Role of human β-defensins in human burn wounds.

Mobin Syed

School of Life Sciences

UNIVERSITY OF WESTMINSTER
SCHOOL OF BIOSCIENCES
&
QUEEN MARY UNIVERSITY OF LONDON
INSTITUTE OF CELL AND MOLECULAR SCIENCE

ROLE OF HUMAN β- DEFENSINS IN HUMAN BURN WOUNDS

BY
MR MOBIN SYED

Thesis submitted in partial fulfilment of the requirements for PhD at the University of Westminster
March 2009

CONTENTS

STATEMENT OF ORIGINALITY

I declare that this thesis was composed in its entirety by me. All experimental work and results reported are my own except where due reference has been made and and help provided has been acknowledged.

Mobinulla Syed

ACKNOWLEDGEMENTS

I take this opputunity to thank my supervisors Dr Pamela Greenwell, Professor Harshad Navsaria and Dr David Perrry for their advice, time and constructive criticism during the course of my PhD. I would specially like to thank Dr Greenwell for her immense support, guidance and angelic patience during this period.

I would like to thank and congratulate Fadi Al Safadi and Khurshid Alam who successfully completed their MSc projects under my supervision, undertaking part of the work in chapters 4 and 6 for their thesis projects.

I owe special thanks to Mr Martin Taube, Mr Brian Davies and Dr Ben O'Donohue at West Wales General Hospital for their support and encouragement in times of distress and despair and guiding me through to this stage.

I am also thankful to Dr Sanjiv Rughooputh and Dr Frank Hucklebridge for help in various aspects of PhD.

Finally I would like to thank my Parents, my brother and sister for their generous guidance, help, endless encouragement and unrelenting moral support throughout my life and career.

List of Acronyms & Abbreviations

AB	antibody
ANGF	Angiogenesis Factors
BD	**β** - defensin
CCR	Chemokine receptor
cDNA	Complementary Deoxyribonucleic Acid
CRP	C - Reactive Protein
CTGF	Connective Tissue Growth Factor
DAB	3, 3’ Diaminobenzidine
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside triphosphate
ECM	Extracellular Matrix
E.colil	*Escherichia coli*
EpGF	Epidermal Growth Factor
EGF	Endothelial Cell Growth Factor
ERK	Extracellular Signal Regulated Kinase
GAG	Glycosaminoglycans
HBD	Human β defensin
HIF	Hypoxia Induced Factor
HNP	Human Neutrophil Peptide
IFN	Interferon

INF-γ-	Interferon-alpha
IL	Interleukin
ILGF	Insulin like Growth Factor
LPS	Lipopolysaccharide
MCP	Monocyte Chemoattractant Protein
MIF	Macrophage Migration inhibitory factor
MIT-K	Mitogen Activated Protein(MAP)Kinase
MMP	Matrix Metalloproteinase
mRNA	Messenger Ribonucleic Acid
MRSA	Methicillin resistant *Staphylococcus aureus*
NaCl	Sodium Chloride
NF	Nuclear factor
NDF	Normal Dermal Fibroblast
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDGF	Platelet derived growth Factor
PFA	Paraformaldehyde
RCM	Robertsons cooked meat medium
rRNA	Ribosomal Ribonucleic Acid
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SD	Standard Deviation
SNP	Single Nucleotide Polymorphism

STAT	Signal Transducer and Activator of Transcription
TBE	Tris Borate / EDTA Buffer
TNF	Tumour Necrosis Factor
TUNEL	Terminal Deoxynucleotide transferase-mediated d UTP nickend labeling
UK	United Kingdom
UTP	Uridine Triphosphatase
VEGF	Vascular Endothelial Growth Factor

Published abstracts and Presentations arising out of the current PhD

Role of β- Defensins in the keloid Pathogenesis

Oral Presentation International Society for Burn Injuries – 25-29 September, Brazil 2006

Time Dependent variation of β- Defensins in Burn Wounds

Oral Presentation ETRS-Pisa, Italy, September 2006

The Progress of Dermal Matrices in Cutaneous Wound Repair

Oral Presentation, V congress of Central European Burns -18 - 20 May, Slovakia, 2006

6 year review of the Keloid Management –Relevance of Steroid Therapy

Oral Presentation 7th European Trauma Congress-14 -17 May, Ljubljana, Slovaenia, 2006

Cortisol Circadian Rhythm in Critically ill patients – Can we modulate it?

Poster presentation -196th meeting of the Society of Endocrinology; London Nov 7-9 2005

β Defensin 1,2,3 expression in burn wounds-Correlation to bacteria

Oral Presentation - European Burns Association Congress Sept 21-24Estoril, Portugal, 2005

Papers in Preparation from the current PhD

1. Longitudinal Quantitative Human **β-** Defensin Expression in Deep Burn Wounds –Is there an attenuation of Innate Immune Responses?

2. Keloid Pathogenesis –Do Human **β-** Defensins play a role?

3. The Role of Human **β-** Defensins in Systemic Infections.

4. Human **β-** Defensins and Burns –A comprehensive review.

5. Buccal Human **β**-Defensin expression in Peri-operative conditions-Does Stress Influence defensin expression?

List of tables

List of figures

Abstract

Background

Burns is a complex condition requiring assessment and addressing of both the wound and the patient in a holistic way. In spite of tremendous improvements in burn care, infection continues to remain an important cause of morbidity and mortality. Human B Defensins (HBD) are a group of recently discovered antimicrobial peptides. The main subtypes include HBD1, 2 and 3 and are individually known to have other functions apart from being anti-microbial. Some of these are inherently expressed while others are induced in response to microbial challenge.

Aims

The aim of the current PhD was to understand the pattern of expression of HBDs in acute burns, their source of expression, and factors influencing the expression, with a view to use these peptides as therapeutic agents in future.

Methods

The expression of HBD1, 2 & 3 was determined at mRNA and protein levels in acute burn wounds of different burn durations, using real time rt-PCR and immunohistochemistry, respectively. The influence of type and quantity of bacteria, contribution from blood cells and the influence of stress on HBD expression was determined in separate clinical situations similar to those seen in major burns.

Results

The results show that HBD1, 2 and 3 mRNA is highly expressed in both early and late burns, but a parallel increase is not reciprocated at protein levels. The bacteria isolated from the burn wounds showed a trend changing from colonising organisms to more resistant forms in time, however no significant correlation with HBD was established.

Peripheral blood cells produced HBD in response to inflammatory mediator's *in-vivo*, thus suggesting to a possible contribution of HBD 1, 2 and 3 from blood cells in granulating burn wounds.

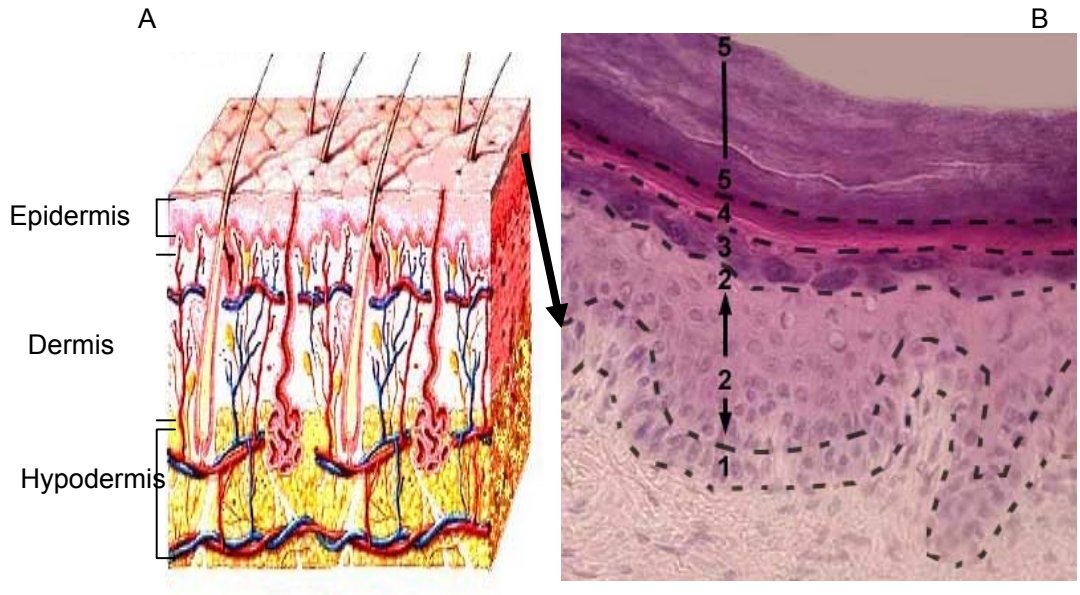
There was no down regulation of HBD1, 2 and 3 in the presence of increased cortisol levels – a reflection of heightened stress as seen in burns. HBD1 and 3 mRNA expressions showed an early up-regulation, followed by elevation in HBD2 mRNA levels. There was no HBD2 mRNA and protein expression in keloid tissue specimens from various parts of the body. The absence of HBD2 – a unique intrinsic peptide with an ability to trigger the anti-fibrotic cytokines suggests to the possibility that HBD's are implicated in the pathogenesis of keloids, which are usually associated with burn scars.

Conclusion

The studies suggest a possible role of HBD in the pathogenesis of burn wounds and the potential to use these as therapeutic peptides to prevent infections and scarring associated with burns.

Chapter 1

General Introduction



1.1 *Skin*

Figure 1.1 Diagrammatic representation of skin

Adapted from www.nlm.nih.gov/.../ency/imagepages/8912.htm and www.histol.chuvashia.com/.../epithelia-en.htm. Part A of the figure shows the diagrammatic representation of the different layers of skin. Part B shows the epidermis stained with Haematoxylin and Eosin stain, showing the different layers of epidermis. 1-stratum basale, 2-stratum spinosum,3-granular layer, 4-lucidal layer,5-cornified layer.

Skin is considered to be the heaviest organ in the body accounting for nearly 16% of the total body weight and extending over to an area of 1.2-2.3 m^2.It consists of 2 main layers, the epidermis and dermis (figure1.1). The layer beneath the dermis, namely the hypodermis is not considered a part of the skin.

Epidermis (cuticle or scarf skin)

The epidermis is an avascular layer composed of stratified cells predominantly squamous in nature and comprises mainly of keratinocytes (85%). The other cell types consist of

Langerhans cells, melanocytes and Merkel cells. The cells in the basal layer proliferate to generate the cells, which are constantly lost from above. This movement from the basal layer to the environment takes about 47-48 days (Iizuka, 1994) and is called the “epidermal turnover time”. The cells differentiate from the basal layers as they reach the top layer to mature cells. Skin consists of five layers from deep to superficial; the *stratum basale, stratum spinosum, stratum granulosum, stratum lucidum* and the *stratum corneum*. The *stratum basale* is the deepest of the 5 layers and is composed of single layer of proliferating cuboidal (or columnar) cells over the basement membrane. Connective tissue elements called hemidesmosomes and desomosomes attach it to the basement membrane, and to each other respectively. Specialised cells such as melanocytes and Merkel cells are scattered within this layer. Both these cell types are of neural crest origin but have varied functions. The melanocytes produce a pigment melanin which serves to absorb the UV rays and protect the skin while the Merkel cells aid in the sensory perception of light touch. Langerhans cells are derived from bone marrow and serve as antigen presenting cells and are distributed in the basal, spinous and granular layers of epidermis (Don R Revis Jr, 2006).

The next layer after the basal layer is the s*tratum spinosum* that is composed of multiple layers of proliferating cuboidal or polygonal cells with a central euchromatic nucleus. The cytoplasm is composed of dense connective tissue elements called tonofilaments which project as spines into desmosomes, hence this layer is called the *stratum spinosum*. Interspersed in this layer are giant cells called Langerhans cells. Sometimes the term *stratum germinativum* is used for the above 2 layers.

The *stratum granulosum* consists of 3-5 layers of flattened polygonal cells. Nuclei are present but there is no active cell proliferation. They are characterised by an abundance of

keratohyaline granules composed of phosphorylated proteins, hence the term *stratum granulosum*. The cells also contain lamellar granules rich in protein and lipids. The contents of these granules are secreted over the surface making it waterproof. The *stratum lucidum* is a thin layer composed of translucent eosinophilic cells.

The outermost layer of skin, the *stratum corneum* consists of dead cells arranged in 4-5 layers and these are shed as new cells are added from below. It serves as a barrier regulating the entry and exit of substances into and out of the skin.

Nutrition of the Epidermis

Since the epidermis is avascular, nutrition is obtained by diffusion from the underlying vascular dermis. The cells of epidermis progressively die as they migrate upwards from the basal layer hence the epidermis, except in the *stratum basale* and *stratum spinosum* is composed of dead cells.

Dermis

This can be further divided into the papillary layer and the reticular layer. The papillary layer consists of the rich network of blood vessels and also houses the free nerve endings in the form of eminences called papillae. The rich vasculature aids in thermoregulation and in delivering the nutrients to the avascular epidermis while the nerve endings help in tactile sensation either directly or by forming specialised touch receptors called Meissners corpuscles. The reticular layer is composed of predominantly white fibrous tissue and some fibres of yellow elastic tissue with other connective tissues.

Functions of skin

Some of the important functions of skin are listed in Table 1.1.

Table 1.1 The functions of skin

1	Mechanical and biological protection
2	Maintenance of internal homeostasis by preventing loss of water, electrolytes and macro-macromolecules
3	Sensory organ
4	Vitamin D and hormonal synthesis
5	Thermoregulation
6	Lubrication and waterproofing

1.2 Burn wounds

Classifications of burns

Burns result in destruction of skin and subcutaneous tissues and can result from a variety of causes. The burn wounds can be classified either in relation to the depth of the injury or the cause of the injury(Sheridan et al., 1995a, Sheridan et al., 1995b). A brief and simplified classification and summary of the management and outcome is outline below(Johnson and Richard, 2003).

Superficial

The two major causes are ultraviolet radiation from sun or transient contact with hot objects. The patient presents with pain, erythema, absence of blistering and oedema. The burn injury is confined to epidermis and localised vasodilation of the vascular plexus at the dermo-epidermal junction results in erythema. Apart from analgesia, specific management includes application of topical Aloe vera gel and usually within 5 days, complete healing is seen without scarring.

Partial thickness burns

These can be divided into two types, superficial and deep.

Superficial partial thickness

The aetiology is varied and may result from thermal contact, electrical, chemical, radiation or friction injuries. The patient presents with pain, erythema, hypersensitivity, blistering and oedema. In this case, the burn injury extends beyond the epidermis into the papillary dermis. Localised vasodilation of the vascular plexus at the dermo-epidermal junction results in erythema. The patient is given analgesia and specific management includes dressings aimed to keep the wound moist. Complete healing is noted without scarring within 5-7days.

Deep partial thickness

The aetiology is varied and the burn may result from thermal contact, electrical or friction injuries. The patients present with altered sensation, poor or absent capillary refill, mixed red or white lesions, oedema and an absence of blisters. The burn injury extends to the level of

reticular dermis or areolar tissue deep to dermis. The patient is given analgesia and topical antiseptics and antibiotics are used. Surgical debridement and grafting are commonly required.

Full thickness and Sub dermal burns

The aetiology is varied and burns may result from thermal contact, electric burns or friction injuries. The burnt area is insensitive to touch but sensitive to pressure, capillary refill is absent. The burn is white or black in appearance with an absence of blisters, the patient has thrombosed vessels, poor distal circulation and the area is dry, rigid and leathery. In this case, the burn injury extends beyond dermis. The patient is given fluid replacement and the burn is treated with antibiotics and dressings. Skin grafting may be necessary and the burns have a high potential for scarring.

Subdermal burns

The aetiology is varied and is the result of a severe insult. The skin is charred and the underlying tissue exposed. The patient has little sensation in the area. In this case, all layers of the skin and hypodermis are burnt with subcutaneous and underlying tissues exposed. All the nerve endings and sensory cutaneous receptors are destroyed.

Classification based on the aetiology of burn wound.

Table 1.2 Categories of burns based on aetiology

1	Thermal burns
2	Chemical burns
3	Electrical burns
4	Friction burns
5	Ultraviolet radiation induced burns

Physiological effects of Burn injury

Burn injury results in both local and systemic responses. These responses can be variable depending on the nature and the extent of the burn injury.

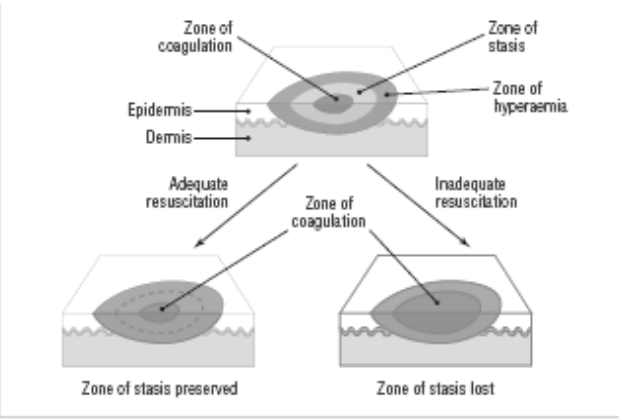


Figure 1.2 The local response to burn injury and the Jacksonian zones surrounding the burn injury site. Adapted from (Hettiaratchy and Dziewulski, 2004b)

The ***local responses*** are summarised in the figure 1.2 and result in tissue damage which can be of variable degree depending on the distance from the area of insult. Jackson in 1947 classified the area of damage into three zones, the zone of coagulation, the zone of stasis and the zone of hyperaemia. However burn results in damage to some or all the layers of skin and thus variable loss of function. The **systemic changes** involve a number of organ systems as summarised in the figure 3 below. The responses are more pronounced with the increase in the percentage of the total burn surface area (TBSA) and are seen when the TBSA approaches 30%(Hettiaratchy and Dziewulski, 2004b). These effects are mediated by the release of cytokines and inflammatory mediators IL-1,TNF-α, IL-6 etc which are elevated in the post burn scenario(Drost et al., 1993).

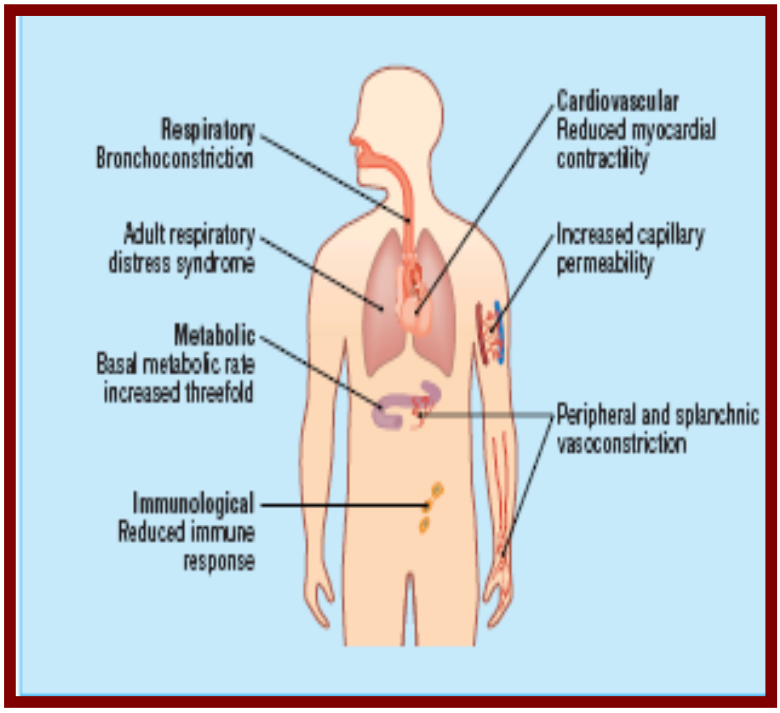


Figure 1.3 –The multiple systemic affects following a burn injury.(Adapted from (Hettiaratchy and Dziewulski, 2004b)

Cardiovascular changes and Respiratory Changes

The changes are reflected in the form of increased capillary permeability, resulting in the loss of intravascular proteins and fluids to the interstitium. This is associated with compensated splanchnic and peripheral vasoconstriction. There is also decreased cardiac contractility secondary to release of cytokines like Tumour necrosis alpha (TNF-α). Loss of fluid from the burn wound, from intravascular compartment and decreased contractility result in systemic hypotension and organ hypo-perfusion resulting in end organ failure.

The respiratory effects are in the form of broncho-constriction and in severe cases result in adult respiratory distress syndrome.

Metabolic changes

Burns result in a hypermetabolic state with the basal metabolic rate (BMR) increasing to twice the normal in burns of 40% TBSA. This increased metabolic rate with ongoing tissue regeneration and loss of intravascular proteins increases the metabolic demand and need for nutritional supplementation.

Immunological changes

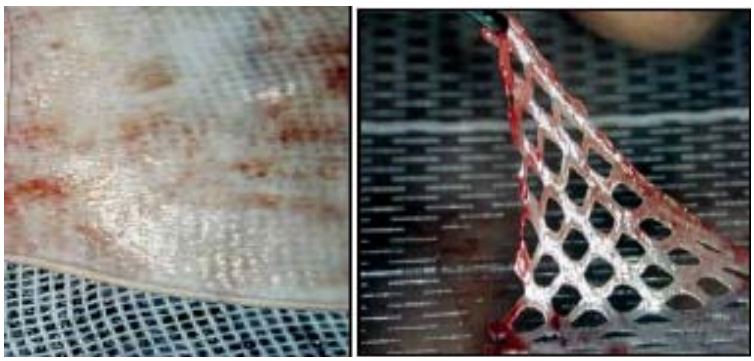
It is reported that there is a non –specific down- regulation of both adaptive and innate immunity following a major burn injury. There are alterations in the arachidonic acid pathway and the cytokine cascade. There have also been reports of neuro-endocrine axis dysregulation

and changes affecting all the cells of the immune system. Changes in the humoral immunity have also been reported(Munster, 1994).

Management of burn wounds

The management of major burns needs to be aggressive to achieve the best outcome. The ***systemic management*** of the patient should be according to the ATLS (acute trauma and life support) and EMSB (Emergency Management of Severe burns) guidelines. The patient should be adequately resuscitated and subsequently special attention should be given to the nutrition of the patient as burn is a hypermetabolic state.

Local management of a burn wound depends on the depth of the burn. It can be either conservative using a variety of dressings aimed at providing a sterile cover and an environment to promote healing; this mode of management however relies on the presence of adnexal structures in the burnt area from where re-epithealisation begins.



Unmeshed graft ***Meshed graft***

Figure 1.4 An illustration of unmeshed and meshed skin grafts

Surgical-Debridement and grafting

This is generally undertaken for deep dermal and full thickness burn injuries and is aimed at removing the dead tissues and at providing epithelial cover over the burn area. The epithelial cover can be provided in the form of partial thickness skin graft either meshed or unmeshed (figure 1.4) Systemic management comprises of adequate fluid resuscitation and supportive care in the form of nutritional and psychological support.

Complications associated with burn injuries

There can be a number of complications associated with burn injuries; the important ones are listed in Table 1.3. Some of these are directly related to the burn, others are related to the physiological changes associated with these types of injuries. The most common complication associated with burns is infection in the early stages and scarring in the delayed state.

Table 1.3- Complications of burn injuries.

Immediate /early	**Delayed**
Infection: local and /or systemic	Scarring and disfigurement Contractures/keloids/hypertrophic scars
Dehydration	
Hypothermia	
Shock:hypovolaemic and/or septic	
Multi-organ failure	

The current study addresses some of these complications in relation to anti-microbial peptides specifically β defensins.

1.3 Skin antimicrobial peptides

There have been tremendous advances in the management of burns in the last decade. However infection continues to be one of the most important causes of morbidity and mortality in burn patients. A variety of dressings have been developed aimed at providing the best mechanical cover over the burn wound. Controlled temperature, humidity and laminar air flow in burn wards and theatres provide an environment which helps prevent infection, and provide conditions aimed at compensating for the loss of mechanical cover provided by the skin. Replacing the skin by grafting is the most effective way of managing burn wounds; however this option is not suitable if the areas involved are large and it also requires availability of specialist skills.

Since loss of skin is common to all burns wounds, it is envisaged that the loss of mechanical and biological protection provided by the skin is partly responsible for this high incidence of infections. Although a variety of dressings have been developed which to some extent provide a good mechanical cover, the biological properties of the skin need to be addressed. The biological properties of the remnant epithelial cells in the burn wound vicinity, especially those in the zone of statsis, is unclear. In the current study we endeavoured to study the antimicrobial peptide expression in the burn wounds.

Antimicrobial peptides (AMP) constitute a group of polypeptides, each less than 100 amino acids (10-50 amino acids) in length, found in the body which have antimicrobial properties

under physiological conditions in the tissues of their origin(Ganz, 2003). Since the antimicrobial effects of these peptides are mediated directly and indirectly *in vivo* they are referred to also as host defence peptides (HDP)(Sahl et al., 2005). About a hundred antimicrobial peptides have been described to date, however cathelidins and defensins are the two most important members of this group.

1.3.1 Defensins

Defensins are a unique family of structured peptides found in vertebrates. They are characterised by their high cationic charge, β-sheet fold and cysteine rich residues interlinked by six disulphide bridges(Selsted et al., 1985). They are all synthesised as pre-propeptides and undergo further processing depending on the site of expression and the type of defensin(Selsted and Ouellette, 2005). The molecular weights of defensins are in the range of 3-5 kDa. Similar peptides containing 6-8 interlinked cysteine residues found in non-vertebrates and plants, and are also known as defensins. The family is divided into subclasses α (alpha) and β (β), based on differences in structure, the lengths of the peptide segments between the cysteine residues and the pairing of the residues(Hoover et al., 2000).

Recently a third group of θ- defensins has been described in primates, which are cyclical peptides, probably derived by fusion of α-defensin-like precursor peptides. These peptides are not found in humans as their synthesis is disrupted by the formation of premature stop codons(Trabi et al., 2001).

Functionally the defensins have a broad spectrum of antimicrobial activity against gram positive and gram-negative bacteria, fungi and some viruses(Huttner and Bevins, 1999). They

mediate the killing of the microbes by forming pores in their membranes(Raj and Dentino, 2002). Six different α and about 28 different β defensins (BD) have been described in humans so far, however only four β-defensins have been characterised.

1.3.2 α –Defensins

Skeleton Structure

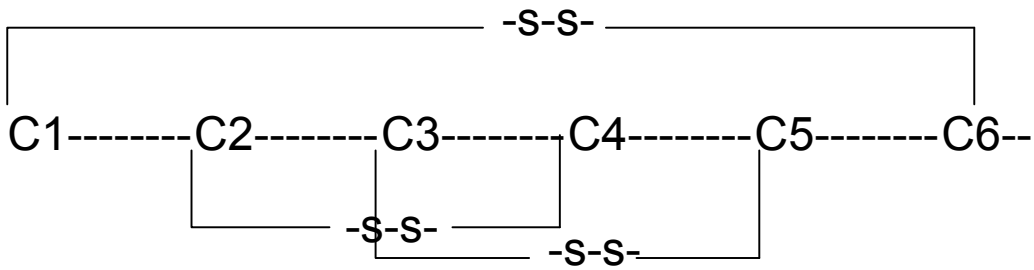


Figure 1.5. A representative skeleton structure of α –defensins;S-S represent the disulphide linkages to the carbons in the skeleton structure.

Characteristics

These are cationic peptides 29 –35 amino acids in length and differ from β defensins in terms of the position of the cysteine residues and the disulphide motifs(Raj and Dentino, 2002). The six cysteines residues are linked at positions 1-6, 2-4, 3-5. They have been further divided into subtypes HNP1-4, HD5 and HD6.

Genes and synthesis

The gene for α-defensin synthesis is located along with the *BD* gene cluster at chromosome 8p23. This gene cluster is associated with polymorphisms with the number of copies of defensin genes varying between individuals(Mars et al., 1995). They are synthesised in promyelocytes in the bone marrow as tripartite pre-pro-peptides containing an amino terminal signal sequence, an anionic pro-piece, and mature defensins at the carboxy terminal end(Daher et al., 1988, Valore and Ganz, 1992). Mature neutrophils contain large amounts of defensins but do not actually synthesise the peptide, the actual peptide is synthesised in the bone marrow when the neutrophils are in the state of pro-myelocytes(Ganz, 2003).

Distribution

α-Defensins have been found in the neutrophils of most mammalian species, in the intestinal paneth cells of humans and rodents(Risso, 2000) and the female genital tract of humans(Quayle et al., 1998). The concentrations of defensins in granules of leukocytes and the crypts of small intestine have been found to be >10mg/ml(Ganz et al., 1985, Selsted et al., 1983, Ayabe et al., 2000).

Spectrum of activity and mechanism of action

Alpha defensins have a wide spectrum of anti microbial activity *in vitro* against bacteria, fungi and enveloped viruses(Selsted et al., 1983, Ganz et al., 1985, Lehrer et al., 1985). Their mechanism of antimicrobial activity is considered to be electro- statically mediated and specific against prokaryotic cells(Weinberg et al., 1998, Ganz, 2003).

1.3.3 β-Defensins (BD)/Human B Defensins (HBD's)

1.3.4 Structures

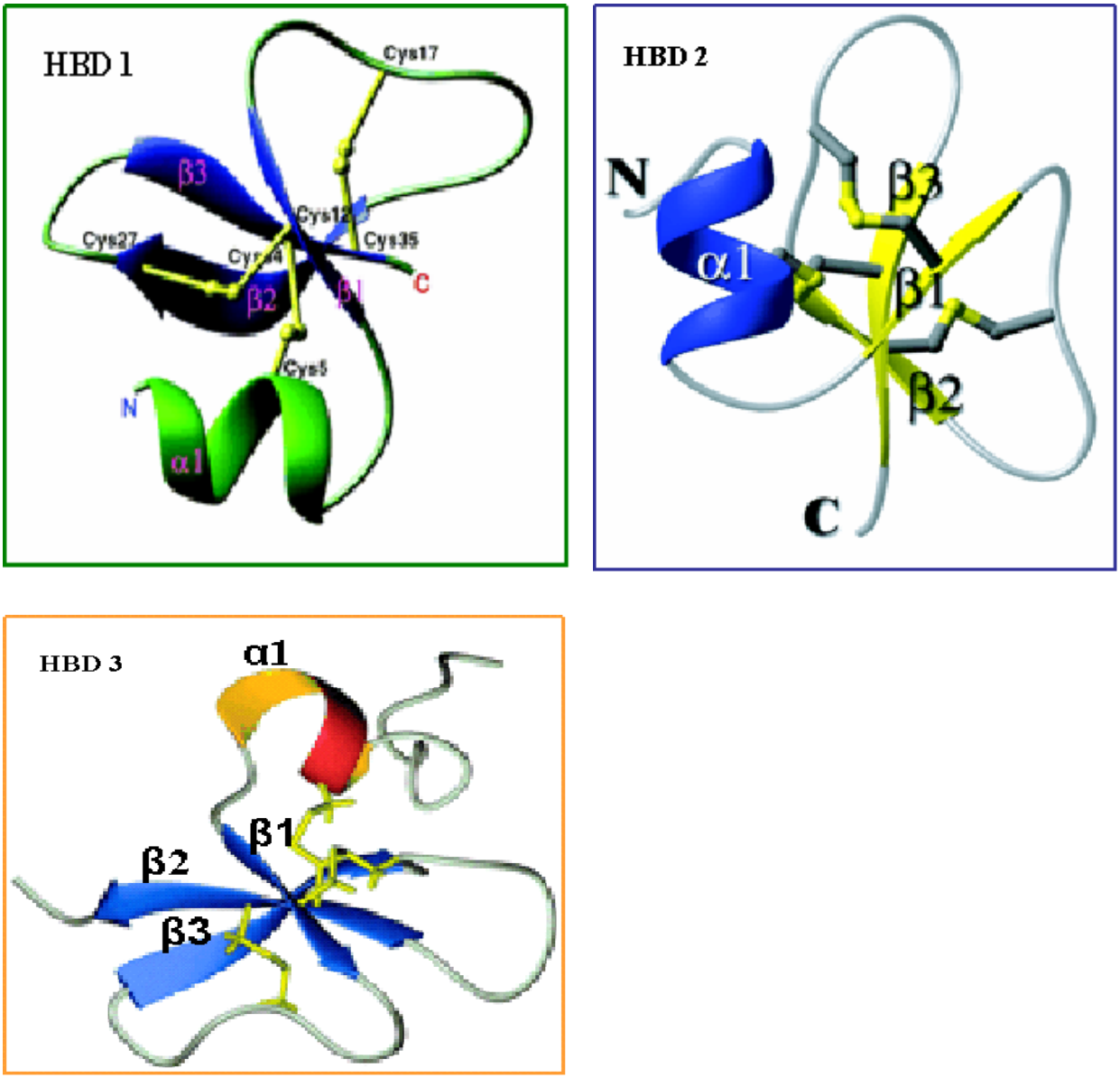


Figure 1.6 Three dimensional structures of β defensins- in approximately similar orientation, with three β sheet and three disulphide bonds (shown in yellow) (Adapted from(Hoover et al., 2002, Hoover et al., 2000, Schibli et al., 2002).

Skeleton structure

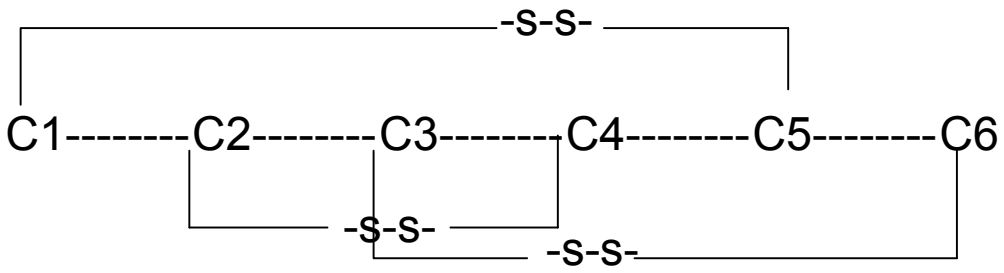


Figure1.7 Structure of human β-Defensins (HBD; S-S represent the disulphide linkages to the carbons in the skeleton structure.

Characteristics

Human B defensins (HBD) are characterised by the presence of cysteine residues linked in 1-5, 2-4 and 3-6 positions as seen in the figures 1.6 and 1.7 above. Despite the difference in the linkages of the cysteine residues between α and β-defensins, they a have common three-dimensional structure with the three disulphide bonds stabilising the three anti-parallel β strands. Though initially they were given different names, they were finally classified as β defensins based on their common properties.

Immunological properties, genes and synthesis

HBD has also been shown to stimulate the adaptive immune response and thus may be a link between the primitive innate immunity and the advanced adaptive immunity(Yang et al., 1999). *HBD* genes are located on 8p23. Further studies showed that the encoded peptides differed from each other in features such as sequence similarity, size of introns, site of expression and elements of genetic regulation, and hence were further subdivided. Four different varieties of BD, human β defensin 1 (HBD1), human β defensin 2 (HBD2), human β defensin 3 (HBD3)

and human β defensin 4 (HBD4), have been isolated and extensively studied, although a total 28 different types of β-defensins have been described.

Distribution

β- Defensins have been isolated from a variety of organisms ranging from plants to invertebrates, vertebrates and mammals, including man. In man they are expressed, by the epithelial cells of the respiratory tract, skin and the urogenital tract, in response to inflammatory mediators and the microbes. This response has been shown *in vitro* and *in vivo*.

Human β defensin 1 (HBD1)

The genito-urinary tract is the principal site of production of HBD1. However it has been reported to be produced by epithelial cells at a number of sites including skin, kidney, gut, oral cavity,lung(Zhao et al., 1996, Ali et al., 2001) and macrophages(Alp et al., 2005). The production of HBD1 is not influenced by inflammatory activity and the challenge with microbes although there are consensus sites for nuclear factor (NF) interleukin (IL)-6 and γ-interferon at the 5` flanking region of HBD1, suggesting that inflammation may mediate its expression(Valore et al., 1998). HBD1 shows antimicrobial activity more markedly against gram negative bacteria as compared to gram positive bacteria.

Human β defensin 2 (HBD2)

Human β defensin 2 was the first reported inducible antimicrobial peptide. It was first isolated from psoriatic scales(Harder et al., 1997). It was shown to be induced both *in vitro* and *in vivo* by a number of inflammatory mediators and bacteria(Liu et al., 2002a). HBD2 is active against gram-negative bacteria such as *Escherichia coli, Pseudomonas aeruginosa* and yeasts such as *Candida albicans* . It is also shown to be bacteriostatic at higher concentrations against *S. aureus*(Schroder and Harder, 1999).

Human β defensin 3 (HBD 3)

HBD 3 is another important inducible 45 amino acid long antimicrobial peptide first isolated from psoriatic scales. It is produced at various sites in the body(Jia et al., 2001) in response to bacterial challenge and TNF -alpha. HBD3 has shown a wide spectrum of antimicrobial activity against multi-drug resistant *S .aureus* and vancomycin resistant *Enterococcus faecium*. and also has chemo attractant properties(Harder et al., 2001).

Human β defensin 4 (HBD 4)

Recently HBD4 has also been identified and it has been found to be tissue specific. There are reports that it has been isolated from testis, uterus, thyroid gland, lung and kidney during infection(Garcia et al., 2001b). It is thought to influence monocyte migration. They have been shown to demonstrate the antimicrobial activity in genetically modified keratinocytes(Smiley et al., 2007).

1.4 Overview of the PhD

The current study was carried out to investigate the various aspects related to HBD expression in relation to conditions found in burn wounds. Five clinical studies were conducted in either burns patients or in non –burn patients with trauma similar to that found in burn patients.

Studies1 and 2- Chapter 3

Title: Human β-Defensin expression in burn wounds and correlation to bacteria and duration of burn wound.

The primary study was to analyse the expression of HBD 1, 2 & 3 in debrided burn wounds at different time points. Estimation of bacteria present, both qualitatively and quantitatively was also performed in these wounds and this was correlated to the HBD1, 2 & 3 expression at mRNA and protein levels

Study 3-Chapter 4

Title: Expression analysis of B –Defensins (HBD1, 2&3) in peripheral blood cells: comparison of profiles between septic (microbiologically positive and negative) and healthy individuals.

The aims of the study were to understand, the contribution of blood cells *in vivo* to HBD expression in a bacteria-challenged environment and the role of HBD in states of systemic sepsis

Study 4-Chapter 5

Title: Buccal HBD 1, 2and 3 expression profile in peri-operative states: correlation to cortisol levels

The aim of this part of the work was to understand the effect of stress on the expression of HBD in an *in vivo* environment similar to that experienced by a severely burnt patient.

Study 5 –Chapter 6

Title: The role of human β Defensins in the pathogenesis of keloids

In this study, the expression of HBD in keloids was analysed to determine whether HBD play a role in the pathogenesis of keloids.

Chapter 2

General Methodology

2.1 Collection of clinical samples

Collection of samples is the most important and the most difficult part of a clinical research project. It is technically demanding as it involves co-ordinating between the surgeons, theatres, patients and the laboratory. It is also important to ensure that the samples are collected in the optimised way for the kind of experiment being planned as collecting samples in an un-optimised manner can result in un-interpretable and void results; resulting in wastage of resources and time.

The samples for the current projects were collected after obtaining Ethics Committee approval and consent from individual patients (refer to Appendix).The details of the methods and the problems encountered during collections for individual experiments will be discussed in chapters related to specific experiments.

2.2 Immunohistochemistry

This is the technique of localisation of antigens in a tissue specimen using specific antibodies which are in turn visualised using different reporter technologies such as enzymatic reactions, fluorescent dyes and radioactive elements. The technique is based on the underlying principle that antibodies bind to specific antigens.

Antibodies

Antibodies can be polyclonal or monoclonal, the type being based on the clone of cells from which they are produced. As the name suggests, polyclonal antibodies are produced by multiple clones of cells and hence they are immunologically dissimilar and recognise different epitopes on the same antigen (figure 2.1A). Monoclonal antibodies on the other hand are

produced by a single clone of cells, are therefore immunologically similar and thus bind to the same epitope (figure 2.1B).

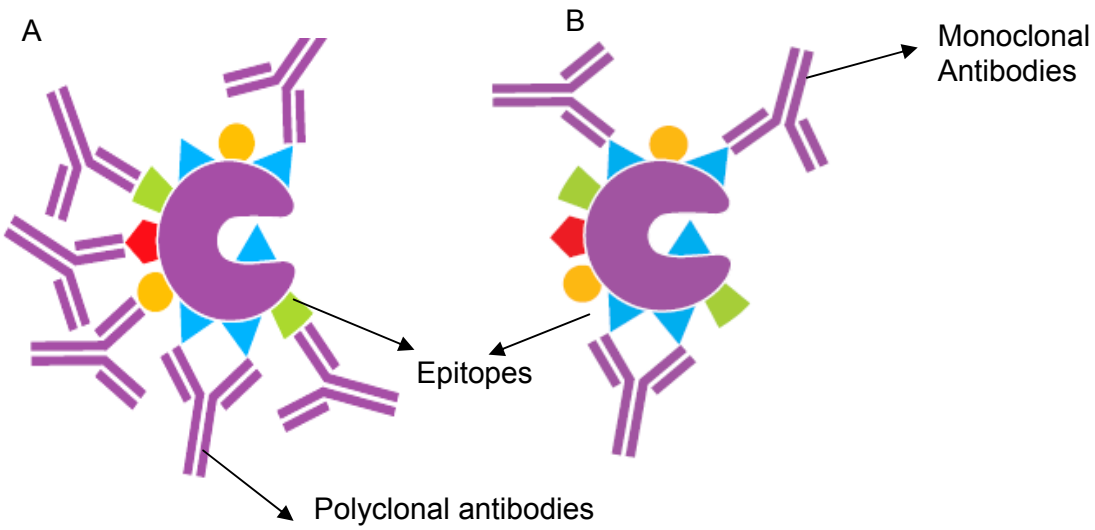


Figure 2.1 The principle of immunohistochemistry with polyclonal (part A) and monoclonal (part B) antibodies.

Availability of a specific pre-tested antibody is an important prerequisite for successful completion of immunohistochemical techniques in a short time. All the antibodies used were obtained commercially. Specific antibody search engines such as Abcam (http://www.abcam.com/) and Linscotts Directory of Immunological and Biological Reagents (http://www.abcam.com/, http://www.linscottsdirectory.com/) were used to locate the required antibodies. The searches revealed that there was no existing commercially available antibody specific to human β defensin 3. There were polyclonal antibodies available which detected human β defensin 1, 2 and 3 but since our study was looking at the differential expression of each of these peptides it was decided that using such non-specific antibodies would be futile. Further searches revealed that there were specific rabbit polyclonal antibodies to human β defensin 1and 2 but not to defensin 3. Professor Navsaria (Centre for cutaneous research)

generously agreed to provide these two antibodies. Since there was no antibody targeted to human β defensin 3, the closest possible commercially available alternative, the rabbit antibody to mouse β defensin 3 was purchased from Autogen Bioclear. Though there is more than 50% homology between mouse and human β defensin 3(Bourcier et al., 2004) there was no information available as to whether the anti mouse antibody could be used on human material. This antibody had not been previously tested in immunohistochemical techniques. So this antibody was used on human tissues using immunohistochemistry techniques as the first part of the project. Our results showed that the anti mouse β defensin 3 antibody showed specific binding to human tissues (figure 2.2).

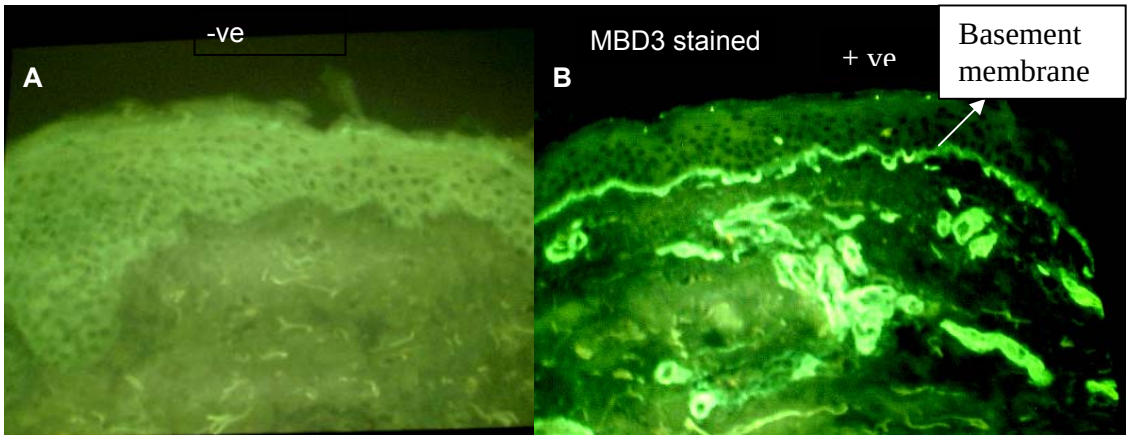


Figure 2.2 Representative skin sections stained MBD3 and visualised using FITC.

Part A of the figure shows the background staining and part B shows the fluorescence in the basement membrane and the lining of the glands.

However subsequently commercial antibodies were also marketed for Human β defensin 3 (HBD3) which were used for the later immunohistochemistry studies.

Tissue processing for embedding in paraffin blocks

Fixation and dehydration

Fixation is the process by which the cell contents are prevented from being lost as a result of storage or further chemical treatment. The mechanism of fixation varies, depending on the fixative used. A number of chemical fixatives can be used such as alcohol, acetone, glutaraldehyde, formalin as well as combined fixatives such as B5. The most common fixative, formalin, can be used in various concentrations (http://www.ihcworld.com/_books/Dako_Handbook.pdf). Formalin treatment results in fixation by covalently cross-linking proteins between α and ε amino groups. This results invariably in the alteration of the antigenicity which is especially significant when the tissue is being used for the detection of proteins. The degree of antigenic variation induced depends on the composition of the fixative, duration of fixation, concentration of the fixative and the size of the tissue specimen. In order to compensate for the change in the antigenic structure various antigen retrieval techniques are used which restore at least partially the antigenicity of the proteins. Some of the common antigen retrieval techniques are digestion with enzymes like pepsin or trypsin or incubation with citrate buffer in a microwave oven.

Graded dehydration, the taking of the tissue through various concentrations of alcohols and other organic solvents such as xylene or chloroform, allows the sample to be dehydrated.

Embedding

Embedding was carried on weekly basis using batches of tissue samples. 10% formal- PBS was used for fixation. Though ideally the tissues are better not left in formalin for more than 24 hours they were usually processed after fixation for periods ranging from 48-72 hours. An

account of time duration of fixation was made for each of the sample. During antibody staining, assessment was made to determine the effect of time on staining pattern. Each batch consisted of about 4 to 10 specimens, the number being dependent on the availability of patient tissues. The embedding process was done manually following a 3-day standard protocol as described in Carleton's Histological Techniques.

Tissue processing for frozen material

Cooling of the tissue usually results in some degree of fixation although chemical fixatives like methanol, ethanol or acetone can be used to supplement this. No other special measures are required apart from storage of the tissue at temperatures below –70^{o} C for long-term storage.

Cutting of tissue sections

Paraffin blocks

Sections of 5-micron thickness were cut from paraffin blocks using a standard microtome (Medcon microtome, USA). The technique of taking the section from the stage of the microtome to the time the section is set on the slide varies between laboratories. The techniques followed in our laboratory along with practical considerations are summarised. Before starting the procedure it was ensured that a water bath set at 52^{o} C (a little below the melting point of wax) was available. It was found that the sections were easier to cut if they were left in a freezer for a short time before being cut; hence they were left at - 20^{o} C for half an hour before use. The tissue sections stuck to each other forming long chains. Chains of 4-6 sections were the best to handle and they were separated using pointed forceps after being floated on a large slide of alcohol. Separating sections directly in the water bath was more

successful if a small nick was made in the edge of the block, from where the separation can be initiated.

All the sections were orientated in the same direction while transferring them onto the slide; this was useful when visualising the slide under the microscope since changing the field for each slide was not required. Once the section was mounted on the slide it was left overnight in an incubator maintained at 37^{o}C .The slides were then ready for use.

Cutting cryo-sections

Sections of 5-micron thickness were cut using a standard cryostat (Bright Instrument Co Ltd, England). While mounting the tissues on the cryostat, the long axis of the tissue was orientated perpendicular to the blade. This made it easier to take the tissue on the slide. The temperatures of the cryostat were maintained between –20 to –30^{o}C during the entire process and the tissue samples were taken out in turns from the –70 ^{o}C freezer. The slides were then appropriately labelled and stored in plastic bags at -70 ^{o} C until required.

Immunostaining 1

Two step indirect immunofluorescence was chosen owing to its 10 fold higher sensitivity as compared to the direct immunoflourescence techniques. Since the anti mouse β defensin 3 had not been previously tested in immunohistochemistry, it was tested on mouse sections and human tissues. Since there was no information available from the manufacturer regarding the dilution to be used and the duration of staining, a protocol was devised. The use of the antibody on paraffin embedded tissues required pre-treatment to facilitate the retrieval of the epitopes, which might have been altered during the processing of the tissue. This gives scope for variation in the results depending on incomplete antigen retrieval or the antibody not being

effective on human tissue. Since there was no need for any pre-treatment in case of frozen tissue, the antibody was first tested on frozen tissue.

Immunostaining-2

Antibodies for HBD3 became commercially available and it was therefore decided to use these antibodies along with those to HBD1 and HBD2 for estimation of protein expression of BD. To avoid the problems posed by the immunofluorescence such as loss of fluorescence and the costs involved, colorimetric detection of the bound antibodies was used. The steps involved in the immunohistochemical analysis with antibodies(Taylor CR, 1994) are described below. A two step indirect method was used (figure 2.3). Dewaxing involved removing wax from the section and rehydrating the tissues for further staining. The slides were taken through a series of solutions as shown (table 2.1).

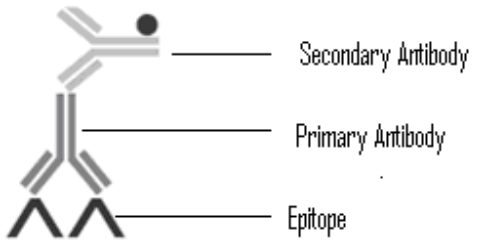


Figure 2.3 principle of Indirect immunoflourescence.

The primary antibody binds to the epitope and the secondary antibody, which is linked to the fluorescent or enzyme linked substrate, binds to the primary antibody. The presence of specific epitopes causes binding of the primary and secondary antibodies, resulting in activation of the substrate or fluorescent tag, leading to detection.

Table 2.1 Summary of the steps of the rehydration of paraffin sections

Purpose	Solution	Duration	No of cycles
De-paraffinising	Xylene	5 minutes	2
Graded rehydration	Absolute alcohol	3 minutes	2
Graded rehydration	90% alcohol	3 minutes	1
Graded rehydration	70% alcohol	3 minutes	1
Rehydration	Distilled water	Until further use	1

Antigen retrieval

No antigen retrieval was required for immunohistochemistry using β defensin antibodies since it was demonstrated in previous experiments that similar results were obtained with or without antigen retrieval (data not shown).

Blocking endogenous peroxidase activity

The presence of endogenous peroxidase activity in cells or tissues to be stained using horse radish peroxidase labelled antibodies (either primary or secondary) can result in non-specific background staining. To counteract this endogenous peroxidase activity, the slides were pre-treated with saturating amounts of hydrogen peroxide. This resulted in irreversible inactivation of endogenous peroxidase; and thereby reduction of the degree of background staining.

The slides were immersed in a solution of freshly prepared 3% (v/v) ice cold hydrogen peroxide in methanol for 10 minutes. The slides were then immersed in PBS and kept in PBS until further use. The sections were treated (blocked) with normal serum derived from the host species from which the secondary antibody was derived; the serum contains antibodies, which bind to undesirable antigens to which the secondary antibody can potentially bind and give a non-specific result. For mouse and rabbit antibodies blocking was done using horse serum and rabbit serum was used for goat antibodies. Freshly thawed serum was added over the smear, keeping the slides in a moist chamber to cover the smear. This was left to stand for 30 minutes before the serum was removed from the slide. No washing was done after this step as the serum may have been washed off.

Primary antibody binding

Primary antibody specific to the antigen (protein) under question, i.e. HBD1, HBD2, HBD3 was added respectively to each of the slides being stained for each of the antigens detected. The optimum dilution was found using a trial run with different dilutions. The optimum dilutions for each of the antibody were as follows (table 2.2);

Table 2.2 –The optimised dilutions of the three HBD antibodies.

Antibody type	**Dilution**
HBD1	1:800
HBD2	1:500
HBD3	1:300

Circles were made with a wax pen around the tissue sections to prevent the flowing of the antibody into neighbouring sections. The slides were then left in a cooling chamber at 4ºC overnight for incubation with the primary antibody for 18 hours.

Secondary antibody stain

The following day the secondary antibody was added. For HBD1 and HBD2 anti goat antibody (Vector laboratories Ltd, UK) was used and for HBD3 Universal vectastatin ABC kit (Vector laboratories Ltd, UK) was used. The slides were stained with secondary antibody after two PBS washes each lasting 10 minutes. Secondary antibody was incubated for 30 minutes at room temperature.

Tertiary antibody and colour development with DAB (Diamino benzidine dye complex)

Two washes of PBS each lasting for 10 minutes were undertaken before adding the tertiary antibody. Tertiary antibody was prepared as per the manufacturers instructions and 30µl were added for each section. The slides were incubated for 30 minutes at room temperature. DAB complex was freshly prepared as per manufacturers instructions and added to the slides. Slides were visualised under the microscope and once optimum colour development was observed they were washed with PBS. The duration of colour development allowed for each of the antibodies at the concentrations above was as follows for the staining of tissue sections:

HBD1-1 minute 30 seconds

HBD2 –4 minute 15 seconds

HBD3- 1 minute 30 seconds

Counterstaining with haematoxylin

The slides were immersed in eosin for a brief period of about 30 seconds, followed by immersion in haematoxylin for 1 minute.

Dehydrating and mounting

The slides were taken through a series of graded alcohols and xylene to dehydrate the sections, mounted using petroleum based jelly and cover slips and, after allowing them to dry, visualised under the microscope.

Grading-stoichiometric analysis

A colleague in the laboratory not associated with current experiment blindly scored slides on a scale of 0-4 (least to most staining).

Immunocytochemistry

This involves the same principle as immunohistochemistry; however the term is applied to the technique used on cells rather than tissues and was thus aimed at *in situ* localisation of proteins within cells. Details of the method of collection of different types of cells will be given in relevant chapters.

Fixation of cells

The cells were fixed using 4% para formaldehyde or 10% acetone-alcohol depending on the availability of fixative and nature of the cells.

Preparation of slides

Slides were prepared using a cytospin centrifuge (Shandon, UK). Each cell suspension was concentrated by spinning in a centrifuge at 1000 rpm for 10 minutes. The cell pellet was re-suspended in 1ml of fixative. Seventy µl of this concentrated suspension was used in the preparation of each slide. Between 10 and 12 slides were prepared from each patient sample. The slides were then air dried and stored in racks until required for further staining.

