DEVELOPMENT OF A NOVEL THERAPY FOR THE PREVENTION OR REDUCTION OF CUTANEOUS SCARRING

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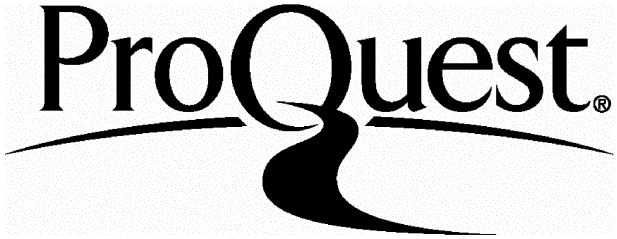

Abstract

There currently exists no effective therapy for either the prophylaxis or treatment of cutaneous scarring. The myofibroblast has been implicated in the aetiology of pathological scars, and therefore the development of therapies to prevent scarring in man have focussed upon the prevention of the myofibroblast phenotype. The aim of this thesis was to investigate whether insulin could prevent dermal fibroblast - myofibroblast differentiation *in vitro* and *in vivo*.

Three preparations of insulin that are in common clinical usage were evaluated for their efficacy in reducing fibroblast - myofibroblast differentiation in human dermal fibroblast cell cultures. Using immunohistochemical and western blotting techniques, insulin was shown to be effective in reducing myofibroblast number.

The effect of insulin on fibroblast proliferation, total protein and collagen synthesis was also determined. In addition, the effect of insulin on the contraction of 3D collagen gels was evaluated.

Using immunohistochemical techniques, the effect of growth factors known to inhibit apoptosis were evaluated for their abilities to reduce fibroblast - myofibroblast differentiation. In addition, prior to commencement of *in vivo* studies, the effectiveness of insulin in the presence of its physiological antagonists was established.

To investigate the effect of insulin *in vivo*, the compound was tested on healing murine incisional and excisional wounds. Incisional wounds proved to be the most reliable for interpretation. Myofibroblast numbers were assessed using both immunohistochemical and western blotting.

Progress towards the development of a novel therapy for the prevention or reduction of cutaneous scarring has been achieved by assessing the effects of insulin both *in vitro* and *in vivo*.

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Table of Contents

Abstract 2
Acknowledgements 3
Table of Contents 4
Index of Tables and Figures 12
List of Abbreviations 19
Chapter 1
Introduction 21
1.1 Introduction 22
1.2 Anatomy of skin 23
1.2.1 The epidermis 24
1.2.2 The dermis 24
1.3 Wound healing 25
1.3.1 Inflammatory (lag) phase 26
1.3.2 Proliferative phase 27
1.3.3 Remodelling 30
1.3.4 Control of Wound Healing 31
1.4 Scarring 33
1.4.1 Inadequate wound healing 33
1.4.2 Excessive wound healing 34
Keloid scars 35
Hypertrophic scars 36
1.5 The Fibroblast and Myofibroblast 36
1.5.1 History / discovery of the myofibroblast 38
1.5.2 Definition 38
1.5.3 Origin of the myofibroblast 39
1.5.4 Histological appearance 40
1.5.5 Association with pathological fibrosis 41
Skin 41
Ocular scarring 41
Oral scarring 42
Lung 42

Kidney 43
Liver 43
1.5.6 Factors that influence myofibroblast differentiation 43
1.5.6.1 Growth Factors 44
1.5.6.2 Interactions with the extracellular matrix 46
1.6 Management of the unsatisfactory scar 47
1.6.1 Conservative 47
Pressure Dressings 47
Silicone dressings 48
Hyaluronan 49
Zinc 49
1.6.2 Medical / Adjuvant 49
Steroids 49
Radiotherapy 50
1.6.3 Surgical 50
Approaches to Reduce the Formation of Cutaneous Scarring 51
Surgical Therapy of the Established Scar 52
1.7 Potential therapies proposed to reduce or prevent cutaneous scarring 54
1.7.1 Manipulation of the wound environment 54
1.7.2 Addition of exogenous substances 55
1.8 Insulin as a potential anti-scarring therapy 58
1.8.1 Synthesis and Secretion of Insulin 58
1.8.2 Actions of insulin 59
1.8.3 Insulin and fibroblast – myofibroblast differentiation 60
1.9 Aims of this thesis 60
1.10 Hypothesis 60
Chapter 2
Materials and Methods 62
2.1 Materials 63
2.1.1 Animals 63
2.1.2 Anaesthetic Equipment and Drugs 63
2.1.3 Theatre Consumables 63
Animal Procedures 63

2.1.4 Tissue Culture 63
2.1.5 Tissue Analysis 64
Light microscopy 64
Immunohistochemistry 64
2.1.6 Image Acquisition and Analysis 65
Histology 65
Tissue Analysis 65
2.2 Methods - General Tissue Culture 65
2.2.1 Media 65
Normal Growth Medium 65
Growth Factor Dialysed Medium 65
2.2.2 Fibroblast Isolation and Culture 66
2.2.3 Fibroblast culture for Immunohistochemistry 66
2.2.4 Cryopreservation of cells 67
2.2.5 Cell Numbers 68
2.3 Methods – Cell / Tissue Analysis 68
2.3.1 Immunohistochemistry 68
2.3.2 Western Blotting 69
Preparation of protein extracts from cells 69
Western Blot Analysis 69
Staining of Gel 70
Detection of αSMA 70
Staining for total protein 71
2.3.3 Crystal Violet Proliferation Assay 71
Initiation and routine care 71
Staining and reading 72
Validation of assay 72
2.3.4 Protein Assay 73
2.3.5 Collagen Assay 75
Initiation of cultures 75
Collagen assay 75
2.3.6 3D Collagen gels 76
Extraction of Rat Tail Collagen 76

Preparation of 3D collagen gels 76
Analysis of contraction....77
2.3.7 Detection of Apoptosis 77
2.4 Methods - Murine Scarring Model....78
2.4.1 Husbandry....78
2.4.2 Anaesthesia....79
2.4.3 Operative Procedures....80
Incisional Wound 80
Excisional Wound 80
Local Infiltration....80
2.4.4 Wound Evaluation 81
2.4.5 Wound Harvest 81
Cell culture 81
Histology 82
Protein Preparation 82
Tenisometry....82
2.4.6 Murine Fibroblast Culture 82
2.4.7 Histological Evaluation of Fixed Tissue 83
2.4.8 Immunohistochemistry 83
2.4.9 Analysis of murine wounds 84
2.4.10 Tensiometry 85
2.5 Statistics 85
Chapter 3
Determination of the effect of insulin on fibroblast differentiation *in vitro* 87
3.1 Introduction....88
Aims 89
3.2 Results....90
3.2.1 Bovine Insulin....90
3.2.2 Human Insulin 94
Immunohistochemistry....94
Western Blotting....98
3.3 Discussion 102
3.4 Conclusions....107

Chapter 4

The effect of insulin on aspects of fibroblast behaviour important during wound healing 108

4.1 Introduction 109

Hypothesis: 110

Aims: 110

4.2 Results 111

4.2.1 Proliferation 111

4.2.2 Protein Production 115

4.2.3 Collagen Production 117

4.2.4 Collagen Production as a ratio of total protein production 119

4.2.5 Contraction of collagen gels 120

4.3 Discussion 122

4.3.1 Proliferation 123

4.3.2 Synthesis of Extracellular Matrix Components 124

4.3.3 Contraction of collagen gels 126

4.4 Conclusions 129

Chapter 5

Do polypeptides that share certain biological properties with insulin affect fibroblast - myofibroblast differentiation? 131

5.1 Introduction 132

Aims: 134

5.2 Results 135

5.2.1 Basal apoptosis rates in fibroblast culture medium 135

Colorimetry 135

Microscopy 135

5.2.2 The Addition of Factors Known To Reduce Apoptosis 136

Insulin Like Growth Factor 1 (IGF-I) 137

Insulin Like Growth Factor 2 (IGF-II) 139

Basic Fibroblast Growth Factor (bFGF) 141

Platelet Derived Growth Factor (PDGF) 143

Wortmannin, an Inhibitor of PI 3-kinase 146

5.3 Discussion 149

Insulin like Growth Factors 150
Basic Fibroblast Growth Factor 151
Platelet Derived Growth Factor 151
Common signalling pathways 152
Conclusions 153
Chapter 6
The effect of substances with physiologically antagonistic roles to insulin on insulin's efficacy in reducing fibroblast - myofibroblast differentiation 154
6.1 Introduction 155
Aims: 156
6.2 Results 157
6.2.1 Glucagon 157
In isolation 157
Effect on insulin action 158
6.2.2 Dexamethasone 159
In isolation 159
Effect on insulin action 160
6.2.3 Adrenaline 161
In isolation 161
Effect on insulin action 162
6.2.4 Noradrenaline 163
In isolation 163
Effect on insulin action 164
6.3 Discussion 166
6.3.1 Glucagon 166
6.3.2 Dexamethasone 168
6.3.3 Adrenaline and Noradrenaline 169
6.3.4 The effect of insulin in combination with its physiological antagonists 169
6.4 Conclusions: 170
Chapter 7
The effectiveness of insulin on fibroblast - myofibroblast differentiation during wound healing *in vivo* 171
7.1 Introduction 172

Aims 174
7.2 Results 175
7.2.1 Confirmation of the effectiveness of insulin on murine derived fibroblasts . 175
Immunohistochemistry 175
Western Blotting 178
7.2.2 Incisional wounds 179
7.2.3 Excisional model 186
7.2.4 Time course 187
Immunohistochemistry 189
Western Blotting 190
7.2.5 Tensiometry 192
7.2.6 Alternative methods of administration 193
7.3 Discussion 196
7.3.1 The effect of insulin on murine derived fibroblasts 196
7.3.2 *In vivo* models 197
7.3.3 The effect of insulin on *in vivo* fibroblast - myofibroblast differentiation 198
The effect of insulin of multiple doses of insulin 198
The credibility of controls 199
A time course of myofibroblast events *in vivo* 200
Immunohistochemistry versus western blotting 201
7.3.4 Tensiometry 202
7.3.5 Alternative methods of insulin administration 202
7.4 Conclusions 204
Chapter 8
General Discussion 205
8.1 Proof or refutation of hypotheses 207
8.1.1 Determination of the effect of insulin on fibroblast differentiation *in vitro* .. 207
8.1.2 The effect of insulin on aspects of fibroblast behaviour important during wound healing 208
8.1.3 Do polypeptides that share certain biological properties with insulin affect fibroblast - myofibroblast differentiation? 209
8.1.4 The effect of substances with physiologically antagonistic roles to insulin on insulin's efficacy in reducing fibroblast - myofibroblast differentiation 209

8.1.5 The effectiveness of insulin on fibroblast - myofibroblast differentiation during wound healing *in vivo* 210
8.2 Critique 210
8.2.1 General considerations 211
8.2.2 *In vitro* experiments 212
8.2.3 *In vivo* experiments 213
8.3 Proposals for future studies 214
References 216
Appendices 242
Appendix I Composition of Solutions 243
Appendix II - Suppliers 249

Index of Tables and Figures

Fig 1.1 Diagrammatic representation of the skin (taken from Grays Anatomy (Williams, Bannister, Berry, and Collins,1995) 23

Table 1.1 Growth factors involved in wound healing (Taken from Bennett and Schultz (1993)) 32

Fig 1.2: Langers lines on the lateral aspect of the head and neck. (Image taken from Grays anatomy (Williams, Bannister et al., 1995)) 52

Fig 2.3 Graph showing the relationship between cell density and optical density (measured at 595 nm) following staining of cultures with crystal violet stain. 72

Table 2.1 Composition of dermal fibroblast Pre-incubation and Incubation media for the experiments to determine protein and collagen production. 74

Fig 2.4: Anaesthetic machine showing vaporiser and anaesthetic chamber used for mouse anaesthesia. 79

Table 2.2 Table showing the grading system used for the analysis of alpha smooth muscle actin staining in murine scars (200 x). 86

Fig 3.1 Unstained fibroblasts growing on glass coverslips as viewed using an inverted microscope (200 x). 90

Fig 3.2: Immunohistochemical staining of dermal fibroblasts cultured in GFD medium (**A**) and GFD medium supplemented with insulin (**B**) demonstrating alpha smooth muscle actin. Green stress fibres may be observed in positive cells (myofibroblasts). Propidium Iodide has been used to counterstain nuclei in order to identify all cells present and therefore calculate myofibroblast proportion (200 x). 91

Fig 3.4: Graph showing the percentage change in myofibroblast proportion from that seen in GFD alone for all three dermal fibroblast cell lines tested. Error bars are standard deviations from the mean of 3 different cell lines. 93

Fig 3.5: Graph showing the percentage change in myofibroblast proportion from that seen in GFD alone for all six dermal fibroblast cell lines tested. Error bars are the standard deviations from the mean of 6 different cell lines. 95

Fig 3.6: Graph showing the percentage change in myofibroblast proportion from that seen in GFD alone for all six dermal fibroblast cell lines tested. Error bars are the standard deviations from the mean of 6 different cell lines. 95

Fig 3.7: Graph showing the percentage change in myofibroblast proportion from that seen in GFD alone for all six dermal fibroblast cell lines tested. Error bars are the standard deviations from the mean of 6 different cell lines. 97

Fig 3.8: Representative Western blot showing expression of αSMA in a single dermal fibroblast cell line cultured in either NGM, GFD or GFD supplemented with Insulatard®. Insulatard was added at concentrations of 1 IU/ml, 0.1 IU/ml and 0.01 IU/ml. The amount of protein sample loaded was varied to represent a standardized cell number for each sample ($2x10^5$ cells). 98

Fig 3.9 Mean percentage reduction in αSMA expression in dermal fibroblast cultures initiated from 4 patients cultured in NGM, GFD or GFD supplemented with Actrapid. The graph displays the percentage expression of αSMA as compared to that measured in GFD medium. 99

Fig 3.10 Mean percentage reduction in αSMA expression (compared with that of GFD treated cultures) in dermal fibroblast cultures initiated from 4 patients cultured in NGM, GFD or GFD supplemented with Mixtard. The graph displays the percentage reduction as compared to the expression in GFD medium. 100

Fig 3.11 Mean percentage reduction in αSMA expression (as compared to that seen in GFD treated cultures) in dermal fibroblast cultures initiated from 4 patients cultured in NGM, GFD or GFD supplemented with Insulatard. The graph displays the expression of αSMA as a percentage of that seen in cultures grown in GFD medium. 101

Fig 4.1 Graph showing the change in mean cell number (directly proportional to OD) for 3 dermal fibroblast cell lines cultured in either NGM or GFD media over a 14 day period as assessed using the crystal violet assay. Error bars represent the standard deviation from the mean. 112

