storage and dissipation. Elastic fibres perform the function of energy storage while the viscous elements of skin (collagen mainly and proteoglycans) carry out the mechanical response of energy dissipation [19]. Energy storage by elastic fibres ensures tissue shape recovery from deformation caused by applied mechanical load. The proportional dissipation of energy occurred by the sliding of collagen fibres in the viscous matrix during their realignment in the direction of the applied force, resulting in the reorientation of collagen fibres which largely contributes to the extensibility of skin [19]. As there is a lack or absence of elastic fibres in keloids [21], the important function of removal of forces of deformation at low strain levels (up to 0.3) and restoring shape recovery after deformation, are not accomplished. In the absence of elastic fibres, the cells, microvessels and other constituents of the ECM are constantly exposed to low internal strains of (up to 0.3). This upsets the normal physiological balance between external gravitational force and internal tensile forces which maintain certain connective tissue (CT) cell phenotypes and their level of activity. An increase of internal strain caused by the absence of elastic fibres results in increased loading on cells and the ECM; the cells display anabolic effects, showing increased levels of gene expression, collagen synthesis and excretion and mitotic activity [22]. Increased internal tension extending collagen fibres attached to dermal cells via integrins [5] triggers stretching of cell membranes which activates ion channels located in the cell membrane [22]. The disfiguring dermal deformations manifesting as keloids may, therefore, be a gradual accumulation of the effects of stress on dermal tissue lacking elastic fibres. With the lack of elastic fibres, the proportion of energy dissipated by the sliding of collagen fibres in the viscous matrix is increased. Concurrent with this process is the realignment of collagen in the direction of the applied force, resulting in the reorientation of collagen fibres. An increase in internal energy to be dissipated would, therefore, result in the realignment of a larger number of collagen fibres or bundles in the direction of the applied force. This is an important attribute that imparts to lesional skin the capability of being overextended, leading, sometimes, to keloids of grotesque proportions. The defects caused by the lack of elastic fibres i.e. failure of tissue to recover shape after deformation and effects of increased tensile stress were morphologically clearly evident as keloidal growths, the overproduction of different collagen types and proliferation of fibroblastic cells and blood vessels in the various regions forming the lesion [16].

Elastic and collagen fibres resist deforming mechanical forces by stretching and sliding

Elastic fibres are dominant in the stretch mechanism during dermal resistance to deforming mechanical forces while collagen fibres aid with offering resistance by sliding mainly and minimal stretch. With the lack of elastic fibres in keloids the partial assimilation of mechanical forces by energy storage does not occur, nor the recovery of tissue shape after deformation by the applied force. Elastic fibres respond to external loads at low strain levels (up to 0.3), by stretching while collagen fibres extend by uncoiling with minimal stretch and slippage [5,23]. At medium strains (0.3–0.6) collagen responds to external loads by stretching from former wavy shape into a straightened configuration [23]. The flexible domains of collagen fibres will start uncoiling and stiffening (40% of stretch here is due to microfibril *D* spacing extension from 67 to 69 nm) [23]. Stretching may also occur by the deformation of intermolecular cross links between tropocollagen tiers stacked to form collagen fibrils [23]. Collagen stretch is also facilitated by the folding-over and lengthening of the glycosaminoglycan (GAG) crosslinks [23]. As the collagen fibres stretch they become stiffer; stiffness increases and is maximum when the fibres are stretched to their limit. Beyond the stretch limit, individual collagen fibrils begin to fail with continued loading; as more fibrils fail, damage within the collagen fibre accumulates and stiffness is reduced [23]. Collagen fibres stiffen at larger strains to ensure cell and tissue integrity [20] but, at elevated strains collagen failure and reduction in stiffness leads to tissue damage [24]. At the end of the stretch capacity of collagen, the fibres are no longer resilient to deformation and tearing generally occurs [23]. All of the above events are morphologically illustrated in keloids as areas of dense regular connective tissue (collagen fibres stretched to limit), areas of collagen lysis (collagen damage) and fibrous tubular areas (collagen failure and reduction of stiffness) [16]. In fibrous tubular areas collagen failure under elevated strain would manifest as snapped collagen fibres with reduced stiffness which would entwine in various configurations and directions. This is clearly shown in areas where fibrous fringes emanate from tubular stalks, where aggregates of tubules entwine to form stalks and where aggregates of intertwined tubules attach to stalks [16]. These areas of tissue failure also exhibit degenerate microvessels and degenerate and necrotic cells including pyknotic spindle cells, aggregates of necrotic fuzzy epitheloid cells, degenerate spindle cells drawn into entwining eosinophilic tubules and enwrapped necrotic spindle cells [16].

Crimped and wavy collagen form at low strains

Collagen fibres respond to external loads at low strain levels (up to 0.3), by extending like an uncoiling spring. During this time the collagen fibrils are folded and minimal collagen stretching and slippage occur with deformation. The flexible domains of collagen will stretch by gradually uncoiling and will reversibly store elastic energy during the process. During uncoiling collagen fibrils may buckle at the "hinge" region found between consecutive flexible–non-flexible regions. Gutsmann et al. [25] indicated that tendon collagen fibrils subject to axial compression can only withstand a very limited bow prior to buckling. One way in which wavy collagen fibres form is by buckling at the "hinge" region of fibrils; another way is by the crimping action of contracting myofibroblasts on collagen. The crimped structure of collagen is characterized by rectilinear segments connected by sharp kinks [25]. At a molecular level, the crimped pattern is reported to form by intermolecular splitting in a certain direction influenced by the angle at which the anisotropic collagen molecules lie to the fibril axis, even under modest lateral stress [26]. Areas of dense regular wavy connective tissue, where buckling of collagen fibrils occurred at the hinge region, were morphologically illustrated in keloids [16]. Areas of crimped collagen were not as plentiful and were usually found closer to the lateral borders of the keloid [16].

Collagen in some keloid regions resembles tendon collagen

In keloid regions of dense regular wavy and dense regular connective tissue the collagen fibres were extremely long when compared with other keloid regions [16]. The arrangement and length of collagen fibres in the dense regular connective tissue regions were similar to that in tendon, suggesting that it was a structural attempt to cope with the increasing mechanical load. The collagen in tendon is structurally modified to cope with the increased

tensile forces exerted by muscle and bone. Tendon collagen has an elastic spring constant of 8 GPa whereas in skin the value is 4.4 GPa [10]. Collagen fibril lengths for dermis are about 50 μm compared to between 400 and 900 μm for tendon [10]. The higher value of the elastic spring constant and very long fibril length for collagen in tendon compared to that found in skin shows that tendon can withstand greater tensile loads than skin. The production, in skin, of collagen that is structurally similar to that in tendon, indicates that skin is exposed to high mechanical loads and that collagen structure is modified in order to cope with the stress imposed.

Collagen stress strain curve and functionality

In a typical stress strain curve for collagen there are three major regions: (1) the toe or toe-in region, (2) the linear region and (3) the yield and failure region [27]. During physiologic activity, most collagen and collagenous structures such as ligaments and tendons exist in the toe and a bit in the linear region [27]. These constitute a nonlinear stress strain curve. Crimp or waviness in collagen is a structural characteristic that plays a significant role in the mechanics by contributing significantly to the nonlinear stress strain relationship for all soft collagenous tissues. "Un-crimping" of the crimp occurs in the collagen fibrils in the nonlinear stress strain curve. This part of the stress strain curve shows a relatively low stiffness since it is easier to stretch out the crimp of the collagen fibrils [10]. After the collagen fibrils become uncrimped, the collagen fibril itself is stretched, giving rise to a stiffer material. As individual fibrils begin to fail, damage within the collagen fibre accumulates, stiffness is reduced and the collagen failure occurs; collagen is then unable to perform its primary roles in tissue, which are to prevent premature mechanical failure and to help store, transmit and dissipate energy imparted either by the musculoskeleton or as a result of externally applied forces [10]. Collagen fibres are functional in the morphological range between the various degrees of crimp, straightened and fully stretched structures.

Keloids form in fragile skin of greatest thickness

Thin skin varies in thickness from 0.5 mm on the eyelids to 4 mm or more on the shoulders [28]. According to the Obagi Skin Classification System the skin type of patients with keloids or a history of scarring after mild trauma is fragile skin; skin type according to fragility is further divided into thin, medium thick and thick skin [29]. Keloid prone areas of the body were shown to have the greatest thin skin thickness when compared with non-keloid forming thin skin [29]. Some measurements illustrating the thick quality of keloid prone skin are: shoulder-blade skin (mean thicknesses in mm: males 3.07/ females 2.47); the upper back (m, 3.04/f, 2.85); the chin (m, 3.04/f, 2.69); the lower lip (m, 2.66/f,2.50) and lower back (m, 2.64/f, 2.39); whereas non-keloid forming skin was especially thin skin such as skin found in the upper eyelid (m, 0.59/f, 0.64); the lower eyelid (m, 0.99/f, 0.86); in the groin (m. 0.97/f, 0.90); in the armpit (m, 1.01/f, 1.09) and on the forearm (m, 1.13/f. 1.00 mm) [30]. In addition to variation in thickness in fragile skin, there was also racial variation in the occurrence pattern of this type of skin; it was found to be more common in Asians and Blacks [29]. This complies with the finding that keloids form predominantly in darker skinned individuals [31].

Skin covering connective tissue overlying bony prominences in the upper torso is prone to keloid formation

Generally keloid prone areas with elevated muscle and skin tension overlie bony surfaces such as the sternum, deltoid regions of the upper arm and upper back and the head, neck and mandibular border. Important extrinsic stresses that directly affect this area are pressure and shear (mechanical load), which internally are carried by tissue to the skeleton and are maximized at the boney prominence [32]. The uneven pressure distribution that occurs around boney prominences is defined as pinch shear stress [32].When there is sliding or the potential for motion between two surfaces the associated force is called parallel shear stress; pressure (force per unit area) is the force applied perpendicular to the surface [32]. In addition to external forces, the elevated muscle and skin tension in keloid prone skin overlying bony surfaces makes the potential for parallel and pinch shear high. Both parallel and pinch stresses (forces) cause strain which manifests as distorted tissue. Parallel and pinch stresses may cause distortion in the same direction (additive) or they may reduce or cancel each other's distortion when applied in opposite directions [32]. The distortion associated with pressure is compression. The applied forces causing distortion of tissue are elevated post injury, by tensile forces generated by wound healing fibroblasts. Elevated forces cause greater strain, increasing deformation and compression of dermal tissue, leading to compression and collapse of microvessels and thus, induction of ischaemic conditions and tissue failure. Closure of capillaries leads to a rise in arteriolar blood pressure; if this rises above the threshold level, blood flow to capillaries is restricted or blocked, exacerbating ischaemia and tissue damage. The presence of compressed and occluded blood vessels was a common feature in all keloids examined [16]. Damage to vasculature under mechanical load may also occur as a result of damage to peripheral nerves in skin; regulation of local blood circulation under mechanical load was thought to be controlled by the nervous system [33]. The neurogenic factors released stimulate the proliferation of new blood vessels that are fragile and can cause bleeding and scar tissue formation [33]. Restoration of circulation after an ischaemic episode does not usually reinstate normal function; rather, it results in inflammation and oxidative damage through the induction of oxidative stress [34]. White blood cells, carried to the area during reperfusion by blood, release various inflammatory factors as well as free radicals in response to tissue damage. Thus, tissue damage caused by deprivation of oxygen and nutrients during ischaemia, stimulated an inflammatory response which contributed to the reperfusion injury. Dermal tissue injury where cellular and vascular damage was pronounced was observed in keloids [16].

Compressive forces amplified at the soft and hard tissue interface are implicated in ear lobe keloid formation

Ear lobe keloid formation secondary to ear lobe piercing is a common occurrence, especially in females. The earlobe is usually affected on the posteromedial surface in association with the posterior positioning of the earring post. This suggests that a critical factor in the development of these keloids must be pressure applied by the earring post and the back of the earring which compresses the soft tissue of the ear lobe between two firm surfaces. This applies large external forces on the small area of the ear lobe; as there is very little tissue to dissipate the stress, internal stress load is greatly elevated and the ear lobe encounters the full effects of the resulting strain. This is aggravated by the amplification of the elevated stress at the interface between the highly compliant soft tissue of ear lobe skin and the hard cartilage of the external ear. Normal stress is highest in the interface region between soft and hard tissue; in addition, normal stress also combines with other forces (parallel and pinch shear) at this interface region [32]. This is similar to the situation at the bony prominence/soft connective tissue interface where stress levels are the highest as is the distortion (strain) [32]. The tendency for keloids to form on the posteromedial surface of the earlobe, post ear piercing, may occur as a result of application of pressure to the ear lobe in

the anterio-posterior direction in patients who sleep primarily on one side for too long, subjecting the ear lobe to long periods of increased external pressure and subsequent compression of microvessels and insufficient circulation.

Elevated normal and shear stress, microvessel compression, ischaemia and reperfusion injury implicated in ear lobe keloids

Both the duration of stress application and the degree of pressure applied are important factors determining the extent of distortion (strain) and its effects in tissue. It was found that when pressure on internal tissues exceeds CCP of 32 mm Hg for more than 2 h, blood supply to tissue is compromised, resulting in anoxia and death of tissue [35]. Experiments in which 300 mm Hg compressions were applied continuously for 24 h showed only necrosis caused by ischaemia, whereas repeated-compression resulted in the formation of ischaemic areas and the aggravation of wounds and damaged tissues [35]. The later is the more likely situation in ear lobe keloid formation where after ear piercing repeated compression is imposed by the earring post and the back of the earring; added to this are the effects of friction, shear and gravitational forces. The stress levels are elevated in the ear lobes because there is very little tissue to dissipate the stresses and because of the amplification of normal stress at the interface between soft tissue (ear lobe skin) and hard tissue (cartilage). Elevated normal and shear stress cause tissue compression and distortion, respectively. In the process blood flow is restricted or blocked in areas in the dermis of the earlobe. The resulting ischaemia leads to degeneration and necrosis of tissue. How do we then explain the paradoxical situation of keloidal growth? Growth is stimulated during reperfusion when blood supply returns to the tissue after stresses are released in the post-ischaemia period. Growth factors are produced by activated platelets that accumulated within vascular beds during ischaemia in preparation for their release early after reperfusion [36]. In addition to growth stimulation by reperfusion, an increase of internal stress also leads to cell stimulation and an increase in their levels of gene expression, collagen synthesis and mitotic activity [22].

Higher blood haemoglobin content in the epidermis of a keloid prone region

An interesting result reported by Sandby-Møller [30] was the higher blood haemoglobin content in the epidermis of the shoulder (31.1%), which is a keloid prone region, when compared with that of the forearm (22.4%) and buttock (29.6%) which are keloid protected areas. As the epidermis is avascular, the only way that red blood cells can enter the epidermis is via vascular and sub epidermal basement membrane damage. What factors would cause such damage in normal skin? A plausible reason would be the adverse effects of frictional shearing forces on skin relayed to capillaries and post capillary collecting venules which have the thinnest walls when compared with other blood vessels. This force would also cause stretching of the basement membrane in a direction perpendicular to the force. As rete ridges, which provide additional resistance and stronger tethering against shearing frictional forces, are not as well developed in thin as in glabrous skin, the basement membrane has less play and therefore, is liable to stretch and damage. If there is blood vessel damage and leakage of RBC into the epidermis, then there must also be leakage of plasma into this area. As the protective stratum corneum is thinner in keloid prone skin and as bacteria and fungi live on the skin surface, it is likely that even a minor breach of the stratum corneum will permit their entry and invasion of the epidermis and dermis where the bacteria and fungi will flourish in the nutrient rich milieu of damaged blood vessels. As indicated previously, infection in skin was proposed to be one of the three factors involved in the aetiopathogenesis of keloid, with genetic factors and suture location and tension being the other two factors [14].

How vellus hairs on keloid prone skin may contribute to keloid formation

Keloid prone skin is covered with fine, short vellus hair whereas keloid protected areas, viz., scalp, axilla and bearded and genital areas, are covered with thick long terminal hair. What is the association between skin with vellus hair follicles and keloid formation? Vellus hair follicles are small and are rooted in the papillary or upper reticular dermis whereas terminal hair follicles reach into the deep dermis and their bulbs are located in the subcutaneous fat. When stimulated by fear or cold the arrector pili muscle that attaches the sheath of the hair follicle (just below the sebaceous gland level) to the papillary dermis, contracts and the hair stands erect. During arrector pili muscle contraction and relaxation tissue fluid is displaced against dense collagen bundles of the reticular dermis, resulting in shear and tensile stress effects. Vellus hair roots are exposed to these shear and tensile stress effects, as, unlike terminal hair roots, they are not protected by a surrounding cushion of subcutaneous adipose tissue. Additional shear effects arise in the following way: Tensile stress between two objects (collagen bundles and hair roots) with fluid between, will force inward flow between the surfaces, and the non-slip conditions at the liquid–solid interfaces will result in the occurrence of shear [37]. Another possible factor increasing shear is the inefficient functioning or absence of the Arao-Perkins body which is a pad of elastic tissue under the dermal papilla of vellus hairs [38]. The prime function of this body is the absorption of forces of deformation, especially during contraction of arrector pili muscles. Wound healing tissue lacks elastic fibres [39], this deficiency denotes poor formation or absence of the Arao-Perkins body in scar tissue; this also applies to keloids which are formed during excessive wound healing processes [16]. Without the Arao-Perkins body forces of deformation, shear and tensile stress persist and are augmented with every contraction of arrector pili muscles. Stress effects will cause uncrimping, straightening and stretching of collagen. Uncrimping of collagen under tensile stress causes internal friction within bundles as the fibres slide through the thick viscous proteoglycan gel [40]. Within the elastic range there is a linear increase in deformations related to the applied load. This region represents the elastic modulus of the collagen bundle [40]. Within this elastic range of loads the collagen bundle will return to its original length when unloaded. Beyond this is the region of primary failure called the plastic region where permanent changes in collagen fibre length occur and this is often associated with microtrauma [41]. If load application continues the collagen fibres will reach a point of total failure [42].

Keloid microstructure exhibited the above stages of collagen integrity as dense regular collagen bundles, dense regular wavy collagen bundles with waves of varying width and amplitude, compressed microvessels and cells and areas of tissue lysis [16]. Dermal papillae at the root of vellus hair follicles typically do not have capillaries and their supply of nutrients and oxygen diffuses from blood vessels around the vellus hair follicles. In keloids this blood supply is deficient because of the compression of microvessels during tissue deformation caused by excessive internal stress. Without its blood supply the vellus hair differentiation is impaired; this may be the reason why skin covering keloids is generally hairless and smooth.

Discussion

The main factor predisposing the healing upper torso skin, post injury, to keloid formation, is the elevated dermal stress level

caused by stress-amplification at the soft and hard supportive connective tissue interface e.g. soft dermal tissue and bony prominences in the deltoid, chest and mandibular areas or cartilage and dermis in the ear lobe. Other factors contributing to internal stress elevation in the late wound healing stages are inherent skin characteristics such as the bundled arrangement of collagen in the reticular dermis, the existent high tension, the low elastic modulus, low stretch ability and contractile forces of wound healing fibroblastic cells. In addition to elevated stress levels, other factors predisposing healing upper torso skin to keloid formation are the thinner epidermis, the presence of vellus hairs, thick fragile quality of the thin skin, the absence of protective multiple layers of keratin in the epidermis, etc. Stress promotes keloid formation by causing dermal distortion and compression which subsequently stimulate proliferation and enhanced protein synthesis in wound healing fibroblastic cells. Distortion and compression of the dermis also compress and occlude microvessels, causing ischaemic effects and reperfusion injury which stimulate growth when blood rich in growth factors returns to the tissue and releases these growth factors. The growth promoting effects, caused primarily by increased internal stress and by growth factors released by reperfusing blood, manifest in keloid formation.

In contrast to keloid prone areas, keloid protected areas exhibit many properties that increase stress tolerance, e.g. lax and creased nature of the skin, inherent low prestress levels, unbundled and loose arrangement of dermal collagen fibres as in eyelid skin, etc. In contrast, keloid prone skin is taut with high skin tension and collagen fibres are assembled in dense irregularly arranged bundles which promote shear, friction and compressive forces, ultimately causing tissue distortion and compression. Another feature in keloid protected skin (eyelid) is the absence of dermal fat and decreased hypodermal fat; this effectively reduces cell surface area in the dermis and therefore, the cell interface-collagen fibril- interaction and associated force generation and transduction are also reduced.

Collagen and elastic fibres in the dermal layer of the skin are responsible for the mechanical properties of skin such as its tensile strength and elasticity. Collagen and elastic fibres resist deforming mechanical forces by stretching and sliding mechanisms. When skin is injured, normal collagen is replaced by scar collagen and the original highly organized structure of collagen in dermal connective tissue is not regained [43]. Thus, healing skin has decreased tensile strength when compared with normal skin. Tensile strength is usually associated with the content, organization and physical properties of the collagen and elastic fibre networks. During the wound healing process in skin, the intermolecular covalent cross-linking pattern within collagen fibrils is altered [44] and collagen-ground substance interactions are also altered [45].

Collagen reorganization into larger bundles and collagen maturation occurred during the late remodeling stage of wound healing and this increased the tensile strength of the wound. In keloids both fibre types that play a role in withstanding deformational stress i.e. collagen and elastic fibres are defective and/or lacking, respectively [21]. As keloids are formed by excessive connective tissue deposition during ongoing wound healing processes, the original quality of collagen, its highly organized structure and tensile strength as in normal dermis are not attained. Keloids are excessive scar tissue formations therefore, the temporary lack of elastic fibres occuring in normal scars persists in keloids. Elastic fibre defects found in keloids were that deposition of elastin and DANCE (also known as fibulin-5) on microfibrils were abolished and the distribution of fibrillin-1 was abnormal [46]. Morphological studies by Bux and Madaree illustrated the defective nature of collagen and elastic fibres in areas of connective tissue rupture as well as in areas where collagen and elastic fibres were disheveled and degenerate [21]. They also showed a range of collagen fibre arrangements in bundles from deeply wavy, crimped to straight and regular as in tendons; this reflects collagen adaptation to withstand increasing levels of stress exerted in the dermis of keloid lesions.

Stress causes dermal distortion and compression which subsequently stimulate proliferation and enhanced protein synthesis in wound healing fibroblastic cells. Strain caused by stress also compresses and occludes microvessels causing ischaemic effects and reperfusion injury which stimulate growth when blood rich in growth factors returns to the tissue. The growth promoting effects of increased internal stress, primarily and growth factors released by reperfusing blood manifest in keloid formation. Thus elevated stress caused by external and internal forces on the upper torso dermis, inherent morphological characteristics of the upper torso of the body and infection are implicated in the pathogenesis of keloids.

Predictions of hypothesis

From the above hypothesis it can be emphasized that the following precautionary measures or adjuvant treatment options be taken in order to minimise the risk of keloid formation in predisposed individuals or recurrence after surgical removal:

(1) Surgical incisions must be made parallel to Langer's lines of tension [47], where possible, during surgery. Langer's lines are relaxed skin tension or cleavage lines. In incisions made parallel with these lines, most of the bundles of collagen in the reticular dermis are longitudinally orientated to the incision and the wound heals with a fine linear scar, while in incisions made perpendicular to these lines, the bundles are cut in cross section and irregular tensions are created which may result in abnormal scar tissue formation (hypertrophic or keloid scars).
(2) When pressure therapy is used in the management of keloid scars, the amount and direction of pressure should be controlled to reduce the risk of complications caused by the application of excessive pressure e.g., promotion of keloidal growth or ulceration.
(3) After surgical excision of keloids the wound should be closed by and large, without tension.
(4) Pressure gradient garments should be used as an adjunct in the postoperative treatment of keloids to help prevent recurrence. The protective mechanism here is to prevent elevation of stress levels caused by friction, shear and pressure.
(5) Prevention should be the first rule of keloid therapy.
(6) Unneeded aesthetic surgery should not be performed on individuals prone to keloid formation. Incisions in areas covered with high tension skin as in the upper torso should be avoided whenever possible.