Staining of slides

Haematoxylin and Eosin staining was carried out for each of the slides to understand the morphology before proceeding to immunohistochemical staining with antibodies.

Analysis of stained slides

The slides were analysed under the microscope at different magnifications and staining and comparison drawn between different samples and controls.

2.3 Extraction of RNA

This was done using the choloroform-isopropanol method, RNAzol B or SVtotal RNA isolation kit (Promega corp. UK).

Choloroform-Isopropanol method

Each frozen tissue specimen was cut into 5µ sections using a cryotome and then the tissue was transferred to a glass homogeniser (Jencons England, UK) and 1ml of RNAzol (Cinna/Biotex lab Inc, UK) was added to it. The tissue was carefully homogenised until no intact tissue was visible. The contents of the homogeniser were then transferred to a sterile,

freshly autoclaved eppendorf tube. To this 80µl of chloroform was added and the contents were mixed. The tube was centrifuged at 5000rpm for 10 minutes. The upper layer was then collected in a fresh autoclaved eppendorf tube containing 1 ml of isopropanol .The contents were mixed well and left at –20 ^{o} C for 1 hour. The contents were subsequently spun at 10,000rpm for 10 minutes, keeping the tubes in a known orientation.

The supernatant was discarded and the pellet dried. The pellet was then redissolved in 20µl of nuclease free water. 10µl of this was added to 2µl of loading dye, which was run on a 1% agarose gel in 1x TBE for 1 hour, stained with ethidium bromide (0.05µg/ml) (Sigma, UK) and visualised under UV light to determine the integrity of the RNA.

***RNAzol ^{TM} B Method*:**

The RNAzol method was used to extract total RNA from isolated cells-leukocytes using RNAzol B reagent (Biogene,UK) which is a monophase solution containing phenol and guanidinium thiocyanate(Chomczynski and Sacchi, 1987).However the same method can be used for isolation of RNA from other cell types with small modifications. The following description is for the isolation of RNA from leukocytes. The suspended leukocytes in the eppendorf tubes were spun in a micro-centrifuge for 10 minutes at 1000 rpm. Supernatants were discarded and the pellets, which contained leukocytes, were used for total RNA isolation.

For each sample of 1 X 10^6 cells/ml, 1ml of RNAzol reagent was added. After leukocytes were counted, approximately 1ml of RNAzol was added to each leukocyte pellet resulting from 700µl of leukocyte suspension. Suspended leukocytes in RNAzol were stored at – 70° C and were

processed further in small batches. Since two pellets were obtained in two different eppendorf tubes from each blood sample for RNA isolation, one of them was re-suspended and stored with RNAzol at –70° C to be used as a back-up in case of error occurring during RNA extraction procedures. The other pellet was used for RNA isolation.

After RNAzol had been added, 80μl of chloroform was added per 1ml of RNAzol reagent and the mixture was mixed vigorously by vortexing for 1 – 2 minutes. The mixture was stored at –70° C for 10 minutes to facilitate the lysis of leukocytes in order to release the RNA from them. Centrifugation of the chloroform RNAzol mixture was performed at 10,000 rpm for 20 minutes. As a result of the centrifugation, the sample formed the lower blue phenol-chloroform phase, the interface which contained DNA and proteins, and the upper colourless aqueous phase which exclusively contained total leukocyte RNA. The upper aqueous phase was transferred to a fresh autoclaved eppendorf tube and 1ml of iso-propanol was added in order to precipitate the RNA from the aqueous phase.

The sample was stored at –70° C for 10 minutes to facilitate the precipitation followed by centrifugation of the sample at 10,000 rpm for 10 minutes. The RNA precipitate formed a white pellet at the bottom of the eppendorf tube. The supernatant was removed and the pellet was dried for 30 minutes and re-suspended in 20 μl of diethyl pyrocarbonate (DECP) treated water (RNase free water) and stored at –70° C to minimise RNA degradation until the subsequent steps. The above steps are summarised in the figure 2.4.

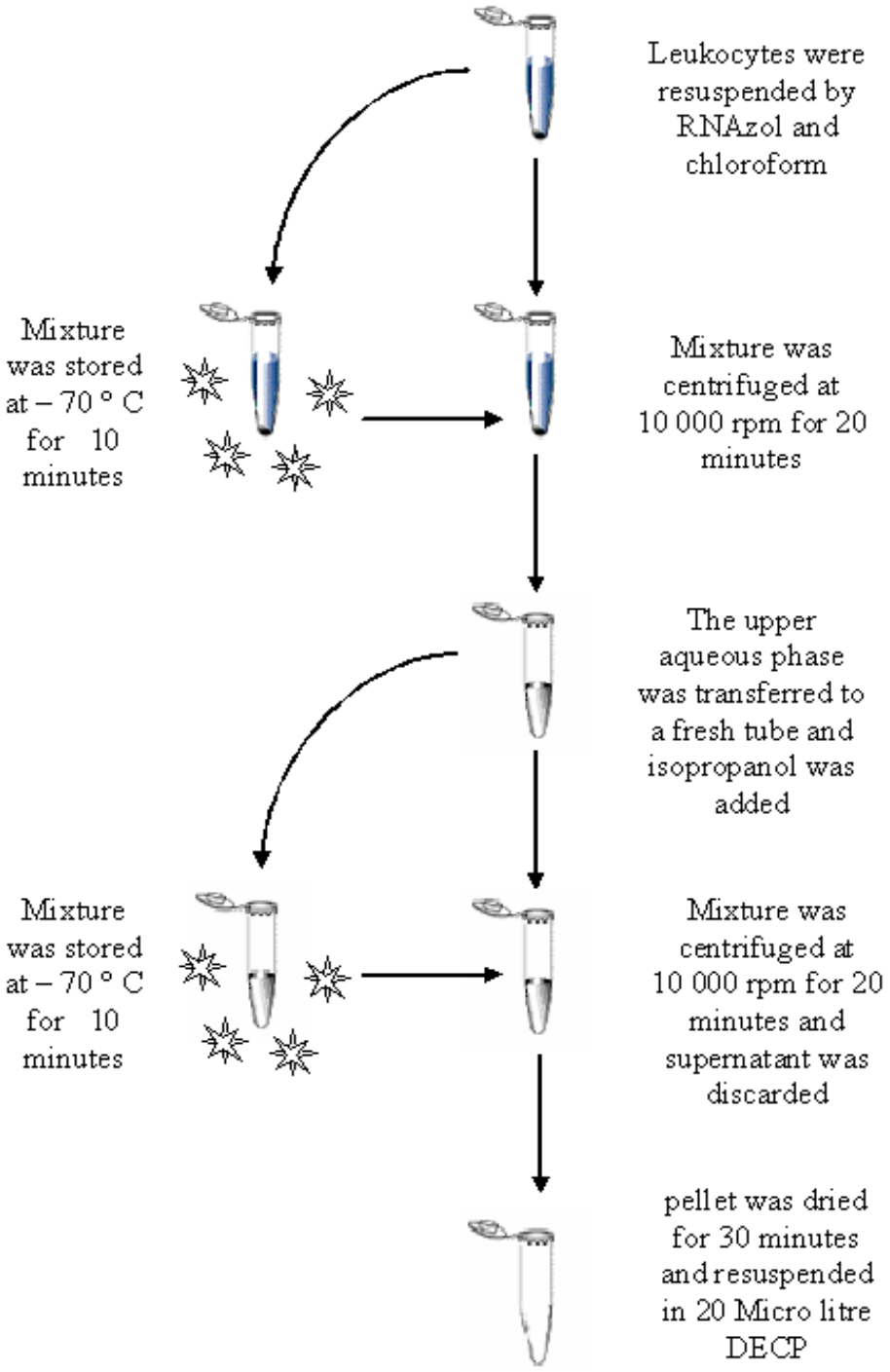


Figure 2.4: Schematic representation of the total RNA isolation from peripheral blood leukocytes by RNAzol B method.

The purity and integrity of the RNA is critical to the success of any RNA based analysis. In this study, the quantity and the quality of the extracted RNA from each sample were determined by means of spectrophotometry and gel electrophoresis.

Extraction of RNA by SV Total RNA Isolation system ^{TM} (Promega; Z3100,UK)

All standard precautions were taken to ensure the environment was free of RNAses and that all instruments and the work bench were pretreated with RNAZap (Promega;UK). The frozen tissue was put in a sterile porcelain bowl and was ground to fine powder with liquid nitrogen added to the bowl. The powdered tissue material, after allowing the liquid nitrogen to evaporate, was then transferred to a pre-weighed eppendorf tube according to the manufacturer's protocol(http://www.promega.com/tbs/tm048/tm048.pdf). Lysis buffer was added, 175µl per 30mg tissue. The contents of the eppendorf tube were homogenised using glass homogenisers. 175µl of lysate was retained for further purification and any excess was frozen at –70 °C. Subsequent steps were as per manufacturers protocol, and are summarised in figure 2.5.

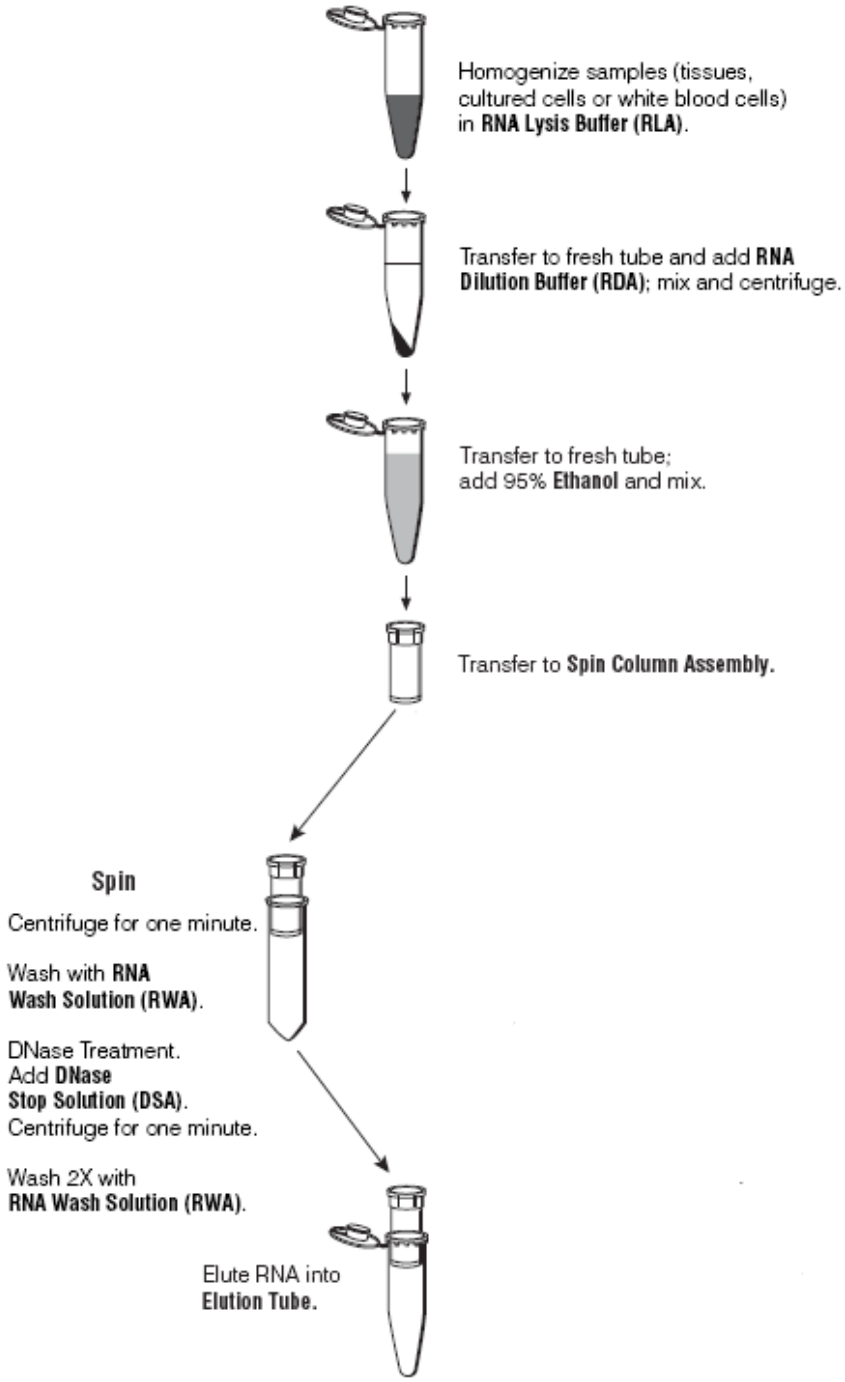


Figure 2.5: Diagramatic illustration of the steps involved in the isolation of RNA using the SV total RNA isolation system.

To 175µl of lysate, 350µl of dilution buffer was added and the contents mixed by inversion. This was then placed for 3 minutes in a heat block maintained at 70°C; the tubes were then centrifuged for 10 minutes at 13,000 rpm. Lysate was then transferred to the spin column assembly (supplied by the manufacturer) without disturbing the pellet. To the column, 200µl of 95% ethanol was added; after mixing gently for 3-4 times, the contents were spun at 13,000 rpm for 1 minute. To the column 600µl of SV RNA wash solution was added after discarding the liquid in the collection tube. This was again centrifuged at 130,000rpm for 1 minute. After emptying the collection tube, 50µl of freshly prepared DNase incubation mix was added to spin column assembly. DNase incubation mix was prepared according to the manufacturer's protocol by mixing 40µl of core buffer, 5µl of 0.09M $MnCl_2$ and 5µl of DNase 1 enzyme mix. After adding the incubation mix, the contents of the spin column assembly were incubated for 15 minutes at 25°C; following this 200µl of SV DNase stop solution was added and the spin column assembly spun at 13,000 rpm for 1 minute. Subsequently, 600µl of SV RNA wash solution was added and a further spin of 13,000 rpm given for 1minute. After emptying the collection tube and adding 250µl of SV RNA wash solution, the assembly was spun at 13,000rpm for 2 minutes. The spin basket was transferred from the collection tube to elution tube and the new assembly spun at 13,000rpm for 1 min after adding 100µl of water. The RNA, which was collected in the elution tube, was assessed by spectrophotometric analysis for purity and stored at –70°C until further processing.

Quantifying RNA

Measuring the optical density (OD) at 260nm and 280nm wavelengths in a spectrophotometer determined purity and concentration of the extracted RNA in the sample. 1OD unit measured

at 260nm (A_{260}) corresponds to 40μg of RNA per 1ml whereas the value of OD measured at 280nm (A_{280}) corresponds to the amount of the protein in the sample. The ratio of A_{260} / A_{280} is indicative of the purity of the sample which in the case of RNA should be between 1.6-1.9. The procedure was achieved by taking 2μl of the resuspended RNA in DEPC treated water and diluting with 98μl of sterile distilled water. The amount of RNA per 1μl sample was calculated as follows:

$[RNA]_{\mu g/ml}$ = A_{260} X 40μg/ml X Dilution Factor 50 (1:50 = 2μl RNA to 98μl sterilised distilled water)

For example one of the samples has A_{260} = 2.4230, the amount of the RNA in 1μl of this sample is:

$[RNA]_{\mu g/ml}$ = 2.4230 X 40μg / ml X 50 = 4847.0μg/ml = 4.8470μg/μl.

For extractions where it was anticipated that the RNA quantity was expected to be low, the dilution factor was decreased to 1 in 20 or at times neat RNA was also used.

RNA Quality

Quantifying RNA by spectrophotometry does not indicate the degree of RNA degradation in the sample, so that the assessment of RNA quality is an important step to be determined prior the conversion of RNA into cDNA.

The quality of RNA was analysed by using gel electrophoresis (3-4 % agarose gel in 1x TBE) and ethidium bromide staining. Two sharp bands should appear on the gel which represents 28S and 18S ribosomal RNA bands with a streak of mRNA from 200-2000 bp that in turn reflects the quality of the extracted RNA. To be visualised using ethidium bromide, at least 200ng of RNA must be loaded onto gel. However other more sensitive nucleic acid stains such

as SYBR® gold or SYBR® green (Invitrogen; Molecular probes, UK) may be used which enable detection of 1-2ng of RNA. For the current set of experiments only ethidium bromide was used owing to limited resources. In some instances where the quantity of RNA was expected to be low, although the ribosomal RNA bands were not obtained, the conversion of RNA into cDNA was continued and PCR products were visualised and analysed.

2.4 cDNA conversion

Reverse transcription was performed using ImProm II Reverse Transcriptase (RT) kit (Promega corp UK). The method was based on the manufacturer's two-step RT protocol. After checking the optical density, the initial template was taken such that 1µg of RNA was present in each 20µl reaction. Experiments were carried out using both oligo dT and random hexamer primers. The best results were obtained using a combination of both primers. After confirming that the experiments were working, 40µl reactions were carried out to ensure enough cDNA was available for further PCR reactions from the same conversion. This was done to avoid variations due to inter-experimental error.

Steps:

Template-	1-4µl (based on the optical density)
Oligo dT primers	0.5µl(1 µM final concentration)
Random hexamers	0.5µl (1 µM final concentration)
Nuclease free H_20	0-3µl
Total volume	5µl

The reaction tubes were transferred to a pre-heated heat block at 70 ºC for 5 min and were then kept on ice ice 5 min. The tubes were then centrifuged at 13 000 rpm for 10 sec and then transferred to ice, until the reaction mix was added.

Reaction Mix (Per 5µl reaction)

5× Reaction mix	4µl
25(mM) $MgCl_2$	4µl
dNTP mix(0.5mM)	1µl
Reverse Transcriptase	1µl
Nuclease free H_20	5µl
Total volume	15µl

The mixture was incubated at 25 ºC for 5 min, then 42 º C for 1 hour, and finally at 70 ºC for 15 minutes. The cDNA thus obtained was stored at –70 ºC until required for further use.

2.5 Polymerase chain reaction (PCR)

Reverse transcriptase polymerase chain reaction (RT–PCR) is one of the commonly used techniques for the quantification of transcription. There are other methods such as *in situ* hybridisation, Northern blotting and RNAse protection assays which can be used for mRNA quantification. However, PCR is the most sensitive of the available assays.

PCR optimisation

PCR was carried out initially on a Perkin Elmer thermal cycler to optimise the conditions of PCR for the burn samples. A range of combinations of annealing temperatures, denaturing temperature, duration and number of cycles were trialled. The best results were obtained at the following conditions:

Initial Denaturation	94 ºC	2 min	
Denaturation	94 ºC	30 sec	
Annealing	60 ºC	1 min	--- 40 Cycles
Extension	68 ºC	2 min	
Final Extension	68 ºC	7 min	
Soak	4 ºC	to end	

All the experiments included a negative and positive control. The negative control was water as template and a negative control for cDNA conversion was also included. The amplicons were run on a 3% agarose gel in 1xTBE and the size of the bands estimated by comparison with 1 Kb standard marker.

Real time PCR

Having confirmed that a single product was obtained and conditions for PCR had been optimised, the expression of the *defensin* genes was assessed quantitatively in real time using SYBR green on an Opticon QT-PCR machine (GRI systems, UK). Real time or quantitative PCR has the advantage of allowing quantitation as the amount of product is calculated by the system after each cycle. The conditions of PCR had to be re-optimised for real time analysis but best results were obtained at annealing temperatures of 60ºC and 40-cycle reaction, as above. Quantitative PCR was carried out using Dynamo SYBR green qt PCR kit (Finnzymes F-400L, UK). The reactions were undertaken following the manufacturers protocol.

For experiments in which the quantity of RNA was low, a further modification of PCR, Duplex PCR, was performed.

Duplex PCR

This involves amplifying two genes simultaneously in one reaction and helps in conserving the limited resources. This was done using Qiagen Multiplex PCR no ROX kit (Qiagen; UK). Each duplex reaction was composed of the housekeeping gene *β actin* and the gene of interest i.e. *HBD1, HBD2* or *HBD3.*

Primer design

The primer design is one of the key factors influencing the specificity and sensitivity of the duplex PCR. The primers require to have similar amplification efficiencies and should not form primer dimmers. This can be achieved by designing primers with similar annealing temperatures, GC content between 35-60% and by choosing primers which lack homology within themselves or with one another. These factors were considered and the primers were designed using the software on the Qiagen Website (https://customassays.qiagen.com/design/inputsequences.asp). Preliminary experiments to optimise the duplex assay were performed, which included comparison of primer efficiencies and optimisation of the annealing temperatures. Subsequently the individual experiments were carried out in triplicate and the mean of the Ct values (the point at which the amplification curve crosses a threshold of amplification) was taken to analyse the results.

2.6 Microbial estimation

Microbial estimation was done on a day-to-day basis. The sample was transported as described above taking all necessary aseptic precautions. 1µl and 10µl of the sample that had been transported in PBS were plated on day 1 on blood agar, Neomycin blood agar and Mac Conkey plates, and incubated at 37 ^{o} C under aerobic and anaerobic conditions. The plates incubated under aerobic conditions were observed after 24 hours and those incubated under anaerobic conditions were observed after 48 hours. On day 2 a further 1µl or 10µl were plated depending on whether or not there was growth or no growth on plates from day 1. These plates were used to further analyse the growth in terms of counts and types of bacteria and also to obtain distinct colonies so that further tests could be done.

The aliquots of the tissue transported in Robertson's cooked meat medium (RCM) was also plated in a similar way. Robertson's cooked meat medium served for enrichment of organisms present in small numbers, as transport medium for the anaerobes, and also to dilute the effects of any antibiotics that might have been used. The containers used were weighed before and after tissue addition to ascertain the weight of the tissue within. The colonies were counted manually if they were distinct, otherwise they were approximated as shown in the diagram, after being plated by streak method of serial dilution (figure 2.6). (http://www.microbiologyprocedure.com/microbiological-methods/streak-plate-method.htm)

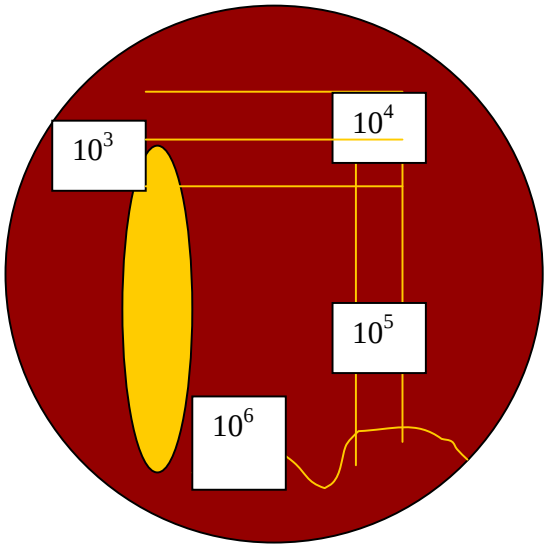


***Figure 2.6**. An illustration showing the approximation of bacterial counts on an agar plate*

The bacterial count of each of the plates was recorded. The counts from day 1 PBS plates were regarded as most significant unless the counts on day 1 from 1 µl RCM plates were drastically different, when the average of the two counts was taken. Gram staining was performed on all colonies taking care that a distinct colony was picked up.

Preparation of a bacterial smear on the slide

A new slide was taken, cleaned with alcohol and labelled to identify from which plate the colony was taken. 1µl of sterile PBS was taken with a sterile loop and transferred to the slide. Taking a sterile loop, a small aliquot of a distinct colony was transferred to the PBS on the slide. The colony was mixed gently to form an even smear and then the slide was transferred to a hot plate to allow the smear to dry. Once the smear was adequately dry it was removed

from the hotplate and stored for future use. The slide could be stored normally in any rack and no special conditions were required.

Gram Staining

Bacteria can be classified as gram positive or gram negative based on the properties of the cell wall that affects whether the bacteria retain the primary stain, or lose it and take up the secondary stain. In gram negative bacteria, due to the permeability of the cell wall, alcohol washes off the primary stain whereas in gram positive bacteria the stain is retained.

Crystal violet was used as the primary stain, which was left on the slide for a minute. The slide was then washed with distilled water and the mordant Lugols iodine (QCM Laboratories, UK) was applied for a minute. This was followed by a wash with distilled water. Decolourising was achieved using 3% acid alcohol (QCM Laboratories, UK) and lasted no more than 30 seconds. Safranin (QCM Laboratories,UK) was used as counter stain and the step lasted for a minute (http://www.ganfyd.org/index.php?title=Gram_stain). After this the slides were washed with distilled water to remove the excess stain and after being dried the slides were visualised under oil immersion. The physical characteristics of the colonies such as shape, colour, haemolysis, mucuoid nature, spreading pattern and any characteristic smell were noted. After this the bacteria were characterised using confirmatory tests such as catalase, coagulase and oxidase activity as needed (http://www.ganfyd.org/index.php?title=Gram_stain, http://www.splammo.net/JLbactsite.html).

2.7 Cell Culture

mRNA and protein extracted from cultured cells were used to optimise the conditions for real time rt-PCR and immunohistochemistry. This was done to ensure that invaluable patient material is not lost in optimising the techniques. Psoriatic cell lines provided the positive control and the specificities of the commercially available antibodies was determined using western blot, on the protein extracted from these cell lines.

Keratinocytes are the principal cellular components of the epidermis while fibroblasts constitute the principal cellular type in the dermis. Experiments were carried out on the primary fibroblasts derived from normal skin, keratinocyte and psoriatic cell lines.

Keratinocyte cell lines rTS31b, which are immortalized squamous cell carcinoma cells lines and nTERT, which are keratinocytes immortalised by fusion with telomerase positive cells were provided by Professor Navsaria (Centre for cutaneous research). Psoriatic cell lines – PSA 1 and PSA 2, were originally developed at the Institute of Cell and Molecular Science (ICMS) by Professor Navsaria's group. These cell lines were produced from the lesional psoriatic scale cells after fusion with telomerase positive cells.

Cells were cultured for at least two passages and enough stocks were produced before starting the experiments to ensure availability of similar passage stock cells for all the experiments.

The keratinocyte cell lines were grown in E4+ F12 medium with 10% foetal calf serum; E4 + F12 is a mixture of 3 parts DMEM (Dulbeco's modified Eagles medium) and 1 part Hams medium and supplied pre-made by the Cancer Research UK at the ICMS.

The E4+F12 medium was supplied in 400ml bottles and the final medium was prepared according to the following protocol.

Protocol for preparation of the medium

To 400ml of E4 +T12 (usually stored at 4ºC in cold room) 4 ml of glutamine (1% final concentration of the 100x stock) and 40 ml of freshly thawed foetal calf serum (FCS) were added and the solution was mixed well. The solution was sterilised using a sterile vacuum filter and then stored at 4ºC. For primary fibroblasts and keratinocyte culture an additional supplement 4 ml of RM+ (Appendix) was added to the media.

Protocols for plating the cryopreserved cells

All the work was carried out in sterile environment inside a Class II tissue culture cabinet, using sterile disposable pipettes. The cabinet was cleaned each time with either 95% alcohol or other cleaning solutions depending on the protocol of the laboratory.

The media was prepared and warmed to 37 °C in a water bath. About 20 ml of warm medium was transferred into a 50ml falcon tube. The vial containing frozen cells was quickly thawed in a water bath at 37°C as rapid thawing was considered to give better yields of cells than gradual thawing, as cells spend less time in DMSO environment. Better results were obtained by gently shaking the tube than by pipetting, which can kill the cells. The cells were centrifuged at 1000rpm for 5 minutes; the supernatant pipetted out, ensuring the pellet was not disturbed, leaving about 0.5ml of medium along with cell pellet.

The cells could have been washed once again with the medium as described above; however it was advisable to seed the cells at this stage as more cells were lost in re-suspending and

spinning the pellet. The pellet was resuspended in 2 ml of warm medium and transferred into a 75ml flask. The flask was labelled with the name of the cells, passage number, user name and date seeded. 20ml of warm medium was added before transferring the cells to the flask. The cells were checked under the microscope at low power (5 x magnifications) to ensure presence of cells, which at this stage would still be floating. The flask was placed in an incubator at 37 °C with 10% CO_2 and cells were checked the following day to ensure the cells had attached and there was no bacterial contamination.

The medium was initially changed once every 5 days and once the cells were about 50% confluent, the media was changed every 2^{nd} or 3^{rd}. If cells were dying, or there was suspicion of contamination then the medium was changed earlier. The cells were split at about 70-80% confluence. It has been observed that when cells are allowed to grow in more abundance they undergo phenotypic changes and may also undergo mutations. Such changes can give rise to varied results especially when different experiments are planned from this cell stock.

Tissue culture -protocol for sub-culturing (splitting) the keratinocytes

The normal splitting ratio was 1:5 but this was varied as required. The media was aspirated and 5ml of versene (an aqueous solution of EDTA a chelating agent; GIBCO-Versene, Invitrogen UK) was added to each 75ml flask and incubated at 37°C for 10 minutes. The versene was aspirated and the cells were washed twice with 10ml of warm PBSA each time. 10ml of PBSA was then added and flasks were incubated at 37°C for 20min. The PBSA was aspirated and 10ml of a 1:1 Trypsin/Versene (at 37°C) solution was added and flasks were incubated for 8-10 minutes. After this time, the cells were viewed under the microscope and tapped on the sides to facilitate cells into suspension. When cells appeared to round up, the

solution was neutralised with an equal volume of culture media, the contents of the flask pipetted up and down and then transferred to a Falcon tube.

If some cells were still adherent, the trypsin step was repeated to facilitate complete removal. The cell suspension was vortexed at low speed to produce a homogeneous suspension and the cells counted.

Performing cell count

A Neubaeurs counting chamber and the thick cover slip were cleaned with alcohol and the cover slip was placed over the H of the counting chamber. About 10µl of homogeneous cell suspension was inserted into the space to cover the mirrored surface.

The 4 corner squares and the central square in each counting chamber were counted in 40X magnification (4 x objective). The total count was divided by the umber of squares counted and as the depth of the counting chamber was 0.1 mm, the count was divided by 0.1 which gave the number of cells /mm^3, and multiplied by 1000 to yield the number of cells per ml.

A million cells were added per flask, together with fresh media, and the flasks were placed in an incubator with 10% CO_2 at 37°C. Cells were observed as described previously for viability and infection. The cultured cells were further processed for extraction of RNA or protein.

Protein extraction from cultured cells

For protein extraction, cells were grown on a 100mm plate. When the cells were about 70-80% confluent protein was extracted. This is preferred as at this stage the majority of the cells are the same stage of development and hence the extraction would be most representative. The plates were placed flat on ice during the extraction. To wash each plate ice cold PBS (5-10 ml per plate) was added and mixed by swirling the plate around so that all areas were covered, the liquid was then discarded and the wash was repeated twice.

Preparation of the RIPA buffer and proteinase inhibitor mix

For each plate of 100mm size 300µl of RIPA buffer (Prepared as described in the Appendix) containing commercial proteinase inhibitor was added following the manufacturers instructions.

The buffer was swirled around until it covered all the areas on the plate. The plate was then incubated at 4°C for 20 minutes (on ice). Having removed all the PBS, the cells were harvested using a cell scraper and the recovered cells were transferred to an eppendorf tube using a pipette. The contents of the eppendorf tube (low speed) were sonicated. The sonicator probe was washed twice with distilled water before, after and in-between each sonication.

The extracted proteins were then analysed using Western blotting.

2.8 Protein analysis

Bradford Assay; protein quantitation

One ml of Bradford concentrate (BIORAD, UK) was added to 4ml of distilled water. Bovine serum albumin (BSA) (Cambio, UK) was prepared at a concentration of 1ug/ul. One ml of Bradford reagent was added to 2µl of RIPA buffer and the appropriate concentration of BSA. The tubes were mixed and the absorbance read at 595nm.

Different concentrations of BSA (0, 1, 2, 4, 8, 12, 16 and 20µg) were used to construct a standard curve. 1ml of diluted Bradford reagent was added to 2µl sample and the absorbance was assessed at 595nm. The samples were diluted if required in RIPA buffer and protein concentration was read from the standard curve.

SDS-PAGE Western blotting

20µg of protein in a total volume of 16µl was added to 4µl of loading buffer. (For preparation refer Appendix). The samples were incubated for 5 minutes at 90°C. The ladder (Invitrogen, UK) was prepared according to the manufacturers instructions.

Preparation of SDS PAG

The 16% gel was prepared as per the protocol(Sambrook and Russell, 2001)

Once the separating and stacking gels had set, the samples and ladder were loaded. Electrophoresis was carried out at a voltage of 100V until the protein bands began to move and subsequently the voltage was increased to 140 V for 45 minutes.

Western blot

The blot was set up according to the manufacturer's instruction and a. PVDF membrane (VWR international, UK) was used to capture the blotted proteins. Blotting was carried out at 400mA (constant current) for 2 hours for 2 gels of full size.

The membrane was removed and the transfer of the markers was assessed by visualisation of the coloured bands in the standard marker. The membrane was washed on a rotator in 1x TBS–T (T= tween 20) three times, each wash lasting for 5 minutes. The membrane was then blocked with 10% milk (10% milk in TBS–T) for 30 minutes.

Table 2.3- The concentrations of the primary antibodies used for western blotting

Concentration	**Primary Antibody**
1:3000	GAPDH
1:3000	HBD1
1:2000	HBD2
1:5000	HBD3

Incubation with the primary antibody (details of dilution as shown in table 2.3) was done overnight in 5% milk buffer. Antibodies for GAPDH gave good results when incubated for 30 minutes at room temperature. Two washes of 10 min each were performed with 10% milk in TBS-T. Incubation with the suitable secondary was done for 30 min at room temperature in 5% milk in buffer, followed by 2 washes as above. ECL plus reagent (GE-Healthcare, UK) was used for development of the bands. This was performed in a dark room according to the manufacturer's protocol using an X-ray film.

RNA was extracted from the cultured cells according to the following protocol

For RNA extraction the cultured plates were washed with PBS as described earlier. 300µl of SV total RNA lysis buffer (Promega, UK) was added to each plate which was then scraped with a cell scraper. The contents of the plate, which were a mixture of cell lysate and lysis buffer, were then transferred to an eppendorf tube and stored at -80°C.Subsequent extraction of RNA was as described in the section on RNA extraction and PCR.

Chapter 3

Human β-Defensin expression in burn wounds and correlation to bacteria and duration of burn wound.

3.1 Introduction

Wound infections account for high morbidity and mortality in patients with burns(Pruitt et al., 1998, Polavarapu et al., 2008). The loss of skin is considered a major factor in such infections. The skin was thought to protect the body by mainly providing a mechanical cover but more recently other specialised functions of this organ have been described. Recent studies have shown that epithelial cells in various parts of the body can produce a number of antimicrobial chemicals. Many such chemicals have been described and have diverse properties. Defensins, one such family of chemicals, are of interest as they show a promise in defence against infections.

Burn wounds

Effective wound care is paramount for the success of any surgical procedure and in patients who have sustained burn wounds. In these patients wound infection is associated with high mortality and morbidity(Aufwerber et al., 1991, Polavarapu et al., 2008). Burn wounds are prone to colonisation by microbes, which may cause tissue invasion and lead to infection.

Burn wounds are assessed based on the surface area involved and depth of the wound as these are of critical importance in deciding the management plan and predicting the prognosis of a burn injury. Burn wounds are initially colonised in the first 48 hours and subsequently the invasion of the viable tissues occurs resulting in invasive infections. If viable tissues are invaded, disseminated systemic infection can occur. The colonising organisms are usually derived from the skin flora, usually gram positive *cocci* resident in the depths of the sweat glands and hair follicles, and thus escaping the initial injury(Luterman et al., 1986, Mooney and Gamelli, 1989). However the nature and type of the infective organisms are variable and

depend on a number of host and institutional factors(Vindenes and Bjerknes, 1995, Erol et al., 2004). The type of organisms also vary depending on the duration since burn, the type of colonisation at non-burn areas in the host and the nature of the antimicrobial therapy administered(Erol et al., 2004).

The importance of prevention of infection in burn wound care is a well recognised concept and is considered to be one of the most important factors predicting the outcome of the burn injury. Interestingly by investigating the organisms that cause infection in these patients it has been ascertained that these patients have altered immune function, since the majority of the organisms causing infections are of an opportunistic nature(Pruitt and McManus, 1992, Taylor et al., 1992). Also the fact that serious infections in these patients involve sites other than the burn site further confirms this concept of immune dysregulation(Engelich et al., 2001) that influences both innate and adaptive components of the immune system.

The aim of the current study was to investigate whether the high incidence of infections in burns patients was associated with alterations in β-defensin expression and if the type and pattern of HBD correlated with bacterial flora in burn wounds. The expression of HBD 1, 2 and 3 was studied in full thickness burns in relation to microbial flora and the duration of the burn wound.

3.2 Materials and methods

The materials were collected from Burns Theatre at Chelsea and Westminster Hospital; after obtaining ethical committee approval for the study-RREC 2992.

Patient recruitment

The patients admitted to the Burns Unit who were in relatively stable and conversable condition were approached. Children, pregnant women, comatose patients and mentally incapacitated patients were not approached either directly or indirectly because of ethical and practical reasons. Consent was taken after giving enough time for the patient to go through the information sheet and clarifying any doubts that had arisen in due course. This process would have become all the more difficult but for the co-operation from the doctors involved in the treatment of patients. The plan was to recruit patients who were undergoing debridement within 24 hours of trauma. These patients usually have large burns and would have been ideal for the study. However, they could not be recruited since they were in a very stressful situation and the relatives were either not approachable or refused consent.

Collection of material for microbial estimation

Material for microbial estimation was collected in 1.5ml eppendorf tubes containing 1ml of sterile PBS (phosphate buffered saline pH7.4). PBS was chosen as it is considered an inert transport medium which does not favour the proliferation of the microbes. These tubes were weighed before and after the specimens were collected to calculate the mass of the tissue. Since the thickness of the tissues was nearly uniform the mass was taken proportional to the length. A punch biopsy could not be taken to maintain uniformity as regards the amount of

material owing to financial and practical constraints. A small piece of the tissue was transported in Robertson's cooked meat medium (RCM), which served for enrichment of bacteria and dilution of any antibiotics which may have been used. The tissue was placed in the media as rapidly as possible post-collection and the media were streaked onto the respective bacterial nutrient plates at the earliest opportunity, taking all the necessary aseptic precautions. The average time lapse between collection and plating was about 3 hours.

Collection of material for formalin fixation

The material was collected at the earliest opportunity (time lapse not more than 15 minutes) and transferred to a container containing 10% formalin. At the time of collection the burnt tissue was distinguished from the relatively normal tissue present at the edges. The skin taken from the edges was classified as peri-burn. Either the difference in colour or the presence or absence of hair was considered as the criteria for differentiating between the two. Burnt tissue was collected in a different container from that of relatively normal tissue.

Collection of material for snap freezing

Liquid nitrogen was obtained for the process from the hospital Pathology Department after obtaining permission from the appropriate authorities. Different techniques were evaluated before selecting the best technique. Initially the tissue was placed into small cryotubes and the tubes were dropped in liquid nitrogen. It was realised that it was not possible to get the tissue out from such tubes without completely thawing the material as the small drops of fluid were freezing and attaching to the sides of the tube. It was considered better if the tubes and the tissues were frozen separately first and then the tissue transferred to the tubes. Though this

was probably the best method, it was not practical in our case since it required large quantities of liquid nitrogen and long forceps for taking the tissues and tubes from liquid nitrogen flask. It was therefore decided to put the tissues into wider mouthed tubes. Thin walled plastic tubes were selected for use since there was a risk of glass or thick walled tubes would break during freezing.

The tissue has to be thawed before it is mounted on a cork in the proper orientation to be cut on a cryotome. Since it was not considered to be good to repeatedly thaw and freeze the tissue, it was decided to mount the tissue directly on cork before putting it into liquid nitrogen. This was done using the tissue adhesive OCT (Raymond A Lamb Laboratory Supplies; UK), which was applied on the cork before the tissue was laid on it. Since the skin material was thin it would not stand in the correct orientation, it was decided to cut through the cork and hence the skin was laid flat on the cork for adhesion. However, owing to the practical difficulties involved in cutting through the cork it was decided to orient the tissue by stiffening it by partial freezing using an instant freezing spray (iso-pentane) and then putting it on the cork to which OCT was applied. Additional pieces of skin were stored frozen by laying them on cork without application of any OCT. Pre-treatment such as graded cooling using isopentane or hexane are considered better than direct freezing in liquid nitrogen since sudden chilling is likely to make the tissue brittle which in turn poses difficulties while making sections. Also washes with PBS can be given to rid the material of blood. Since it was not practically possible to transport frozen material on a day-to-day basis from the hospital to the university laboratory the samples were batched up and transported either in liquid nitrogen or on solid CO_2.

Thus the final protocol was as follows: tissue material for RNA work was collected in wide mouthed plastic containers, the material for cryo-sections was first hardened using iso-pentane spray and then applied to OCT coated cork.

Labelling of materials

All the materials were adequately labelled using a unique coding system so as not to reveal the patient identity. Uniformity of labelling was maintained with regards to the tissues being stored and processed by different methods.

Immunohistochemistry

The tissue sections were processed by immuno-fluorescence techniques as described earlier and were analysed by stoichiometric methods.

Microbial estimation

A qualitative and quantitative microbiological analysis was performed as described in the methods chapter.

RNA extraction and expression analysis

Since the debrided skin was from areas which was exudative and burnt, RNA extraction was not predicted to be easy. Exudates are considered to be rich in RNAses and the burning process destroys the cells and the RNA. A variety of different methods of RNA extraction were trialled and the best extraction was achieved using SVtotal RNA (Promega, UK; Z3100) isolation system.

RNA was extracted from frozen burn skin material using the following steps. Tissue material was ground into fine powder using a ceramic homogeniser in an atmosphere of liquid nitrogen. All standard precautions were taken to ensure the environment was free of RNAses and that all instruments and the work bench were pre-treated with RNAZap (Promega, UK). The powdered tissue material was then transferred to a pre-weighed eppendorf tube containing 1ml of lysis buffer according to the manufacturer's protocol (SV total RNA extraction kit; Promega, UK) after allowing the liquid nitrogen to evaporate. The eppendorf tube was weighed to estimate the weight of the tissue, which was 30mg per 175µl of lysis buffer. The contents of the eppendorf tube were homogenised using glass homogenisers. Additional lysate beyond 175µl was frozen at –70 °C. To 175µl of lysate 350µl of dilution buffer was added and the contents mixed by inversion. Subsequent extraction steps were as described earlier in the methods chapter. The RNA, which was collected in the elution tube, was assessed by spectrophotometric analysis for purity and stored at –70° C until further processing.

Table 3.1 NCBI accession numbers of the genes analysed, and the sequence of the specific forward and reverse primers

Gene	Accession number	Sequence;5'-3'
HBD1	U73945	Forward- AATCCTGAGTGTTGCCTG Reverse- GCGTCATTTCTTCTGGTC
HBD2	Z71389	Forward- CAGCCATCAGCCATGAGGGT Reverse- GGAGCCCTTTCTGAATCCGCA
HBD3	AJ237673	Forward- GCCTAGCAGCTATGAGGATC Reverse- CTTCGGCAGCATTTTGCGCCA
B Actin	NM_001101	Forward- CCAACAGAGAGAAGATGAC Reverse- AGGAAGGAAGCCTGCAAC

cDNA conversion

mRNA was converted to cDNA using ImProm II Reverse Transcriptase (RT) kit (Promega;UK). Conversion was based following the manufacturers protocol as described earlier. The samples were run on the gel and the size of the bands estimated by comparison with 1Kb standard marker.

Real time PCR

Real time (quantitative) PCR analysis was carried after optimization as described earlier using Opticon II quantitative thermal cycler (GRI Biosystems; UK). The set of primers as shown in Table 3.1, used for RT-PCR were used for quantitative PCR.

3.3 Data Analysis and Results

The patients were subdivided into two groups; B and C. Group B included patients with burn wounds of less than 2 weeks duration at the time of debridement and group C comprised of patients with burn wounds of more than 2 weeks duration. The details of the patients are shown in table 2.1. The mean age was 48.44 years with a range-24-88 years, the sex distribution was 8:10; male: female. The mean percentage burn was 7.05%. This varied between the groups, the mean percentage burn for B group was 3.73 (min-1%, max-15%) for group C was 8.88%, (min-1%, max-30%). The range of burns was 1-30%. The distribution of burns in our study population was very varied as seen in table 3.2 and figure 3.1. The most common site was arm followed by the forearm.

Table 3.2 Patient details

Patient code	Sex	Age (Years)	Percentage of burn	Duration of burn in days	Site of burn
1B	M	29	1	7	Leg
2B	F	70	8	3	Arm
3B	F	67	2	5	Abdomen
4B	F	24	1	7	Arm
5B	M	30	1	10	Hand
6B	F	41	2	2	Fore arm
7B	M	42	4	3	Groin
8B	M	47	5	7	Back
9B	F	50	1	2	Finger
10B	F	47	15	3	Fore arm
11B	M	67	1	6	Fore arm
1C	M	Not noted	4	14	Not noted
2C	F	36	6	14	Chest
3C	F	38	2	16	Arm
4C	M	64	6	14	Axilla
5C	M	88	1	60	Sole
6C	F	27	20	30	Back
7C	M	58	30	21	Arm
8C	F	47	2	14	Forearm

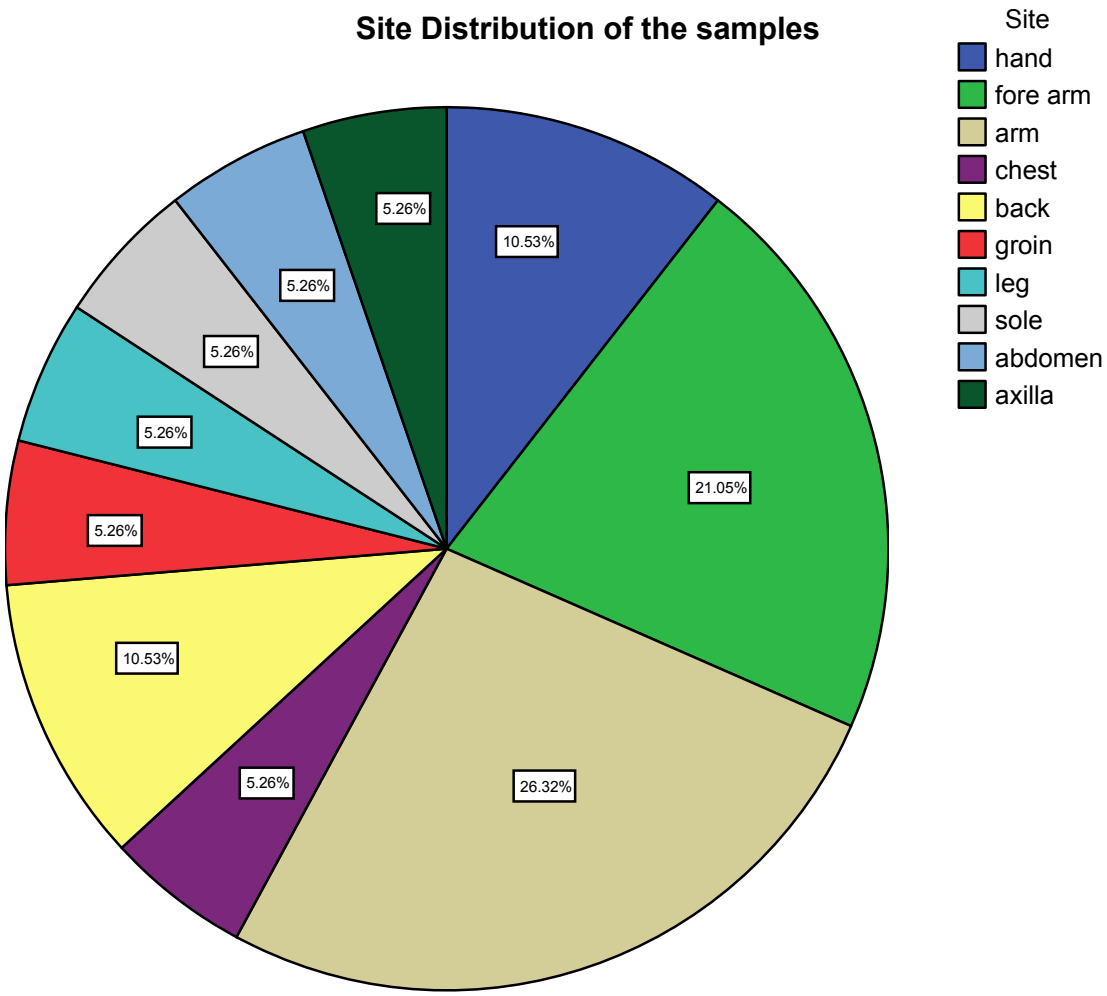
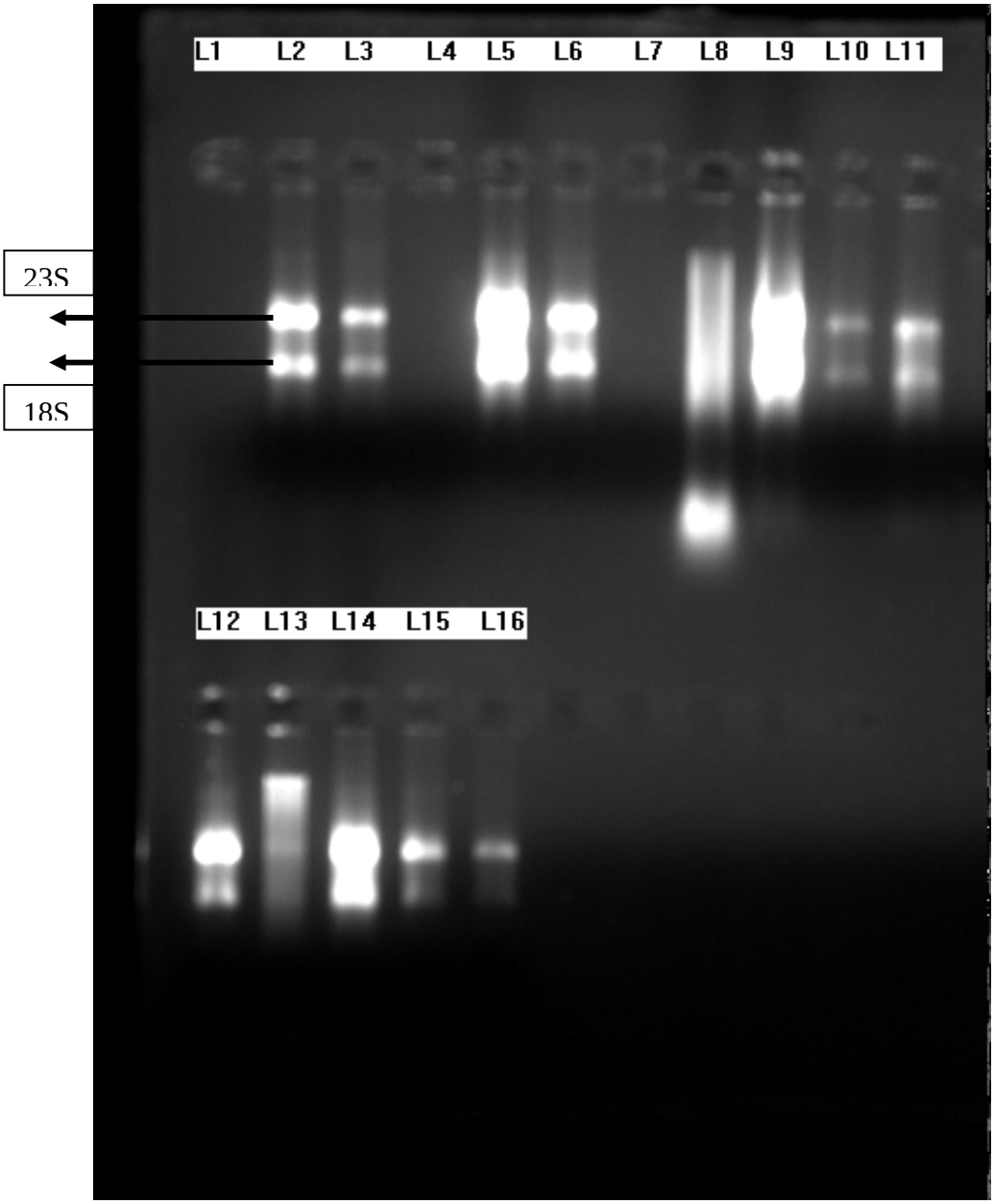


Figure 3.1 Summary of the burn sites in the patients.



L1-empty, L2 –psoriatic sample, L3-Normal Skin, L4-empty, L5-16 patient samples

Figure 3.2 shows the analysis of RNA extracted from skin and separated on a 1% agarose gel. Two bands (arrows), 23s (upper band) and 18s (lower band) rRNA were noted in the patient samples and the psoriatic and normal skin specimens. The quality of the total RNA extracted varied between the patient samples with some samples (6 and 11) showing good quality RNA (distinct 23S and 18s bands) whereas others (15 and 16) giving a hazy smear.

Real time PCR data

The experiments with each sample were done in triplicate and Table 3 shows the final values, which were taken for further calculation of the relative expression. The samples that did not show clear peaks on melting curves for the products were not considered and the experiment repeated. Those showing slight alteration in the melting temperature of the product away from the control were considered as significant if they showed clear single peak in melting curve. Some samples were run on the gel to confirm the presence of the product.

Table 3.3 Quantitative PCR data for HBD1 and housekeeping gene-β- Actin (Ba)

Patient code	Mean C(T)-B1	Mean Tm	Mean C(T) Ba	Mean Tm
7B/burn B1	20.5	80.1	29.0	86.6
7B/periburn B1	19.6	80.8	23.4	86.3
8B/burn B1	22.0	81.2	17.9	86.2
8B/periburn B1	16.1	81.6	16.9	86.5
9B/burn B1	16.2	80.8	21.2	82.5
9B/periburn B1	20.9	83.5	28.6	86.4
10B/burn B1	9.71	82.9	11.7	85.8
10B/periburn B1	18.2	78.4	17.9	85.4
11B/burn B1	20.6	81.8	26.8	78.0
11B/periburn B1	17.4	83.4	19.3	86.2
1C/periburn B1	20.1	86.1	24.8	85.7
2C/burn B1	17.7	84.3	17.2	86.3
2C/periburn B1	17.7	82.7	15.4	85.9
4C/burn B1	24.5	83	28.5	86.8
4C/periburn B1	20.8	82.4	29.5	86.7
5C/burn B1	23.5	81.5	21.2	86.0
5C/periburn B1	20.9	82.2	21.0	85.9
6C/burn B1	23.9	80.2	26.4	78.0
6C/periburnB1	23.6	84.4	24.3	78.4
7C/burn B1	20.3	81.8	22.2	85.7
7C/periburn B1	21.3	83.4	23.5	86.8
8C/burnB1	21.1	82.7	27.5	85.9
8C/periburn B1	19.8	82.2	26.8	86.5
Psoriatic Skin	16.8	84.8	14.8	87.8
Normal Skin B1	28.2	83.6	13.0	78.8
Mean SD	3.4	1.7	5.2	3.0
Mean		82.3		84.8

Table 3.4-Real time PCR data for HBD 2 gene and housekeeping gene-β- Actin (Ba).