Fig 4.2 Three graphs showing the change in mean cell number for 3 dermal fibroblast cell lines cultured over a 14 day period as assessed using the crystal violet assay. Graphs represent results of culture in media supplemented with Insulatard (**A**), Mixtard (**B**) and Actrapid (**C**). Error bars have not been included for reasons of clarity; however, the maximal standard deviation from the mean was no more than 10% of the mean throughout 114

Fig 4.2 (continued) Graphs showing the change in mean cell number for 3 dermal fibroblast cell lines cultured over a 14 day period as assessed using the crystal

violet assay. The results of culture in media supplemented with Actrapid (**C**) is shown. Error bars have not been included for reasons of clarity; however, the maximal standard deviation from the mean was no more than 10% of the mean throughout 115

Fig 4.3 Graph showing the amount of secreted soluble protein produced per 10000 dermal fibroblasts. Cells were cultured in either NGM, GFD or GFD supplemented with 1 IU/ml Insulatard. Mean protein production for 3 cell lines is displayed with error bars showing standard deviations. 116

Fig 4.4 Graph showing amount of collagen production per 10000 dermal fibroblasts. Cells were cultured in either NGM, GFD or GFD supplemented with 1 IU/ml Insulatard®. The mean collagen production for 3 cell lines is displayed, error bars represent the standard deviation from the mean. 118

Fig 4.5 Graph showing the amount of collagen production by 3 dermal fibroblast cell lines when cultured in GFD or GFD supplemented with insulin, expressed as a percentage of the amount of collagen produced when cultured in NGM. Error bars represent the standard deviations from the mean. 119

Fig 4.6 Graph showing the mean and standard deviations for the ratio of collagen / protein production for 3 dermal fibroblast cell lines. Fibroblasts were cultured in either NGM, GFD or GFD supplemented with 1 IU/ml Insulatard. Error bars represent standard deviations from the mean. 120

Fig 4.7a Representative dermal fibroblast cell line seeded in collagen gels, visualised after 72 hours. All wells were pre coated with 2% BSA. Media added to each well was NGM (N), GFD (G), Insulatard 1 IU/ml in GFD (IG) or Serum Free Medium (SFM). 121

Fig 4.7b Graph showing the mean contraction of 3D collagen gels for 3 dermal fibroblast cell lines over a 72 hour period expressed as a percentage of the surface area of those gels cultured in SFM. Gels were cultured in SFM, NGM, GFD or GFD supplemented with Insulin (Insulatard 1 IU/ml). 121

Fig 5.1 Graph showing the mean and standard deviation for apoptosis in 3 dermal fibroblast cell lines. Apoptosis was determined using the Apopercentage Apoptosis assay (Biocolor). Increasing OD correlates with increasing numbers of apoptosing cells. 136

Fig 5.3 Graph showing mean and standard deviation for the percentage reduction in myofibroblast numbers (when compared to GFD). Dermal fibroblast cultures were maintained for 14 days in NGM, GFD or GFD supplemented with either 10 ng/ml or 100 ng/ml IGF-I, or insulin 1 IU/ml. 138
Fig 5.4 Graph showing the change in proliferation rate when dermal fibroblasts are cultured in medium containing IGF-II. Control medium was minimal % of FCS (0.4%) to maintain the cells in a quiescent state but not induce apoptosis (personal communication C.Linge, RAFT). The graph represents data from 6 replicates of 6 cell lines, expressed as the percentage increase in OD (650 nm, crystal violet assay) from the negative control (0.4% FCS only) 140
Fig 5.5 Graph showing mean and standard deviation for the percentage reduction in myofibroblast numbers (when compared to GFD). Dermal fibroblast cultures were maintained for 14 days in NGM, GFD or GFD supplemented with either 10 ng/ml or 100 ng/ml IGF-II or insulin 1 IU/ml. 140
Fig 5.6 Graph showing the change in proliferation rate when dermal fibroblasts are cultured in medium containing bFGF. Control medium was minimal % of FCS (0.4%) so as to maintain the cells in a quiescent state but not induce apoptosis (personal communication C.Linge, RAFT). The graph represents data from 6 replicates of 6 cell lines, expressed as the percentage increase in OD (650 nm, crystal violet assay) from the negative control (0.4% FCS only) 142
Fig 5.7 Graph showing mean and standard deviation for the percentage reduction in myofibroblast numbers (when compared to GFD). Dermal fibroblast cultures were maintained for 14 days in NGM, GFD or GFD supplemented with 20 ng/ml bFGF or insulin 1 IU/ml. 142
Fig 5.8 Graph showing the change in proliferation rate when dermal fibroblasts are cultured in medium containing PDGF. Control medium was minimal % of FCS (0.4%) so as to maintain the cells in a quiescent state but not induce apoptosis (personal communication C.Linge, RAFT). The graph represents data from 6 replicates of 6 cell lines, expressed as the percentage increase in OD (650 nm, crystal violet assay) from the negative control (0.4% FCS only) 145
Fig 5.9 Graph showing mean and standard deviation for the percentage reduction in myofibroblast numbers (when compared to GFD). Dermal fibroblast cultures were

maintained for 14 days in either NGM, GFD or GFD supplemented with either 10 ng/ml or 100 ng/ml PDGF or insulin 1 IU/ml. 145
Fig 5.10 Graph illustrating the mean percentage change in myofibroblast number (and standard deviation) for 3 dermal fibroblast cell lines, from that seen when cultured in GFD alone, with addition of wortmannin to GFD medium. 148
Fig 6.1 Graph showing the mean percentage reduction (and standard deviation) in myofibroblast number for 3 dermal fibroblast cell lines as compared to that seen in GFD medium. Fibroblasts were cultured in GFD or GFD supplemented with glucagon at 10μg/ml or 1μg/ml. 158
Fig 6.2 Graph showing the mean percentage reduction (and standard deviation) in myofibroblast number for 3 dermal fibroblast cell lines as compared to that seen in GFD medium. Fibroblasts were cultured in GFD or GFD supplemented with glucagon at 10μg/ml and 1μg/ml both with the addition of Insulatard at 1 IU/ml159
Fig 6.3 Graph showing the mean percentage reduction (and standard deviation) in myofibroblast number for 3 dermal fibroblast cell lines as compared to that observed in GFD medium. Dermal fibroblasts were cultured in GFD or GFD supplemented with dexamethasone at 100 ng/ml and 10 ng/ml. 160
Fig 6.4 Graph showing mean percentage reduction (and standard deviation) in myofibroblast number for 3 dermal fibroblast cell lines. Fibroblasts were cultured in GFD, GFD supplemented with adrenaline at 10, 1, 0.1 ng/ml and in GFD supplemented with 1 IU/ml Insulatard. 162
Fig 6.5 Graph showing mean percentage reduction (and standard deviation) in myofibroblast number for 3 dermal fibroblast cell lines. Fibroblasts were cultured in GFD or GFD supplemented with adrenaline at 1 ng/ml and 0.1 ng/ml with the addition of insulin (Insulatard 1 IU/ml). 163
Fig 6.6 Graph showing mean percentage reduction (and standard deviation) in myofibroblast number for 3 dermal fibroblast cell lines. Fibroblasts were cultured in GFD or GFD supplemented with noradrenaline at 10 ng/ml and 1 ng/ml. 164
Fig 6.7 Graph showing mean percentage reduction (and standard deviation) in myofibroblast number for 3 dermal fibroblast cell lines. Fibroblasts were cultured in GFD, GFD supplemented with insulin alone (Insulatard 1 IU/ml) or GFD supplemented with both insulin (Insulatard 1 IU/ml) and noradrenaline at either 10 ng/ml or 1 ng/ml. 165

Fig 7.1 Photographs showing murine fibroblasts in culture. Fibroblasts have been stained immunohistochemically to demonstrate αSMA, and are hence myofibroblasts (Green). A. Murine fibroblasts cultured with NGM, B. Murine fibroblasts cultured with GFD, C. Murine fibroblasts cultured with 1 IU/ml Insulatard (200 x). 176

Fig 7.2 Graph showing, for murine dermal fibroblast cultures, the mean percentage reduction in the number of myofibroblasts present in various media (as compared to those of dermal fibroblasts cultured in GFD medium). Dermal fibroblasts from 3 mice were cultured in either NGM, GFD or GFD supplemented with 1 IU/ml insulin (either Laboratory Grade insulin (Sigma), Actrapid, Mixtard 30 or Insulatard) for 14 days. Error bars represent the standard deviation..... 177

Fig 7.3 Two Western blots showing expression of alpha smooth muscle actin in murine dermal fibroblasts cultured in NGM, GFD or GFD supplemented with insulin (Insulatard, Mixtard or Actrapid). Optical density readings are given for each well. Each lane was loaded with adequate protein equivalent to equal cell number in each well..... 178

Table 7.1 Table showing treatment groups for the investigation of the effect of insulin on incisional wound healing. The figures in parentheses represent the days on which substances were administered to murine wounds..... 179

Fig 7.5 Diagram showing the immunohistochemical method used to detect the presence of αSMA in murine tissue sections..... 182

Fig 7.6 Graph showing mean "myofibroblast score" and standard deviation for each treatment applied to a murine incisional wound healing model. Scores were derived from the analysis of 8 wounds per treatment group with each scar examined at 3 random points along its length..... 184

Fig 7.7 Immunohistochemical staining of murine incisional wound scars to demonstrate the expression of αSMA. Presence of myofibroblasts (αSMA) identified by red stain. Scar having received (A) single administration of saline (control), (B) single dose administration of insulin (0.15 IU) (C) two doses of insulin (D) four doses of insulin (200 x). 185

Table 7.2 Table showing treatment groups for the investigation of the effect of insulin on excisional wound healing. The figures in parentheses represent the days on which substances were administered to murine wounds..... 186

Table 7.3: Table showing number of mice culled at each time point during the 28 day incisional wound healing time course. 189
Fig 7.8 Graph showing mean "myofibroblast score" and standard error over a 28 day time course for each treatment applied to a murine incisional wound healing model. Scores were derived from the analysis of 4 wounds per treatment group with each scar examined at 3 random points along its length. 190
Table 7.4: Table showing number of mice culled at each time point during the 28 day incisional wound healing time course. 191
Fig 7.9 Graph showing mean (and standard error) expression of alpha smooth muscle actin in single dose insulin treated vs. single dose saline treated full thickness incisional wounds. All treatments were applied at the time of injury and scars were harvested at days 7, 14, 21 and 28. The expression of αSMA was determined by western blotting. 192
Fig 7.10 Graph showing mean and standard deviation for the stress (in MPa) required to disrupt murine incisional scars. Scars had previously been treated with a single dose or multiple dose administrations of insulin or saline (control). 193
Fig 7.11 Graph showing mean "myofibroblast score" and standard deviation for each treatment when applied to a murine incisional wound healing model. Insulin (0.15 IU Insulatard) was applied either as a single application systemically, topically (onto the wound), as a local infiltration into the wound edges or as a multiple application applied systemically. Scores were derived from the analysis of 8 wounds per treatment group with each scar examined at 3 random points along its length. 195

List of Abbreviations

αSMA	Alpha Smooth Muscle Actin
AP	Alkaline Phosphatase
APES	Aminopropyltriethoxy-silane
bFGF	Basic Fibroblast Growth Factor
Da	Dalton
DABCO	14-Diazo dicyclo 2,2,2 octane
DF	Dermal Fibroblast
dH_2O	Distilled Water
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Di-methyl Sulfoxide
EGF	Epidermal Growth Factor
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
GAG	Glycosaminoglycan
GF	Growth Factor
GFD	Growth Factor Dialysed
GFDI	Growth Factor Dialysed medium + insulin
H&E	Haematoxylin and Eosin
IGF-I	Insulin-like Growth Factor I
IGF-II	Insulin-like Growth Factor II
IU	International Units
MOM block	"Mouse on Mouse" Block
MMP	Matrix metallo-proteinase
NGM	Normal Growth Medium
OD	Optical Density
P0	Cells freshly isolated from skin
P1	Cells cultured for one passage
PBS	Phosphate Buffered Saline
PDE	Phosphodiesterase

PDGF	Platelet Derived Growth Factor
PG	Proteoglycan
PI 3-kinase	Phosphatidylinositol 3-kinase
RSTL	Relaxed Skin Tension Lines
SMA	Smooth Muscle Actin
T:V	Trypsin:Versene
TBS	Tris Buffered Saline
TGFβ	Transforming Growth Factor beta
TTBS	Tween-Tris Buffered Saline
U	Unit
UV	Ultra Violet

Chapter 1

Introduction

1.1 Introduction

Cutaneous injury to man frequently results in a defect of the integument that requires repair. In an attempt to repair such damage, the processes of wound healing are initiated. Unfortunately, these reparative processes are not without fault and in man result not in perfect repair but in cutaneous scarring.

Scarring occurs because of the reparative rather than regenerative nature of wound healing (Anderson, 1988). Instead of regenerating the damaged architecture of the skin, wound healing results in the deposition of replacement tissue bulk. This newly formed tissue frequently causes problems and may be unsightly, leading patients to seek help in improving the appearance of their scars. In addition to cosmetic concerns, many scars may be the cause of discomfort, with symptoms including hyper-aesthesia and pruritis (Alster & West, 1997). Scars that occur over joints may cause further problems by limiting movement and impeding everyday activities.

Due to the problems that scarring can cause, there is much interest in the development of a therapy that may be able to prevent or reduce cutaneous scarring. Scarless healing is the ultimate goal, and is known to be possible since it occurs in the human foetus up to the middle of the second trimester of pregnancy (Longaker & Adzick, 1991). It is still largely unknown precisely which differences exist between foetal and adult wound healing that result in perfect regeneration in the former and "imperfect" scarring in the latter (Samuels & Tan, 1999). It has thus been necessary to return to the basic biology behind the wound healing response in order to develop novel therapies to combat cutaneous scarring.

The work contained within this thesis explores a novel mechanism by which scarring may be reduced or prevented. However, before discussion of the current strategies available to reduce or prevent cutaneous scarring, it is necessary to understand the structure of the skin and the many processes involved in wound healing.

1.2 Anatomy of skin

The skin is the largest organ of the human body, exceeding $2m^2$ on an average human and accounting for almost one sixth of the total body weight (Hunter, Savin, and Dahl, 1989). It comprises of a sheet-like investment of the entire body that adapts to its contours and conforms to the movements of the organism within. It acts as a physical barrier to prevent desiccation from evaporation, is able to withstand mechanical, chemical and microbial insults and has important thermoregulatory and sensory roles (Quarmby, West et al., 2002).

