Effect of growth factors on T-lymphocyte induced keratinocyte apoptosis

Ilse Sofia Daehn

Department of Medicine-Biotechnology
Flinders University of South Australia

A thesis submitted for the degree of
Doctor of Philosophy
January 2007
For my family and Tom

“Happy is he who gets to know the reasons for things.”

Virgil (70-19 BCE)
Roman poet.
Table of Contents

Declaration .......................................................................................................................... 11
Acknowledgements ........................................................................................................... 12
Publications arising from this project ........................................................................ 14
Abbreviations .................................................................................................................. 15
Thesis summary: ............................................................................................................. 17

CHAPTER 1

Introduction

1.1 The skin’s barrier function ......................................................................................... 20
1.2 Keratinocyte differentiation ....................................................................................... 22
  1.2.1 Keratinocyte cell death ......................................................................................... 24
1.3 Death receptor pathways ............................................................................................ 26
  1.3.1 Fas induced apoptosis ......................................................................................... 26
  1.3.2 The Caspase Cascade ......................................................................................... 27
1.4 Atopic eczema ............................................................................................................. 28
  1.4.1 Impact of atopic eczema ....................................................................................... 30
  1.4.2 Prevalence and diagnosis of atopic eczema ......................................................... 30
  1.4.3 T-lymphocyte response ....................................................................................... 33
  1.4.4 The T-lymphocyte response in atopic eczema ..................................................... 35
1.4.5 Dysregulated immune response in atopic eczema ....................... 37
1.4.6 Keratinocyte apoptosis in atopic eczema ................................. 38
1.4.7 The role of keratinocytes in atopic eczema ............................... 40

1.5 Current therapies for atopic eczema ............................................. 41
1.5.1 Anti-inflammatory therapies targeting T-lymphocyte activation .... 42
1.5.2 Can keratinocyte apoptosis be a therapeutic target? ..................... 43

1.6 Growth Factors in skin homeostasis ............................................. 44
1.6.1 The role of IGF-I in skin ............................................................. 46
1.6.2 The role of TGFβ in skin ............................................................. 47
1.6.3 Growth factor effects in keratinocyte apoptosis ......................... 48
1.6.4 Whey growth factor extract (WGFE) as source of IGF-I and TGFβ .. 51

1.7 Thesis Hypothesis ........................................................................ 52
1.7.1 Thesis aims .............................................................................. 52

Chapter 2
MATERIALS AND METHODS

2.1 Buffers and solutions .................................................................... 54
2.1.1 Phosphate buffered saline (PBS) ................................................. 54

2.2 Antibodies and staining reagents .................................................. 57

2.3 Cell culture .................................................................................. 62
2.3.1 HaCaT Keratinocytes ............................................................... 62
2.3.2 Normal human epidermal keratinocytes .................................... 62
2.3.3 Jurkat T-lymphocytes ................................................................. 63
2.3.4 Primary T-lymphocytes ................................................................. 64
2.3.5 Cryopreservation ......................................................................... 65

2.4 Functional studies co-culture experiments .................................... 66
2.4.1 Mitogen activation of T-lymphocyte ........................................... 66
2.4.2 Jurkat T-lymphocyte conditioned media ..................................... 66
2.4.3 Jurkat T-lymphocyte and HaCaT co-culture ............................... 67
2.4.4 Primary T-lymphocyte co-culture with HaCaTs or NHEKs .......... 67

2.5 Flow cytometry ............................................................................. 67
2.5.1 Cell viability and apoptosis ......................................................... 68
2.5.2 Cell surface immunofluorescence staining ............................... 68
2.5.3 Intracellular Immunofluorescence staining ............................... 70

2.6 DNA fragmentation studies ............................................................ 70
2.6.1 HOECHST staining ................................................................. 70

2.7 Caspase activity assay ................................................................. 71

2.8 Western blotting ........................................................................... 72
2.8.1 Protein quantification - Bradford Assay ................................. 73
2.8.2 SDS-PAGE Separating Gels ...................................................... 74