Label	Mean C(T) B 2	Mean Tm	Mean Ba	Mean Tm Ba
7B/burn B2	21.7	82.2	29.0	86.6
7B/periburn B2	20.7	82.3	23.4	86.6
8B/burn B2	21.0	81.9	18.0	86.4
8B/periburn B2	16.0	82.0	16.9	87.0
9B/burn B2	18.8	80.1	21.2	86.6
9B/periburn B2	21.8	81.9	28.6	86.6
10B/burn B2	10.4	82.1	11.7	86.0
10B/periburn B2	21.4	79.8	17.9	86.0
11B/burn B2	23.6	81.3	26.8	78.0
11B/periburn B2	17.5	81.0	19.3	86.2
1C/burn B2	24.1	82.1	28.2	86.6
1C/periburn B2	23.6	82.5	20.2	86.4
2C/burn B2	17.2	82.3	17.2	86.8
2C/periburn B2	16.5	81.5	15.5	86.0
4C/burn B2	20.5	82.0	28.5	86.0
4C/periburn B2	20.2	82.2	29.5	86.0
5C/burn B2	14.0	81.2	21.2	86.0
5C/periburn B2	13.3	80.3	21.1	86.6
6C/burn B2	28.6	78.4	26.4	78.0
6C/periburnB2	25.9	83.6	24.3	78.4
7C/burn B2	17.4	80.8	20.8	86.2
7C/periburn B2	21.6	86.2	23.5	86.8
8C/burn B2	16.4	83.0	27.5	86.6
8C/ periburn B2	21.8	81.5	26.8	86.4
Psoriatic skin	15.3	83.2	14.8	87.8
Normal skin	23.2	78.4	13.1	78.8
Mean SD	3.4	2.4	5.4	2.9
Mean		81.5		85.0

Table 3.5-Real time PCR data for HBD3 gene and housekeeping gene- B Actin (Ba).

Label	Mean C(T) B3	Mean Tm	Mean Ba	Mean Tm
7B/burn B3	24.4	78.2	29.0	86.6
7B/periburn B3	22.7	79.1	23.4	86.3
8B/burn B3	20.0	81.5	18.0	86.2
8B/periburn B3	21.0	82.6	16.9	86.5
9B/burn B3	19.2	84.8	25.0	86.6
9B/periburn B3	24.1	84.8	28.6	86.4
10b/burn B3	14.2	85.0	11.7	85.8
10b/periburn B3	21.8	78.4	22.0	86.0
11B/burn B3	25.0	81.8	20.1	78.4
11B/periburn B3	20.4	84.6	19.3	86.2
1C/burn B3	26.7	78.5	28.2	83.3
1C/periburn B3	23.2	83.1	24.8	85.7
2C/burn B3	18.1	81.4	17.2	86.2
2C/periburn B3	18.4	81.4	15.5	85.9
4C/burn B3	23.8	78.9	28.5	86.8
4C/periburn B3	24.2	82.0	29.5	86.7
5C/burn B3	19.2	84.6	20.8	86.1
5C/periburn B3	18.7	84.3	21.1	85.9
6C/burn B3	23.4	80.4	26.4	78.0
6c/peri-burn B3	24.1	80.2	24.3	78.4
7C/periburn B3	20.8	79.6	20.8	86.2
7C/graft B3	20.1	84.6	23.5	86.8
8c/periburn B3	16.2	82.7	27.5	85.9
8c/graft B3	25.7	78.9	26.8	86.5
Psoriatic Skin	16.6	78.4	14.8	87.8
Normal skin	25.6	79.2	13.1	79.0
Mean SD	3.4	2.4	5.4	2.9
Mean		81.5		85.0

Tables 3.3, 3.4 and 3.5 show the mean threshold cycle [C(T)] and mean melting temperature of the product (Tm) for each of the samples for the *HBD(B1,B2 &B3)* and *B Actin(Ba*) genes. The patient codes indicate the patient serial number followed by B or C which indicates whether the patient belonged to the <2 weeks group or >2 weeks group respectively. Only those pairs have been listed from whose RNA successful amplification was possible. B1-denotes *HBD1*, B2-denotes *HBD2*, B3-*HBD3* and Ba-*B Actin.*

Variability of Tm was seen in some samples. The mean Tm wereas follows: for *HBD1*-82.3ºC (SD-1.6ºC), *HBD2*-81.5ºC (SD- 2.4ºC), *HBD3*-81.5ºC (SD- 2.4ºC), *B Actin*-85ºC (SD-3ºC).

The individual patient samples showed slight variation in melting temperatures as indicated by the standard deviations. The melting temperature for the products was lower in the burns patient's samples than in the normal skin sample; this is probably related to the difference in salt concentrations required for normal and burn skin samples.

The Tm for *HBD3* varied between 78/ and 79ºC or 82ºC and 83ºC, this could be related to the presence of a yet undescribed polymorphisms in the *HBD3* gene. It is intended to sequence the product of the amplification to confirm this finding.

RNA and Protein expression

RNA and protein expression for individual patient samples is listed in Table 4. RNA expression is expressed in the form of 2^{-DdCt}. Dct is calculated as difference between Ct (cycle number when the product appeared) of the *defensin* gene and the *actin* gene. Taking a reference sample among the samples (in this experiment psoriatic sample) and subtracting the Dct of sample from Dct of psoriatic tissue gives Ddct. This is then expressed as 2^{-DdCt}. This gives the expression of RNA of the concerning gene relative to the reference gene normalised to the housekeeping gene. Psoriatic tissue was chosen as the reference sample for this study and the reference gene was *β–actin*. Protein expression, detected by immunohistochemistry was graded blindly (stoichiometric analysis) on a scale of 0-4 with 0 being the lowest expression corresponding to no expression and 4 being the maximal expression (table 3.6).

Table 3.6 mRNA and protein expression.

Number	B1-RNA Expression 2^{-Ddct} B1	B2-RNA Expression 2^{-Ddct} B2	B3-RNA Expression 2^{-Ddct} B3	Protein Expression B1	Protein Expression B2	Protein Expression B3
1B/burn	No RNA	No RNA	No RNA	2	2	3
1B/periburn	No RNA	No RNA	No RNA	1	1	3
2B/burn	No RNA	No RNA	No RNA	2	0	2
2B/periburn	No RNA	No RNA	No RNA	3	1	2
3B/burn	No RNA	No RNA	No RNA	2	0	1
3B/periburn	No RNA	No RNA	No RNA	3	0	2
4B/burn	No RNA	No RNA	No RNA	1	0	2
4B/periburn	No RNA	No RNA	No RNA	2	4	3
5B/burn	No RNA	No RNA	No RNA	1	0	3
5B/periburn	No RNA	No RNA	No RNA	2	1	1
6B/burn	No RNA	No RNA	No RNA	3	1	2
6B/periburn	No RNA	No RNA	No RNA	1	0	1
7B/burn	2^{12}	2^{6}	2^{9}	2	0	3
7B/periburn	2^{9}	2^{4}	2^{6}	2	3	2
8B/burn	2^{3}	2^{2}	2^{7}	1	0	3
8B/periburn	2^{3}	2^{7}	2^{7}	2	3	4
9B/burn	2^{10}	2^{6}	2^{8}	2	2	2
9B/periburn	2^{9}	2^{7}	2^{5}	4	1	4
10B/burn	2^{9}	2^{5}	2^{6}	2	4	3
10B/periburn	2^{4}	2^{2}	2^{7}	2	4	1
11B/burn	2^{4}	2^{7}	2^{4}	1	1	2
11B/periburn	2^{4}	2^{1}	2^{1}	2	5	3
1C/burn	2^{12}	2^{8}	2^{6}	0	0	3
1C/periburn	2^{0}	2^{0}	$1/2^{1}$	2	0	2
2C/burn	2^{6}	2^{5}	2^{3}	0	0	1
2C/periburn	2^{1}	2^{1}	2^{1}	1	0	2
3C/burn	No RNA	No RNA	No RNA	0	0	1
3C/periburn	No RNA	No RNA	No RNA	1	1	2
4C/burn	$1/2^{1}$	2^{7}	2^{3}	1	0	2
4C/periburn	2^{3}	2^{5}	2^{1}	3	0	3
5C/burn	$1/2^{2}$	2^{10}	2^{6}	1	0	2
5C/periburn	2^{4}	2^{7}	2^{4}	1	0	1
6C/burn	2^{5}	$1/2^{2}$	2^{5}	4	2	2
6C/periburn	2^{3}	$1/2^{1}$	2^{2}	2	4	3
7C/burn	2^{4}	2^{5}	2^{4}	1	0	1
7C/periburn	2^{2}	2^{4}	2^{2}	1	1	2
8C/burn	2^{8}	2^{5}	2^{5}	2	1	1
8C/periburn	2^{6}	2^{9}	2^{11}	2	2	2
7C/graft	2^{6}	2^{2}	2^{4}	3	3	3
8C/graft	2^{6}	2^{3}	2^{4}	4	2	4

AmplifiableRNA could not be extracted from 7patient samples. These include 6 samples from the subset "B" and 1 from subset "C". Protein expression could be visualised in all the patient samples in both B and C groups. B subgroup indicates samples<2 week's duration while C subgroup indicates samples > 2 week's duration.

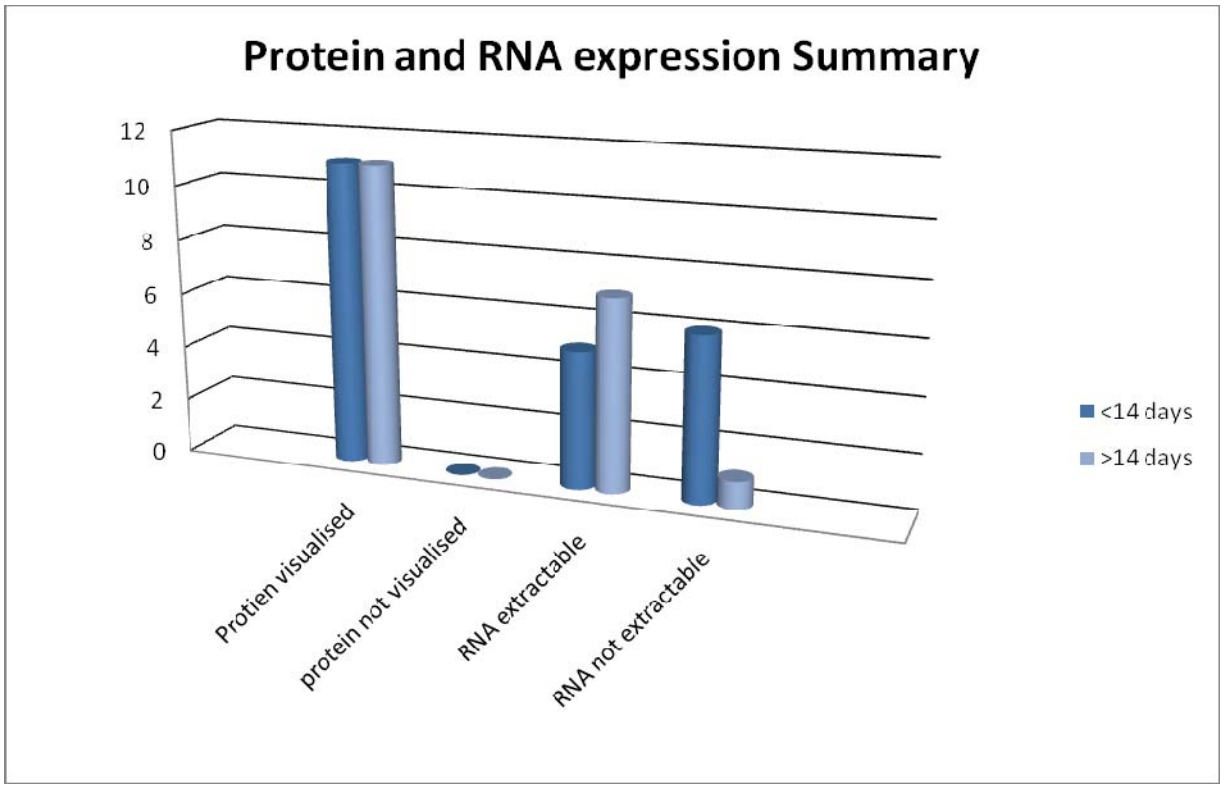


Figure 3.3 Summary of RNA and protein extraction from patient samples

The Y –axis denotes the number of patients while the X axis denotes the protein or RNA extraction. All samples from group B and C patients expressed protein which could be visualised, whereas only in about 40% of those with burns less than 14 days and 75% of those with burns more than 14 days was RNA extractable (tables 3.6 and figure 3.3).

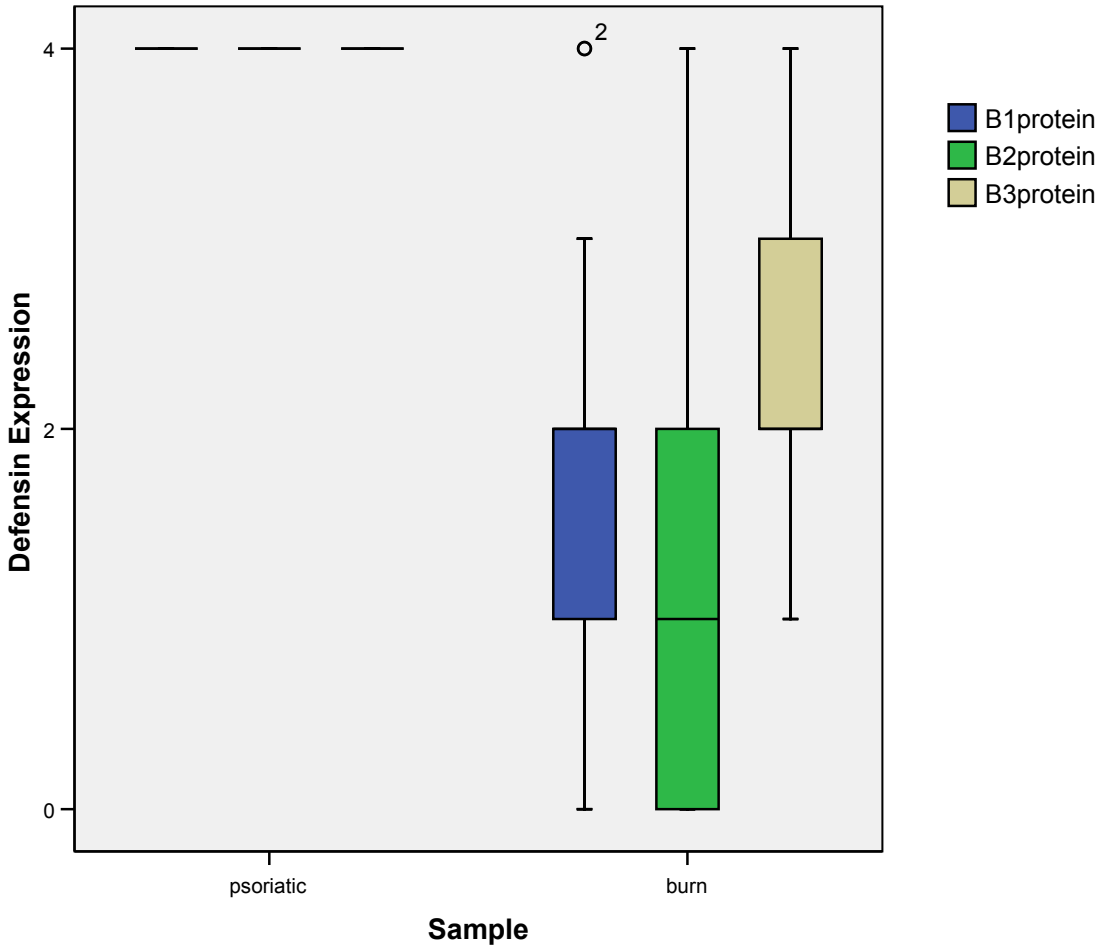


Figure 3.4 Boxplot of the protein expression in psoratic and burns tissues

The median protein expression in psoriatic samples was higher than the median protein expression of the HBD1,2 and 3 in the burn patient samples(both burn and peri-burn).(B1,2&3 refer to the β defensin expression).The scale on the Y-axis represents an arbitrary grading scale.

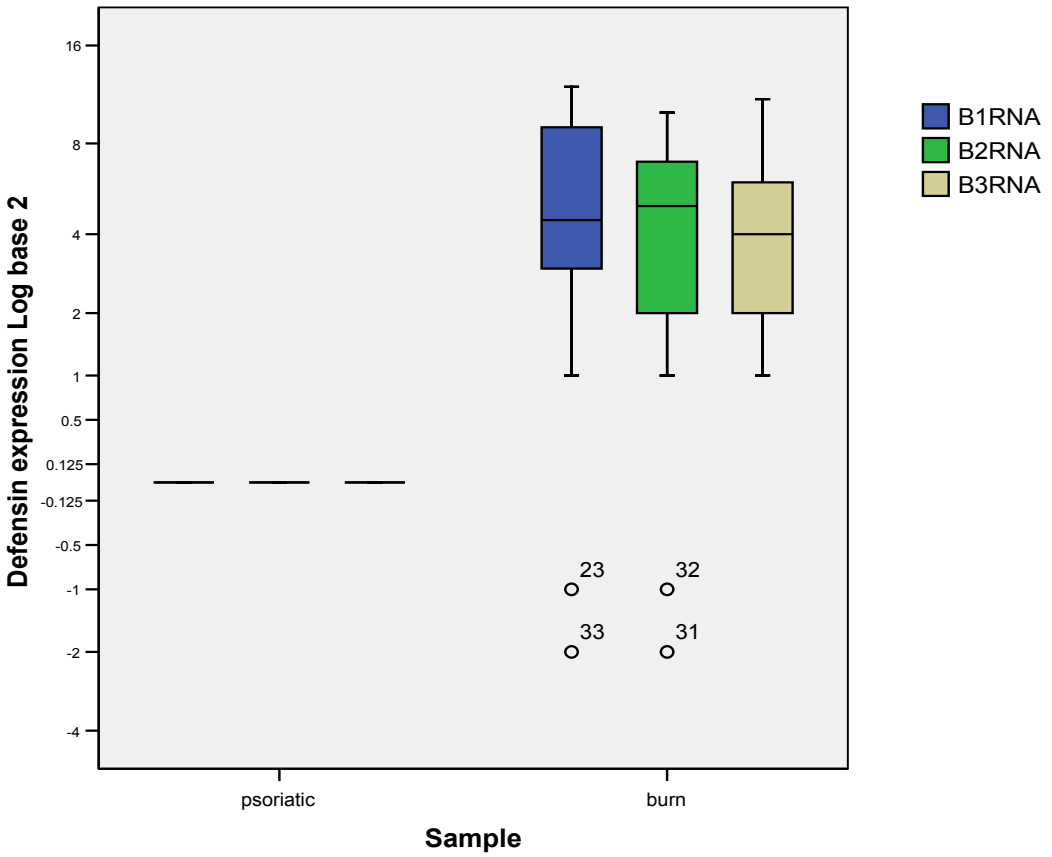


Figure 3.5 Boxplot of the mRNA expression in psoratic and burns tissues

The boxplot comparing the expression demonstrates that the median HBD1,2 & 3 RNA expression (shown by the black line inside the box) in the burn patient samples (both burn and periburn) was higher as compared to the expression in psoriatic tissue. This difference was statistically significant as shown by the independent sample t-test (Table 7). The small circles at the bottom of the figure denote the outlying values where the expression was less than that of psoriatic sample.

Table 3.7: Independent samples t-test-comparing HBD1,2& 3 RNA and protein expression between psoriatic and burn samples(All burn samples included both burn and periburn)

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	T	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
B1RNA	Equal variances assumed	4.2	0.1	-1.9	22.0	0.1	-5.4	2.8	-11.1	0.4
	Equal variances not assumed			-6.6	21.0	0.0	-5.4	0.8	-7.1	-3.7
B2RNA	Equal variances assumed	3.1	0.1	-2.1	22.0	0.0	-4.6	2.2	-9.2	-0.1
	Equal variances not assumed			-7.1	21.0	0.0	-4.6	0.7	-6.0	-3.3
B3RNA	Equal variances assumed	3.0	0.1	-2.2	22.0	0.0	-4.3	1.9	-8.3	-0.3
	Equal variances not assumed			-7.5	21.0	0.0	-4.3	0.6	-5.5	-3.1
B1protein	Equal variances assumed	3.1	0.1	3.3	38.0	0.0	2.3	0.7	0.9	3.6
	Equal variances not assumed			14.7	37.0	0.0	2.3	0.2	2.0	2.6
B2protein	Equal variances assumed	4.4	0.0	2.7	38.0	0.0	2.8	1.0	0.7	4.9
	Equal variances not assumed			12.0	37.0	0.0	2.8	0.2	2.3	3.3
B3protein	Equal variances assumed	3.0	0.1	3.1	38.0	0.0	1.8	0.6	0.6	3.0
	Equal variances not assumed			13.8	37.0	0.0	1.8	0.1	1.6	2.1

As shown (highlighted column) in Table 3.7 the difference between protein and RNA expression in the psoriatic and burn tissue was statistically significant for all the three HBDs.

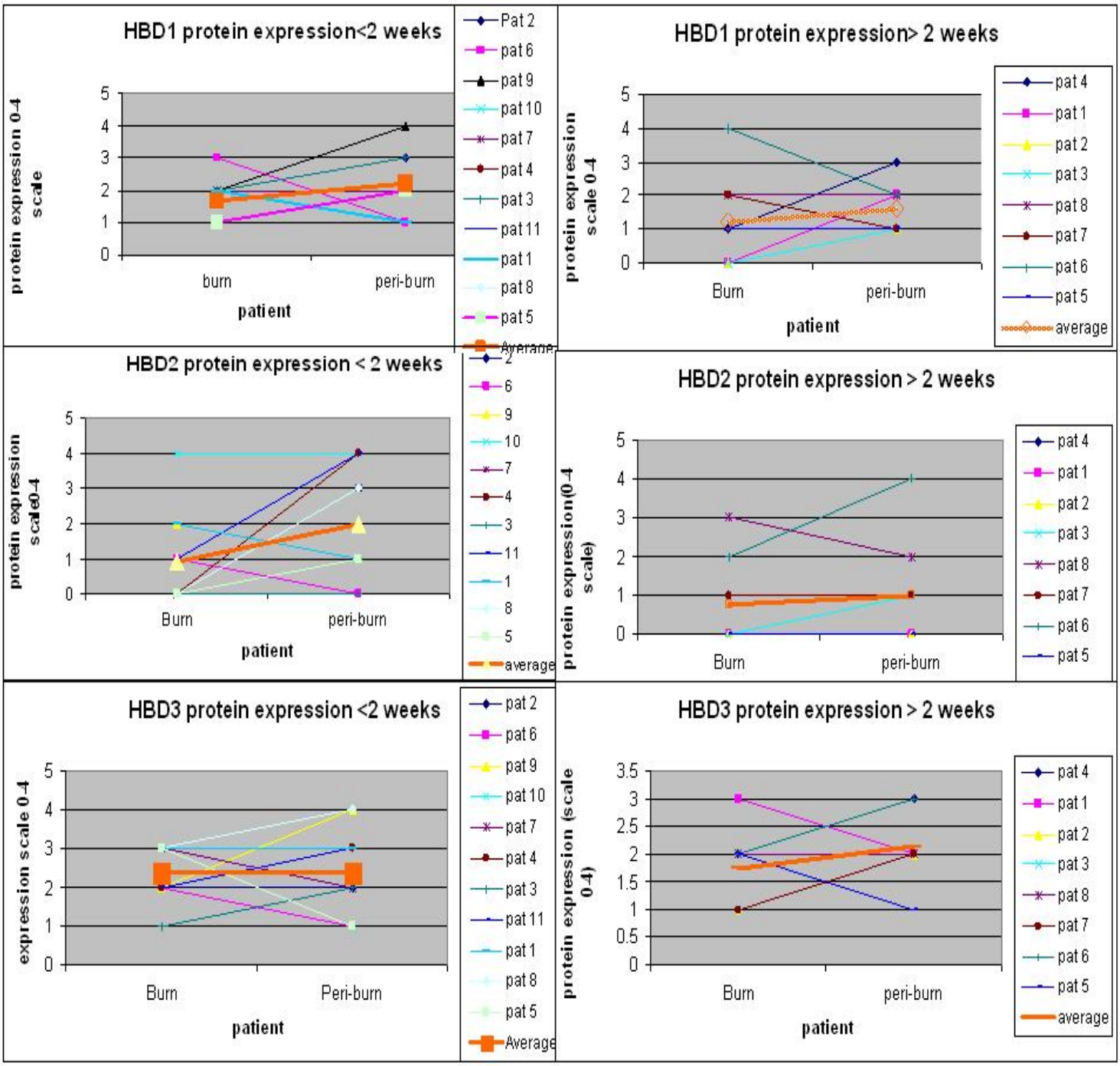


Figure 3.6 ***Individual protein expression in both the B(< 2weeks) and the C(>2 weeks) groups.***

The expression in both the burn and the peri- burn area is shown in each sample with the mean expression of all the samples in the group shown with a thick orange line.

As seen in the figure 3.6 the periburn HBD1, 2 & 3 protein expression in the B group (ie < 2weeks) was higher as compared to the defensin expression of the burns in most of the samples (e.g. 9 of 11). Samples 6 and 11 showed expression contrary to the general pattern for all the three defensins. The expression of HBD 1, 2 & 3 proteins in the C group (>14 days group) was predominantly higher or equal in peri-burn area to that in the burn group except in samples 3,7 for HBD1, 8 for HBD2 & 1,5 for HBD3. In these samples the expression was higher in the burn area compared to the peri-burn areas.

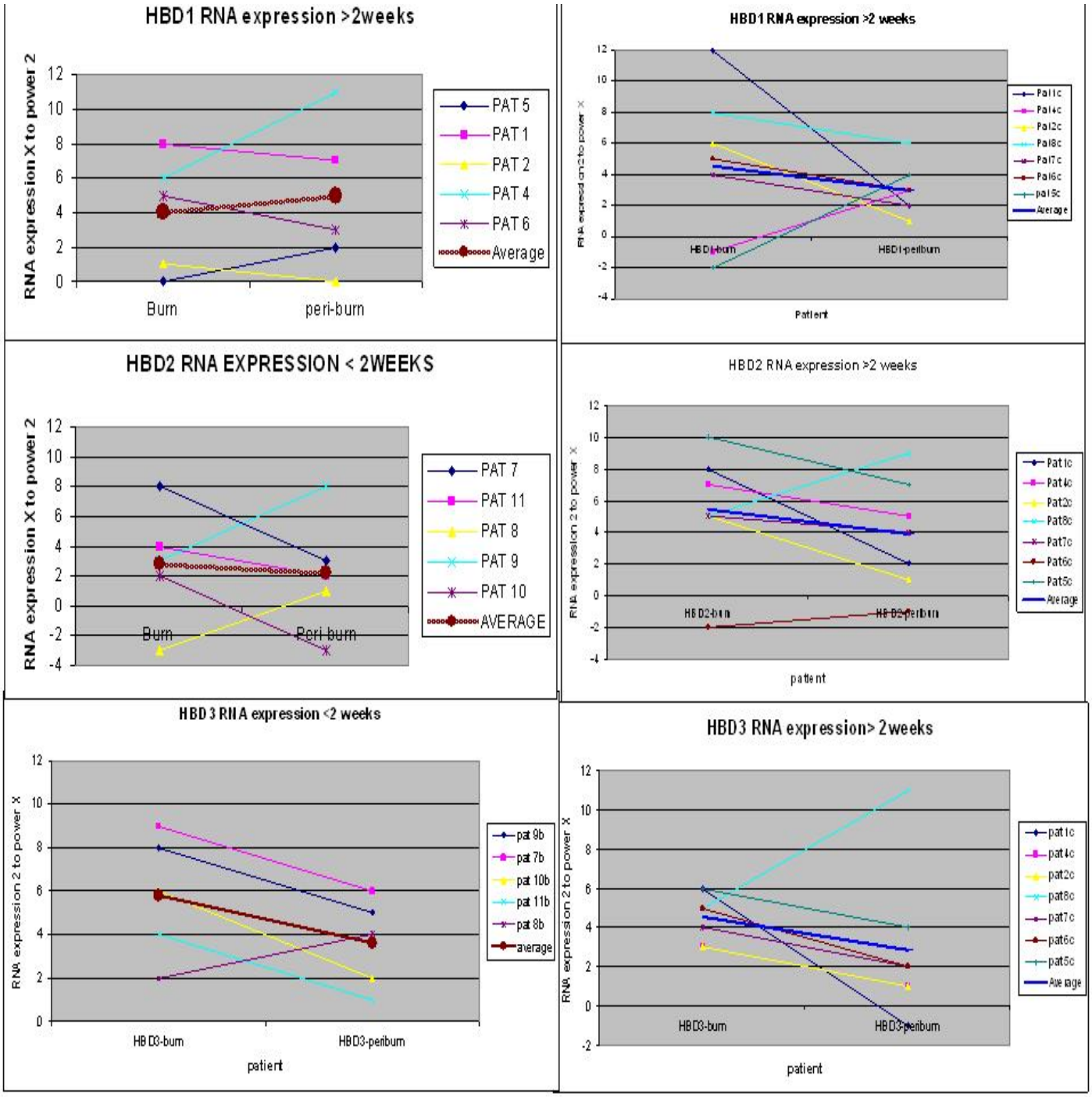


Figure 3.7 Individual expression of HBD1, 2 & 3 RNA in both the B and the C groups.

The individual expressions in both the burn and the peri-burn areas are shown with the mean expression in the group depicted by the thicker line.

As seen in the figure 3.7, the RNA expression of all three *HBD*s in B group (< 2 weeks) the expression was predominantly higher in the burn group as compared to the peri-burn group except in the sample 9b which showed expression opposite to the general norm for all the three β defensins.

The *HBD1, 2& 3* RNA expression in the patients of C group (> 2 weeks) was predominantly higher in the burn area as compared to the peri-burn area. There were however individual variations to this trend in that the expression was higher in the peri-burn areas in samples 4c, 5c for *HBD1* RNA, 6c, 8c for *HBD2* RNA and 8c for *HBD3*. The RNA expression among individual samples was elevated as compared to psoriatic tissue expression except in samples 1c, 4c, 5c, and 6c. In sample 1c the periburn area showed a decreased expression of *HBD3* RNA. In samples 4c and 5c the *HBD1* expression in burn area was less than the psoriatic tissue expression. In sample 6c both the burn and periburn area showed a decreased expression of *HBD2* RNA.

Thus to summarise, as seen in figures 3.8 and 3.10, the mean expression of *HBD1,2& 3* mRNA was higher in the burn areas compared to peri-burn areas in burn wounds of both less than 2 weeks and more than 2 weeks duration. However the protein expression was higher in the peri-burn areas in burns of both less than and greater than 2 weeks as seen in summary figure 3.10.

Figure 3.9 shows representative sections stained with HBD1, 2, and 3 antibodies.

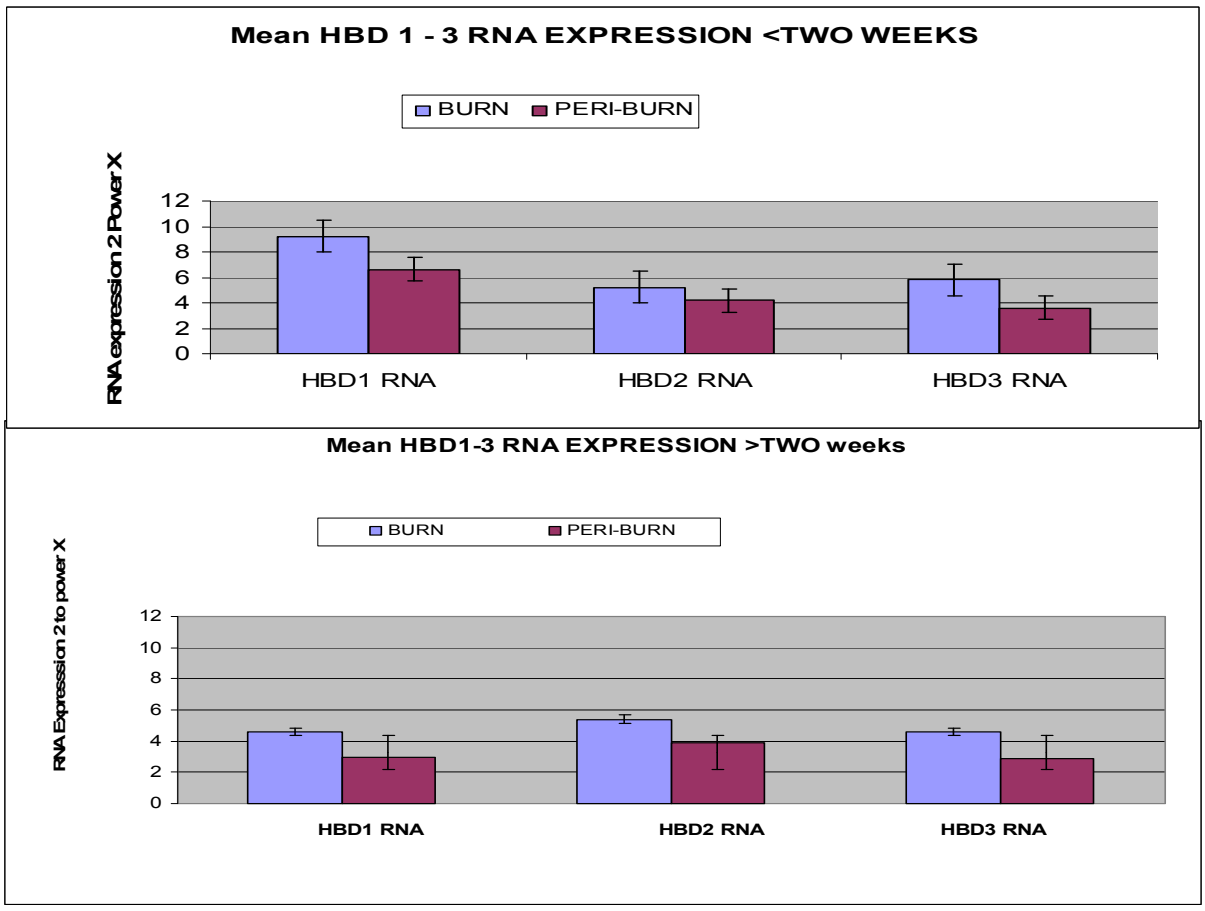


Figure 3.8 Mean HBD1,2 &3 RNA expression in the Burn and the Peri-burn area.

The upper panel shows the expression in the early (<2 weeks or B group), while the lower panel shows the expression in the late (>2 weeks or C group). The bars represent the standard error.

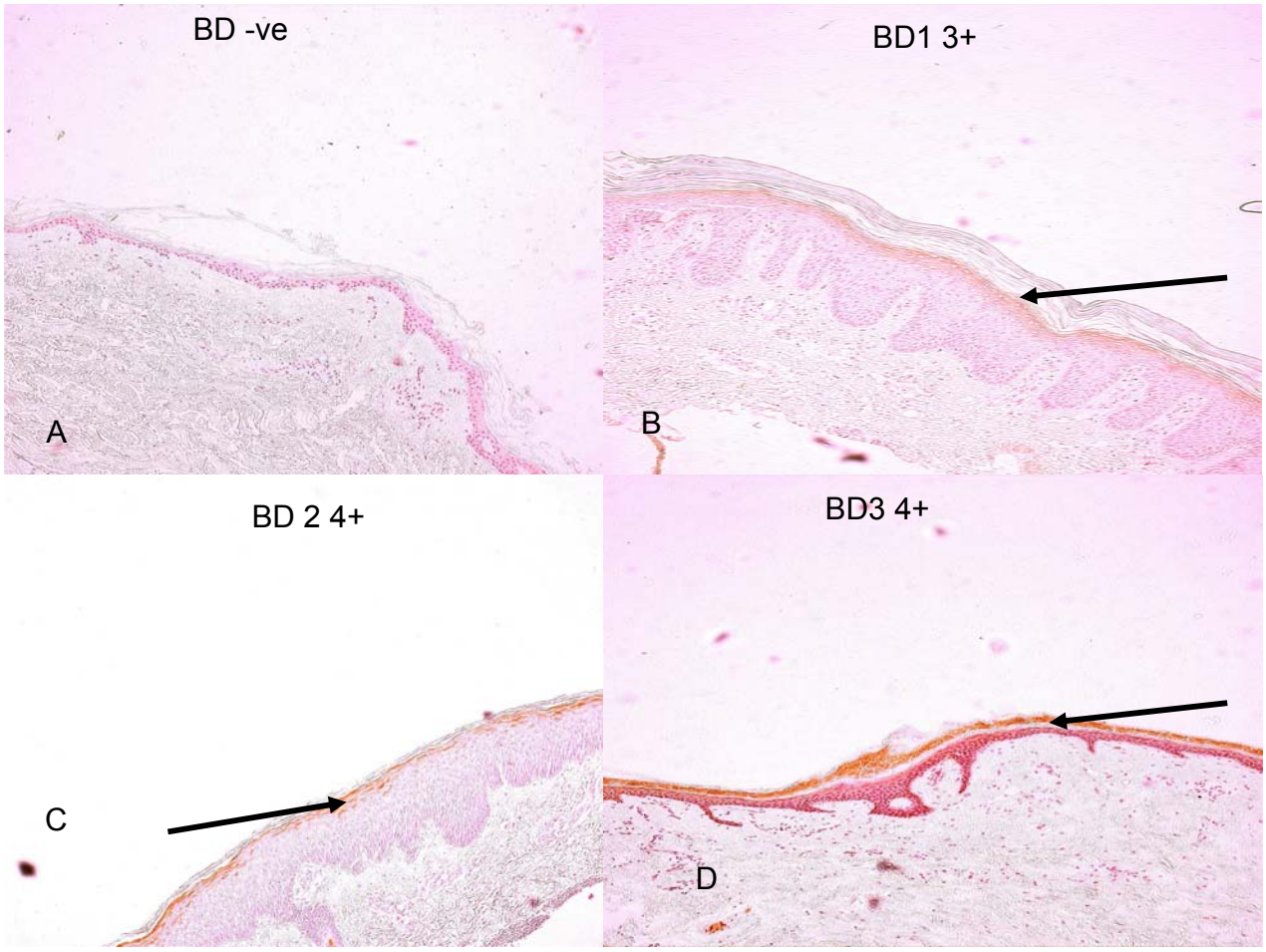


Figure 3.9 Immunostaining of the burn skin sections stained with the HBD antibodies and the developed with DAB.

The arrows show the immunolocalisation of the individual HBD. Panel A shows the no primary antibody –ve control; Panel B, C, D show the HBD1, 2 and 3 localisation respectively graded by stoichiometric methods.

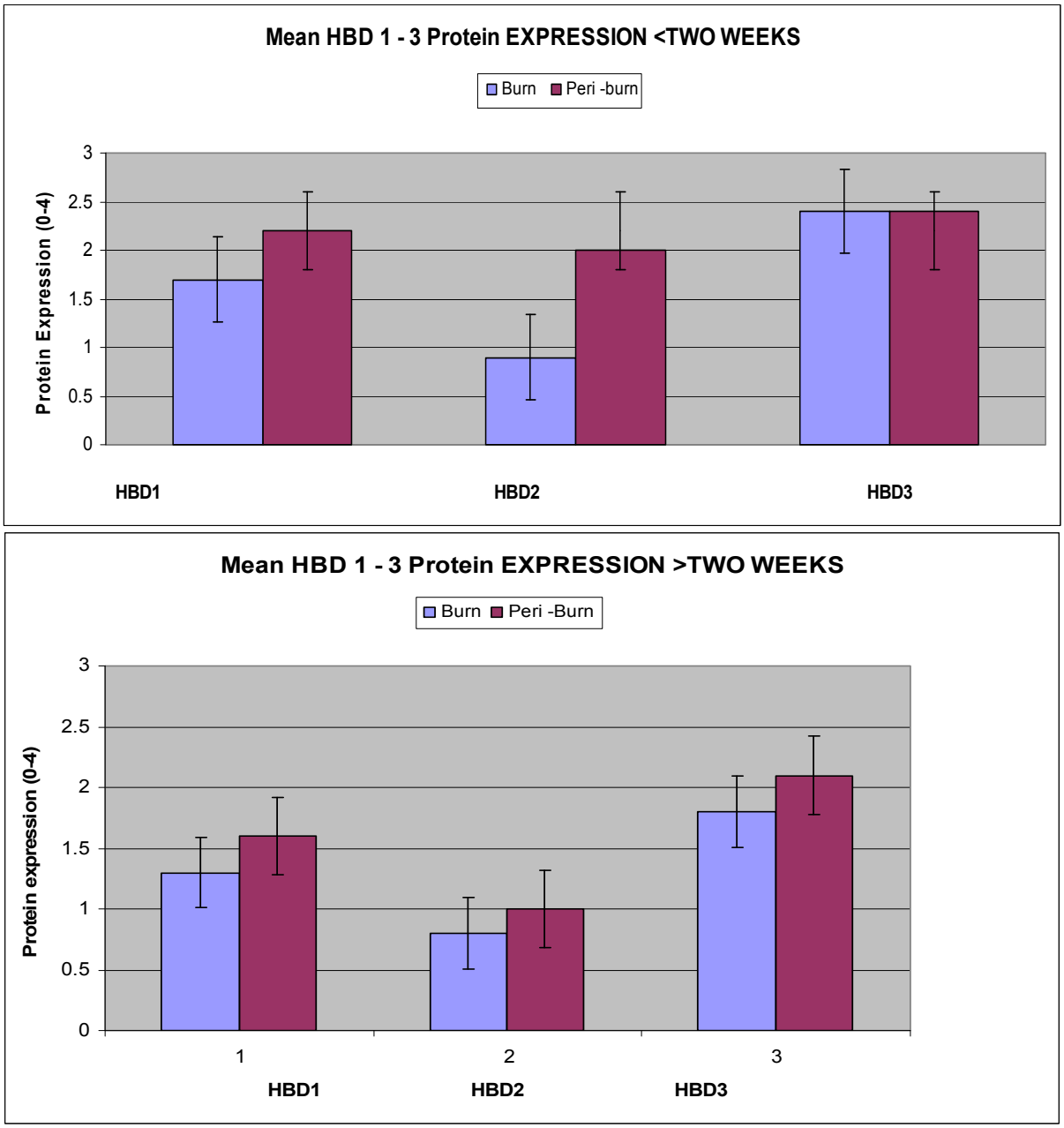


Figure 3.10 The mean HBD1,2 &3 protein expression in the Burn and the Peri-burn area

The upper panel shows the expression in the early (<2 weeks or B group), while the lower panel shows the expression in the late (>2 weeks or C group). The bars represent the standard error.

Table 3.8: Paired samples test-comparing the RNA and protein expression between the burn and peri-burn samples in both the early and late burn groups

		Paired Differences					t	Df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	burnB1RNA - periburnB1RNA	1.3	4.3	1.3	-1.6	4.2	1.0	10.0	0.4
Pair 2	BurnB2RNA - PeriburnB2RNA	0.9	3.3	1.0	-1.3	3.1	0.9	10.0	0.4
Pair 3	BurnB3RNA - PeriburnB3RNA	1.5	2.9	0.9	-0.5	3.4	1.7	10.0	0.1
Pair 4	BurnproteinB1 - PeriburnproteinB1	-0.4	1.2	0.3	-1.0	0.2	-1.5	18.0	0.1
Pair 5	burnproteinB2 - periburnproetinB2	-0.7	1.6	0.4	-1.5	0.0	-2.1	18.0	0.1
Pair 6	burnproteinB3 – periburnpreoteinB3	-0.1	1.2	0.3	-0.7	0.5	-0.4	18.0	0.7

Table 3.9: Paired samples test; comparing the RNA and protein expression of HBD1,2& 3 between burn and the peri-burn samples in the early (< 2 weeks) samples

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	burnB1RNA - periburnB1RNA	2.6	4.5	2.0	-3.0	8.2	1.3	4.0	0.3
Pair 2	BurnB2RNA - PeriburnB2RNA	1.0	4.1	1.8	-4.0	6.0	0.6	4.0	0.6
Pair 3	BurnB3RNA - PeriburnB3RNA	2.2	2.4	1.1	-0.8	5.2	2.1	4.0	0.1
Pair 4	BurnproteinB1 - PeriburnproteinB1	-0.5	1.1	0.3	-1.2	0.3	-1.3	10.0	0.2
Pair 5	burnproteinB2 - periburnproetinB2	-1.1	1.9	0.6	-2.3	0.2	-1.9	10.0	0.1
Pair 6	burnproteinB3 - periburnpreoteinB3	0.1	1.4	0.4	-0.8	1.0	0.2	10.0	0.8

Table 3.10 :Paired samples test-comparing the burn and peri-burn RNA and protein expression between late burn samples

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	burnB1RNA - periburnB1RNA	0.2	4.2	1.7	-4.3	4.6	0.1	5.0	0.9
Pair 2	BurnB2RNA - PeriburnB2RNA	0.8	2.9	1.2	-2.2	3.9	0.7	5.0	0.5
Pair 3	BurnB3RNA - PeriburnB3RNA	0.8	3.4	1.4	-2.7	4.4	0.6	5.0	0.6
Pair 4	BurnproteinB1 - PeriburnproteinB1	-0.4	1.4	0.5	-1.6	0.8	-0.8	7.0	0.5
Pair 5	burnproteinB2 - periburnproetinB2	-0.3	0.9	0.3	-1.0	0.5	-0.8	7.0	0.5
Pair 6	burnproteinB3 - periburnpreoteinB3	-0.4	0.9	0.3	-1.1	0.4	-1.2	7.0	0.3

The tables 3.8, 3.9 & 3.10 show the results of paired sample t-tests comparing the β defensin 1, 2 & 3 protein and RNA expression between burn and periburn samples, early and late burns. The difference between the groups was not statistically significant.

Table 3.11. Microbiological analysis

Patient code	Age	% Burn	Duration Of Burn	Variety of organisms	Organism 1	Organism 2	CFU/gram
1B	29	1	7	2	Coagulase negative *Staphylococci*	*Klebsiella.sp.*	10^7
2B	70	8	3	1	*S. aureus*		10^2
3B	67	2	5	0	None		
4B	24	1	7	0	None		
5B	30	1	10	0	None		
6B	41	2	2	3	Coagulase negative *Staphylococci*		10^3
7B	42	4	3	2	*E.coli*	Coagulase negative *Staphylococci*	10^5
8B	47	5	7	2	*S. aureus*	Coagulase negative *Staphylococci*	10^7
9B	50	1	2	2	*Coryneform* bacteria		10^5
10B	47	15	3	2	*S. aureus*	Coagulase negative *Staphylococci*	10^6
11B	67	1	6	2	Coagulase negative *Staphylococci*	*E.coli*	10^6
2C	36	6	14	1	Coagulase negative *Staphylococci*		10^3
3C	38	2	16	1	*MRSA*		10^6
4C	64	6	14	1	Coagulase negative *Staphylococci*		10^6
5C	88	1	60	4	Coagulase negative *Staphylococci*	*Proteus. sp.*	10^5
6C	27	20	30	1	*Pseudomonas.sp.*		10^5
7C	58	30	21	2	Coagulase negative *Staphylococci*	*E.coli*	10^7
8C	47	2	14	1	*Bacillus sps.*		10^6

The results presented in table 3.11 show that 3 patients had no detectable micro organisms and 7 had 2 types of organism present. The table also shows the duration of the burn and the organisms are presented as the number of colony forming units per gram of tissue.

Table 3.12. Correlation between microbes and % burn

Percentage of burn	Duration of Burn(days)	Organism 1	Organsim 2
1	7	*Coagulase-ve Staphylococci*	Klebsiella.sp
1	10	*Coagulase-ve Staphylococci*	*E.coli*
1	7		
1	10		
1	60	*Coagulase-ve Staphylococci*	*Others-Proteus*
1	2	*Others-coryneform bacteria*	
2	5		
2	*16*	*Methicillin resistant S.aureus*	
2	14	*Others-Bacillus sps*	
4	3	*E.coli*	*Coagulase-ve Staphylococci*
5	7	*S. aureus*	*Coagulase-ve Staphylococci*
6	14	*Coagulase-ve Staphylococci*	
6	14	*Coagulase-ve Staphylococci*	
8	3	*S. aureus*	
15	3	*S. aureus*	*Coagulase-ve Staphylococci*
20	30	*Pseudomonas.sp*	
30	21	*Coagulase-ve Staphylococci*	*E.coli*

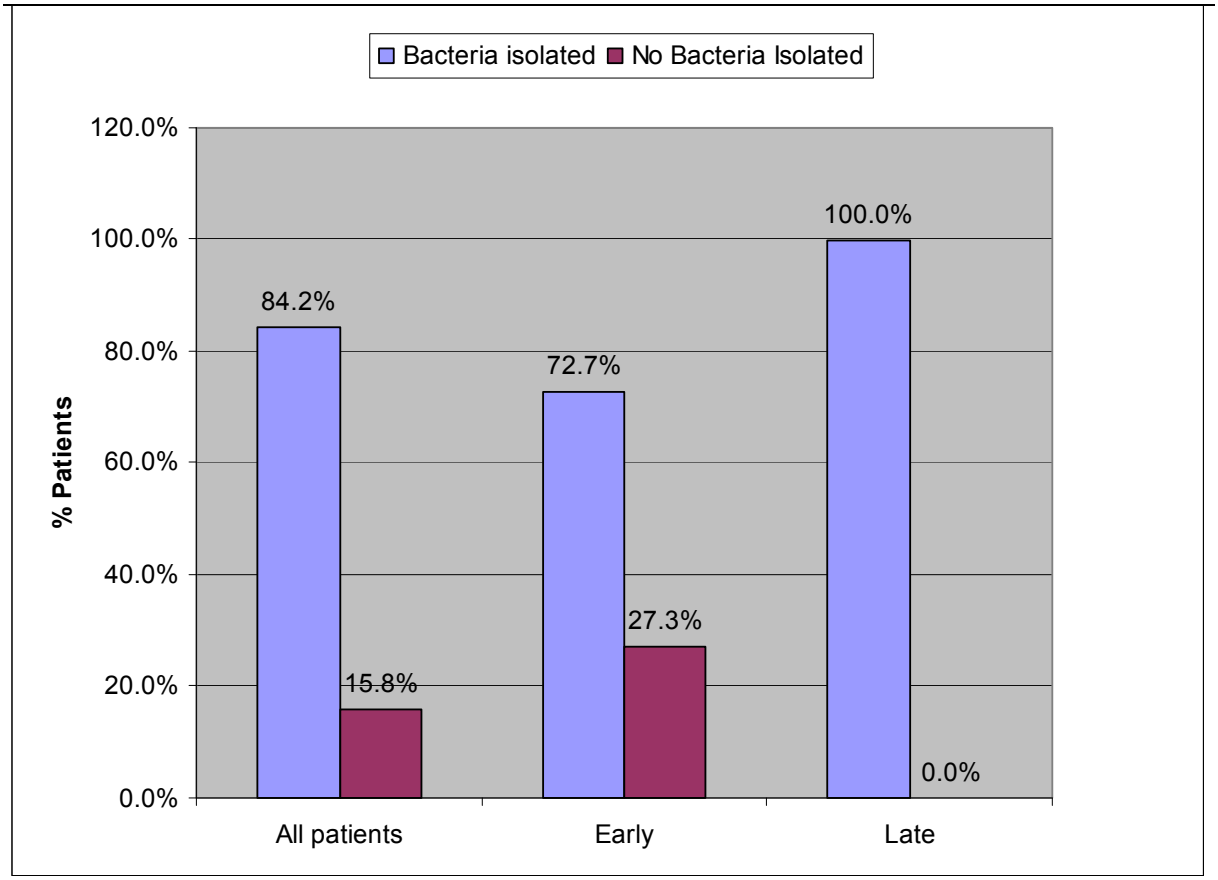


Figure 3.11 Summary of the samples from which bacteria were isolated and in what numbers.

As can be seen from the figure 3.11, bacteria were isolated from all the late burn patients whereas only 72.7% of the early group patients showed the presence of bacteria.

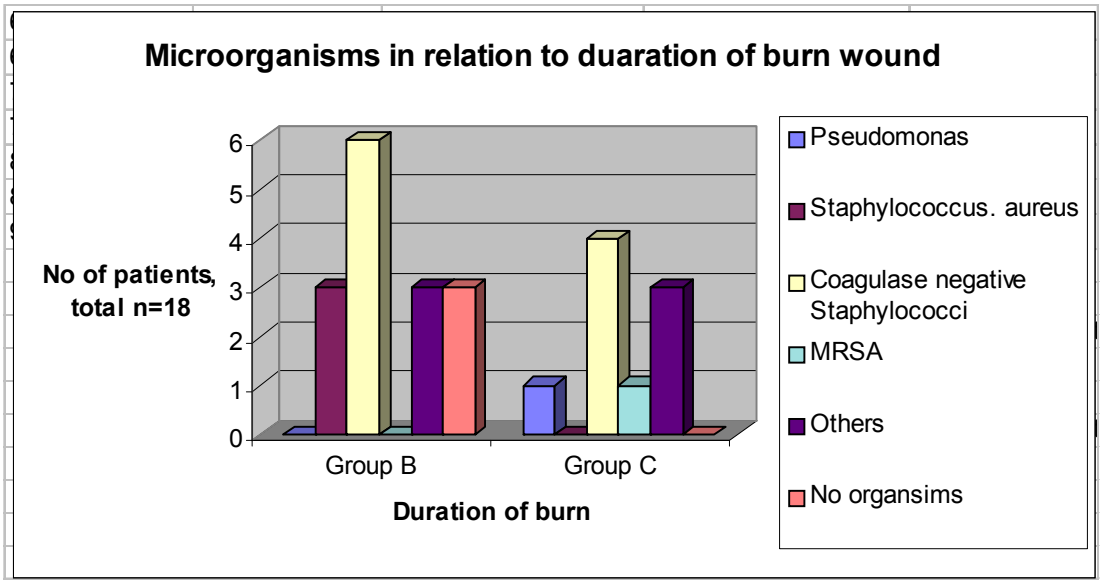


Figure 3.12- The change in the type of flora with the duration of the burn wound.

As seen in figure 3.12 the predominant organisms change from skin commensals (e.g Coagulase negative *Staphylococci*) into more resistant forms (e.g, Methicillin resistant *Staphylococcus aureus* (MRSA)) with increase in duration of the burn wound.