Skin is composed of two distinct layers that are strongly attached to each other, a superficial epidermis and a deeper dermis (See Fig 1.1). The following account of skin anatomy is based upon Ebling and co-authors unless otherwise stated (Ebling, Eady et al., 1992):

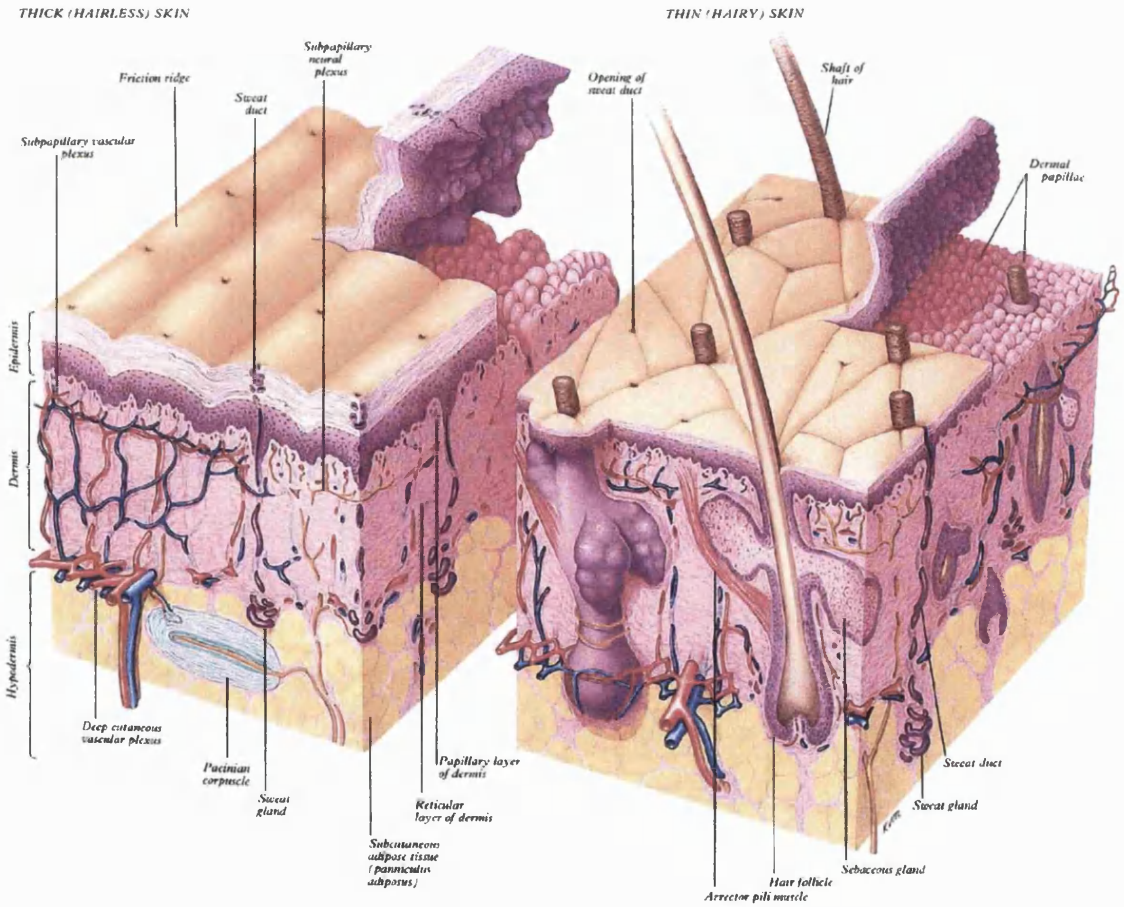


Fig 1.1 Diagrammatic representation of the skin (taken from Grays Anatomy (Williams, Bannister, Berry, and Collins, 1995)

1.2.1 The epidermis

The epidermis is composed mainly of keratinocytes (so called because they produce proteins known as keratins). It may be sub-divided into 4 layers based on the microscopic appearance of the keratinocytes:

i) Stratum germinativum or basal layer is a single germinative layer of cells, where the keratinocytes are cuboidal in shape, which lies on the basal lamina (a thin sheet of extracellular matrix by which the epidermis attaches to the dermis)
ii) Stratum spinosum or spinous layer containing several layers of polyhedral cells held tightly together via the intracellular connections, desmosomes – giving them their characteristic "spined" appearance
iii) Stratum granulosum or granular layer containing flattened, nucleated cells with distinctive cytoplasmic keratohyalin granules
iv) Stratum corneum or cornified layer containing thin, flat, anucleate cells that are the end point of differentiation

The outer keratinised layer (stratum corneum) is continuously shed and replaced by progressive movement and maturation of cells from the innermost layers, the stratum germinativum. In order to maintain skin thickness, the rate of cell shedding is equivalent to the rate of cell division in the underlying layers.

1.2.2 The dermis

The dermis is the deeper of the 2 layers of skin and is a fibro-elastic tissue derived from the embryological mesoderm. It is composed of interwoven collagen fibres, mostly Collagen Type I and III, and accounts for the tensile strength of skin. The majority of dermal collagen is type I collagen, with approximately 15% being type III (Cormack, 1987). In addition, an array of elastic fibres helps to provide skin with its deformable properties. The dermis is subdivided into an upper papillary dermis, in contact with the epidermis, and a deeper reticular dermis. The defining boundary between the two dermal layers is the horizontal sub-papillary plexus of arterioles and post-capillary

venules. The collagen and elastic fibres within the papillary dermis are finer and sparser than those in the reticular dermis.

Interspersed between the fibro-elastic fibres of the dermis is an amorphous ground substance composed of polysaccharides, known as glycosaminoglycans (GAGs), various salts and water. The major GAGs in dermis are hyaluronic acid and dermatan sulphate. In addition, there are lesser amounts of chondroitin-6-sulphate, chondroitin-4-sulphate and heparin sulphate. GAGs are often found covalently attached to proteins, thus forming proteoglycan molecules that are referred to as matrix proteins. In addition, many skin appendage organs, such as hair follicles, sweat glands and sebaceous glands are situated within the dermal layers and sometimes extend into the underlying subcutaneous fat. The principle cell type found within the dermis is the fibroblast. Although this cell type is abundant and found in connective tissues throughout the body, it seems that not all populations of fibroblasts are homogeneous (Sappino et al., 1990; Schmitt-Graff et al., 1994), with some populations expressing features of smooth muscle differentiation. Such smooth muscle-like fibroblasts have been termed myofibroblasts (Section 1.5). The dermis also contains rich vascular, lymphatic and nerve networks (Foster, Alberti et al., 1982).

1.3 Wound healing

The term "wound healing" refers to the collection of processes initiated following injury that result in the eventual repair of damage to the integument. Normal repair process is dependent upon protein synthesis, matrix deposition, cellular migration and replication (Galliano, Zhao et al., 1996), traditionally it has been subdivided as occurring in three phases (Peled, Chin et al., 2000) although temporally these overlap:

i) "inflammatory" (or lag)
ii) "proliferative"
iii) "remodelling"

Regulation of wound healing requires the co-ordination of a number of cellular activities via a multifactorial control system, relying on the actions of growth factors

and interactions with the extracellular matrix (Robertson, Belford et al., 1999). The following description of wound healing is taken in part from Peled et al. (2000), unless otherwise indicated. Whilst this description is primarily concerned with cutaneous wound healing, the processes involved are similar throughout all tissues in the body.

1.3.1 Inflammatory (lag) phase

Tissue injury results in the disruption of blood vessels and initiation of the coagulation cascade, culminating in the formation of a clot over the wound. This re-establishes haemostasis and provides a provisional Extra-Cellular Matrix (ECM) for cellular migration. During coagulation, platelets release the contents of their alpha granules, discharging many growth factors into the wound e.g. Platelet Derived Growth Factor (PDGF), Insulin Like Growth Factor-I (IGF-I), Epidermal Growth Factor (EGF) and Transforming Growth Factor-beta (TGFβ) (Bennett & Schultz, 1993). These factors initiate the wound healing response proper, by attracting and activating fibroblasts, endothelial cells and macrophages. The coagulation process is therefore vital to wound healing, both releasing important mediators that co-ordinate the remainder of the healing process and forming the fibrin scaffold which is important for the migration of cells responsible for the repair of the tissue defect.

This phase of wound healing is also characterised by the inflammatory response which is initiated through activation of the complement cascade, and may be loosely divided into early and late periods (Kössi, Peltonen et al., 2001). In the early phase, the predominant cell types present are leukocytes and macrophages, whereas during the late phase, fibroblasts tend to be more numerous.

The first inflammatory cells to respond to this signal are the granulocytes, which infiltrate the wound within 24-48 hours of injury. These cells are attracted by the variety of agents present within the wound, complement components (principally C5a), bacterial products and TGFβ. Shortly after injury, granulocytes adhere to the endothelial cells in blood vessels adjacent to the site of injury (margination) and then actively move through the walls of the blood vessels (diapedesis). After leaving the circulation, the granulocytes migrate through tissue, via chemotaxis. Once within the wound,

granulocytes are responsible for the removal of damaged and foreign material, helping to reduce the incidence of infection. Despite this seemingly important function, wound healing can continue even in the absence of granulocytes (Aston et al. 1997).

After 48-72 hours, the macrophage phenotype can be detected within the healing wound. In addition to having a phagocytic role, macrophages produce many of the growth factors that are responsible for the control of wound healing (Winston, Krein et al., 1999). These processes include the production and proliferation of the extra-cellular matrix by fibroblasts, proliferation of smooth muscle cells and proliferation of endothelial cells (important for later angiogenesis). After 72 hours, another population of white blood cells, the lymphocytes, can be identified within the healing wound. Lymphocytes are thought to be pivotal in both collagen and ECM remodelling, but to date conclusive evidence supporting this role during wound healing is not available (Greenfield, 1993).

Following the conclusion of the inflammatory phase of healing, the wound is populated principally by white blood cells and fibroblasts. A temporary scaffold comprised of fibrin has been erected which permits migration of many other cells responsible for healing into the wound.

1.3.2 Proliferative phase

During the proliferative phase of wound healing, mesenchymal cells proliferate, angiogenesis commences and re-epithelialization occurs. Growth factors (previously produced principally by the inflammatory cells) are produced by fibroblasts, keratinocytes and endothelial cells (Bennett & Schultz, 1993) and coordinate much of the cellular activity of this phase of healing. Successful healing requires the migration of fibroblasts into the wound and the subsequent production of ECM. Fibroblast migration occurs relatively early within wound healing such that by day 7, fibroblasts are the predominant cell type present. The fibroblasts migrate into the wound by organising polymerised actin into a filamentous network at the cell's leading edge (Ehrlich, Keefer et al., 1999), a process known as cell "crawling". Once in the wound, fibroblasts commence the synthesis of ECM, replacing the provisional matrix with a

collagenous matrix. In addition to proliferation, fibroblasts differentiate into what is known as the "myofibroblast" phenotype approximately 3 days after injury. The myofibroblast is thought to be important in the production of scar tissue and wound contraction and as such is described in detail later in this thesis (Section 1.5). Wound contraction begins approximately 4 to 5 days post-injury.

The role of wound contraction is questionable in man when compared to wound healing in other animals. Indeed, in the healing of neatly opposed surgical wounds this process is thought to be of little significance. However, in mammals with skin that is loosely attached to the underlying tissue, wound contracture plays a significant role, resulting in rapid closure and minimal scarring with little or no loss of function. In man, where the skin is more firmly attached to underlying tissues, the benefits of wound contraction are less. This results in a broad spectrum of scar severity, ranging from minimal cosmetic abnormality to loss of limb motion or even major deformity (Grinnell, 1994).

Initially, myofibroblasts were thought to be the sole mediators of wound contraction, causing the wound to contract by 0.6 to 0.75 mm per day in man (Koopmann, 1995). However, other cells have been shown to cause contraction *in vitro*, for example keratinocytes (Chakrabarty, Heaton et al., 2001), resulting in the theory that wound contraction is probably due to interplay between the different cell types.

Even in animal models where cellular contraction is thought to play a minor role, e.g. sutured incisional wounds, the amount of scar deposited during healing is limited because normal skin is "pulled" into the defect. This "pull" is achieved through the continued reorganization of collagen fibres in granulation tissue (Ehrlich et al., 1999) rather than through cellular contraction.

The epidermis serves as a barrier between the internal and external environments, and as such, it is important that when damaged, it is repaired quickly and efficiently. Although closure of the wound in most animals is largely achieved through contraction, some migration and proliferation of epidermal keratinocytes is still required to completely heal the breach. These latter processes take on a particularly important role in cutaneous healing in humans. In full thickness wounds, mitosis of epidermal cells begins 48 hours

following injury, with the epithelium then advancing across the defect. Several theories have been proposed to explain the mechanism of keratinocyte migration, including the "leap frog" model (Krawczyk, 1971) in which cells above and behind the leading cell stream over the latter to attach to the wound bed. Alternative theories include the "chain" model whereby chains of cells advance over the wound (Daly, 1990) (in Kloth LC Wound healing: Alternatives in management). Keratinocyte migration is increased if the wound does not require debridement, if the wound is kept moist and if the basal lamina remains intact. In addition, re-epithelialization may be accelerated by many growth factors known to promote epidermal growth and chemotaxis, e.g. EGF (Nanney, Paulsen et al., 2000), Fibroblast Growth Factor (Pandit, Ashar et al., 1998) and Keratinocyte Growth Factor (Xia, Zhao et al., 1999).

In addition to cellular migration and proliferation, the production of collagen commences approximately 3-5 days after injury with the rate of production increasing rapidly and remaining accelerated for 2-4 weeks. Collagens account for approximately 70-80% of the dry weight of the dermis and are the most abundant family of proteins in man. Initially, during healing, collagen type III is produced to reconstruct the extracellular matrix, but as the wound matures, this is replaced by collagen type I. Collagens can be considered to be key in wound healing, seemingly having a role at every point in the healing process. During the initial phases of injury, exposed collagens promote platelet aggregation and activation of chemotactic factors important in co-ordinating the cellular processes. Later, during the proliferative phase of wound healing, migrating epidermal cells degrade collagen using collagenases in order to separate the desiccated eschar from viable tissue (Singer & Clark, 1999). The collagen scaffold is also necessary for wound contraction and important during angiogenesis (Singer & Clark, 1999).