2.9 Slot blot ......................................................................................... 74
2.10 Cytospins .................................................................................... 75

2.11 Quantification of cytokines ......................................................... 75
2.12 Statistics ..................................................................................... 76
Chapter 3
Sodium butyrate induced HaCaT apoptosis

3.1 Introduction.............................................................................................. 77
3.2 Methods ................................................................................................... 82
  3.2.1 Butyrate treatment of HaCaT ............................................................ 82
  3.2.2 Measurements of apoptosis............................................................... 82
  3.2.3 Measurements of differentiation....................................................... 83
3.3 Results ..................................................................................................... 84
  3.3.1 Induction of HaCaT apoptosis by Sodium butyrate ....................... 84
  3.3.2 Sodium butyrate induced morphological features of apoptosis and nuclear fragmentation................................................................. 85
  3.3.3 Activation of the caspase cascade by sodium butyrate treated HaCaTs 89
  3.3.4 Sodium butyrate induced Fas expression HaCaTs. ......................... 94
  3.3.5 Caspase 3 inhibitor did not inhibit butyrate induced apoptosis ....... 94
  3.3.6 Sodium butyrate did not induce HaCaT differentiation ................. 98
3.4 Summary................................................................................................ 101

Chapter 4
Jurkat induced HaCaT apoptosis

4.1 Introduction.............................................................................................. 108
4.2 Methods .......................................................................................................................... 110
  4.2.1 Jurkat conditioned media ...................................................................................... 110
  4.2.2 Jurkat T-lymphocyte and HaCaT co-cultures ...................................................... 110
  4.2.3 Assessment of HaCaT apoptosis ........................................................................... 111
  4.2.4 Mechanistic assessments ..................................................................................... 111
4.3 Results .......................................................................................................................... 112
  4.3.1 Jurkat activation .................................................................................................... 112
  4.3.2 Jurkat co-culture induced HaCaT apoptosis ....................................................... 112
  4.3.3 Jurkat co-culture induced HaCaT caspase 3 activity .......................................... 117
  4.3.4 Jurkat induced HaCaT apoptosis by Fas ............................................................ 118
  4.3.5 Effect of IFNγ on Jurkat induced HaCaT apoptosis ............................................. 123
  4.3.6 Effect of IFNγ on HaCaT Fas expression ........................................................... 124
  4.3.7 Jurkat co-culture induced HaCaT ICAM-1 expression ........................................ 124
4.4 Summary ..................................................................................................................... 130

Chapter 5

Primary T-lymphocyte induced keratinocyte apoptosis

5.1 Introduction .................................................................................................................... 133
5.2 Methods ........................................................................................................................ 137
  5.2.1 Primary CD4+ T-lymphocyte and HaCaT co-culture ....................................... 137
  5.2.2 Primary CD4+ T-lymphocyte and primary keratinocyte co-culture ............ 138
  5.2.3 Cytokine measurement ....................................................................................... 138
5.2.4 Keratinocyte differentiation ............................................................ 139
5.3 Results ................................................................................................... 140
  5.3.1 Primary CD4+ T-lymphocytes induced HaCaT apoptosis .......... 140
  5.3.2 FasL expression by CD4+ T-lymphocytes increases with activation 145
  5.3.3 T-lymphocyte induced HaCaT apoptosis was mediated by Fas ...... 145
  5.3.4 Co-culture induced adhesion molecule expression ......................... 146
  5.3.5 T-lymphocytes induced Fas mediated apoptosis of normal human epidermal keratinocytes................................................................. 151
  5.3.6 IFNγ is release during co-culture..................................................... 155
  5.3.7 IFNγ increased T-lymphocyte induced keratinocyte apoptosis ....... 156
  5.3.8 IFNγ potentiated T-lymphocyte induced Fas expression of HaCaTs 159
  5.3.9 T-lymphocyte co-culture induced HaCaT early differentiation....... 162
  5.3.10 T-lymphocyte induced keratinocyte apoptosis was associated with α6-
        dim expression .................................................................................. 167
5.4 Summary ................................................................................................. 169