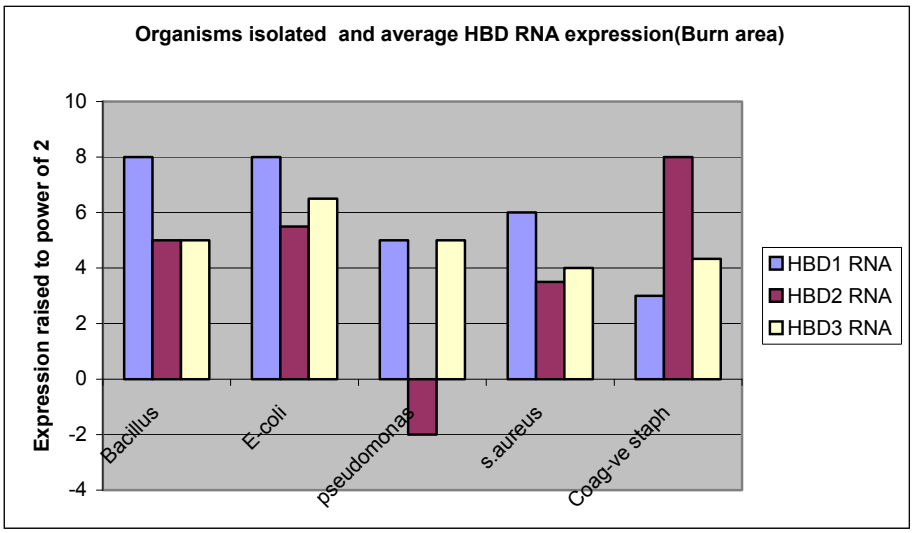


Figure 3.13 The average HBD mRNA expression in relation to micro-organisms present.

All three β defensins were expressed in samples from which organisms were isolated as shown in figure 3.13. It was seen that the HBD1 was relatively highly expressed in samples from which *Bacillus* sp.and *E.coli* were isolated. HBD2 expression was markedly decreased in samples from which *Pseudomonas* was isolated and was highly expressed in samples from which coagulase negative *Staphylococci* were isolated. *HBD3* RNA was highly expressed in all samples irrespective of the type of organism present. The expression was relatively higher in samples with *Bacillus, E.coli, Pseudomonas* and lower in samples with *Staphylococci*.

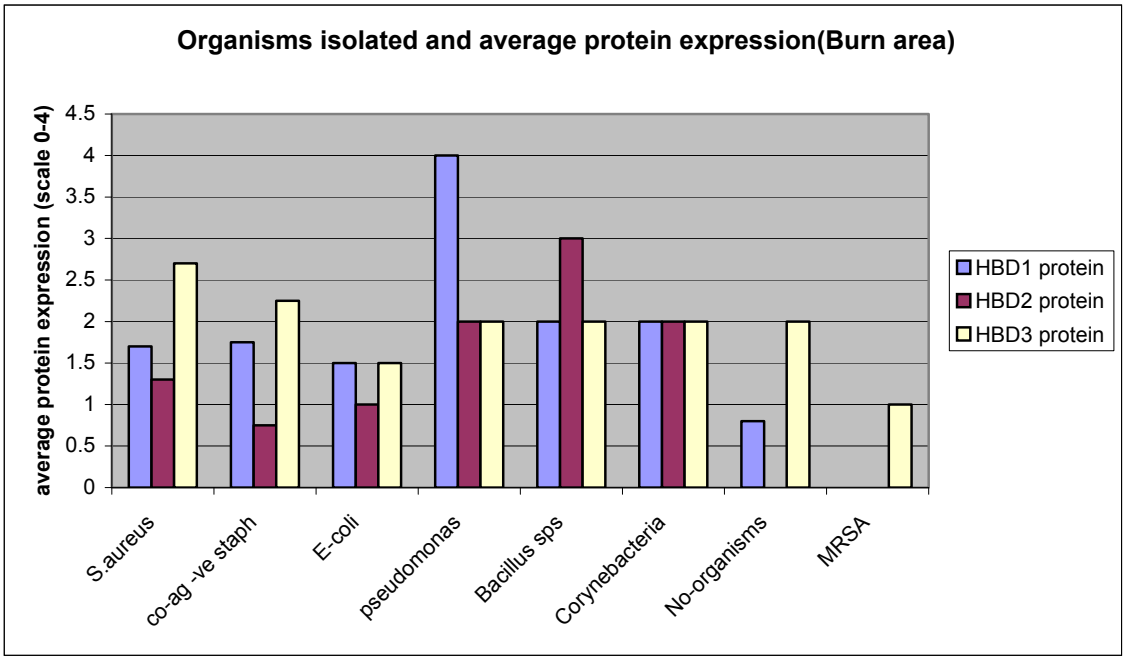


Figure 3.14 The average HBD protein expression and organisms isolated from the patient burns.

It can be seen from figure 3.14, that with the exception of samples in which MRSA was isolated, all the three HBD proteins were expressed in all the patient samples from which organisms were isolated. There was no peptide expression of HBD1 and 2 in this sample in

the sample colonised with MRSA. The samples in which no organisms were isolated showed no expression of HBD2 protein and HBD1 was expressed in relatively lower amounts.

3.4 Discussion

Infection is one of the important causes of mortality and morbidity in burns patients. This is related to loss of skin, which serves as a mechanical and biological barrier. Alteration in the expression of HBDs, which form a part of the biological cover, may play a role in burn wound infections. It has been shown previously that the expression of *HBD2* is reduced at mRNA and protein level in burn skin(Milner and Ortega, 1999, Bick et al., 2004). The expression of *HBD3* which is specifically active against S*taphylococci* has never been studied in burn wounds.

The common organisms implicated in burn wound infections are resident skin flora in early infections followed by resistant organisms such as *Pseudomonas* sp and MRSA with increase in the duration of the burn wound. Burn wounds are characterised by systemic and local responses. Local responses vary within the extent of the burn. Three zones namely zone of coagulation, zone of stasis and zone of hyperaemia have been described in a burn by Jackson in 1947 (figure.3.15).

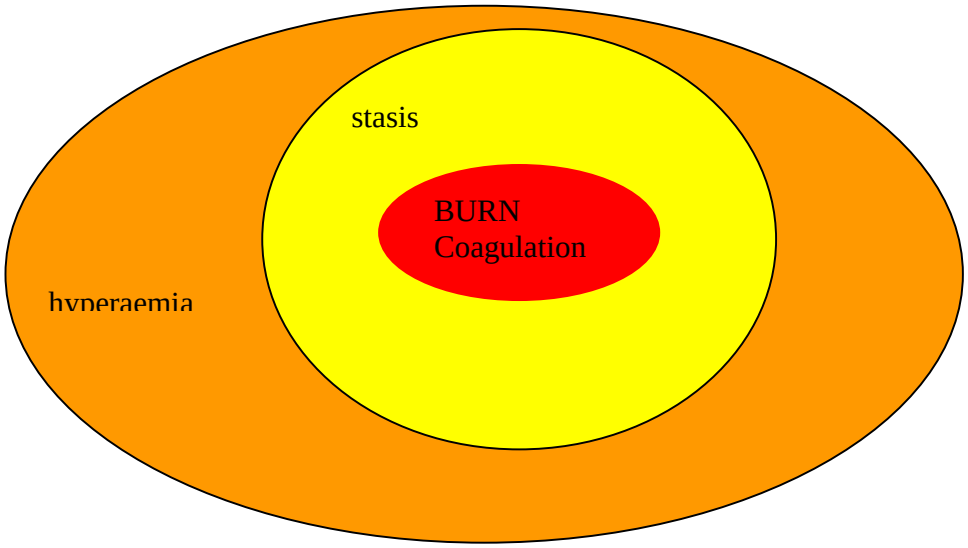


Figure 3.15. Diagrammatic representation of the 3 zones of of a burn

The central zone is characterised by coagulation of the proteins and is the actual part which has been in contact with the hot surface. The zone of stasis has decreased perfusion but the tissues themselves are salvageable and no irreversible damage has occured in this zone. How the tissues in this area recover depends on the management and presence of complications like infection. The tissues may completely recover or progress to a state of irreversible damage. The zone of hyperaemia is characterised by increased perfusion and intense inflammatory activity, but the tissues themselves recover with time unless secondary tissue loss following infection occurs(Hettiaratchy and Dziewulski, 2004b). The burn area in our study would include zone of coagulation and variable amounts of zone of stasis, which would be specific to the nature of the insult causing burn and the patient. The periburn area would comprise zone of stasis to a variable extent and the zone of hyperaemia; thus being characterised by vasodilatation and influx of inflammatory mediators.

This is the first study looking at the expression of *HBD 1, 2, 3* at protein and mRNA levels simultaneously with quantitative analysis of the flora in the burn wounds of different durations. Our study demonstrates that all the three defensins are expressed in burn wounds at mRNA level in all patient samples in which RNA was extractable. This suggests that living cells in the burn and periburn areas have increased expression of *HBD* mRNA. Burn injury is followed by release of inflammatory mediators such as IL1 and TNF-α . These mediators, *in vitro,* are known to enhance the expression of HBDs(Bick et al., 2004). The expression of *HBD* was studied in relation to psoriatic tissue expression normalised to the housekeeping gene β*-Actin*. The expression of mRNA in most of the samples was elevated and much higher than the expression in psoriatic tissue. It is known from previous studies that psoriatic tissue has elevated level of HBD 1, 2 and 3 expression(Harder et al., 2001). There was variation in the level of expression between the patient samples; this was expected as the expression of HBDs is influenced by a number of factors such as the degree of immunosuppression, percentage of burn, age of the patient and the site of burn. Although it would have been ideal to have a number of psoriatic samples for comparison purposes it was practical to get only one and this was provided by the Dermatology Department at Chelsea and Westminster Hospital. The ideal controls for this type of study would have been burn wound specimen and tissue from the corresponding non-burn area from the same person, as HBD expression is known to vary between individuals, and between different sites in the same person(Ali et al., 2001). Since this is unethical it was decided to take samples from the burn area and the peri burn area; the closest available analogue of the near normal skin. We considered using graft skin as a specimen of normal skin; but they were usually taken from the thigh which, being close to groin, may have a greater degree of colonisation by micro-organisms. Also the process of

taking a split skin thickness graft is traumatising to the tissue and may serve as a triggering factor for the stimulation of inflammatory mediators and cytokines, enhancing the expression of HBD. We analysed two graft tissue samples taken from thigh of the patients and the expression of the three HBD was found to be elevated and higher than the expression in psoriatic tissue.

The distinction between burn and periburn is relative, being observer dependant. To avoid such bias all the samples were collected by a single observer and the patients included in the study were under the care of one of the two consultant surgeons working in the unit. The other possibility for controls would have skin samples from patients from different sites; this was also not practically possible although we obtained a normal abdominal skin sample from one patient to use as a control. Since the expression of HBD is inducible and hence may vary for normal skin depending on the nature of the flora inhabiting the area, we decided to discuss the expression of our experimental samples in relation to the psoriatic sample.

RNA was not isolated from some of the patient samples either from the burn or periburn area. Thus, unless the samples had paired burn and peri-burn RNA they were not considered for expression analysis at RNA level. It is probable that in the burn area there were no living cells or any living cells in the remnant hair follicles. Since the description of burn and periburn area is relative it is possible that the periburn area was also affected by the burn injury and the RNA was destroyed. Also the necrotic slough and the exudates present in the wound are rich in RNAses, which might have resulted in the destruction of RNA thus affecting the RNA extraction. The number of living cells in the tissue is lower compared to the healthy normal

tissues and the extraction of RNA depended on meticulous extraction technique and stringent methods of avoiding RNAses. The tissues were stored at optimum temperatures for storage of material for RNA extraction. However any slight lapses in the transport and storage of the tissue might have impacted on the final RNA extraction.

The quality of the extracted RNA was variable with clear bands seen for some samples demonstrating good quality RNA while others had only a smear. All the samples showing some RNA were processed further for cDNA conversion and amplification of the specific genes. Some of the samples, which had low quality RNA, and hence did not show clear peaks for the products of the *HBD* genes or the housekeeping genes, were taken as samples from which RNA could not be isolated.

The melting temperature of the products within the same experiment varied within 2°C for most of the samples, however some samples showed a single peak for the product, but a melting temperature about four degree lower than the expected melting temperatures. This could be either due to the presence of chelating salts or presence of polymorphisms in the sample showing alteration of Tm. The other possibility of contamination was also considered but the Tm altered between one of the two melting temperatures and a single peak was observed(Ririe et al., 1997).

The expression of *HBD3* in the samples was characterised by one of the two melting temperatures either 79-80ºC or 84ºC. Since this alteration of Tm was consistent it is possible

that HBD3 is characterised by a common polymorphism, which can account for this alteration of Tm.

The expression of *HBD1, 2, 3* mRNA for most of the samples was seen to be markedly elevated as compared to the expression in the psoriatic tissue. The protein expression of HBD1, 2 and 3 however was relatively lower than the psoriatic tissue expression. The reason for the difference in the relative expression at mRNA and protein levels is unclear. This may be related either to failure of the conversion of the RNA message to protein due to failure or underdeveloped post transcriptional machinery, or to the coagulation of the proteins following the burn injury. The inability of the RNA message to be translated into the protein message may result in low expression of the HBD. This effect is more pronounced as not all the cells are viable and among the viable cells the expression is altered. Thus both these factors can account for higher incidence of infections in burn patients.

Our study confirms the findings of previous researchers that there is a drift towards more resistant organisms with increase in duration of burn wounds. The study also demonstrates the expression of *HBD1, 2, 3* at mRNA level is increased in relation to the presence of different organisms. The expression of *HBD2* RNA was decreased relatively in samples from which pseudomonas is isolated. Although the expression of HBD2 was present at protein level the low mRNA may indicate failure of the cell machinery to enhance the expression further and thus may be related to high incidence of *pseudomonas* infections in burn patients. The cause of this decreased expression is unclear. The expression of HBD2 protein was absent in burn samples where either no organisms were isolated or MRSA was isolated. In the sample with MRSA there was no expression of HBD 1. This is perplexing, as HBD 1 is known to be

constitutively expressed. This expression could not be confirmed at mRNA level as no RNA could be extracted from this sample. Either the expression of the protein may have been low or coagulation of the proteins may account for the failure of detection. The low expression of HBD1 and the presence of MRSA can be correlated to failure of cell differentiation resulting in decreased expression of antimicrobial peptides leading to susceptibility to infection by resistant organisms as HBD1 is known to play a role in cell differentiation(Frye et al., 2001).

Conclusion

Thus we conclude that among surviving cells in the burn and in the periburn area the mRNA expression of *HBD 1, 2* and *3* is increased to levels comparable or higher than psoriatic tissue levels. The expression at mRNA level is relatively higher in the burn area relative to the periburn area. The protein level expression does not relate with RNA expression levels; the overall expression being lower and relatively expression being higher in the periburn area as compared to the burn area. This low level of protein expression need to be confirmed on larger *in vitro* studies with prospects of preventing the burn wound infections by supplementing the HBD protein in burn wounds.

Chapter 4

Expression analysis of HBDs (HBD1, 2 & 3) in peripheral blood cells; comparison of profiles between septic (microbiologically positive and negative) and healthy individuals.

4.1 Introduction

The increasing development of bacterial resistance to traditional antibiotics has required the development of new antimicrobial agents. Defensins, which are natural antimicrobial peptides, have been shown to be key elements in the innate and adaptive immune system of many organisms representing the first line of defence against invading microbes. These natural antibiotics possess novel mechanisms of action and different cellular targets compared with existing antibiotics.

The basic role of mammalian defensins is direct antimicrobial activity which occurs mainly in the phagocytic vacuoles of macrophages and on the surface of the skin and mucosa through the formation of multimeric pores within the bacterial cell membrane causing lysis(Hoover et al., 2000). These antimicrobial activities present new opportunities of using defensins as effective antibiotics or for construction of their more effective derivatives.

Human β defensins

The first HBD was isolated from hemofiltrate in 1995. Subsequently, four other HBDs were identified as human epithelial defensins that have been suggested to play a pivotal role in the innate and adaptive immune responses in epithelial tissues.

***Functions of human β defensin**:*

The main function of HBD is as a regulator of innate antimicrobial immunity seems to be either the killing of bacteria and fungi on the epithelial surface of higher organisms or of phagocytosed microorganisms within phagolysosomes of phagocytes. HBDs also play an important role in adaptive immunity.

A recent study indicates that human β defensin 1 (HBD-1) is chemotactic for immature dendritic cells and HBD-2 is chemotactic for memory CD 4^+ T-cells(Garcia et al., 2001a). HBD-3 is also chemotactic for freshly isolated peripheral blood monocytes which initiate in turn the adaptive immunity(Yang et al., 2001). Thus, HBDs may promote adaptive immune response by attracting and recruiting dendritic cells and memory T-cells to the site of microbial invasion through the interaction between HBDs and chemokine receptor 6 (CCR6) on the surface of the cells of adaptive immunity (Yang et al., 1999).

Tissue expression of human β defensins

Human β defensin 1 (HBD-1) and human β defensin 2 (HBD-2) are constitutively expressed by epithelial and mucosal cells in many organs such as kidney, pancreas, gingival, airway tracts, and female reproductive tracts(Valore et al., 1998, Seo et al., 2001, Krisanaprakornkit et al., 1998, Dale et al., 2001, Bals, 2000, Alp et al., 2005). HBD-2 expression is up-regulated by exposure to bacterial cell wall components such as lipopolysaccharides (LPS) and proinflammatory cytokines such as tumour necrosis factor (TNFα). HBD-3, which was first isolated from psoriatic scales, is expressed in both epithelial and non epithelial cells and is considered to have diverse functions(Harder et al., 2001, Dhople et al., 2006). Recently, HBD-

4 was identified and its expression was observed in human testis(Garcia et al., 2001b) and genetically modified keratinocytes(Smiley et al., 2007).

Recently, the expression of HBD-1 and HBD-2 has been detected in monocytes and macrophages and their expression increased after exposing cells to bacterial cell membrane components such as LPS or proinflammatory cytokines such as IFNγ in an *ex vivo* environment(Duits et al., 2002).

To date, it is not known how the expression of HBDs in peripheral blood leukocytes is altered *in vivo* in response to systemic inflammatory disease or by invading bacteria (bacteraemia and septicaemia) and whether different types of HBDs are induced in response to different types of septicaemia.

Peripheral blood leukocytes are among the first cells to be recruited to sites of infection and inflammation following platelet aggregation. It is not known if these act by producing HBDs as well as being directly phagocytic. Fang *et al.* demonstrated that HBDs were produced by leukocytes on *ex-vivo* challenge by bacteria(Fang et al., 2003) and recently a study showed that leucocytes with patients with septicaemia have decreased inducibility to BD2(Book et al., 2007). Each of the three HBD's seems to have a more specialised function and also more specific activity against particular subgroups of microbes. As sepsis and infections result from a variety of bacteria we investigated the *in vivo* expression of HBD 1, 2 and 3 in patients with microbiologically positive and microbiologically negative sepsis (systemic inflammatory response syndrome) and compared the expression with that of healthy volunteers. There have been no studies to date ascertaining the expression of these peptides *in vivo* in relation to the circulating bacteria.

Thus the aim of this study was to analyse peripheral blood leukocytes from patients with septicaemia and compare the expression profiles of HBD 1, 2 and 3 with those of normal healthy subjects in order to determine whether the expression levels were increased in peripheral blood leukocytes in response to systemic infections.

The future aim of this study is to differentiate individuals who are more susceptible to develop septicaemia from those who have lesser predisposition to septicaemia dependent on the natural expression pattern of HBDs in their peripheral blood leukocytes. Moreover, HBD as natural antibiotics could be used as therapeutic agents to combat the bacterial infections in patients with septicaemia when bacterial strains have not yet developed resistance against these natural antibiotics.

4.2 Materials and Methods

The blood samples of patients and control groups were collected at West Wales General Hospital, Carmarthenshire NHS Trust, Carmarthen, Wales. The study was undertaken after obtaining a favourable ethical committee approval.

Samples were collected from clinically septic patients and peripheral blood counts and blood cultures were performed at the same session. Leukocytes were isolated from fresh peripheral blood from patients and control groups, to be analysed at RNA and protein level, using density gradient centrifugation with Histopaque^{TM}. For RNA extraction the leukocytes were frozen at –80°C in SV total RNA lysis buffer (Promega, UK), and for immunohistochemical purposes they were fixed in 4% paraformaldehyde in PBS. The details of the methods were as described in the Chapter 2 – General Methodology. After processing 20 samples, only 3 were

found to have a positive blood culture even after 48 -72 hours; leading to doubts as to whether organisms had been present in the samples or if the plates had been contaminated. To utilise the resources more efficiently the method was modified and the samples which were microbiologically positive within 24 hours were taken as positive. One ml of the blood was aliquoted and the leukocytes were isolated after lysing the RBC's with SV RNA lysis solution (Promega, UK). The microbiologically positive samples were then processed at the end of 24 hours. The drawback with this method was that the blood sample had to be stored at 4°C for 24 hours and it is well documented that this does result in decrease of RNA yield. However considering the paucity of resources and given that PCR is a very sensitive technique it was decided to proceed with the modification. The leucocytes were pelleted and re-suspended in 175µl of SV total RNA lysis buffer (Promega, UK), and stored at –80°C until further processing. The remaining blood sample was snap frozen and stored at –80°C.

Thus the final inclusion criteria for the study were:

For the microbiologically positive group

- Clinical suspicion of sepsis
- Microbiologically positive blood cultures with 24 hours of incubation
- Adults
- Able to give an informed consent
- Not on immunosuppressive medication and no history of haematological and immunosuppressive diseases.

For the microbiologically negative group

- Clinical suspicion of sepsis
- Microbiologically negative blood cultures after 5 days of incubation
- Adults
- Able to give an informed consent
- Not on immunosuppressive medication and no history of haematological and immunosuppressive diseases

For healthy controls

- Healthy without any acute or chronic symptoms
- Adults
- Not on any medications
- Not suffering from immunosuppressive conditions

HBD gene expression*:*

HBD gene expression was analysed at RNA and protein levels by reverse transcriptase PCR and immuno-cytochemistry respectively.

HBD gene expression analysis at RNA level

In this study, the expression of three different β defensins (*HBD-1, HBD-2* and *HBD-3*) was analysed at mRNA level by extracting total RNA from isolated leukocytes and converting it into cDNA. The concentration of RNA in each of the samples and the OD at 260/280 (a reflection of the quality of the RNA) is shown in table 4.1. This was then analysed using RT-PCR and quantitative RT-PCR. The methods were as described in Chapter 2 – general methodology.

Table 4.1 The OD 260/280 ratio and the concentration of the extracted RNA in ng/μl from each patient sample.

Patient number	*260/280 ratio*	*RNA concentration (ng) per 1 μl*
BE1	1.85	17.43
BE3	1.82	9.83
BE4	1.97	31.66
BE5	1.96	26.65
BE6	1.95	27.87
BE7	1.8	24.1
BE8	1.66	21.5
BE9	1.67	13.3
BE10	1.72	38.4
BE11	1.72	44.0
BE12	1.85	29.3
BE13	1.94	36.7
BE14	1.89	29.1
BE15	1.81	31.7
BE16	1.86	35.3
BE17	1.94	43.1
BE18	1.93	46.4
BE19	1.90	66.7
BE20	1.90	34.6
BE21	1.95	95.9

Patient number	*260/280 ratio*	*RNA concentration (ng) per 1 μl*
BC1	1.98	57.6
BC2	1.79	18.2
BC2A	1.89	51.7
BC3	1.57	110.8
BC3A	1.79	22.1
BC4	1.89	49.9
BC4A	1.89	46.7
BC5	1.75	45.3
BC5A	1.83	36.0
BC6	1.84	15.8
BC7	1.79	28.5
BC7A	1.73	16.7
BC8	2.0	31.9
BC8A	1.77	28.6
BC9	1.80	30.7
BC9A	1.71	39
BC10	1.72	33.9
BC11	1.96	49.4
HC1	1.73	36.93
HC2	1.68	40.23
HC3	1.72	67.45

RNA was diluted in 10µl of nuclease free water. Two µl of the sample was used to check OD and to calculate the concentration. The volume added per reverse transcriptase reaction was adjusted so that the concentration of mRNA was 200-500ng per reaction.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR):

RT-PCR is a sensitive technique to detect mRNA expression of specific gene using specific primers and cDNA as a template. After cDNA synthesis had been performed, PCR was carried out for three different *defensin* genes using three different primer pairs for *β defensins 1, 2* and *3*. Expression of the *β-actin* gene was used as internal control to ensure that the cDNA, which was served as a template, was synthesised correctly and PCR was carried out properly.

Classical RT-PCR was initially performed and the samples were run on 2% agarose gel stained with ethidium bromide (EtBr). *B actin* gene expression was detected but there was no detecable expression of the *defensin* genes. The minimum amount of DNA required for detection using EtBr and photography is 2ng in a 0.5cm wide band(Joseph Sambrook and David W. Russell., 2001). Since the expected copy number of these genes was likely to be low, it was decided to use the more sensitive quantitative RT-PCR to detect any possible expression compared to the controls. To keep the intra-experimental errors to minimum it was decided to perform a duplex real time PCR combining primers for the housekeeping gene *β actin*, and the *HBD* gene in each of the tubes and also the PCR was performed for 45 cycles. Some of the products were re-amplified and run on gels to confirm the products. Tables 4.2 and 4.3 show the sample set up and the cycling conditions for quantitative duplex RT-PCR reaction respectively.

Table 4.2 : Sample mixtures for quantitative duplex PCR.

Tube contents	***Tube 1 β defensin 1+β actin***	***Tube 2 β defensin 2 +β actin***	***Tube 3 β defensin 3+β actin***
PCR Master Mix	12.5 μl	12.5 μl	12.5 μl
Nuclease-free* water	5.5 μl	5.5 μl	5.5 μl
cDNA*	2 μl	2 μl	2 μl
Defensin Primer+Probe mix (TET)	2.5 μl	2.5 μl	2.5 μl
Actin Primer+Probe mix(FAM)	2.5 μl	2.5 μl	2.5 μl
Total	25μl	25μl	25μl

**volumes were adjusted to ensure optimal template cDNA concentration*

Table 4.3. Optimised cycling conditions for the duplex PCR

Description	*Number of Cycles*	*Temperature° C*	*Time*
Hotstart activation	**1**	**95**	**15 minutes**
Amplification cycles	**44**	**94 (Denaturation)**	**45 seconds**
		56 (Annealing)	**45 seconds**
		72 (Extension)	**45 seconds**
Final extension	**1**	**72**	**5 minutes**

Immunocytochemistry

The leucocytes were fixed in 4% paraformaldehyde and a Cytospin® 3 Cytocentrifuge (Shandon, UK) was used to deposit a monolayer of leukocytes on slides while maintaining cellular integrity for further staining by immunocytochemistry.

Cytospin procedure

After sample chambers had been assembled in the following order – labelled slides with the samples number, filters and sample chambers – 40 μl of suspended fixed leukocytes were added to the sample chamber and spun at 1000 rpm for 5 minutes at low acceleration. Six slides were made for each patient and control sample to be stained with three different β defensins specific antibodies by immunocytochemistry.

Optimising leukocytes slides:

Leukocytes slides that had been obtained by cytospin preparation were checked under the microscope before the immunocytochemistry labelling in order to assure the proper distribution of the cells on the slides. One slide out of six from each sample was stained with Hematoxylin or Giemsa(Lillie, 1977). The distribution and morphology of the leukocytes on the slides were examined by conventional light microscope at 10X (Figure 4.1) and 40 X (Figure 4.2) magnifications.

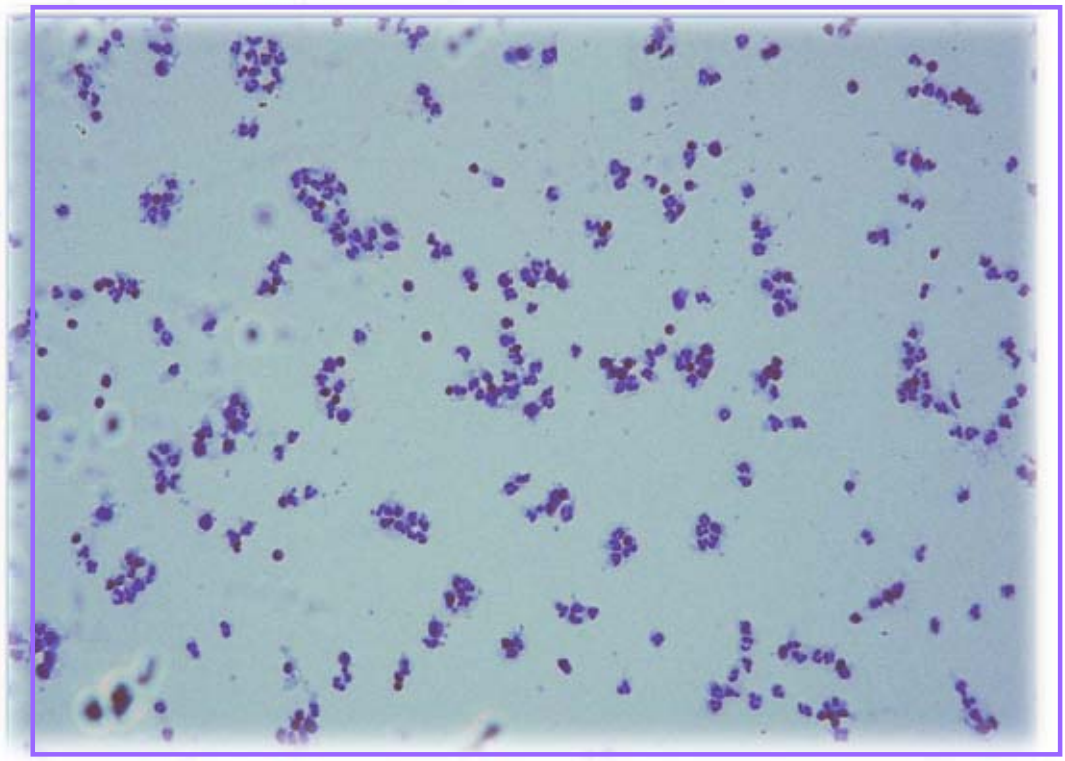


Figure 4.1 Distribution of leukocytes in the leukocyte smear

Leukocytes nuclei appear purple under Giemsa staining. Objective 10X.

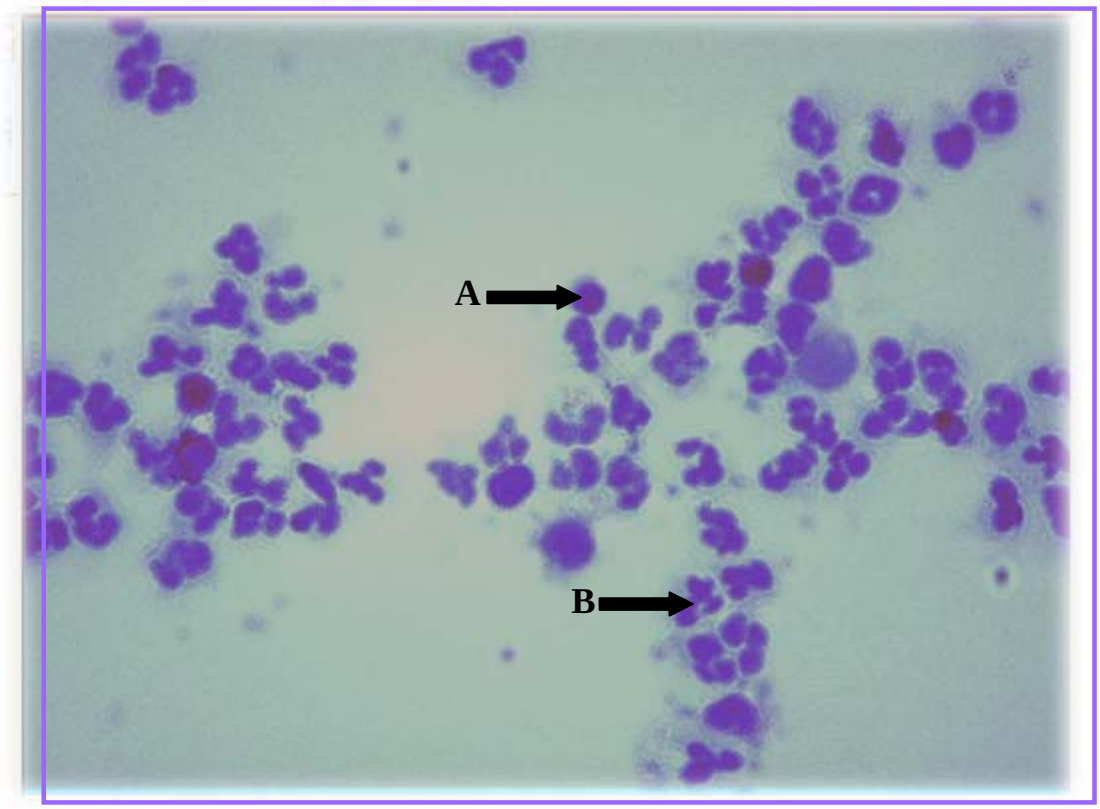


Figure 4.2. A healthy control smear stained with Giemsa

This shows different types of leukocytes in the smear of healthy control sample under Giemsa staining. A: Lymphocyte which can be characterised by dark stained nucleus occupied most of the cells with thin rim of pale cytoplasm around it. B: polymorphonuclear leukocyte (granulocyte) in this case a neutrophil which can be identified by multi-lobed dark stained nucleus. Objective 40X.

Immunocytochemistry labelling and microscopic analysis:

After confirming the morphology of the cells by staining with H&E and Geimsa stains; immunocytochemistry was performed using the commercially available antibodies as described earlier (Chapter 2).

3.3 Results

Twenty patients (male to female ratio 1:1) whose blood cultures were judged positive within 24 hours of incubation were included in the study (Table 4.4). In the blood culture negative group there were 18 patients recruited of which 11 were males and 7 females. The mean age in the microbiologically positive group was 60.65 years and that of the negative group was 60.5 years. The blood samples from 3 healthy volunteers were also included in the study. All three were male and the mean age was 35.3 years. The healthy volunteers were chosen from the younger age group in order to control for the fact that there is scope for a blunted innate immune response in the elderly population. In this experiment, even if the response to infection or inflammation was minimal it would be detected as all results were expressed relative to the healthy controls. The mean body temperatures in the microbial positive and negative groups was 38.46°C and 38.42°C respectively while in the healthy volunteer group it was 36.8°C.The mean duration of symptoms in the two groups were similar; 2.8 days in the positive group and 2.7 days in the negative group respectively.

Table 4.4.shows the Demographics of the Patients and organisms identified

Patient code	**Sex**	**Age(years)**	**Temperature(°C)**	**Duration of symptoms (days)**	**Organisms identified**
BE1	M	51	38.2	3	Streptococcus
BE3	F	57	37.9	4	Staphylococci
BE4	F	82	38.5	2	Staphylococci
BE5	M	61	36.4	5	yeast
BE6	F	65	38.4	2	Staphylococci
BE7	F	88	37.9	2	Streptococci
BE8	M	19	39.2	3	Staphylococci+ Diphtheroids
BE9	M	39	38.2	4	Staphylococci+ Diphtheroids

Patient code	Sex	Age(years)	Temperature(°C)	Duration of symptoms (days)	Organisms identified
BE10	M	88	39.4	2	Staphylococci
BE11	F	78	37.8	1	Staphylococci
BE12	M	64	38.8	3	Streptococcus
BE13	F	69	39.1	4	Staphylococci
BE14	M	75	38.5	2	Staphylococci
BE15	M	72	39.1	2	Streptococci
BE16	M	56	37.8	3	Gram negative bacilli
BE17	F	76	37.9	3	Streptococci
BE18	F	32	39.1	4	Gram negative bacilli
BE19	F	40	39	2	Streptococci
BE20	M	80	37.8	2	Streptoccoi
BE21	F	21	40.1	2	Meningococci
Mean		**60.65**	**38.46**	**2.8**	
BC1	M	65	38.1	2	None
BC2	M	62	39.2	3	None
BC3	F	47	38.2	3	None
BC4	M	65	38.4	2	None
BC5	F	68	37.9	4	None
BC6	M	69	38.1	2	None
BC7	M	40	37.8	4	None
BC8	F	30	38.4	2	None
BC9	M	35	37.9	2	None
BC10	M	70	38.6	5	None
BC11	M	61	39.1	2	None
BC12	F	66	37.9	2	None
BC13	F	50	38	4	None
BC14	F	56	39.2	2	None
BC15	M	75	37.8	3	None
BC16	M	87	39.1	3	None
BC17	F	61	39.7	2	None
BC18	M	82	38.1	2	None
Mean		**60.5**	**38.42**	**2.7**	
HC1	M	29	36.4		None
HC2	M	35	37.1		None
HC3	M	42	36.9		None
Mean		**35.33**	**36.8**		

BE represented samples from culture positive group whereas BC were the culture negative group. HC1,2 and 3 were the healthy controls.

The quality of the extracted RNA was tested by spectroscopic analysis (Table 4.1). RNA was converted to cDNA using an Improm-II^{TM} reverse transcriptase kit (Promega, UK) and quatitative PCR analysis was performed on an Opticon II QPCR machine as described previously. The samples were analysed using the software supplied by the manufacturer and the $2^{-\Delta\Delta Ct}$ method was for expression analysis(Livak and Schmittgen, 2001). It was decided to use relative quantification rather than absolute quantification as the quality of RNA was an important factor in the experiment. As we were working with extremely small volumes of blood determining the expression of the *defensin* genes relative to an internal control measured in the same tube (duplex) with similar primer efficiencies was considered ideal.

Some representative samples were run on 4% agarose gel to confirm the presence of the correct size products. Prior to performing the duplex assay the primers and probes were tested and optimised both individually and in combinations, and the correct size products confirmed. Some of the results of the duplex quantitative RT –PCR, run on gel electrophoresis are shown in Figure 4.3; the top panel shows the assay with HBD1 and actin primers. The *HBD* expression was lower than the *actin* expression as can be seen from the faint HBD1bands. The second panel shows the *HBD2* and *actin* expression while panel 3 shows the *HBD3* and *actin* expression. The *HBD3* expression was clearly higher than *actin* expression.

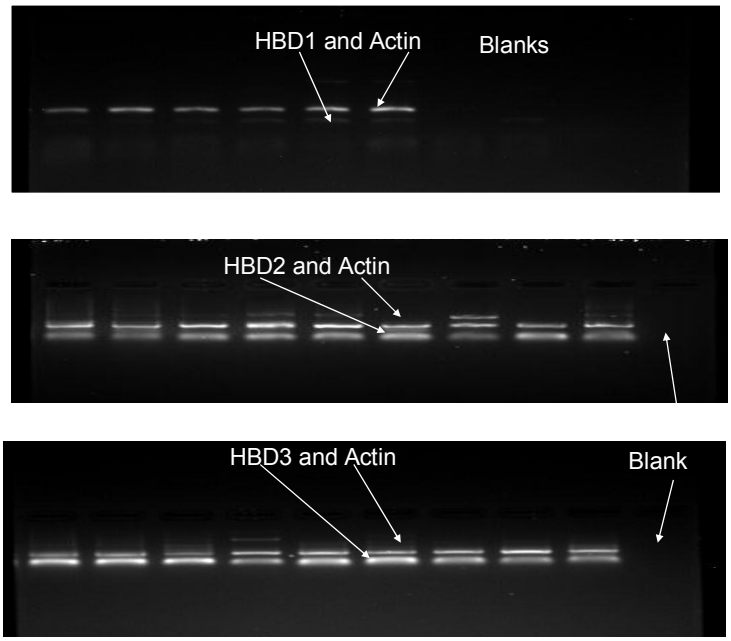


Figure 4.3 The analysis of the amplicons produced in duplex assays of defensins and actin

Table 4.5 The mean HBD expression along with the details of the bacteria isolated in patients in the microbiologically positive group.

Patient code	Microbiology	HBD1RNA Expression ($2^{-\Delta\Delta Ct}$)	HBD2RNA Expression ($2^{-\Delta\Delta Ct}$)	HBD3RNA Expression ($2^{-\Delta\Delta Ct}$)
BE1	Streptococcus	0.3	20.8	504.5
BE3	Staphylococci	1.9	19.2	1254.3
BE4	Staphylococci	1.9	22.1	2351.9
BE5	Yeast	0.8	19.5	1293.6
BE6	Staphylococci	0.7	13.5	2885.6
BE7	Streptococci	11.3	11.8	7889.2
BE8	Staphylococci+ Diphtheroids	32.6	18.8	2687.7
BE9	Staphylococci+ Diphtheroids	0.6	17.7	1382.1
BE10	Staphylococci	4.0	15.5	1750.0
BE11	Staphylococci	2.6	20.1	1754.2
BE12	Streptococcus	8.4	14.6	1747.0
BE13	Staphylococci	6.0	13.1	551.10
BE14	Staphylococci	2.6	16.8	921.71
BE15	Streptococci	6.8	17.7	1949.8
BE16	Gram negative bacilli	9.1	20.7	2487.8
BE17	Streptococci	6.2	16.2	843.7
BE18	Gram negative bacilli	4.8	13.5	465.3
BE19	Streptococci	4.4	15.4	2625.1
BE20	Streptoccoi	4.5	15.4	1097.2
BE21	Meningococci	5.2	13.6	190.5
Mean		**5.7**	**16.8**	**1831.6**
SD		**7.0**	**3.0**	**1638.9**

The patient code is indicated as BE. The end of the table shows the summary statistics in the form of overall mean and the estimate of variability in the form of Standard deviation (SD).

Table 4.5 above shows the summary of the microbiological and PCR findings. As seen all the three *HBDs* were expressed in all the samples irrespective of the type of micro-organisms isolated. The expression of *HBD3* was markedly elevated (mean 1831 times compared to healthy controls normalised to the house keeping gene.)

Table 4.6 ***The mean HBD expression along with the details of the bacteria isolated in patients in the microbiologically negative group.***

Patient code	HBD1RNA Expression ($2^{-\Delta\Delta Ct}$)	HBD2RNA Expression ($2^{-\Delta\Delta Ct}$)	RNA Expression ($2^{-\Delta\Delta Ct}$)
BC1	4.2	12.6	1404.3
BC2	1.7	15.9	461.8
BC3	9.0	20.4	2453.6
BC4	10.8	16.4	5629.0
BC5	1.0	17.1	392.9
BC6	15.2	14.1	1452.8
BC7	30.1	15.0	920.7
BC8	1.9	20.5	378.3
BC9	0.1	14.8	987.5
BC10	0.6	23.4	1126.9
BC11	0.6	17.1	6033.0
BC12	0.1	21.6	860.3
BC13	1.2	17.6	1260.4
BC14	1.2	19.2	608.3
BC15	4.2	16.1	806.0
BC16	0.2	18.0	389.0
BC17	0.3	16.6	468.3
BC18	2.1	14.2	470.9
Mean	**4.7**	**17.3**	**1450.2**
SD	**7.6**	**2.8**	**1678.1**

The patient code is indicated as BC. The end of the table shows the summary statistics in the form of overall mean and the estimate of variability in the form of Standard deviation (SD).

Table 4.6 above shows the summary of PCR findings in microbiologically negative patients. The expression of all the three *HBD*s was seen in all the samples. The expression of *HBD3* was markedly elevated (mean expression was 1450 times expression in healthy normal controls normalised to the housekeeping gene *β actin)*.

Immunocytochemistry

Immunocytochemistry was performed using three different specific commercially available primary antibodies against three different β defensins HBD1, HBD2 and HBD3. The binding was visualised using DAB (Diamino-Benzidine) (DAKO, UK). The methods were as described in Chapter 2. Negative controls were designed where no primary antibodies were added, instead horse or rabbit serum was used. Positive controls were in the form of skin and buccal epithelial samples which were previously shown to express the HBD.

The aim of the immunohistochemical staining was to localise the source of HBD production in *vivo* HBD's are known to be secretory proteins and on stimulations more than 70% of the protein is released into circulation from the cells. Depending on the degree of stimulation, variable amounts of the synthesised peptide are released into the circulation and hence individual cells were expected to be variably stained depending on the degree of synthesis and the peptide released into circulation. In our patients however no protein could be distinctly visualised in the polymorphonuclear cells. There was a halo of DAB around individual cells and this was considered as background stain (Figure 4.4). However this was variable and more marked around some cells compared to others. This variability was persistent in spite of using different concentrations of the antibody and variation in times of development with DAB. Since HBD's are cytoplasmic proteins and are secretory in nature, it can be expected that the protein would be localised in the perinuclear region. Due to variability in the intensity of this halo in the perinuclear region it can be considered that some of the cells expressed HBD1, 2 & 3 proteins. However this staining was of very low intensity suggesting that even if the HBD's were produced by the peripheral blood cells, most of it was secreted out. Thus, the HBD1, 2 and 3 expression was not localised to polymorphonuclear cells, lymphocytes or monocytes.

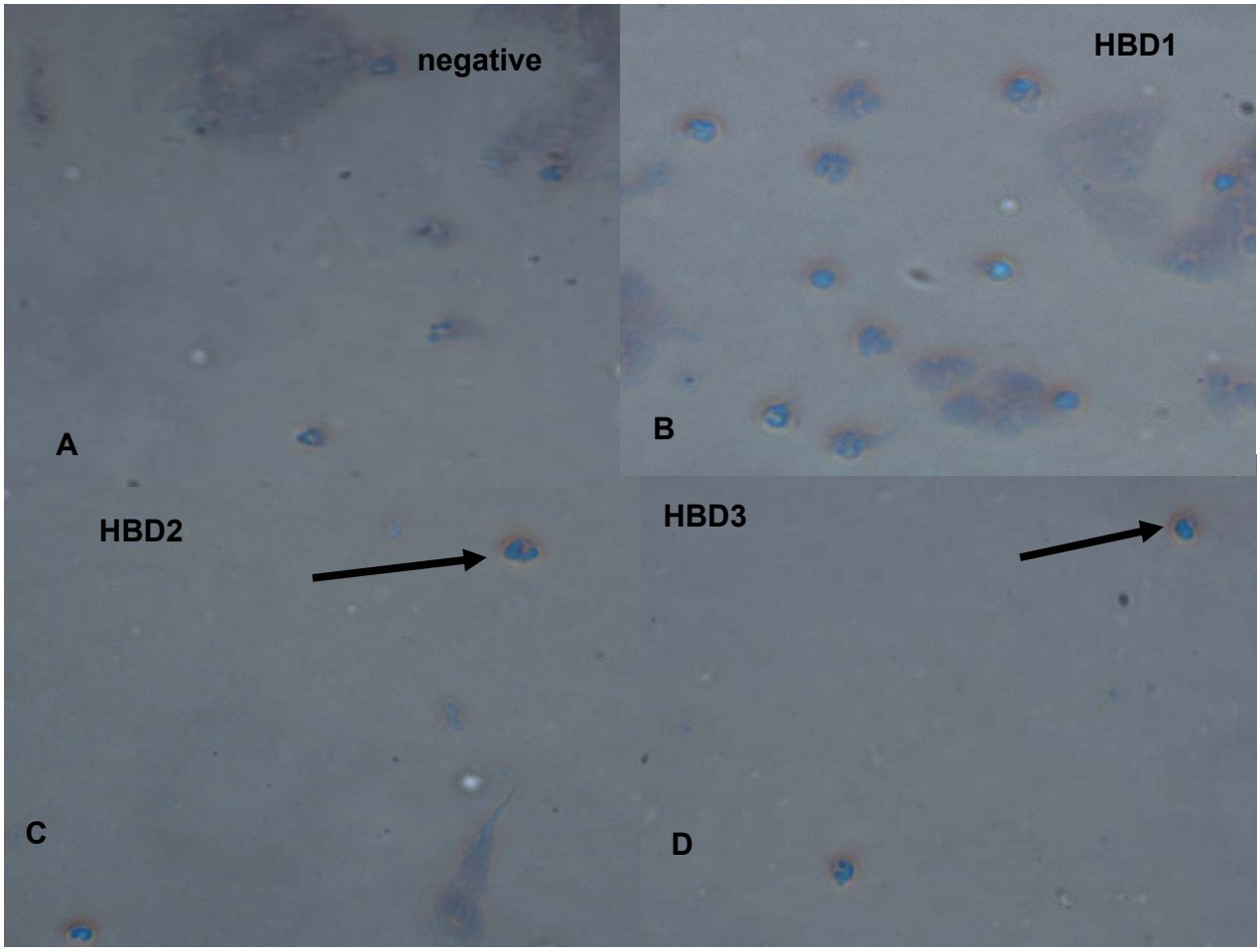


Figure 4.4 The results of immunostaining with HBD antibodies and developed using DAB. *Magnification 20x ; Panel A shows the; no primary –ve , Panels B,C, D shows the slides stained with HBD1,2 & 3 antibodies respectively. Arrow in panel C shows a polymorphocytic leucocyte, while the arrow in panel D shows a lymphocyte.*

Discussion

Sepsis has been defined as systemic illness resulting from the microbial invasion of normally sterile parts of the body(Lever and Mackenzie, 2007). The condition has an estimated mortality of 30-50 per 100,000 population per year(Eisen et al., 2006), and is ranked among the top 10 causes of death. The incidence of sepsis is put at 3 per 1000 population per year in USA(Angus et al., 2001). A similar clinical picture does occur in a number of non-microbial conditions. The similarity between the microbial and non–microbial states is the nature of the cytokines and immune regulatory peptides present; although the term sepsis should not be used for these conditions. Sometimes there can be a mixed picture wherein there may be a suspicion of microbial stimulus in the background of systemic inflammatory activity.

Sepsis results from the entry of the pathogen into the blood stream which in turn results from the breach of immunological or physical barrier. Sepsis can proceed to severe sepsis which is defined as presence of hypoperfusion or dysfunction of one of the organ systems; or to septic shock characterised by refractory hypotension despite adequate fluid resuscitation and requiring the need for vasopressor agents. Why some people develop full blown septic shock associated with high mortality while others respond and improve from the initial stage of sepsis is unclear and a number of pathogen and patient factors are implicated. It is possible that the innate responses within the blood stream including the variable HBD expression may play an important role in containing the progress of the sepsis. Leucocytes and mainly the neutrophils being the major immune cells in the blood stream do play an important role in controlling sepsis. This is probably related to the fact that neutrophilia is one of the primary responses to inflammation or sepsis. The effects of leucocytes are either directly mediated by their own

phagocytic action or by the release of mediators such as alpha or possibly β defensins from their granules.

The present project was an endeavour to ascertain the *in vivo* status of the leucocytes in relation to the production of the HBD1, 2 and 3. B defensins are cationic antimicrobial peptides originally isolated from haemofiltrate in the case of HBD1(Bensch et al., 1995) and from psoriatic scales in the case of HBD2 and 3(Harder et al., 2001). Their expression was considered to be mainly localised to keratinocytes but recently they have been isolated from variety of different tissues. The other interesting feature of HBD has been the inducibility in response a number of microbial and non-microbial stimuli. Since blood and its components form an important part of the immune system, we envisaged that leucocytes which are the primary defence cells may also act by inducing the expression of β defensins.

Our study demonstrates that the leucocytes have an enhanced expression of *HBD2* mRNA in patients with sepsis but there was no significant difference between the patients who were microbiologically positive and negative (unpaired *t*-test 2 tailed significance between the groups 0.638). Our findings are similar to those of Book and colleagues(Book et al., 2007) as regards enhanced expression in a septic population. The expression of *HBD3* in leucocytes has never been investigated before; we demonstrate for the first time that the leucocytes in patients with sepsis show very high levels of *HBD3* mRNA expression much higher as compared to normal healthy individuals. The *HBD1* expression was variable in individual patient samples.

Blood is composed of plasma and the cellular elements. The cellular elements are mainly composed of red blood cells or erythrocytes and the white blood cells or leucocytes. Since mature RBCs lack a functional nucleus they are not capable of transcription and thereby

cannot produce any inducible factors in response to stimulation. Thus the leucocytes are the only cells in the blood capable of responding to stimulation and producing various inducible factors. Functionally leucocytes are considered to be an inherent part of the immune system. Among the leucocytes the neutrophils or the polymorphonuclear cells comprise the majority (70%) of the population, while lymphocytes and monocytes comprise the remaining 30%. HNP or alpha defensins are a class of antimicrobial peptides which have been shown to be constitutively produced by neutrophils. It has been shown previously that neutrophils do not have a basal expression of HBD however they do produce HBD1, and 2 in response to ex-*vivo* stimulation. We demonstrate that the expression of HBD also occurs *in vivo* in response to the appropriate stimulation.

Proteins similar to HBD have been isolated from bovine neutrophils (BNP)(Selsted et al., 1993). HBD1 was originally isolated from the haemofiltrates, but since those patients were suffering from end stage renal failure it was considered that these were produced as a urogenital epithelial barrier and secreted into the blood stream. The studies in the past have shown no demonstrable basal expression of *HBD1, 2,* or *3* in leucocytes by reverse transcriptase PCR in blood of normal people(Fang et al., 2003). It has however been shown by Duits *et al* (2002) that freshly extracted monocytes show expression of both *HBD1* and *HBD2,* which was further enhanced by stimulation suggesting that some of the leucocytes have a basal level of *HBD* expression. In consensus with Duits *et al* we demonstrate a low basal expression of *HBD* in individuals (Mean±2SD HBD1-1.13±0.57; HBD2 1.50±1.37; HBD3 3.51±5.58). The difference in the sensitivity of the detection procedures that is traditional PCR versus quantitative PCR probably accounts for the difference between our findings and those of our predecessors.

The promoter region of *HBD1* contains consensus sites for NF-IL-6 and Interferon (IFN)-γ suggesting the potential of inducibility of these peptides(Liu et al., 1997). *HBD2* has a number of consensus sites in its promoter region, these include NF-κβ (Nuclear factor κβ), Activator protein -1 (Ap-1) and NF-IL-6 (Nuclear factor IL-6). *HBD3* is induced in keratinocytes by TGF-α(Sorensen et al., 2003), TNF- α(Harder et al., 2001), but not by IL-1 or IL-6 and neither does the promoter have consensus sites for NF-κβ, suggesting differential ways of regulation of these two inducible *HBD*s(Garcia et al., 2001a).