Concluding remarks

The high recurrence rate of keloids and their poor response to therapy present a great challenge to surgeons. Currently the best treatment option used is excision followed by postoperative chemotherapy, radiotherapy or use of other adjuvants. The numerous therapeutic regimens demonstrate that to date, there is no single therapy that is absolutely successful. Thus, it is necessary that research on keloid pathogenesis and therapy persists. The hypothesis presented in this paper presents many new research avenues on the promotion of keloid formation by elevated internal stress caused by pertinent dermal connective tissue characteristics, the effects of hard tissue in close proximity to the dermis, contractile forces exerted by wound healing fibroblastic cells and external forces. These

parameters provoke keloid pathogenesis by causing dermal distortion and compression which subsequently stimulate proliferation and enhanced protein synthesis in wound healing fibroblastic cells. It is hoped that these new research avenues are explored with the aim of progressing with the quest to elucidate the factors implicated in the aetiopathogenesis of keloids with the long term view of providing unfailing specific effective treatment.

Conflict of interest statement

None declared.

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TITLE
THE SIGNIFICANCE OF HYALINIZED TISSUE IN THE PATHOGENESIS OF KELOIDS

Authors:
Shamin Bux BSc, MMedSc, Department of Physiology, Faculty of Health Sciences, Nelson R Mandela School of Medicine, University of Kwazulu-Natal, Durban, South Africa.

Anil Madaree FRCS (1), FCS (Plastics) (South Africa). Department of Plastic and Reconstructive Surgery, Faculty of Clinical Sciences, Nelson R Mandela School of Medicine, University of Kwazulu-Natal, Durban, South Africa

Corresponding author and author to receive reprints:
Shamin Bux, Dept of Physiology, Faculty of Health Sciences, Nelson R Mandela School of Medicine, University of Kwazulu-Natal, Private Bag 7, Congella 4013, Durban, South Africa
Tel: 031-2604362 Fax: 031-2604455 e-mail: Bux@ukzn.ac.za

Running Title: Histological assessment of the hyalinization process in keloids

Keywords: Keloids, hyalinization, collagen bundles, wound healing, mast cells, fibroblastic phenotypes, cell death

Abstract
Aims: To critically examine the region of hyalinizing collagen bundles (HCB) in keloids to understand why the hyalinization process occurs in human tissues and to decipher the role of this process in the pathogenesis of disease.
Methods: The HCB region in forty nine keloid specimens from twenty eight patients were processed for routine haematoxylin and eosin and differential staining with Mason's trichrome, reticulin, elastic, sulphated Alcian blue, Luxol fast blue, and Van Kossa's stains. Immunohistochemical staining to localize α smooth muscle actin (α SMA), vimentin, desmin and fibronectin was also performed on these specimens.
Results: Hyalinizing collagen bundles were composed of varying amounts of different connective tissue and cellular elements including protein fibres (collagen and reticular), microfilaments (α SMA), intermediate filaments (vimentin), glycoproteins (fibronectin), fibrin, calcium salt deposits and cellular debris. As cells approached the HCB area they underwent morphological and behavioural changes and modification such as aggregation, attachment, alignment in different patterns, lytic change and necrosis. They also exhibited altered functional characteristics such as space formation. The hyalinisation process incorporated the involvement of mast cells, myofibroblastic cells and other fibroblastic phenotypes; the presence of reticular fibre enwrapped cellular material and cellular debris in HCB; and cell degeneration, early apoptosis, autophagic cell death and necrosis associated with HCB.
Conclusion: The hyalinization process is hypothesized to isolate certain degenerating wound healing fibroblastic phenotypes; to play a protective role by sequestrating mast cell cytokines and proteases; and to prevent autoimmune attack by segregating injured cells that might not have gained prior tolerance.

Keywords: Keloids, hyalinization, collagen bundles, wound healing, mast cells, fibroblastic phenotypes, cell death

Introduction

Hyalinization is a widespread degenerative process characterized by the replacement of some normal tissue components with amorphous collagen and other connective tissue (CT) elements. The hyalinization process is thought to be initiated in the stroma between smooth muscle cells (SMC) before progressing to the replacement of SMC (2) by amorphous collagenous tissue of a poorly defined nature. Hyalinization, also called hyaline change, describes tissue with a glassy, amorphous eosinophillic appearance when stained with hematoxylin and eosin (H&E). Hyalinization occurs in a wide spectrum of tissue types which include skin; nerve (3); lung (4); gastro-intestinal tract (5); uterine wall (6), thyroid gland (7), breast (8); kidney (8, 9); liver (10); testes (11); muscle (12), lymph nodes (13), periodontal ligament (PDL) (14), and Islets of Langerhans in the pancreas (15). In many of these tissues like nerve, Islets of Langerhans, PDL, lymph nodes etc., SMC, a reported prerequisite for hyalinization, are absent except in the walls of blood vessels. Therefore, in these tissues hyalinization was most likely initiated in the walls of blood vessels. Hyaline change is produced in various types of pathological conditions, including amyloidosis (16), hypertension (17), and scar formation (18). Varying degrees of hyalinization have been described in diverse types of pathology such as hyalinizing spindle cell tumours with giant rosettes (19), pulmonary hyalinizing granuloma (20), hyalinizing trabecular tumours (7), chronic cyclosporine nephrotoxicity (21), eosinophilic esophagitis (22) and around seminiferous tubules in testes (23). The widespread occurrence of hyalinization in various tissues and pathological conditions indicates the importance of this process in pathology. Yet little is known about the hyalinization process. Why does hyalinization occur? Is it merely a degenerative process where degenerate SMC are replaced with hyalinised connective tissue elements? Why is hyalinization associated with SMC? Do SMC alone, contribute to the hyalinization process? Which cells are responsible for the production of the connective tissue elements that become hyalinised? Many questions about hyalinization remain unanswered. This study attempts to elucidate the process of hyalinization by examining keloids where a characteristic, diagnostic feature is the presence of numerous bundles of hyalinized collagen. Hyalinised collagen bundles (HCB) are pathognomonic of keloids which are fibro-proliferative tumours produced by an excessive wound healing process (24). In a recent article we reported the significant features observed in the HCB region of keloids; these included the presence of fibroblastic, epitheloid and mast cells; features of cellular and microvascular degeneration and necrosis and HCB variation in shape, size and adjacent stromal characteristics (24). It is expected that a critical examination of the morphology of keloids treated with various differential stains and antibodies will provide more insight into the hyalinization process in human tissues.

Patients and Methods

Surgical biopsies obtained from 28 patients undergoing surgery for the removal of keloids located in one or more of the following areas: the head, neck, pre-sternal, deltoid and supra-pubic areas (total of 49 specimens), were placed in 10% formalin saline (4% formaldehyde) and processed routinely for wax embedment. The research protocol for the study was approved by the Nelson R Mandela Faculty of Medicine Ethics Committee and informed consent was obtained from all patients. Multiple sections were cut of each specimen at a thickness of 3-5 μm for differential staining methods and at a thickness of 2-3 μm for immunocytochemistry. Routine haematoxylin and eosin (H&E) staining was performed on all slides. The sections for immunocytochemistry and some of the differential staining procedures were picked up on poly-L-lysine coated slides. The poly-L-lysine coating was an adhesive that prevented sections from floating off the slide when treated with harsh substances or when subjected to long staining or incubation periods.

Problems were encountered with the sectioning of the keloid samples. This was attributed to the dense nature of the fibrous tissue. These problems were overcome by:

- Using numerous new knives
- Seeking the assistance of expert histology technicians

- Persevering, optimising block temperature, angle of cutting etc., and even resorting to uncurling sections that otherwise could not be mounted on slides.

Indirect immunohistochemical staining was performed on the tissue sections after employing microwave antigen-retrieval in citrate buffer (pH 6.00) at 98^0C for 10 minutes (25). The primary, secondary and tertiary antibodies and chromogen used as well as other details of the imunocytochemical study are listed in Table 2. Differential stains employed and other relevant details are listed in Table 3.

Results

The region of hyalinizing collagen bundles contained many HCB, each of which was composed of thick, dense, aggregates of collagen bundles and other connective tissue elements including cells (Figs 1A, B). The bundles had a glassy appearance and were usually located in the upper reticular dermis below the sub-papillary plexus (Fig. 1A). They were often separated by fibrous cellular stroma containing flattened elongated fibroblastic cells adjacent to the outer edge of the bundle (Fig. 1 B). Hyalinizing collagen bundles showed diversity of shape, size, orientation, appearance, content, and morphology of surrounding stroma (Figs 1A, B, C, and D). The appearance of HCB ranged from irregular, elongated tubular, whorled, small spherical, smaller less compact bundles to merging bundles (Fig. 1A, C, D).

Isolated hyalinizing collagen bundles

Some HCB occurred as small single bundles or as a small group (2-5 bundles) surrounded by a fibrous to myxoid stroma with spindle or fibrohistiocytic cells (Fig. 1E). These appeared to be newly formed early HCB. Early HCB formed within fine fibrous cellular regions of keloids and the frequent location adjacent to wavy collagenous stroma with wavy slender elongated fibroblastic cells (Fig. 1E) or small blood vessels (venules) was suggestive of an association with differentiation of tissue. Early HCB displayed shapes ranging from elongated tubular to fan-shaped HCB to irregular. The cellular content of the stroma around early HCB varied from moderate to hypercellular and the cells displayed varying morphology which included epitheloid, fibrohistiocytic, spindle and elongated fibroblastic cell types. Cells in the vicinity of early HCB exhibited varying degrees of degeneration and many were necrotic. On approaching early HCB the cells often showed a palisading arrangement (Fig. 1E, 2A) and very degenerate to necrotic features like very swollen nuclei with ruptured nuclear membranes, cellular lysis and dissolution (Fig. 1F). Fibrohistiocytic cells with indistinct nuclei and large spherical cells and spindle cells with pyknotic nuclei were associated with space formation; triangular cells with long processes were occasionally found among these space forming cells (Fig. 1E, 2A, B). The pyknotic nuclei of spherical cells occasionally exhibited features of apoptosis (Fig. 2A). Fibrohistiocytic cells often showed attachment to each other in the vicinity of early HCB (Fig. 2B, C) and fibroblastic cells became very slender and elongated when associated with oedematous clefts around early HCB (Fig. 2C). Some elongated fibroblastic cells in wide extracellular spaces with sparse myxoid stroma displayed unipolar or bipolar morphology, and were occasionally in intimate contact with mast cells (Fig. 2D). This relationship was also observed between attached fibrohistiocytic and mast cells (Fig. 2E). Small fibrinous and dense deposits were scattered in areas of close association between mast cells and elongated fibroblastic cells (Fig. 2E). Mast cells in close proximity to large fuzzy cells with indistinct nuclear and cytoplasmic boundaries displayed calcified nuclei (Fig. 2F). Calcified nuclei were also present in most quiescent and degranulated mast cells in close proximity to early HCB (Fig. 3A). Mast cell granules were found within fine tubular structures or scattered in the milieu of early HCB. In the vicinity of scattered mast cell granules, cells and microtubular structures showed

degenerate and necrotic features (Fig. 3B). The main cell type showing degenerate features and necrosis associated with MC granules were attached fibrohistiocytic cells (Fig. 3B). Cells around early HCB could be differentiated into two populations on staining with silver stains; the cells showing pleomorphic to elongated morphology and frequent attachment to each other, in keeping with features of fibrohistiocytic cells, stained positively for reticulin and they were closely associated with elongated fibroblastic cells that were negative for silver staining (Fig. 3C). This stain also revealed the presence of a large number of reticular fibres in early HCB (Fig. 3D); other components found in early HCB included fibrin (Fig. 3E) necrotic cellular material (Fig. 3F) and calcium deposits (Fig. 4A).

Immunocytochemical staining in the early HCB area showed some positivity for vimentin, α-SMA and fibronectin while immunostaining for myosin and desmin was absent. Early HCB showed pale positivity for vimentin while in the cells around HCB vimentin positivity was found in short cellular processes which often showed curvilinear forms and in pyknotic cells (Fig. 4B). Cells with vimentin positive processes were usually pyknotic or showed pyknotic nuclei, while cells displaying pale vimentin staining were swollen and necrotic; many tubular structures were present adjacent to, or in close proximity to or entwined around components of positive cells (Fig. 4B). Many vimentin positive apoptotic bodies were scattered in the vicinity of early HCB (Fig. 4B).
Positivity for α-SMA was found in the mural cells of microvessels which were often compressed, in slender fibroblastic cells in the vicinity of microvessels, in fibroblastic cells between early HCB and in cellular debris scattered in the early HCB milieu (Fig. 4C). Occasionally, squiggly strands of cellular debris showing strong α-SMA immunoreaction were scattered around microvessels with calcified nuclei in some mural cells; these positive squiggly strands were closely associated with the outermost mural cells presumably, pericytes (Fig. 4D). The squiggly strands of cellular debris positive for α-SMA were also found within lymphatic vessels, often at the periphery of the lumen, in narrow and wide clefts around early HCB and within early HCB (Fig. 4D). Fibronectin immunoreaction in the early HCB area was found in elongated fibroblastic cells with tapering or blunt ends and around microtubular structures arranged in clusters adjacent to HCB or forming the outer edge of HCB; areas of tissue lysis were usually found adjacent to these microtubular structures (Fig. 4E, F). Many of the fibronectin positive cells showed features of degeneration mainly as swollen organelles (Fig. 4E). Fibronectin immunoreactivity was also found within HCB or at the edges of HCB; in the latter case a majority of cells in the milieu were negative for fibronectin whereas when fibronectin positivity in HCB was decreased, more cells in the milieu were reactive for fibronectin (Fig. 4E, F).

Areas with aggregates of hyalinizing collagen bundles
As opposed to early HCB, the HCB region contained numerous aggregates of HCB with an irregular branching pattern, showing a tendency towards nodular formation when the HCB exhibit increased eosinophilia and prominent cleft formation. Various stages of nodule and cleft formation were observed and these were associated with varying degrees of eosinophilia and cell viability. Generally, less HCB were separated by fibrous CT containing unbundled collagen fibres and moderate numbers of spindle cells (Fig. 5A), elongated fibroblastic bipolar or tripolar cells (Fig. 5B) and epitheloid cells (Fig. 5C). Often less HCB were separated by narrow slits lined by pyknotic spindle cells or elongated fibroblastic cells with condensed cytoplasm, vesicular nuclei and prominent nucleoli (Fig. 5D). These nuclear features were also displayed by spindle cells in close proximity; many of these spindle cells displayed varying stages of nuclear and cytoplasmic condensation, pyknosis and necrosis (Fig. 5D). Mast cells surrounded by a clear zone were scattered in this milieu (Fig. 5D). Some degranulated mast cells with pyknotic nuclei were found in close proximity to areas of

CT lysis associated with necrotic cells and tadpole-like fibroblastic cells containing vesicular nuclei and prominent nucleoli (Fig. 5E).

Whilst some HCB aggregates were less eosinophillic, others were intensely stained and associated with cleft formation (Fig. 5F). The clefts around HCB contained elongated fibroblastic cells which often were attached in varying patterns of arrangement including linear, hinged or Indian file arrays (Fig. 6A). The clefts also showed the presence of shadow cells; shadow cells were plump, spindle, spherical to irregular in shape and were condensed or atrophic (Fig. 6A,B). Few atrophic cells were found in less compact connective tissue adjacent to clefts or HCB. Pyknotic elongated fibroblastic cells and fibrohistiocytic cells were frequently found at the edge of HCB or in HCB (Fig. 6B). Fibrohistiocytic cells displayed vesicular nuclei, prominent nucleoli, cytoplasm and attachment to each other (Fig. 6B). Elongated fibroblastic cells were flattened, contained condensed elongated nuclei and long slender polar processes that were often curved or split into complementary curves to surround very pale amorphous material within clefts between HCB; the processes frequently crossed clefts at a 45^0 angle to the long axis of the HCB (Fig. 6C). Some elongated fibroblastic cells exhibited processes displaying polar curving or splitting with the terminal processes crossing clefts at a 45^0 angle (Fig. 6C). Morphological evidence suggests that the material present within HCB was secreted by spindle, fibroblastic, fibrohistiocytic, "tadpole" cells and mast cells (Fig. 6D, E).
Many stromal cells around HCB, especially fibroblastic and fibrohistiocytic cells showed features of mild to severe degeneration and necrosis (Fig. 3B, 2F). These included cytoplasmic and nuclear membrane damage. The nuclei of degenerate and necrotic cells often displayed merging of the nuclear chromatin network with cytoplasmic contents and necrotic cells appeared as large fuzzy cells with indistinct nuclear and cytoplasmic boundaries (Fig. 2F).

Another cell type ubiquitously found in the HCB area was the mast cell. In the HCB area mast cells displayed varying morphology in accordance with its quiescent or degranulation state. These ranged from quiescent mast cells in a rounded pyknotic state with very condensed cytoplasm and calcified nuclei (Fig. 6E, 7A); to elongated, polygonal and irregular forms in different stages of degranulation (Fig. 7B). Degranulated or degranulating mast cells were closely associated with cellular necrosis (Fig. 7C) and often their granules were found within fine delicate or more robust processes or within the nucleoplasm and cytoplasm of afflicted cells (Fig. 7D). Free mast cell granules were observed to be strewn within HCB and they were often associated with dissolution necrosis of mast cells, aggregates of necrotic fibrohistiocytic cells and fragmented microtubular structures (Fig. 7F). In the vicinity of quiescent mast cells the cell types usually found were shadow cells, atrophic cells and occasional spindle cells, some with vesicular nuclei and prominent nucleoli and others with condensed nuclei and cytoplasm (Fig. 6B). Often mast cells were partially or completely surrounded by space (Fig. 5D, E; 6A; 7A-D) and few were connected to stromal cells by fine processes (Fig. 7B-D). Mast cells in the vicinity of degenerate microvessels and disaggregating degenerate fibrohistiocytic cells were usually associated with fibrinous exudate (Fig. 6E, 7A).

Mast cells were often found in close proximity to HCB, shadow cells and occasional spindle cells, some with vesicular nuclei and prominent nucleoli and others with condensed nuclei and cytoplasm (Fig. 6B). Aggregates of elongated fibroblastic cells attached by many processes and bordered by prominent, long curvilinear cellular processes were also found in these areas of HCB and mast cells (6E). In some areas of early cleft formation less dense and less stroma was attached to the HCB containing flattened, curvilinear cells, spindle cells with

hyperchromatic nuclei, fibrohistiocytic cells, atrophic cells, fuzzy cells and occasional microvessels with hyalinized walls (Fig. 8A).

With regard to the vascular supply of the HCB area, it was generally poorly vascularised to avascular. Blood vessels found in the HCB area were microvessels, mainly venules. They appeared unhealthy with pathological changes becoming progressively worse from the outer edge to the innermost part of the HCB aggregate. Common pathological changes in venules in the outer HCB area included swollen endothelial cells and obliterated lumina and paravenular cleft formation with the presence of calcified granular aggregates within the clefts (Fig. 8 B). Venules in septa of connective tissue (CT) between HCB exhibited proliferation and swelling of mural cells and degenerate and necrotic pericytes (Fig. 8C). Further into the HCB area the venules showed degenerate and necrotic mural cells, areas of paravenular clearing containing pyknotic cells and cellular debris and fibrinous exudate in lumens (Fig. 8D).

Minimal staining for elastic fibres was found in the HCB area within or adjacent to some HCB. In HCB the elastic fibres were degenerate and appeared as dispersed or sploshed positivity for elastin (Fig. 8E, F). Occasionally it was observed that slender long linear strands of elastic fibres were closely associated with aggregates of elongated tubular structures adjacent to HCB (Fig. 9A). Fragmented microtubular structures overlying necrotic cellular material and necrotic ECM material were scattered in the vicinity of the elastic fibres adjacent to HCB (Fig. 9A). Some elastic fibres adjacent to HCB were short and thick and they appeared to cover thick tubular structures (Fig. 9B). Occasionally elastic fibril positivity was evident as isolated spots or aggregates of spots in close proximity to degenerate cells and blood vessels at the edge of HCB; these spots appeared to merge into blotches of elastin (Fig. 9C).

Reticular fibres composed of collagen type III, fibronectin and reticulin were generally found within HCB, as a network supporting cells between HCB and around blood vessels in the HCB area (Fig. 9D). Reticular fibres were usually found adjacent to elongated fibroblastic cells, around the outer mural cells of microvessels (Fig. 9E) and covering processes or parts of fibroblastic and fibrohistiocytic cells (Fig. 9F). These reticular fibre covered cellular components appeared to be incorporated into the HCB (Fig. 9F). It was noted in blood vessels that in mural parts lacking reticular fibres the cells were degenerative whereas in parts where these fibres were present the cells were intact (Fig. 10A). In HCB areas with wide space between HCB large fibrohistiocytic cells covered with reticular fibres were found within the space or attached to HCB (Fig. 10B). Other cell types enwrapped by reticular fibres in the HCB region were epitheloid, pleomorphic, spindle and slender fibroblastic cells; these cells were closely associated with unwrapped spindle, fibroblastic and epitheloid cells (Fig. 10C). Occasional aggregates of space forming cells with reticular fibres aligned around the spaces were found between HCB (Fig. 10D). In some HCB areas many aggregates of reticular fibres were scattered in the HCB, while in adjacent areas lysis of these reticular fibres and HCB were evident (Fig. 10E). Lysis of the extracellular matrix elements appeared to be initiated in the spaces between the HCB (Fig. 10F).

Immunocytochemical staining of the HCB region with αSMA showed prominent positivity in the mural cells of microvessels, mainly venules and occasional capillaries (Fig. 11 A); these vessels were compressed to the extent that their lumina were obstructed. Less prominent αSMA staining was observed in elongated fibroblastic cells between HCB and clefts and in spindle cells in the stroma between HCB (Fig. 11 A). Other cells positive for αSMA in the HCB area were large fibrohistiocytic and epitheloid cells associated with space formation and

found within lymphatic vessels and elongated fibroblastic and epitheloid cells connected to palliating cells (Fig. 11 B, C). Some αSMA positive fibrohistiocytic cells linked with space formation or present within lymphatic vessels were attached to each other (Fig. 11 C, D). Cellular debris showing positivity for αSMA was found within some HCB associated with cleft formation or at the edge of these HCB around which cleft formation occurred; αSMA positive cells present at the edge of these HCB were fibrohistiocytic, epitheloid and fibroblastic cells (Fig. 11 D).

Fibronectin immunoreaction was found in fibroblastic, epitheloid, fibrohistiocytic, pleomorphic and necrotic cells in the stroma between HCB (Fig. 11 E). Mild fibronectin positivity was found in processes of epitheloid cells and in necrotic cells (Fig. 11 E). Attached fibrohistiocytic cells, degenerate cells and some HCB also displayed fibronectin positivity (Fig. 11 F).

Vimentin immunopositivity in the HCB area was observed in fibroblastic, spindle and fibrohistiocytic cells and in cellular processes (Fig. 12 A). The positive spindle and fibrohistiocytic cells often were attached to each other and some of the vimentin positive cellular processes were very long (Fig. 12 A). Vimentin immunoreactive processes and cells were surrounded by areas of tissue lysis and some cells showed features of degeneration; the latter cells showed pale vimentin staining. Vimentin positive cellular debris was scattered in or adjacent to HCB (Fig. 12 A, B).

Several “tadpole” cells in the stroma around HCB displayed vimentin positive processes that often attached to similar cells. Frequently vimentin positive cells and processes and necrotic cells were arranged around HCB (Fig. 12 B, C). Many positive necrotic and degenerating cells still showed attachment to their necrotic counterparts (Fig. 12 C). Vimentin immunoreactive “tadpole” cells were also associated with lysis of adjacent tissue (Fig. 12 B).
Differential staining with Masson's trichrome showed cytoplasm, fibrin and muscle in red and collagen in green. Fibrin deposits in the HCB area were present, generally, where the stroma around HCB was more cellular. Here fibrin deposits and/or aggregates of cellular debris surrounded by collagen formed the HCB (Fig. 12 D). Fibrin deposition in HCB was usually associated with degenerating vessels and degenerate attached palliating or aggregated fibrohistiocytic cells (Fig. 12 D). As a result of the pronounced degeneration of cells and blood vessels in the HCB area there was an accumulation of excessive amounts of phospholipids within HCB which was evident on staining with Luxol fast blue (Fig. 12 E) Also associated with the degeneration and necrosis was the occasional presence of calcium deposits within HCB in the form of dense deposits and/or aggregates of small particulate or granular deposits (Fig. 12 F).