Chapter 6

TGFβ and IGF-I effects on T-lymphocyte induced keratinocyte apoptosis

6.1 Introduction................................................................................................ 173
6.2 Methods ..................................................................................................... 176
  6.2.1 Co-culture treatment with IGF-1, TGFβ1 or LR3-IGF .................... 176
6.3 Results ........................................................................................................ 177
6.3.1 Effect of growth factors on T-lymphocyte induced HaCaT apoptosis

6.3.2 A combination of TGF\(\beta_1\) and IGF-I decreased T-lymphocyte induced HaCaT apoptosis

6.3.3 Post-treatment with TGF\(\beta_1\) and IGF-I did not rescue T-lymphocyte induced HaCaT apoptosis

6.3.4 TGF\(\beta_1\) and IGF-I inhibited the release of IFN\(\gamma\) in co-culture

6.3.5 Effect of TGF\(\beta\) and IGF-I on HaCaT Fas expression

6.3.6 TGF\(\beta_1\) and IGF-I prevented T-lymphocyte induced keratinocyte differentiation

6.3.7 IGF-I prevents T-lymphocyte induced apoptosis of NHEKs

6.3.8 Effect of IGF-I on NHEK Fas expression and early differentiation

6.4 Summary

Chapter 7

Effects of WGFE on T-lymphocyte induced keratinocyte apoptosis

7.1 Introduction

7.2 Methods

7.2.1 WGFE

7.3 Results

7.3.1 WGFE prevented T-lymphocyte induced HaCaT apoptosis

7.3.2 Effect of WGFE on IFN\(\gamma\) release and Fas expression
7.3.3 WGFE prevents T-lymphocyte induced early differentiation........... 217
7.3.4 WGFE did not prevent T-lymphocyte induced apoptosis of NHEKs 221
7.3.5 Effect of IGF-I enriched WGFE (UFO2N010) on T-lymphocyte induced NHEK apoptosis ................................................................. 221
7.3.6 UFO2N010 prevents T-lymphocyte induced early differentiation of normal human epidermal keratinocytes ......................................................... 222

7.4 Summary ...................................................................................... 226

Chapter 8
DISCUSSION

8.1 T-lymphocyte induced keratinocyte apoptosis ................................. 229
  8.1.1 T-lymphocyte induced Fas mediated apoptosis of keratinocyte ...... 230
  8.1.2 T-lymphocyte induced keratinocyte apoptosis is mediated by IFNγ stimulated upregulation of keratinocyte Fas and subsequent activation of caspase 3 231

8.2 Growth factors protected keratinocytes from T-lymphocyte induced apoptosis ...................................................................................... 235
  8.2.1 Growth factors effects Fas .............................................................. 237
  8.2.2 Potential pathways mediating keratinocyte survival induced by growth factors 238

8.3 T-lymphocyte induced early keratinocyte differentiation ...................... 241
  8.3.1 Loss of α6 integrin by apoptotic keratinocytes .............................. 241
8.3.2 α6 integrin mediated survival of keratinocytes ............................................. 243
8.4 Growth factor mediated keratinocyte survival............................................. 245
8.5 Application of thesis outcomes and future work .................................... 248
  8.5.1 Growth factor based therapies as potential treatments for inflammatory
       skin disorders................................................................................................ 248
  8.5.2 Milk derived growth factor based therapies as potential treatments for
       atopic eczema................................................................................................ 250
8.6 Conclusion ............................................................................................... 254

Appendices ........................................................................................................... 255

BIBLIOGRAPHY .............................................................................................. 267
Declaration

“I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text”

Ilse S. Daehn

January 2007
I would like to take this opportunity to thank a number of people without whose help and support this thesis would have never been possible. A special acknowledgment to my supervisor Dr Tim Rayner, for the valuable guidance and support over these last few years, without whose knowledge and assistance this study would not have been successful. I will take the upside down triangle ▼ approach wherever I go! I would like to thank Dr Antiopi Varelias for the encouragement, advice and friendship. I also express my gratitude for Dr Peter Macardle and Dr Allison Cowin who have provided valuable advice and feedback to this thesis. In addition I would like to thank Dairy Australia and The Queen Elizabeth Hospital Research Foundation for providing me with financial support to conduct my studies.