Other matrix molecules deposited early in wound healing are the fibronectins. These molecules are involved in wound contraction, cell-cell and cell-matrix interactions, cell migration and epithelialization. In addition, they act as a scaffold for collagen deposition. The importance of fibronectin in wound healing is thought to be demonstrated by the mortality of fibronectin knockout mice. Such mice have deficient mesoderm formation, possibly indicating the role of fibronectin in the creation of a

"scaffold" for emigrating cells, a function that is of some importance in wound healing as well as embryogenesis (Hochscheid, Jaques et al., 2000). During wound healing, fibronectins are able to attach to the fibrin clot (the result of coagulation) and facilitate fibroblast attachment. The quantity of fibronectin fibres increases as the wound matures and type I collagen replaces type III.

An additional important component of the wound matrix is the ground substance, composed of proteoglycans (PGs) and glycosaminoglycans (GAGs). The role of PGs within wound healing is currently poorly understood. However, they seem to create a charged, hydrated environment that facilitates cell mobility. They are also vital in restoring the visco-elastic properties of the repaired tissue

1.3.3 Remodelling

Approximately 21 days following injury in man, the remodelling phase is entered (defined as the point at which the rates of collagen synthesis and breakdown equilibrate). This phase of wound healing is characterised by a reduction in the number of cells present (fibroblasts, myofibroblasts and macrophages) and also in vascularity. The decrease in cellularity during this stage of healing has been demonstrated to be due to apoptosis (Desmouliere, Redard et al., 1995). Prior to the reduction in cell number within the wound, collagen is degraded by several proteolytic enzymes that are secreted by macrophages, epidermal cells, endothelial cells and fibroblasts. Over the first 3 weeks of wound repair, only 20% of the final strength of repair is gained, with the remainder accumulating with continued wound remodelling (Singer, Quinn et al., 2002). The newly synthesised collagen I that replaces the earlier deposited collagen III forms multiple intra- and inter-molecular bonds with other collagen fibres, contributing to the increase in tensile wound strength seen during this phase. Despite extensive remodelling and collagen deposition, wounds will only reach approximately 70% of the strength of normal skin following completion of repair.

1.3.4 Control of Wound Healing

Much of the coordination of the healing process is undertaken by a subclass of cytokines termed "growth factors" (Greenhalgh, 1996). Typically, these molecules are proteins, and generally weigh between 4,000 and 60,000 Daltons. They are able to impart a particular biochemical message to a specific target cell through binding to a specific membrane receptor even when present in minute concentrations.

Despite being termed "growth factors", these molecules are capable of affecting many aspects of cellular behaviour. All growth factors can stimulate mitosis in one or more of the cell types involved in wound healing, but do exhibit some degree of selectivity. Growth factors are also capable of attracting (via chemotaxis) inflammatory cells and fibroblasts into the wound. They are important for the stimulation of angiogenesis, the production and degradation of the extracellular matrix and may even influence the synthesis of cytokines and other growth factors by cells either in or adjacent to the wound (Aston et al., 1997).

Many of the growth factors of importance during wound healing operate in either an autocrine or paracrine manner. The better characterized growth factors involved in the healing process include EGF, PDGF, acidic and basic FGF, IGF-I, IGF-II, TGFβ, TGFα, IL-1, and TNFα (Greenhalgh, 1996). These are summarized in Table 1.1.

Investigation of wound healing in "knockout" mice, developed to lack a specific growth factor, have illustrated a redundancy in the actions of each growth factor. Wound healing proceeds as normal in many knockout mice (e.g. EGF (Luetteke, Qiu et al., 1999), KGF (Guo, Degenstein et al., 1996)) demonstrating that normal tissue repair can proceed even in the absence of a single cytokine. However, although genetic manipulation of laboratory animals can be useful, frequent embryonic lethality has restricted many *in vivo* wound healing studies (Scheid, Meuli et al., 2000).

Growth factor	MW (kDa)	Structure	Found in	Biologic effect	Family
Amphiregulin	9.8	Monomer	Breast cancer cells, keratinocytes(?)	Mitogenic for some cell lines, inhibits others	
EGF	6	Monomer	Almost all body fluid, platelets	Mitogenic for most epithelial tissues, fibroblasts, endothelial cells	The EGF family includes EGF, TGF-α, AR, VEGF and HBEGF
FGF (acidic and basic)	16-18	Monomer	Fibroblasts, astrocytes, endothelial cells, bone cells, smooth muscle	Mesenchymal and neural tissue mitogen	The FGF family includes aFGF, bFGF, KGF, hst/KS3, int-2, FGF-5, FGF-6. only the first 3 have been extensively studied
Heparin-binding EGF	22	Monomer	Macrophages	Mitogenic for keratinocytes, fibroblasts	
IGF-I	7.5	Monomer	Most tissues; fibroblasts, macrophages	Mitogenic for fibroblasts, bone cells, neural tissues, haematopoietic cells, endothelial cells	The IGF family includes IGF-I and IGF-II. Both have similar structures and effects. IGF-I is representative of the group
IGF-II		Monomer	Most tissues; fibroblasts, macrophages	Mitogenic for fibroblasts	
Insulin	6	Monomer	β cells islets of Langerhans of the pancreas	Mitogenic for many cell types. Major hormone of glucose homeostasis	Insulin family
KGF	28	Monomer	Fibroblasts	Mitogenic for epithelial cells, but not fibroblasts or endothelial cells	A member of the FGF family
PDGF	28-35	Dimer, A and B chains	Endothelial cells, platelets, macrophages, fibroblasts	Mitogenic for vascular smooth muscle, fibroblasts	The PDGF family consists of PDGF and VEGF
TGFβ	25	Dimer of 2 identical chains	Macrophages, lymphocytes, fibroblasts, bone cells, keratinocytes, platelets	Inhibits replication of most cells *in vitro*, including keratinocytes, endothelial cells, lymphocytes and macrophages; may inhibit or stimulate fibroblasts	TGFβ family is comprised of TGFβ-1-5. Only TGFβ-1-3 are found in mammalian cells, and have similar effects. TGFβ1 is representative of the group
TGFα	5-20	Monomer	Macrophages, eosinophils, keratinocytes	Similar to EGF, but more potent angiogenesis factor	
VEGF	45	Dimer of 2 identical chains	Pituitary cells	Mitogenic for endothelial cells, but not keratinocytes, smooth muscle or fibroblasts	

Table 1.1 Growth factors involved in wound healing (Taken from Bennett and Schultz (1993))

1.4 Scarring

Following completion of wound healing, the continuity of the integument is re-established. Due to the reparative rather than regenerative nature of this repair, obvious differences exist between the newly formed tissue (the "scar") and the original tissue.

Unfortunately, wound healing is not a homogeneous process, and differences exist between individuals in both the healing responses, and resulting scars. In fact, although the processes occurring during healing seem identical in all wounds, significant differences can exist in the outcome of healing even between different sites in the same organism and in different portions of the same wound (Rockwell, Cohen et al., 1988). The resulting scar can be considered to be the culmination of a series of cellular and molecular processes, some of which act to promote scar formation and others that act to promote scar resolution (Marcus, Tyrone et al., 2000). Imbalances between these processes result in abnormalities of healing, which then may be considered either inadequate (Section 1.4.1) or excessive (Section 1.4.2).

1.4.1 Inadequate wound healing

Failed or delayed wound healing is commonly encountered and thought to be due to a number of problems. To date, some of the factors known to result in this form of wound healing include the nutritional status of the patient and the presence of certain vitamin deficiencies, notably that of vitamins A and C (Kirk, Mansfield et al., 1996). Local factors such as the presence of infection (overt or sub clinical) and hypoxia can also impede wound healing and thus strenuous efforts are made to prevent these with careful clinical management (Linares, 1996). Many therapeutic interventions, such as steroid therapy and radiotherapy have also been shown to be detrimental.

The age of the patient seems to have a bearing upon the rate or outcome of healing, with wounds in the elderly frequently noted to heal more slowly or even inadequately (Marcus et al., 2000). *In vivo* models have corroborated these findings, demonstrating a significant delay in epithelialization in aged animals. However, whereas delayed epithelialization results in pronounced scarring in the younger animal, in the elderly, scar formation is diminished once healing is complete (Marcus et al., 2000).

Probably the most commonly encountered cause for impaired wound healing in clinical practice is in the diabetic patient. Although diabetes causes a number of abnormalities that may lead to impaired wound healing, such as micro and macro-angiopathies (Chiarelli, Santilli et al., 2000), studies on animal wounds have shown that the lack of insulin may itself impair wound healing (Greenfield, 1993).

1.4.2 Excessive wound healing

Wound healing that results in the over-formation of tissue is often termed "excessive wound healing". Such resulting scars may be sub-divided into hypertrophic and keloid scars. These two abnormal forms of excessive wound healing occur in up to 15% of all wounds (Peled, et al., 2000) and are 15 times more common in non-Caucasians (Aston et al., 1997). The distinction between these two forms of excessive wound healing is sometimes difficult. Fundamentally, hypertrophic scars are those that heal excessively but remain within the original confines of the original wound, where any increase in size has to be achieved by pushing out its margins and not by "invasion" of the surrounding tissue (Anderson, 1988). However, whenever the healed wound contains excess connective tissue that extends beyond the original wound, it is termed a keloid. Some differences have been noted in the development of these scars, with hypertrophic scars generally forming in the initial weeks after injury whereas keloid scars may develop much later following cessation of healing (Datubo-Brown, 1990).

Despite these differences, both of these extreme-scarring conditions share the common characteristics of excessive deposition of extracellular matrix components and hypercellularity. Nevertheless, the precise causes of abnormal scarring are still unknown with multiple hypotheses still valid.

Although it is not within the remit of this thesis to discuss the relative merits of these hypotheses in detail, they can generally be categorized into three mechanisms as follows:

1) Exogenous Factors:

where external factors such as foreign matter influences the normal wound healing process – extending it and thus resulting in excessive scarring. This is typically thought to be the result of infection, either bacterial or viral.

2) Abnormal extracellular signalling:

where the signals involved in normal wound healing have gone "awry" or become abnormal for some reason. This mechanism has been suggested by McCauley et al. (McCauley, Chopra et al., 1992) who found increased levels of TNF-α, IL-6 and IFN-β during wound healing with subsequent keloid scarring.

3) Abnormal cellular response:

where the cellular responses to normal wound healing signals are abnormal. This mechanism is suggested by the distinctly different behaviour of fibroblasts obtained from pathological scar as compared to those obtained from normal skin and normal scar (Garner, Karmiol et al., 1993).

It is important to realize that none of these hypotheses are mutually exclusive.

Although the precise cause of abnormal scarring is still unknown, histological analyses of many pathological scars has revealed, in addition to hypercellularity, the persistence of the myofibroblast phenotype (Desmouliere, 1995). The significance of this cell will be discussed in Section 1.5, but its prevalence in pathological scarring has lead many workers to consider its prevention (or reduction) the key to the development of novel anti-scarring therapies. Diminution of the numbers of myofibroblasts during (and following) wound healing has thus become the goal.

Keloid scars

Keloid scars were first described in the Smith papyrus (thought to originate from 13-1700 BC) although Alibert proposed the current name in 1802, as reviewed in Rockwell et al. (Rockwell, Cohen, & Ehrlich, 1988). Despite improvements in our understanding of wound healing, the precise aetiology of both hypertrophic and keloid scars remain unknown (Linares, 1996). Keloids are known to occur more frequently in certain races.

More deeply pigmented people are thought to have a greater risk of keloid formation, with the tendency lowest in Caucasians (Aston et al., 1997). In addition to race, certain anatomical sites are more prone to developing keloid scars e.g. deltoid, pre-sternal, upper back and ear lobe. Certain areas, for example the genitalia, eyelids, palms and soles seem not to form these types of scars (Chipev, Simman et al., 2000). One of the many hypotheses proposed to explain the occurrence of keloids suggests that they occur secondary to increased skin tension within the scar (Rockwell, Cohen, & Ehrlich, 1988). However, the high incidence of keloid scars on the ear lobe might suggest otherwise.

Hypertrophic scars

Both keloid and hypertrophic scars differ from normal skin and normal scar by the presence of a rich vascular network and a high number of mesenchymal cells. The incidence of hypertrophic scars varies from 29-68% after surgery and, following burns (depending upon the depth of the injury) between 33 and 91% (Peled et al., 2000). Hypertrophic scars are characterized by overexhuberant ECM deposition with collagen fibrils organised in swirls and abundant mucinous ground substance. Histologically, hypertrophic scars are difficult to distinguish from keloids, with distinction between the two frequently only possible using the electron microscope (Rockwell, Cohen, & Ehrlich, 1988).

1.5 The Fibroblast and Myofibroblast

The fibroblast is the major cell type found within the dermis and thought to play an important role in wound healing and scarring (both normal and abnormal). Despite its abundance within the body, until recently it was thought that all fibroblasts behaved the same, irrespective of anatomic location. However, Komuro described different sub-populations with specialized functions in 1990 (Komuro, 1990) and phenotypic heterogeneity amongst this cell type is now accepted. Histologically, such diversity can be demonstrated by the use of specific cytoskeletal markers and by their production of specific types of collagen (Sappino et al., 1990).

Further evidence for fibroblast phenotype diversity was suggested by morphological differences *in vitro* and *in vivo*. *In vivo*, fibroblasts seldom establish contact and have

few microfilaments (Sappino et al., 1990). In contrast, cultured fibroblasts have a flattened, polarized shape with numerous stress fibres and gap junctions. Fibroblast cultures express low levels of αSMA and are negative for keratin and factor VIII, indicating that they do not originate from epithelial or endothelial cells (Arora and McCulloch, 1999).

The effects of aging upon fibroblast behaviour are well established and it may be expected that the myofibroblast (being derived from the fibroblast) may behave in a similar manner. Although evaluating rat dermal fibroblasts *in vitro*, work by Mays et al. (1995) has demonstrated that fibroblasts can continue to proliferate until passage 34 - 40. In addition, with increasing population doublings, a decline in both collagen and total protein synthesis is noted. An inverse correlation between proliferative capacity of fibroblasts *in vitro* and age of donor has also been described (Martin et al, 1970). No data is presently available that evaluates changes in the rate of fibrobast – myofibroblast differentiation with aging either *in vitro* or *in vivo*. It may be expected, however, that such rates diminish commensurate with the general decline in cellular activity with increasing passage. It has thus been accepted that work assessing cellular behaviour and phenotype be performed on "young" cells, with most authors using cells of passage 12 or less (Yokozeki et al., 1999; Masur et al., 1996; Vaughan et al., 2000).

Functionally, fibroblasts have tradionally been viewed as quiescent cells, but are now known to actively produce collagen and to respond to cytokines produced by the immune system, even producing their own pleiotropic growth factors (Sporn and Roberts, 1986). Fibroblasts are known to be capable of differentiation into the myofibroblast phenotype. Histological studies of the excessive forms of wound healing, hypertrophic and keloid scarring, have shown a persistence of the myofibroblast cell type (Gabbiani, 1996). As these cells are known to disappear from normal wounds after the proliferative phase of healing is complete, control of the myofibroblast phenotype has been suggested to be key for the control of cutaneous scarring (Walker, Guerrero et al., 2001).