Discussion

The results of this study showed that HCB were composed of varying amounts of different connective tissue and cellular elements including extracellular protein fibres (collagen and reticular fibres mostly with minimal elastic fibres), microfilaments (α SMA), intermediate filaments (vimentin), glycoproteins (fibronectin), fibrin and cellular debris. Cellular fragments appeared to be the undigested products of necrotic cells observed within HCB; the cell types involved were spindle, fibroblastic, epitheloid and fibrohistiocytic cells. Spindle and fibroblastic cells were abundant in the fine fibrous cellular areas surrounding and infiltrating the HCB area. As these proliferating cells approach the HCB they appear to go through various modifications such as aggregation, attachment, alignment in different patterns (palisading mainly and Indian file), lytic change, etc. Altered functional

characteristics of the cells were also noted in the HCB milieu; this included space formation by spindle, fibroblastic, epitheloid and fibrohistiocytic cell types. Space formation in the HCB area was observed to be closely associated with cell death as ascertained by features of necrosis and early apoptosis (condensed cytoplasm and nuclei). Previous research on space (aerenchyma) formation in ground parenchymas of plants showed that oxygen deficiency and ethylene signals initiate physiological processes commencing cell death and cellular degeneration leading to the creation of intercellular spaces (26). Cellular degeneration and death were pronounced in the HCB area and space formation occurred both intracellularly and extracellularly. These phenomena, as in plants, could well be an outcome of hypoxia which arises because of the poor vascular supply instigated by the collapsed, constricted and degenerating microvessels (24). With the diminished vascular supply to the HCB area the oxygen supply will fall to critical levels as cellular metabolism continues; with the exhaustion of oxygen supplies there is increased anaerobic metabolism. An excess of acid in and around the cells is deleterious to cells and tissue; life-essential functions, like movement of potassium (K^+) and sodium (Na^+) across the cell membrane are hampered because their respective channels are inactivated by acidosis. Inactivation of the sodium-potassium pump affects important cellular functions such as maintainance of resting potential, providing transport channels and regulation of cell volume. In the HCB area morphological changes in cell volume were heterogeneous ranging from swollen to condensed cells, illustrating the effects of osmotic diffusion of solvent into and out of cells from hypotonic and from cells into hypertonic tissue fluid, respectively. Acidosis activates MAP kinases, possibly by altered regulation of pH-homeostasis and modified signaling induced by ROS (27). The effects of activated MAP kinases (28) are evident in the HCB area in that cell proliferation and cell death are marked while defective/deficient MAP kinase function was indicated by the absence of differentiated dermal structures such as hair follicles, sweat glands and peripheral nerves and deficient cell differentiation, as assessed by the presence of vimentin immunostaining which is characteristic of mesenchymal cells.

Under normal circumstances surplus acid is removed or neutralized trough the vascular, respiratory, and renal systems as well as through the sweat glands or the acid-alkaline balance is restored by increasing mineral alkaline reserves. The poor vascular supply and the lack of sweat glands in the HCB area of keloids minimize elimination of excess acid via these routes and restoration of acid-alkaline balance occurs by increasing mineral alkaline reserves, mainly calcium which is obtained from blood, tissues and then from bones. Thus cytosolic calcium concentration is decreased during acidosis (27) and levels of ionized calcium in the ECM are increased, mainly due to reduced protein-binding that occurs with the lowering of the pH (29). Calcium salt deposits were evident in the HCB area as small granular or larger deposits. Granular deposits were frequently found in aggregates and it appeared as if these coalesced to form the larger deposits. Calcium salt deposition in the ECM also occurred through cellular necrosis with the spillage of cellular debris into the ECM and subsequent release of calcium ions bound to proteins.

Cellular lysis in the HCB area was also closely associated with the presence of mast cells which were ubiquitous in the HCB area. Many mast cells were in a degranulating state and they were intimately associated with lysing cells. A previous study linking mast cells and cellular lysis pioneered the finding that mast cell chymase caused cell death (30). Chymase, a serine protease, is also found within cytotoxic granules of cytotoxic lymphocytes and is implicated in perforin-mediated cellular lysis (31). The previous statements explain that in keloids during the intimate association of many degranulating mast cells with lysing cells, abundant chymase was released. Further, Hara et al (30) proposed that mechanical overload promoted the production of mast cell chymase, causing cardiac myocyte loss and fibrosis.

These criteria are also implicated in the pathology of keloids: they include the presence of many degranulating mast cells in various keloid regions (24) and elevated internal stress caused by pertinent dermal connective tissue characteristics, the effects of hard tissue in close proximity to the dermis, contractile forces exerted by wound healing fibroblastic cells and external forces (32). The widespread fibrosis with varying patterns of collagen arrangement in different regions and extensive cellular lysis were dominant features in keloids (24).

Another effect of proteases released by degranulating mast cells is the modification of the extracellular matrix (ECM), either by direct effects on components of the ECM or by regulating enzymes involved with production of these ECM components. Tryptase catalyzes the degradation of procollagen, type VI collagen and denatured collagen, while chymase catalyzes the degradation of procollagen, among other ECM constituents. The spaces generally observed around mast cells in the HCB area may thus be formed by the dissolution of ECM components by mast cell enzymes. Also, both tryptase and chymase have been shown to degrade fibronectin. This was evident in the HCB area where clusters of fibronectin –positive microtubular structures were associated with areas of tissue lysis. In the late stages of normal wound healing fibronectin forms a three-dimensional matrix which (1) regulates cell signalling by sequestering growth factors and associated proteins; (2) regulates the deposition of ECM proteins, including collagen types I and III and fibrinogen and (3) permits the assembly of a more mature and stable ECM network on this fibronectin-matrix (33). The fibronectin –positive microtubular clusters associated with areas of tissue lysis indicated that the fibronectin three-dimensional matrix was degraded by mast cell tryptase and chymase, impeding the important late wound healing functions of this matrix. Even fibroblastic cells that produce the fibronectin were in a degenerate state, further hampering the function of fibronectin in the wound healing process.

It was often observed that quiescent mast cells were connected by fine processes to some fibroblastic stromal cells, indicating that a close association existed between the two cell types. With regard to wound healing fibroblastic cells, it has been reported that activated, myofibroblasts express adhesion molecules such as intracellular adhesion molecule-1 and vascular cell adhesion molecule and that mast cells associate with myofibroblasts via the appropriate adhesion molecules and participate in well-controlled immunological reactions (34). As αSMA staining in elongated fibroblastic cells was observed adjacent to HCB it is likely that the fibroblastic cells attached to quiescent mast cells were in fact, myofibroblasts. Although the mast cells appeared quiescent, their granules were found within processes attaching them to myofibroblasts. The efficiency of such a system would ensure mast cell granule functionality such as stimulating proliferation and collagen production of myofibroblasts; both these effects were evident in the HCB area.

In the HCB region, additional to αSMA staining in elongated fibroblastic cells, it was also found in spindle cells in the stroma between HCB, and in large fibrohistiocytic and epitheloid cells associated with space formation and often found within lymphatic vessels. It is very likely that these αSMA-positive cells are variations of the fibroblastic/myofibroblastic phenotypes since keloids are a prototype of excessive wound healing. The spectrum of fibroblastic/myofibroblastic phenotypes range from non-contractile fibroblasts to contractile myofibroblast with many intermediate phenotypes (35). Space formation close to fibroblastic/myofibroblastic cells may be an ECM clearance function to facilitate their association with mast cells via cellular processes and adhesion molecules to modulate fibroblastic differentiation and collagen production. Mast cells in coculture with fibroblasts were reported to induce alpha-smooth muscle actin expression by fibroblasts and to stimulate fibroblast contraction of collagen gels (36). Based on this it is likely that in the HCB area,

mast cells stimulate fibroblastic cells to contract collagen fibres together to form the HCB. Linked with this is the finding that mast cell proteases promoted ECM accumulation by acting as mitogens for fibroblasts (37). Mast cell histamines induced an increase in alpha-smooth muscle actin expression by fibroblasts (36). The presence of degenerating myofibroblastic cells or their cellular debris shows that myofibroblastic differentiation in the HCB area is depressed probably by switching off the production of histamine. A feature supporting this is the absence of inflammation in the HCB area; **histamine** indirectly contributes to inflammation by affecting the functions of leukocytes in the area. The presence of αSMA positive cells within lymphatic vessels and αSMA positive cellular debris within HCB is indicative of removal of degenerate myofibroblastic cells by lymphatic vessels and/or sequestration of their necrotic remnants within HCB, respectively. The picture created is that mast cells suppress myofibroblastic differentiation to promote their prompt removal by lymphatic vessels or to sequestrate myofibroblastic necrotic remnants by contracting collagen fibres around them. During these occurrences inflammation appears to be suppressed. In this way autoimmune insult to myofibroblast phenotypes is prevented. This highlights the importance of myofibroblasts and the vital roles they play in regulating ECM processes, interstitial fluid volume and pressure, wound healing, etc.

Fibroblastic cell demise occurred via different pathways as revealed by pathological changes observed in non-viable cells; these included features of swelling, pyknosis, atrophy and severe necrosis. Generally, early fibroblastic and spindle cells displayed condensed nuclei and cytoplasm, consistent with early signs of apoptosis, while fibrohistiocytic and epitheloid cells exhibited features of cell swelling associated with necrosis. The fibroblastic and spindle cells initiated apoptotic programmed cell death (condensed nucleus and cytoplasm) or they atrophied through the process of programmed autophagic cell death. These cells appeared to be less affected by factors causing cellular necrosis. The fibrohistiocytic and epitheloid cell phenotypes experienced either programmed autophagic or necrotic cell death. It is speculated that the tendency of these cells to aggregate when degenerating was probably an attempt to speed up the autophagic process by permitting the efficient action of numerous lysosomes on the aggregated cells. From the results it is assumed that another method of inducing cell death by not involving immune cell activity was by the wrapping of tubular elements around cells as a method of strangulating cells to help with demise of certain slender fibroblastic cell types.

Despite the differences among cells in the HCB area all were of mesenchymal origin as indicated by their positivity for vimentin. It was noteworthy that strong positivity for vimentin was displayed by some cellular processes that remained intact in the HCB milieu of space formation and cell lysis. These processes were enwrapped by fibres; one type identified was reticular fibres. The implication here is that reticular fibres play a protective role also, by wrapping around cellular processes. Another apparent protective role provided to cells in the HCB area is protection of mesenchymal cells and elements against auto immune reactivity, by stimulating autolysis or by sequestering, within HCB, necrotic cells that might not have gained prior tolerance. The absence of immune cells in the HCB area bears testimony to the successful isolation and sequestration of degenerating and necrotic fibroblastic-myofibroblastic phenotypes from immune attack.
In summary, this morphological and immunocytochemical assessment of HCB in keloids provides information about the content of HCB and features associated with the hyalinization of tissue: such as variation of fibroblastic phenotypes with differing characteristics and varying states of viability; another feature was the distinctive relationship between fibroblastic cells and mast cells. Important aspects observed and discussed are the involvement of mast cells, myofibroblastic cells and other fibroblastic phenotypes in the

hyalinization process; the presence of collagen types I and III and cellular debris in HCB as well as cell degeneration, early apoptosis, autophagic cell death and necrosis of cells associated with HCB. Possible reasons for the occurrence of the hyalinization process are to provide a protective role by sequestrating cytokines and proteases produced by mast cells and to prevent autoimmune attack by providing a safe isolated region where injured cells, that might not have gained prior tolerance, can demise without the danger of exposure to immune reactivity. These cells include degenerating and necrotic myofibroblastic cells and other fibroblastic phenotypes. The wrapping of tubular elements around cells suggests that a method of strangulation of cells might exist in the HCB area to help with the "protected" demise of certain slender fibroblastic cell types.

Unlike reported information specifying that hyalinization is initiated adjacent to SMC before replacing the SMC, in this study, intact SMC were not found in the HCB area. The closest phenotypes to SMC were few αSMA and vimentin immunopositive cells. However, the presence of αSMA and desmin immunopositive cellular debris in and around HCB implies rapid breakdown and replacement of differentiating smooth muscle phenotypes in the HCB area. A speculative reason for the rapid removal of the SMC phenotype is prevention of autoimmune assault. These cells may belong to a system that gained immune privilege in the foetal stages and this immune tolerance may be lacking in their regenerative forms present in the ongoing wound healing process within keloids.

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Conflict of interest

The authors declare that they have no conflict of interest.

Table 1 Details of keloid biopsies

Patient number	Race	Sex	Age	Diagnosis, location and size	Nature of initial injury and number of biopsies
1.	A	M	21	Keloid. Left ear lobe.	Ear piercing. Bx,1
2.	A	F	15	Keloid. Right ear lobe	Ear piercing. Bx,1
3.	A	F	18	Keloids. Left and right ear lobes.	Ear piercing. Bx,2
4.	A	F	16	Keloids. Left and right ears lobes.	Ear piercing. Bx,2
5.	A	F	26	Keloids. Left and right ear lobes.	Ear piercing. Bx,2
6.	A	F	16	Keloid Left and right ear lobes	Ear piercing. Bx,2
7.	A	F	27	Multiple keloids. Neck, back, presternal, chest.	Plastic burns. Bx,8
8.	A	F	29	Keloid. Suprapubic area.	Ceasarian section scar. Bx,1
9.	A	F	33	Keloids. Left and right ears lobes. Recurrent.	Ear piercing. Bx,2
10.	A	F	18	Keloids. Left breast, neck.	Neck, wire injury. Breast, abscess injury. Bx,2
11.	A	F	21	Keloids. Left and right ear lobes. Recurrent	Ear- piercing. Bx,2
12.	A	F	23	Keloid. Left ear lobe	Ear-piercing. Bx,1
13.	A	F	18	Bilateral ear lobes	Ear piercing. Bx,2
14.	A	F	26	Left ear lobe, shoulder. Recurrent.	Ear piercing. Shoulder, NA. Bx,2
15.	A	F	22	Left ear lobe. Recurrent	Ear piercing. Bx,1
16.	A	M	24	Left and right ear lobes	Ear piercing. Bx,2
17.	A	M	49	Left ear	Stab wound. Bx,1
18.	A	F	17	Right ear	Ear piercing. Bx,1
19.	A	F	27	Left and right ears lobes. Recurrent for 3rd time	Ear piercing. Bx,2
20.	A	M	24	Left ear lobe.	Accident. Bx.1
21.	A	F	18	Left ear lobe	Ear piercing. Bx,1
22.	A	F	26	Left ear lobe	Ear piercing. Bx,1
23.	A	M	26	Left ear lobe, posterior	Ear piercing. Bx,1
24.	A	F	18	Bilateral ear lobes	Ear piercing. Bx,2
25.	A	M	28	Neck (below right ear), submandibular area	NA. Bx,2
26.	I	F	5	Neck (below chin)	NA. Bx,1
27.	A	M	24	Left and right ear lobes	Ear piercing. Bx,2
28.	A	F	24	Right ear lobe	Ear piercing. Bx,1

C – Caucasian
A – African
M – Male
F – Female

Bx - Biopsy
RHS - Right hand side
NA - Not available

Table 2 Differential staining techniques, colour reactions and main features identified

Staining technique	Tissue/ component	Colour reaction	Reference
Mason's trichrome (MT)	Nuclei	Blue-black	Bancroft and Gamble [26]
	Cytoplasm, muscle and fibrin	Red	
	Collagen, mucin	Green	
Gordon & Sweets' method for reticulin (Retic)	Reticular fibres	Black	Bancroft and Gamble [26]
	Nuclei	Unstained	
	Other elements	Red	
Miller's elastic stain (MES)	Nuclei	Blue-black	Bancroft and Gamble [26]
	Elastic fibres	Black	
	Collagen	Red	
	Other tissue	Yellow	
Sulfated Alcian blue (SAB)	Mast cells	Green	
Giemsa	Mast cells	Dark blue	
	Nuclei	Blue	
Luxol fast blue (LFB)	Myelin/phospholipids	Blue/black	Kiernan [27]
	Nuclei	Red	
Van Kossa's silver deposition method (VK)	Calcium salts	Black	Carleton and Drury [28]
Giemsa	Mast cell granules	Purple	Luna [29]

Table 3. Primary antibody details: Antibody/clone/species/target tissue or component/dilutions/pre-treatment and source

Antibody	Clone/Lot	Donor species	Target cell/tissue component	Dilution range	Pre-treatment	Source
Primary antibodies						
αSmooth muscle actin	1A4	M	Myofibroblasts	Pre-diluted	MW	Bio-genex
Vimentin	L058h4825	G	Mesenchymal cells	1:20-1:40	MW	Sigma
Myosin	F1.652	M	Smooth-muscle cells	1:10	MW	DSHB
Desmin	D-33	M	Smooth-muscle cells	1:200	MW	Sigma
Fibronectin	F3648	R	Cellular and ECM fibronectin	1:400	MW	Sigma
Secondary antibodies	Clone/Lot	Donor species	Dilution range			Source
Mouse IgG	M6898	G	1:50-1:200			Sigma
Goat IgG	GT-34	M	1:20-1:100			Sigma
Rabbit IgG	B8895	G	1:800			Sigma
Tertiary antibodies	Clone/Lot	Donor species	Enzyme	Dilution range		Source
PAP (antibody-enzyme complex)	P1901	G	PAP	1:100-1:200		Sigma
PAP (antibody-enzyme complex)	P6-38	M	PAP	1:200-1:400		Sigma
Chromogen	Clone/Lot	Substrate Conjugate				Source
DAB	K3466	PAP, LSAB				Dako
Fast DAB with metal enhancer	D 0426	PAP, LSAB				Sigma

M - Mouse
G - Goat
R - Rabbit
ECM – Extracellular matrix
PAP - Peroxidase-anti-peroxidase
DAB -Three,3'diaminobenzidine tetrahydrochloride
LSAB- Labeled streptavidin-biotin

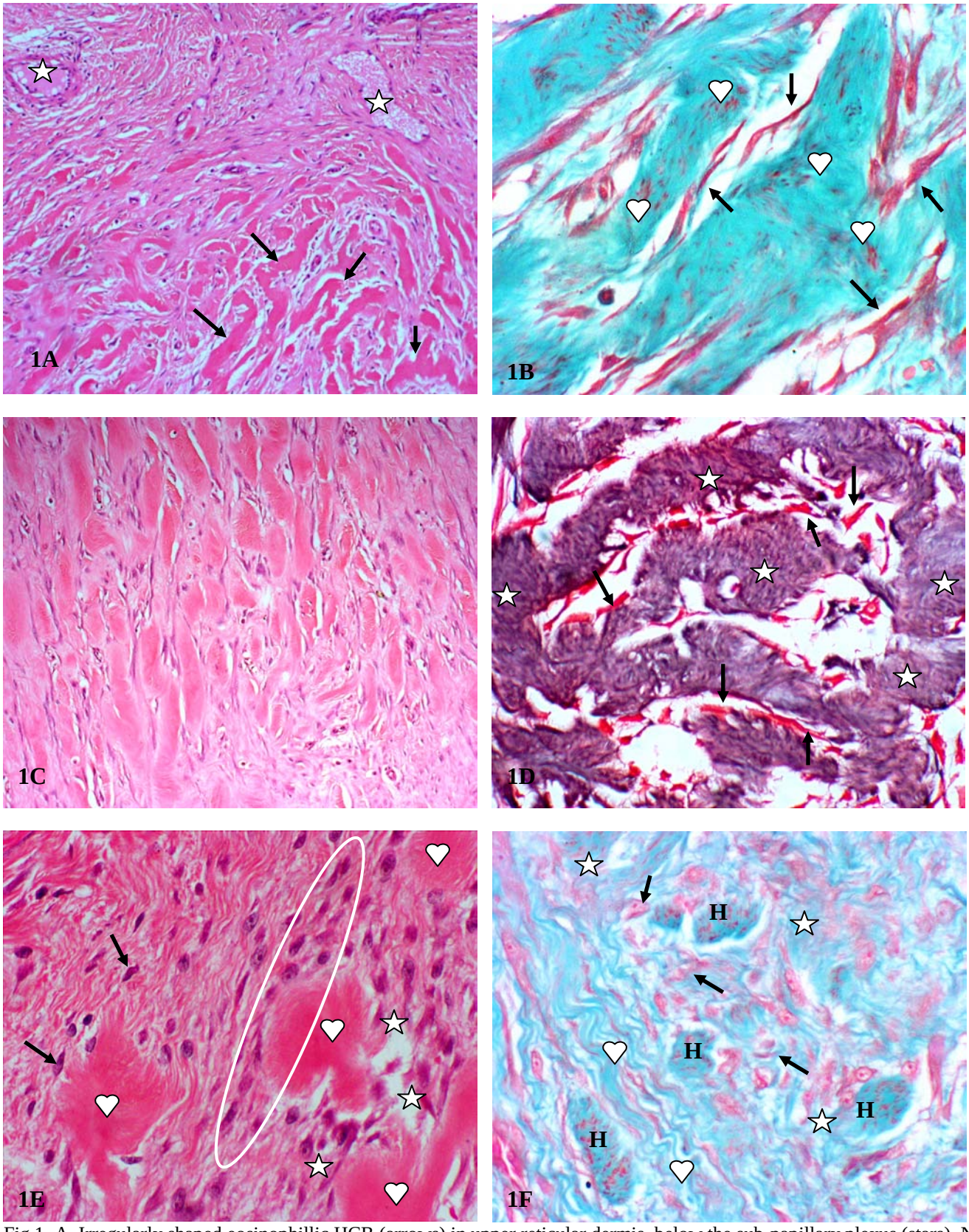

Fig.1. A. Irregularly shaped eosinophillic HCB (arrows) in upper reticular dermis, below the sub-papillary plexus (stars). Note fibrous stroma separating HCB (H&E X500). B. Presence of cellular components in HCB (hearts) and elongated flattened fibroblastic cells (arrows) adjacent to the outer edge of HCB (MT X1500). C. Elongated tubular HCB separated by fibro-cellular stroma (H&E X500). D. Whorled pattern of HCB (stars) separated by large spaces containing elongated flattened fibroblastic cells (arrows) (Retic X1000). E. Early HCB (hearts) surrounded by fibrous to myxoid stroma with spindle (arrows) and space forming fibrohistiocytic cells (stars). Note palisading cells within ellipse (H&E X1000). F. Early HCB (H) adjacent to wavy collagenous stroma with wavy elongated fibroblastic cells (hearts). Note swollen nuclei with ruptured nuclear membranes (arrows), lysis and dissolution (stars) (MT X1000)

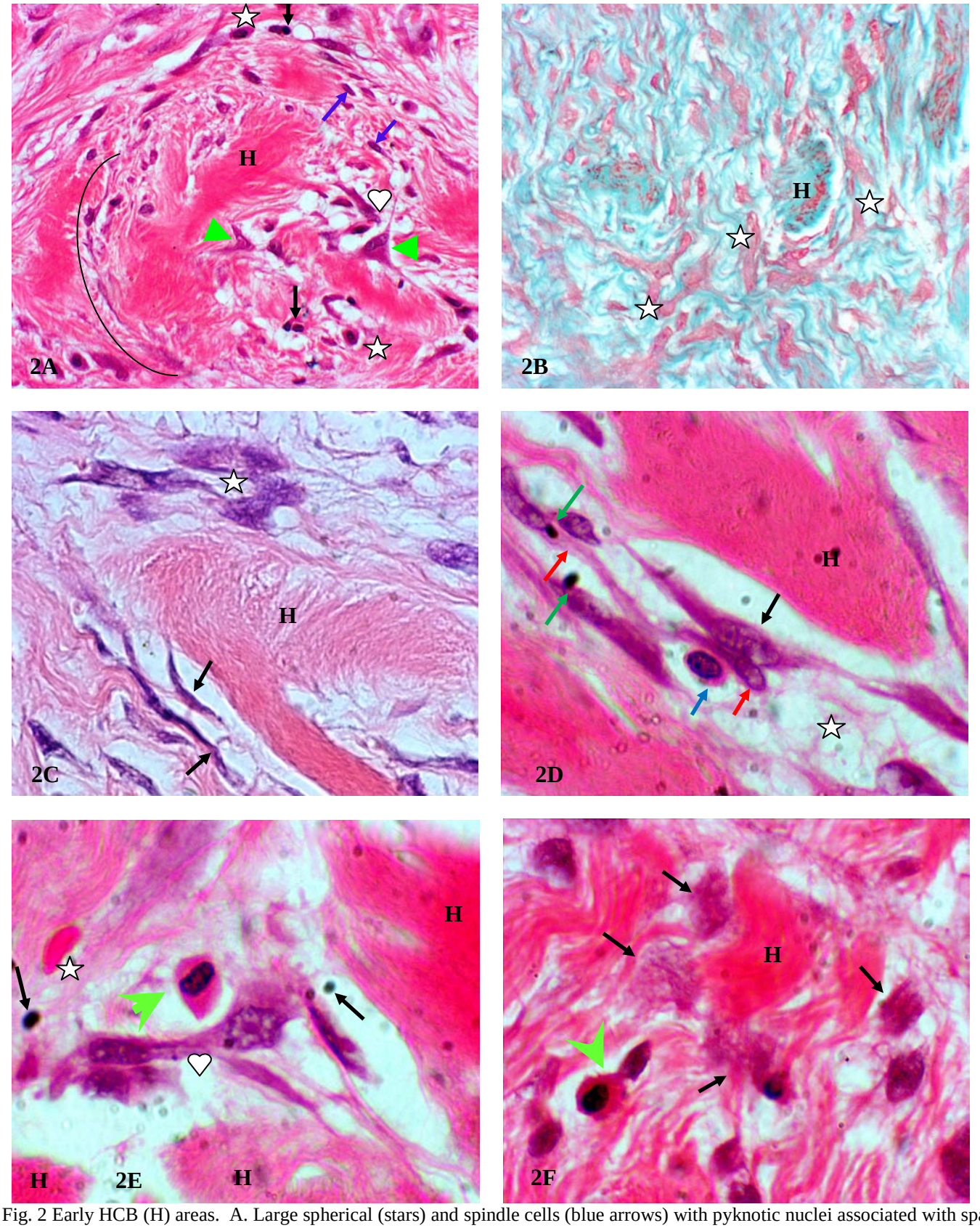

Fig. 2 Early HCB (H) areas. A. Large spherical (stars) and spindle cells (blue arrows) with pyknotic nuclei associated with space formation. Note apoptosing nuclei (black arrows), space formation associated with fibrohistiocytic cells (heart), palisading cells (curved line) and tri-polar cells (arrowhead) (H&E X1000). B. Fibrohistiocytic cells (stars) showing indistinct nuclei and attachment to each other in the para- early HCB area. (MT X1000). C. Attached fibrohistiocytic cells with indistinct nuclei (star) and very slender elongated fibroblastic cells (arrows) associated with oedematous clefts around early HCB. (PAS/Alcian blue, X 2500). D. Elongated unipolar (red arrow) and bipolar cells (black arrow) in close contact with mast cells (blue arrow). Note sparse myxoid stroma (star), wide extracellular spaces and dense deposits (green arrow). (H&E X2500). E. Mast cell (arrowhead) associated with attached fibrohistiocytic cells (heart). Note presence of small fibrinous (star) and dense deposits (arrows) (H&E X2500). F. Quiescent mast cell with calcified nucleus (arrowhead) and large fuzzy necrotic cells with indistinct nuclear and cytoplasmic boundaries (arrows) in close proximity to HCB (H&E X2500).