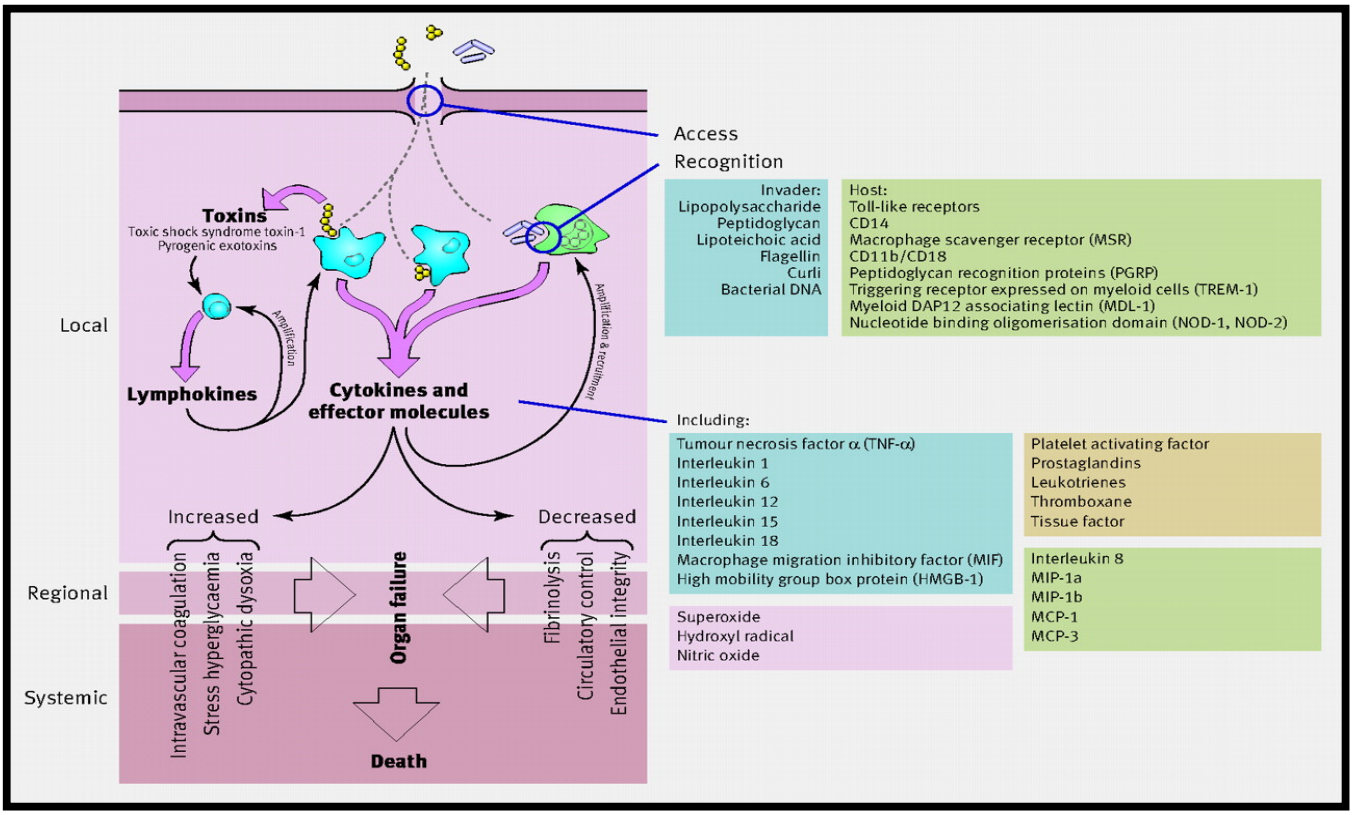


Figure 4.5 ***The patho-physiological mediators of sepsis. (****Adapted from Lever, A. et al.(Lever and Mackenzie, 2007)*

Sepsis is characterised by the presence of microbes and their toxins. These in turn trigger the immune pathways with the aid of a number of pathogen recognition receptors(Bochud and

Calandra, 2003), that in turn result in the release of number of cytokines, lymphokines and also activation of a number of transcriptional pathways as seen in the figure 4.5.

The most important cytokines that are implicated in the triggering of the sepsis syndrome are the “proximal”or the early cytokines namely TNF-α and IL-1β. These in turn with their myriad effects and along with the microbial products mediate the release of the “distal” or late cytokines which include IL-6 and IL-8(Blackwell and Christman, 1996). It is considered that cytokines are not stored in a “ready format” but are synthesised in response to the challenge. Studies have shown that the primary cytokines like TNF- α peaked in about 60 minutes following stimulation(Michie et al., 1988).

It is interesting to note that IL-1β and IL-6 are potent inducers of *HBD2* while TNF- α induces *HBD3* expression. Thus it likely that these cytokines play an important role in the induction of these peptides from leucocytes. The degree of induction is dependent on the degree of triggering factors and also on the leucocyte count and a number of patient factors which influence the translational machinery of the patient. As regards the individual cells which are responsible for production, our study is flawed by the fact that individual leucocyte cell populations were not separately analysed for their expression. Given the fact that all the patients in the study had raised neutrophils counts and that immunocytochemical studies suggest the possible localisation of *HBD*’s to leucocytes, it can be hypothesised that the main source of production were neutrophils and lymphocytes; the two main cell populations among leucocytes. Since polymorphonuclear cells are the predominant cell population of leucocytes and also the main effector cells in case of bacterial sepsis, it can further be derived that the polymorphonuclear cells are the major cell population producing *HBD* in the inflammatory and bacteria induced sepsis conditions.

In our study the mean expression of *HBD* between the microbiologically positive and negative group was similar, suggesting that the regulation of these factors is governed by other inflammatory mediators apart from microbial stimuli. Thus the possible mechanism of induction of these peptides in leucocytes is mediated by cytokines and also involves the translational machinery as these peptides not stored in the leucocytes. This also draws attention to the possibility that these peptides may be more than simply antimicrobial and may actually play a part in immune regulation. The relative preponderance of *HBD3* as compared to *HBD2* is unclear but it can be hypothesised that relatively lower *HBD2* may itself be a factor in the pathogenesis of sepsis, as it has been shown that in patients with sepsis the inducibility of *HBD2* is reduced(Book et al., 2007). However this warrants further investigation and larger studies both *in vitro* and *in vivo*.

It is being increasingly perceived that increased levels of cytokines by themselves are detrimental to the overall outcome and this has brought forth the concept of anti-cytokine therapies for the treatment of sepsis syndrome(Suffredini, 1994). It has also been perceived that because of the complexity of the interactions involved and with individual cytokines, it is better to focus on the immunomodulatory or even anti-inflammatory cytokines like IL-10. It has been shown by Boniotto *et al* that HBD2 is a potent inducer of a number of cytokines including the pro-inflammatory and anti-inflammatory groups. Although and HBD1 and HBD3 have been shown to induce IL-8, they seem to lack the capacity to cause a more generalised response(Boniotto et al., 2006). These findings together with our findings suggest other important functions of immunomodulation of HBD2. We propose that HBD3, with its universal salt insensitive antimicrobial property and broad spectrum of antimicrobial activity, is the prime antimicrobial peptide induced in states of sepsis and its action is partly supported by HBD1

and 2. However HBD2 further mediates more important actions of orchestrating the cytokine response by way of its cellular receptors; those currently identified include CCR6 and TLR4.

Future studies need to be undertaken to understand the inter-relationships among these antimicrobial peptides, and to decipher yet unknown functions of these important group of peptides. Studies also need to be undertaken to separate out the various leucocytes cell fractions and individually analyse the protein and RNA concentrations of HBD's. Also plasma defensin levels should be estimated to get better overall picture. This information would used in formulating novel therapeutic strategies utilising the potential of these peptides as antimicrobial and immunoregulatory molecules.

Chapter 5

Buccal HBD 1, 2 and 3 expression profiles in peri-operative states – correlation to cortisol levels.

5.1 Introduction

Stress has been defined as “a constellation of events, which begins with a stimulus (stressor) that precipitates a reaction in the brain (stress perception), which subsequently activates physiologic systems in the body (stress response)”(Dhabhar, 2000). Stress can result from negative life events which have been broadly classified into either objective discrete events like bereavements, trauma , surgery and infections, or self reported list of events like daily hassles and life events(Herbert and Cohen, 1993). Stress in a hospital setting can result from major polytrauma, burns, infections or surgical procedures: the accompanying physiological responses can impact both positively and adversely on an individual. Stress responses on one hand help the individual to prepare and survive the stressful event effectively while on other had the mediators of stress response have been implicated to have damaging effects. A number of studies have shown that stress can be immunosuppressive and thus can be damaging to health(Khansari et al., 1990, Marucha et al., 1998, Herbert and Cohen, 1993). There have been other studies which have suggested that stress responses are important to help patients survive the stressful stimulus. Studies by Dhabhar *et al.* have shown that acute stress actually stimulates the skin immune function while chronic stress suppresses it(Dhabhar and McEwen, 1999).

Human β defensins (HBD) are a family of cationic innate defense peptides which are primarily considered antimicrobial. Three subtypes of these HBD1, 2 and 3 have been identified in the buccal mucosa(Saitoh et al., 2004). HBD1 is constitutive while HBD2 and HBD3 are inducible in response to a number of microbial and inflammatory stimuli. The inflammatory stimuli are a part of the cytokine cascade which is also activated during stress. However it is not known how

the stress hormones which are also activated during stress influence the expression of these peptides *in-vivo*.

Steroids are also considered an important aspect of treatment of critically ill patients and it has been a matter of perennial debate as to the risks, benefits and timing for supplementing steroids in the treatment of these patients. One of the reasons of the controversy has been the down-regulation of the immune system by the steroids. It has been shown by Duits *et al.* that HBD3 expression is down regulated in cultured bronchial epithelial cells and mononuclear phagocytes. There have been no studies looking at the influence of steroids on the *in vivo* expression of these peptides. The current experiment was planned to determine the variation of HBD expression compared with expression of the innate stress hormone cortisol.

Patients in high stress clinical conditions such as burns, polytrauma and major surgical procedures are prone to infections. HBDs are antimicrobial peptides that are considered an important component of innate host defence system. We studied the expression of HBD in the buccal mucosa in relation to cortisol levels in patients undergoing major surgical resection for malignancies.

5.2 Materials and Methods

The study was carried out at the West Wales General Hospital, Carmarthenshire NHS trust in Wales, UK after getting a favourable ethical committee approval. Patients undergoing major surgical procedures which would require them to be admitted to the critical care areas i.e. HDU or ITU during the post-operative period as part of the hospital policy were recruited for the study. The patients were recruited, after obtaining an informed consent, over a period of 1 year.

Inclusion criteria

- Patients undergoing major surgery
- Admission to the hospital night prior to surgery
- Post –operatively planned to be admitted to critical care area
- Not on steroids or immunosuppressive therapy
- Not suffering from immunosuppressive conditions
- Expected mean hospital stay of about 1 week as per the hospital policy/departmental policy.

Exclusion criteria

- Children
- Patients on steroids or immunosuppressive therapy
- Patients with lesions or conditions involving oral cavity
- Patients unable to consent

The common procedures among the recruited patients were open radical prostatectomies (complete removal of the prostate gland to treat extensive prostate cancer), cystectomies (removal of the urinary bladder for treatment of bladder cancer) with or without neo-bladder reconstruction and operations for extensive bowel malignancies.

Prior to undertaking the actual study of correlating cortisol and buccal HBD levels and a preliminary study (Study 1) was performed.

The objectives of the preliminary study were to determine:

- If there was any specific pattern of cortisol expression in critically ill patients
- If it was possible to successfully and consistently detect cortisol levels using saliva
- the factors influencing the cortisol levels

Over a 6 month period 12 patients were recruited; the recruitment criteria were the same as above. Patients had 4 hourly salivary and blood cortisol samples. A record of a number of parameters and significant activity during these 24 hours was maintained.

Study 1- Assessment of salivary cortisol

Salivary cortisol assessment was done using commercially available ELISA based assay (Salimetrics, Cat log No-1-0102/1-0112, USA) according to the manufacturers protocol. The tests were done in triplicate to ensure reliability.

5.3 Results

12 patients were recruited in the study, of which four were females and 8 males. The mean age of the recruited patients was 55.5 years with a range of 19-78 years.

Correlation between sedation, serum cortisol and plasma cortisol levels

There was no significant correlation found between the salivary and serum cortisol results. However there was significant correlation between serum cortisol and degree of sedation as assessed by Ramsay sedation score (refer to table 5.1 below), irrespective of whether only paired samples of salivary cortisol were used or not. The increase in sedation score suggestive of the patient being more sedated was associated with lower serum cortisol levels and vice versa as seen in figure 5.1. Table 5.1 shows the correlation between all the cortisol samples and the sedation score while the table 5.2 shows the correlation between the *paired* samples; sedation scores with samples contaning enough saliva to analyse were only considered and sedation scores at time points where there was not enough cortisol to be sampled were eliminated.

Table 5.1The Pearson correlation co-efficient between sedation score and the cortisol levels (both salivary and serum)

		Serum cortisol nmol/L	Salivary cortisol nmol/L	Sedation score
Serum cortisol (nmol/L)	Pearson Correlation	1	-0.1	-.496(**)
	Sig. (2-tailed)		0.4	0.0
	N	70	33.0	70.0
Salivary cortisol (nmol/L)	Pearson Correlation	-0.1	1.0	-0.1
	Sig. (2-tailed)	0.4		0.5
	N	33.0	34.0	34.0
Sedation score	Pearson Correlation	-.496(**)	-0.1	1.0
	Sig. (2-tailed)	**0**	0.5	
	N	70	34.0	71.0

** Correlation is significant at the 0.01 level (2-tailed).

Not enough saliva was obtainable for assay in all samples and only 33 of the 70 samples had enough saliva (47%) for analysis.

Table 5.2 The Pearson correlation co-efficient between sedation score and the cortisol levels (both salivary and serum).

		Salivary cortisol nmol/L	Serum cortisol nmol/L	Sedation score
Salivary cortisol nmol/L	Pearson Correlation	1	-0.1	-0.1
	Sig. (2-tailed)		0.4	0.5
	N	34	33.0	34.0
Serum cortisol nmol/L	Pearson Correlation	-0.1	1.0	-.450(**)
	Sig. (2-tailed)	0.416		0.0
	N	33	33.0	33.0
sedationscore	Pearson Correlation	-0.1	-.450(**)	1.0
	Sig. (2-tailed)	0.45	0.0	
	N	34	33.0	34.0

** Correlation is significant at the 0.01 level (2-tailed).

The missing values have been eliminated and only paired samples have been considered.
There was no significant correlation between the salivary and serum cortisol even after replacing the missing values using the linear trend method in SPSS 14 (Refer to table 5.3). Similarly there was no correlation between salivary cortisol and sedation (Table 5.3).

Table 5.3 shows the correlation between the sedation score and the cortisol levels (both salivary and serum) after replacing the missing cortisol values using the linear trend method in SPSS 14.

		Sedation score	TREND(salivary cortisol)	TREND(serum cortisol)
Sedation score	Pearson Correlation	1	-0.1	-.487(**)
	Sig. (2-tailed)		0.5	0.0
	N	72	72.0	72.0
TREND(salivary cortisol)	Pearson Correlation	-0.1	1.0	-0.1
	Sig. (2-tailed)	0.5		0.5
	N	72	72.0	72.0
TREND(serum cortisol)	Pearson Correlation	-.487(**)	-0.1	1.0
	Sig. (2-tailed)	0	0.5	
	N	72	72.0	72.0

** Correlation is significant at the 0.01 level (2-tailed).

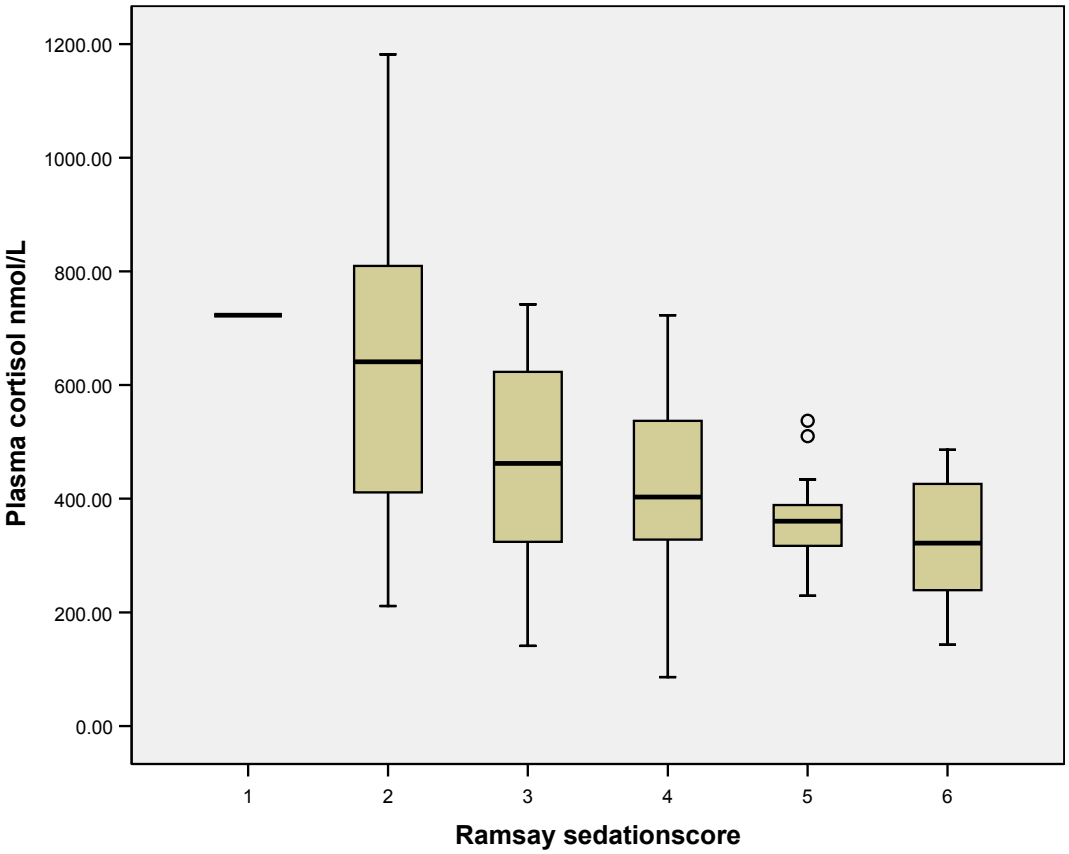


Figure 5.1 Box and whiskers plot demonstrating the association between the Plasma cortisol levels and the Ramsay sedation score.

It can be seen from figure 5.1 that an increasing sedation score is associated with a fall in the median plasma cortisol levels.

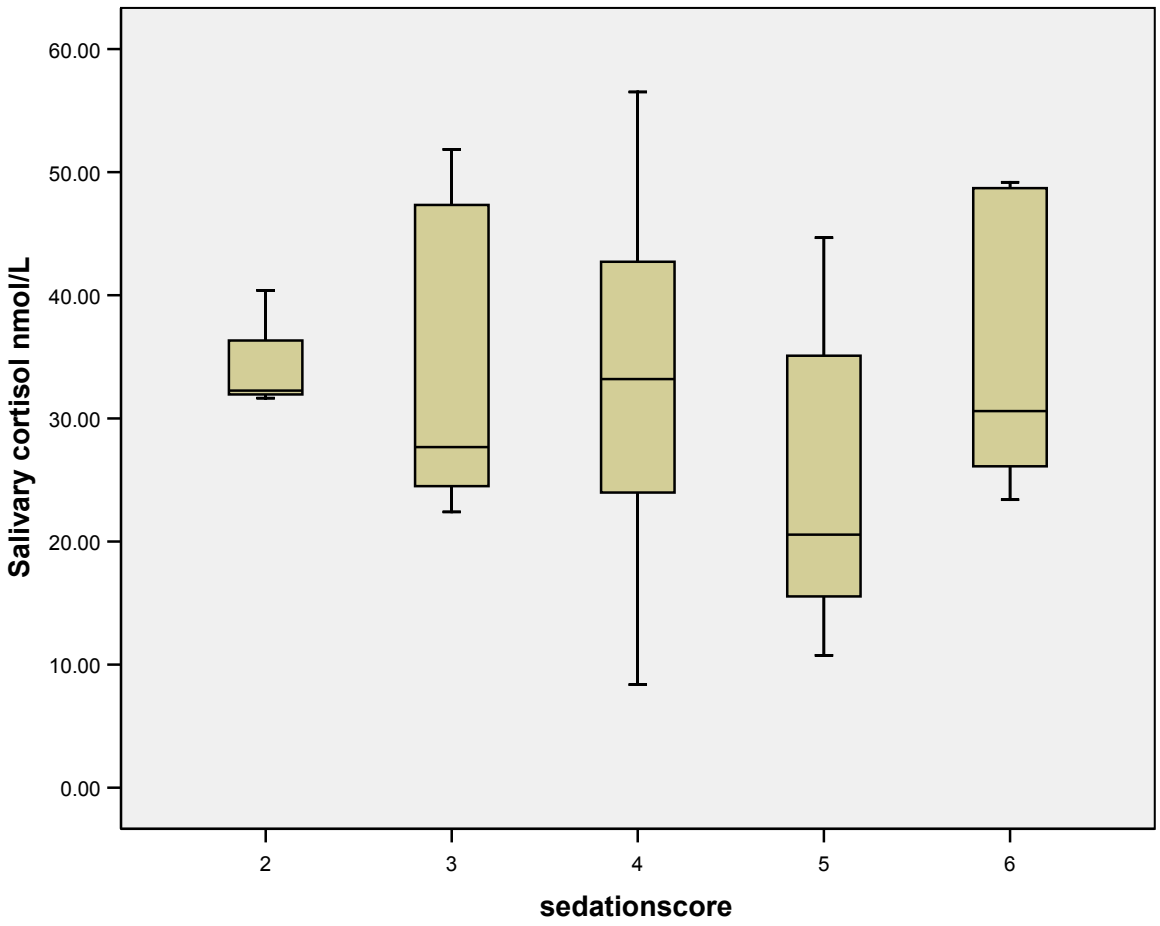


Figure 5.2. Box and whiskers plot demonstrating the lack of association between the salivary cortisol levels and the Ramsay sedation score.

It can be seen that there is no specific identifiable association.

Circadian rhythm

Circadian rhythm is a cyclical variation of biological activity based on a 24 hour internal biological clock for example the sleep-awakeness cycle. In healthy individuals the serum cortisol levels show a circadian rhythm associated with an early morning surge in the levels of cortisol. There was no definite pattern of circadian rhythm seen in salivary cortisol samples (Figures 5.2, 5.3), while the mean serum cortisol showed a peak around 8 am. This however correlated with the decreased level of sedation at this time in these patients (Figures 5.2, 5.3).

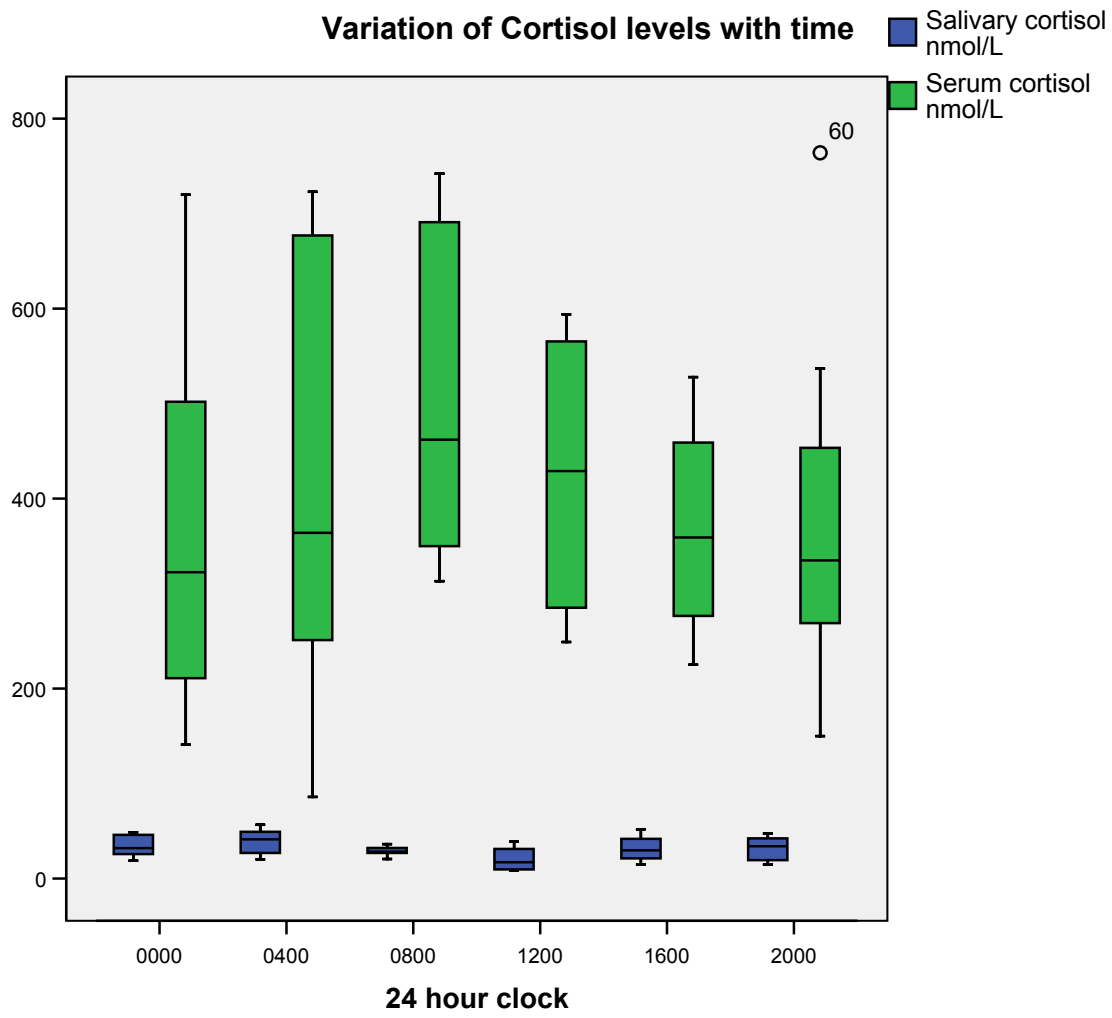


Figure 5.3 Box plot showing the cortisol levels (both salivary and Serum) on Y axis and its variation with time on x axis.

The dark lines in the middle of the boxes represent the median value. It can be seen that median serum cortisol levels peeks at 8am while mean salivary cortisol shows little variation.

Affect of activity on cortisol levels

There was no demonstrable effect of variation in cortisol levels with activities such as starting feeds or altering physical positions (data not shown).

Study 2: Effect of cortisol on the secretion of HBD in the oral mucosa

Methods

Since there was no real rhythm in the salivary cortisol secretion, the sample was taken early in the morning. The first sample was taken on the day of surgery just after dawn. The second sample was taken on the first day post-surgery. If it was not possible due to practical considerations then the second sample was taken on the second day post-surgery. The third sample was taken on the day of patients discharge between days 7-10 days from the day of surgery; depending on when the patient was being discharged.

Each sample included a salivary sample collected using a salivette™ (Starstedt, USA), cheek scrapings were obtained using a cell scraper (BD Flacon™, UK). The cheek scrapings were taken from the buccal surface of both cheeks. A part of the cheek scrapings was to be used for immunocytochemistry, this was fixed in 4% paraformaldehyde; the other part for real time PCR analysis was dispersed in sterile PBS. This was then pelleted and re-suspended in SV RNA lysis (Promega; UK) buffer and subsequently stored at -80°C until further processing.

RNA extraction, cDNA conversion & real time PCR analysis

RNA extraction was done using SV total RNA extraction system (Promega; UK) as described earlier. The cDNA conversion was done using ImProm-II™ (Promega; UK) reverse transcriptase kit. The RNA concentration was estimated using spectroscopic analysis and

between 200 to 500ng of RNA was converted per reaction. Duplex quantitative PCR analysis was performed using Opticon II real time machine (Bio-rad, UK) with *β actin* as internal control (house keeping) gene.

Results

Table 5.4. Demographics of the patients recruited for study 2.

		Patient id.	Sex	Age(years)
1		1	male	52
2		2	male	63
3		3	male	70
4		4	male	72
5		6	male	54
6		7	male	70
7		8	female	77
8		9	male	71
9		10	male	78
10		11	female	72
11		12	male	55
12		14	male	73
13		15	female	75
14		16	male	78
15		17	female	70
16		18	male	75
17		19	female	75
18		20	male	69
Total	**N**	18		
	Mean			69.39

There were 18 patients recruited over a period of 6 months. The male: female ratio was 15: 3 and the mean age as seen in the table was 69.39 years (Table 5.4)

.

Table 5.5. Mean cortisol values and their standard deviations in the three phases of the study.

		cortisol			
		Mean	Standard Deviation	Total N	Missing
Timing	pre-operative	17.107	8.207	18	3
	post-operative	16.129	9.342	18	8
	pre-discharge	12.554	4.654	18	8

From each patient, 3 sets of samples were to be collected. However some samples were insufficient, while some samples could not be collected as the patient had been discharged (Table 5.5). Three sets of samples, pre-operative, post-operative and pre–discharge were collected from 18 patients. There was no perceived need for additional controls as the pre-operative samples would serve as baseline while immediate post-operative would serve as post-stressor, acute response phase, while the pre-discharge samples would serve as post–stressor phase.

Among the salivary cortisol samples, 35% (19) samples were missing or insufficient. 16 of the insufficient samples were from the post–operative and the pre-discharge group. Most of these patients provided blood cortisol samples as well, but these were not considered due to lack of correlation in these circumstances as free cortisol index (FCI) was not available. Free cortisol index is the ratio of serum total cortisol level to cortisol binding globulin level. It is considered predictor of the true cortisol levels in blood especially in patients who may be hypo-proteinaemic.

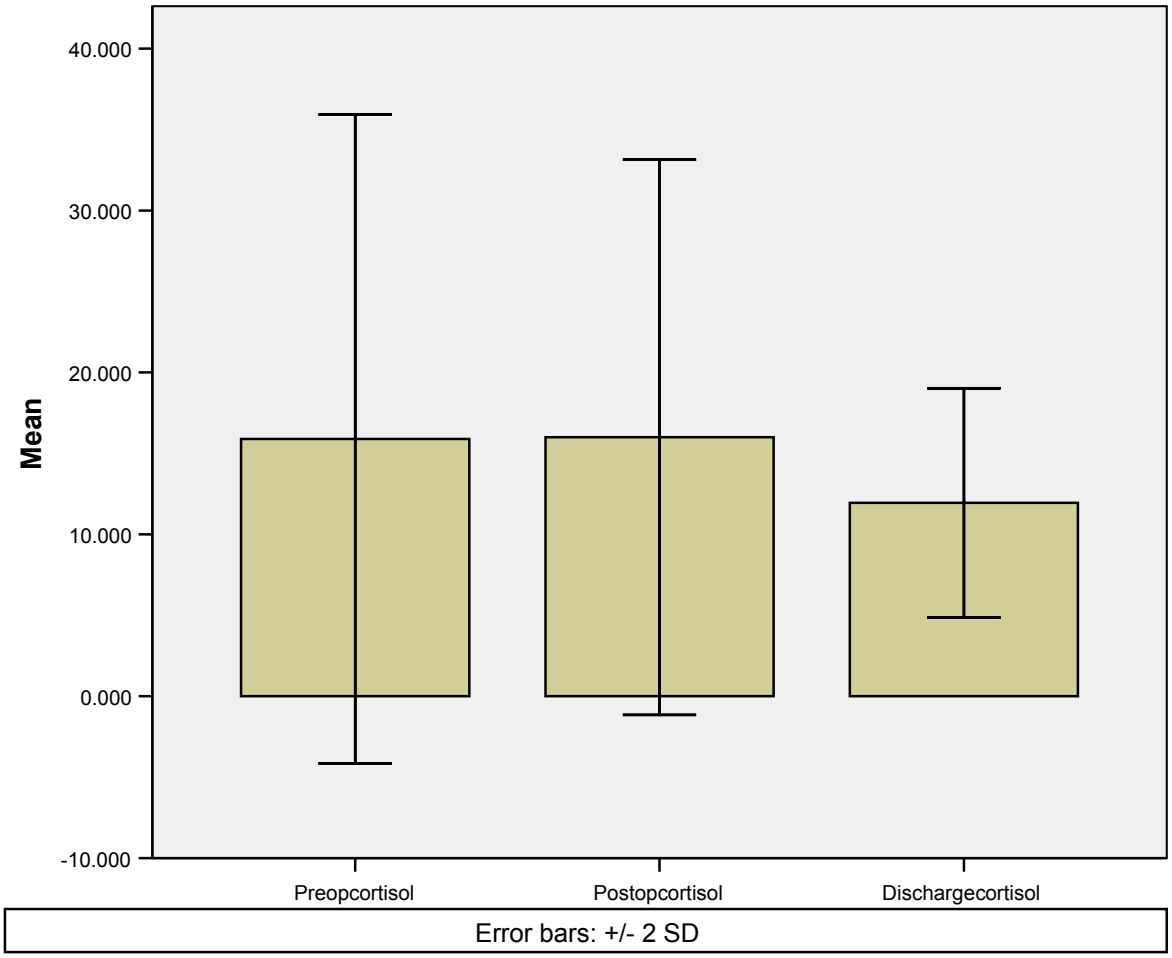


Figure 5.4 Bar charts with error bars depicting the mean salivary cortisol at the pre-operative phase, immediate post –operative phase and discharge phase.

The mean cortisol was highest in the pre-operative group in our set of patients; however there was a considerable variation between the individual patients as evidenced by the error bars in the figure 5.4. The mean cortisol levels progressively decreased in the immediate post – operative phase to its lowest in the pre-discharge group. As seen the variation was also much less in the pre-discharge group and most of the samples showed a trend towards decreasing cortisol response compared to the pre-operative situation. The difference between the groups was not statistically significant when compared by paired *t*–test (Refer to table 5.6).

Table 5.6 The results of the paired sample t-test between the pre-operative samples and post-operative or pre-discharge salivary cortisol samples.

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Preoperative cortisol – Discharge cortisol	4.9	9.7	3.1	-2.1	11.9	1.6	9.0	0.1
Pair 2	Preoperative cortisol – Postoperative cortisol	-0.2	10.3	3.2	-7.6	7.1	-0.1	9.0	0.9

The table shows the results with actual number of samples available.

The missing samples values were replaced using linear trend method in SPSS 14 and paired t-test did not show any significant difference between the time points.(Refer to table 5.7)

Table 5.7 Results of the paired sample t-test between the pre-operative samples and post-operative or pre-discharge salivary cortisol samples.

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	LINT(Preoperative cortisol) - LINT(Postoperative cortisol)	-0.8	10.4	2.8	-6.8	5.3	-0.3	13.0	0.8
Pair 2	LINT(Preoperative cortisol) - LINT(Dischargecortisol)	3.0	8.6	2.1	-1.4	7.5	1.5	16.0	0.2

Table 5.8 shows the mean *HBD1,2* and *3* expression at different time points. As seen in Table 5.8 and Figure 5.5 the mean *HBD1* and *3* expression were higher in the pre-operative patients and progressively decreased in the post-operative and pre-discharge stages. The *HBD2* expression however peaked at the post–operative time. There was considerable individual variation in the *HBD1* levels in samples taken in the pre and post–operative periods as seen by the standard deviation. The variation in the individual patients in pre-discharge period was least for all *HBD*'s as seen.

Table 5.8. ***Mean HBD1, 2 and 3 expression and the standard deviation (SD) at different time points***.

		HBD1		HBD2		HBD3	
		Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
Timing	pre-operative	2.3	3.7	1.4	1.2	1.5	1.4
	post-operative	1.9	4.2	1.6	1.5	0.8	0.6
	pre-discharge	0.7	0.7	1.2	0.7	0.8	0.3

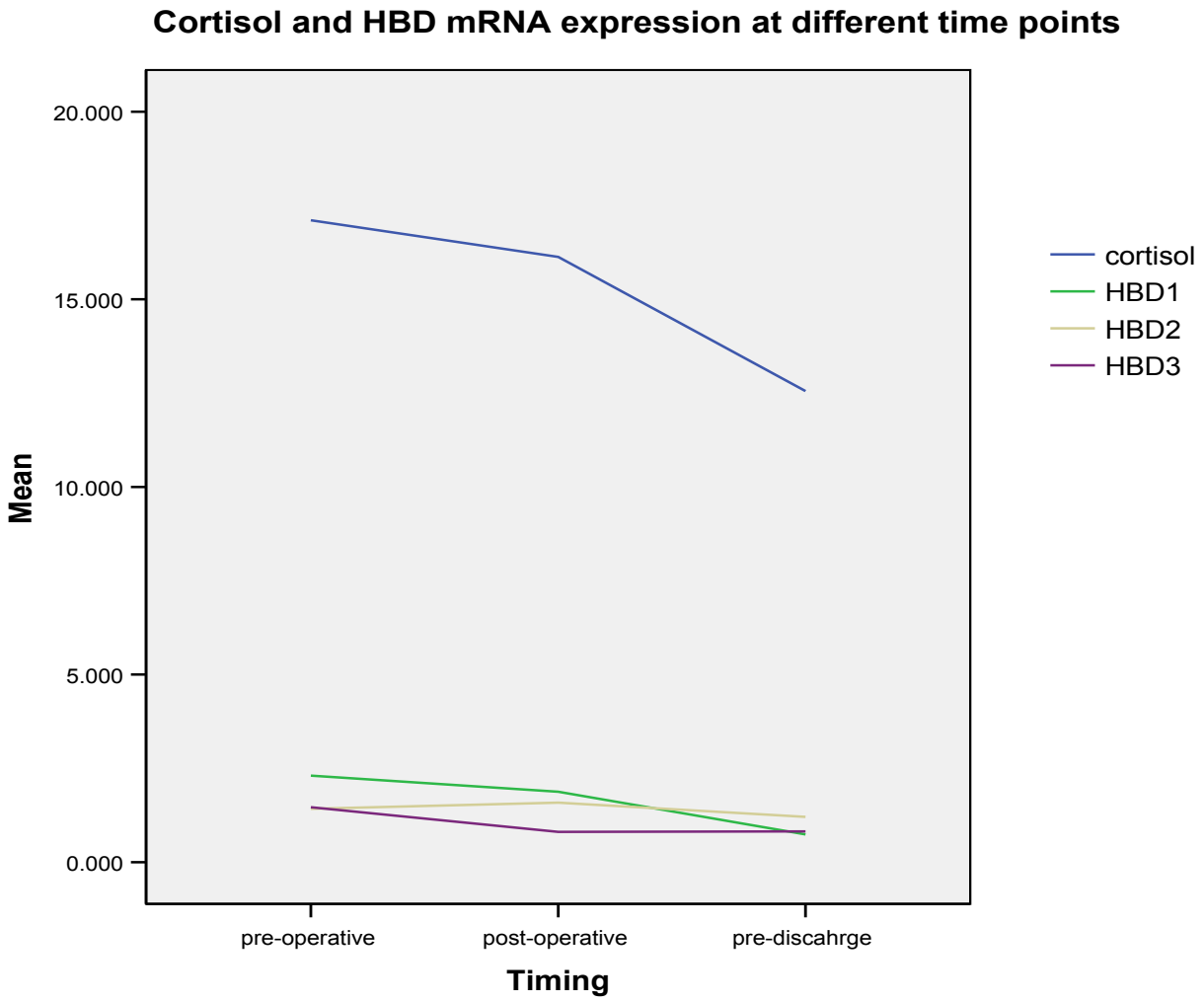


Figure 5.5 Graphical representation of the variation of Cortisol and HBD1,2 & 3 RNA expression at the different time points in the study, namely pre-operative, post – operative and pre-discharge stages.

Table 5.9. The mean difference between the HBD 1,2 and 3 mRNA expression between different time points

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	**PreopHBD1 - PostopHBD1**	0.5	6.1	1.6	-2.9	3.9	0.3	14.0	0.8
Pair 2	**PreopHBD1 - DischargeHBD1**	2.2	4.3	1.3	-0.6	5.1	1.7	10.0	0.1
Pair 3	**PreopHBD2 - PostopHBD2**	-0.1	1.6	0.4	-1.0	0.8	-0.3	14.0	0.8
Pair 4	**PreopHBD2 - DischargeHBD2**	0.4	1.6	0.5	-0.7	1.5	0.8	10.0	0.4
Pair 5	**PreopHBD3 - PostopHBD3**	0.7	1.6	0.4	-0.1	1.6	1.8	14.0	0.1
Pair 6	**PreopHBD3 - DischargeHBD3**	1.0	1.4	0.4	0.1	1.9	2.4	10.0	0.0

The results were not statistically significant.

The difference in the expression of the HBD1, 2 & 3 RNA at different time points was assessed using paired *t*-test (Table 5.9). The paired *t*-test was used to see if there was statistically significant difference between the pre-operative expression of these peptides as compared to the post –operative and the pre-discharge samples. As seen in Table 5.8, the difference in the *HBD* expression was not significant for any of the HBDs as compared to the basal, pre-operative, expression.

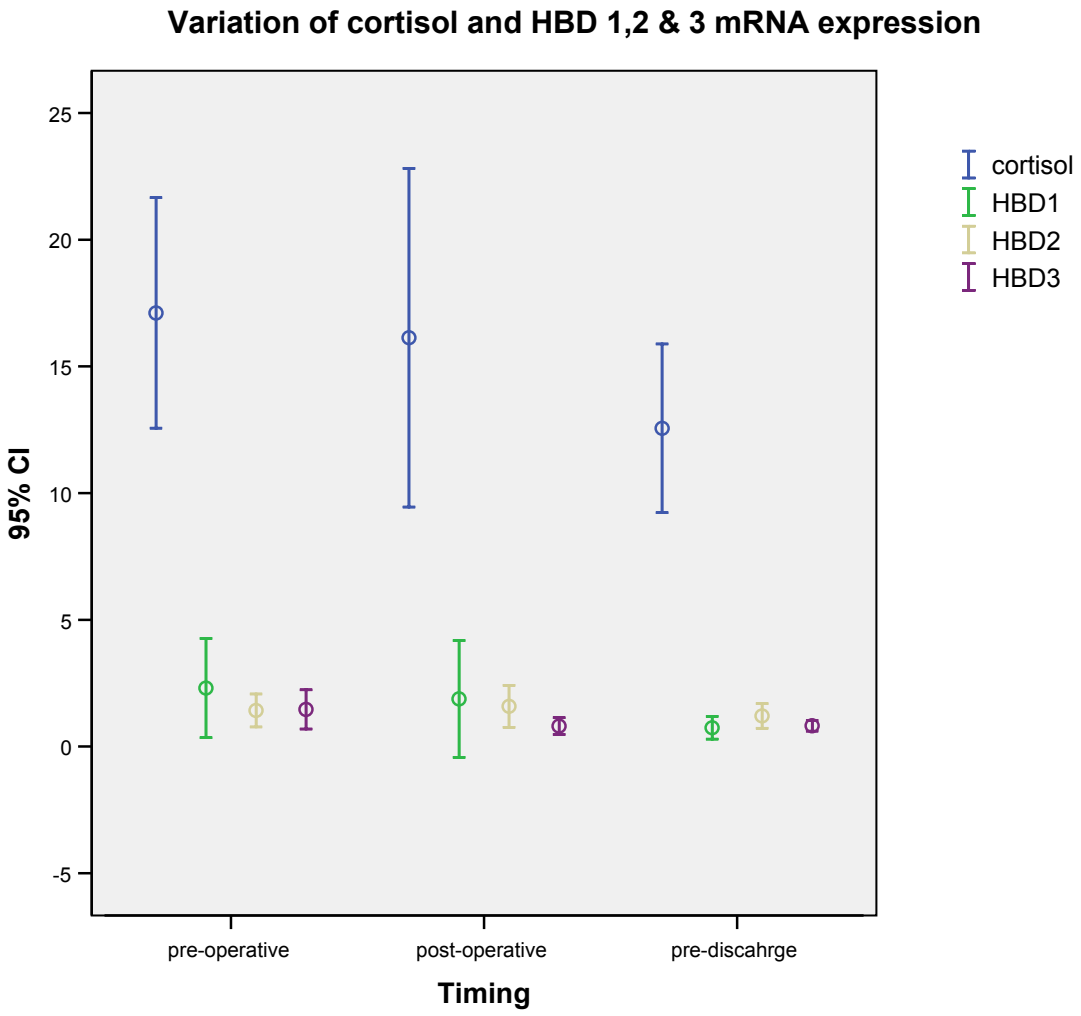


Figure 5.6 The variation in the cortisol levels and the HBD1,2 &3 mRNA levels at different time points. *The Y axis shows the mean with 95% confidence intervals (CI) and the X axis represents the different time points.* As seen in Fig 5.6 there was considerable variation of cortisol levels and HBD1, 2 &3 between the patients. The variation was most marked in immediate post-operative cortisol levels and HBD1mRNA levels followed then by the levels in the pre-operative states. The variation in both the cortisol levels and the levels of HBD1, 2&3 was least in the pre-discharge state.

Variation of Cortisol and HBD levels- Table 5.10

		CORTISOL			HBD1			HBD2			HBD3		
		P1	P2	D	P1	P2	D	P1	P2	D	P1	P2	D
P1 cortisol	PC	1	0.3	0.1	-0.4	0.1	-0.6	-0.2	-0.3	-0.4	0.1	-0.1	0.3
	Sig.		0.3	0.7	0.2	0.8	0.1	0.4	0.3	0.2	0.9	0.7	0.4
	N	15	10.0	10.0	13.0	12.0	10.0	13.0	12.0	10.0	13.0	12.0	10.0
P2 cortisol	PC	0.3	1.0	0.3	-0.7	0.3	-0.5	0.5	-0.6	0.0	-0.1	-0.6	0.3
	Sig.	0.3		0.6	0.1	0.6	0.3	0.3	0.2	1.0	0.9	0.1	0.6
	N	10.0	10.0	6.0	8.0	7.0	6.0	8.0	7.0	6.0	8.0	7.0	6.0
DC	PC	0.1	0.3	1.0	0.1	0.3	0.2	-0.3	-0.4	-0.1	-0.4	0.3	-0.6
	Sig.	0.7	0.6		0.9	0.5	0.7	0.4	0.3	0.7	0.3	0.4	0.1
	N	10.0	6.0	10.0	10.0	9.0	10.0	10.0	9.0	10.0	10.0	9.0	10.0
P1 HBD1	PC	-0.4	-0.7	0.1	1.0	-0.2	0.1	0.4	0.2	0.0	**.498(*)**	0.2	0.2
	Sig.	0.2	0.1	0.9		0.6	0.8	0.1	0.4	0.9	**0.1**	0.4	0.5
	N	13.0	8.0	10.	16.	15.0	11.0	16.0	15.0	11.0	16.0	15.0	11.0
P2 HBD1	PC	0.1	0.3	0.3	-0.2	1.0	0.4	-0.2	-0.2	-0.4	-0.2	**.795(**)**	-0.1
	Sig.	0.8	0.6	0.5	0.6		0.2	0.6	0.6	0.2	0.5	**0.0**	0.9
	N	12.0	7.0	9.0	15.	15.0	10.0	15.	15.0	10.0	15.0	15.0	10.0
DHBD1	PC	-0.6	-0.5	0.2	0.1	0.4	1.0	-0.2	-0.1	0.0	0.2	0.0	-0.5
	Sig.	0.07	0.3	0.6	0.7	0.2		0.41	0.74	0.9	0.4	0.9	0.1
	N	10	6	10	11	10	11	11	10	11	11	10	11
P1 HBD2	PC	-0.2	0.4	-0.2	0.4	-0.1	-0.2	1	0.3	-0.0	0.2	-0.079	0.25
	Sig.	0.433	0.2	0.4	0.1	0.5	0.471		0.2	0.983	0.3	0.7	0.4
	N	13	8	10	16	15	11	16	15	11	16	15	11
P2 HBD2	PC	-0.3	-0.5	-0.3	0.2	-0.1	-0.1	0.3	1	-0.166	-0.036	0.014	-0.025
	Sig.	0.3	0.1	0.3	0.4	0.5	0.7	0.2		0.647	0.9	0.96	0.945
	N	12	7	9	15	15	10	15	15	10	15	15	10
DHBD2	PC	-0.4	-0.0	-0.1	0.0	-0.4	0.0	-0.0	-0.1	1	-0.1	0.03	0.112
	Sig.	0.2	0.9	0.7	0.8	0.2	0.9	0.9	0.6		0.713	0.935	0.742
	N	10	6	10	11	10	11	11	10	11	11	10	11
P1 HBD3	PC	0.0	-0.0	-0.3	**.498(*)**	-0.2	-0.2	0.26	-0.036	-	1	0.084	**.862(**)**

										0.126			
	Sig.	0.8	0.8	0.2	**0.05**	0.461	0.489	0.331	0.9	0.713		0.765	**0.001**
	N	13	8	10	16	15	11	16	15	11	16	15	11
P2HBD3	**PC**	-0.1	-0.6	0.3	0.2	**.795(**)**	0.039	-0.079	0.014	0.03	0.084	1	0.229
	Sig.	0.743	0.1	0.4	0.4	**0**	0.9	0.78	0.96	0.9	0.765		0.524
	N	12	7	9	15	15	10	15	15	10	15	15	10
D HBD3		0.276	0.2	-0.5	0.2	-0.062	-0.4	0.25	-0.025	0.1	**.862(**)**	0.229	1
	Sig.	0.44	0.6	0.0	0.5	0.866	0.1	0.458	0.945	0.7	**0.001**	0.524	
	N	10	6	10	11	10	11	11	10	11	11	10	11

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

Key P1- Pre operative, P2- post operative, D –discharge, PC-Pearson's correlation coefficient,Sig- Significance 2 tailed

Table 5.10 ***Results of Pearson's correlation between the HBD1,2 & 3 RNA expression and the cortisol expression at the different phases respectively.***

There was significant positive correlation between the pre and post-operative mean *HBD1* and *HBD3* mRNA levels. No significant correlation was seen between the cortisol levels and *HBD* levels (Table 5.10).

Immunocytochemistry

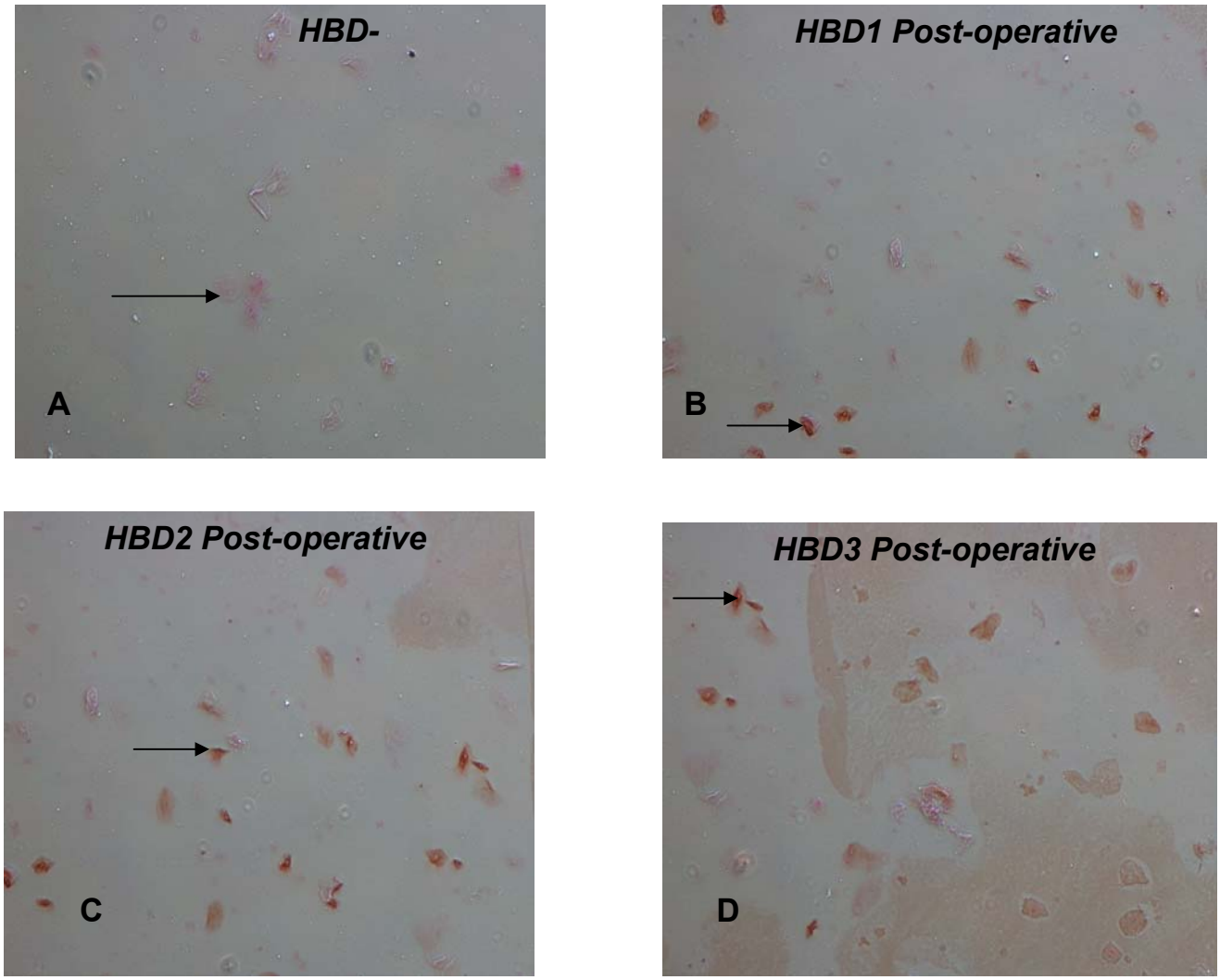


Figure 5.10- Immuno-localisation of HBD1, 2 & 3 proteins.

Slides were stained with specific primary antibodies and developed with DAB. Panel A shows no primary antibody negative control , arrows shows buccal epithelial cells ; as seen no DAB stain is seen. Panels B,C,D show the slides stained with specific HBD1, 2 & 3 antibodies respectively, arrows point to cells which show localisation of the protein.

The HBD1, 2 and 3 protein localised in the buccal epithelial cells was detected by immunocytochemistry. Immunocytochemistry was only used to localise the HBD proteins to ascertain their source in the oral cavity to be buccal epithelial cells (5.10). HBD are secretory proteins and depending on the maturity of the individual epithelial cell and the degree of stimulation, they can have variable amount of defensin peptide. Hence it is expected that there would be variation in the peptide level among the individual cells. The number of cells obtained per patient sample for immunocytochemistry was about 50. These cells were to be re-distributed between 3-4 slides, thus the number of cells available on each slide per patient per HBD was about 15. The low cell number per slide made any kind of objective, statistically significant expression analysis impossible, hence immunocytochemistry was used only as a tool to localise the proteins. Saliva was considered as a better source of quantifying the HBD peptide expression in the oral cavity using high pressure liquid chromatography (HPLC) techniques. Although saliva was collected, it was considered outside the remit of the current PhD to perform peptide quantification using HPLC.