1.5.1 History / discovery of the myofibroblast

In 1971, Gabbiani et al. (Gabbiani, Ryan et al., 1971) described a new cell phenotype within granulation tissue. The "modified" fibroblasts displayed many features of smooth muscle cells including the expression of actin filament bundles, in particular alpha smooth muscle actin (αSMA). Later in 1972, Gabbiani et al. (Gabbiani, Hirschel et al., 1972) went on to demonstrate that isolated strips of granulation tissue were able to undergo contraction *in vitro*, a property thought to be due to the presence of these modified fibroblasts. Cells of this phenotype were termed "myofibroblasts" and proposed to be responsible for the generation of force within the wound leading to wound contraction. This function was in keeping with that of other cells in which αSMA is known to be expressed (smooth muscle cells and myo-epithelial cells) (Serini & Gabbiani, 1999). Since their discovery, myofibroblasts have been noted in a diverse array of tissues such as skin, lung, liver and kidney (Powell, Mifflin et al., 1999).

1.5.2 Definition

Myofibroblasts have been defined as "smooth muscle like cells" or "activated smooth muscle cells" although such vague definitions have been criticised for being too lax (Powell et al., 1999). Currently, the accepted minimal requirement for a cell to be termed a myofibroblast is for a fibroblast-like appearance but also expressing αSMA (Sappino, Schurch et al., 1990). However, this criteria is also met by other cells that have been independently termed pericytes, Rouget cells, mural cells or perivascular cells (Sims, 1991), (Levy, Joyner et al., 2001). Some authors now consider that these cells are also myofibroblasts.

Perhaps the most important distinction for myofibroblasts is to distinguish them from skeletal muscle myoblasts. This may be achieved by the myofibroblast inability to differentiate into muscle or to withdraw from the cell cycle, a common trait of skeletal muscle cells.

1.5.3 Origin of the myofibroblast

The cellular origin of the myofibroblast has been the subject of much debate, with many candidate progenitor cells proposed such as fibroblasts (Gabbiani, 1996), macrophages (Bhawan & Majno, 1989) and muscle cells (Martin, Benson et al., 1993). Desmoulier (Desmouliere, Geinoz et al., 1993) showed in 1993 that fibroblast cultures treated with TGFβ *in vitro* contain many cells that stain positive for αSMA. In comparison, normal untreated fibroblast cultures have been shown to contain a small number of cells that are positive for αSMA (Clarke, 1996). Based on this *in vitro* work, it has become accepted that fibroblasts can be activated and differentiated into myofibroblasts by the addition of TGFβ, suggesting that myofibroblasts originate from fibroblasts *in vivo*. However, the myofibroblast has been shown to be a diverse cell type with varying ultrastructural characteristics (Desmouliere & Gabbiani, 1996), a finding that historically lead some investigators to propose differing progenitor cells for different myofibroblast subtypes.

Presently, it is thought that all myofibroblasts, irrespective of the tissue in which they reside, share a common ancestor but then differentiate into a specific myofibroblast phenotype that is dictated by their micro-environment. This theory has been supported by analyses of the expression of several proteins common in myofibroblasts, as comprehensively reviewed by Walker et al. (Walker, Guerrero, & Leinwand, 2001). Here protein expression was found to be dependent upon the characteristics of the microenvironment in particular, whether derived from pathological or normal tissue, and organ or origin.

Myofibroblasts have proven to have a highly plastic and diverse phenotype. However, analyses of different subtypes of myofibroblasts has revealed many common features, including the expression of muscle and non-muscle structural and regulatory proteins, contractile proteins and extracellular matrix constituents (Walker, Guerrero, & Leinwand, 2001). These proteins include vimentin, desmin and αSMA. Myofibroblasts are now known to play a major role in both the inflammatory response and in wound healing through the production of growth factors, cytokines and other soluble mediators (Gailit, Marchese et al., 2001).

Many studies have examined the cellular behaviour and history of the myofibroblast and speculated that under normal conditions, the process of myofibroblastic differentiation ends with the death of the cell (through apoptosis) (Desmouliere, Badid et al., 1997). This terminal event is thought to occur either as a direct result of cytokine action or due to cytokine withdrawal.

1.5.4 Histological appearance

Ultrastucturally, myofibroblasts are large cells with long cytoplasmic extensions which differ from fibroblasts by their expression of bundles of microfilaments with dense bodies similar to those found in smooth muscle cells. They also contain a well-developed Golgi apparatus and dilated rough endoplasmic reticulum. Although originally αSMA was considered to be expressed by practically all myofibroblastic populations, especially *in vivo* (Schmitt-Graff, Desmouliere et al., 1994), more recent work has identified a number of different myofibroblast phenotypes co-expressing proteins in addition to cytoplasmic actin isoforms (Desmouliere & Gabbiani, 1996):

a) vimentin
b) vimentin and desmin
c) vimentin and αSMA
d) vimentin, desmin and αSMA
e) vimentin, αSMA and smooth muscle myosin heavy chains
f) vimentin, αSMA, desmin and smooth muscle myosin heavy chains

Myofibroblasts are interconnected *in vivo* by gap junctions and connected to the extracellular membrane at discrete adhesive sites termed fibronexi. Adhesion to the ECM induces integrin clustering at focal contacts, and results in signal transduction pathways triggered by the combination of structural molecules and regulatory enzymes interacting with the actin cytoskeleton (Darby, Bisucci et al., 1997). These signalling pathways are known to act downstream of growth factor receptor tyrosine kinases. In addition, many ECM ligands are able to directly activate receptor tyrosine kinases (Vogel, Gish et al., 1997). Growth factors have been demonstrated to affect the way in which the myofibroblast interacts with the extra-cellular matrix. For example TGFβ is

able to affect the interactions with the ECM by alteration of the integrin expression pattern (Zambruno, Marchisio et al., 1995) and by the synthesis of ligands such as fibronectin and collagen. Physiological differences between fibroblasts and myofibroblasts include matrix-metalloproteinase (MMP) secretion patterns, response to phorbol esters and integrin expression (Masur et al., 1996).

1.5.5 Association with pathological fibrosis

Skin

The analysis of pathological forms of wound healing, such as hypertrophic and keloid scars, has suggested that the cell type known as the myofibroblast may play a significant role in their aetiology. Dermal fibroblasts are normally quiescent, but following cutaneous wounding can become activated. This causes the fibroblasts to migrate towards the wound interface, to proliferate, to synthesize a new collagen-containing extra-cellular matrix (called granulation tissue) and to differentiate into myofibroblasts.

The myofibroblast has been demonstrated to appear in the healing human wound approximately 48-72 hours following injury. In normal scars, analysis of wounds has revealed that this cell type begin to disappear at approximately 14 days (reviewed by Desmouliere and Gabbiani, 1996), following completion of the proliferative phase of wound healing. However, analysis of abnormal wounds (in particular hypertrophic and keloid scars) has shown that this phenotype may be present many years following injury (Nedelec, Ghahary et al., 2000). Indeed, in a study of foetal sheep wounds, Cass et al. (1997) noted that myofibroblasts typically disappeared from the healing wound by day 14 in scarless healing. Persistence of this phenotype beyond 14 days was typically associated with the formation of a cutaneous scar. In view of this, the myofibroblast is thought to be the cellular culprit in the formation of both normal and pathological scarring. However, whether the myofibroblast's presence is secondary to scarring, or is the actual cause of the scarring is unknown.

Ocular scarring

Within the eye, minimal scarring is desirable in order to maintain the refractive properties of the tissues and reduce any impact on vision and acuity. As is the case in

cutaneous scarring, the myofibroblast is considered to be the principle cell for the target of novel therapies to prevent or reduce ocular scarring (Jester, Petroll et al., 1999). It has been noted that myofibroblasts disappear with completion of healing within the eye, but that their persistence is characteristic of abnormal healing as seen in fibrotic lesions (Masur, Dewal et al., 1996) and in the development of visual complications post surgery (Mohan, Hutcheon et al., 2003). Additionally, the myofibroblast is thought to be instrumental in the failure of filtration procedures (Reddick, Merritt et al., 1985).

Oral scarring

Scarring of the oral mucosa is thought to be responsible for the impairment of maxillary growth following cleft palate surgery (Cornelissen, Maltha et al., 2000). Palatal fibroblasts are able to differentiate into myofibroblasts when cultured, with those derived from sites of palatal scars possessing more myofibroblast characteristics (including higher collagen synthesis and α-SMA) than normal palatal fibroblasts (Yokozeki, Baba et al., 1999). It has therefore been suggested that myofibroblasts play important roles in palatal scar formation, although whether myofibroblasts form in response to the scar or are actually the cause of the scarring is unknown.

Lung

In addition to its role in cutaneous scarring and fibrosis, the myofibroblast has been implicated in the pathogenisis of pulmonary fibrosis. In iatrogenic lung injury (e.g. administration of GM-CSF to rat alveoli) a fibrotic response was initiated that was characterised by the presence of myofibroblasts (Andreutti, Gabbiani et al., 1998). In lung injury induced by hypoxia (Durr, Dubaybo et al., 1987) a significant increase in myofibroblast numbers was noted. Similarly, histological examination of lung biopsies found myofibroblasts to be present at sites of diffuse alveolar damage (Pache, Christakos et al., 1998). Since their discovery, myofibroblasts have been identified in many pulmonary diseases and current research efforts are endeavouring to understand the biology of this cell in order to devise new therapies for respiratory diseases (Pache, Christakos et al., 1998).

Kidney

Myofibroblast differentiation has been shown to play a significant role in the development of renal failure (Badid, Mounier et al., 2000). All forms of renal disease that eventually proceed to renal failure have, as their final common pathway, fibrosis which corresponds to an irreversible scar within the kidney. Such fibrosis has been shown to correlate to the number of myofibroblasts present (Badid, Desmouliere et al., 1999) and has lead to suggestions that myofibroblast evaluation be undertaken on renal biopsies to assess clinical prognosis.

Liver

The liver probably represents the most common site for tissue fibrosis. Following injury, the hepatic stellate cells differentiate into cells resembling myofibroblasts (Brenner, Waterboer et al., 2000) thereafter proliferating and increasing the synthesis of extracellular matrix components. As with other tissues, control of myofibroblast development may lead to new therapies to prevent hepatic fibrosis.

1.5.6 Factors that influence myofibroblast differentiation

Aside from the initial work that strove to prove the cellular origin of myofibroblasts, much interest has been focussed upon discovering how fibroblast - myofibroblast differentiation is controlled. The lack of a homogeneous culture of myofibroblasts has made such work more difficult as differences in culture conditions *in vitro* seem able to affect the relative proportion of myofibroblasts present. Masur et al. (1996) demonstrated higher proportions of myofibroblasts when fibrobasts were plated at low densities. The concentration of serum has also been shown to affect myofibroblast proportions (Arora and McCulloch, 1999) with higher numbers found in serum free conditions.

Determination of the factors that allow or induce the formation of the myofibroblastic phenotype may ultimately identify mechanisms that can be targeted in order to prevent the formation of this phenotype. It has become apparent that fibroblast - myofibroblast differentiation may be initiated and controlled through the action of growth factors or via cellular interactions with the extra-cellular matrix.

1.5.6.1 Growth Factors

Many growth factors are known to be important in wound healing and therefore their roles upon myofibroblast differentiation have been studied. Growth factors are generally pleiotopic regulators of cell behaviour which, in addition to modulating cell functions such as proliferation and gene expression, have been shown to play an important role in controlling cellular migration. Growth factors have been demonstrated to either inhibit or stimulate fibroblast – myofibroblast differentiation *in vitro:*

Inhibitors of Fibroblast - Myofibroblast Differentiation

Many studies have been performed to identify compounds that can reduce fibroblast - myofibroblast differentiation. Of the many compounds investigated to date, γ-interferon has been demonstrated to reduce myofibroblast numbers both *in vitro* and *in vivo* (Pittet, Rubbia Brandt et al., 1994). Although these studies were performed on hypertrophic scar and Dupuytren's disease, similar studies performed on liver fibrosis have yielded comparable results (Mallat, Preaux et al., 1995) in the reduction of myofibroblast numbers.

Furthermore, i*n vitro* studies performed by Maltseva et al. (Maltseva, Folger et al., 2001) have highlighted the role played by FGF during fibroblast – myofibroblast differentiation. Here, treatment of corneal myofibroblasts with FGF-1, FGF-2 or 10% Foetal Calf Serum (a common constituent of fibroblast culture medium) reduced the number of myofibroblasts.

Stimulators of Fibroblast - Myofibroblast Differentiation

From studies of the effects of growth factors on fibroblast – myofibroblast differentiation, it has become apparent that in addition to being able to inhibit such differentiation, growth factors are also able to stimulate this process. Despite the number of other active growth factors during wound healing, most interest has concentrated upon the role played by the interactions of TGFβ's and the Extracellular Matrix (ECM).

TGFβ is considered to be a direct inducer of the myofibroblast phenotype and has been demonstrated to increase fibroblast production of α-smooth muscle actin and collagen both *in vitro* and *in vivo* (Desmouliere et al., 1993). This 25 kDa protein is an important regulator of inflammation and is involved in both recruitment of macrophages and the inhibition of some lymphocyte functions (Mckay & Leigh, 1991). In mammals, TGFβ is found in three isoforms, $TGF\beta_1$, $TGF\beta_2$ or $TGF\beta_3$, each with differing spatial and temporal patterns of expression during development, leading many to believe that they have distinct biological functions (Ellis, Banyard et al., 1999). Although the different isoforms show 70-80% homology, they have also been shown to have differing effects on myofibroblast formation and behaviour (Serini & Gabbiani, 1999). $TGF\beta_1$ and $TGF\beta_2$ have been shown to induce myofibroblast formation whilst $TGF\beta_3$ can act as a negative regulator of myofibroblasts (Shah, Foreman et al., 1995). Interestingly, the different activities of the TGFβ isoforms have only been demonstrated *in vivo* (Graycar, Miller et al., 1989). The differences in the activities of TGFβ isoforms *in vitro* compared to their effects *in vivo* is thought to suggest that interaction with other growth factors is important in the modulation of the fibroblast.

The different activities of the TGFβ isoforms has lead many investigators to consider this growth factor to be a "master control" for wound repair, with dis-regulation of its production being known to produce fibrosis in many different tissues (lung (Sime, Xing et al., 1997), liver (Gressner, 1991), kidney (Diamond, van Goor et al., 1995)).