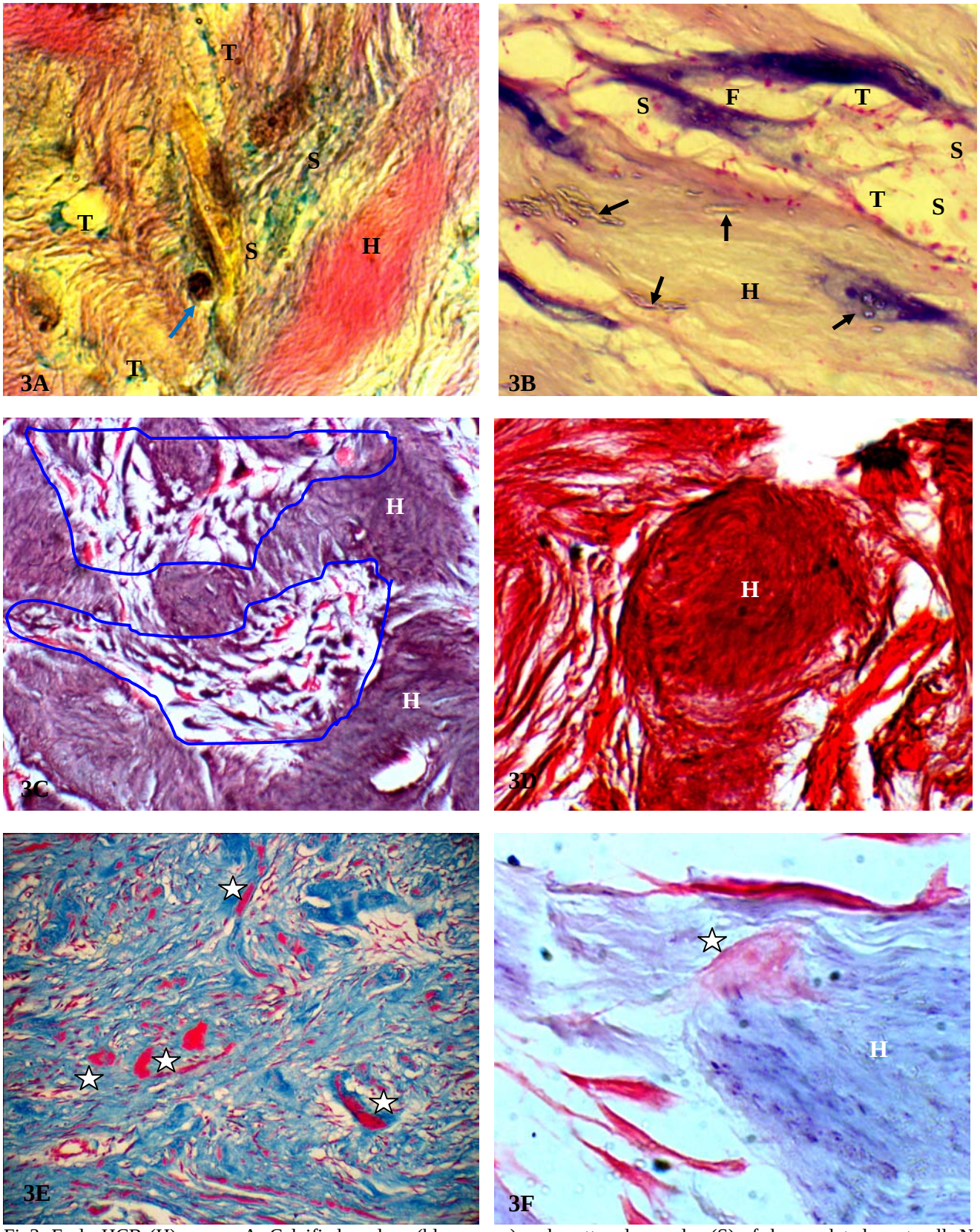

Fig3. Early HCB (H) areas. A. Calcified nucleus (blue arrow) and scattered granules (S) of degranulated mast cell. Note granules within fine tubules (T). (SAB X2500). B. Degenerate and swollen fibrohistiocytic cells (F) and microtubular structures (arrows) in the vicinity of scattered mast cell granules (S). (Giemsa X2500). C. Fibrohistiocytic cells showing positivity for silver stain (black) closely associated with red staining fibroblastic cells (blue autoshape). (Reticulin X1000). D. Numerous reticular fibres (black fibres) in early HCB (Reticulin X1000). E. Early HCB showing presence of fibrin (stars) (MSB X500). F. Necrotic cellular material incorporated into early HCB (MSB X2500).

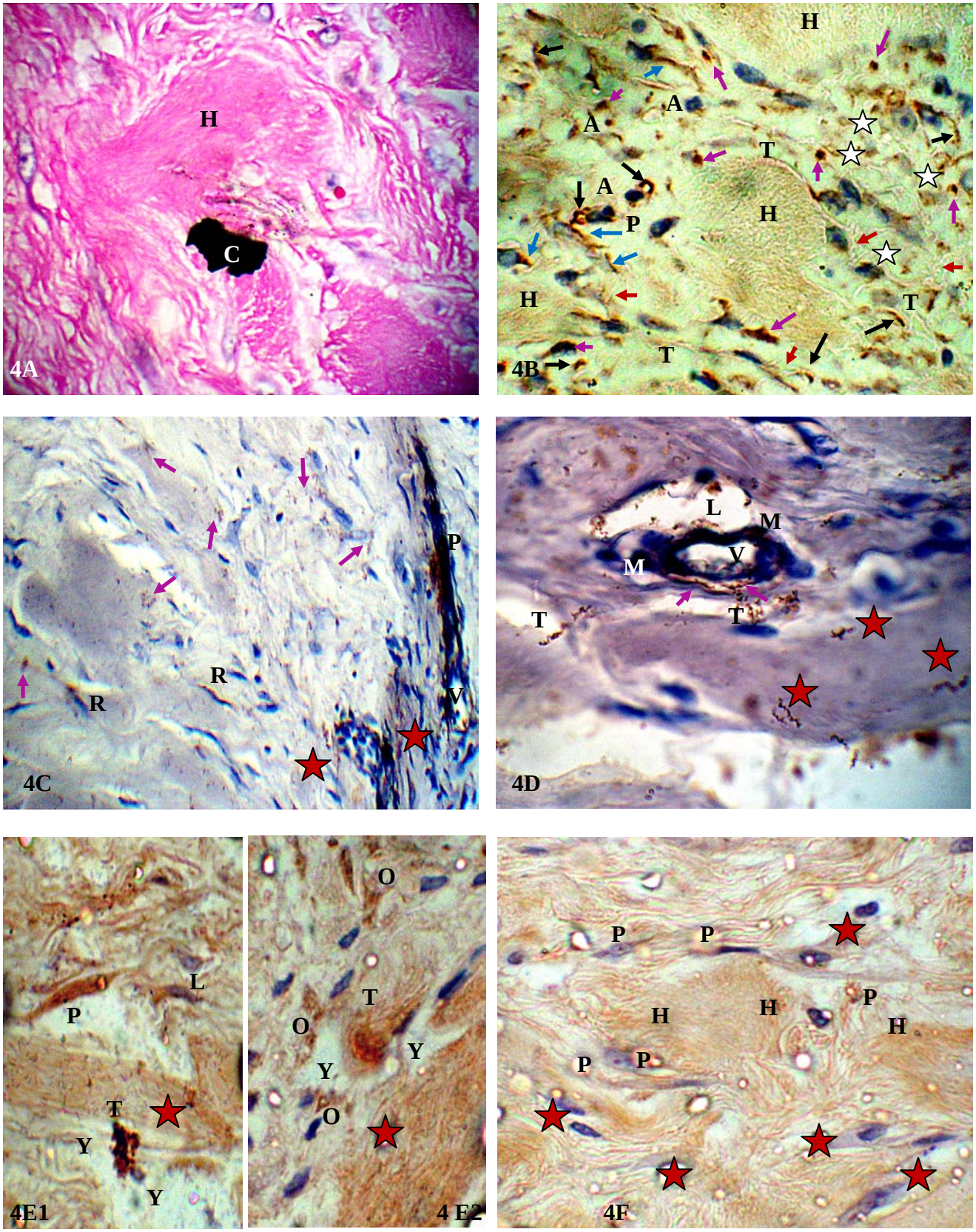

Fig.4. A. Large calcium deposit (C) in early HCB (H) (Van Kossa's silver X1000). B. Vimentin positivity in early HCB (H), cell processes (blue arrows), {some curvilinear (black arrows)} and in pyknotic cells (pink arrows). Note pyknotic nuclei (P) of cells with vimentin positive processes, pale vimentin staining in swollen and necrotic cells (stars) and in tubular structures (T), some in an entwining state (red arrow). Also note apoptotic bodies scattered in the area (A). (Vimentin X 2000). C. α-SMA positivity microvessel walls (V) {note compressed part (P)}, in slender fibroblastic cells around microcrovessels (red stars), in fibroblastic cells between early HCB (R) and in cellular debris (arrows) in the early HCB milieu. (α-SMA X 1000). D. α-SMA positivity in squiggly strands of cellular debris (arrows) scattered around microvessels (V) {Note calcified nuclei in some mural cells (M)}, in lymphatic vessels (L), in clefts around early HCB (T) and within early HCB (stars) (α-SMA X 2500). E.1 Fibronectin positivity in elongated fibroblastic cells with tapering (P) or blunt ends (L), and around microtubules arranged in clusters (T) (Note areas of tissue lysis (Y) adjacent to these clusters). 2. Degenerate fibronectin positive cells showing swollen organelles (O) and areas of tissue lysis (L) adjacent to positive tubular cluster (T). Note fibronectin positivity in HCB (stars). (Fibronectin X 2200). F. Fibronectin positivity at the edges of HCB (H) and pale staining in few cells (P). Note many cells are negative for fibronectin (stars). (Fibronectin X 2300).

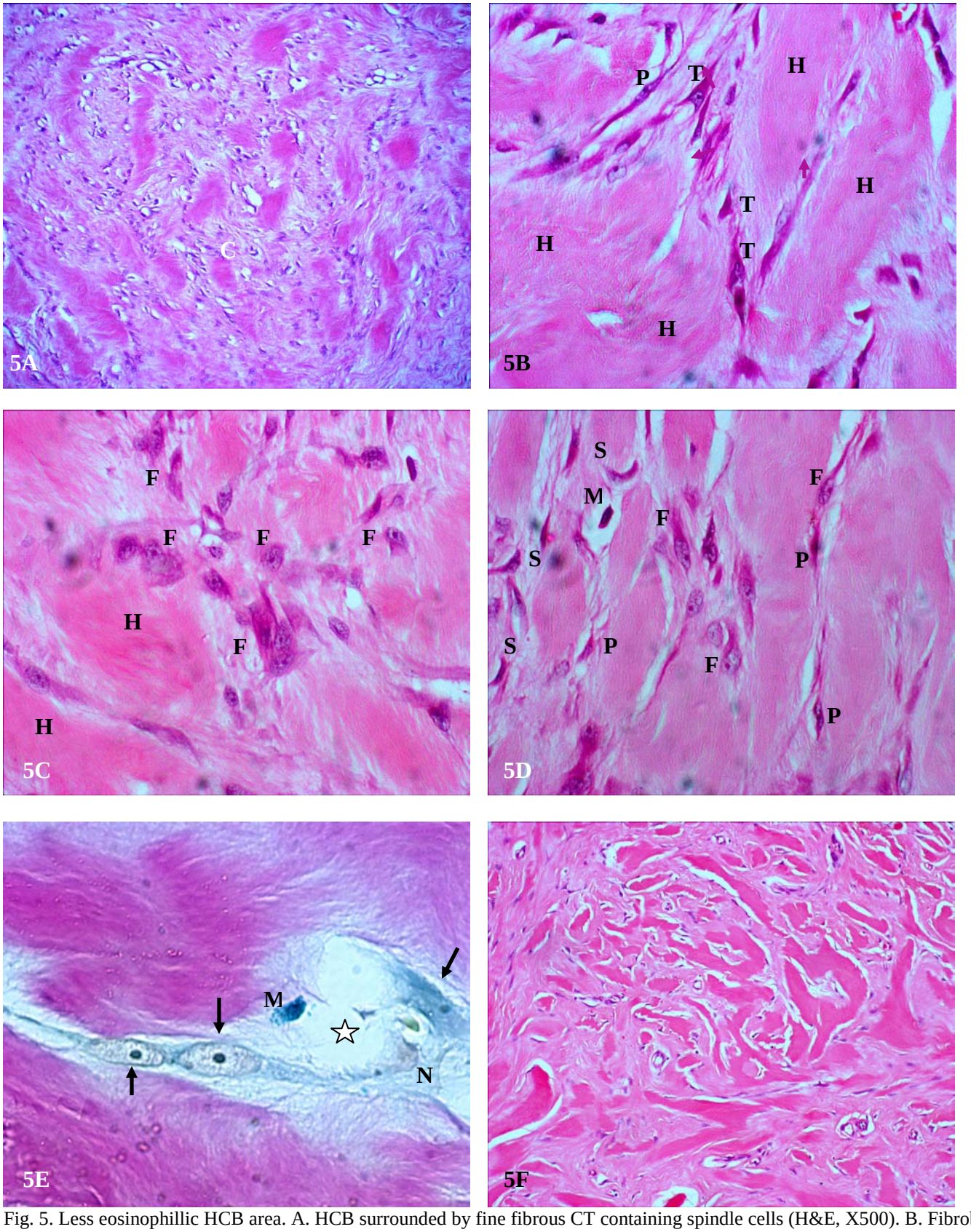

Fig. 5. Less eosinophillic HCB area. A. HCB surrounded by fine fibrous CT containing spindle cells (H&E, X500). B. Fibroblastic bipolar (P) or tripolar cells (T) in CT around HCB (H&E, X1000). C. Fine fibrous CT containing fibrohistiocytic cells (F) (H&E, X1000). D. Pyknotic spindle cells (P) and fibroblastic cells with condensed cytoplasm, vesicular nuclei and prominent nucleoli associated with space and slit formation (F) Note presence of mast cells (M) surrounded by a clear zone and spindle cells showing varying stages of nuclear and cytoplasmic condensation (green arrows), pyknosis (red arrow) and necrosis (blue arrow) (H&E, X500). E. Area of CT lysis (star) surrounded by tadpole- like fibroblastic cells with vesicular nuclei and prominent nucleoli (black arrows), necrotic cell (N) and degranulated mast cell with pyknotic nucleus (M) (SAB, X2500). F. Intensely eosinophillic HCB associated with cleft formation around or adjacent to it (H&E, X500)

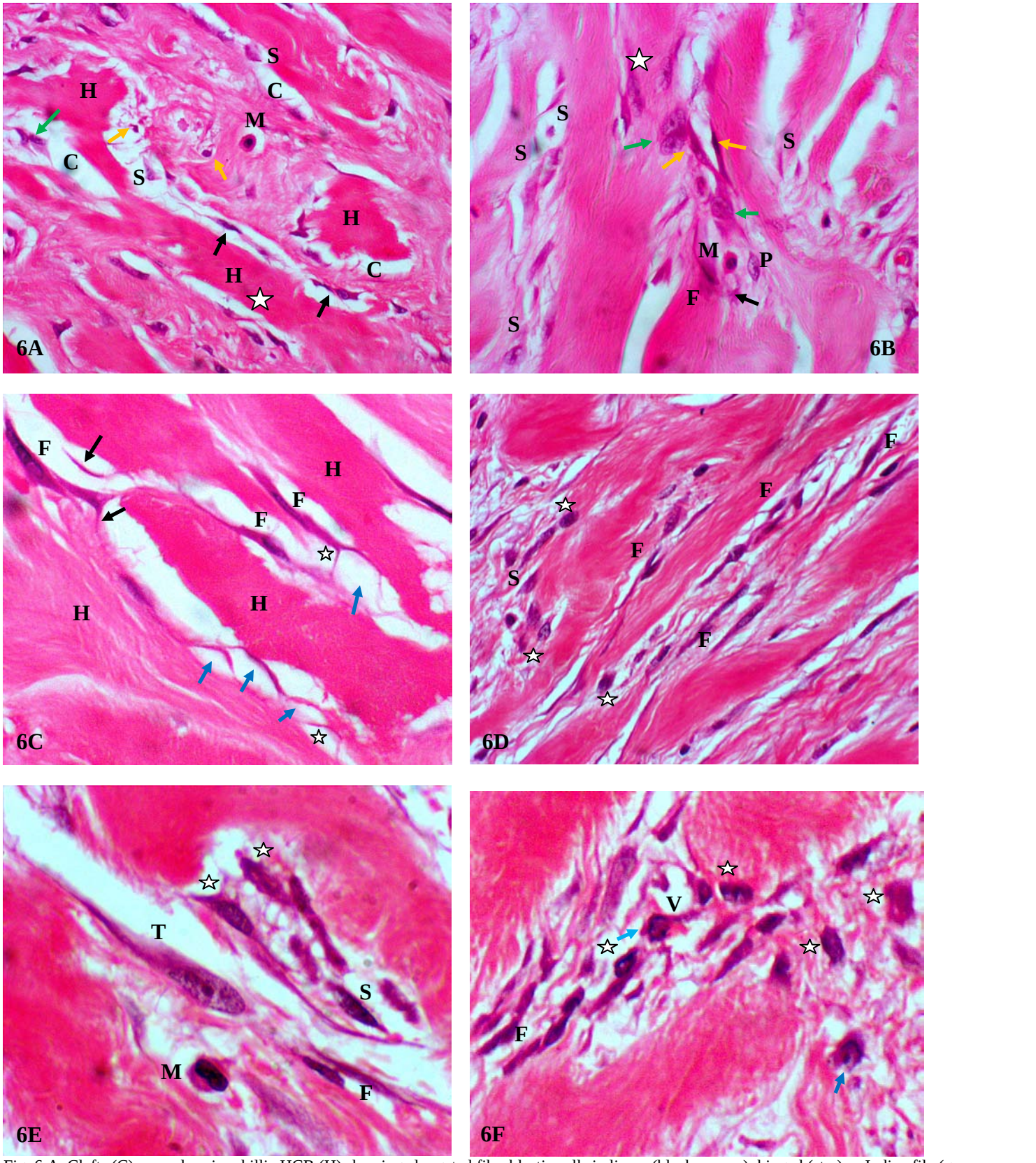

Fig. 6 A. Clefts (C) around eosinophillic HCB (H) showing elongated fibroblastic cells in linear (black arrows), hinged (star) or Indian file (green arrow) attachment. Note presence of shadow cells in clefts (S), mast cell adjacent to a clear zone (M), atrophic cells (yellow arrows) (H&E, X1000). B. Pyknotic elongated fibroblastic at edge of HCB (yellow arrows) or in HCB (F) and fibrohistiocytic cells with vesicular nuclei, prominent nucleoli and eosinophillic cytoplasm at edge of HCB (green arrows) or in HCB (star). Note presence of shadow cells in clefts (S), mast cell (M) in close proximity to shadow cell (black arrow), pyknotic cell (F), fibrohistiocytic cell (green arrow) and spindle cell with vesicular nuclei and prominent nucleoli (P) , atrophic cells (H&E, X1000). C. Elongated fibroblastic cells with condensed nuclei (F) showing slender polar processes that are curved (black arrows) or split into complementary curves that surround pale amorphous material (blue arrows) within clefts between eosinophillic HCB (H). Note processes crossing clefts at a 45^0 angle (stars) (H&E, X1500). D&E. Eosinophillic material adjacent to spindle (S), fibroblastic (F), fibrohistiocytic (star), "tadpole" cells (T) and mast cells (M) (H&E, D: X1000, E: X2300). F. Fibroblastic (F) and fibrohistiocytic (stars) cells embedded in eosinophillic material and showing nuclear and cytoplasmic membrane damage (blue arrows). Note fibrinous material around mural cells of venule (V) (H&E, X2000).