5.4 Discussion

Salivary cortisol gives the direct measurement of serum free cortisol. Cortisol in a healthy person exists in three different types: type a) 10% physiologically active free unbound form, type b) 20% bound to albumin and type c) 70% bound to cortisol binding globulin. Types b and c are physiologically inactive. Serum cortisol represents total cortisol which includes the free and bound cortisol forms. However a number of factors influence protein binding and levels and hence serum free cortisol measurement is considered a better predictor of adrenal function in critically ill patients(Umeda et al., 1981, Hamrahian et al., 2004). Salivary cortisol

measurement is a direct reflection of serum free cortisol and is considered useful for measurement of free cortisol levels(Umeda et al., 1981). In patients with extensive inflammation and hyper-metabolic states such as sepsis, poly-trauma and post –operatively there are changes in the globulin, albumin and the protein content, hence measuring total serum cortisol does not give a true measure of stress response evoked in these situations. These patients are known to have acute phase responses which can result in variations in cortisol-binding globulin levels and combined with nutritional status and other factors such as hydration status can also result in the alteration of albumin and total protein levels(Pugeat et al., 1989). A number of physio-pathological changes occur during acute phase response that result in alteration of globulin and albumin levels; these include increased permeability of the blood vessels, decreased hepatic synthesis*(Ruot et al., 2000)* and increased degradation of globulin by elastase secreted from activated neutrophils(Bladon et al., 1996). Since these effects could be transient depending on a number of factors including variation in cytokine levels and hormones it is difficult to interpret the total free cortisol levels as both the globulin and albumin levels are extremely variable. In these circumstances salivary cortisol or serum free cortisol is taken as a better predictor of true cortisol levels and to reflect the cortisol response(le Roux et al., 2003). This may explain our findings of lack of correlation between the salivary and serum cortisol in critically ill patients.

In states of sedation our results demonstrate lower levels of serum total cortisol while the salivary cortisol does not show significant changes. Propofol was the predominant agent used for maintaining sedation in our cohort of patients. It is well documented that this drug causes increased vascular permeability mediated by nitric oxide signalling pathways(Chen et al.,

2007). The increased vascular permeability is associated with loss of intravascular proteins which in turn causes a decrease in the apparent total cortisol levels, i.e serum total cortisol levels. This explains our observation of a significant negative correlation between sedation score and serum cortisol levels. However as shown by the lack of correlation between the salivary cortisol and sedation levels, this does not reflect true variation in cortisol response but is a reflection of transient variation in albumin and cortisol binding globulin levels.

Thus our study reinforces the message that measurement that salivary cortisol, which reflects the active form, that is serum free cortisol, is a better measure of true cortisol levels especially in critically ill patients as it is not influenced by alterations of the protein levels or by physical activity or sedation. The collection of saliva is also non-invasive and hence can be used even in children and people who have needle phobia.

The drawbacks of salivary cortisol

It should however be borne in mind that measurement of salivary cortisol is not routinely available in the clinical setting. Currently it is predominantly used as a research tool, however it is utilised for diagnostic purposes in some hospitals. The most important drawback associated with salivary cortisol measurement is insufficiency of the salivary sample. As salivation is controlled by the autonomic nervous system, most of the patients in critically ill state or those with activated stress responses do not salivate. The problem is exacerbated as some of these patients are dehydrated or hypo-volaemic. This problem can be circumvented to some extent by increasing the duration for which the cotton plug used for salivary collection is kept in the oral cavity and also by using special cotton plugs which contain stimulants in the form of citric

acid. The plugs in our study were kept for the cavity for 2 minutes. Longer durations were not used as patients did not tolerate the plugs which resulted in feelings of nausea. Care must also be taken to avoid the risk of aspiration of the plug especially if these are used in children or in comatose patients.

Salivary cortisol and surgery

This is the first study comparing the pre and post operative the salivary cortisol levels in patients undergoing major surgery for malignancies. Our study demonstrated a progressive fall in the mean salivary cortisol levels in our group patients from day 0 pre-operative (day of surgery) to day of discharge. It has been recognised that the surgery itself is a stress factor and does cause stress response. The studies previously demonstrated an increase in the serum cortisol levels immediately after surgery as compared to the pre-surgical values(Bellon et al., 1998, Glaser et al., 1995). The degree of response was proportional to the degree of tissue trauma during surgery. However these studies were done in truly elective conditions wherein there was no ongoing pathological process; for example the study by Glaser *et al* compared elective laproscopic and open cholecystectomies, and by default elective cholecytectomy is contraindicated in the presence of any signs of inflammation of the gall bladder. One of the inclusion criteria in our study was that patients needed to have major surgery which required them to be admitted to the critical care area as per the hospital policy. In our study the patients recruited had extensive malignancies with local spread, requiring radical procedures to eradicate the malignancy. Some tumours can themselves evoke a stress response while in some types of cancers such as lung cancers(Lichter and Sirett, 1968) elevated cortisol levels may be a paraneoplastic feature. In our study the types of malignancies

were either urological or gastrointestinal; there have been studies suggestive of elevated cortisol as a result of direct effect of these malignancies. The workup to surgery and the psychological impact of the whole process is likely to have an influence on the cortisol levels, however it is possible that these malignancies may directly cause elevated cortisol levels by yet undescribed pathways. Thus it is not surprising that most of individual patients and the mean cortisol in the pre-operative state were highest. The inflammation and the stress responses associated with surgery and also the malignancy are likely to show a down trend with time. This is reflected in our group of patients as a progressive fall of mean salivary cortisol form pre-operative to post –operative and pre discharge states. The patients who had a lower cortisol levels pre-operative stage had a peak at the post-operative period followed by a fall at the pre-discharge stage. This trend however was not seen in 2 patients (patient ID 7, 17). These patients had similar cortisol levels during the pre- and post- operative periods and their cortisol levels progressively increased to the time of discharge. It is possible that these patients had residual pathology *in situ* which did not surface to the time that they were discharged. The difference in the cortisol levels between the groups that is in the post-operative and the pre-discharge state as compared to the pre-operative state were not statistically significant as assessed by paired *t*-tests. This could be accounted for by the marked individual variations and insufficient number of successful samples (having enough saliva for evaluation).

Human B –Defensins (HBD) and Cortisol

Although our study did not demonstrate a statistically significant correlation between the salivary cortisol levels and the *HBD1, 2,* and *3* expression, it is the first study of its kind and

helps understand the physiological response of the innate immune system in preparation and during a major stress event. Our study showed an increase in the *HBD1* and *3* mRNA expression in the pre-operative state and progressive tapering down of the expression similar to that of the cortisol pattern. There was statistically significant correlation between the *HBD1* and *HBD3* mRNA levels in the pre-operative and the post–operative groups. The *HBD2* mRNA expression unlike *HBD1 & 3* mRNA levels peaked in the immediate post-operative period before falling to less than pre-operative levels by the pre-discharge state.

HBD1, 2 & 3 are expressed in the oral cavity and serve as the first line of defense against microbial invasion(Dale and Krisanaprakornkit, 2001, Dunsche et al., 2002). In oral pathological conditions and inflammatory states the *HBD's* are variably expressed with *HBD2 and or 3* being more elevated compared to HBD1(Dommisch et al., 2005). These antimicrobial peptides in the oral cavity are considered to be stimulated in the presence of inflammation by the inflammatory cytokines specially TNF-α(Saitoh et al., 2004).

We have demonstrate for the first time a significant correlation between the *HBD1* and *HBD3* mRNA levels when the levels of *HBD1* and *3* were high; this suggests to a possibility of crosstalk between these peptides which until now have been considered primarily antimicrobial. Increased levels of *HBD1* and *3* in the pre-operative and the early post operative phase also demonstrate the heightened level of innate immune defences at the mucosal front in preparation for the stress event. This further co-relates with the fact that the majority of the oral cavity pathogens belong to the *Staphylococcal* group against which *HBD3* is specifically active. Increased levels of *HBD2* in the immediate post –operative period supports the increasing lobby of evidence suggesting to the more diverse role of *HBD2* apart from being

antimicrobial. Following the trauma of surgery the pro-inflammatory cascade is activated which includes IL-1 and IL-6 which are known to stimulate the *HBD2* expression. Some of the inflammatory mediators like TNF-α induce the expression of *HBD3*. These cytokine responses prevail for variable amounts of time following surgery, being dependent on a number of patient dependent factors. IL-6 is the most important mediator in the early post–operative phase which mediates activities like acute phase response(Sheeran and Hall, 1997), IL-6 is also a very potent inducer for *HBD2*. This response is however soon suppressed by the immunosuppressive influence of the cortisol(Desborough, 2000). This probably explains the peak *HBD2* levels in the post –operative phase. The other cytokines gradually decrease in time and this combined with microbial stimuli in the oral cavity determine the progressive fall in the *HBD2* and *HBD3* expression.

There was considerable variation in the individual levels of cortisol and the inherently expressed HBD1 in the pre and the post operative state, being more marked in the immediate post operative state. The levels of cortisol depend on the level of stress and its mediators while the factors influencing the levels of HBD1, 2 & 3 can be variable. In the pre-operative state the level of stress is expected to be variable depending on the psychological response to the malignancy and surgery. Also as suggested earlier the direct effect of malignancy may also influence the cortisol levels. Again in the immediate post-operative phase depending on the extent of the tissue trauma and the scale of surgery the cortisol levels can be extremely variable. This was demonstrable in our study as variation in the levels of cortisol demonstrated by 2SD of 9.34 in the immediate post-operative phase, while this was 8.2 in the pre-operative phase. As expected the variability decreased in the pre-discharge phase as levels of cortisol returned to normalcy with decreasing stress and stress mediators. HBD1 is normally inherently

expressed at a number of different sites of the body while HBD2 and 3 are normally inducible peptides. In the oral cavity presumably as it is perennially inhabited by some inherent flora all the three peptides are inherently expressed to variable levels depending on the flora. In our study the variability of HBD1, 2 & 3 was highest in the immediate post-operative phase as compared to the pre-operative phase.

The primary function of the innate immune system is the first line of defence against the invading micro-organisms, as secondary function components of the innate immune system also trigger the adaptive immune system to serve as a secondary line of defence again. In the oral cavity *HBD* serve as the first line of defence to prevent infections possibly by maintaining a homeostatic environment by keeping the flora in the oral cavity under check. The current study was an endeavour to understand how the body responds during an imminent major stress event in relation to expression of innate defence peptides *HBD1, 2 & 3.* The increased HBD1 & 3 mRNA expression in the pre-operative phase suggests to the activation of the innate immune responses to prepare for the stress event, especially given the fact that the oral cavity flora are sensitive to the *HBD 1 & 3.* As the stressors intensify by the actual onset of the tissue trauma the expression of these peptides is further induced at the mRNA level as evidenced by the increased mRNA expression. *HBD2* and *HBD3* have been shown to serve as a link between the adaptive and the innate immune system. The marked increase in the *HBD2* along with an increase in *HBD3* in the immediate post-operative phase suggest to the activation of the signalling pathways which can trigger the adaptive immune system to serve as a secondary defence mechanisms if the primary defence capacity is exceeded. The localisation of peptides by immunocytochemistry to the buccal epithelial cells suggests to the successful translation mechanisms.

Conclusion

Our study demonstrates increased expression of antimicrobial peptides *HBD1, 2, and 3* at the mRNA level in the early peri-operative period. This probably reflects the body's natural response at the mucosal front for a major stress event. The outcome of the stress event is however is based on the complex interplay between the degree of microbial challenge, duration of the stressor, the level of tissue and protein breakdown, the nutritional and the reserve capacity of the patient. The study also demonstrates for the first time the possibility of cross talk between the different *HBDs.* This requires further scrutiny but will be an important milestone in determining the scope of using these peptides for modulating the immune response to prevent infections in patients undergoing major stress events.

Chapter 6

Expression analysis of Human B Defensins 1, 2 & 3 in keloid scars

6.1 Introduction

Keloids are abnormal scars wherein the scar tissue takes a disfiguring appearance and extends over and around the original wound. The Smith Papyrus first described a disfiguring scar around 300BC however it was Jean Louis Alibert who coined the term ‘cheloide (Greek words; chele-crabs claw, oid-like) in 1806(Yang et al., 2003) to describe the crab claw like extension of the lesion into normal skin. The first systemic review of keloids appeared in literature in 1961 by Cosman and co-workers describing presentation, characteristics and possible treatment of keloids; subsequently Peacock and colleagues described the difference between keloids and hypertrophic scars(Peacock et al., 1970).

Keloids are considered to result from an exuberant healing response and abnormal dermal fibroblast proliferation(Calderon et al., 1996), secondary to cutaneous injury(Xia et al., 2006) with persistent growth of the wound tissue even after re-epithelisation and extension beyond the original boundary of injuring stimulus; being pathognomonic features.

Incidence and distribution

Keloids are benign or partially benign dermal mesenchymal tumours occurring on almost any part of the body; being less common in cornea(Bourcier et al., 2004) . They were initially thought not to occur on the weight bearing areas of the body namely palms and soles(Chipev et al., 2000) however there have been cases reported from these areas as well(LeFlore and Antoine, 1991). The incidence of keloids is higher among africans and asians(Tuan and Nichter, 1998) with equal sex distribution(Oluwasanmi, 1974). These lesions have been described in children with Lowe’s syndrome and Rubinstein-Taybi syndrome(Bourcier et al.,

2004). The tendency to form scars increases with the increasing gestational age and cellular maturity after 60-90 days gestation(Cass et al., 1997), and the incidence of keloids is higher around puberty and during pregnancy.

Symptoms and clinical presentation

Itch (alloknesis) and pain (allodynia) are common symptoms of keloid patients. Itching is common around the edge whereas pain is common around the centre of keloid. These two sensations are carried by small nerve fibres and may result from small nerve neuropathy(Lee et al., 2004) or due to the increased expression of neuropeptides like β CGRP and Substance P. The other common symptom is the disfiguring appearance of the scar. Apart from the physical symptoms there are immense psychological issues owing to the disfigurement associated with these lesions.

Aetiology

The aetiology of keloids can be variable however the common pathway is via the cutaneous insult. This can be in the form of a piercing for cosmetic reason for example ear lobule keloids, or surgical incision or trauma and burns. Other conditions like chicken pox and acne which can cause vesicles or breach of skin and infection or inflammation can cause keloids. The incidence of keloids following burns can be variable ranging from 4-16%.

Pathogenesis

Keloid was initially considered to be a dermal pathology but this view has changed with the unfolding knowledge. The patho-physiological mechanisms involved are complex and unclear and involve dermo-epidermal interactions. Keloids are associated with a significant morbidity from both a physical and psychological perspective and an effective treatment continues to evade this condition(McGrouther, 1994). The current treatments are ineffective and unsatisfactory and are associated with a high recurrence rates. More efforts and resources are needed to evaluate Keloid pathogenesis to have effective control of this disease entity(McGrouther, 1994). Current research reveals that in keloids, the epidermis shows abnormalities of keratinocyte proliferation and differentiation(He et al., 2004). There is dysfunctional regulation of the extracellular matrix (ECM) which contains hyaluronic acid, fibronectin(Diegelmann et al., 1979, Babu et al., 1989) and bi-glycans but no elastin(Bhangoo et al., 1976) or cellular components of normal scar milieu resulting in disproportionate extra-cellular matrix accumulation and tissue fibrosis. Keloids thus are characterised by histological features of prolonged inflammatory fibrotic activity marked by the presence of inflammatory cells like Mast cells and an environment rich in cytokines(Zhang et al., 2006). Keloids are histologically characterized by the presence of abundant collagen types 1 and 3(Abergel et al., 1985, Naitoh et al., 2001) and high metabolic rate(Ueda et al., 1999) mainly due to anabolic glycolysis(Ueda et al., 2004). The mechanism of keloid formation probably includes alteration in growth factors, collagen turnover, tension alignment and genetic and immunologic contributions(Al-Attar et al., 2006).

Keloid and Cellular Components

Keloid fibroblast show no significant differences either in morphology or growth kinetics to normal skin fibroblasts *in vitro*, although in a keloid they are arranged in a an irregular crisscross and overlapping pattern suggesting loss of polarity(Huang et al., 1998). Like other skin-derived cells they maintain a monolayer and display contact inhibition in cultures(Koch et al., 1997). Basal and superficial keloid fibroblasts show increased doubling time compared to the central keloid fibroblasts (Luo et al., 2001). Keloids are a local pathological process with no systemic influence as suggested by an *in vitro* study wherein the growth kinetics of keloid derived fibroblasts and normal dermal fibroblasts remained similar irrespective of culturing in the presence of keloid or normal patient sera(McCoy and Cohen, 1981). Keloid fibroblasts have localised hyper-androgen metabolism and are composed of polyclonal fibroblasts disproving the concept of over- proliferation of a single clone of cells(Chevray and Manson, 2004, Moulton-Levy et al., 1984).

Role of apoptosis

Keloid fibroblasts have increased proliferation rate due to lower inherent threshold for S phase entry(Calderon et al., 1996) and have higher rate of programmed cell death, (apoptosis)(Akasaka et al., 2001) and reduced survival(Akasaka et al., 2005) which is reflected *in vitro* by expression of caspase 2,3,9 and terminal deoxynucleotide transferase-mediated dUTP nick-end labelling (TUNEL). This apoptotic activity becomes more aggressive if the Keloid fibroblasts (KF) are deprived of serum and can be inhibited significantly by the caspase-3 inhibitor, Ac-Asp-Glu-Val-Asp-fluoromethyl ketone (DEVD-FMK)(Akasaka et al., 2000). Fas

protein is expressed excessively in Keloid and peri-keloid normal skin(Lu et al., 2003), and is mainly expressed in the central hypocellular region of Keloid with localisation along the cell surfaces. In contrast P53 and bcl-2 expression is localised to hypercellular, peripheral areas of Keloid in a peri-nuclear pattern. This focal up-regulation of p53and bcl-2 may produce a combination of increased fibroblast proliferation and decreased cell death in the younger, hypercellular areas of Keloid and this phenotype is reversed in older areas of Keloid. Such a distribution difference may be responsible for preventing malignant changes in Keloids(Palladino et al., 2003, Ladin et al., 1998). Subsequent studies showed that Keloid fibroblast are resistant to Fas mediated apoptosis; this resistance is reversed by neutralisation of autogenous TGF-β-1 thus suggesting a role of TGF-β-1 in resistance of Keloid fibroblasts to apoptosis(Chodon et al., 2000). Similarly Keloid fibroblast have enhanced expression of IGF-1 receptors which helps them to resist etoposide induced apoptosis(Sell et al., 1995) and also enhances their invasive activity(Ishihara et al., 2000). Keloid fibroblast proliferation can be inhibited by all isoforms of retinoid especially Tretinoin(Daly and Weston, 1986).

Fibrosis

Fibrosis is the key pathological event in Keloid pathogenesis. Keloid fibroblasts have an inherent capacity to synthesize more collagen(Diegelmann et al., 1979). Also increased fibrosis in keloids may be due to influence of multiple factors which are still unclear. It is generally perceived that the reparative fibrotic process progresses to pathological fibrosis due to chronic influence of pro-inflammatory cytokines which are known to exist in keloids. Increased expression of the *propiomelanocortin* gene and lack of regulation of cytokines like TGF-β is suggested in pathogenesis(Teofoli et al., 1997). An imbalance in the inflammatory

cell subpopulation of macrophages and lymphocytes particularly CD4 and CD8 lymphocytes and fibroblast may also play a role(Boyce et al., 2001). Similarly Keloid fibroblasts show resistance to macrophage/lymphocyte cytokine- oncostatin M which is a stimulator of normal fibrotic repair via activation of collagen and glycosaminoglycan synthesis in normal dermal fibroblast; thus leading to abnormal fibrosis(Duncan et al., 1995). Epidermal keratinocytes are also suggested to play a role through paracrine and double paracrine effects(Funayama et al., 2003) and epidermal-mesenchymal(Lim et al., 2001) signalling to promote dermal fibroblast proliferation and decrease apoptosis. The co-culture of Keloid keratinocytes and Keloid fibroblasts expressed more TGF-β-1,3 and TFG-β-1 receptors on both cell lines thus suggesting to cross talk between the cell types and complex interactions(Xia et al., 2004). The cellular difference between Keloids and hypertrophic scar was thought to be due to the presence of myofibroblast in hypertrophic scar but study of Keloid ultra structure has disproved this by showing the presence of myofibroblast in Keloid(Lehmann et al., 2002).

Keloid and Extracellular Matrix

Keloido-genesis is considered to be a fibrotic pathology due to the excessive extra-cellular matrix (ECT) production. Keloid collagen is distributed in the form of nodules which form the structural unit of Keloid(Kischer and Bailey, 1972). These nodules in turn are composed of compactly packed uni-directionally arranged collagen fibrils(Kischer and Brody, 1981) which can be visualised by transmission electron microscopy after toluidine blue O stain(Kischer, 1984). Collagen deposition increases with time from the base to surface and is concentrated along the lateral branches of new vessels Keloids are thus considered products of granulation tissue(Kischer et al., 1990). The Keloid matrix contains increased amount of collagen I and III

packed into nodules(Prathiba and Suryanarayanan, 1999) occluded vessels, fibronectin(Kischer and Hendrix, 1983), immunoglobulin, chondroitin-4-sulphate, integrin alpha 1,2(Szulgit et al., 2002) and higher partial pressure of carbon dioxide with low partial pressures of oxygen. Procollagen type-1 carboxyterminal propeptide (P1CP) represents type I collagen metabolism in fibroblasts and Keloid fibroblasts (KF) expressed more P1CP on application of histamine,TGF-B-1 and EGF than control normal fibroblast (NF). This increased metabolism of collagen type-1 was suppressed by gamma interferon(Kikuchi et al., 1995). This enhanced ECT production is considered to be due to suppressed activity of cyclooxygenase-2 in KF as compared to NF. The activity of cyclooxygenase was enhanced by macrophage migration inhibitory factor (MIF), a pluripotent cytokine in NF as compared to KF. MIF also induced increased production of prostaglandin E2 an anti- fibrogenic molecule in NF as compared to KF thus suggesting a role of prostaglandins in the pathogenesis of Keloids(Hayashi et al., 2006). Keloid fibroblast show increased production of ECT proteins such as fibronectin, tenascin C(Han et al., 2005, He and Han, 2004) and undulin(Dalkowski et al., 1999) both at RNA and protein levels as compared to NF. This activity is further enhanced by the TGF isofrom TGF-β-1(Babu et al., 1992, Bettinger et al., 1996). Similarly free radicals such as nitrous oxide can simulate Keloid fibroblasts to produce excessive collagen(Cobbold, 2001). The production of extracellular proteins and glycoproteins such as fibronectin, glycosaminoglycans (GAG) and collagen by Keloid fibroblasts can be inhibited *in vitro* by pentoxifylline, an analogue of methylxanthine theobromine, in a dose dependent manner(Berman and Duncan, 1989, Habib et al., 2003). Low energy neodymiun:YAG laser decreased collagen production *in vitro*; suggesting their use in modifying ECT in keloids(Castro et al., 1983).

Hyaluronic acid (HA) an important glycosaminoglycan and a component of the ECT has been found to form a pericellular coat around the cells. This tends to localise cytokines which induces collagen production. Alaish and co-workers demonstrated that Keloid fibroblasts synthesised higher amounts of hyaluronic acid compared to normal fibroblasts and thus in turn synthesised more collagen(Alaish et al., 1995). This increased synthesis of HA is reversed by triamcinolone acetonide which also softens and reduces Keloid size possibly due to enhanced degradation of collagen(Cohen et al., 1977). The Keloid matrix has similar types (type I and III) of collagen as normal skin(Clore et al., 1979) but high levels of tenascin C, an ECT glycoprotein that has been proposed as a marker for keloids(Dalkowski et al., 1999).

Keloid Vasculature

Keloids not only have an abnormal local microenvironment but also have altered vasculature It was previously thought that the Keloids had decreased vascular density and that the resulting hypoxia played an important role in pathogenesis(Beer et al., 1998). Recent studies demonstrated increased vascular density(Amadeu et al., 2003) and that the new microvasculature grows from below to the free surface in an environment rich in inflammatory cells and fibroblasts(Kischer et al., 1990). This increased angiogenesis is due to the increased expression of TGF-1 and vascular endothelial growth factors (VEGF).in Keloid fibroblasts(Fujiwara et al., 2005b). The major source of VEGF is epidermis rather than dermis(Gira et al., 2004). VEGF secretion could be blocked *in vitro* by verapamil, a calcium channel antagonist which also decreases the rate of proliferation of fibroblasts(Giugliano et al., 2003). The local invasive nature of Keloids is probably due to angiogenesis factors like vascular endothelial growth factor, basic fibroblast growth factor and platelet derived growth

factor and their respective receptors(Jiang et al., 2004). Keloids contain significant microvascular occlusion which begins during the granulation phase of wound healing. Thus occlusion produces a hypoxic environment that results in Keloids secondary to excessive collagen production(Kischer, 1992). This collagen forms nodules which contain only a few micro-vessels, most of which are occluded(Kischer and Bailey, 1972) due to increase in the number of endothelial cells. The micro-vessels are mainly peripheral at the edges of the nodules(Kischer and Brody, 1981). Keloids reveal an increase in subepidermal venous vessels which correlates very closely to the hyperaemic nature of Keloid(Lametschwandtner and Staindl, 1990). The neovascularisation in Keloids is promoted by increased expression of macrophage inflammatory protien-1(MIP-1), monocyte chemoattractant protien-1(MCP-1),interleuken-8(IL-8) and heme oxygenase(Zhu et al., 2005).

Keloid and Growth Factors.

The molecular basis of Keloid formation is governed by interplay of cellular signal pathways, specific target gene activation and the natural microenvironment resulting in prolonged inflammation and an altered balance in extra-cellular matrix metabolism(Le et al., 2004).

Keloids have increased expression of TGF-β1 and TGF-β-2 without any increase in TGF-β-3(Lee et al., 1999, Borel and Maquart, 1998) and this auto-production of TGF-β-I is minimised by truncated TGF β receptor II(Liu et al., 2002b). Similarly Keloids have an increased expression of TGF receptors I,II (TGG-R1/TGF-R11) and basic fibroblast growth factor(Hanasono et al., 2003).The increased expression of TGF β –I was inhibited *in vitro* by

tamoxifen(Mikulec et al., 2001) TGF-β stimulates the release of connective tissue growth factor CTGF) which is considered as a downstream mediator of TGF-β scarring and fibrotic activity. Both keloid and hypertrophic scars have increased intrinsic expression and exaggerated expression of CTGF in response to TGF –β stimulation(Colwell et al., 2005). Insulin like growth fFactor (ILGF) has synergetic effects with TGF-β-1 thus enhancing the production of ECT proteins 25 fold(Daian et al., 2003). The increased production of TGF-β 2 is mediated via p38 mitogen activated protein (MAP) kinase(Xia et al., 2006). KF cultured on collagen gel contracts the gel to a greater extent than the gel populated with NF, and this difference can be eliminated by TGF-β application to NF cultured in a gel(Kamamoto et al., 2003). Such contraction can be minimised by the application of TGF-β-1 inhibitor called SB-431542(Hasegawa et al., 2005).

SMAD-3 signalling

The SMAD-3 signalling pathway also plays an important role in Keloid pathogenesis through epithelial mesenchymal interaction(Tsujita-Kyutoku et al., 2005, Phan et al., 2005). The effect of TGF-β is terminated by negative feedback mechanism through SMAD6,7(Yu et al., 2005). These proteins are under-expressed in Keloid fibroblast.

Keloids also show increased expression of heat shock protein 47(Chen and Cen, 2005), free radicals(Selsted et al., 1996) and thrombospondin-1. The upregulation of plasminogen/plasmin system possibly mediates Keloid formation by thrombospondin-1(Centeno et al., 2002). Keloids exhibit increased phosphorylation through tyrosine kinase which is linked to many cytokine receptors like epidermal growth factor (EGF), discoidin

domain (DD), shc and adaptor protein. EGF stimulates the growth of Keloid fibroblasts (Harper, 1989) and Keloid fibroblasts have increased collagen synthesis(Diegelmann et al., 1979), this probably is related to increased expression of collagenase inhibitors alpha-1 antitrypsin and alpha-2-macroglubulin(Diegelmann et al., 1977). TNF-α is a potent pro-inflammatory agent involved in activation of signal events and transcriptional programs such as NF kappa β. TNF-α up-regulates NF kappa β in KF 15 times more than NF and Keloid tissue also expresses higher levels of TNF-receptor associated factors-TRAF1,TRAF2-TNF-alpha, inhibitor of apoptosis (c-1AP-1), and NF kappa β than normal skin(Messadi et al., 2004).

The Keloid fibroblasts produce type-1 collagen, matrix metalloproteinase-1(MMP-1) (interstitial collagenase), matrix metalloproteinase-2 (MMP-2) (gelatinase-A), matrix metalloproteinase-9(Neely et al., 1999) and tissue inhibitor of matrix metalloproteinase (TIMP-1). Increased production of MMPs has role in the high migratory activity of cultured Keloid fibroblasts(Fujiwara et al., 2005a). Keloid derived fibroblasts show enhanced proliferation in the presence of platelet derived growth factor (PDGF) due to increased expression of PDGF receptors(Haisa et al., 1994) on the fibroblast surface. PDGF exerts its role through ERK(Liu et al., 2003). Similarly the invasive property of Keloid fibroblasts can be explained by increased expression of insulin like growth factor-1 (ILGF-1) and its receptors on Keloid fibroblasts, and such invasive properties of KF can inhibited by neutralizing antibody to ILGF-1(Ohtsuru et al., 2000, Yoshimoto et al., 1999).

Interferon-gamma 2b (IFN-γ-2b) normalises the excessive synthesis of collagen, glycosaminoglycans and collagenase by KF and reduces Keloid reoccurrence by enhancing expression of p53 and apoptosis(Ladin et al., 1998, Jacob et al., 2003). Imiquimod is a rapid

and potent inducer of IFN-γ-2b and imiquimod treated Keloids showed significant down regulation of caspase-3 and up regulation of DFFA(Jacob et al., 2003).

Keloids are considered to have some immunological aspects due to the expression of antinuclear antibodies mainly directed against fibroblasts and the presence of raised levels of immunoglobulins A,G and M (Kischer et al., 1983).

The urokinase –mediated plasminogen (uPA) activation and inhibitory system is known to play a role in wound remodelling, angiogenesis and cell migration. Studies have shown a strong expression of uPA in the extracellular matrix of Keloids suggesting a role in the expansion of the Keloid beyond the wound margins(Leake et al., 2003). However other studies have shown that Keloid fibroblasts expressed low levels of urokinase plasminogen activator (uPA) and high levels of plasminogen activator inhibitor(PA-I) thus favouring the collagen accumulation(Tuan et al., 2003, Tuan et al., 1996). This increased expression of PA-I is stimulated by increased expression of VEGF(Wu et al., 2004).

Epidermal growth factor receptors (EGF-R) can induce the fibroblast motility in normal wound healing but this response is somewhat diminished in Keloid fibroblasts due to the rapid loss of EGF-R upon exposure to EGF(Satish et al., 2004).

Hypoxia produced at the healing wound either by surgical techniques or poor vasculature can induce Keloid formation possibly due to the over- expression of vascular endothelial growth fFactor (VEGF)(Steinbrech et al., 1999) and hypoxia induced factor-1(HIF-1)(Zhang et al.,

2003). This expression of VEFG and HIF-1 is augmented when Keloid fibroblasts are co-cultured with mast cells (Zhang et al., 2006).

The acidic and basic fibroblast growth factor (FGF) can decrease the biosynthesis of collagen-1 by down-regulating gene expression. The effect of acidic FGF is enhanced in the presence of heparin but basic FGF does not need heparin to inhibit the collagen biosynthesis(Tan et al., 1993).

Prevention of Keloid

Keloids remain one of the best examples to the dictum that “prevention is better than cure”. There is no effective treatment and hence all possible steps should be taken to prevent the formation a Keloid. It is evident that inflammation plays an important role in Keloid pathogenesis and monofilament suture initiates less inflammation than multifilamentous suture like vicryl rapide leading to less scaring and Keloid formation(Niessen et al., 1997).

Formation of Keloids can be minimised by accurately apposing the wound edges, placing the suture under minimal tension, everting the skin edges, placing the wound in Langer’s lines, adequately supporting the wound and ensuring the wound is free of infection and haematoma(Wallace et al., 2006). The inflammatory phase of wound can be modified pharmacologically by anti-inflammatory agents, fibroblast proliferation can be slowed by radiotherapy and collagen bundle orientation by pressure application(Ketchum, 1977).

Keloids and current treatments

Occlusive therapies include silicone and such other occlusive dressings. There is no clear evidence in relation to these therapies that are thought to act by promoting local hydration. However they can cause adverse effects in the form of local maceration and irritation.

Compression therapy involves using local pressure with or without Silicone. There is no clear evidence as to mechanism of action of this therapy, but concepts such as fibroblast degeneration from local hypoxia or temperature differences have been proposed.

Pharmacological therapies

Triamcinolone acetonide(Carroll et al., 2002) and heparin(Carroll and Koch, 2003) have been shown to decrease the cellular proliferation and collagen production by dermal fibroblasts. Triamcinolone acts by mediating the release of bFGF and lowering the release of TGF-b1 while heparin increases both of bFGF and TGF-b. The response to triamcinolone acetonide inhibition on keloid fibroblast is far less than on normal skin fibroblasts(Russell et al., 1982). Administration of hydrocortisone decreases hyaluron levels by approximately 70%(Meyer et al., 2000). Flashlamp Pulse-Dye Laser (PDL) which is another modality used to treat Keloids suppresses the Keloid proliferation, induces Keloids fibroblast apoptosis and up-regulates extracellular signal regulated kinase (ERK), p38 mitogen-activated proteins kinase (p38MAP kinase)(Kuo et al., 2005a) and down- regulates TGF-β1(Kuo et al., 2005b). Interferon has shown impressive reduction is size and collagen production of Keloids(Low and Moy, 1992). Phan and co-workers (2003) showed that Quercet, a dietary compound which has strong antioxidant and anticancer properties, can block the TGF-β/SMAD-signalling pathway(Phan et

al., 2004) and ILGF-1 signalling transduction(Phan et al., 2003), resulting in decreased Keloid fibrosis. Similarly endothelial cell growth factor (ECGF) and heparin minimise Keloid fibrosis by inhibiting collagen production by Keloid fibroblasts(Bontems et al., 1991). 5-Fluorouracil , a pyrimidine analogue and a common cancer chemotherapeutic drug, has been used in Keloid treatment. The mode of action is probably by antagonising the effect of TGF-β and it also inhibits both SMAD3and SMAD4 transduction and formation of SMAD/DNA complex induced by TGF-β(Wendling et al., 2003). Other pharmacological treatments include bleomycin, doxurubicin, tamoxifen, tacrolimus, imiquinoid, retinoic acid, and verapamil.

Human β-Defensins and Keloids

Keloids appear to have a multi-factorial pathogenesis where a number of factors interplay to form these lesions in individuals with suitable genetic predisposition. The multi-factorial pathogenesis can be summarised as either an increased exposure to, and/or expression of the inflammatory mediators. These inflammatory mediators influence the fibroblasts either directly or indirectly to produce excessive collagen or decrease collagen breakdown resulting in the formation of the Keloids. Human β defensins (HBD) are a group of peptides which were originally considered to be primarily anti-microbial in function. Up to 28 HBDs have been described although the more extensively studied are HBD 1, 2, and 3. As the research and understanding of these peptides has progressed, a number of other functions have been attributed to them. HBD1 has been shown to play a role in cell differentiation(Frye et al., 2001) while HBD2 has been shown to be a link between the innate and the adaptive immune system(Yang et al., 1999). HBD2 has a number of indirect effects and mediates release of a number of inflammatory mediators(Niyonsaba et al., 2001). To date there is no information on

the expression pattern of HBDs in Keloid lesions. Since Keloids result from increased exposure to inflammatory mediators, the expression of HBD 1, 2 and 3 in Keloid lesions at both RNA and protein levels was investigated.

6.2 Materials and Methods

The clinical part of the study was undertaken at The Royal London Hospital of the Barts and the London NHS trust. Keloid samples from various body sites of 10 Afro-carribean patients were collected (Ethical Committee approval Ref.05/Q06603/22). Normal skin samples were obtained from people undergoing elective plastic surgical procedures after obtaining an informed consent.

Table 6.1-Demographics of the keloid patients

Sample	Ethnicity	Gender	Age	Anatomical Site
Keloid-1	Afro-carribean	M	27	Ear lobule
Keloid-2	Afro-carribean	M	30	Chest
Keloid-3	Afro-carribean	F	20	Ear lobule
Keloid-4	Afro-carribean	F	22	Ear lobule
Keloid-5	Afro-carribean	F	19	Abdomen
Keloid-6	Afro-carribean	M	22	shoulder
Keloid-7	Afro-carribean	F	19	Ear lobule
Keloid-8	Afro-carribean	F	20	Ear lobule
Keloid-9	Afro-carribean	F	21	Abdomen
Keloid-10	Afro-carribean	M	20	Ear lobule

The patients were given all the required information under Tissue Act April.2006 and a valid informed consent were taken from each patient. Tissue was transport from Theatre to Laboratory in saline soaked gauze. The tissue sample was registered in Tissue Register kept in laboratory for future record. On arrival the skin and keloid samples were cut into blocks of approximately 2cm x 2cm x1 cm using a scalpel with a size 22 blade (Swan-Morton, Sheffield U.K.). Then the cut skin or Keloid samples were snap frozen in liquid nitrogen and stored at -80^{0}C until needed.

Snap frozen pieces of skin and Keloid were mounted in Cryo-M-Bed (Bright, Huntington, UK) and 7 μm thick sections cut on a cryostat (Slee, Mainz, Germany) at -25^{0}C .Sections were thaw mounted on Superfrost® plus microscope slides (Knittel Glaser, Germany) and air-dried for 30 minutes, before storage at -80^{0}C until required.

HBD 1,2 and 3 Protein expression Immuno-histochemistry

Immuno-histochemical analysis was performed to study the expression analysis at the protein level. The methods were similar to those described earlier in chapter 2 (general methodology). The anti-bodies optimised previously were used in the same dilutions as described earlier.

The frozen sections were thawed and once dried, fixed in acetone alcohol for 10 minutes. The slides were then washed with water and processed as described in chapter 2. The slides were developed with DAB and subsequently analysed after counter staining with haematoxylin and eosin.

RNA expression and real time PCR analysis.

RNA was extracted from snap frozen tissue specimens using SV total RNA isolation kit (Promega, UK) as described (in chapter 2 – General methodology) according to the manufacturer's protocol. The RNA obtained was reconstituted after concentration to obtain the desired concentration for cDNA conversion and DNA amplification. Less than 1µg of total RNA was used per reaction for cDNA conversion and 2µl of template cDNA was used per reaction of PCR. Real time PCR was performed using Opticon II PCR machine. The amplification was carried out using gene specific intron spanning primers and labelled minor groove binding probes as described in the chapter on general methodology.

6.3 Results

All the 10 patients recruited in the study were of Afro-Carribean origin and the male to female ratio was 4:6. The mean age of the patients was 22 years with a range between 19-30 years. As seen in table 6.1, the commonest site of the Keloids was ear lobules in our group; while the other sites analysed included abdomen, shoulder and chest. The most common aetiology for the Keloids in our group of patients was piercing for cosmetic purposes.

Protein expression

Immunohistochemical analysis showed that HBD1 and 3 were expressed at varying levels in all Keloid samples whereas none of the samples showed any detectable HBD2 expression.

HBD1 expression

HBD1 was seen almost evenly throughout all layers of epidermis and in some samples the protein was more localised in the stratum corneum. The mean expression of HBD 1 in the Keloid samples was 1.9 while mean in normal skin was 1.5.

HBD2 expression

There was no expression of HBD 2 seen in any of the samples. However the normal skin samples showed variable expression while the negative controls without the primary antibody did not show any staining. The expression of HBD2 in normal samples was mainly localised in the stratum corneum.

HBD3 expression

The Keloid samples showed higher expression of HBD3 than normal controls. The expression was predominantly seen in the stratum corneum and other superficial layers of epidermis. The mean HBD3 expression in keloids was 2.4 while the mean in normal skin was 1.5.

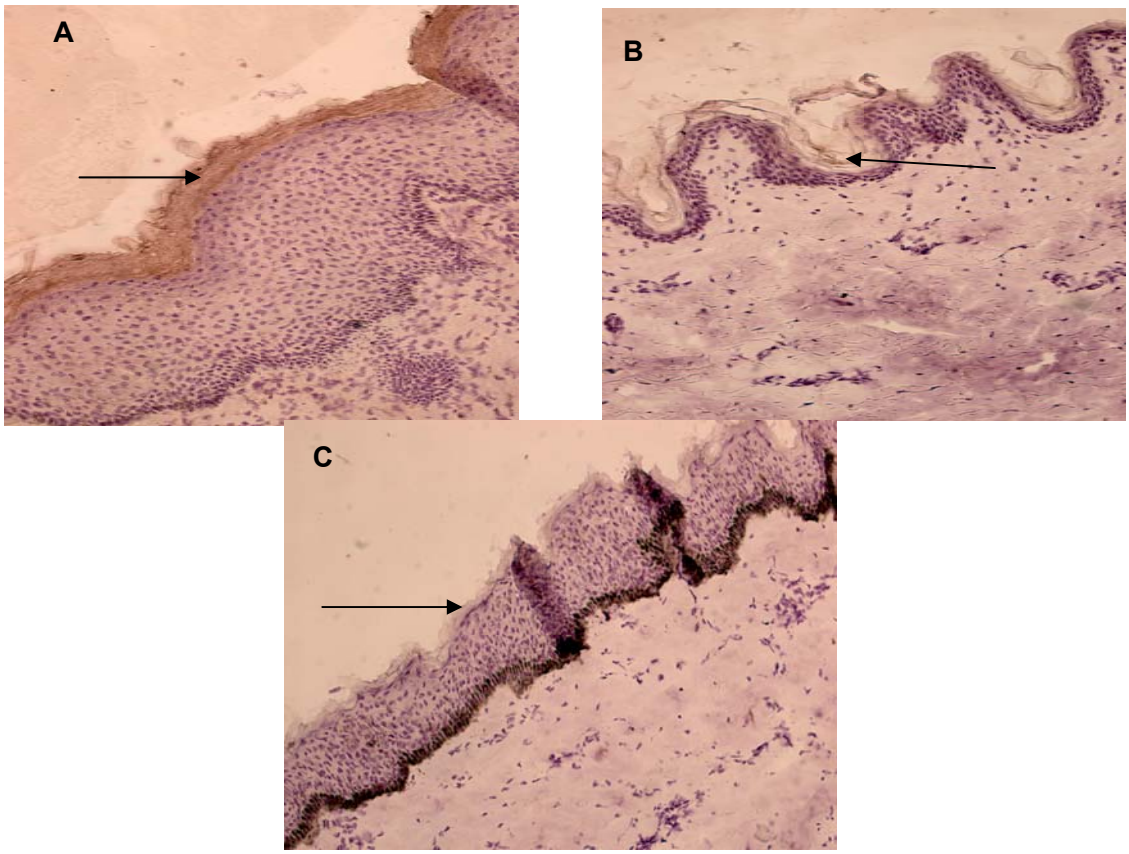


Figure 6.1, shows the representative sections stained with HBD1 antibody and developed using DAB (magnification 20x). Panel A- shows the section from patient 2 (pre-sternal keloid); the arrow shows the localisation of HBD1 in stratum corneum using specific antibody and DAB, Panel B –shows the normal skin section stained with HBD1 and the arrow shows the localisation of the protein in stratum corneum. Panel C-shows the keloid skin sample stained with no primary antibody. The arrow points to stratum corneum and it can be seen that there is no HBD1 localisation. Melanocytes containing melanin can be seen in the basement membrane.

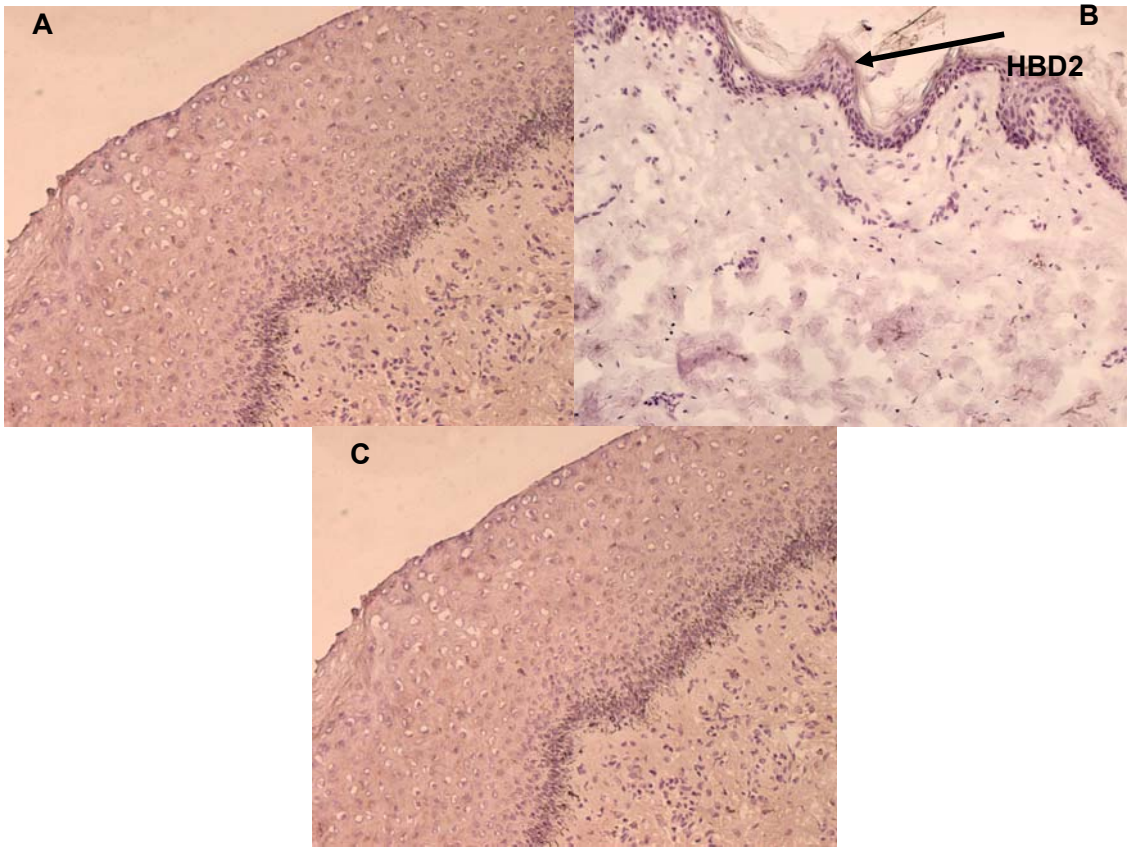


Figure 6.2 –Shows B Defensin-2 expression in Keloid and site matched normal skin sample (magnification 20x). Panel A shows the ear lobule keloid stained with HBD2 antibody. No HBD2 was localised in the keloid sample. Panel B shows a normal skin around ear (face lift) stained with HBD2 antibody. As seen HBD2 protein was localised to epidermis, mainly stratum corneum (arrow). Panel C shows the same ear lobule keloid not stained with the primary antibody (negative control).

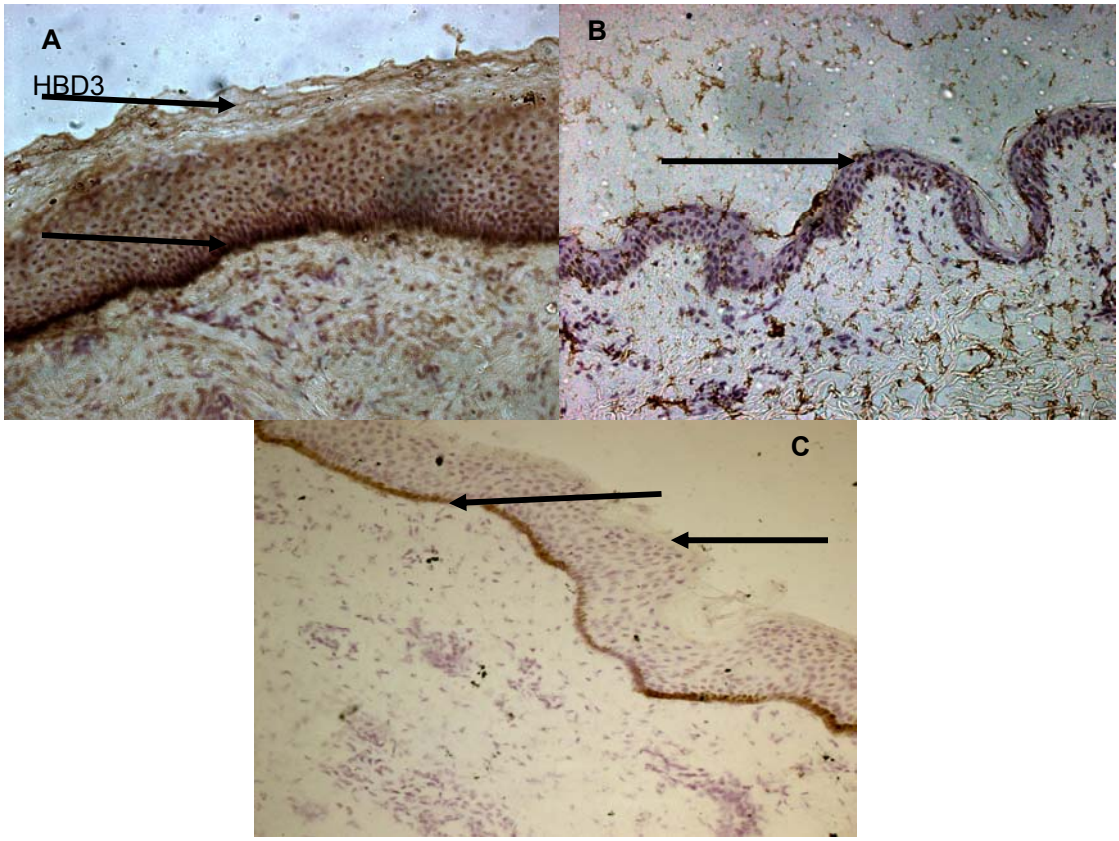


Figure 6.3 B Defensin-3 expression in Keloid from shoulder region (Patient K6)and site specific Human Skin (chest). Panel A shows the localisation of HBD3 to stratum corneum and cells of basement membrane (arrows). In the basement membrane the melanocytic pigmentation can also be seen as the darker pigment. Panel B shows the normal skin section stained with HBD3. The brown stain represents the HBD3. Panel C shows the keloid sample stained without the Primary HBD3 antibody (-ve control. As seen there is no protein localisation in the stratum corneum (top arrow) or in the basement membrane. The brown/black pigment seen in the basement membrane is the melanocytic pigmentation.

The expression of HBD1 and 3 was variable in keloids from different sites as seen in figures 6.1.6.3 and 6.4 and table 6.3. There was no HBD2 peptide expression in any of the keloid samples (Figure 6.2 and Table) 6.3.

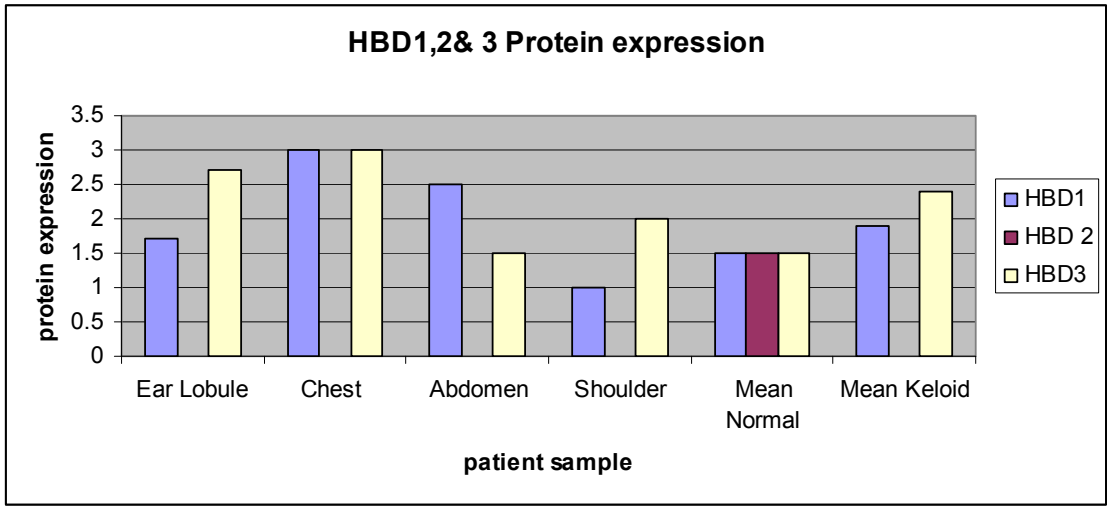


Figure 6.4 shows the distribution of the HBD1, 2 and 3 proteins in the keloid tissue samples. The mean expression in the normal and the keloid samples is shown to wards the end of the graph. As seen none of the keloid samples actually showed HBD2 protein expression

Table 6.2 summarises the stoichiometric analysis of sections stained with HBD1,2 and 3 antibodies

Site	Sample	HBD1	HBD2	HBD3
Ear lobule	K 1	2	0	3
Chest	K 2	3	0	0
Ear lobule	K 3	1	0	3
Ear lobule	K4	2	0	3
Abdomen	K5	3	0	2
Shoulder	K6	1	0	2
Ear lobule	K7	2	0	3
Ear lobule	K8	2	0	2
Abdomen	K9	2	0	1
Ear lobule	K10	1	0	2
Mean	All Keloids	1.9	0	2.4
Normal skin				
Face	Face	1	2	2
Breast	Breast	2	1	1
Mean Normal skin		1.5	1.5	1.5

The results of qualitative Immunoreactivity of HBD1,2 and 3 antibodies on keloid samples has been summarised in Table 6.3.

Type of Defensin-β	Immunoreactivity	Distribution of expression of Defensin -β
HBD1	Positive, variable Expression at Different sites	Dense at Stratum Corneum and faint through out the Epidermis other layers of epidermis
HBD2	Negative	No Expression in any layer of epidermis
HBD3	Positive	Dense expression at Stratum Corneum and Some expression in the basal layer

Real time PCR analysis

Gene expression was calculated by ΔΔCt method. Briefly the method can be explained as follows: The Ct of the gene of interest and the housekeeping gene was determined

The difference between the Ct of the housekeeping gene and the gene of interest (ΔCt) is calculated. This gives the expression of the gene of interest in each sample relative to the

housekeeping gene (*β actin*). In order to normalise the expression, the ΔCt of the individual sample is subtracted from the mean ΔCt of the normal skin to obtain the ΔΔCt for each of the genes. Subsequently -ΔΔCt is determined and the individual expression is expressed as $2^{-\Delta\Delta Ct}$. This gives the expression of the gene of interest relative to the *β actin* gene normalised to the expression in normal skin. Table 6.4 shows the ΔCt values for each of the samples and the statistical significance (standard deviation (SD)).

Table 6.4 summarises the mean ∆Ct values for each of the HBD's. B1 stans as BD1, B2 for BD2 while B3 stands for BD3. IF the Ct was beyond 40 cycles, the expression was disregarded and the expression was taken as zero. In these situations eg as in BD2 in keloid samples ∆Ct was taken as zero.