Special thanks to Mrs Silvia Nobbs for her invaluable expertise and help with the flow cytometry work. I also thank WCH Haematology department for bleeding me, yes there is literally blood, sweat and tears in the making of this thesis… My appreciation also goes to the staff at CHRI and TGR-Biosciences for their allowing me to perform experiments using their equipment, for their valuable intellectual input and constructive criticism.

A special acknowledgment to Ken and Adrian from the TQEH animal house, as well as Madeline and Ashley, for making the days at TQEH so much fun. A super thanks to the
CHRI crew: Marko, Prodel, Mic, Donato, Pallave, Naoms, Rogy, Walter and LJ with whom we shared so many funny moments including those unforgettable conversations…., the Torrens runs, coffee schemes, the pub o’clocks, those crazy pubcrawls... I would like to also thank everyone involved in the making of the CHRI calendar! Thanks to the BioHazardous and the Sand-Sationals. Special thank you to those close friends who I can always count on and have contributed to my sanity in so many ways; my Vec, Dave and Olgi… I love you guys.

I cannot end without thanking my parents, whose courage and determination in life will always inspire me. To my sisters whom I love and finally to my husband Tom, who’s constant support, encouragement and love, helped me finish this thesis. A part of you all is in here, in some form, somewhere… I am extremely grateful, I could not have done it with out you, so it is to you that I dedicate this work.
Publications arising from this project

(see Appendix)

Daehn I, Varelias A, Rayner T. Sodium butyrate induced keratinocyte apoptosis.

Ruzehaji G, Daehn I, Varelias A, Rayner T. Exploring cellular interactions relevant to
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>α6</td>
<td>alpha-6 integrin</td>
</tr>
<tr>
<td>ACD</td>
<td>allergic contact dermatitis</td>
</tr>
<tr>
<td>AE</td>
<td>atopic eczema</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DMEM</td>
<td>dulbecco's modified eagle's medium</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorter</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>HLDA</td>
<td>human cell differentiation antigens</td>
</tr>
<tr>
<td>HDI</td>
<td>histone deacetylase inhibitor</td>
</tr>
<tr>
<td>hrs</td>
<td>hours</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intracellular adhesion molecule</td>
</tr>
<tr>
<td>IGF-I</td>
<td>insulin-like growth factor-I</td>
</tr>
<tr>
<td>IGFBP</td>
<td>IGF-binding proteins</td>
</tr>
<tr>
<td>IFNγ</td>
<td>interferon γ</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LFA-1</td>
<td>lymphocyte function-associated antigen-1</td>
</tr>
<tr>
<td>LR3-IGF</td>
<td>LONG™R3 IGF-I</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>mins</td>
<td>minutes</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>NF-κβ</td>
<td>nuclear factor κβ</td>
</tr>
<tr>
<td>NHEK</td>
<td>normal human epidermal keratinocytes</td>
</tr>
<tr>
<td>PARP</td>
<td>poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol myristate acetate</td>
</tr>
<tr>
<td>PS</td>
<td>phosphotidylserine</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TIMs</td>
<td>topical macrolide immunomodulators</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumour necrosis factor α</td>
</tr>
<tr>
<td>TRAIL</td>
<td>tumour necrosis related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>WGFE</td>
<td>whey growth factor extract</td>
</tr>
<tr>
<td>x g</td>
<td>relative centrifugal force (g-force)</td>
</tr>
</tbody>
</table>
Atopic eczema is a T-lymphocyte mediated chronic inflammatory skin disorder. The interaction of CD4+ T-lymphocytes with epidermal keratinocytes results in dysregulated, chronic inflammation and altered barrier function. T-lymphocyte induced keratinocyte apoptosis has been proposed as a mechanism by which epidermal integrity is impaired in eczema. Apoptosis of keratinocytes is thought to result from T-lymphocyte associated Fas ligand (FasL) binding to the death receptor Fas on keratinocytes. The primary aim of this project was to characterize the induction of keratinocyte apoptosis by T-lymphocytes and address the hypothesis that insulin-like growth factor-I (IGF-1), transforming growth factor β1 (TGFβ1) and a milk derived growth factor extract containing TGFβ and IGF-I (whey growth factor extract; WGFE) protect keratinocytes from T-lymphocyte mediated apoptosis.