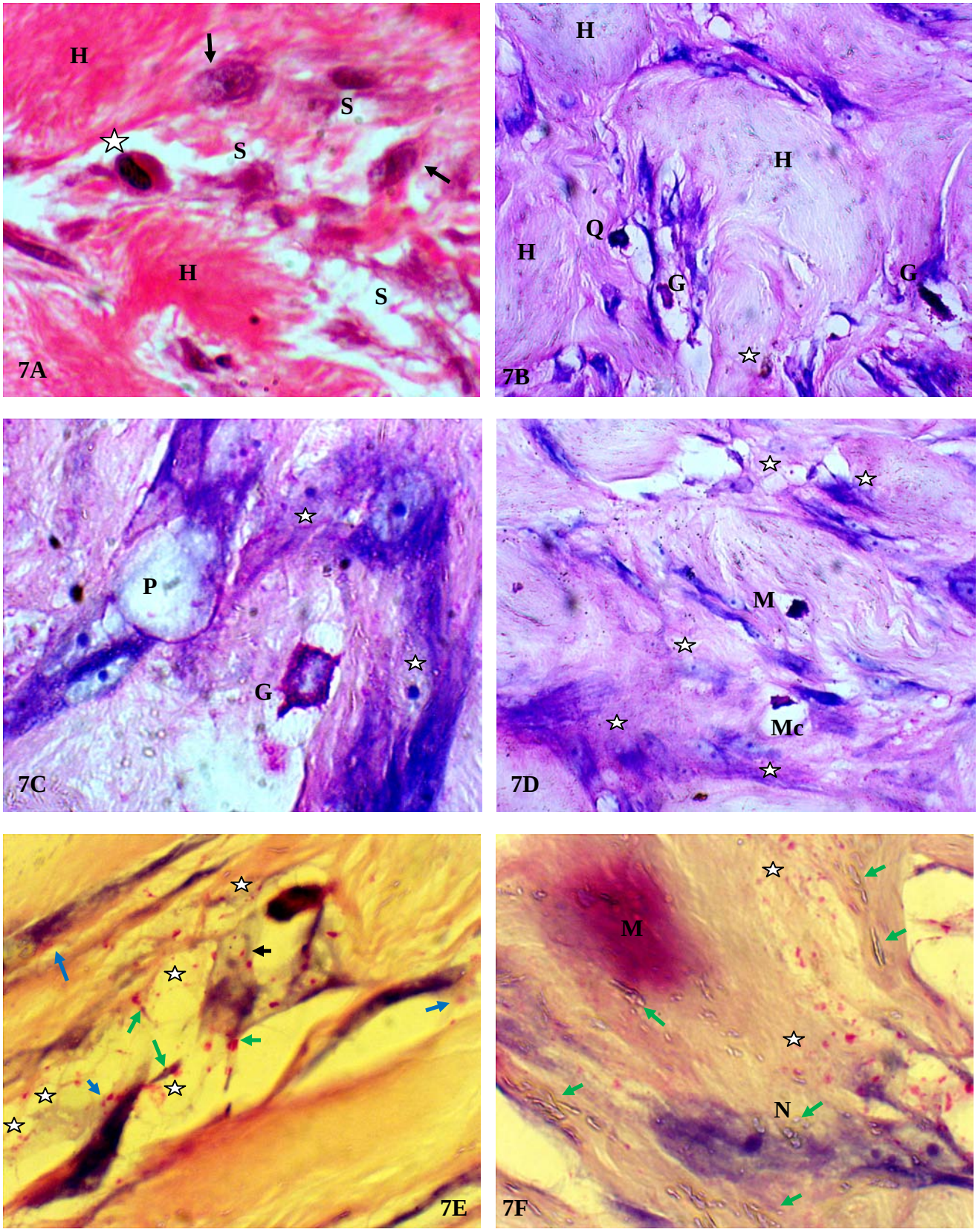

Fig. 7. A. Mast cell with calcified nucleus and adjacent atrophic cell and fibrin deposit in degenerate microvessel and (star). Note polygonal and irregular cells displaying eosinophillic cytoplasm and mild (black arrows) and severe (S) nuclear and cytoplasmic membrane damage (H&E, X2500). B. Quescient (Q), degranulating (G) and degranulated (star) mast cells showing rounded, elongated and polygonal forms, respectively. Note prescence of degenerate cells close to degranulating mast cells (G) and processes attaching mast cell to fibroblastic cells (Q) (Giemsa X1500). C. Nuclear (star) and cytoplasmic (P) membrane injury in necrotic cells around degranulating mast cell (G). Note processes attaching mast cell (G) to necrotic cells (Giemsa, X2500). D. Degranulating mast cells (Mc, M) in close proximity to aggregates of necrotic cells (star). Note process attaching mast cell (Mc) to spindle cell (Giemsa, X1500). E. Mast cell granules within delicate (stars) and more robust (green arrows) processes, within the nucleoplasm (black arrow) and cytoplasm (blue arrow) of afflicted cells (Giemsa, X2500). F. Free mast cell granules strewn in HCB area (stars) associated with lysed mast cell (M), necrotic fibrohistiocytic cells (N) and fragmented microchannels (green arrows) (Giemsa, X2600).

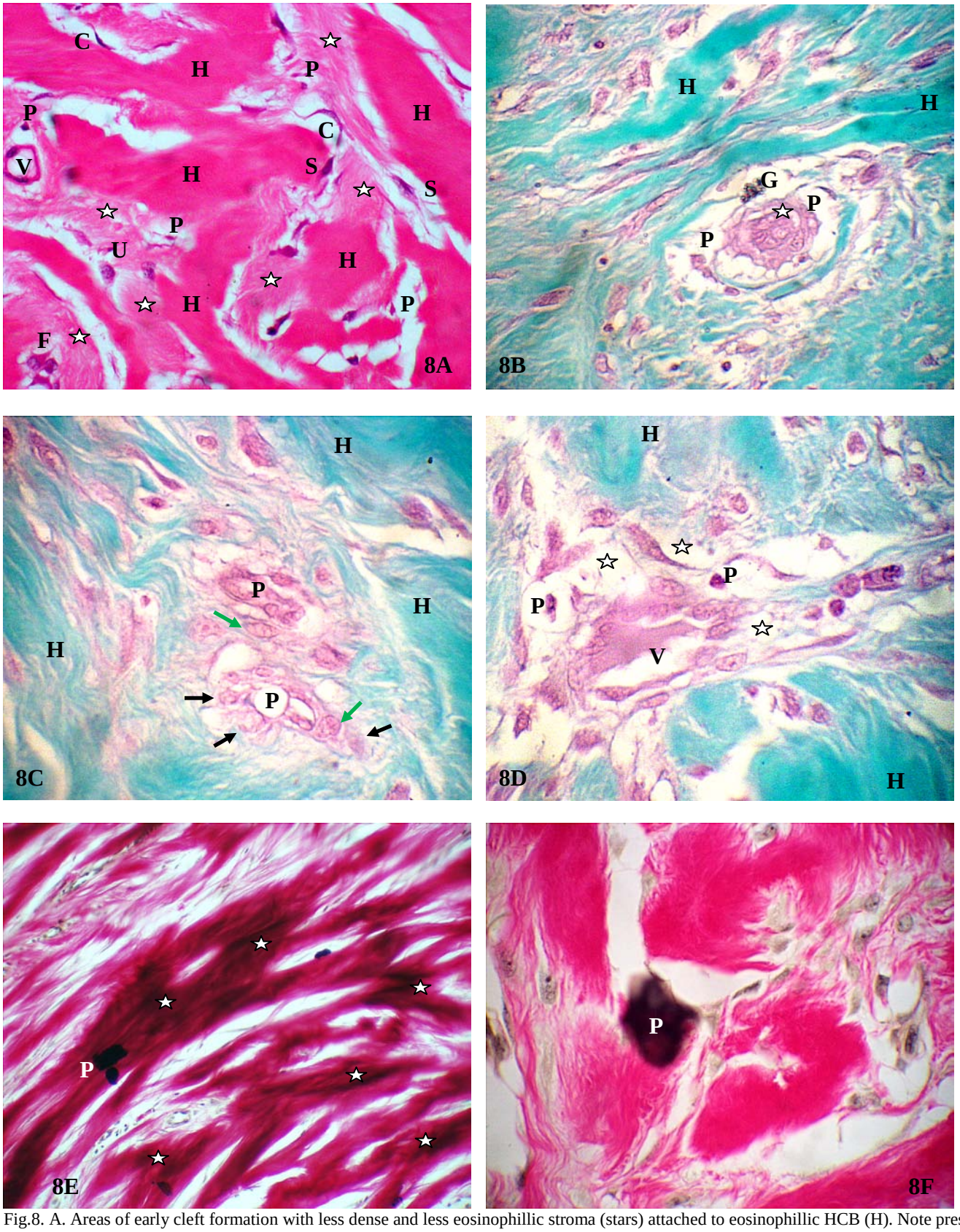

Fig.8. A. Areas of early cleft formation with less dense and less eosinophillic stroma (stars) attached to eosinophillic HCB (H). Note presence of curvilinear (C), spindle (S), fibrohistiocytic (F), atrophic (P) and smudge (U) cells and microvessel with hyalinized wall (V). B. Venules in outer HCB area (H) displaying swollen endothelial cells and luminal obliteration (star), paravenular clefts (P) and dense granular aggregate (G) (MT X 1000). C. Venules in septa of CT between HCB (H) exhibiting proliferation and swelling of mural cells (P) and degenerate (black arrow) and necrotic (green arrow) pericytes ((MT X 2000). D. Venule (V) with degenerate and necrotic mural cells and areas of paravenular clearing containing pyknotic (P) cells and cellular debris (stars). Note fibrious exudate in lumen of venule (V) (MT X 2500). E, F. Degenerate elastic fibre staining present in HCB as dispersed (stars) or blotched (P) patches of elastin {Millers, X 1000 (E) and X2000 (F)}.

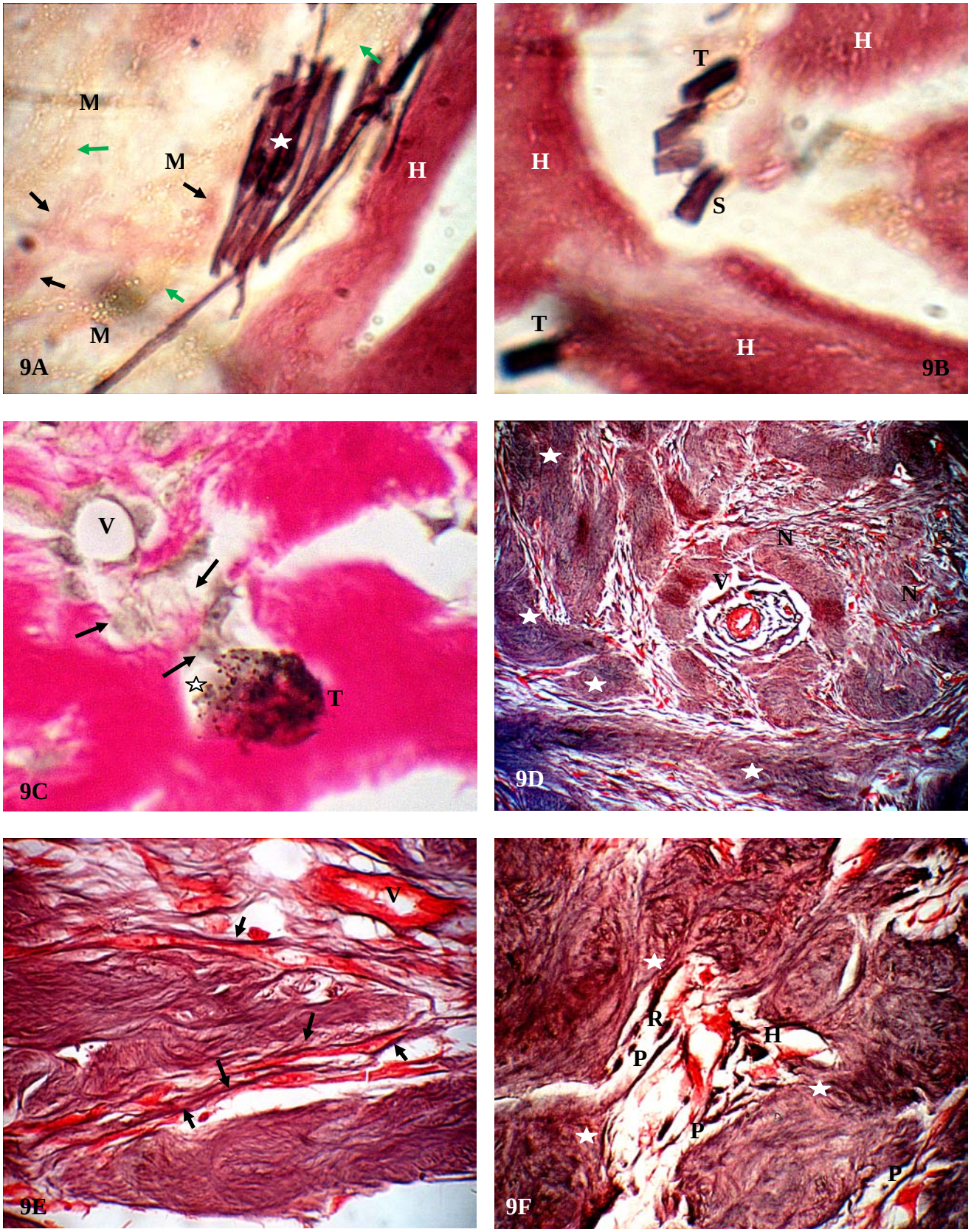

Fig. 9. A. Long linear strands of elastic fibres closely associated with aggregates of elongated tubular structures (star) adjacent to HCB. Note fragmented microtubular structures (M) overlying necrotic cellular material (green arrows) and necrotic ECM material (arrows) in the vicinity of elastic fibres. (EVG,2000). B. Short and thick elastic fibres (T) covering thick tubular structures (S) adjacent to HCB. (EVG, X2000).C. Elastic fibrils evident as aggregates of spots (star) in close proximity to degenerate cells (arrows) and blood vessel (V) at the edge of HCB. Note spots merge into blotch of elastin (T). (Millers, X2000). D. Reticular fibres within HCB (stars), adjacent to cells (N) and around blood vessels (V) in the HCB area (Retic, X1000). E. Reticular fibres adjacent to elongated fibroblastic cells (arrows) and around the outer mural cells of microvessels (V) (Retic, X2600). F. Reticular fibres covering processes (P) or parts of fibroblastic (R) and fibrohistiocytic (H) cells. Note reticular fibre covered cellular components being incorporated into the HCB (stars). (Retic, X2000).

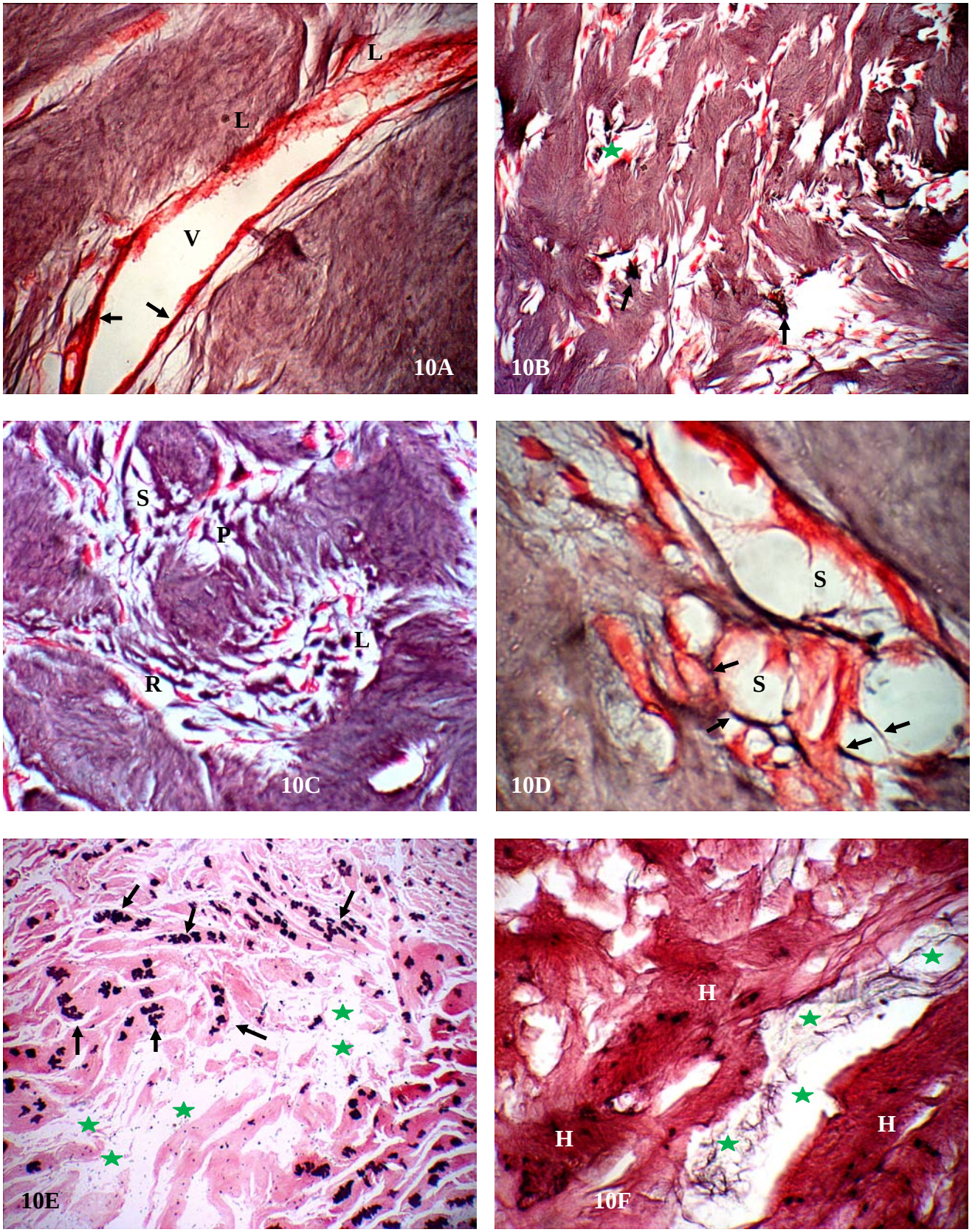

Fig. 10. A. Venule (V) showing mural parts lacking reticular fibres with degenerate cells (L) and intact parts where reticular fibres were present (arrows). (Retic, X2000). B. Fibrohistiocytic cells covered with reticular fibres in spaces between HCB (arrows) or attached to HCB (stars). (Retic, X1000). C. Epitheloid (L), pleomorphic (P), spindle (S) and slender fibroblastic (R) cells enwrapped by reticular fibres and closely associated with unwrapped counterparts (red cells) in the HCB region. (Retic, X1500). D. Aggregate of space forming cells (S) with reticular fibres aligned around spaces (arrows). (Retic, X2600). E. Many aggregates of reticular fibres scattered in the HCB (arrows), and adjacent areas showing lysis of reticular fibres and HCB (stars). (Gordon and Sweet's X1000). F. Lysis of reticular fibres and other extracellular matrix elements (stars) in spaces between HCB (H), (Gordon and Sweet's X2000)

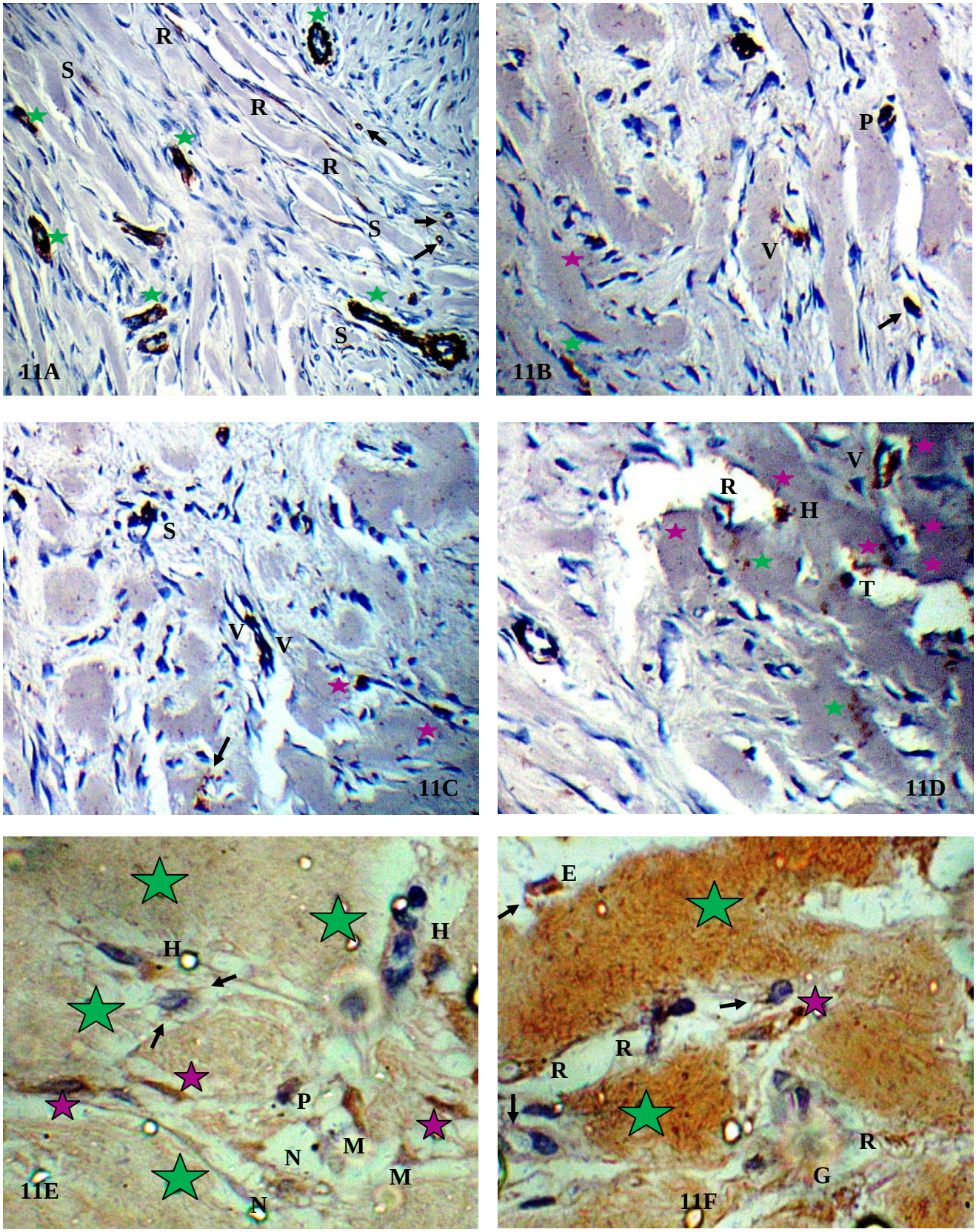

Fig. 11. A. αSMA positivity in mural cells of compressed venules (stars) and capillaries (arrows); in elongated fibroblastic cells (R) adjacent to HCB (H) and in spindle cells (S). (αSMA X 1000). B. αSMA positive large fibrohistiocytic and epitheloid cells associated with space formation (pink star, arrow, resp.) and within lymphatic vessels (V, P, resp.). Note (αSMA elongated fibroblastic cell connected to palisading cells (green star). (αSMA X 1500). C. αSMA positive epitheloid cells connected to palisading cells (pink stars) and attached fibrohistiocytic cells in space formed (S) or in lymphatic vessel (V). Note αSMA positive cellular debris at the edge of HCB (arrow). (αSMA X 1600). D. αSMA positive cellular debris at the edge of HCB (pink stars) and within HCB (green stars). Note αSMA positive attached fibrohistiocytic cells in lymphatic vessel (V) and αSMA positive fibrohistiocytic (H), epitheloid (T) and fibroblastic (R) cells close to αSMA positive debris in HCB. (αSMA X 1800). E. Fibronectin immunoreaction in fibroblastic (pink stars), epitheloid (P), fibrohistiocytic (H), pleomorphic (M) and necrotic (N) cells in stroma between HCB (green stars). Note mild fibronectin positivity in processes of epitheloid cells (arrows). (Fibronectin X 2500). F. Fibronectin positivity in epitheloid (T), attached fibrohistiocytic (pink star) and degenerate (G) cells and in HCB (green stars). Note fibronectin positive processes of epitheloid (arrows) and fibroblastic (R) cells. (Fibronectin X 2600).