Site	Sample	Mean *B1* ∆Ct(Ct *actin* –Ct *B1*)	SD	Mean *B2* ∆Ct(Ct *actin* – Ct *B2*)	SD	Mean *B3* ∆Ct(*Ct* actin – Ct *B3*)	SD
Ear lobule	K 1	2.3	0.6	0.00	0.00	9.7	0.1
Chest	K 2	-3.1	1.3	0.00	0.00	5.9	0.3
Ear lobule	K 3	0.8	0.2	0.00	0.00	10.5	0.7
Ear lobule	K4	2.6	0.3	0.00	0.00	9.9	0.1
Abdomen	K5	1.5	0.2	0.00	0.00	10.1	0.3
Shoulder	K6	2.3	0.4	0.00	0.00	10.2	0.4
Ear lobule	K7	1.7	0.2	0.00	0.00	10.3	0.1
Ear lobule	K8	1.7	0.3	0.00	0.00	6.2	0.2
Abdomen	K9	1.4	0.3	0.00	0.00	8.9	0.1
Ear lobule	K10	1.8	0.1	0.00	0.00	9.3	0.8
Mean	All keloids	1.3	0.4	0.00	0.00	9.1	0.2
Normal skin							
Mean Face		1.1	0.3	5.0	1.5	7.4	0.2
Mean Breast		-0.6	1.5	2.2	0.4	6.9	0.8
Mean Normal skin		0.2	0.9	3.6	0.9	7.2	0.5

***Table 6.5- shows the mRNA expression in the keloid and normal skin samples The mRNA expression is presented normalised to house keeping gene Beta actin relative to the mean expression in normal skin.* |**

	Sample	*B1*RNA Log $2^{-\Delta\Delta CT}$	*B2*RNA Log $2^{-\Delta\Delta CT}$	*B3*RNA Log $2^{-\Delta\Delta CT}$
Ear lobule	K 1	2^{2}	0	2^{2}
Chest	K 2	2^{-3}	0	2^{-1}
Ear lobule	K 3	2^{1}	0	2^{3}
Ear lobule	K4	2^{2}	0	2^{3}
Abdomen	K5	2^{1}	0	2^{3}
Shoulder	K6	2^{2}	0	2^{3}
Ear lobule	K7	2^{2}	0	2^{3}
Ear lobule	K8	2^{1}	0	2^{-1}
Abdomen	K9	2^{1}	0	2^{2}
Ear lobule	K10	2^{2}	0	2^{2}
Normal skin				
Mean Face	Face	2^{1}	2^{1}	2^{0}
Mean Breast	Breast	2^{-1}	2^{-1}	2^{0}
t-test		0.230		0.002

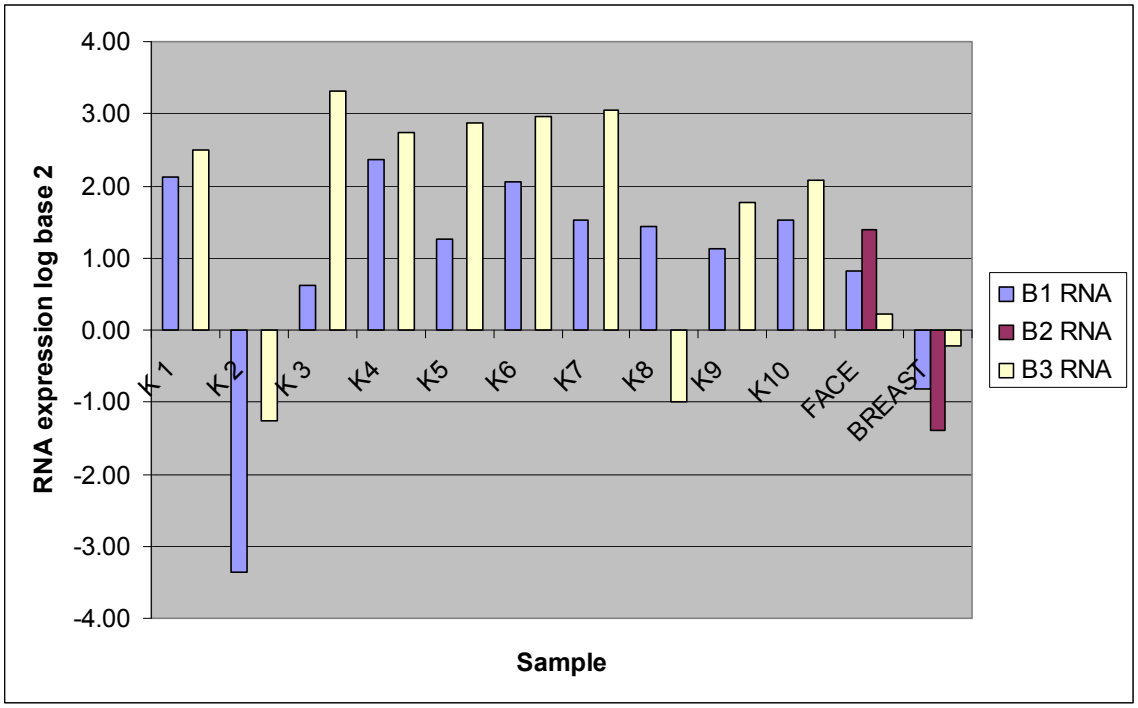


Figure 6.5 shows the graphical representation of the mRNA expression in the keloid and normal skin samples. The mRNA expression is shown on the Y-axis in log scale and is normalised to the housekeeping gene Beta actin and relative to the mean expression in normal skin.

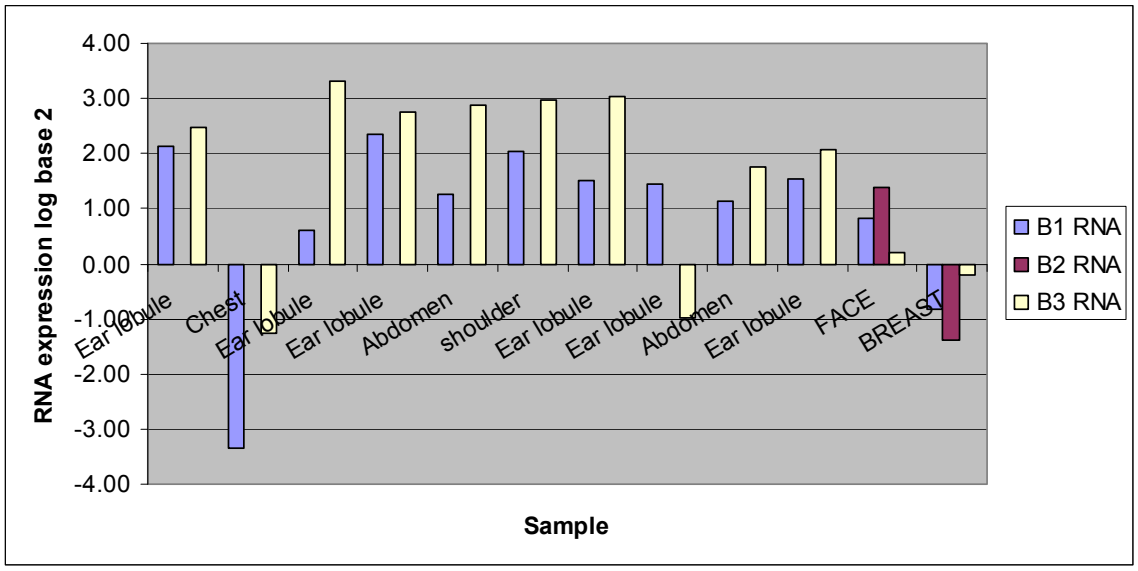


Figure 6.6 shows the sites of the different keloid samples and their mRNA expressions

The mRNA expression of HBD1 and 3 was variable in different keloid samples and also was varied depending on the site. There was no detectable HBD2 mRNA expression in any of the keloid samples while the all the three HBD's were expressed to variable extent in the normal skin specimens. The keloid from chest region had less HBD1 and 3 expression compared to mean HBD1 &3 expression in normal skin as seen in figures 6.6 and 6.7.

HBD 1 RNA expression

The expression of H*BD1* was variable in the individual Keloid samples as seen in the table a. number. Except for the Keloid from the chest (K2), the expression of H*BD1* in Keloid samples was higher (mean 3 times the expression in normal) than the mean H*BD1* expression in normal samples. The difference in the expression of H*BD1* between the normal samples and the Keloid samples was not significant (Unpaired T –Test 2 tailed significance =0.230).

HBD2 RNA expression

There was no detectable product for H*BD2* in any of the Keloid samples; product that was detected after 40 cycles was taken as insignificant. There was variable expression of H*BD2* in normal breast and face skin samples. The H*BD2* expression in the normal breast sample was 25% lower than the mean H*BD2* expression in normal skin samples.

HBD3 RNA expression

The *HBD3* expression was variable in Keloid samples from different sites. The *HBD3* was predominantly higher (mean Keloid HBD3 was 5.3 times normal) as compared to the mean H*BD3* expression in normal skin samples. This difference in the expression was statistically significant (unpaired *t*-test 2 tailed statistical significance 0.002).

6.4 Discussion:-

Wound healing is one of the most primitive but important innate immune response exhibited by the human body. The reparative process results in the fibrotic scar formation. The process of cutaneous wound healing can be divided into three phases: inflammation, proliferation which comprises re-epithelialisation, granulation tissue formation and neovascularisation, and maturation. Such a complex process is governed by well organized secretion of cytokines, growth factors and chemokines(Werner and Grose, 2003). The Keloids represent an abnormal healing process and are associated with a complex interplay of cytokines and growth factors which influence the cellular components and extracellular components to form these benign disfiguring lesions. Though fibroblasts are the main cellular components

involved causing the fibrotic process, it is proposed that dermo-epidermal interactions play an important role in the pathogenesis(Lim et al., 2002). The morphology of a Keloid is associated with a hyper-proliferative dermal and epidermal component. Keratinocytes being the principal component of epidermis and also the main source of *HBD*; their expression was investigated for the first time in this study.

Throughout the three phases, different cellular and humoral components interplay to bridge the epithelial gap and to lay down the collagen tissue to provide adequate strength. During the maturation phase there is remodelling of collagen wherein there is change of type III collagen to type I and also a process of degradation and synthesis to achieve optimal scarring. Thus the final outcome is a result of the balance between the syntheis and breakdown of collagen which in turn results from a balance between the profibrotic and anti-fibrotic cytokines. There are a number of growth factors and cytokines secreted during the wound healing process but the most important profibrotic mediators are TGF-β, SMAD-3, CTGF, ED-A fibronectin and the anti-fibrotic mediators are TNF-α, IFN-γ(Leask and Abraham, 2004). Thus, the final outcome of scarring process is a result of balance between these two sets of cytokines and their downstream mediators.

HBD are cationic mucosal and skin proteins of innate immune system(Donnarumma et al., 2004) expressed by the keratinocytes and found in the interstices between the cells. They are primarily considered antimicrobial as they are localised at sites of environmental interfaces and are induced by infective and inflammatory agents. The role of antimicrobial peptides is being clearly recognised in the innate defence of mammals at the epithelial

front(Huttner and Bevins, 1999). As new functions of these peptides are uncovered it is becoming obvious that HBD may have additional important roles.

HBD1 is a 3.9 kDa peptide of 36 amino acids residues and is mainly produced by various epithelial tissues including urogenital(Bals et al., 1998), respiratory system(Alp et al., 2005) and human skin. This isomer of β defensin, although constitutively expressed,can be induced by microbial derived molecules such as lipopolysaccharide (LPS)(Bohling et al., 2006, Becker et al., 2000) and peptidoglycan(Bals, 2000). The expression of BD1 is also stimulated by pro-inflammatory cytokines such as IFN-γ(Duits et al., 2002) and TNF- α however the relative stimulation is much less compared to other HBDs(Lehmann et al., 2002). Other studies have shown that BD1 expression is not enhanced by TGF- α, TGF- β, IL-1, IL-2, 4, 6, 8, 17 and TNF- α(Sorensen et al., 2005). HBD1 is expressed consistently in skin and besides its antimicrobial property it is a strong chemo-attractant in the inflammatory milieu and is salt sensitive. It has been shown to promote differentiation of keratinocytes(Frye et al., 2001) and the type of HBD expression by keratinocytes depends on the stage of their differentiation(Chadebech et al., 2003) and extracellular and intracellular concentration of calcium ions.

The expression of HBD1 in Keloids has never been investigated and this study demonstrates that the HBD1 is expressed in all Keloid samples tested. The expression was variable in Keloids from different sites and this is similar to normal skin wherein there is a site specific variation(Ali et al., 2001). There was no significant statistical difference in the expression between the Keloid samples and the normal skin samples although the expression in Keloid samples is relatively high (mRNA expression 3 times normal). Keloids are associated with

increased levels of TGF- β(Lee et al., 1999) and also increased levels of IL-6, TNF- α, IFN-β(McCauley et al., 1992) and increased sensitivity of Keloid tissue to inflammatory mediators. These inflammatory mediators either do not increase the expression of HBD1 or cause a relatively small increase in the HBD1 expression. The promoter region of HBD1 contains consensus sites for NF-IL-6 and interferon IFN-γ(Liu et al., 1997). Although constitutive, the presence of these consensus sites suggests to possibility of enhanced expression and transcriptional regulation by these cytokines. However since the cytokines which are increased in Keloids are not the ones that trigger any of these mediators the lack of increased HBD1 can be explained.

HBD2 consists of 41 amino acids and has 4.3 kDa peptide, is mainly expressed in psoriatic skin and is intensely induced by interleukin (IL-1), tumour necrosis factor alpha (TNF-α), interferon gamma (IFN-γ) and bacteria(Abiko et al., 2002). In normal skin, where it is variably expressed, HBD2(Ali et al., 2001) is localized to uppermost layer of epidermis called the Stratum corneum and is inherently expressed in chronic wounds(Butmarc et al., 2004). HBD2 is synthesized and stored in laminar bodies of the stimulated keratinocytes of the spinous and granular layer. The laminar bodies may then release their content during differentiation of keratinocytes and dendretic cell maturation(Biragyn et al., 2002), and after barrier disruption into the intercellular spaces of the epidermis layers where high amounts HBD2 are detected. The BD2 acts through toll-like receptors 4 (TLR-4)(Biragyn et al., 2002) and TLR-2(Birchler et al., 2001, Hertz et al., 2003) and is salt sensitive which can be overcome by its increase concentration.

Apart from antimicrobial activity HBD2 activates mast cells(Niyonsaba et al., 2001) to degenerate and generate prostaglandin D2, has a chemoattractive role for mast cell and stimulate keratinocytes to produce interleukin 8, 18 and 20. Furthermore the production of hBD2 by IL-1 and IL-18 is under the control of MAP kinase p38 MAPK and ERK. The promoter region of HBD2 contains consensus sites for NF-kβ activator protein 1 (AP-1) and NF-IL-6(Liu et al., 1998). It has been shown previously that HBD2 is stimulated only in the presence of NF-kβ and IL-1 mediated stimulation; isolated NF-kβ activation does not result in trigger stimulation(Takahashi et al., 2001).

We have shown in this study that there is no demonstrable protein or RNA expression of HBD2 in Keloids. The normal skin showed variable levels of HBD2 expression depending on the site of body and local factors such as hygiene and bacterial colonisation. The Keloid samples in our cohort were obtained from different body sites and none of them expressed HBD2. As seen above, the known mechanisms of induction of HBD2 include NF-kβ, AP-1 or NF-IL-6 pathways. The anti-fibrotic cytokines namely TNF-α and IFN-γ activate the pathways and induce production of HBD2, but these are not the predominant cytokines in Keloids in which the pro-fibrotic cytokines, which do not activate the HBD2, dominate. HBD2 was originally isolated form psoriatic scales and psoriasis is a hyper-inflammatory non–infective diseased state characterised by high levels of pro-inflammatory cytokines such as TNF-α (TRAF-2) , IFN-γ , IL-1 (TRAF-6) andIL-6(Nickoloff et al., 2007). These cytokines have a capacity to stimulate production of BD2 owing to presence of consensus sites on the promoter region. This probably explains increased levels of HBD2 although there is no bacterial stimulus. It can also perceived that the role of these peptides is more protective and

prophylactic as they are produced in the absence of bacterial mediators also they may have yet un-described functions. As regards to the lack of HBD2 expression in Keloids is probably due to absence of mediators; microbial and non–microbial (cytokines) and thus there are no factors to induce HBD2 expression.

HBD3 is a 5.1 kDa protein and has 45 amino acids residues. HBD3 is an inducible protein expressed significantly in keratinocytes and tonsillar tissue and to a variable extent in small amount is expressed in epithelium of respiratory, gastrointestinal tract (GIT) and genito-urinary tract (GUT). HBD3 has strong antimicrobial activity against gram negative and gram positive bacteria including fungi. Its expression is induced by bacterial contact and TNF-α(Harder et al., 2001) and IFN-γ It has been shown recently that IGF-1 and TGF-α selectively increase the expression of BD3 in human keratinocytes(Sorensen et al., 2005). BD1 and 3 have been shown to be selectively induced in differentiated keratinocytes in an *in vitro* environment.(Abiko et al., 2003) In our study we demonstrated an increased expression of HBD3 in keloids at both RNA (5.3 times normal skin expression) and protein levels compared to normal skin samples. As seen above there are a number of infective and non-infective factors which can induce HBD3 expression and HBD2 and 3 are induced by different pathways. The increased expression in Keloids is probably mediated by the IGF-1 as Keloids have been shown to have increased expression of IGF receptors(Daian et al., 2003) or is a consequence of multilayers of differentiated keratinocytes producing HBD3 which are seen in the Keloid scar.

Keloids- epidermal pathology influencing the dermis.

Keloids result from a cutaneous insult either in the form of a severe or trivial injury. Although it is suggested that Keloids can occur spontaneously, it is considered that these are a result of insignificant trauma that is ignored(Pitche, 2006). The cutaneous injury results in platelet aggregation and activation of coagulation cascade and release of inflammatory mediators. These mediators attract other cell populations namely macrophages, PMN and T lymphocytes. These cell populations produce cytokines and growth factors which result in epithelialisation, synthesis of ECM, and neo-vascularisation. As the wound healing proceeds to the maturation phase the final outcome is dependent on the balance between the factors influencing the degradation and synthesis of ECM.

The platelets attracted to the site of injury produce the cytokines which trigger the inducible component of HBD. HBD 2, and 3 in turn re-inforce the chemotactic influence mediated by the cytokines produced by platelets and attract the inflammatory cells. It has been shown that HBD1 and 3 expression is stimulated in differentiated keratinocytes(Abiko et al., 2003). HBD2 is enhanced in differentiated keratinocytes by the pro-inflammatory cytokines such as IL-1α(Liu et al., 2002a). However there is no expression of HBD2 in Keloids probably due to the absence of cytokines which can induce HBD2 expression. We propose that HBD2 has a role in regulation of anti-fibrotic cytokines as it has been shown to be important in regulating other immune factors(Dommisch et al., 2007) and thus its absence results in a pro-fibrotic state. The current known cellular receptors for HBD2 include TLR4 and CCR-6 while the cellular receptors for HBD1 and HBD3 are yet to be identified(Boniotto et al., 2006). Differential expression of HBD1 and 3 is related to variation in differentiation of epidermal cells at different

sites as HBD expression shows sitewise variation and the expression influences differentiation of keratino(Bourcier et al., 2004)cytes(Abiko et al., 2003).

Previous studies have shown the differential expression of HBD1 and 2 on facial and foreskin at Stratum corneum and the Malpighian layer of the epidermis(Ali et al., 2001). The unique expression of these proteins helps the cellular differentiation of keratinocytes(Abiko et al., 2003).

Once HBD proteins are expressed, these can induce the formation of cytokines such as Interleukin-8 and monocyte chemoattractant protiens-1(MCP-1)(Boniotto et al., 2006) by monocytes in the inflammatory milieu. BD are known to induce tumour vasculogenesis by increase expression of vascular endothelial growth factor (VEGF) on dendritic cell (DC) precursors(Conejo-Garcia et al., 2004) and act as chemoattractant for monocytes, macrophages, DC and T lymphocytes. As all these factors are key players in keloid formation; it is likely that the absence of immuomodulatory HBD2 and increased expression of HBD3 which influences keratinocyte differentiation plays a role in Keloid pathogenesis.

Thus this study brings forth a novel aspect to Keloid pathogenesis, and also suggests that HBD2 and 3 are more than antimicrobial peptides. We believe that these peptides are important mediators of the cytokine cascade and understanding their regulation may hold(McCoy and Cohen, 1981) the key to a number of pathological conditions associated with altered cytokine and growth factor profiles.

Chapter 7

General Discussion

7.1 β Defensins- A unique class of antimicrobials

Antimicrobial peptides are polypeptides with less than 100 amino acids characterised by inherent antimicrobial activity at physiological conditions in the tissue of their origin(Ganz, 2003).They have been an area of active interest and about 750 different eukaryotic antimicrobial peptides have been reported(http://www.bbcm.univ.trieste.it/%7Etossi/pag5.htm, accessed 07/ 2008, Brogden et al., 2003). These peptides have been grouped basing on similarities in their 3D structure, charge, functional similarity and sequence homology. Broadly they can be classifies as shown in fig 7.1.

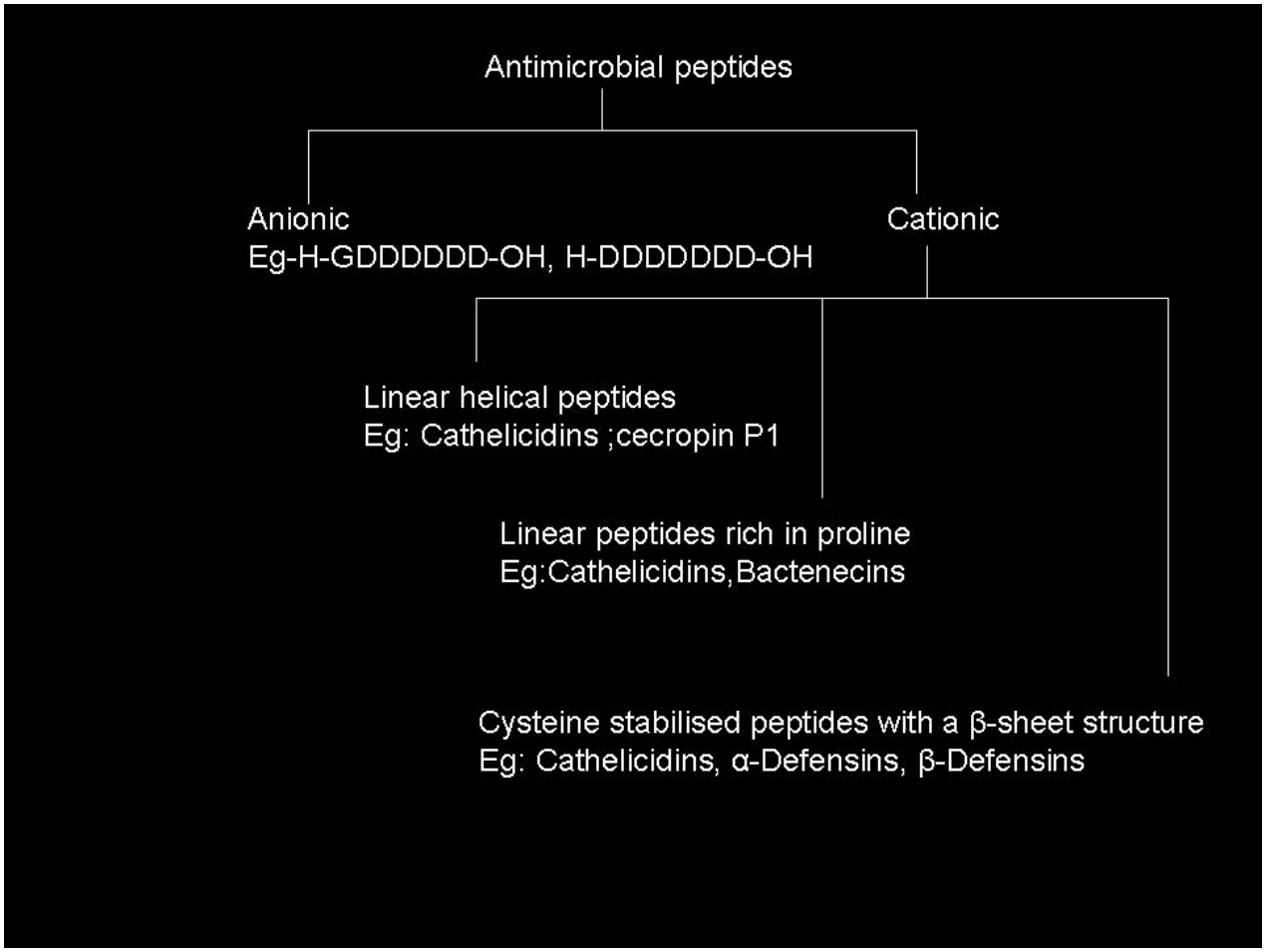


Figure 7.1- Showing the broad classification of antimicrobial peptides in animals.

The two important classes of antimicrobial peptides in humans are defensins and cathelicidins. As seen from figure 7.1 HBD (Human β Defensins) are cysteine stabilised cationic peptides with a β-sheet and are characterised by their unique tertiary structure. They also have some other characteristics which make these peptides of special interest.

Origin

HBD are evolutionarily conserved throughout the plant and the animal kingdom(Brogden et al., 2003, Ganz, 2003). Similar proteins as are found in humans have been identified among plants and a number of other organisms in the animal kingdom. The primitive nature of these peptides gives evidence to the fact that these peptides have continued to remain useful throughout evolutionary process and hence there was minimal need for change. These continue to remain effective as antimicrobials in plants, lower animals and humans.

Synthesis and structure

HBD along with α-Defensins are among the smallest of the cationic antimicrobial peptides ranging from 38-42 amino acid residues and having a weight of 4-6 KD. The HBD precursor lacks or has a small pro-piece unlike the other defensins(Selsted and Ouellette, 2005). The possible reason for this is unclear but might explain the rapid induction and synthesis of these peptides when stimulated.as post translational processing requires single peptidase only(Ganz, 2003). Owing to their unique tertiary structure and their charge, HBD are known to act as antimicrobials by forming pores in the cell membranes of microbes explained by the carpet worm hole model(Ganz, 2003). This antimicrobial activity is pronounced at non-ionic or low ionic conditions, and can be seen at extremely low HBD concentrations of 1-10μg/ml.The

microbial activity is competitively inhibited by increasing salt and plasma protein concentrations. Thus HBD have more marked anti-bacterial activity at physiological conditions thereby serving a preventive function in the first instance. Subsequently at times of challenge, they are induced both in type and quantity thereby serving a secondary therapeutic function.

Pattern of expression

HBD's as seen earlier are of different types and each of them has more marked activity against specific micro-organisms. The pattern of expression of each type of HBD seems to be variable between different sites on the body .In most of the sites there is a basal level of expression of HBD1sometimes along with other HBD's and upon stimulation there is enhanced expression of HBD 1 and other HBD depending on the nature of stimulation. Thus it appears that apart from the difference in the activity with the change of environment, the innate defence provided by HBD is 2 staged as evidenced by the pattern of expression. The primary defence is in the form of the basal expression of particular type of HBD or HBD's which upon need is enhanced either on its own or in combination with other HBD.

It is possible that different HBD act synergistically potentiating the action of each other either directly or indirectly.

Human β Defensins –more than just antimicrobials

Since the discovery of HBD in 1995 when HBD1 was isolated from human blood filtrate of patients undergoing dialysis(Bensch et al., 1995), scientific knowledge has expanded significantly in the last thirteen years in relation to these peptides. In the early years following discovery of these peptides they were considered to be primarily antimicrobial being the first

line of defence against microbial invasion and forming a part of the innate immune system. However with increasing understanding of the structure and functions of these peptides, they are now recognised as being more than antimicrobial.

The antibacterial activity of HBD was the first to be established in a number of *in-vitro* and *in-vivo* studies. Subsequent studies demonstrated their activity against viruses(Sun et al., 2005) and fungi. All these studies provided evidence to these peptides forming a part of the innate defence barrier. The other non-antimicrobial functions are progressively being unravelled as additional studies demonstrate more diverse functions.

HBD1 has been shown to promote cell differentiation in keratinocytes(Frye et al., 2001). HBD 2 has been shown to act as signalling molecule to trigger the adaptive immune system by recruiting immature dendritic cells and memory T cells using the CCR6 receptor(Yang et al., 1999). HBD3 has also been shown to have chemo attractant properties to T-lymphocytes and immature dendritic cells(Dhople et al., 2006). The chemotactic properties have been linked to the disulphide bridges in the structure(Wu et al., 2003). It has also been shown BD2, and 3 induce secretion of pro-inflammatory cytokine IL-18 in keratinocytes(Niyonsaba et al., 2005), and also mediate induction of tissue remodelling proteins such as matrix metalloproteinases (MMP)(Varoga et al., 2005) and reduction of MMP inhibitors.

Burn wounds – more than just loss of skin cover

Burns are one of the most complex injuries encountered in medical practice. These injuries are associated with multiple co-morbidities and a multidisciplinary care addressing the multiple problems faced by these patients is required to achieve a favourable outcome(Hettiaratchy and Dziewulski, 2004a). The injuries in a burn patient can be broadly sub-classified into:

1. Physical
2. Psychological
3. Biological

The physical injury relates to the actual burn. This could be of variable depth from superficial to full thickness and results in partial to complete loss of skin cover respectively. Basing on the % total burn surface area (TBSA) and the depth of the burn; management is planned as described previously. However this physical aspect of injury related to loss of skin is associated with psychological and biological injury, which is more pronounced with the increase in the percentage and depth of burn wound. The psychological injury relates to the trauma associated with the actual incident resulting in the burn, and also the social stigma associated with the injury, scarring etc and its related co-morbidities. The biological injury relates to the alteration in the myriad biological functions of the body to maintain an internal homeostatic environment following the insult. These result from the release of inflammatory mediators and cytokines following the burn injury, and also from the loss of biological function of the skin. These effects are usually seen when the TBSA exceeds 30%(Hettiaratchy and Dziewulski, 2004b). Thus the burn injury results in more than of loss of skin physical barrier.

Human β Defensins and burn wounds

A great interest has developed in the recent years in HBD inview of their unique properties especially in relation the burn wounds. HBD mRNA expression has been shown to be upregulated in burn wounds in some of the previous studies(Poindexter et al., 2006, Kaus et al., 2008).

In the current PhD the role of HBD was further investigated in relation to a number of factors seen in burn injury. The first few experiments (chapter 3) were aimed at understanding the expression profile at protein and RNA levels in burn wounds, and the influence of local factors like duration since burn, type and quantity of bacteria present in wounds.

Expression profile of HBD in burn wounds:

- Expression in relation to duration of burn wound.
- Expression in relation to site of burn wound.
- Expression in relation to the type of bacteria present in burn wound.
- Expression in relation to the number of bacteria present in burn wounds.

The result of the study showed an increased expression of HBD1, 2, 3 mRNA in burn wounds relative to psoriatic skin. Similar levels were not demonstrable at protein stage (Chapter 3).

Having understood the expression profile an attempt was made to establish the **source of HBD**. It was known from our own study and previous studies that keratinocytes were the main source of HBD expression. Since granulation tissue with predominance of leucocytes, macrophages forms a major part of the healing burn wounds especially in the early stages following injury. It was decided to investigate if these blood cells contributed to the HBD expression in vivo. It was shown by Fang *et al* that human peripheral blood has no expression of HBD mRNA *in-vivo*. However on *ex-vivio* stimulation of leucocytes by LPS or dead bacteria HBD1 and 2 mRNA was detectable but not HBD3. In order to find out whether this was the scenario in-vivo i.e. if the peripheral blood cells contributed to HBD expression in the presence of inflammatory mediators or bacteria or bacterial products, which is the environment seen in burn wounds.

Since it was not practically and ethically possible to recruit patients with major burns and extract leucocytes from the granulating wound beds an alternative model was looked into where similar conditions existed in peripheral blood. A study was planned looking into the expression of HBD in patients with microbiologically positive and negative blood cultures and comparing the expression between the two groups to healthy volunteers. Chapter 4 gives the full details of the study.

The results of our study demonstrated that the expression of HBD1, 2 and 3 was enhanced to similar levels in both the microbiologically positive and negative groups at both the RNA and the protein stage. Thus suggesting that the enhance expression was mediated by the inflammatory mediators like cytokines than the by the microbes itself. It can be further derived from the study that the blood cells contribute to the HBD1, 2 and 3 mRNA and protein expression.

It is a well recognised fact that major burns described as those involving more than 25-30% of TBSA are associated with catabolic hypermetabolic state, negative nitrogen balance and loss of lean body mass(Meyer et al., 1994, James et al., 2002). These are considered to be secondary to alterations in the hypothalo-pituatary adrenal axis following release of cytokines(Woloski et al., 1985). This in-turn causes release of stress hormones mainly cortisol, which has been shown to be 10 times higher than in normal individuals(Garrel et al., 1995). It has also been reported that there is loss of circadian rhythm in burn patients similar to that seen in critically ill patients(Molteni et al., 1979). In our previous study (chapter 5) we have demonstrated that critically ill patients in ITU/HDU do not have a circadian rhythm of cortisol expression. This increase in stress hormones is useful to help tide over the stressful situation however is also associated with a number of changes in metabolism and immune system

some of which can be deleterious. It is considered that during such major stress events there is down regulation of the innate immune system resulting in oppurtunitistic infections in these groups of patients.

HBD are considered a part of the innate immune defence and serve not only as the first line of defence, but also a link connecting the adaptive and the innate defence mechanisms. Due to their pivotal role in the defence mechanisms, it was decided to investigate if the expression of HBD is influenced by the high cortisol levels which in turn are a reflection of the stress in patients. As it was practically difficult to obtain enough patients to have a meaningful study with majors burns (>30%) and it was not possible to have controls, especially the baseline if the burns patients were recruited. Hence a model was chosen in the form of patients undergoing major surgical procedures who, as per their clinical protocol, were expected to be nursed in the critical care environment in the immediate post-operative period. The study was planned such that a sample of cortisol and HBD could be studied at three stages; a baseline (pre-operative), day 1 post–operative and Day 9/10 post-operative or pre-discharge sample. It has been shown in previous studies that patients undergoing major surgical procedures have a stress response manifested by lack of circadian rhythm which is usually restored in about 8-10 days time following surgery. This model appeared to be closely mimicking the conditions seen in burns in a controlled way and over a shorter time span and hence was decided upon.

The results of the study (chapter 5) showed that the HBD1 and 3 were high in the pre-operative period compared to normal controls, and this was further elevated in the immediate post-operative period. There was statistically significant positive correlation between the HBD1 and 3 levels. HBD2 was found elevated during the immediate post-operative period and

progressively with time all the three HBD showed a down-trend. There was no significant negative correlation between the cortisol levels and the HBD levels.

Thus drawing up from this study it can be suggested that in major stress conditions like burns the natural response of the body is to augment the immunity at the mucosal and skin surfaces. There is initially enhanced expression of inherently expression HBD1 and this is associated with increased HBD3 as there appears to be a cross talk between these two defensins. It is important to note that HBD1 and HBD3 have specific activity against staphylococcal species (which are the organisms initially responsible for wound infections). While HBD1 is inherently expressed and is active at physiological pH and salt concentrations, HBD3 is active at high salt concentrations and is predominantly induced. Thus the defence at muco-cutaneous levels is mediated as a 3 tier system. The primary tier comprises the inherently expressed antimicrobial peptide HBD1, a predominantly inherent antimicrobial peptide at physiological pH which is also inducible. The next tier comprises HBD3, an inducible but mainly locally acting antimicrobial peptide, both under physiological and pathological conditions. The third tier consists of HBD2, an inducible peptide with inherent antimicrobial activity, but also serving as triggering molecule to the adaptive immune system. In our group of patients, cortisol did not seem to adversely effect the HBD expression.

Scars are an inherent component of burns, leaving a permanent mark on these patients after the acute sequelae of burn settles down. Quite often in major burn patients the scars can be in the form of keloids or hypertrophic scars. These type of scars, particularly keloid, are difficult to treat as the mechanisms underlying the formation of keloids are not clear. The aetiology of keloids is considered to be multi-factorial subsequent to a cutaneous insult. A number of

inflammatory and immune mediators have been implicated in the pathogenesis and it is considered that an imbalance between the pro and anti-fibrotic cytokines leads to the keloid formation. It is however not known what triggers the imbalance between the pro and anti-fibrotic mediators shifting the balance to the pro-fibrotic state. Since HBD spear to have more diverse functions and to complete the investigation into the role of HBD in burn pathogenesis, it was decided to investigate the expression profile of HBD in keloids (chapter 6).

The results of study (chapter 6) show that there was no HBD2 expression at both mRNA and protein levels in keloids. The HBD1 and 3 mRNA was 3 and 5 times higher, respectively, in keloids compared to the mean expression in normal skin samples.

In summary the study suggests to a possible role of HBD's mainly lack of HBD2; an anti-fibrotic cytokine inducer in keloid pathogenesis.

Summary

The studies undertaken during the PhD demonstrate for the first time that HBD1, 2 and 3 are induced to very high levels at mRNA levels in burn patients, but parallel levels were not seen at protein levels.

The bacteria isolated from the burn wounds showed a trend changing from colonising organisms to more resistant forms in time.

The peripheral blood leucocytes demonstrated an increased expression of HBD 1, 2 and 3 mRNA in clinically septic patients (both micro-biologically negative and positive patients), compared to normal controls. The study suggests that peripheral blood cells produce HBD in response to inflammatory mediator's invivo, thus alluding to a possible contribution of HBD 1, 2 and 3 in granulation tissue in burn wounds.

There was no down regulation of HBD1, 2 and 3 in the presence of increased cortisol levels, a reflection of heightened stress as seen in burns. HBD1 and 3 mRNA expressions showed an early up-regulation followed by elevation in HBD2 mRNA levels. All the three HBD proteins were localised to buccal epithelial cells.

There was no HBD2 mRNA and protein expression in keloid tissue specimens from different parts of the body. The absence of HBD2- a unique intrinsic peptide with an ability to trigger the anti-fibrotic cytokines suggests to the possibility that HBD's are implicated in the pathogenesis of keloids.

7.2 Conclusion & Scope for clinical application

The current work was an endeavour to investigate and review the role of HBD 1, 2 and 3 in burn wounds. The results of the studies undertaken show that the natural response of the body in the epithelial remnants in burn wounds, the periburn tissue and the peripheral blood cells is to enhance the mRNA expression of HBD. Protein was not localised to similar high levels, suggestive of extensive consumption of this secretory protein or potential deficiencies in the translational mechanisms in the immature cells. The high stress and the accompanying immuno-suppression do not seem to directly down-regulate the HBD expression. The keloid scars do not have any expression of HBD2 either at protein and mRNA level.

The treatment of burn patients has tremendously improved with improvement in the critical care support and better understanding of the pathogenesis in burns. However infection still continues to be an important problem in major burns, and the development of ever new generation of the existing antibiotics seems to be hampered by the increasing bacterial

resistance. In this era of increasing bacterial resistance designing novel therapies using primitive, conserved antimicrobial peptides like HBD seems like a promising alternative.

HBD's as has been discussed show a wide spectrum of antimicrobial activity against varieties of bacteria, fungi and viruses and each of the subtypes have more marked activity against specific microbes. They have unique mechanism of action and inspite being in the plant and animal kingdom from times immemorial, they successfully continue to be lethal to microbes.

The current study provides more information which is useful to plan and streamline such therapeutic strategies. Drawing from the results of the studies on burn wound samples it can be suggested that the HBD's should be supplemented as proteins which could be incorporated into wound dressings or collagen scaffolds. If the idea was to use cultured skin substitutes, then the the HBD, s can be made to overexpress invitro before transferring the skin onto the patient. Novel therapeutic strategies for the treatment of keloids can also be planned by considering enhancing the invivo expression of HBD2 mRNA or supplementation of HBD2 proteins. The results of these studies can also be applied to the non-burn scenarios like chronic non-healing wounds where similar microbes as in delayed burn wounds have been found. Such wounds can also be supplemented with HBD to promote healing and prevent infection. The individual HBD used has to be tailored to the duration and the nature of the wound, for instance acute wounds would benefit from supplementation of HBD1 and 3, while HBD2 should be used for chronic wounds. However such experiments should be carefully trialled given the possibility that these HBD peptides may have many more yet undescribed and undiscovered properties.

7.3 Future work

Having established the *in-vivo* expression profile of HBD in burn wounds and the conditions seen surrounding these complex wounds, the next step would be to apply the results for potential therapeutic use. The project can be taken forward in different directions as follows:

- *In-vitro* studies relating to enhanced expression of HBD 1, 2 and 3 both individually and separately in organotypic skin models and analysing their influence on epithelisation, differentiation and prevention of infection.
- Large scale synthesis of these peptides using recombinant techniques; and subsequent direct topical use of these peptides in an aqueous base.
- Use of stimulants of HBD mRNA expression for topical application in oil base or direct injection into the lesion. Dermal scaffolds with incorporated HBD2 can also be used following excision and grafting of keloid lesion to prevent recurrence.
- Application of HBD supplemented Dermal or CSS (cultured skin substitutes) to non-burn chronic wounds including diabetic or venous ulcers.

References

ABERGEL, R. P., PIZZURRO, D., MEEKER, C. A., LASK, G., MATSUOKA, L. Y., MINOR, R. R., CHU, M. L. & UITTO, J. (1985) Biochemical composition of the connective tissue in keloids and analysis of collagen metabolism in keloid fibroblast cultures. *J Invest Dermatol,* 84**,** 384-90.

ABIKO, Y., JINBU, Y., NOGUCHI, T., NISHIMURA, M., KUSANO, K., AMARATUNGA, P., SHIBATA, T. & KAKU, T. (2002) Upregulation of human beta-defensin 2 peptide expression in oral lichen planus, leukoplakia and candidiasis. an immunohistochemical study. *Pathol Res Pract,* 198**,** 537-42.

ABIKO, Y., NISHIMURA, M., KUSANO, K., YAMAZAKI, M., ARAKAWA, T., TAKUMA, T. & KAKU, T. (2003) Upregulated expression of human beta defensin-1 and -3 mRNA during differentiation of keratinocyte immortalized cell lines, HaCaT and PHK16-0b. *J Dermatol Sci,* 31**,** 225-8.

AKASAKA, Y., FUJITA, K., ISHIKAWA, Y., ASUWA, N., INUZUKA, K., ISHIHARA, M., ITO, M., MASUDA, T., AKISHIMA, Y., ZHANG, L., ITO, K. & ISHII, T. (2001) Detection of apoptosis in keloids and a comparative study on apoptosis between keloids, hypertrophic scars, normal healed flat scars, and dermatofibroma. *Wound Repair Regen,* 9**,** 501-6.

AKASAKA, Y., ISHIKAWA, Y., ONO, I., FUJITA, K., MASUDA, T., ASUWA, N., INUZUKA, K., KIGUCHI, H. & ISHII, T. (2000) Enhanced expression of caspase-3 in hypertrophic scars and keloid: induction of caspase-3 and apoptosis in keloid fibroblasts in vitro. *Lab Invest,* 80**,** 345-57.

AKASAKA, Y., ITO, K., FUJITA, K., KOMIYAMA, K., ONO, I., ISHIKAWA, Y., AKISHIMA, Y., SATO, H. & ISHII, T. (2005) Activated caspase expression and apoptosis increase in keloids: cytochrome c release and caspase-9 activation during the apoptosis of keloid fibroblast lines. *Wound Repair Regen,* 13**,** 373-82.

AL-ATTAR, A., MESS, S., THOMASSEN, J. M., KAUFFMAN, C. L. & DAVISON, S. P. (2006) Keloid pathogenesis and treatment. *Plast Reconstr Surg,* 117**,** 286-300.

ALAISH, S. M., YAGER, D. R., DIEGELMANN, R. F. & COHEN, I. K. (1995) Hyaluronic acid metabolism in keloid fibroblasts. *J Pediatr Surg,* 30**,** 949-52.

ALI, R. S., FALCONER, A., IKRAM, M., BISSETT, C. E., CERIO, R. & QUINN, A. G. (2001) Expression of the peptide antibiotics human beta defensin-1 and human beta defensin-2 in normal human skin. *J Invest Dermatol,* 117**,** 106-11.

ALP, S., SKRYGAN, M., SCHLOTTMANN, R., KREUTER, A., OTTE, J. M., SCHMIDT, W. E., BROCKMEYER, N. H. & BASTIAN, A. (2005) Expression of beta-defensin 1 and 2 in nasal epithelial cells and alveolar macrophages from HIV-infected patients. *Eur J Med Res,* 10**,** 1-6.

AMADEU, T., BRAUNE, A., MANDARIM-DE-LACERDA, C., PORTO, L. C., DESMOULIERE, A. & COSTA, A. (2003) Vascularization pattern in hypertrophic scars and keloids: a stereological analysis. *Pathol Res Pract,* 199**,** 469-73.

ANGUS, D. C., LINDE-ZWIRBLE, W. T., LIDICKER, J., CLERMONT, G., CARCILLO, J. & PINSKY, M. R. (2001) Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Crit Care Med,* 29**,** 1303-10.

AUFWERBER, E., RINGERTZ, S. & RANSJO, U. (1991) Routine semiquantitative cultures and central venous catheter-related bacteremia. *Apmis,* 99**,** 627-30.

AYABE, T., SATCHELL, D. P., WILSON, C. L., PARKS, W. C., SELSTED, M. E. & OUELLETTE, A. J. (2000) Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria. *Nat Immunol,* 1**,** 113-8.

BABU, M., DIEGELMANN, R. & OLIVER, N. (1989) Fibronectin is overproduced by keloid fibroblasts during abnormal wound healing. *Mol Cell Biol,* 9**,** 1642-50.

BABU, M., DIEGELMANN, R. & OLIVER, N. (1992) Keloid fibroblasts exhibit an altered response to TGF-beta. *J Invest Dermatol,* 99**,** 650-5.

BALS, R. (2000) Epithelial antimicrobial peptides in host defense against infection. *Respir Res,* 1**,** 141-50.

BALS, R., GOLDMAN, M. J. & WILSON, J. M. (1998) Mouse beta-defensin 1 is a salt-sensitive antimicrobial peptide present in epithelia of the lung and urogenital tract. *Infect Immun,* 66**,** 1225-32.

BECKER, M. N., DIAMOND, G., VERGHESE, M. W. & RANDELL, S. H. (2000) CD14-dependent lipopolysaccharide-induced beta-defensin-2 expression in human tracheobronchial epithelium. *J Biol Chem,* 275**,** 29731-6.

BEER, T. W., BALDWIN, H. C., GODDARD, J. R., GALLAGHER, P. J. & WRIGHT, D. H. (1998) Angiogenesis in pathological and surgical scars. *Hum Pathol,* 29**,** 1273-8.

BELLON, J. M., MANZANO, L., LARRAD, A., HONDUVILLA, G. N., BUJAN, J. & ALVAREZ-MON, M. (1998) Endocrine and immune response to injury after open and laparoscopic cholecystectomy. *Int Surg,* 83**,** 24-7.

BENSCH, K. W., RAIDA, M., MAGERT, H. J., SCHULZ-KNAPPE, P. & FORSSMANN, W. G. (1995) hBD-1: a novel beta-defensin from human plasma. *FEBS Lett,* 368**,** 331-5.

BERMAN, B. & DUNCAN, M. R. (1989) Pentoxifylline inhibits normal human dermal fibroblast in vitro proliferation, collagen, glycosaminoglycan, and fibronectin production, and increases collagenase activity. *J Invest Dermatol,* 92**,** 605-10.

BETTINGER, D. A., YAGER, D. R., DIEGELMANN, R. F. & COHEN, I. K. (1996) The effect of TGF-beta on keloid fibroblast proliferation and collagen synthesis. *Plast Reconstr Surg,* 98**,** 827-33.

BHANGOO, K. S., QUINLIVAN, J. K. & CONNELLY, J. R. (1976) Elastin fibers in scar tissue. *Plast Reconstr Surg,* 57**,** 308-13.

BICK, R. J., POINDEXTER, B. J., BHAT, S., GULATI, S., BUJA, M. & MILNER, S. M. (2004) Effects of cytokines and heat shock on defensin levels of cultured keratinocytes. *Burns,* 30**,** 329-33.

BIRAGYN, A., RUFFINI, P. A., LEIFER, C. A., KLYUSHNENKOVA, E., SHAKHOV, A., CHERTOV, O., SHIRAKAWA, A. K., FARBER, J. M., SEGAL, D. M., OPPENHEIM, J. J. & KWAK, L. W. (2002) Toll-like receptor 4-dependent activation of dendritic cells by beta-defensin 2. *Science,* 298**,** 1025-9.

BIRCHLER, T., SEIBL, R., BUCHNER, K., LOELIGER, S., SEGER, R., HOSSLE, J. P., AGUZZI, A. & LAUENER, R. P. (2001) Human Toll-like receptor 2 mediates induction of the antimicrobial peptide human beta-defensin 2 in response to bacterial lipoprotein. *Eur J Immunol,* 31**,** 3131-7.

BLACKWELL, T. S. & CHRISTMAN, J. W. (1996) Sepsis and cytokines: current status. *Br J Anaesth,* 77**,** 110-7.

BLADON, P. T., ROWLANDS, T. E., WHITTAKER, J. A. & OAKEY, R. E. (1996) Serum cortisol binding capacity measured with Concanavalin A-Sepharose in patients with a recent inflammatory response. *Clin Chim Acta,* 253**,** 9-20.

BOCHUD, P. Y. & CALANDRA, T. (2003) Pathogenesis of sepsis: new concepts and implications for future treatment. *Bmj,* 326**,** 262-6.
BOHLING, A., HAGGE, S. O., ROES, S., PODSCHUN, R., SAHLY, H., HARDER, J., SCHRODER, J. M., GROTZINGER, J., SEYDEL, U. & GUTSMANN, T. (2006) Lipid-specific membrane activity of human beta-defensin-3. *Biochemistry,* 45**,** 5663-70.
BONIOTTO, M., JORDAN, W. J., ESKDALE, J., TOSSI, A., ANTCHEVA, N., CROVELLA, S., CONNELL, N. D. & GALLAGHER, G. (2006) Human beta-defensin 2 induces a vigorous cytokine response in peripheral blood mononuclear cells. *Antimicrob Agents Chemother,* 50**,** 1433-41.
BONTEMS, F., ROUMESTAND, C., GILQUIN, B., MENEZ, A. & TOMA, F. (1991) Refined structure of charybdotoxin: common motifs in scorpion toxins and insect defensins. *Science,* 254**,** 1521-3.
BOOK, M., CHEN, Q., LEHMANN, L. E., KLASCHIK, S., WEBER, S., SCHEWE, J. C., LUEPERTZ, M., HOEFT, A. & STUBER, F. (2007) Inducibility of the endogenous antibiotic peptide beta-defensin 2 is impaired in patients with severe sepsis. *Crit Care,* 11**,** R19.
BOREL, J. P. & MAQUART, F. X. (1998) [Molecular mechanisms of wound scarring]. *Ann Biol Clin (Paris),* 56**,** 11-9.
BOURCIER, T., BAUDRIMONT, M., BOUTBOUL, S., THOMAS, F., BORDERIE, V. & LAROCHE, L. (2004) Corneal keloid: clinical, ultrasonographic, and ultrastructural characteristics. *J Cataract Refract Surg,* 30**,** 921-4.
BOYCE, D. E., CIAMPOLINI, J., RUGE, F., MURISON, M. S. & HARDING, K. G. (2001) Inflammatory-cell subpopulations in keloid scars. *Br J Plast Surg,* 54**,** 511-6.
BROGDEN, K. A., ACKERMANN, M., MCCRAY, P. B., JR. & TACK, B. F. (2003) Antimicrobial peptides in animals and their role in host defences. *Int J Antimicrob Agents,* 22**,** 465-78.
BUTMARC, J., YUFIT, T., CARSON, P. & FALANGA, V. (2004) Human beta-defensin-2 expression is increased in chronic wounds. *Wound Repair Regen,* 12**,** 439-43.
CALDERON, M., LAWRENCE, W. T. & BANES, A. J. (1996) Increased proliferation in keloid fibroblasts wounded in vitro. *J Surg Res,* 61**,** 343-7.
CARROLL, L. A., HANASONO, M. M., MIKULEC, A. A., KITA, M. & KOCH, R. J. (2002) Triamcinolone stimulates bFGF production and inhibits TGF-beta1 production by human dermal fibroblasts. *Dermatol Surg,* 28**,** 704-9.
CARROLL, L. A. & KOCH, R. J. (2003) Heparin stimulates production of bFGF and TGF-beta 1 by human normal, keloid, and fetal dermal fibroblasts. *Med Sci Monit,* 9**,** BR97-108.
CASS, D. L., BULLARD, K. M., SYLVESTER, K. G., YANG, E. Y., LONGAKER, M. T. & ADZICK, N. S. (1997) Wound size and gestational age modulate scar formation in fetal wound repair. *J Pediatr Surg,* 32**,** 411-5.
CASTRO, D. J., ABERGEL, R. P., MEEKER, C., DWYER, R. M., LESAVOY, M. A. & UITTO, J. (1983) Effects of the Nd:YAG laser on DNA synthesis and collagen production in human skin fibroblast cultures. *Ann Plast Surg,* 11**,** 214-22.
CENTENO, R. F., ALBO, D., ROTHMAN, V. L., GRANICK, M. S. & TUSZYNSKI, G. P. (2002) Thrombospondin-1 may modulate keloid formation through up-regulation of the matrix-associated plasminogen/plasmin system. *Plast Reconstr Surg,* 110**,** 363-5.
CHADEBECH, P., GOIDIN, D., JACQUET, C., VIAC, J., SCHMITT, D. & STAQUET, M. J. (2003) Use of human reconstructed epidermis to analyze the regulation of beta-defensin hBD-1, hBD-2, and hBD-3 expression in response to LPS. *Cell Biol Toxicol,* 19**,** 313-24.

CHEN, J. & CEN, Y. (2005) [Detection of expression of heat shock protein 47 mRNA in pathological scar tissue by using real-time fluorescent quantitative RT-PCR]. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi,* 19**,** 613-6.

CHEN, Y. S., CHEN, K. H., LIU, C. C., LEE, C. T., YANG, C. H., CHUANG, K. C. & LIN, C. R. (2007) Propofol-induced vascular permeability change is related to the nitric oxide signaling pathway and occludin phosphorylation. *J Biomed Sci,* 14**,** 629-36.

CHEVRAY, P. M. & MANSON, P. N. (2004) Keloid scars are formed by polyclonal fibroblasts. *Ann Plast Surg,* 52**,** 605-8.

CHIPEV, C. C., SIMMAN, R., HATCH, G., KATZ, A. E., SIEGEL, D. M. & SIMON, M. (2000) Myofibroblast phenotype and apoptosis in keloid and palmar fibroblasts in vitro. *Cell Death Differ,* 7**,** 166-76.