Recent work has suggested that αSMA promotes contractile force generation within the wound. Given that TGFβ is known to increase the cellular expression of αSMA, it was therefore hypothesized that addition of this growth factor to fibroblast cultures *in vitro* would promote the generation of contractile force. Work performed on human fibroblasts *in vitro* has since confirmed that TGFβ increases contractile force in a dose dependent manner and that this is correlated with an increase in cellular expression of αSMA (Vaughan, Howard et al., 2000).

Interestingly, $TGF\beta_1$ has been linked to an increase in scarring after cutaneous wound healing. In an analysis of human foetal skin wounds, Bullard et al. discovered that the addition of $TGF\beta_1$ not only decreased the activity of collagenases during healing but

also resulted in an increase in scarring (Bullard, Cass et al., 1997). In a similar manner, assessing the effects of neutralising antibodies to $TGF\beta_1$ on rodent wounds, a reduction in scarring was noted although the addition of neutralising antibodies directed at all 3 TGFβ isoforms had no discernable effects (Shah, Foreman, & Ferguson, 1995). In view of these findings, one could hypothesise that the pathological forms of scarring (keloid and hypertrophic scar) were due to overproduction, or indeed increased sensitivity of TGFβ. However, attempts to correlate pathological scarring with TGFβ production have been contradictory. Indeed, a study by McCauley et al. (McCauley et al., 1992) has measured lower levels of TGFβ in black patients with keloids than in normal controls.

1.5.6.2 Interactions with the extracellular matrix

Many studies agree that the bioactivity of cytokines is defined by the context of its microenvironment (Schor & Schor, 1987), (Tuan & Nichter, 1998). Indeed some investigators have furthered this concept by suggesting that the bioactivity of cytokines is formulated in the context of a "tissue response unit". This comprises of the target cell population maintained at a particular activated state, the macromolecular matrix in contact with those cells and the full complement of the cytokines present in that microenvironment (Pickering, Uniyal et al., 1997). The role of the ECM in fibroblast - myofibroblast differentiation has been supported by the sequence of events occurring during wound healing. Prior to myofibroblast differentiation, ECM deposition has invariably occurred, suggesting that fibroblast - myofibroblast differentiation may be an adaptive response to modifications of the ECM (Desmoulier, Darby et al., 1997).

Of the macromolecular components of the ECM, much interest has focussed upon the role of fibronectin. This molecule exists in a number of different forms that arise from a single mRNA transcript that can be alternatively spliced in three regions (Badid, Mounier et al., 2000). mRNA for specific fibronectin transcripts have been identified in fibroblasts during wound healing. These fibronectin transcripts have also been shown to be necessary for the induction of fibroblast - myofibroblast differentiation by $TGF\beta_1$ *in vitro* (Serini, Bochaton-Piallat et al., 1998) and have also been shown to be produced in response to stimulation with $TGF\beta_1$ (Balza, Borsi et al., 1988).

1.6 Management of the unsatisfactory scar

Dermal tissue injury usually results, after healing is complete, in the formation of a permanent cutaneous "abnormality", the scar. Although the passage of time frequently improves the appearance of many scars, once an injury has occurred, there is little that can be undertaken to guarantee an improvement in its appearance. Occasionally intervention is possible to attempt to improve the cosmesis of a scar. These methods can be conservative measures (Section 1.6.1), medical and pharmacological therapies (Section 1.6.2) or surgery (Section 1.6.3). The numerous approaches available to improve cutaneous scarring might suggest that there is no reliably effective method for use in the majority of patients (Wilson, 2000).

1.6.1 Conservative

Unsurprisingly, many different conservative methods are available for the treatment of cutaneous scars. Such measures usually involve the application of dressings to the wound or established scar, but also include ultrasound (Rockwell, Cohen, & Ehrlich, 1988) and the administration of various creams e.g. application of vitamin E (Baumann & Spencer, 1999). The most common conservative interventions currently undertaken to improve the appearance of cutaneous scars i.e. pressure dressings, silicone, hyaluronan and zinc are described below:

Pressure Dressings

Pressure dressings have been utilised for the reduction of cutaneous scarring since the work of Pare in the sixteenth century. However, such treatments had not gained universal support until the Shriners Burns Institute examined their use in burn scarring (Linares, 1996). Following encouraging reductions in the incidence of hypertrophic scars following burns by the use of pressure dressings, the use of regimens combining dressings with positioning, splinting and exercise was explored. Subsequent studies measuring the pressure produced below pressure dressings have shown "good results" with pressures as low as 4mmHg (Robertson, Hodgson et al., 1980). Current recommendations suggest a pressure be maintained between 24 and 30 mmHg for 6-12 months for this therapeutic approach to be effective (Mustoe, Cooter et al., 2002). For optimum results, the garments should be worn for 24 hours a day until satisfaction with

the scar is achieved. The duration of treatment is thus dependent upon the patient and the improvement required within the scar itself. The prolonged duration required for appreciable results with this form of scar therapy requires significant compliance from the patient.

Pressure therapy is currently thought to succeed through causing realignment of collagen bundles within the substance of the scar. In addition, scar vascularity is reduced, leading to areas of relative ischaemia and subsequent fibroblast death. This alters the balance of collagen metabolism so that catabolism becomes dominant (Johnson, 1984). Microscopic examination of scars treated with pressure dressings has shown a reduction in the cohesiveness of the collagen fibres lending support to this theory.

Silicone dressings

During the early studies on the effects of pressure dressings, silicon pads were often used beneath the dressings to provide consistent pressure during movement and limb positioning (Quinn, Evans et al., 1985). However, as it can prove difficult to apply pads to certain areas of the body, silicone gel was used e.g. over joints. Subsequent work has now shown that silicone gel sheeting (and indeed silicone gel) is a safe and effective management option that, even when used in isolation, can reduce the incidence of both hypertrophic and keloid scars (Poston, 2000). In order to treat scars with silicone, patients are ideally required to wear the gel for 24 hours per day, although beneficial effects have been reported with only 12 hours per day (Alster & West, 1997). The mechanism by which silicone reduces scarring is still unknown. Investigations aimed at defining how scarring is reduced have thus far excluded effects on local pressure, temperature or oxygen tension (Quinn, 1987). Theories suggesting a reliance on silicone leakage into the dermis have likewise been discounted (Chang, Kuo et al., 1995). Presently, silicone is thought to increase cutaneous hydration, principally within the stratum corneum. This in turn leads to a "moist milieu" throughout the wound and results in decreased capillary activity, hyperaemia and collagen deposition (Niessen, Spauwen et al., 1998).

Hyaluronan

Hyaluronan is a high molecular weight polysaccharide which exists as a matrix component in all connective tissues and has been shown to improve the outcome of healing in studies in man (Hellstrom & Laurent, 1987). During wound healing, hyaluronan has been shown to exert a number of roles, modulating cell function (Turley, Bowman et al., 1985) stimulating the production of keratin and in addition increasing chemotaxis for inflammatory cells (Hakansson, Hallgren et al., 1980).

Zinc

Following observations that both hypertrophic and keloid scars that had been dressed with zinc oxide tape showed lower recurrence rates, (Söderberg, Hallmans et al., 1982), zinc has been shown to be beneficial in the healing of other wounds. Early work on the role of zinc tape during wound healing suggested that scar prominence was reduced as an effect of local splintage. However, application of zinc tape to the wound has also been shown to reduce water loss by evaporation, leading to increased levels of hydration within the skin (Hallmans, 1977). Animal studies have also shown that percutaneous absorption of zinc can occur, a potentially beneficial effect as zinc is known to decrease synthesis of collagen by fibroblasts (Waters, Moore et al., 1971), collagen cross linkage (Chvapil & Walsh, 1973) and to be a major component of collagenases.

1.6.2 Medical / Adjuvant

In addition to conservative measures, the administration of medication (either systemically or locally) has been found to be beneficial to wound healing and to reduce the severity of cutaneous scarring in some cases. Many treatments have been assessed including the administration of substances such as retinoic acid (DeLimpens, 1980). However, the most effective treatments for established excessive cutaneous scarring are considered to be intralesional steroid injection and radiotherapy (Mustoe, Cooter et al., 2002). These latter treatments are described in more detail below:

Steroids

Probably the most common medical intervention used to improve established cutaneous scarring is the administration of steroids. Although few randomised prospective studies

exist that confirm the effectiveness of steroid injections in scar management, there is broad consensus that injected steroid is efficacious for the treatment of keloid scars (Mustoe, Cooter et al., 2002). Steroids, e.g. triamcinolone, are usually applied directly into the scars at 4 to 6 week intervals until the scar has regressed to the satisfaction of both the patient and clinician. Typically, response rates vary from 50-100% with recurrence rates quoted at 9-50% (Niessen, Spauwen et al., 1999). Administration of steroids is usually painful and up to 63% of patients experience side effects that include skin atrophy, depigmentation and telangiectasis (Sproat, Dalcin et al., 1992). This results in many patients being unable to complete the recommended course of treatments, reducing the usefulness of this therapy. Topical steroid creams have been used but seem inferior to injected steroid (Mustoe, Cooter et al., 2002).

Radiotherapy

Other, more radical therapies have been used to reduce pathological scarring. In 1906, Debgeurman and Gougerot discovered that radiotherapy could be used for the treatment of keloids (Rockwell, Cohen, & Ehrlich, 1988). It is now thought that ionising radiation kills the fibroblasts present within the keloid scar and swings the balance of the processes involved in wound healing towards collagen degradation (Rockwell, Cohen, & Ehrlich, 1988). Levy et al. (Levy, Salter et al., 1976) achieved an 88% success in preventing keloid recurrence, although the length of follow up for this work was only 2 years. However, debate continues about the possible side effects that this therapy may cause, in particular the risk of carcinogenesis, although to date there are only 2 recorded cases of carcinoma following radiotherapy for keloid scarring (Alster & West, 1997) Currently this therapy is reserved for use in adults with keloids resistant to other management modalities (Mustoe, Cooter et al., 2002) and is not considered a routine treatment for keloid scarring.

1.6.3 Surgical

Surgical management of a "normal scar" is rarely indicated. Although surgery cannot completely remove a scar as it simply substitutes one scar for another, it can be useful in altering the scar appearance, position, alignment or shape e.g. releasing joint contractures (Parsons, 1977). The surgical treatment of pathological scars is empirical

and despite increasing use of conservative methods of scar reduction following scar excision, recurrence rates remain high, ranging from 45 to 100% for surgical revision of keloid scars (Mustoe, Cooter et al., 2002). The use of excision alone has now been relegated mainly to the management of lesions that are unresponsive to non-surgical therapy. The surgical treatment of unsatisfactory cutaneous scarring may be subdivided into measures that may be taken to reduce the formation of scars and those measures that may be utilised to reduce scarring once it has occurred. The optimal surgical therapy for scarring remains unclear and, at present, there is no simple cure.

Approaches to Reduce the Formation of Cutaneous Scarring

Before performing an incision, the surgeon should consider both the visibility and presumed behaviour of the resulting scar (Hinderer, 1977). Although unsatisfactory scarring cannot always be avoided, careful surgical technique may result in the formation of a more inconspicuous scar. Prior to the creation of a wound, several considerations must be made. For example, for many operative procedures, it is possible to place the scar within concealed areas e.g. intra-oral approach to the mandible or periareolar incisions for breast surgery. For those incisions that lie in un-concealed skin, orientation of the wound along the Relaxed Skin Tension Line (RSTL) will improve the final scar result (Parsons, 1977).

RSTL (Borges & Alexander, 1959) are lines that follow the collagen fibres of the reticular dermis, and result in "microfolds". They were first identified by Langer in 1861 and are often referred to as "Langer's lines". In the main, the RTSL run opposite the direction of contraction of the underlying musculature. These lines are readily visible in the aging face and are shown in Fig 1.2.

In addition to considering the orientation and placement of the wound, a number of intra-operative factors may influence the resultant scar. In a multi-centre secondary analysis of 924 wounds, Singer et al. investigated a number of factors thought to predispose towards unfavourable wound outcome (Singer, Quinn et al., 2002). Incorrectly apposed or wide excisional wounds were found to lead towards the poor cosmetic result. In addition, tissue trauma, either pre-operative or via "heavy handed" tissue handling and the use of electrocautery (common to achieve haemostasis) resulted

in an increased risk of poor cosmesis. The presence of infection can also affect the outcome of wound healing as it is associated with a greater inflammatory response and tends to result in the formation of a wider, slower maturing scar (Parsons, 1977). Finally, the presence of particles of dark coloured foreign material within traumatic wounds will, if not removed, result in a permanent tattoo effect, also adversely affecting the cosmetic appearance of the final scar.

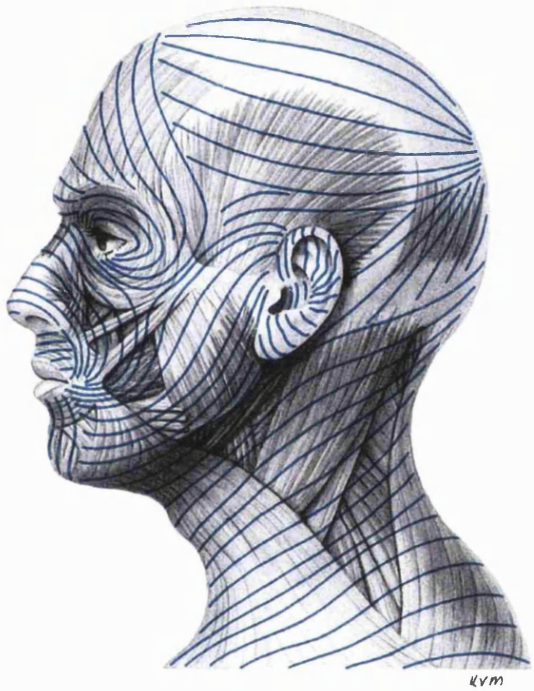


Fig 1.2: Langers lines on the lateral aspect of the head and neck. (Image taken from Grays anatomy (Williams, Bannister et al., 1995))

Surgical Therapy of the Established Scar

Despite measures taken to reduce the occurrence of cutaneous scarring, every wound that penetrates the papillary dermis leaves a scar. Scars appear at their worst between 2 weeks and 2 months following creation, and gradually improve as the scar continues to mature. This maturation phase may be considered to be due to a decrease in vascularity and the disappearance of excess collagen (Parsons, 1977).