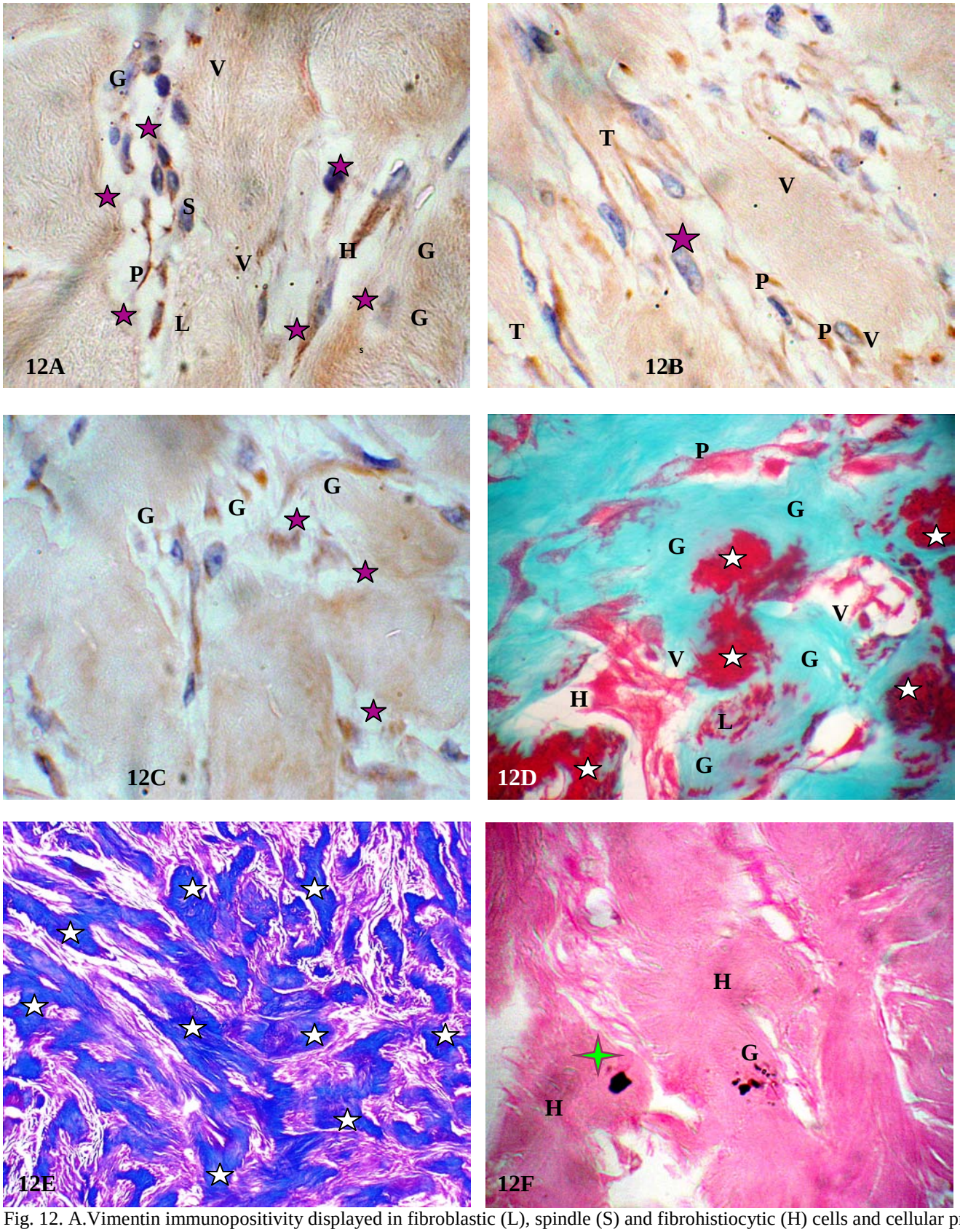

Fig. 12. A.Vimentin immunopositivity displayed in fibroblastic (L), spindle (S) and fibrohistiocytic (H) cells and cellular processes (P). Note attached spindle (S) and fibrohistiocytic (H) cells and long positive cellular processes (P) surrounded by areas of tissue lysis (stars). Note also pale staining degenerate cells (G) and vimentin positive cellular debris in and adjacent to HCB (V). (Vimentin X 2000). B. "Tadpole" (T) cells around HCB with vimentin positive processes, some of which attached to other "tadpole" cells (stars). Note positive cells (V) and processes (P) and necrotic cells (N) arranged around HCB. (Vimentin X 2200). C. Attached vimentin positive necrotic (N) and degenerate (G) cells (Vimentin X 2200). D. HCB with fibrin deposits (stars) and aggregate of cellular debris (L) surrounded by collagen (G). Note degenerate vessels (V) and degenerate attached palisading (P) or aggregated (H) fibrohistiocytic cells (MT, X2700).. E. Accumulation of phospholipids within HCB (stars) (LFB, X600). F. Dense calcified deposit (star) and aggregate of small and granular calcium deposits (G) within HCB (H) (Von Kossa, X1000).

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IMPLICATION OF THE LACK OF ELASTIC FIBERS IN THE PATHOGENESIS OF KELOIDS

S. Bux[1] and A. Madaree[2]

Disciplines of [1]Physiology and [2]Plastic Surgery, Nelson R. Mandela School of Medicine, University of KZN

As keloids are a prototype of excessive wound healing, traction force is continuously generated by wound healing fibroblasts on elements of the extracellular matrix, especially collagen, to facilitate migration. During this process, the collagen network is deformed and may become aligned to provide contact guidance for 3D migration wherein cells move bidirectionally along an axis of fiber alignment, which may result in nonuniform cell distribution[1]. The regional variation of connective tissue elements recently described[2], showing varied distribution of cells, fibres, and blood vessels in keloid may be governed by traction forces. Keloids are a manifestation of deformations in the reticular dermis; such permanent change occurs when traction force is excessive and exceeds the elastic limit. As elastic fibres in connective tissue permit recovery of shape after deformation, we hypothesise that in keloids there is a decrease of elastic fibres, reducing the elastic limit and increasing tissue deformation. To assess this, we histologically assessed elastic fibre content and histomorphometrically quantified and compared the mean (± SEM) percentage area of elastic fibres in lesional and non-lesional skin of patients with keloids.

Histological assessment of the elastic fiber content in lesional and non-lesional skin from patients with keloids showed that generally, elastic fibres in keloids were increased in the border regions and they were long and thick or short and stubby (Fig 1). At the papillary dermis/keloid border increased numbers of elastic fibres were found adjacent to inflamed vessels of the subpapillary plexus or in the inflammatory infiltrate (Fig. 2). At the border, keloid zones such as the fibrous tubular and dense regular wavy areas, contained abundant elastic fibres (Fig. 3), whereas matched areas within the keloid were devoid of elastic fibres or had few frail fibres. Keloids showed a paucity of elastic fibers and this was restricted to certain keloid zones, mainly, fibrous tubular and dense regular wavy areas (Fig. 4). A consistent finding was the absence of elastic fibres in very cellular keloid regions. Statistical analysis (Wilcoxon signed ranks test) showed significant differences ($p < 0.05$) in elastic fiber content between non-lesional dermis and keloid areas as well as elastic and collagen fiber content in keloids and the keloid border, when compared with non-lesional dermis.

In the absence of dermal elastic fibers the accumulative effect of deformation forces is compaction of fibres and stiffening of tissue, which biomechanically, promotes mitogenesis and cell contractility, exacerbating matrix stiffness and cell contraction[3]. Stress beyond the elastic limit also modifies cell differentiation and DNA and protein synthesis and increases collagen biosynthesis[4]. As keloids display all of the above effects, we propose that in the absence of elastic fibers, persistent forces of deformation of increasing magnitude play a crucial role in the pathogenesis of keloids.

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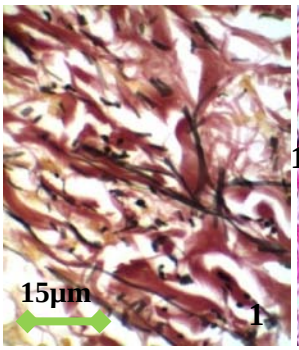

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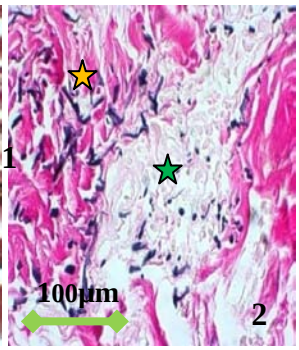

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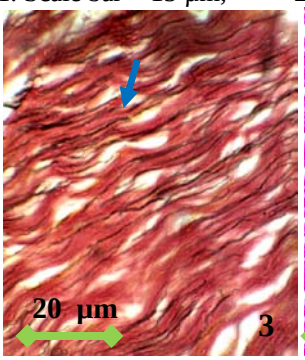

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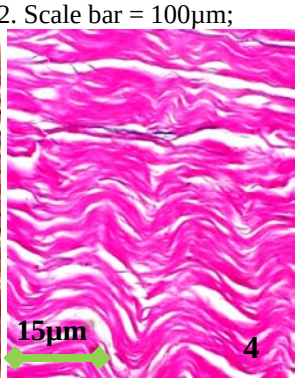

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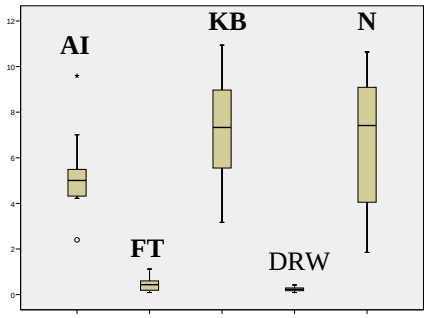

Elastic fibres: **1** at papillary dermis/keloid border; **2** in and adjacent to inflamed subpapillary plexus (stars); **3** in dense regular wavy area at the border and in the keloid **(4)**. Fig. **5** Elastic fiber content in areas of inflammation (AI), fibrous tubular (FT), keloid border (KB), dense regular wavy (DRW) and normal dermis (N). Results are shown as mean percentage area of ten fields/specimen (n = 10). *p<0.05 compared with control.

KELOIDS SHOW REGIONAL DISTRIBUTION OF PROLIFERATIVE AND DEGENERATE CONNECTIVE TISSUE ELEMENTS

S. Bux[1] and A. Madaree[2]

Disciplines of [1]Physiology and [2]Plastic Surgery, Nelson R. Mandela School of Medicine, University of Kwa-Zulu Natal

Keloids are tumours formed by the deposition of excessive scar tissue which occurs three months to many years post injury. They are characterised by the presence of thick hyalinised collagen bundles and an overproduction of collagen. Other morphological features reported are often contradictory; these include hyperproliferation of fibroblasts versus few fibroblasts, rich opposed to poor vascularization, dilated against occluded microvessels, distinct collagen nodules versus its absence and elevated levels of types I and III collagen opposed to no change [1]. This study attempted to clarify the controversies concerning keloid morphology by examining entire keloids and establishing its baseline histological characteristics.

Keloid biopsies obtained from thirty two patients, some with multiple lesions, were processed for paraffin wax vacuum embedment and for routine haematoxylin and eosin staining. Sections were also stained with Giemsa for the identification of mast cells. The sections were examined, features pertinent to the study were photographed and stored images were studied, using the Camedia graphics processing programme.

The results of this study showed that keloids comprise an assortment of proliferative connective tissue elements which displayed a regional pattern of arrangement; the microvascular supply to each of these regions was impaired. Even in the areas of angiogenesis the vascular supply was inefficient as very few differentiated functional blood vessels were found here. The regions identified in keloids were categorized as: the zone of hyalinising collagen bundles (Fig. 1), fine fibrous areas (Fig. 2), fibrous tubular areas (Fig. 3), area of angiogenesis (Fig. 4), area of inflammation (Fig.6), nodular fibrous area and zone of dense regular connective tissue. The microvascular supply to each of these regions was impaired and features of degeneration and necrosis of keloid fibroblastic cells (Fig. 5) and microvessels (Fig. 6) were ubiquitous. Impairment of the healing stage of chronic inflammation, inefficient healing by fibrosis, multiple and exaggerated phases of vascular and fibrous granulation and remodelling stages manifest in keloid formation. The uneven distribution of cells may be due to the generation of traction forces by keloid cells; these forces also modify DNA and protein synthesis, leading to an overproduction of extracellular matrix components[2] which is evident in keloids. The regions of varying tissue architecture identified in this study provides a structural basis to fathom past controversy regarding keloid morphology and to utilize in future ultrastructural and immunocytochemical research on keloids and other fibro-proliferative disorders.

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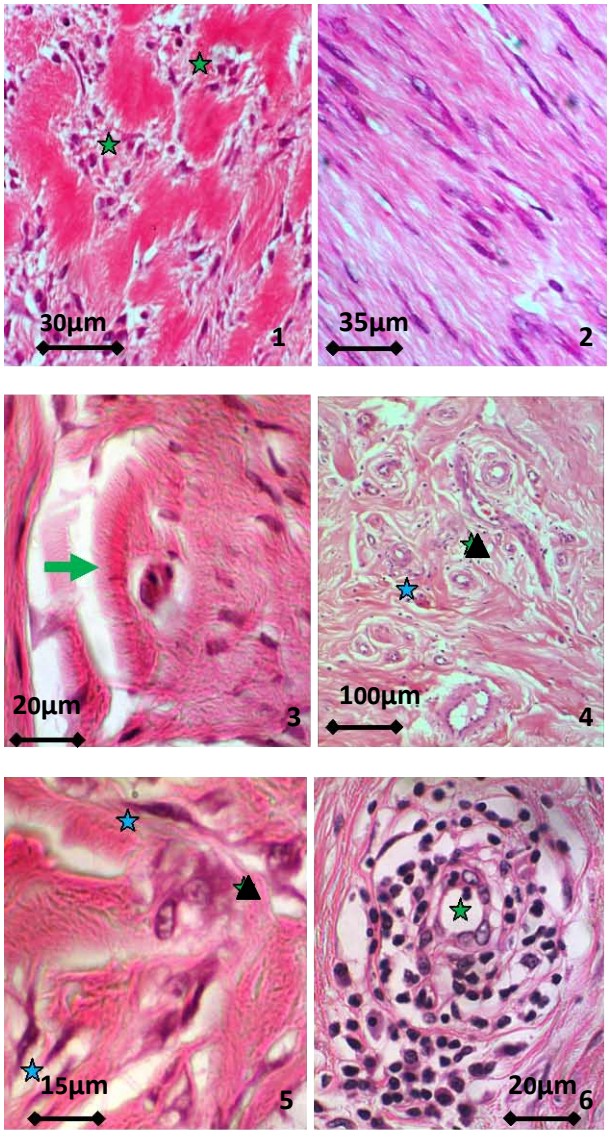

Fig. 1 HCB area showing spherical cells with condensed nuclei (stars); **2** Fine fibrous areas showing elongated flattened fibroblastic cells aligned parallel to collagen fibres; **3** Fibrous tubular area showing stalk enwrapped by tubules (arrow); **4** Area of angiogenesis showing coagulation (star) and dissolution (triangle) necrosis of micro-vessels; **5** Pyknotic spindle cells (stars) and aggregate of necrotic epitheloid cells in myxoid stroma (triangle) in fibrous tubular area; **6** Inflamed microvessel (star) with swollen and necrotic endothelial cells surrounded by wide extracellular spaces

THE SIGNIFICANCE OF HYALINISED COLLAGEN BUNDLES IN KELOIDS

S. Bux and A. Madaree*

Departments of Physiology and *Plastic Surgery
Faculty of Medicine, University of Natal, Durban, South Africa

Many of the problems associated with the treatment of keloids may be related to the incomplete understanding of the cellular components involved in the pathogenesis of the lesion. The hallmark of keloids is the presence of excessive collagen in the dermis, mainly in the form of glassy hyalinised bundles. An important prerequisite to establishing the pathogenesis of keloids is the critical examination of the connective tissue elements in the dermal area where the glassy bundles of collagen are present. We therefore undertook this study to establish the composition of the glassy collagen bundles and to investigate which cells are associated with the production and enlargement of these bundles.

Keloidal specimens from 30 patients located in one or more of the following areas: the head, neck, pre-sternal and deltoid areas, were processed for light and electron microscopy using conventional methods. The primary antibodies used in the imunocytochemical study were anti-collagen, types I and III, von Willebrandt factor, anti-vimentin and α smooth muscle actin. The differential stains used were: Giemsa, sulfated alcian blue, elastic van Gieson, Mason's trichrome and reticulin.

Microscopical examination showed that aggregates of glassy, hyalinised collagen bundles were located in the upper reticular dermis, below an exaggerated and altered papillary dermis. These collagen bundles showed diversity of shape, size and orientation. They contained aggregates of tubular structures, collagen types I and III and cellular debris (Fig. 1). The bundles were separated by septae of fibrous tissue that contained small blood vessels and various cell types. Two types of fibroblasts were identified in this area: small fibroblasts with spherical dark-staining nuclei and large elongated fibroblasts with long tapering processes. Immunocytochemical studies showed that the elongated fibroblasts were positive for both actin {intermediate filaments}(Fig. 2) and vimentin {microfilaments}. Ultrastructurally, these elongated cells exhibited peripheral filamentous focal densities in the cytoplasm, a fragmented basement membrane and irregular nuclei that occasionally showed concertina-like folds. Based on the morphological features described, these elongated fibroblasts were classified as myofibroblasts[1]. Another type of cell that was present in the vicinity of hyalinised collagen bundles was mast cells, which were partially surrounded by a space and were often related to myofibroblasts aligned along one side (Fig. 3). Mast cells were found to be in various stages of degranulation. There was a conspicuous absence of an inflammatory cell infiltrate and elastic fibres in the milieu of glassy collagen bundles.

Generally, the vascularity of the hyalinised collagen bundles decreased from the outer peripheral to the innermost parts. The size of cells and collagen bundles increased from the outer to the inner aspect. Very often the bundles were surrounded by degenerate cells (myofibroblasts and fibroblasts) that displayed swelling of their processes which, when gross, appeared as large spaces adjacent to the bundles of collagen (Fig. 4). Where the cells appeared intact and viable, the spaces adjacent to the collagen bundles were smaller and increased amounts of unhyalinised collagen fibres were present around the cells.

From the results of the study, hyalinised collagen bundles appear to be a product of fibroblasts and myofibroblasts. What triggers off the overproduction of collagen types I and III by these cells needs to be investigated. The presence of degenerate cells adjacent to the bundles and of cellular debris within the bundles is indicative of the degenerative process being associated with the accumulation of the different fibre types. Degeneration of cells could be an effect of proteases from degranulating mast cells[2]. Also, the decreased vascularity or the lack of a blood supply in the inner aspect of a collection of

bundles may contribute to the deterioration of the cells. Another secretory product of mast cells, transforming growth factor β, stimulates collagen production[3], but, presumably in viable cells only. The association between degeneration of fibroblasts and myofibroblasts, their excessive collagen production and the hyalinisation of the collagen aggregates has to be ascertained in order to establish the significance of glassy collagen bundles in the pathogenesis of keloids.

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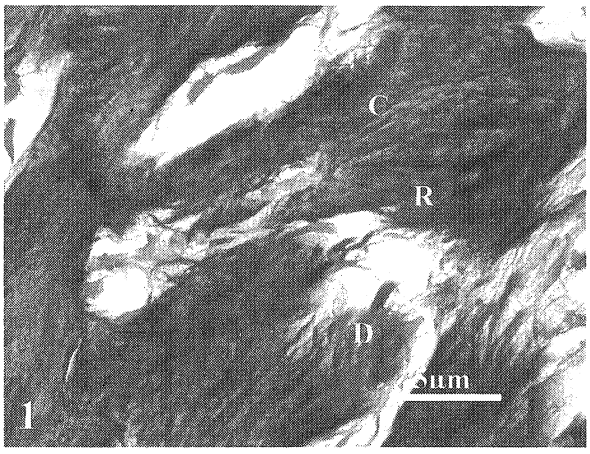

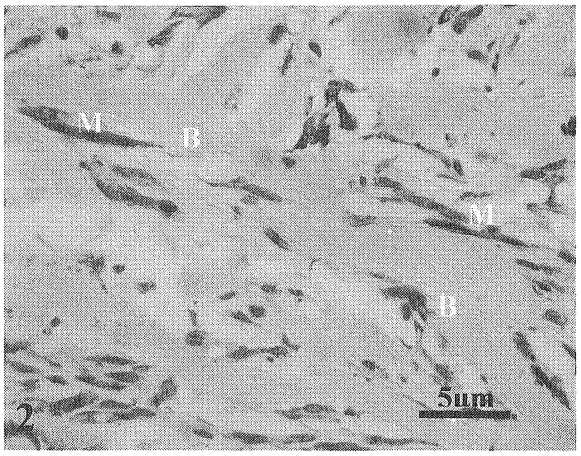

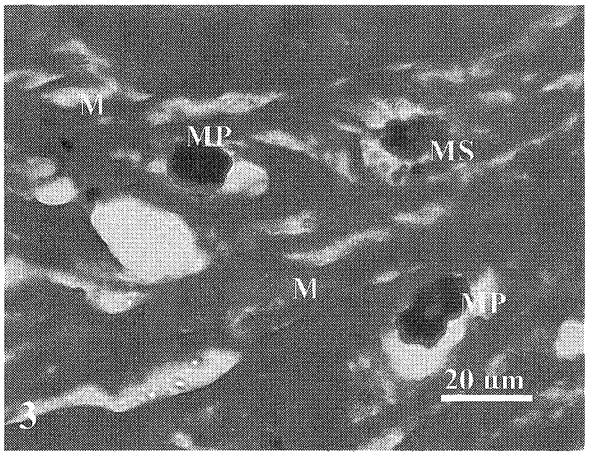

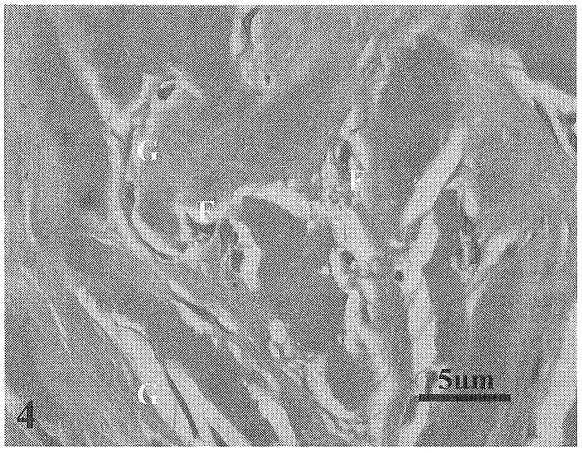

Fig. 1. Photomicrograph of reticulin-stained hyalinised collagen bundles showing black reticular fibres (R), purple collagen type I collagen fibres (C) and pink cellular debris (D).
Fig. 2. Photomicrograph of myofibroblasts (M) displaying positivity for α smooth muscle actin, adjacent to bundles of glassy collagen aggregates (B).
Fig. 3. Photomicrograph of Giemsa-stained mast cells surrounded by a space (MS) with some (MP) showing a close association with myofibroblasts (M) aligned along one side.
Fig. 4. Photomicrograph of degenerating myofibroblasts and fibroblasts adjacent to the glassy collagen bundles, exhibiting mild (F) or gross (G) swelling of their processes.

MAST CELL INVOLVEMENT IN KELOID FORMATION

S. Bux, A. Madaree*, P.K. Ramdial#, M.F. Dutton and A. Chuturgoon

Departments of Physiology, *Plastic Surgery and #Anatomical Pathology,
Faculty of Medicine, University of Natal, Durban, South Africa

The development of keloids which comprise excessive scar tissue, is an unsolved problem in the process of wound healing. Previous studies provide support for the role of inflammation in the pathogenesis of keloid formation[1]. The exact mechanism of this process remains uncertain. In this study the involvement of inflammatory and resident connective tissue cells in keloid formation was investigated using light and electron microscopy.

Wedge biopsies were obtained from keloids (Group A) and adjacent apparently normal skin (Group B) from 15 patients after surgical removal of the lesion. Control specimens of normal skin (Group C) were procured from healthy individuals who had breast reductions. Biopsies were processed for histological differential staining and electron microscopy using conventional techniques. Giemsa and sulfated alcian blue staining techniques were used.

Microscopical examination showed the presence of mild to moderate paravascular chronic inflammatory cell infiltration comprising plasma cells and few lymphocytes in 70% of the keloid specimens. This was not observed in the normal control tissue nor in the non-lesional skin (Group B) of the patients with keloids. A striking difference in the extracellular matrix (ECM) cell population between keloid specimens (Groups A&B) and normal skin (Group C) was the elevated numbers of mast cells in lesional as well as non-lesional skin (Groups A&B). There was a 3-7 fold increase in mast cell numbers in both biopsies from keloid patients. Whereas mast cells in uninvolved skin were inactive, those in keloids were in an active degranulating state. Numerous mast cell granules or remnants thereof were scattered in the ECM among the collagen fibres (Fig. 1). Many electron dense nuclei of degranulated mast cells were scattered throughout the ECM (Fig. 2). There was degeneration of cells and loss of integrity of components of the ECM in the vicinity of degranulating mast cells. Masses of intermingled fibrillar material and cellular debris formed the bulk of keloids.