CHODON, T., SUGIHARA, T., IGAWA, H. H., FUNAYAMA, E. & FURUKAWA, H. (2000) Keloid-derived fibroblasts are refractory to Fas-mediated apoptosis and neutralization of autocrine transforming growth factor-beta1 can abrogate this resistance. *Am J Pathol,* 157**,** 1661-9.

CHOMCZYNSKI, P. & SACCHI, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem,* 162**,** 156-9.

CLORE, J. N., COHEN, I. K. & DIEGELMANN, R. F. (1979) Quantitative assay of types I and III collagen synthesized by keloid biopsyes and fibroblasts. *Biochim Biophys Acta,* 586**,** 384-90.

COBBOLD, C. A. (2001) The role of nitric oxide in the formation of keloid and hypertrophic lesions. *Med Hypotheses,* 57**,** 497-502.

COHEN, I. K., DIEGELMANN, R. F. & JOHNSON, M. L. (1977) Effect of corticosteroids on collagen synthesis. *Surgery,* 82**,** 15-20.

COLWELL, A. S., PHAN, T. T., KONG, W., LONGAKER, M. T. & LORENZ, P. H. (2005) Hypertrophic scar fibroblasts have increased connective tissue growth factor expression after transforming growth factor-beta stimulation. *Plast Reconstr Surg,* 116**,** 1387-90; discussion 1391-2.

CONEJO-GARCIA, J. R., BENENCIA, F., COURREGES, M. C., KANG, E., MOHAMED-HADLEY, A., BUCKANOVICH, R. J., HOLTZ, D. O., JENKINS, A., NA, H., ZHANG, L., WAGNER, D. S., KATSAROS, D., CAROLL, R. & COUKOS, G. (2004) Tumor-infiltrating dendritic cell precursors recruited by a beta-defensin contribute to vasculogenesis under the influence of Vegf-A. *Nat Med,* 10**,** 950-8.

DAHER, K. A., LEHRER, R. I., GANZ, T. & KRONENBERG, M. (1988) Isolation and characterization of human defensin cDNA clones. *Proc Natl Acad Sci U S A,* 85**,** 7327-31.

DAIAN, T., OHTSURU, A., ROGOUNOVITCH, T., ISHIHARA, H., HIRANO, A., AKIYAMA-UCHIDA, Y., SAENKO, V., FUJII, T. & YAMASHITA, S. (2003) Insulin-like growth factor-I enhances transforming growth factor-beta-induced extracellular matrix protein production through the P38/activating transcription factor-2 signaling pathway in keloid fibroblasts. *J Invest Dermatol,* 120**,** 956-62.

DALE, B. A., KIMBALL, J. R., KRISANAPRAKORNKIT, S., ROBERTS, F., ROBINOVITCH, M., O'NEAL, R., VALORE, E. V., GANZ, T., ANDERSON, G. M. & WEINBERG, A. (2001) Localized antimicrobial peptide expression in human gingiva. *J Periodontal Res,* 36**,** 285-94.

DALE, B. A. & KRISANAPRAKORNKIT, S. (2001) Defensin antimicrobial peptides in the oral cavity. *J Oral Pathol Med,* 30**,** 321-7.

DALKOWSKI, A., SCHUPPAN, D., ORFANOS, C. E. & ZOUBOULIS, C. C. (1999) Increased expression of tenascin C by keloids in vivo and in vitro. *Br J Dermatol,* 141**,** 50-6.

DALY, T. J. & WESTON, W. L. (1986) Retinoid effects on fibroblast proliferation and collagen synthesis in vitro and on fibrotic disease in vivo. *J Am Acad Dermatol,* 15**,** 900-2.
DESBOROUGH, J. P. (2000) The stress response to trauma and surgery. *Br J Anaesth,* 85**,** 109-17.
DHABHAR, F. S. (2000) Acute stress enhances while chronic stress suppresses skin immunity. The role of stress hormones and leukocyte trafficking. *Ann N Y Acad Sci,* 917**,** 876-93.
DHABHAR, F. S. & MCEWEN, B. S. (1999) Enhancing versus suppressive effects of stress hormones on skin immune function. *Proc Natl Acad Sci U S A,* 96**,** 1059-64.
DHOPLE, V., KRUKEMEYER, A. & RAMAMOORTHY, A. (2006) The human beta-defensin-3, an antibacterial peptide with multiple biological functions. *Biochim Biophys Acta,* 1758**,** 1499-512.
DIEGELMANN, R. F., BRYANT, C. P. & COHEN, I. K. (1977) Tissue alpha-globulins in keloid formation. *Plast Reconstr Surg,* 59**,** 418-23.
DIEGELMANN, R. F., COHEN, I. K. & MCCOY, B. J. (1979) Growth kinetics and collagen synthesis of normal skin, normal scar and keloid fibroblasts in vitro. *J Cell Physiol,* 98**,** 341-6.
DOMMISCH, H., ACIL, Y., DUNSCHE, A., WINTER, J. & JEPSEN, S. (2005) Differential gene expression of human beta-defensins (hBD-1, -2, -3) in inflammatory gingival diseases. *Oral Microbiol Immunol,* 20**,** 186-90.
DOMMISCH, H., WINTER, J., WILLEBRAND, C., EBERHARD, J. & JEPSEN, S. (2007) Immune regulatory functions of human beta-defensin-2 in odontoblast-like cells. *Int Endod J,* 40**,** 300-7.
DON R REVIS JR, M. B. S. (2006) Skin, Anatomy.
DONNARUMMA, G., PAOLETTI, I., BUOMMINO, E., ORLANDO, M., TUFANO, M. A. & BARONI, A. (2004) Malassezia furfur induces the expression of beta-defensin-2 in human keratinocytes in a protein kinase C-dependent manner. *Arch Dermatol Res,* 295**,** 474-81.
DROST, A. C., BURLESON, D. G., CIOFFI, W. G., JR., JORDAN, B. S., MASON, A. D., JR. & PRUITT, B. A., JR. (1993) Plasma cytokines following thermal injury and their relationship with patient mortality, burn size, and time postburn. *J Trauma,* 35**,** 335-9.
DUITS, L. A., RAVENSBERGEN, B., RADEMAKER, M., HIEMSTRA, P. S. & NIBBERING, P. H. (2002) Expression of beta-defensin 1 and 2 mRNA by human monocytes, macrophages and dendritic cells. *Immunology,* 106**,** 517-25.
DUNCAN, M. R., HASAN, A. & BERMAN, B. (1995) Oncostatin M stimulates collagen and glycosaminoglycan production by cultured normal dermal fibroblasts: insensitivity of sclerodermal and keloidal fibroblasts. *J Invest Dermatol,* 104**,** 128-33.
DUNSCHE, A., ACIL, Y., DOMMISCH, H., SIEBERT, R., SCHRODER, J. M. & JEPSEN, S. (2002) The novel human beta-defensin-3 is widely expressed in oral tissues. *Eur J Oral Sci,* 110**,** 121-4.
EISEN, D. P., DEAN, M. M., THOMAS, P., MARSHALL, P., GERNS, N., HEATLEY, S., QUINN, J., MINCHINTON, R. M. & LIPMAN, J. (2006) Low mannose-binding lectin function is associated with sepsis in adult patients. *FEMS Immunol Med Microbiol,* 48**,** 274-82.
ENGELICH, G., WRIGHT, D. G. & HARTSHORN, K. L. (2001) Acquired disorders of phagocyte function complicating medical and surgical illnesses. *Clin Infect Dis,* 33**,** 2040-8.
EROL, S., ALTOPARLAK, U., AKCAY, M. N., CELEBI, F. & PARLAK, M. (2004) Changes of microbial flora and wound colonization in burned patients. *Burns,* 30**,** 357-61.
FANG, X. M., SHU, Q., CHEN, Q. X., BOOK, M., SAHL, H. G., HOEFT, A. & STUBER, F. (2003) Differential expression of alpha- and beta-defensins in human peripheral blood. *Eur J Clin Invest,* 33**,** 82-7.
FRYE, M., BARGON, J. & GROPP, R. (2001) Expression of human beta-defensin-1 promotes differentiation of keratinocytes. *J Mol Med,* 79**,** 275-82.

FUJIWARA, M., MURAGAKI, Y. & OOSHIMA, A. (2005a) Keloid-derived fibroblasts show increased secretion of factors involved in collagen turnover and depend on matrix metalloproteinase for migration. *Br J Dermatol,* 153**,** 295-300.

FUJIWARA, M., MURAGAKI, Y. & OOSHIMA, A. (2005b) Upregulation of transforming growth factor-beta1 and vascular endothelial growth factor in cultured keloid fibroblasts: relevance to angiogenic activity. *Arch Dermatol Res,* 297**,** 161-9.

FUNAYAMA, E., CHODON, T., OYAMA, A. & SUGIHARA, T. (2003) Keratinocytes promote proliferation and inhibit apoptosis of the underlying fibroblasts: an important role in the pathogenesis of keloid. *J Invest Dermatol,* 121**,** 1326-31.

GANZ, T. (2003) Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol,* 3**,** 710-20.

GANZ, T., SELSTED, M. E., SZKLAREK, D., HARWIG, S. S., DAHER, K., BAINTON, D. F. & LEHRER, R. I. (1985) Defensins. Natural peptide antibiotics of human neutrophils. *J Clin Invest,* 76**,** 1427-35.

GARCIA, J. R., JAUMANN, F., SCHULZ, S., KRAUSE, A., RODRIGUEZ-JIMENEZ, J., FORSSMANN, U., ADERMANN, K., KLUVER, E., VOGELMEIER, C., BECKER, D., HEDRICH, R., FORSSMANN, W. G. & BALS, R. (2001a) Identification of a novel, multifunctional beta-defensin (human beta-defensin 3) with specific antimicrobial activity. Its interaction with plasma membranes of Xenopus oocytes and the induction of macrophage chemoattraction. *Cell Tissue Res,* 306**,** 257-64.

GARCIA, J. R., KRAUSE, A., SCHULZ, S., RODRIGUEZ-JIMENEZ, F. J., KLUVER, E., ADERMANN, K., FORSSMANN, U., FRIMPONG-BOATENG, A., BALS, R. & FORSSMANN, W. G. (2001b) Human beta-defensin 4: a novel inducible peptide with a specific salt-sensitive spectrum of antimicrobial activity. *Faseb J,* 15**,** 1819-21.

GARREL, D. R., RAZI, M., LARIVIERE, F., JOBIN, N., NAMAN, N., EMPTOZ-BONNETON, A. & PUGEAT, M. M. (1995) Improved clinical status and length of care with low-fat nutrition support in burn patients. *JPEN J Parenter Enteral Nutr,* 19**,** 482-91.

GIRA, A. K., BROWN, L. F., WASHINGTON, C. V., COHEN, C. & ARBISER, J. L. (2004) Keloids demonstrate high-level epidermal expression of vascular endothelial growth factor. *J Am Acad Dermatol,* 50**,** 850-3.

GIUGLIANO, G., PASQUALI, D., NOTARO, A., BRONGO, S., NICOLETTI, G., D'ANDREA, F., BELLASTELLA, A. & SINISI, A. A. (2003) Verapamil inhibits interleukin-6 and vascular endothelial growth factor production in primary cultures of keloid fibroblasts. *Br J Plast Surg,* 56**,** 804-9.

GLASER, F., SANNWALD, G. A., BUHR, H. J., KUNTZ, C., MAYER, H., KLEE, F. & HERFARTH, C. (1995) General stress response to conventional and laparoscopic cholecystectomy. *Ann Surg,* 221**,** 372-80.

HABIB, G. M., SHI, Z. Z., CUEVAS, A. A. & LIEBERMAN, M. W. (2003) Identification of two additional members of the membrane-bound dipeptidase family. *Faseb J,* 17**,** 1313-5.

HAISA, M., OKOCHI, H. & GROTENDORST, G. R. (1994) Elevated levels of PDGF alpha receptors in keloid fibroblasts contribute to an enhanced response to PDGF. *J Invest Dermatol,* 103**,** 560-3.

HAMRAHIAN, A. H., OSENI, T. S. & ARAFAH, B. M. (2004) Measurements of serum free cortisol in critically ill patients. *N Engl J Med,* 350**,** 1629-38.

HAN, C. M., HE, X. J. & MA, Q. (2005) [The expression of tenascin-C mRNA in keloids and hypertrophic scars]. *Zhonghua Zheng Xing Wai Ke Za Zhi,* 21**,** 40-3.

HANASONO, M. M., KITA, M., MIKULEC, A. A., LONERGAN, D. & KOCH, R. J. (2003) Autocrine growth factor production by fetal, keloid, and normal dermal fibroblasts. *Arch Facial Plast Surg,* 5**,** 26-30.
HARDER, J., BARTELS, J., CHRISTOPHERS, E. & SCHRODER, J. M. (1997) A peptide antibiotic from human skin. *Nature,* 387**,** 861.
HARDER, J., BARTELS, J., CHRISTOPHERS, E. & SCHRODER, J. M. (2001) Isolation and characterization of human beta -defensin-3, a novel human inducible peptide antibiotic. *J Biol Chem,* 276**,** 5707-13.
HARPER, R. A. (1989) Keloid fibroblasts in culture: abnormal growth behaviour and altered response to the epidermal growth factor. *Cell Biol Int Rep,* 13**,** 325-35.
HASEGAWA, T., NAKAO, A., SUMIYOSHI, K., TSUCHIHASHI, H. & OGAWA, H. (2005) SB-431542 inhibits TGF-beta-induced contraction of collagen gel by normal and keloid fibroblasts. *J Dermatol Sci,* 39**,** 33-8.
HAYASHI, T., NISHIHIRA, J., KOYAMA, Y., SASAKI, S. & YAMAMOTO, Y. (2006) Decreased Prostaglandin E(2) Production by Inflammatory Cytokine and Lower Expression of EP2 Receptor Result in Increased Collagen Synthesis in Keloid Fibroblasts. *J Invest Dermatol*.
HE, X. J. & HAN, C. M. (2004) [Study on the expression of tenascin-C in keloid and hyperplastic scar]. *Zhonghua Shao Shang Za Zhi,* 20**,** 79-81.
HE, X. J., HAN, C. M. & PENG, J. P. (2004) [A study of the abnormalities of human epiderm in keloids and hypertrophic scars]. *Zhonghua Wai Ke Za Zhi,* 42**,** 845-8.
HERBERT, T. B. & COHEN, S. (1993) Stress and immunity in humans: a meta-analytic review. *Psychosom Med,* 55**,** 364-79.
HERTZ, C. J., WU, Q., PORTER, E. M., ZHANG, Y. J., WEISMULLER, K. H., GODOWSKI, P. J., GANZ, T., RANDELL, S. H. & MODLIN, R. L. (2003) Activation of Toll-like receptor 2 on human tracheobronchial epithelial cells induces the antimicrobial peptide human beta defensin-2. *J Immunol,* 171**,** 6820-6.
HETTIARATCHY, S. & DZIEWULSKI, P. (2004a) ABC of burns. Introduction. *Bmj,* 328**,** 1366-8.
HETTIARATCHY, S. & DZIEWULSKI, P. (2004b) ABC of burns: pathophysiology and types of burns. *Bmj,* 328**,** 1427-9.
HOOVER, D. M., BOULEGUE, C., YANG, D., OPPENHEIM, J. J., TUCKER, K., LU, W. & LUBKOWSKI, J. (2002) The structure of human macrophage inflammatory protein-3alpha /CCL20. Linking antimicrobial and CC chemokine receptor-6-binding activities with human beta-defensins. *J Biol Chem,* 277**,** 37647-54.
HOOVER, D. M., RAJASHANKAR, K. R., BLUMENTHAL, R., PURI, A., OPPENHEIM, J. J., CHERTOV, O. & LUBKOWSKI, J. (2000) The structure of human beta-defensin-2 shows evidence of higher order oligomerization. *J Biol Chem,* 275**,** 32911-8.
HTTP://WWW.ABCAM.COM/ http://www.abcam.com/.
HTTP://WWW.BBCM.UNIV.TRIESTE.IT/%7ETOSSI/PAG5.HTM (accessed 07/ 2008).
HTTP://WWW.GANFYD.ORG/INDEX.PHP?TITLE=GRAM_STAIN http://www.ganfyd.org/index.php?title=Gram_stain; accessed June 2008.
HTTP://WWW.IHCWORLD.COM/_BOOKS/DAKO_HANDBOOK.PDF *Immunohistochemical staining methods,* 3rd Edition.
HTTP://WWW.LINSCOTTSDIRECTORY.COM/.
HTTP://WWW.MICROBIOLOGYPROCEDURE.COM/MICROBIOLOGICAL-METHODS/STREAK-PLATE-METHOD.HTM.
HTTP://WWW.PROMEGA.COM/TBS/TM048/TM048.PDF.

HTTP://WWW.SPLAMMO.NET/JLBACTSITE.HTML http://www.splammo.net/JLbactsite.html; June 2008.
HTTPS://CUSTOMASSAYS.QIAGEN.COM/DESIGN/INPUTSEQUENCES.ASP.
HUANG, Y., REN, L. & QIN, Y. (1998) [Observation of cicatricial fibroblasts in culture and its biological properties]. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi,* 12**,** 332-5.
HUTTNER, K. M. & BEVINS, C. L. (1999) Antimicrobial peptides as mediators of epithelial host defense. *Pediatr Res,* 45**,** 785-94.
IIZUKA, H. (1994) Epidermal turnover time. *J Dermatol Sci,* 8**,** 215-7.
ISHIHARA, H., YOSHIMOTO, H., FUJIOKA, M., MURAKAMI, R., HIRANO, A., FUJII, T., OHTSURU, A., NAMBA, H. & YAMASHITA, S. (2000) Keloid fibroblasts resist ceramide-induced apoptosis by overexpression of insulin-like growth factor I receptor. *J Invest Dermatol,* 115**,** 1065-71.
JACOB, S. E., BERMAN, B., NASSIRI, M. & VINCEK, V. (2003) Topical application of imiquimod 5% cream to keloids alters expression genes associated with apoptosis. *Br J Dermatol,* 149 Suppl 66**,** 62-5.
JAMES, S. E., GHOSH, S. J., MONTGOMERIE, J., PHILP, B. M. & DZIEWULSKI, P. (2002) Survival of a 75% burn in a patient with longstanding Addison's disease. *Burns,* 28**,** 391-3.
JIA, H. P., SCHUTTE, B. C., SCHUDY, A., LINZMEIER, R., GUTHMILLER, J. M., JOHNSON, G. K., TACK, B. F., MITROS, J. P., ROSENTHAL, A., GANZ, T. & MCCRAY, P. B., JR. (2001) Discovery of new human beta-defensins using a genomics-based approach. *Gene,* 263**,** 211-8.
JIANG, D. Y., FU, X. B., CHEN, W. & SUN, T. Z. (2004) [Relationship of overexpression of angiogenesis factors and their receptors with invasive growth of keloid]. *Zhonghua Zheng Xing Wai Ke Za Zhi,* 20**,** 128-31.
JOHNSON, R. M. & RICHARD, R. (2003) Partial-thickness burns: identification and management. *Adv Skin Wound Care,* 16**,** 178-87; quiz 188-9.
JOSEPH SAMBROOK AND DAVID W. RUSSELL. (2001) *Molecular Cloning,* Cold Spring Harbor, NY, USA, Cold Spring Harbor Laboratory Press.
KAMAMOTO, F., PAGGIARO, A. O., RODAS, A., HERSON, M. R., MATHOR, M. B. & FERREIRA, M. C. (2003) A wound contraction experimental model for studying keloids and wound-healing modulators. *Artif Organs,* 27**,** 701-5.
KAUS, A., JACOBSEN, F., SORKIN, M., RITTIG, A., VOSS, B., DAIGELER, A., SUDHOFF, H., STEINAU, H. U. & STEINSTRAESSER, L. (2008) Host defence peptides in human burns. *Burns,* 34**,** 32-40.
KETCHUM, L. D. (1977) Hypertrophic scars and keloids. *Clin Plast Surg,* 4**,** 301-10.
KHANSARI, D. N., MURGO, A. J. & FAITH, R. E. (1990) Effects of stress on the immune system. *Immunol Today,* 11**,** 170-5.
KIKUCHI, K., KADONO, T. & TAKEHARA, K. (1995) Effects of various growth factors and histamine on cultured keloid fibroblasts. *Dermatology,* 190**,** 4-8.
KISCHER, C. W. (1984) Comparative ultrastructure of hypertrophic scars and keloids. *Scan Electron Microsc***,** 423-31.
KISCHER, C. W. (1992) The microvessels in hypertrophic scars, keloids and related lesions: a review. *J Submicrosc Cytol Pathol,* 24**,** 281-96.
KISCHER, C. W. & BAILEY, J. F. (1972) The mast cell in hypertrophic scars. *Tex Rep Biol Med,* 30**,** 327-38.
KISCHER, C. W. & BRODY, G. S. (1981) Structure of the collagen nodule from hypertrophic scars and keloids. *Scan Electron Microsc***,** 371-6.

KISCHER, C. W. & HENDRIX, M. J. (1983) Fibronectin (FN) in hypertrophic scars and keloids. *Cell Tissue Res,* 231**,** 29-37.
KISCHER, C. W., PINDUR, J., KRASOVITCH, P. & KISCHER, E. (1990) Characteristics of granulation tissue which promote hypertrophic scarring. *Scanning Microsc,* 4**,** 877-87; discussion 887-8.
KISCHER, C. W., SHETLAR, M. R., SHETLAR, C. L. & CHVAPIL, M. (1983) Immunoglobulins in hypertrophic scars and keloids. *Plast Reconstr Surg,* 71**,** 821-5.
KOCH, R. J., GOODE, R. L. & SIMPSON, G. T. (1997) Serum-free keloid fibroblast cell culture: an in vitro model for the study of aberrant wound healing. *Plast Reconstr Surg,* 99**,** 1094-8.
KRISANAPRAKORNKIT, S., WEINBERG, A., PEREZ, C. N. & DALE, B. A. (1998) Expression of the peptide antibiotic human beta-defensin 1 in cultured gingival epithelial cells and gingival tissue. *Infect Immun,* 66**,** 4222-8.
KUO, Y. R., WU, W. S., JENG, S. F., HUANG, H. C., YANG, K. D., SACKS, J. M. & WANG, F. S. (2005a) Activation of ERK and p38 kinase mediated keloid fibroblast apoptosis after flashlamp pulsed-dye laser treatment. *Lasers Surg Med,* 36**,** 31-7.
KUO, Y. R., WU, W. S., JENG, S. F., WANG, F. S., HUANG, H. C., LIN, C. Z. & YANG, K. D. (2005b) Suppressed TGF-beta1 expression is correlated with up-regulation of matrix metalloproteinase-13 in keloid regression after flashlamp pulsed-dye laser treatment. *Lasers Surg Med,* 36**,** 38-42.
LADIN, D. A., HOU, Z., PATEL, D., MCPHAIL, M., OLSON, J. C., SAED, G. M. & FIVENSON, D. P. (1998) p53 and apoptosis alterations in keloids and keloid fibroblasts. *Wound Repair Regen,* 6**,** 28-37.
LAMETSCHWANDTNER, A. & STAINDL, O. (1990) [Angioarchitecture of keloids. A scanning electron microscopy study of a corrosion specimen]. *Hno,* 38**,** 202-7.
LE, A. D., ZHANG, Q., WU, Y., MESSADI, D. V., AKHONDZADEH, A., NGUYEN, A. L., AGHALOO, T. L., KELLY, A. P. & BERTOLAMI, C. N. (2004) Elevated vascular endothelial growth factor in keloids: relevance to tissue fibrosis. *Cells Tissues Organs,* 176**,** 87-94.
LE ROUX, C. W., CHAPMAN, G. A., KONG, W. M., DHILLO, W. S., JONES, J. & ALAGHBAND-ZADEH, J. (2003) Free cortisol index is better than serum total cortisol in determining hypothalamic-pituitary-adrenal status in patients undergoing surgery. *J Clin Endocrinol Metab,* 88**,** 2045-8.
LEAKE, D., DOERR, T. D. & SCOTT, G. (2003) Expression of urokinase-type plasminogen activator and its receptor in keloids. *Arch Otolaryngol Head Neck Surg,* 129**,** 1334-8.
LEASK, A. & ABRAHAM, D. J. (2004) TGF-beta signaling and the fibrotic response. *Faseb J,* 18**,** 816-27.
LEE, S. S., YOSIPOVITCH, G., CHAN, Y. H. & GOH, C. L. (2004) Pruritus, pain, and small nerve fiber function in keloids: a controlled study. *J Am Acad Dermatol,* 51**,** 1002-6.
LEE, T. Y., CHIN, G. S., KIM, W. J., CHAU, D., GITTES, G. K. & LONGAKER, M. T. (1999) Expression of transforming growth factor beta 1, 2, and 3 proteins in keloids. *Ann Plast Surg,* 43**,** 179-84.
LEFLORE, I. & ANTOINE, G. A. (1991) Keloid formation on palmar surface of hand. *J Natl Med Assoc,* 83**,** 463-4.
LEHMANN, J., RETZ, M., HARDER, J., KRAMS, M., KELLNER, U., HARTMANN, J., HOHGRAWE, K., RAFFENBERG, U., GERBER, M., LOCH, T., WEICHERT-JACOBSEN, K. & STOCKLE, M. (2002) Expression of human beta-defensins 1 and 2 in kidneys with chronic bacterial infection. *BMC Infect Dis,* 2**,** 20.

LEHRER, R. I., DAHER, K., GANZ, T. & SELSTED, M. E. (1985) Direct inactivation of viruses by MCP-1 and MCP-2, natural peptide antibiotics from rabbit leukocytes. *J Virol,* 54**,** 467-72.
LEVER, A. & MACKENZIE, I. (2007) Sepsis: definition, epidemiology, and diagnosis. *Bmj,* 335**,** 879-83.
LICHTER, I. & SIRETT, N. E. (1968) Plasma cortisol levels in lung cancer. *Br Med J,* 2**,** 154-6.
LILLIE, R. (1977) *H.J. Conn's Biological Stains,* Baltimore, Lippincott Williams and Wilkins Company.
LIM, I. J., PHAN, T. T., BAY, B. H., QI, R., HUYNH, H., TAN, W. T., LEE, S. T. & LONGAKER, M. T. (2002) Fibroblasts cocultured with keloid keratinocytes: normal fibroblasts secrete collagen in a keloidlike manner. *Am J Physiol Cell Physiol,* 283**,** C212-22.
LIM, I. J., PHAN, T. T., SONG, C., TAN, W. T. & LONGAKER, M. T. (2001) Investigation of the influence of keloid-derived keratinocytes on fibroblast growth and proliferation in vitro. *Plast Reconstr Surg,* 107**,** 797-808.
LIU, A. Y., DESTOUMIEUX, D., WONG, A. V., PARK, C. H., VALORE, E. V., LIU, L. & GANZ, T. (2002a) Human beta-defensin-2 production in keratinocytes is regulated by interleukin-1, bacteria, and the state of differentiation. *J Invest Dermatol,* 118**,** 275-81.
LIU, C. J., TAHARA, S. & GAO, S. (2003) Phosphorylation of extracellular signal-regulated protein kinase in cultured keloid fibroblasts when stimulated by platelet-derived growth factor BB. *Scand J Plast Reconstr Surg Hand Surg,* 37**,** 321-4.
LIU, L., WANG, L., JIA, H. P., ZHAO, C., HENG, H. H., SCHUTTE, B. C., MCCRAY, P. B., JR. & GANZ, T. (1998) Structure and mapping of the human beta-defensin HBD-2 gene and its expression at sites of inflammation. *Gene,* 222**,** 237-44.
LIU, L., ZHAO, C., HENG, H. H. & GANZ, T. (1997) The human beta-defensin-1 and alpha-defensins are encoded by adjacent genes: two peptide families with differing disulfide topology share a common ancestry. *Genomics,* 43**,** 316-20.
LIU, W., CAI, Z., WANG, D., WU, X., CUI, L., SHANG, Q., QIAN, Y. & CAO, Y. (2002b) Blocking transforming growth factor-beta receptor signaling down-regulates transforming growth factor-beta1 autoproduction in keloid fibroblasts. *Chin J Traumatol,* 5**,** 77-81.
LIVAK, K. J. & SCHMITTGEN, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods,* 25**,** 402-8.
LOW, S. Q. & MOY, R. L. (1992) Scar wars strategies. Target collagen. *J Dermatol Surg Oncol,* 18**,** 981-6.
LU, F., GAO, J. H. & LI, X. J. (2003) Immunohistochemical analysis of Fas protein expression in hypertrophic scar and keloid. *Di Yi Jun Yi Da Xue Xue Bao,* 23**,** 228-9, 232.
LUO, S., BENATHAN, M., RAFFOUL, W., PANIZZON, R. G. & EGLOFF, D. V. (2001) Abnormal balance between proliferation and apoptotic cell death in fibroblasts derived from keloid lesions. *Plast Reconstr Surg,* 107**,** 87-96.
LUTERMAN, A., DACSO, C. C. & CURRERI, P. W. (1986) Infections in burn patients. *Am J Med,* 81**,** 45-52.
MARS, W. M., PATMASIRIWAT, P., MAITY, T., HUFF, V., WEIL, M. M. & SAUNDERS, G. F. (1995) Inheritance of unequal numbers of the genes encoding the human neutrophil defensins HP-1 and HP-3. *J Biol Chem,* 270**,** 30371-6.
MARUCHA, P. T., KIECOLT-GLASER, J. K. & FAVAGEHI, M. (1998) Mucosal wound healing is impaired by examination stress. *Psychosom Med,* 60**,** 362-5.
MCCAULEY, R. L., CHOPRA, V., LI, Y. Y., HERNDON, D. N. & ROBSON, M. C. (1992) Altered cytokine production in black patients with keloids. *J Clin Immunol,* 12**,** 300-8.

MCCOY, B. J. & COHEN, I. K. (1981) Effects of various sera on growth kinetics and collagen synthesis by keloid and normal dermal fibroblasts. *Plast Reconstr Surg,* 67**,** 505-10.
MCGROUTHER, D. A. (1994) Hypertrophic or keloid scars? *Eye,* 8 (Pt 2)**,** 200-3.
MESSADI, D. V., DOUNG, H. S., ZHANG, Q., KELLY, A. P., TUAN, T. L., REICHENBERGER, E. & LE, A. D. (2004) Activation of NFkappaB signal pathways in keloid fibroblasts. *Arch Dermatol Res,* 296**,** 125-33.
MEYER, L. J., RUSSELL, S. B., RUSSELL, J. D., TRUPIN, J. S., EGBERT, B. M., SHUSTER, S. & STERN, R. (2000) Reduced hyaluronan in keloid tissue and cultured keloid fibroblasts. *J Invest Dermatol,* 114**,** 953-9.
MEYER, N. A., MULLER, M. J. & HERNDON, D. N. (1994) Nutrient support of the healing wound. *New Horiz,* 2**,** 202-14.
MICHIE, H. R., MANOGUE, K. R., SPRIGGS, D. R., REVHAUG, A., O'DWYER, S., DINARELLO, C. A., CERAMI, A., WOLFF, S. M. & WILMORE, D. W. (1988) Detection of circulating tumor necrosis factor after endotoxin administration. *N Engl J Med,* 318**,** 1481-6.
MIKULEC, A. A., HANASONO, M. M., LUM, J., KADLECK, J. M., KITA, M. & KOCH, R. J. (2001) Effect of tamoxifen on transforming growth factor beta1 production by keloid and fetal fibroblasts. *Arch Facial Plast Surg,* 3**,** 111-4.
MILNER, S. M. & ORTEGA, M. R. (1999) Reduced antimicrobial peptide expression in human burn wounds. *Burns,* 25**,** 411-3.
MOLTENI, A., WARPEHA, R. L., BRIZIO-MOLTENI, L., ALBERTSON, D. F. & KAURS, R. (1979) Circadian rhythms of serum aldosterone, cortisol and plasma renin activity in burn injuries. *Ann Clin Lab Sci,* 9**,** 518-23.
MOONEY, D. P. & GAMELLI, R. L. (1989) Sepsis following thermal injury. *Compr Ther,* 15**,** 22-9.
MOULTON-LEVY, P., JACKSON, C. E., LEVY, H. G. & FIALKOW, P. J. (1984) Multiple cell origin of traumatically induced keloids. *J Am Acad Dermatol,* 10**,** 986-8.
MUNSTER, A. M. (1994) Alteration of the immune system in burns and implications for therapy. *Eur J Pediatr Surg,* 4**,** 231-42.
NAITOH, M., HOSOKAWA, N., KUBOTA, H., TANAKA, T., SHIRANE, H., SAWADA, M., NISHIMURA, Y. & NAGATA, K. (2001) Upregulation of HSP47 and collagen type III in the dermal fibrotic disease, keloid. *Biochem Biophys Res Commun,* 280**,** 1316-22.
NEELY, A. N., CLENDENING, C. E., GARDNER, J., GREENHALGH, D. G. & WARDEN, G. D. (1999) Gelatinase activity in keloids and hypertrophic scars. *Wound Repair Regen,* 7**,** 166-71.
NICKOLOFF, B. J., XIN, H., NESTLE, F. O. & QIN, J. Z. (2007) The cytokine and chemokine network in psoriasis. *Clin Dermatol,* 25**,** 568-73.
NIESSEN, F. B., SPAUWEN, P. H. & KON, M. (1997) The role of suture material in hypertrophic scar formation: Monocryl vs. Vicryl-rapide. *Ann Plast Surg,* 39**,** 254-60.
NIYONSABA, F., SOMEYA, A., HIRATA, M., OGAWA, H. & NAGAOKA, I. (2001) Evaluation of the effects of peptide antibiotics human beta-defensins-1/-2 and LL-37 on histamine release and prostaglandin D(2) production from mast cells. *Eur J Immunol,* 31**,** 1066-75.
NIYONSABA, F., USHIO, H., NAGAOKA, I., OKUMURA, K. & OGAWA, H. (2005) The human beta-defensins (-1, -2, -3, -4) and cathelicidin LL-37 induce IL-18 secretion through p38 and ERK MAPK activation in primary human keratinocytes. *J Immunol,* 175**,** 1776-84.
OHTSURU, A., YOSHIMOTO, H., ISHIHARA, H., NAMBA, H. & YAMASHITA, S. (2000) Insulin-like growth factor-I (IGF-I)/IGF-I receptor axis and increased invasion activity of fibroblasts in keloid. *Endocr J,* 47 Suppl**,** S41-4.
OLUWASANMI, J. O. (1974) Keloids in Ibadan. *Trop Geogr Med,* 26**,** 231-41.

PALLADINO, M. A., MALLONGA, T. A. & MISHRA, M. S. (2003) Messenger RNA (mRNA) expression for the antimicrobial peptides beta-defensin-1 and beta-defensin-2 in the male rat reproductive tract: beta-defensin-1 mRNA in initial segment and caput epididymidis is regulated by androgens and not bacterial lipopolysaccharides. *Biol Reprod,* 68**,** 509-15.
PEACOCK, E. E., JR., MADDEN, J. W. & TRIER, W. C. (1970) Biologic basis for the treatment of keloids and hypertrophic scars. *South Med J,* 63**,** 755-60.
PHAN, T. T., LIM, I. J., AALAMI, O., LORGET, F., KHOO, A., TAN, E. K., MUKHOPADHYAY, A. & LONGAKER, M. T. (2005) Smad3 signalling plays an important role in keloid pathogenesis via epithelial-mesenchymal interactions. *J Pathol,* 207**,** 232-42.
PHAN, T. T., LIM, I. J., CHAN, S. Y., TAN, E. K., LEE, S. T. & LONGAKER, M. T. (2004) Suppression of transforming growth factor beta/smad signaling in keloid-derived fibroblasts by quercetin: implications for the treatment of excessive scars. *J Trauma,* 57**,** 1032-7.
PHAN, T. T., SEE, P., TRAN, E., NGUYEN, T. T., CHAN, S. Y., LEE, S. T. & HUYNH, H. (2003) Suppression of insulin-like growth factor signalling pathway and collagen expression in keloid-derived fibroblasts by quercetin: its therapeutic potential use in the treatment and/or prevention of keloids. *Br J Dermatol,* 148**,** 544-52.
PITCHE, P. (2006) [What is true in "spontaneous" keloids?]. *Ann Dermatol Venereol,* 133**,** 501.
POINDEXTER, B. J., BHAT, S., BUJA, L. M., BICK, R. J. & MILNER, S. M. (2006) Localization of antimicrobial peptides in normal and burned skin. *Burns,* 32**,** 402-7.
POLAVARAPU, N., OGILVIE, M. P. & PANTHAKI, Z. J. (2008) Microbiology of burn wound infections. *J Craniofac Surg,* 19**,** 899-902.
PRATHIBA, V. & SURYANARAYANAN, M. (1999) Biochemical and dynamic studies of collagen from human normal skin and keloid tissue. *Indian J Biochem Biophys,* 36**,** 158-64.
PRUITT, B. A., JR. & MCMANUS, A. T. (1992) The changing epidemiology of infection in burn patients. *World J Surg,* 16**,** 57-67.
PRUITT, B. A., JR., MCMANUS, A. T., KIM, S. H. & GOODWIN, C. W. (1998) Burn wound infections: current status. *World J Surg,* 22**,** 135-45.
PUGEAT, M., BONNETON, A., PERROT, D., ROCLE-NICOLAS, B., LEJEUNE, H., GRENOT, C., DECHAUD, H., BREBANT, C., MOTIN, J. & CUILLERON, C. Y. (1989) Decreased immunoreactivity and binding activity of corticosteroid-binding globulin in serum in septic shock. *Clin Chem,* 35**,** 1675-9.
QUAYLE, A. J., PORTER, E. M., NUSSBAUM, A. A., WANG, Y. M., BRABEC, C., YIP, K. P. & MOK, S. C. (1998) Gene expression, immunolocalization, and secretion of human defensin-5 in human female reproductive tract. *Am J Pathol,* 152**,** 1247-58.
RAJ, P. A. & DENTINO, A. R. (2002) Current status of defensins and their role in innate and adaptive immunity. *FEMS Microbiol Lett,* 206**,** 9-18.
RIRIE, K. M., RASMUSSEN, R. P. & WITTWER, C. T. (1997) Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal Biochem,* 245**,** 154-60.
RISSO, A. (2000) Leukocyte antimicrobial peptides: multifunctional effector molecules of innate immunity. *J Leukoc Biol,* 68**,** 785-92.
RUOT, B., BREUILLE, D., RAMBOURDIN, F., BAYLE, G., CAPITAN, P. & OBLED, C. (2000) Synthesis rate of plasma albumin is a good indicator of liver albumin synthesis in sepsis. *Am J Physiol Endocrinol Metab,* 279**,** E244-51.
RUSSELL, J. D., RUSSELL, S. B. & TRUPIN, K. M. (1982) Fibroblast heterogeneity in glucocorticoid regulation of collagen metabolism: genetic or epigenetic? *In Vitro,* 18**,** 557-64.

SAHL, H. G., PAG, U., BONNESS, S., WAGNER, S., ANTCHEVA, N. & TOSSI, A. (2005) Mammalian defensins: structures and mechanism of antibiotic activity. *J Leukoc Biol,* 77**,** 466-75.

SAITOH, M., ABIKO, Y., SHIMABUKURO, S., KUSANO, K., NISHIMURA, M., ARAKAWA, T., NAKASHIMA, K., TAKUMA, T., KAKU, T. & IGARASHI, S. (2004) Correlated expression of human beta defensin-1, -2 and -3 mRNAs in gingival tissues of young children. *Arch Oral Biol,* 49**,** 799-803.

SAMBROOK, J. & RUSSELL, D. (2001) *Molecular Cloning: A Laboratory Manual,* CSHL Press.

SATISH, L., BABU, M., TRAN, K. T., HEBDA, P. A. & WELLS, A. (2004) Keloid fibroblast responsiveness to epidermal growth factor and activation of downstream intracellular signaling pathways. *Wound Repair Regen,* 12**,** 183-92.

SCHIBLI, D. J., HUNTER, H. N., ASEYEV, V., STARNER, T. D., WIENCEK, J. M., MCCRAY, P. B., JR., TACK, B. F. & VOGEL, H. J. (2002) The solution structures of the human beta-defensins lead to a better understanding of the potent bactericidal activity of HBD3 against Staphylococcus aureus. *J Biol Chem,* 277**,** 8279-89.

SCHRODER, J. M. & HARDER, J. (1999) Human beta-defensin-2. *Int J Biochem Cell Biol,* 31**,** 645-51.

SELL, C., BASERGA, R. & RUBIN, R. (1995) Insulin-like growth factor I (IGF-I) and the IGF-I receptor prevent etoposide-induced apoptosis. *Cancer Res,* 55**,** 303-6.

SELSTED, M. E., BROWN, D. M., DELANGE, R. J., HARWIG, S. S. & LEHRER, R. I. (1985) Primary structures of six antimicrobial peptides of rabbit peritoneal neutrophils. *J Biol Chem,* 260**,** 4579-84.

SELSTED, M. E., BROWN, D. M., DELANGE, R. J. & LEHRER, R. I. (1983) Primary structures of MCP-1 and MCP-2, natural peptide antibiotics of rabbit lung macrophages. *J Biol Chem,* 258**,** 14485-9.

SELSTED, M. E. & OUELLETTE, A. J. (2005) Mammalian defensins in the antimicrobial immune response. *Nat Immunol,* 6**,** 551-7.

SELSTED, M. E., TANG, Y. Q., MORRIS, W. L., MCGUIRE, P. A., NOVOTNY, M. J., SMITH, W., HENSCHEN, A. H. & CULLOR, J. S. (1993) Purification, primary structures, and antibacterial activities of beta-defensins, a new family of antimicrobial peptides from bovine neutrophils. *J Biol Chem,* 268**,** 6641-8.

SELSTED, M. E., TANG, Y. Q., MORRIS, W. L., MCGUIRE, P. A., NOVOTNY, M. J., SMITH, W., HENSCHEN, A. H. & CULLOR, J. S. (1996) Purification, primary structures, and antibacterial activities of beta-defensins, a new family of antimicrobial peptides from bovine neutrophils. *J Biol Chem,* 271**,** 16430.

SEO, S. J., AHN, S. W., HONG, C. K. & RO, B. I. (2001) Expressions of beta-defensins in human keratinocyte cell lines. *J Dermatol Sci,* 27**,** 183-91.

SHEERAN, P. & HALL, G. M. (1997) Cytokines in anaesthesia. *Br J Anaesth,* 78**,** 201-19.

SHERIDAN, R. L., PETRAS, L., BASHA, G., SALVO, P., CIFRINO, C., HINSON, M., MCCABE, M., FALLON, J. & TOMPKINS, R. G. (1995a) Planimetry study of the percent of body surface represented by the hand and palm: sizing irregular burns is more accurately done with the palm. *J Burn Care Rehabil,* 16**,** 605-6.

SHERIDAN, R. L., SCHOMAKER, K. T., LUCCHINA, L. C., HURLEY, J., YIN, L. M., TOMPKINS, R. G., JERATH, M., TORRI, A., GREAVES, K. W. & BUA, D. P. (1995b) Burn depth estimation by use of indocyanine green fluorescence: initial human trial. *J Burn Care Rehabil,* 16**,** 602-4.

SMILEY, A. K., GARDNER, J., KLINGENBERG, J. M., NEELY, A. N. & SUPP, D. M. (2007) Expression of human beta defensin 4 in genetically modified keratinocytes enhances antimicrobial activity. *J Burn Care Res,* 28**,** 127-32.

SORENSEN, O. E., COWLAND, J. B., THEILGAARD-MONCH, K., LIU, L., GANZ, T. & BORREGAARD, N. (2003) Wound healing and expression of antimicrobial peptides/polypeptides in human keratinocytes, a consequence of common growth factors. *J Immunol,* 170**,** 5583-9.

SORENSEN, O. E., THAPA, D. R., ROSENTHAL, A., LIU, L., ROBERTS, A. A. & GANZ, T. (2005) Differential regulation of beta-defensin expression in human skin by microbial stimuli. *J Immunol,* 174**,** 4870-9.

STEINBRECH, D. S., MEHRARA, B. J., CHAU, D., ROWE, N. M., CHIN, G., LEE, T., SAADEH, P. B., GITTES, G. K. & LONGAKER, M. T. (1999) Hypoxia upregulates VEGF production in keloid fibroblasts. *Ann Plast Surg,* 42**,** 514-9; discussion 519-20.

SUFFREDINI, A. F. (1994) Current prospects for the treatment of clinical sepsis. *Crit Care Med,* 22**,** S12-8.

SUN, L., FINNEGAN, C. M., KISH-CATALONE, T., BLUMENTHAL, R., GARZINO-DEMO, P., LA TERRA MAGGIORE, G. M., BERRONE, S., KLEINMAN, C., WU, Z., ABDELWAHAB, S., LU, W. & GARZINO-DEMO, A. (2005) Human beta-defensins suppress human immunodeficiency virus infection: potential role in mucosal protection. *J Virol,* 79**,** 14318-29.

SZULGIT, G., RUDOLPH, R., WANDEL, A., TENENHAUS, M., PANOS, R. & GARDNER, H. (2002) Alterations in fibroblast alpha1beta1 integrin collagen receptor expression in keloids and hypertrophic scars. *J Invest Dermatol,* 118**,** 409-15.

TAKAHASHI, A., WADA, A., OGUSHI, K., MAEDA, K., KAWAHARA, T., MAWATARI, K., KURAZONO, H., MOSS, J., HIRAYAMA, T. & NAKAYA, Y. (2001) Production of beta-defensin-2 by human colonic epithelial cells induced by Salmonella enteritidis flagella filament structural protein. *FEBS Lett,* 508**,** 484-8.

TAN, E. M., ROUDA, S., GREENBAUM, S. S., MOORE, J. H., JR., FOX, J. W. T. & SOLLBERG, S. (1993) Acidic and basic fibroblast growth factors down-regulate collagen gene expression in keloid fibroblasts. *Am J Pathol,* 142**,** 463-70.

TAYLOR CR, C. R. (1994) *Immunomicroscopy: A Diagnostic Tool for the Surgical Pathologist,* 2nd ed. Philadelphia, London, Toronto,WB Saunders.

TAYLOR, G. D., KIBSEY, P., KIRKLAND, T., BURROUGHS, E. & TREDGET, E. (1992) Predominance of staphylococcal organisms in infections occurring in a burns intensive care unit. *Burns,* 18**,** 332-5.

TEOFOLI, P., MOTOKI, K., LOTTI, T. M., UITTO, J. & MAUVIEL, A. (1997) Propiomelanocortin (POMC) gene expression by normal skin and keloid fibroblasts in culture: modulation by cytokines. *Exp Dermatol,* 6**,** 111-5.

TRABI, M., SCHIRRA, H. J. & CRAIK, D. J. (2001) Three-dimensional structure of RTD-1, a cyclic antimicrobial defensin from Rhesus macaque leukocytes. *Biochemistry,* 40**,** 4211-21.

TSUJITA-KYUTOKU, M., UEHARA, N., MATSUOKA, Y., KYUTOKU, S., OGAWA, Y. & TSUBURA, A. (2005) Comparison of transforming growth factor-beta/Smad signaling between normal dermal fibroblasts and fibroblasts derived from central and peripheral areas of keloid lesions. *In Vivo,* 19**,** 959-63.

TUAN, T. L. & NICHTER, L. S. (1998) The molecular basis of keloid and hypertrophic scar formation. *Mol Med Today,* 4**,** 19-24.

TUAN, T. L., WU, H., HUANG, E. Y., CHONG, S. S., LAUG, W., MESSADI, D., KELLY, P. & LE, A. (2003) Increased plasminogen activator inhibitor-1 in keloid fibroblasts may account for their elevated collagen accumulation in fibrin gel cultures. *Am J Pathol,* 162**,** 1579-89.

TUAN, T. L., ZHU, J. Y., SUN, B., NICHTER, L. S., NIMNI, M. E. & LAUG, W. E. (1996) Elevated levels of plasminogen activator inhibitor-1 may account for the altered fibrinolysis by keloid fibroblasts. *J Invest Dermatol,* 106**,** 1007-11.

UEDA, K., FURUYA, E., YASUDA, Y., OBA, S. & TAJIMA, S. (1999) Keloids have continuous high metabolic activity. *Plast Reconstr Surg,* 104**,** 694-8.

UEDA, K., YASUDA, Y., FURUYA, E. & OBA, S. (2004) Inadequate blood supply persists in keloids. *Scand J Plast Reconstr Surg Hand Surg,* 38**,** 267-71.

UMEDA, T., HIRAMATSU, R., IWAOKA, T., SHIMADA, T., MIURA, F. & SATO, T. (1981) Use of saliva for monitoring unbound free cortisol levels in serum. *Clin Chim Acta,* 110**,** 245-53.

VALORE, E. V. & GANZ, T. (1992) Posttranslational processing of defensins in immature human myeloid cells. *Blood,* 79**,** 1538-44.

VALORE, E. V., PARK, C. H., QUAYLE, A. J., WILES, K. R., MCCRAY, P. B., JR. & GANZ, T. (1998) Human beta-defensin-1: an antimicrobial peptide of urogenital tissues. *J Clin Invest,* 101**,** 1633-42.

VAROGA, D., PUFE, T., HARDER, J., SCHRODER, J. M., MENTLEIN, R., MEYER-HOFFERT, U., GOLDRING, M. B., TILLMANN, B., HASSENPFLUG, J. & PAULSEN, F. (2005) Human beta-defensin 3 mediates tissue remodeling processes in articular cartilage by increasing levels of metalloproteinases and reducing levels of their endogenous inhibitors. *Arthritis Rheum,* 52**,** 1736-45.

VINDENES, H. & BJERKNES, R. (1995) Microbial colonization of large wounds. *Burns,* 21**,** 575-9.

WALLACE, A. M., HE, J. Q., BURKETT, K. M., RUAN, J., CONNETT, J. E., ANTHONISEN, N. R., PARE, P. D. & SANDFORD, A. J. (2006) Contribution of alpha- and beta-defensins to lung function decline and infection in smokers: an association study. *Respir Res,* 7**,** 76.

WEINBERG, A., KRISANAPRAKORNKIT, S. & DALE, B. A. (1998) Epithelial antimicrobial peptides: review and significance for oral applications. *Crit Rev Oral Biol Med,* 9**,** 399-414.

WENDLING, J., MARCHAND, A., MAUVIEL, A. & VERRECCHIA, F. (2003) 5-fluorouracil blocks transforming growth factor-beta-induced alpha 2 type I collagen gene (COL1A2) expression in human fibroblasts via c-Jun NH2-terminal kinase/activator protein-1 activation. *Mol Pharmacol,* 64**,** 707-13.

WERNER, S. & GROSE, R. (2003) Regulation of wound healing by growth factors and cytokines. *Physiol Rev,* 83**,** 835-70.

WOLOSKI, B. M., SMITH, E. M., MEYER, W. J., 3RD, FULLER, G. M. & BLALOCK, J. E. (1985) Corticotropin-releasing activity of monokines. *Science,* 230**,** 1035-7.

WU, Y., ZHANG, Q., ANN, D. K., AKHONDZADEH, A., DUONG, H. S., MESSADI, D. V. & LE, A. D. (2004) Increased vascular endothelial growth factor may account for elevated level of plasminogen activator inhibitor-1 via activating ERK1/2 in keloid fibroblasts. *Am J Physiol Cell Physiol,* 286**,** C905-12.

WU, Z., HOOVER, D. M., YANG, D., BOULEGUE, C., SANTAMARIA, F., OPPENHEIM, J. J., LUBKOWSKI, J. & LU, W. (2003) Engineering disulfide bridges to dissect antimicrobial and chemotactic activities of human beta-defensin 3. *Proc Natl Acad Sci U S A,* 100**,** 8880-5.

XIA, W., LONGAKER, M. T. & YANG, G. P. (2006) P38 MAP kinase mediates transforming growth factor-beta2 transcription in human keloid fibroblasts. *Am J Physiol Regul Integr Comp Physiol,* 290**,** R501-8.

XIA, W., PHAN, T. T., LIM, I. J., LONGAKER, M. T. & YANG, G. P. (2004) Complex epithelial-mesenchymal interactions modulate transforming growth factor-beta expression in keloid-derived cells. *Wound Repair Regen,* 12**,** 546-56.

YANG, D., CHERTOV, O., BYKOVSKAIA, S. N., CHEN, Q., BUFFO, M. J., SHOGAN, J., ANDERSON, M., SCHRODER, J. M., WANG, J. M., HOWARD, O. M. & OPPENHEIM, J. J. (1999) Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. *Science,* 286**,** 525-8.

YANG, D., CHERTOV, O. & OPPENHEIM, J. J. (2001) The role of mammalian antimicrobial peptides and proteins in awakening of innate host defenses and adaptive immunity. *Cell Mol Life Sci,* 58**,** 978-89.

YANG, G. P., LIM, I. J., PHAN, T. T., LORENZ, H. P. & LONGAKER, M. T. (2003) From scarless fetal wounds to keloids: molecular studies in wound healing. *Wound Repair Regen,* 11**,** 411-8.

YOSHIMOTO, H., ISHIHARA, H., OHTSURU, A., AKINO, K., MURAKAMI, R., KURODA, H., NAMBA, H., ITO, M., FUJII, T. & YAMASHITA, S. (1999) Overexpression of insulin-like growth factor-1 (IGF-I) receptor and the invasiveness of cultured keloid fibroblasts. *Am J Pathol,* 154**,** 883-9.

YU, H., BOCK, O., BAYAT, A., FERGUSON, M. W. & MROWIETZ, U. (2005) Decreased expression of inhibitory SMAD6 and SMAD7 in keloid scarring. *Br J Plast Surg*.

ZHANG, Q., OH, C. K., MESSADI, D. V., DUONG, H. S., KELLY, A. P., SOO, C., WANG, L. & LE, A. D. (2006) Hypoxia-induced HIF-1 alpha accumulation is augmented in a co-culture of keloid fibroblasts and human mast cells: involvement of ERK1/2 and PI-3K/Akt. *Exp Cell Res,* 312**,** 145-55.

ZHANG, Q., WU, Y., ANN, D. K., MESSADI, D. V., TUAN, T. L., KELLY, A. P., BERTOLAMI, C. N. & LE, A. D. (2003) Mechanisms of hypoxic regulation of plasminogen activator inhibitor-1 gene expression in keloid fibroblasts. *J Invest Dermatol,* 121**,** 1005-12.

ZHAO, C., WANG, I. & LEHRER, R. I. (1996) Widespread expression of beta-defensin hBD-1 in human secretory glands and epithelial cells. *FEBS Lett,* 396**,** 319-22.

ZHU, J., LUO, C. Q., HE, Q. Y., PENG, H., ZHOU, J. D. & LI, P. (2005) [Relationship between angiogenesis and expression of HO-1 of scar]. *Zhong Nan Da Xue Xue Bao Yi Xue Ban,* 30**,** 335-9.