After a suitable period has passed, or if abnormal scarring such as hypertrophic or keloid scarring has developed, treatment to improve the appearance of the scar may be

considered. The commonest surgical techniques used to improve cutaneous scarring include scar revision, either as a single procedure or as a serial excision (Rudolph, 1987). Whilst this technique may be of benefit for the treatment of some wounds, e.g. traumatic, for pathological scars such as keloids, surgical excision may be futile and may result in the formation of a scar that is as "un-aesthetic" (or worse) than the original. Scar excision should only be considered if a definite difference can be demonstrated between the original conditions for healing and the current clinical situation (Moran, 2001). If no difference exists, then it is highly likely that wound healing will follow a similar course, and result in a similar scar. Serial excisions for the removal of large scars rely on the elasticity of the skin around the scar, with small segments of scar removed and the resulting wound closed directly. Theoretically this results in a thin scar, although if the tension on the wound is too great, then stretching may occur resulting in a larger scar.

For patients in whom the scar causes contracture and restriction of movement, surgical release may be necessary. Through the basic principles of plastic surgery, and the use of simple local flaps, it is possible to lengthen the scar causing the contracture (usually through the use of single or multiple "z"-plasties (Moran, 2001)). This however, does little to address the cosmetic defect, and may conceivably worsen the appearance of the scar by adding to its size. However, the positive effect on mobility gained by removal of the contracture far outweighs such risks.

Other mechanisms for dealing with cutaneous scarring rely on the introduction of new tissue into the scarred area (Mostafapour & Murakami, 2001). This may be achieved via grafting (both split and full thickness skin grafts may be used) or indeed by flap coverage (both local and free). The main disadvantage of these approaches is that skin or tissue needs to be obtained from somewhere else on the body, requiring the creation of wounds (and subsequent scars) in otherwise healthy, and scarless, tissue.

Despite the many techniques that can be used to manage the scarred patient, none are guaranteed and will completely remove the scar. Certainly, surgical methods do have a place in the treatment of the scarred patient; however, if one could reduce the occurrence of the scar in the first place, then subsequent treatment may not be required.

In view of this, many groups are searching for therapies that will reduce or prevent the cutaneous scarring.

In summary, although there is a wide range of clinical approaches aimed at preventing the formation of excessively severe scars, or indeed reducing the severity of established scars, no treatment that is currently available is reliably effective.

1.7 Potential therapies proposed to reduce or prevent cutaneous scarring

Much interest has been concentrated upon the development of a truly effective therapy to reduce or even prevent cutaneous scarring. Chiefly, these have concentrated on utilising growth factors during wound healing, although the majority of this type of work has concentrated on increasing the speed of healing or on increasing the strength of the final repair (Robertson, Belford & Ballard, 1999). Relatively little work has been performed assessing the effects of growth factor administration on scar appearance. In the main, therapies to reduce scarring can be divided into those that manipulate the wound environment (Section 1.7.1) and those that involve the addition of exogenous substances to direct cell behaviour (Section 1.7.2).

1.7.1 Manipulation of the wound environment

Studies have evaluated the wound healing processes in the non-scarring phenotype of the foetus for clues towards alternative methods in which adult wound healing may be manipulated. It is well known that wounds created during early gestation (typically within the first trimester) tend to heal without scar formation (Longaker & Adzick, 1991). Originally this phenomenon was thought to be due to the foetal wound healing environment, but Lorenz et al. (Lorenz, Longaker et al., 1992) demonstrated that foetal skin (when implanted subcutaneously) could heal without scarring in the adult. Further work has highlighted the role of TGFβ in scar formation, with little evidence of active TGFβ being reported by Sullivan et al. in scarless foetal wound repair (Sullivan, Lorenz et al., 1995). Although the cytokine profiles within foetal wound healing differ from

those seen in the adult, with relatively high ratios of TGF-β_3 to TGF-β_1, other differences also exist that may explain scarless healing. Foetal wound healing is characterised by a reduced inflammatory response, sustained deposition of hyaluronic acid (HA) and relatively greater amounts of collagen III formation (Peled, Chin et al., 2000). In an effort to recreate foetal wound conditions, Hellstrom et al. added HA to tympanic membrane wounds (Hellstrom & Laurent, 1987). Here, the severity of scar was reduced. Interestingly, in an attempt to understand why HA should improve wound healing, Pickering et al. demonstrated that TGF-β_3 (but not TGFβ_1 or TGFβ2) can stimulate HA synthesis (Pickering, Uniyal et al., 1997). This may prove to be a mechanism by which TGFβ_3 itself may improve cutaneous scarring (Shah, Foreman, & Ferguson, 1995).

1.7.2 Addition of exogenous substances

Although much is known about the factors that "turn on" tissue repair, very little is known about those that may "turn off" or even control the extent of such reparative mechanisms. From observations of burn wounds, it is known that the longer a wound remains unhealed, the greater the inflammatory response and hence the resulting scar (Deitch, Weelahan et al., 1983). This is thought to be due to the prolonged presence, or indeed imbalance, of growth factors causing the inflammatory response and subsequent excessive collagen deposition. In an attempt to control these processes, many investigators have explored the use of growth factors in wound healing, to improve the speed of healing and to improve the resulting appearance. However, even though multiple studies have been performed, there exist distinct problems in the interpretation studies performed assessing the same growth factor. In the main, these relate to differences in animal models used between experiments (although many researchers currently accept the porcine model to be the best approximation owing to the structure of the skin being similar to that found in man (Gottrup, Agren et al., 2000)). Additionally, the method of application of the growth factors and the method of assessment of wound healing (Mckay & Leigh, 1991) can vary considerably and give conflicting results between studies.

Of those growth factors found to play a role in wound healing, TGFβ is thought to play the major role in scarring. Evidence of the importance of TGFβ in wound healing has been demonstrated by Mustoe (Mustoe, Pierce et al., 1987) and more recently by Beck (Beck, DeGuzman et al., 1993). The addition of TGFβ to dorsal rat incisional wounds significantly accelerates wound healing, resulting in the formation of stronger scars. Similarly, the systemic administration of $TGF\beta_1$ enhances healing in age or glucocorticoid impaired wounds when given shortly after injury.

Whilst the addition of TGFβ has been shown to be beneficial in healing, its role in the formation of myofibroblasts has suggested that modulation of its expression during wound healing may be of use in controlling the amount of fibrosis (Tuan & Nichter, 1998). The most widely accepted approach to the modulation of TGFβ effects during wound healing pivots on the roles that different isoforms of TGFβ play. Shah et al. (1995) demonstrated that addition of $TGF\text{-}\beta_3$ to cutaneous wounds in mice diminished mononuclear cell infiltrate into the wound and reduced subsequent scar formation. Other work by the same group showed that blocking antibodies to both $TGF\text{-}\beta_{1+2}$ also reduced cutaneous scarring (Shah, Foreman et al., 1994).

Despite the encouraging results published by Shah et al., problems have been anticipated with the use of TGFβ antagonists or neutralising molecules during wound healing. Antagonists that block all isoforms of TGFβ could potentially cause the most disruption to wound healing due simply to the pleiotropic nature of these factors. Ellis et al. (1999) have shown that this growth factor is important in stimulating the migration of confluent dermal fibroblasts *in vitro*. As cellular migration (Pickering, Uniyal et al., 1997) is important in all stages of wound healing, removal of this effect may prolong wound healing significantly. In addition, TGFβ also has effects on the keratinocyte, where unfortunately its effects are not clear cut. Garlick et al. showed that supplementation of keratinocyte cultures with $TGF\beta_1$ inhibited keratinocyte proliferation (Garlick & Taichman, 1994) whereas supplementation of *in vivo* porcine wounds with TGFβ (Jones, Curtsinger et al., 1991) had previously been shown to enhance the speed of healing. The use of either exogenous TGFβ or antibodies towards TGFβ may therefore have implications on wound re-epithelialisation.

Inhibiting the effects of TGFβ or its receptor is not without problems. Several studies have shown an increase in autoimmune diseases (Schull, Ormsby et al., 1992) and malignant transformation (Heldin, Miyazono et al., 1997). The use of blocking molecules and inhibitors have thus resulted in unacceptable side-effects leading to the search for other approaches by which the activities of TGFβ during wound healing may be modulated (Vaughan, Howard, & Tomasek, 2000).

An advance in the development of transgenic animals has allowed a greater understanding of the roles of various growth factors in wound healing. Incisional wounds created in mice with high circulating levels of $TGF\beta_1$ have been shown to scar less readily (Hochscheid, Jaques, & Wegmann, 2000). However, in animal models designed to evaluate the early phases of healing, such as the implantation of subcutaneous PVA sponges to stimulate granulation tissue (Diegelmann, Linblad et al., 1986), addition of $TGF\beta_1$ leads to dramatically increased scarring when compared to wild-type controls (Shah, Revis et al., 1999).

Other growth factors, such as interferon gamma (Granstein, Rook et al., 1990) and interferon alpha2b (Tredget, Shen et al., 1993), have also been shown to be of benefit during wound healing, improve the appearance of both hypertrophic and keloid scars. In particular, the ability of interferon-γ to partially abrogate the myofibroblast phenotype, and to reduce the production of proα2(I) collagen and cell contraction has lead Yokozeki et al. (1999) to suggest the use of this growth factor as a therapeutic agent to block scarring in the oral palatal mucosa.

The expression of MMPs during wound repair in normal and abnormal scarring has suggested that the use of these factors may be of benefit in the management of wound healing and scarring. It is known that during wound repair, MMP-1 and MMP-3 (but not MMP-10) are expressed in dermal fibroblasts and participate in the formation and removal of granulation and scar tissue (Herouy, 2001). In addition, activity of MMP-9 has been shown to be low or undetectable in hypertrophic scars, suggesting that it application of this compound may be beneficial to healing wounds (Neely et al., 1999). MMP inhibitors have similarly been shown to be of benefit in the topical therapy of corneal ulcers (Herouy, 2001). However, despite initially encouraging results, little

evidence exists that demonstrates a positive use for these compounds in the management of scarring.

However, if the administration of either exogenous factors or neutralising antibodies to wounds proves to be safe and to have no detriment to wounds in man, the cost involved in the production and purification of recombinant molecules may yet prove inhibitory for large scale clinical use (Benn, Whitsitt et al., 1996).

1.8 Insulin as a potential anti-scarring therapy

Whilst much work has concentrated on understanding the roles that growth factors play in wound healing, insulin has largely been ignored. Wounds treated with antibodies to insulin showed delayed wound healing while insulin itself has been shown to accelerate cutaneous healing when applied topically to wounds in diabetic mice (Berne and Levy, 1996). The lack of insulin has been shown to lead to abnormal proliferation and differentiation in keratinocytes and other tissues *in vitro* (Wertheimer, Spravchikov et al., 2001). Recent work (detailed in Section 1.8.3) performed at the RAFT institute has demonstrated that insulin may play a role in the control of myofibroblast numbers *in vitro* (Linge, Shelton et al., 2004) and may thus form the basis for a therapy for the prevention or reduction of cutaneous scarring. This thesis aims to explore the possible use of insulin as an "anti-scarring" therapy.

1.8.1 Synthesis and Secretion of Insulin

The following description of insulin has been taken, in part, from Berne and Levy (Berne and Levy, 1996). Insulin is a peptide hormone with a molecular mass of approximately 6000 Da and is produced by the β-cell population of the Islets of Langerhans in the pancreas. Following translation of the gene product (resulting in the production of a preprohormone), the signal peptide is removed to yield the single chain proinsulin. This is further modified by excision of a central peptide (the C-peptide) that connects two distinct chains (α and β) allowing disulphide bonds to then form between complementary amino acids. Both insulin and C-peptide are packaged together in secretory granules by the cellular golgi apparatus and then released from the cell by

exocytosis. Secretory granules are rich in zinc, which helps to join insulin molecules together into a hexamer (Berne and Levy, 1996).

The secretion of insulin is governed by a feedback relationship with the exogenous nutrient supply. Principally, this is via the systemic levels of glucose, with little insulin being secreted when levels of glucose fall below 50 mg/dl. As glucose levels rise, the amount of insulin secreted increases also, reaching a maximal level when plasma glucose levels reach 250 mg/dl.

Within the circulation, insulin is known to have a half-life of approximately 6-8 minutes. The majority of insulin is degraded within the kidney, although alternative degradation methods do exist. An example of this includes the internalisation of insulin after it binding to its own receptor, leading to degradation of the internalised hormone.

1.8.2 Actions of insulin

Insulin has many biological activities but functions in the whole to facilitate the storage of nutrients and to inhibit their release. It acts primarily upon the liver, muscle and adipose tissue in order to achieve this role. Insulin stimulates the transport of glucose from plasma into the cytoplasm of muscle and adipose tissues.

In addition, the presence of insulin stimulates the transport of certain amino acids into the cytoplasm of muscle cells in a manner that is independent of glucose transport. Insulin also increases protein production by the cells by stimulating transcription and translation. Such effects are reinforced by the inhibition of proteases and amino acid release. Insulin is well known to play an important role in regulating growth and tissue remodelling / regeneration. In cartilage and osseous tissues, for example, insulin (and the IGFs) enhances the synthesis of proteins, DNA, RNA and other macromolecules.

Despite the myriad of effects that are attributed to insulin, (in particular those upon mesenchymal derived cells), very little is known about its effects on fibroblasts other than that of nutrient transport and storage.

1.8.3 Insulin and fibroblast – myofibroblast differentiation

During studies to produce a more defined medium that supported cell survival, Linge et al. (2004) discovered that foetal calf serum contained an inhibitor of myofibroblast differentiation. Fibroblasts cultured in medium that had been treated to remove growth factor activity showed significantly higher levels of myofibroblasts than those cultured in normal growth medium containing foetal calf serum. Further work aimed at identifying the precise component of serum responsible for the modulation of fibroblast - myofibroblast differentiation identified that the factor responsible was insulin. Addition of bovine insulin to fibroblast cultures *in vitro* reduced myofibroblast numbers with an EC_{50} of approximately 20-40 nM/ml whereas the related growth factors IGF-I and IGF-II did not exhibit this effect when evaluated over a wide range of concentrations.

1.9 Aims of this thesis

This thesis aims to build upon the findings of Linge et al. (2004) and to further define the effects insulin has on dermal fibroblasts both *in vitro* and *in vivo* during wound healing. The original work was undertaken using laboratory grade bovine insulin, however for this phenomenon to be useful clinically similar effects need to be seen with human insulin preparations. Similarly, the effect insulin has on other processes important during wound healing has yet to be evaluated. If this compound is proven effective *in vitro*, *in vivo* studies are necessary to determine its true efficacy during wound healing

1.10 Hypothesis

Human insulin preparations can reduce the myofibroblast differentiation of dermal fibroblasts both *in vitro* and *in vivo* without any deleterious effects on the remaining wound healing processes.