Features of degeneration and the chronic inflammatory cell infiltration observed in keloid specimens may be a response mediated by the secretory products of mast cells. The granules of these cells are known to contain pro-inflammatory mediators[2] and various proteases[3]. The proliferation of collagen and fibroblasts which is characteristic of keloids may also be attributed to over-active mast cells. The secretory products of mast cells include cytokines of which fibroblast growth factor β and transforming growth factor β are mitogenic for fibroblasts and stimulate collagen production respectively[4,5]. The excessive numbers of actively secreting mast cells and the associated pathology present in lesional skin suggest that mast cells may play a key role in the pathogenesis of keloids.

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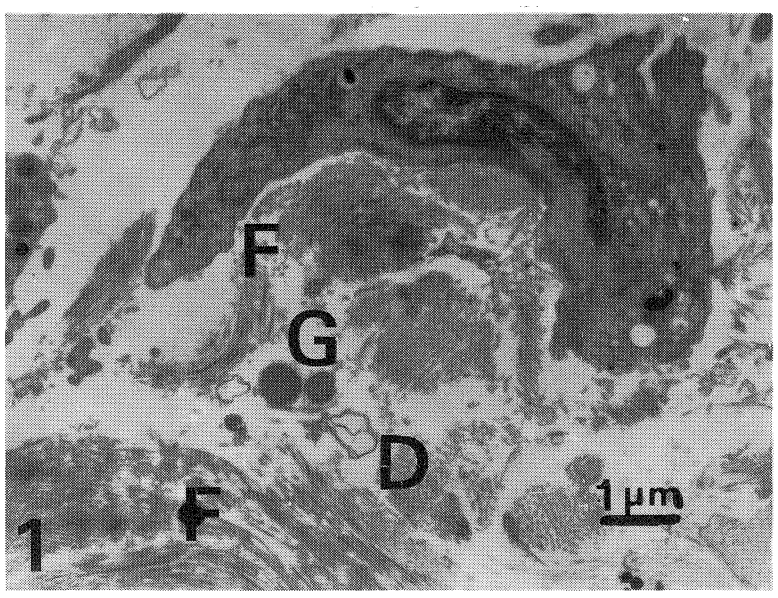

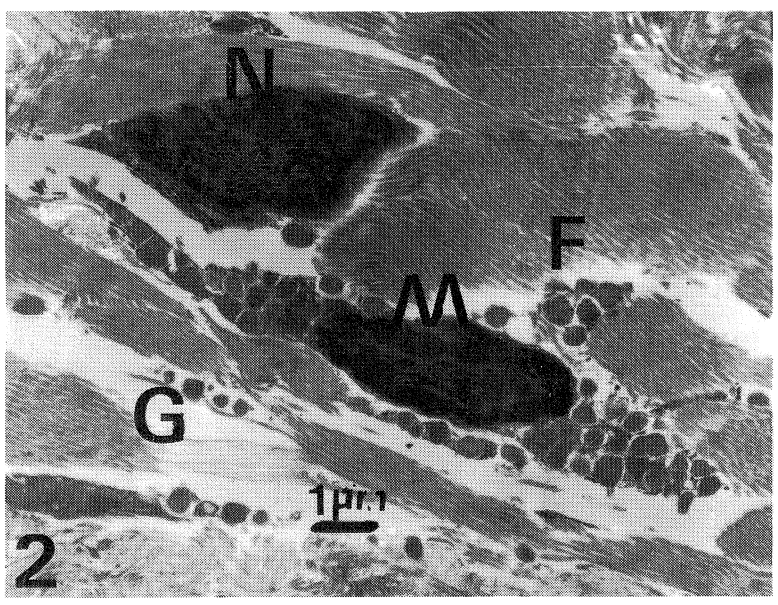

Fig. 1. Electron micrograph of mast cell granules (G) in the keloid ECM among the collagen fibrils (F) and cellular debris (D).

Fig. 2. Electron micrograph of keloid connective tissue showing the nucleus of a degranulated mast cell (N), collagen fibrils (F), mast cell granules (G) and a mast cell (M).

CHARACTERISATION OF KELOIDAL FIBROBLASTS

S. Bux, A. Madaree*, A. Chuturgoon , P.K. Ramdial# and M. Dutton

Departments of Physiology, *Plastic Surgery and #Anatomical Pathology,
Faculty of Medicine, University of Natal, Durban, South Africa

There are conflicting reports in the literature as to the cell types present in keloids. Previous microscopical studies describe the presence of myofibroblasts (MFBs) in keloids while other studies show an absence of MFBs[1] but an increase in the density[2] of fibroblasts. Such controversy may be the result of different areas of keloids biopsied for examination. Clarification of the cell types present in keloids is important as this may help in the interpretation of the clinical behaviour and the pathogenesis of these lesions. The aim of this study was to differentiate cell types present in keloids using morphological and immunohisto-chemical means.

Wedge biopsies of keloids were obtained from 10 patients after surgical removal of the lesion. Biopsies were processed for immunohistochemistry (HC), electron microscopy and electron immunocytochemistry (ICC) using conventional techniques. Primary antibodies used in the ICC procedure were rabbit anti-α smooth muscle actin (SMA) and monoclonal anti-collagen type I. Probes used were PAP for light microscopy and goat anti-mouse and goat anti-rabbit IgG conjugated to 10 and 20nm gold, respectively, for electron microscopy.

Four types of fibroblasts were identified in keloids. The first type was similar to the conventional fibrocyte found in the dermis of normal skin. They were stellate or spindle shaped, exhibited fibrillar cytoplasm and swollen organelles. Aggregates of fine, short collagen fibres surrounded these cells. The second type of keloidal cells were larger than fibrocytes, had long processes, intracellular collagen fibres and several cisternae of rough endoplasmic reticuli (ER) (Fig. 1). Large fibroblasts with very long bilateral branching processes and dark nuclei comprised the third type. Many of these cells displayed unilateral branching processes and dark nuclei displaced to one side giving them a comet-like appearance. Numerous collagen fibres radiated from the branching points between the processes. The ER of the cells were very swollen and a number of vacuoles were present in the processes. Gold -labelled collagen type I material was abundant within the vacuoles and scattered in the cytoplasm (Fig. 2). The fourth type of cells identified were large, elongated and had a fibrillar cytoplasm with occasional focal densities within. The nuclei were indented and contained dense chromatin. These cells often appeared in aggregates, showed the presence of occasional intercellular junctions and were surrounded by a fragmented basement membrane. These features, although not as prominent as in the true counterparts, categorise these cells as MFBs. Immunocytochemically, keloidal MFBs were positive for α SMA and collagen type I (Fig. 3).

Type 3 fibroblasts and MFBs were frequently adjacent to large bundles of hyalinised collagen which result from the fusion and homogenisation of compact collagen indicating ageing stroma. Fibroblasts and MFBs in the vicinity also displayed features of older cells such as convoluted pyknotic nuclei, grossly swollen ER and branched, vacuolated cellular processes. In addition the conspicuous paucity of Golgi bodies, mitochondria and secretory vesicles indicate that these cells are inactive. The presence of intracellular collagen fibres and apparently newly formed collagen between the branching processes may be the result of passage of propeptide peptidases into the cell and tropocollagen out of the cell through the porous plasmalemmae of effete fibroblasts. The three groups of fibroblasts may thus represent younger and progressively older to effete cells while MFBs may represent those from the early wound healing stage.

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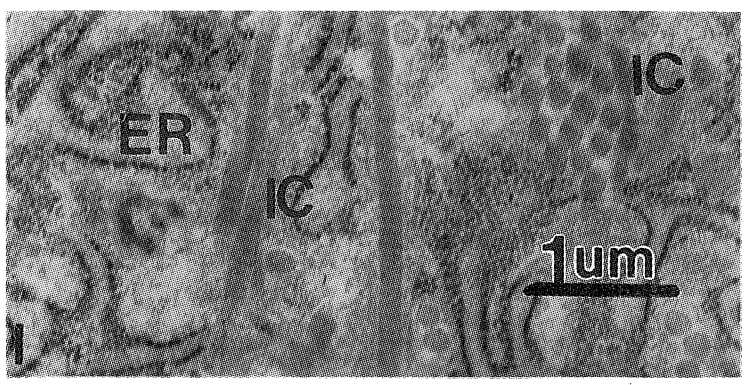

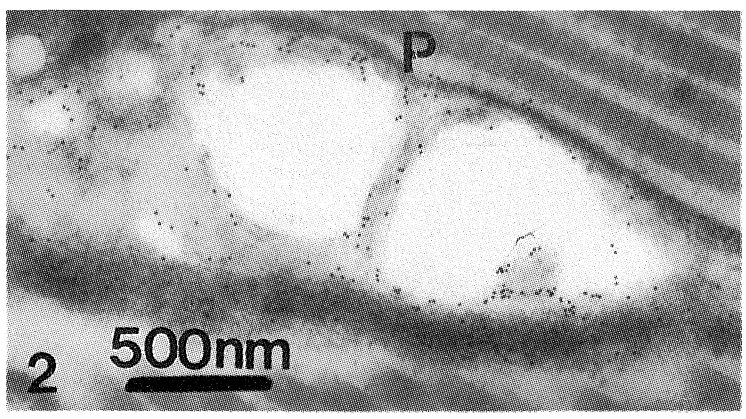

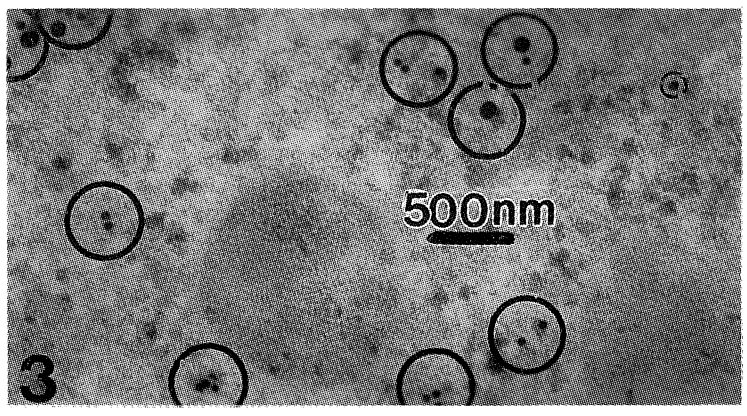

Fig. 1. Electron micrograph of internalised collagen (IC) and ER in fibroblast.
Fig. 2. Electron micrograph of vacuolated process (P) of a fibroblast exhibiting gold label for collagen type I.
Fig. 3. Cytoplasm of myofibroblast showing gold labelled actin (20nm) and collagen type I (10nm).

AMYLOID PROFILE OF KELOIDS AT THE EPIDERMAL EDGE OF THE LESION

J. Giandhari, M, Raboobee, H. Mothi. *A. Madaree and S. Bux

Disciplines of Physiology and *Plastic Surgery, Nelson R. Mandela School of Medicine, University of Kwa-Zulu Natal

Amyloid is a generic term referring to extracellular, fibrillar proteinaceous deposits that are resistant to proteolytic digestion. These deposits accumulate in various tissue types and are associated with disease. The different types of amyloid are grouped according to the type of protein they contain. These groups include AL amyloid (light chain of immunoglobulins), AA amyloid (serum), A prealbumin (transthyretin) and AE (endocrine forms of amyloid). The type of amyloid found in skin is related to keratin.

In some disease conditions such as primary systemic amyloidosis, faintly eosinophilic amorphous amyloid deposits were found in the dermis as well as in the subcutaneous tissue. Also accumulations of amyloid deposits close to the epidermis and around sweat glands and blood vessels were reported by Lever (1983) in systemic amyloidosis[1]. As amyloid has been localized in skin in disease conditions, we considered it important to investigate whether amyloid was also implicated in the aetiopathogenesis of keloid formation. This was reinforced by the following criteria: diseases characterized by amyloid are slow-onset and degenerative (as is the case in keloids); AA amyloid is associated with inflammatory diseases (chronic inflammation is almost always present in keloids, especially at the border of the papillary and reticular dermis). For these reasons and because the amyloid profile of keloids was not assessed previously, we undertook to examine the papillary/reticular dermal edge of keloids for the presence of amyloid.

Wedge biopsies of keloids were obtained from 10 patients after surgical removal of the lesions. Biopsies were processed for routine wax embedment and sections were stained with haematoxylin and eosin and Congo red for amyloid.

Amorphous and fibrillar staining patterns for amyloid were observed at the papillary/reticular dermal edge of keloids. Patches of amorphous staining for amyloid was found in 4 out of 10 specimens and was localised in necrotic areas of inflammation, in cellular areas where the fibroblastic cells were necrotic and in damaged walls of blood vessels. Less intensely-stained fibroblastic areas showed features of increased cell necrosis (Fig.1), whereas in the intensely stained areas there was less necrosis. Amyloid fibrils were present in all keloid specimens and they appeared in small to large clusters with a dense core (Fig.2) or as individual strands. They were generally found in less cellular areas as well as in inflammatory areas where necrosis was minimal or absent.

The results of the study show that amyloid is definitely implicated in the aetiopathogenesis of keloids. Pronounced necrosis in areas of less intense staining for amyloid, less necrosis in areas of intense staining and minimal necrosis in areas where aggregates of mature amyloid fibrils are present suggest that amyloid formation has a protective role. In accordance with this, large amounts of amorphous amyloid appeared to be more efficient than less in their protective role, as were long standing amyloid fibres, as indicated by the relative decreased structural damage observed in the milieu of clusters of fibrils. An age-dependent progression from the amorphous deposits of amyloid to the formation of dense clusters of fibrillar amyloid has been proposed[2]. In this report it was postulated that amorphous amyloid was the precursor to fibrillar amyloid and hence the amorphous type was called "preamyloid."

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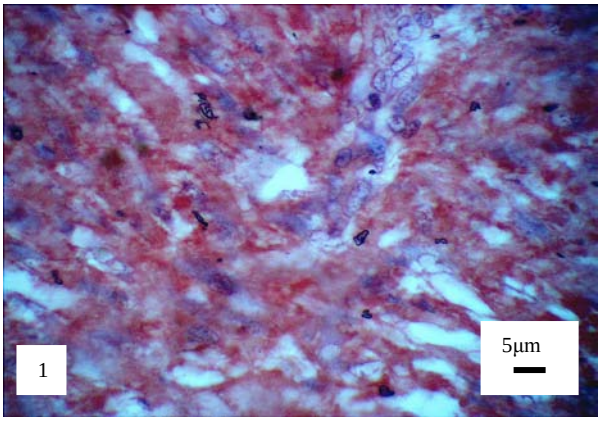

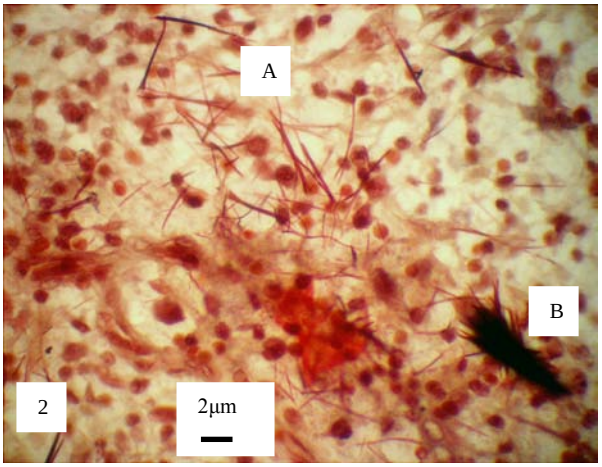

Photomicrographs of the keloid edge showing:
Fig. 1 Less intense amorphous amyloid staining (red) in necrotic fibroblastic areas
Fig. Amyloid fibrils present as large clusters (B) or as individual strands (A) in an area of inflammation

ALTERATIONS IN THE PAPILLARY DERMIS OF KELOID LESIONS

H. Mothi, J. Giandhari, M. Raboobee, *A. Madaree and S. Bux

Disciplines of Physiology and *Plastic Surgery, Nelson R. Mandela School of Medicine, University of Kwa-Zulu Natal

Keloids are a dermal proliferative disorder resembling an enlarged scar. They are formed three months to years after the infliction of injury, in up to 15% of wounds[1]. Keloids have a familial tendency and a predilection to form in dark skinned individuals. They are characterized by increased numbers of fibroblasts, deposition of abundant collagen and a greater degree of inflammation[2]. Previous studies have shown that keloids are formed specifically in the reticular dermis and that the papillary dermis is unaffected. As the papillary dermis is prone to some of the reported predisposing factors associated with keloid formation, it is probable that the papillary dermis is altered. Therefore, this study was undertaken to investigate whether the papillary dermis overlying keloid lesions showed morphological alterations when compared to normal skin.

After informed consent wedge biopsies were obtained from patients treated by the Plastic Surgery Department at the Inkosi Albert Luthuli Central Hospital. Three were from normal skin, and 10 from keloids. The specimens were processed for routine haematoxylin and eosin staining using the H2500 Microwave Processor. In addition, 3 differential stains were used: routine Masson's Trichrome for staining collagen, Giemsa for mast cells and elastic von Gieson for elastic fibres. The sections were examined using an Olympus BH-2 microscope and images were recorded using an Olympus DP-11 camera.

The papillary dermis of keloids generally appeared more cellular than that of normal skin. The fibroblasts and mast cells in the keloid were considerably more active than in the normal skin specimens. The inflammation in keloids was chronic and the degree ranged from moderate to severe (Fig.1). The inflammatory cell infiltrate in keloids contained plasma cells, mast cells and few phagocytes. Eosinophils were found in the infiltrate in 3 keloid specimens and multinucleate giant cells in 2 of these specimens (Fig. 2). Fewer macrophages were present in keloids than in normal skin. Features of note in the keloid vasculature were compressed blood vessels and swollen lymphatics (Fig. 3). In keloids there was an increased deposition the collagen which was arranged in dense bundles. Elastic fibres in normal skin were numerous and scattered throughout the papillary dermis whereas in keloids minimal amounts of elastic fibres were found.

The results of this study show many alterations in the papillary dermis of keloids when compared with that of normal skin. The chronic inflammatory cell response, the presence of eosinophils and multinucleate giant cells and the increased numbers of mast cells in keloids suggest that noxious or infective agents may be implicated in the aetiopathogenesis of keloid formation. The reduced numbers of macrophages in this milieu would hamper the effectiveness of the inflammatory process. The compressed blood vessels and swollen lymphatic vessels indicate interrupted flow to and from the papillary dermis, leading to the inefficient clearance of noxious substances and excess extracellular fluid by the lymphatics. The morphological changes observed in the papillary dermis overlying keloids, support the hypothesis that the aetiology of keloid formation may also provoke alterations in the papillary dermis.

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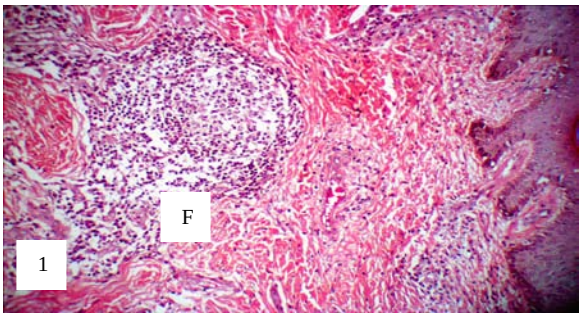

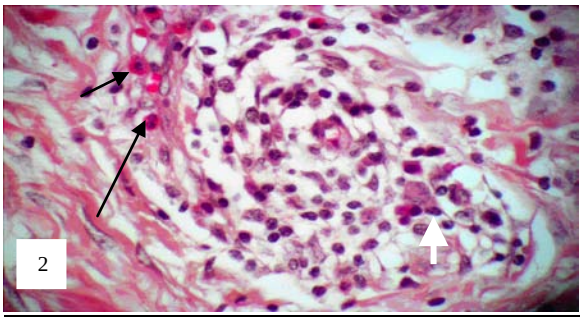

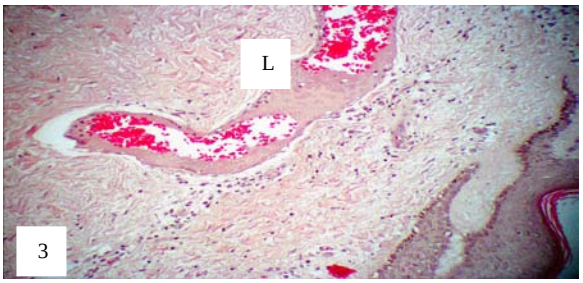

Photomicrographs of the papillary dermis in keloid showing:
Fig. 1 Severe inflammation (F)
Fig. 2 Eosinophils (black arrows) and giant cells (white arrow) in inflammatory cell infiltrate
Fig. 3 Swollen lymphatic vessel (L)

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A MORPHOLOGICAL STUDY OF THE DERMIS OF NORMAL SCAR

M, Raboobee, J. Giandhari, H. Mothi. *A. Madaree and S. Bux

Disciplines of Physiology and *Plastic Surgery, Nelson R. Mandela School of Medicine, University of Kwa-Zulu Natal

Scars are areas of fibrous tissue that replace normal skin after part of the dermis is damaged. It is the sum of the response to injury, repair, and intervention, and is a dynamic process, especially during the first 18 months or until healing is complete. During the normal wound healing process of adult skin, dermal damage is repaired by the deposition of connective tissue forming a scar. Very little is known about the cellular events and the deposition of the various protein fibres of the extracellular matrix in the process of scar formation. It is necessary to understand the pathogenesis of scars for their optimum management. In order to develop a better working knowledge of the process of scar formation, we undertook this study to examine the morphological differences between the dermis of normal skin and normal scar, with special reference to the mast cell, reticular fibre, collagen and elastic fibre profiles.

Biopsies of normal skin and normal scar tissue were obtained from 8 and 10 patients respectively. The age of the scars ranged from 6 weeks to 18 months. The specimens were processed for routine wax blocks and sections were differentially stained with Giemsa for mast cells, Mason's Trichrome for collagen, elastic Von Giesons, and Gordon and Sweets stain for reticulin.

When compared with normal skin, normal scar showed a greater degree of inflammation. In normal skin inflammation was minimal, whereas mild-moderate inflammation was observed in 8 out of 10 normal scar specimens. Normal scar also showed a lack of appendages and prominent rete ridges and dermal papillae. Features of angiogenesis were prominent in normal scar and vascular sprouts were directed vertical to the skin surface. The lymphatic vessels of 2 scar tissue specimens were swollen and were surrounded by lymphocytes. Phagocytes were greater in number in scar tissue than in normal skin. When compared with normal skin, normal scar contained numerous mast cells and their morphology varied from rounded to dendritic (Fig. 1). Collagen in scar tissue was arranged as abundant closely packed fibres or as bundles of fibres. Most of the collagen was arranged as dense bundles. In comparison with normal skin, there was a considerable decrease in the amount of elastic fibres present in normal scar and the diameter of the elastic fibres was greatly reduced There was an increase in reticular fibres in scar tissue. Here, reticular fibres were present alongside collagen bundles, adjacent to elongated fibroblasts, and as a network in foci of inflammation (Fig. 2).

The lack of appendages, features of angiogenesis and progression of collagen arrangement from abundant closely packed fibres into dense bundles in scar tissue indicate that scar tissue is being remodelled to closely resemble the tissue before injury. Macrophages are chemotactically attracted to the site of wound healing and release interleukin I which is chemotactic for lymphocytes[2]. The inflammatory cell infiltrate in scar tissue contained mainly lymphocytes with 3 specimens showing, in addition, few eosinophils. The precise role of mast cells in cutaneous scar reactions is unclear and mast cell numbers may not accurately reflect tissue concentrations of active mast cell products as some mast cells in this study were observed to be in a degranulating state. The positive correlation between the duration of the surgical scar and mast cell concentration in scar tissue found in this study was also reported previously[2]. The decreased elastic fibre content and increased reticular fibre content may be interrelated. The formation of prominent dermal papillae and rete ridges may be associated with the lack of elastic fibres and the subsequent effects of forces of deformation.