To address the aims of this project, an in vitro co-culture model was developed combining T-lymphocytes with keratinocytes. Co-cultures were initially established using human Jurkat T-lymphocytes and human HaCaT keratinocytes with more extensive characterisation undertaken using primary CD4+ T-lymphocytes together with HaCaTs or normal human epidermal keratinocytes (NHEK). Annexin V and propidium iodide staining was established as the primary method for measuring keratinocyte apoptosis with this validated using sodium butyrate a known inducer of apoptosis. Changes in nuclear fragmentation and cell morphology were also examined as a key
feature of apoptosis. The involvement of the Fas pathway was investigated by assessing T-lymphocyte FasL expression, keratinocyte Fas expression and downstream caspase activation. Inflammatory cytokines IFNγ and TNFα were also examined due to their ability to induce Fas expression.

Studies performed with T-lymphocytes demonstrated that keratinocyte apoptosis was induced, with this due primarily to direct T-lymphocytes and keratinocytes interactions, rather than soluble mediators in the co-culture milieu. Activated T-lymphocytes were found to have high levels of FasL and to upregulate keratinocyte Fas expression. The increased keratinocyte Fas was associated with increased IFNγ levels in the co-culture media and activation of the caspase cascade. A Fas blocking antibody prevented T-lymphocyte induced keratinocyte apoptosis demonstrating that this was a Fas dependent event.

As the primary function of keratinocytes is to terminally differentiate, the differentiation status of the cells induced to undergo apoptosis was examined. It was demonstrated that T-lymphocytes decrease the intensity of α6 integrin expression by the keratinocytes. This marker identifies undifferentiated basal cells as high expressors of α6, with cells in the early stages of differentiation pathway found to be low expressors of α6. Co-staining with Annexin V demonstrated that the apoptotic keratinocytes were low expressors of α6 and thus cells committed to the early stages of differentiation. This suggested that the T-lymphocytes initiated the onset of keratinocyte terminal differentiation with this linked
to the cells being more susceptible to death induced by T-lymphocyte by activation of the Fas pathway.

The ability of TGFβ₁, IGF-I and WGFE to inhibit T-lymphocyte induced keratinocyte apoptosis was examined. A combination of recombinant TGFβ (10ng) & IGF-I (100ng) was able to significantly inhibit keratinocyte apoptosis. A similar result was obtained with WGFE, and although these growth factor treatments were able to reduce the elevated IFNγ levels in the co-culture media, they did not reduce T-lymphocyte induced Fas upregulation. The TGFβ₁ and IGF-I combination as well as WGFE did however prevent the T-lymphocyte induced shift from α6 bright to dim expressing keratinocytes. As such, the growth factor combinations appeared to protect the keratinocytes from T-lymphocyte mediated apoptosis by preventing them from committing to terminal differentiation.

The studies in this thesis have characterised the Fas associated mechanisms by which T-lymphocytes induce keratinocyte apoptosis and suggest specific growth factor combinations may have the potential to ameliorate the reduced barrier function associated with inflammatory skin conditions such as atopic eczema.