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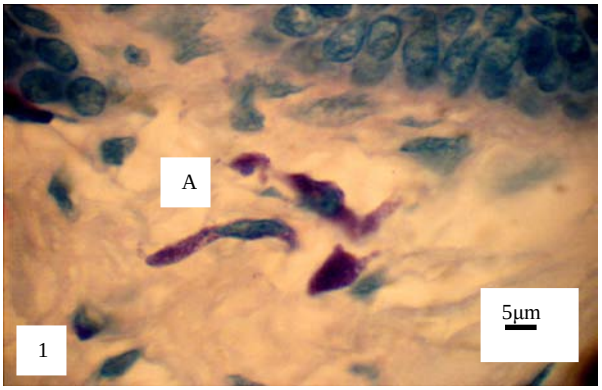

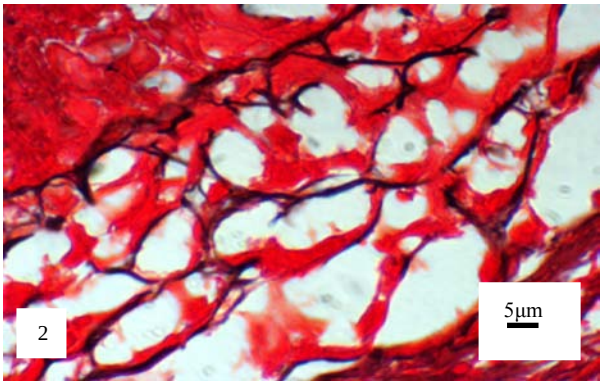

Photomicrographs of normal scar tissue showing:
Fig. 1.Rounded and dendritic mast cells (A) in the papillary dermis
Fig. 2 A network of reticular fibres in an area of inflammation in the dermis

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CHAPTER 3

DISCUSSION

3.1 Integration of work included in the published and submitted papers

The published/submitted papers arising from the keloid study investigated the objectives derived from the various hypotheses formulated. These objectives were:

- To establish baseline morphological characteristics of keloids thorough in depth ultrastructural examination of the entire keloid lesions so as to explain the many past and present contradictions, regarding keloid morphology.
- To establish the elastic fibre distribution in keloids to investigate whether the failure to restore tissue shape after deformation by various forces in keloids, is due to the lack of elastic fibre recoil as this is the main function restoring tissue to normality after deformation.
- To investigate why keloids have a predilection to form in skin covering the upper torso of the body and to assess whether this is related to elevated dermal stress caused by tissue compression and distortion.
- To investigate the significance of hyalinized tissue in the pathogenesis of keloids

The pertinent aspects of these investigations that provide novel information associated with the aetiopathogenesis of keloids are discussed below.

3.1.1 Exaggerated phases of wound healing in keloids

A conspicuous observation when examining keloid specimens was the regional distribution of connective tissue elements. The various regions were deciphered to be exaggerated phases of the following stages of the wound healing process: stages of angiogenesis, inflammation,

remodeling and scar maturation. This is in accordance with keloids being described as a form of an excessive wound healing response. Areas of angiogenesis and the fibrous cellular areas identified may represent extended wound healing phases of vascular and fibrous granulation tissue, respectively. The instigation of such a prolonged and amplified response was interpreted to be the impairment of the wound healing phase of inflammation which had progressed to the chronic stage. Therefore, in the publication entitled “Keloids show regional distribution of proliferative and degenerative connective tissue elements” the description of keloids was expanded to “a form of excessive wound healing that occurs when the healing stage of chronic inflammation is impaired”. All the important factors associated with impaired wound healing, viz., poor blood supply, persisting tissue damage and sequestered dead tissue, were all present in keloids (179). To progress with investigations on the aetiopathogenesis of keloid formation subsequent research areas, should embrace:

- The factors causing chronic inflammation
- The basis of a poor blood supply in keloids
- Causal factors of persistent tissue damage in keloids
- Explanations for a surplus of sequestered dead tissue in keloids.

From observations in the above paper it is presumed that if chronic inflammation was restricted, the keloid would regress and disappear because without chronic inflammation there would be no impaired wound healing resulting in keloid formation. Therefore, the identification and removal of the pathological stimuli causing chronic inflammation should be the prime aim to successfully treat and remove the keloid.

3.1.2 Chronic inflammation in keloids

The main region in keloids where chronic inflammation was pronounced was around differentiated microvessels of the sub-papillary plexus outside the keloid; in comparison,

developing microvessels inside the keloid showed scant to moderate paravascular chronic inflammation. The inciting factor of the inflammation appears to originate in the sub-papillary plexus where the marked inflammation suggests that it is found in higher concentration around these differentiated microvessels when compared with a minimal inflammatory reaction indicative of low concentrations around developing microvessels within keloids. The close proximity of the sub-papillary plexus to the epidermis makes it tempting to suggest that the factor/s provoking chronic inflammation are from an external source, gaining entry through injured parts of the epidermis. However, this has to be excluded as the papillary dermis, which is immediately below the epidermis, is reported to be unaffected.

The presence of multinucleate giant cells, lymphocytes and plasma cells in the chronic inflammatory infiltrate, categorizes the subtype of inflammation as specific chronic inflammation (179); however, as no infectious agent or foreign bodies were identified, it was uncertain as to whether the aetiopathology involved immune or non-immune mechanisms. Specific chronic inflammation appears *de novo* in response to certain types of injurious agents and heals by fibrosis. Of the injurious agents causing chronic inflammation, viz., viral infections, persistent fungal or microbial infections, prolonged exposure to toxic substances and autoimmune diseases, the most likely agent is autoimmune diseases; this is based on the observed chronic inflammatory attack of microvessels with the degree of severity increasing in better differentiated microvessels; and on the lack of evidence of infectious and toxic mediators in keloid specimens.

3.1.3 Keloids as an autoimmune disease

The autoimmune implication in the aetiopathogenesis of keloid formation is evident in the damage and destruction of blood vessels in the areas of inflammation in keloids (179). Also, it is deliberated that hyalinizing collagen bundles (HCB), which are a diagnostic feature of keloids,

are formed to prevent or curtail autoimmune mechanisms in keloids. In the study investigating the characteristics of HCB, reasons for the occurrence of the hyalinization process were thought to:

- Provide protection by sequestrating cytokines and proteases produced by mast cells
- Prevent autoimmune attack by isolating injured cells that might not have gained prior immunological tolerance.

Some examples of immunologically intolerant tissues in the body are the interior of the eye, testes and the brain but, these tissues are concealed behind anatomical barriers that prevent T cells from reaching them. Rupture of the barricades would expose the cordoned-off tissue to autoimmune attack.

In keloids, it is very likely that differentiating wound healing fibroblastic phenotypes, especially pericytes, which are protected within a tubular basement membrane (BM) and are found only around microvessels, are exposed to autoimmune attack when the enveloping protective BM is damaged or still developing during the healing process. The four major components of the BM are type IV collagen, laminin, nidogen/entactin, and perlecan. In keloids there are high levels of matrix metalloproteinase-2 (MMP-2), which are involved in the breakdown of type IV collagen (25), leading to the impairment of BMs (181).

3.1.4 The implication of internal stress in the aetiopathogenesis of keloids

In this study microscopic observations showed collagen fibre damage, degenerative tubular structures and enlarged extracellular spaces in keloid specimens. In addition to MMP-2 and other proteinases are there other factors causing extracellular matrix and tissue damage? From the publication “Involvement of upper torso stress amplification, tissue compression and distortion in the pathogenesis of keloids”, another important causal factor implicated was elevated internal stress caused by:

- Pertinent dermal connective tissue characteristics
- The effects of hard tissue in close proximity to the dermis
- Contractile forces exerted by wound healing fibroblastic cells
- External forces.

This elevated stress caused dermal distortion and compression, occluding microvessels. This initiated ischaemia and tissue damage, leading to impairment of connective tissue barriers protecting healing tissue, causing exposure of regenerating cells to autoimmune attack. In the HCB region the hyalinised tissue provided a place of safety by isolating and enwrapping wound healing cells such as degenerating and necrotic myofibroblastic cells and other fibroblastic phenotypes to prevent autoimmune attack and to provide a safely isolated region where injured regenerating cells, that might not be immunologically tolerant, can demise without the danger of exposure to immune reactivity.

3.1.5 Hypoxia as a factor involved in the aetiopathogenesis of keloids

Poor vascularisation impaired wound healing in keloids because of consequent hypoxia and ischaemia caused by microvessel damage,which was evident in endothelial and mural cell injury and constriction of the lumen. The resultant hypoxic and ischaemic conditions led to tissue damage and necrosis. The ensuing surplus of sequestered dead tissue found in keloids occurred as a result of inadequate clearance mechanisms, most importantly, the lack of macrophages. Tissue degeneration and necrosis was a consequence of the diminished blood supply resulting from poor lesional vascularisation and the impaired nature of these microvessels.

3.1.6 Mechanical factors and site and type of injury are crucial role players in the aetiopathogenesis of keloid formation

In addition to the factors discussed above, other factors such as mechanical factors and site and type of injury were thought to be crucial role players in the aetiopathogenesis of keloid formation. This was linked to the explicit predilection for keloids to form in the upper torso of the body, the main areas being the head (earlobes and mandibular ridge), neck, back, presternal and deltoid areas (178). The reason for the propensity of skin in these regions to form keloids is not known. The inherent characteristics of skin in these regions and the frequent occurrence of ear lobe keloids after ear piercing suggested that the site, type of injury and the effects of intrinsic and extrinsic mechanical factors contributed to this susceptibility. The hypotheses formulated around this concept, together with the morphological observations established after microscopical examination of keloids, normal scar and normal skin biopsies, led to the publication entitled “Involvement of upper torso stress amplification, tissue compression and distortion in the pathogenesis of keloids”. This paper discussed how stress promoted keloid formation by causing dermal distortion and compression which then stimulated proliferation and enhanced protein synthesis in wound healing fibroblastic cells (182). The strain caused by stress also compressed and occluded microvessels causing ischaemic effects and reperfusion injury which stimulated growth when blood rich in growth factors returned to the tissue. All the above features were present in keloid specimens, viz., compressed and occluded microvessels, injured degenerative tissue, proliferated blood vessels, connective tissue cells and extracellular matrix. The article explains how mechanical factors are responsible for causing a diminished blood supply in keloids which then leads to persistent tissue damage and accumulation of dead tissue which is exacerbated by inadequate clearance mechanisms. These occurrences (poor blood supply, persistent tissue damage and a surplus of sequestered dead tissue) impair the healing process at the stage of chronic inflammation (discussed earlier).

3.1.7 Possible causative injurious agents of chronic inflammation in keloids

The most likely causative injurious agent of chronic inflammation founded on the morphological evidence in the study is autoimmune disease. Microscopical studies show that the dermal structure attacked by the immune system was microvessels; the specific components under attack were the mural cells of these vessels. Mural cells include pericytes and smooth muscle cells. Pericytes are multipotent specialized mesenchymal cells closely associated with endothelial cells and they have important supportive roles with regard to the growth and maintenance of microvessels. Therefore, if pericytes or their BM is damaged, blood vessel function and development is defective. The occurrence of impaired microvessels is widespread in keloids. Leakage of pericyte products such as vascular endothelial growth factor (VEGF) occurs; this stimulates angiogenesis and promotes hyperpermeability in vessels (183). In keloids, pericytes under immune attack in the chronically inflamed subpapillary plexus region release VEGF which stimulates angiogenesis and the release of increased amounts of interstitial fluid from blood vessels with augmented permeability; this results in enlarged extracellular spaces. These effects were evident in keloids in areas of angiogenesis and in connective tissue areas with increased extracellular spaces. Enlarged extracellular spaces were found in all keloid regions, frequently around microvessels with degenerative mural cells; this exhibited the effects of pericyte-induced, VEGF- stimulated hyperpermeability. The resulting high interstitial fluid pressure adversely affected tissue by adding to the stress levels caused by intrinsic and extrinsic mechanical factors, causing compression and occlusion of microvessels, tissue injury and proliferation of blood vessels, connective tissue cells and extracellular matrix components. All these features were displayed by keloids (179).

3.1.8 Abnormalities of collagen morphology in keloids

With regard to inherent coping mechanisms to counteract deforming intrinsic mechanical stress in skin, elastic and collagen fibres offer resistance by stretching and sliding. Elastic fibres are dominant in the stretch mechanism while collagen fibres offer resistance mainly by sliding and minimally by stretch. Microscopical examination of keloid specimens showed many abnormalities in collagen morphology which effectively impair the capacity of collagen to resist forces of deformation. The morphologically illustrated collagen aberrations associated with high tension in keloids included: areas of dense regular connective tissue indicative of collagen fibres stretched to the limit, areas of collagen lysis showing collagen damage and dissolution and fibrous tubular areas indicating collagen failure and reduction of stiffness (179). In fibrous tubular areas collagen failure under elevated strain is indicated, manifesting as snapped collagen fibres with reduced stiffness which entwine in various configurations and directions. This is clearly shown in areas where fibrous fringes emanate from tubular stalks, where aggregates of tubules entwine to form stalks and where aggregates of intertwined tubules attach to stalks (179). These areas of tissue failure also exhibit degenerative microvessels and degenerating and necrotic cells including pyknotic enwrapped, and necrotic spindle cells , aggregates of necrotic fuzzy epitheloid cells and degenerative spindle cells drawn into entwining eosinophilic tubules (179).

3.1.9 Lack or absence of elastic fibres in keloids

While collagen fibres provide tensile strength greater than steel to enable skin to withstand forces of deformation, the elastic fibre network provides resilience to skin and resistance to deforming forces by stretching and recoiling. Elastic fibre stretch and recoil are inherent mechanisms to cope with deforming extrinsic and intrinsic mechanical stress in skin. When comparing stress coping mechanisms with collagen, elastic fibres are dominant in the stretch

mechanism, permitting tissues to stretch and recoil without damage. As keloids demonstrate failure of restoration of tissue shape after deformation, which indicates a lack of ability to cope with forces of deformation, and as elastic recoil is the principal function responsible for recovery of deformed tissue, it was a predictable deduction that connective tissue elasticity was decreased in keloids. Therefore, the elastic fibre distribution in keloids was histologically assessed and compared with that of non-lesional skin in the publication entitled “Does the paucity of elastic fibres contribute to the process of keloidogenesis?” (184) . This paper showed that there was a lack or absence of elastic fibres within the various keloid regions, leading to failure in coping with forces of deformation and restoration of tissue shape after deformation. In the absence of elastic fibres, the cells, microvessels and other constituents of the ECM are constantly exposed to low internal strains (up to 0.3), which upsets the normal physiological balance between external gravitational force and internal tensile forces which maintain certain connective tissue cell phenotypes and their level of activity. Increased internal strain results in increased loading on cells and the ECM; the cells display anabolic effects, showing increased levels of gene expression, collagen synthesis and excretion and mitotic activity (185). Increased internal tension extends collagen fibres attached to dermal cells (via integrins), triggering stretch of cell membranes which activates ion channels located in the cell membrane (185). With the lack of elastic fibres, the proportion of energy dissipated by the sliding of collagen fibres in the viscous matrix is increased. Concurrent with this process is the realignment of collagen in the direction of the applied force, resulting in the reorientation of collagen fibres. An increase in internal energy to be dissipated would, therefore, result in the realignment of a larger number of collagen fibres or bundles in the direction of the applied force. This is an important attribute that imparts to skin the capability of being overextended, leading, sometimes, to keloids of grotesque proportions. The defects caused by the lack of elastic fibres i.e. failure of tissue to recover shape after deformation and effects of increased stress were morphologically clearly evident in

keloidal growths where there was an overproduction of different collagen types and the proliferation of fibroblastic cells and blood vessels (179).

3.1.10 Increased elastic fibre content at the borders of keloids

An interesting phenomenon regarding the elastic fibre content of keloids was that at the borders of keloids elastic fibre quantity was increased and the elastic: collagen fibre ratio was 2.4 times higher than in normal skin. In some keloid zones at the border area, viz., fibrous tubular regions and dense regular wavy connective tissue areas, elastic fibres were abundant whereas matched areas within the keloid lesion showed the absence of elastic fibres or contained very few frail fibres. This higher than normal concentration of elastic fibres at the keloid border area was thought to be a proliferative response to cope with deformation forces including traction forces exerted by keloid fibroblasts, extrinsic stresses (pressure and shear) and elevated muscle and skin tension in skin overlying bony or cartilaginous surfaces. The deformation and compression of dermal tissue, led to compression and collapse of microvessels and thus, induction of ischaemic conditions and tissue failure.

3.1.11 The significance of compressed and occluded blood vessels in the pathogenesis of keloids

The presence of compressed and occluded blood vessels was a common feature in all keloids examined (179). Restoration of circulation after an ischaemic episode does not usually reinstate normal function; rather, it results in inflammation and oxidative damage through the induction of oxidative stress (186). Dermal tissue injury where cellular and vascular damage was pronounced was observed in keloids (179). If ischaemia leads to degeneration and necrosis of tissue, how do we then explain the paradoxical situation of keloidal growth? Growth is stimulated during reperfusion when blood supply returns to the tissue after compressive stresses

are released in the post-ischaemia period. Growth factors are produced by activated platelets that accumulated within vascular beds during ischaemia in preparation for their release early after reperfusion (187). This would occur in healthy intact vascular beds around the keloid. As indicated by morphological studies, the healthy proliferating areas were located at the keloid border. In addition to growth stimulation by reperfusion, an increase of internal stress also leads to cell stimulation and an increase in their levels of gene expression, collagen synthesis and mitotic activity (185).

3.2 Synopsis of the pathology and aetiopathogenesis of keloid formation assessed in the study

A widespread pathological feature in keloids was the presence of degenerative, occluded and compressed microvessels, resulting in an impaired vascular supply to each of the keloid regions identified, even in the areas of angiogenesis. This impacted directly on the pathology of keloids where degeneration and necrosis, manifesting the lack of nutrients and oxygen to tissue, were found in all regions. Why was the vascular supply to keloid regions impaired? Inflammation of the microvessels, especially of the sub-papillary plexus and the ensuing damage and destruction of these vessels was the apparent reason, but the inciting factor of the inflammation was unclear and had to be deciphered.

The subdivision of chronic inflammation found in keloids, was identified as specific chronic inflammation, based on the absence of acute inflammation and the presence of multinucleate giant cells, lymphocytes and plasma cells. Specific chronic inflammation appears *de novo* in response to certain types of injurious agents and heals by fibrosis. The most likely agent causing chronic inflammation in keloids was thought to be autoimmune diseases; this is based on the observed chronic inflammatory attack of microvessels with the degree of severity increasing in better differentiated microvessels. Pericytes and wound healing fibroblast

phenotypes that might not have gained prior tolerance to lymphocytes were under attack. Pericytes, found around microvessels, are normally protected within a tubular basement membrane (BM). When the enveloping protective BM is damaged or still developing during the healing process, the mural cells of microvessels are exposed to autoimmune attack. Impairment of the BM is caused by the high levels of matrix metalloproteinase-2 (MMP-2), which are involved in the breakdown of a major component of BM, type IV collagen (25).
Supporting the autoimmune theory was the widespread occurrence of hyalinized collagen bundles. This pathological feature was thought to be a protective consequence to restrict or prevent autoimmune mechanisms in keloids by sequestrating the inciting causative antigenic proteins from injured cells, like pericytes and other wound healing fibroblast phenotypes that might not have gained prior immunological tolerance.

Another important factor implicated in the pathology of keloids was elevated internal stress caused by pertinent dermal connective tissue characteristics; the effects of hard tissue in close proximity to the dermis, contractile forces exerted by wound healing fibroblastic cells and external forces. The strain produced by stress caused dermal distortion and compression which occluded microvessels, initiating ischaemia and tissue damage. In the process connective tissue barriers protecting healing tissue are damaged, exposing regenerating cells (pericytes, fibroblasts, etc.) to autoimmune attack. In the HCB region the hyalinised tissue sequestered antigenic proteins released from wound healing cells such as degenerating and necrotic pericytes, myofibroblastic cells and other fibroblastic phenotypes which prevented autoimmune attack. Also, HCB safely sequestered injured regenerating cells that might not have gained prior tolerance from exposure to immune reactivity.

The effect of internal stress on tissue was exacerbated by the lack or absence of elastic fibres within the various keloid regions, leading to failure in coping with forces of deformation. This led to compression and collapse of microvessels and thus, induction of ischaemic

conditions and tissue failure. In the period when compressive stresses are released, blood rich in growth factors returns to tissue and during this reperfusion phase tissue growth is stimulated. In addition, elevated internal stress also stimulated tissue growth by increasing levels of gene expression, collagen synthesis and mitotic activity (185). This stimulation of growth resulted in keloidal growth.

3.3 List of pathological features and criteria identified in keloids

The following pathological features and criteria were identified in keloids:

- Compressed and occluded microvessels which impaired microvascular supply
- Degenerative and necrotic mural cells of microvessels
- Degeneration and necrosis of fibroblastic phenotypes and connective tissue fibres in all regions
- Severe specific chronic inflammation around microvessels of the sub-papillary plexus and moderate to mild chronic inflammation of developing microvessels within keloids
- Ischaemic injury
- Reperfusion injury
- Anabolic effects of reperfusion
- Presence of various fibroblastic phenotypes in proliferative and degenerative stages. All were vimentin positive, few were actin positive and none showed positivity for desmin.
- Degenerative or calcified nuclei.
- Nuclear and plasma membrane damage, cytoplasmic and nucleoplasmic clearing
- Presence of many degranulated and degranulating mast cells
- Oedematous areas

- Sparse presence of myofibroblastic phenotypes mainly in degenerative stages. These were alpha smooth muscle actin and vimentin positive and desmin negative.
- Overproduction of collagens type I and III
- Lack of elastic fibres in the keloid
- Overproduction of elastic fibres at the keloid border
- Presence of hyalinised collagen bundles
- Exaggerated remodelling stages of dense regular collagen (straight and wavy forms)

3.4 List of aetiopathological factors implicated in the pathogenesis of keloids

The following aetiopathological factors were established to be implicated in the pathogenesis of keloids:

- Antigenic material released from regenerating mural cells through perforated, damaged or absent basement membrane tubes
- Antigenic material released from regenerating fibroblastic phenotypes through perforated, damaged or absent basement membrane tubes
- Lymphocytes and plasma cell activity
- Mast cell activity
- Forces causing deformation of tissue
- Forces causing compression of tissue
- Increased internal stress
- Increased loading on cells
- Ischaemic injury
- Reperfusion injury
- Traction forces exerted by wound healing fibroblastic cells

- Extrinsic stresses (pressure and shear)
- Elevated muscle and skin tension in skin overlying bony or cartilaginous surfaces
- Low stretch ability
- Low elastic modulus
- Mechanical forces exerted by hard tissue in close proximity to the dermis
- Contractile forces exerted by wound healing fibroblastic cells
- Existent high tension in the wound healing milieu of keloids

3.5 Concluding remarks

Keloids present a great challenge to surgeons because of the high recurrence rate and the poor response to therapy. Presently no guaranteed effective treatment exists and keloids, especially recurrent ones, are best treated by multimodal methods. These include surgery, preoperative and postoperative pharmacologic drugs, radiation, wound taping, use of magnetic devices, pressure therapy and silicone gel sheeting or ointment. It is interesting to note that postoperative taping and pressure therapy does significantly reduce the chance of keloid formation. This may be ultimately due to the mechanical theory that has been previously postulated in this study.

Even with the current optimal treatment regimens, there are inevitable side effects such as tissue atrophy, hypopigmentation (triggered by steroids), recurrence with more aggressive growth (provoked by excision) and risk of cancer (prompted by radiation). The availability of many therapeutic regimes, the continual search for novel therapy and the development of new forms of treatment all aim to alleviate the surgeon's frustrating nightmare of recurring and unsuccessfully treated keloids. As, to date, there is no single therapy that is absolutely successful, it is necessary that research on keloid pathogenesis ventures into novel fields to refine or redirect existing therapeutic regimens. The work presented in this thesis is novel and explores new research avenues on the aetiopathogenesis of keloid formation. It is hoped that this

stimulates progress in research to unfold the pathogenesis of keloid growth and development with the long term view of providing unfailing specific effective treatment.

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