To investigate this hypothesis, the following have been undertaken:

- The characterisation of the efficacy of human insulin in reducing myofibroblast differentiation *in vitro.*

- Determination of the optimum commercially available formulation of insulin to reduce fibroblast - myofibroblast differentiation *in vitro*

- Evaluation of the effects of insulin upon some other processes important during wound healing.

- Establishing whether any other growth factors similarly reduce fibroblast - myofibroblast differentiation in growth factor dialysed medium.

- Preliminary examination of insulin signalling pathways to identify alternative substances that will inhibit fibroblast / myofibroblast differentiation.

- Examination of the effect of insulin antagonists on fibroblast - myofibroblast differentiation *in vitro.*

- The characterisation of the efficacy of insulin in reducing myofibroblast differentiation *in vivo.*

Chapter 2

Materials and Methods

2.1 Materials

Unless otherwise specified all tissue culture reagents were supplied by Gibco BRL Life Technologies whereas tissue culture plastics were purchased from Greiner. All chemicals used in this study were the best grade available, supplied by Sigma, BDH or Fisons. All solvents were analytical (AnalR) grade supplied by BDH and Fisons. The recipes for the most commonly used buffers and solutions are detailed in Appendix I.

2.1.1 Animals

Male BALB/c mice of 12 weeks of age were purchased from Harlan. The mice were fed on RMS (expander) breeder diet (W. M. Lillico).

2.1.2 Anaesthetic Equipment and Drugs

Murine anaesthesia was delivered via an M&IE Cavendish Anaesthetic machine with vaporiser. Cylinder oxygen was supplied by BOC. The inhalation agent halothane (Fluothane®) was obtained from Zeneca Ltd.

2.1.3 Theatre Consumables

Animal Procedures

Chlorhexidine 4% surgical scrub and chlorhexidine 0.5% in alcohol were obtained from Adams Healthcare Ltd. Sodium Chloride solution for Irrigation (0.9%) was purchased from Steripak Ltd. Polypropylene monofilament non-absorbable sutures (Prolene® 5/0) was purchased from Ethicon Ltd. Sterile gauze swabs 10 x 10 cm were purchased from Johnson and Johnson. Needles and syringes were purchased from Terumo.

2.1.4 Tissue Culture

Cell culture procedures were performed in sterile Class II laminar air flow hoods (LaminAir) from Heraeus Instruments. Cells were cultured in Heraeus incubators in a humidified atmosphere at 37°C, enriched with 5% CO_2. Cells were centrifuged by use of Heraeus Labofuge. Microscopic examination of live cells was achieved using

Olympus CK2 inverted phase contrast microscope. Cell counting was by use of a Fuchs-Rosenthal haemocytometer (Weber Scientific International).

Dulbecco's phosphate buffered saline (PBS) without Ca and Mg for tissue culture, Dulbecco's Modified Eagles' Medium (DMEM) and foetal calf serum (FCS) were obtained from Gibco Ltd. Other tissue culture products (trypsin, 2.5% ethylenediaminetetra-acetic acid in buffered saline (versene) and di-methyl sulfoxide (DMSO)) were obtained from Sigma Ltd.

2.1.5 Tissue Analysis

Light microscopy

Dehydration stages were performed in a Histokinette 2000 automatic tissue processor. Blocks were embedded using a Raymond A Lamb Blockmaster III and histological sections cut on a Reichert-Jung Microtome. Sections were viewed on a Zeiss Axioskop microscope. All histological solvents, buffered formal saline, toluene, xyline and alcohol were supplied by Genta Medical. Haemotoxylin and Eosin stains (Gurr) were made in house according to protocols published in Bancroft and Stevens (Bancroft & Stevens, 1990). Masson's Trichrome stain was also made in house according to protocols described in Bancroft and Stevens, but using a Methyl Blue (Gurr) counterstain instead of the usual Light Green.

Immunohistochemistry

Monoclonal anti-alpha smooth muscle actin antibody was supplied by Sigma Ltd (Clone 1A4). FITC conjugated rabbit anti-mouse secondary antibodies were supplied by DAKO. Alkaline Phosphatase conjugated secondary antibodies were also supplied by DAKO. Avidin Biotin Block, Vector Red® Alkaline Phosphatase Substrate Kit I and Vector® M.O.M. Immunodetection Kit were supplied by Vector® Laboratories and used according to manufacturers stated guidelines.

2.1.6 Image Acquisition and Analysis

Histology

Both "white light" and "ultra-violet" microscopy images were captured using a Leica DC 200 digital imaging system and Leica Image Management software. Tissue culture images were captured using the same camera.

Tissue Analysis

Macroscopic *in vivo* images were captured using either 35 mm Photography (using Nikon F60 SLR camera, Sigma 28-80 Zoom Lens) or by digital photography using a Casio QV-3500 EX. Colour slide film (Kodak Elite Chrome ASA 100) was purchased from Jessops Photography. Film development was performed by the Medical Illustration Department at Mount Vernon hospital.

Ex vivo photography was performed using UVP Epi Chemi II Darkroom and Labworks image capture software. Image analysis was performed on digital images using Scion Image 4.0.2 (Scion Corporation). Digital images were captured from 35 mm photographic film using a Canon CanoScan FS 2710 film scanner.

2.2 Methods - General Tissue Culture

2.2.1 Media

Normal Growth Medium

Media was made as 500 ml aliquots and stored at 4°C for a maximum of 6 weeks. Normal growth medium for fibroblasts (NGM) consisted of 90% DMEM and 10% FCS supplemented with 2 mM L-glutamine, 100 Units / ml Benzyl penicillin and 100 µg/ml Streptomycin.

Growth Factor Dialysed Medium

Growth Factor Dialysed medium was made as detailed in Appendix 1. It comprises of Dulbecco's Modified Eagles Medium, 10% Growth Factor Dialysed Foetal Calf Serum (GFD-FCS), l-Glutamine and Penicillin / Streptomycin. In order to make GFD-FCS,

foetal calf serum was dialysed in Size 2 (14.3 mm) dialysis membrane (Medicell), MWCO 12-14 kDa, against a 20-fold excess of PBS (without Calcium and Magnesium) for 5 days.

2.2.2 Fibroblast Isolation and Culture

Fibroblasts were cultured from normal human skin using an explant technique. The skin was obtained with both patient and Local Ethical Committee approval. Only tissue that would otherwise be discarded was used. Skin was transported from the Plastic Surgery theatres wrapped in moist saline-soaked swabs in sterile universal containers. It was dissected and any extraneous tissues removed (such as fat and hypodermis) and then minced finely with a scalpel to yield 1-2 mm^3 pieces. Two to three such pieces were placed in a sterile 25 cm^2 tissue culture flask, and allowed to adhere before covering with standard fibroblast growth medium (NGM). Explants were incubated at 37°C, 5% CO_2 for 1 week before changing the media. Thereafter media was changed twice weekly and cultures split when the cells were approaching confluence.

To split fibroblast cultures, fragments of tissue were removed (if present) with sterile forceps. The culture monolayer was washed twice with sterile PBS (without Ca and Mg). Cells were harvested using 1:10 Trypsin:Versene (2.5% trypsin) solution at room temperature, observing for signs of cell detachment from the flask. Upon detachment, the trypsin solution was neutralised with an excess of normal growth media (NGM). If originating from a 25 cm^2 flask the cell suspension was transferred directly to a 75 cm^2 flask and their passage number increased incrementally. In the case of 75 cm^2 flasks, cultures were split 1:3. All cells were used experimentally below passage 6.

2.2.3 Fibroblast culture for Immunohistochemistry

To allow immuno-histochemical analysis, fibroblasts were cultured on 24 mm x 24 mm glass coverslips (Chance-Propper). The coverslips were washed in 70% IMS (Industrial Methylated Spirit – Genta Medical) and allowed to air dry in a laminar flow tissue culture hood. Once dry, one coverslip was placed in each well of a 6-well plate.

Fibroblasts were harvested from tissue culture flasks as in Section 2.2.2 to yield a cell suspension. Cells were counted using a Fuchs-Rosenthal haemocytometer and the number of cells per ml of medium calculated (Section 2.2.5). Cells were centrifuged to form a pellet and resuspended in sufficient volume of Normal Growth Medium to yield 35000 cells per ml. Two millilitres of cell suspension was added to each well and the cells incubated overnight to allow attachment to the glass coverslip (i.e. $7x10^4$ fibroblasts per well).

Each well was washed with PBS (without calcium and magnesium) and 2ml of the appropriate test medium added to each well. All coverslip experiments were performed in triplicate. Media was changed thereafter twice a week and the coverslips harvested after 14 days (or as dictated by experimental protocols).

To harvest coverslips, medium was aspirated and each well washed with cold (4°C) PBS. This was also aspirated and 2 ml of ice-cold methanol added to each well. The plates were incubated at –20°C for 30 minutes before removing the coverslips and air-drying at room temperature on tissue paper. Coverslips were then mounted on glass slides with DPX fixative, ensuring that the fibroblast-colonised side remained uppermost. Slides were stored at –20°C until required.

2.2.4 Cryopreservation of cells

Cells were trypsinised to form a cell suspension before being centrifuged to from a pellet. The supernatant was aspirated and the resulting pellet re-suspended in cryopreservation medium (90% FCS and 10% DMSO). One millilitre aliquots of cells in cryopreservation medium were placed in cryovials, wrapped in 2 layers of tissue paper and transferred to a –80°C freezer, which allows the cells to freeze at approximately –1°C per minute. Vials were later removed from the freezer and transferred to liquid nitrogen for long-term storage.

To recover cells, the cryovial was thawed rapidly in a 37°C water bath. The cell suspension was transferred to a universal container and the cryopreservation mixture diluted through the slow addition of 10mls of fibroblast growth medium. The cells were

centrifuged at 172 x g for 5 minutes to form a pellet, supernatant aspirated and the pellet re-suspended in NGM before transferring to sterile tissue culture flasks.

2.2.5 Cell Numbers

The standard trypan blue exclusion method was used to obtain viable cell numbers where necessary. Briefly, the blue vital dye only enters and thus stains dead and dying cells whose cell membrane has become permeablised. Viable cells are able to exclude the dye, appearing undyed. Single cell suspensions in known volumes of media were mixed in 1:1 ratio with 0.4% trypan blue solution. Cells were counted in a Fuchs-Rosenthal haemocytometer and the number of viable cells calculated accordingly.

2.3 Methods – Cell / Tissue Analysis

2.3.1 Immunohistochemistry

To stain for αSMA, fibroblasts were grown and fixed on coverslips as described in Section 2.2.3. Fixed cultures were ringed with a "grease pen" and rehydrated in PBS for 5 minutes. Monoclonal anti-alpha smooth muscle actin antibody was diluted 1:800 in PBS prior to use. The PBS wash was removed and 100 µl of diluted antibody was added to the centre of each ringed area. The slides were incubated under humidified conditions for 45 minutes, before removing the antibody solution and washing in 3 changes of PBS. During the washing steps, the secondary antibody was prepared for use. FITC conjugated rabbit anti-mouse secondary antibody (DAKO) was diluted 1:400 in PBS. To this, propidium iodide (Sigma, Poole, UK) was added to give a final concentration of 2 µg/ml to allow for the identification of nuclei. 100 µl of this solution was again added to the centre of each ringed area, and the slides incubated in moist conditions in the dark for 1 hour. Following this, antibody solution was again removed before further washes in PBS as above. After washing, excess wash solution was dried from each slide and the coverslips mounted with with an aqueous mountant containing 14-Diazo dicyclo 2,2,2 octane (DABCO, Sigma) (Appendix 1) to preserve the fluorescent signal. The slides were stored at –20°C until required.

Stained coverslips were examined at 400x using a Zeiss Axioskop microscope under UV light via a FITC filter. This rendered the FITC labelled alpha-smooth muscle actin strands green and the propidium iodide counter-stained nuclei red. A minimum of three fields was chosen at random from each coverslip and images captured via the Leica DC 200 digital imaging system for later analysis. During the analysis, the total number of cells and the number of αSMA positive cells (myofibroblasts) were counted.

2.3.2 Western Blotting

Preparation of protein extracts from cells

Adherent cultures of cells were washed once with cold PBS and a volume of lysis buffer (Appendix 1) added (0.3 ml for a 25 cm^2 flask). Flasks were incubated on ice for 30 minutes with occasional rocking to ensure that the lysis buffer covered the entire culture surface which was then scraped free of cells using cell scrapers (Greiner). The extract was then transferred to a sterile microcentrifuge tube (eppendorf), centrifuged at 10000g for 10 minutes and the supernatant transferred to another sterile eppendorf. This was stored at –20°C until required for Western blot analysis. To determine the number of cells present, all cultures were initiated in duplicate, the number of cells in the partner culture being counted to allow for accurate loading onto the gel.

Western Blot Analysis

Protein extracts were thawed at room temperature and 100 µl added to 100 µl of 2x Reducing Sample Buffer (Appendix 1). Prior to loading, samples were heated at 95°C for 3 minutes. The loading volumes were adjusted to give equivalent numbers of cells per lane. Samples were loaded on to a 10% SDS-PAGE resolving gel with a 5% SDS-PAGE stacking gel (Appendix 1) along with 7 µl of Kaleidoscope Prestained Standard (BioRad). The samples were then electophoresed at 200 V for 50 minutes using the BioRad MiniProtean II Western Blotting System and BioRad PowerPak 300.

Protein was transferred to PVDF membrane (Hybond – Amersham Biosciences), prepared as per manufacturers instructions – brief soaking in methanol and 5 minutes washing in water before equilibrating in transfer buffer for a further 5 minutes. The gels were removed from the mounts and equilibrated in fresh transfer buffer for 5 minutes

prior to protein transfer. Protein was transferred in a BioRad Transblot SD (Semi-Dry Blotter) at 25 V and 200 mA for 1 hour. Following protein transfer, the gels were checked for the efficiency of transfer and the membranes placed into blocking buffer (Appendix 1) for a minimum of 1 hour to reduce background during the staining procedure.

Staining of Gel

To evaluate completeness of protein transfer, gels were stained with Coomassie blue. Briefly, gels were incubated with Coomassie stain (Appendix 1) for a minimum of 1 hour before destaining with several changes of Coomassie destain buffer (Appendix 1) until bands became apparent.

Detection of αSMA

Following removal from blocking solution, the membranes were washed 3 times (10 minutes each wash) in Tween-Tris Buffered Saline (TTBS – Appendix I). The membranes were then incubated in monoclonal anti-alpha smooth muscle actin antibody (Sigma Ltd Clone 1A4) diluted 1:1000 in Incubation Buffer (Appendix 1) for 1 hour on an orbital shaker at room temperature. This was followed by three further 10 minute washes in TTBS before addition of the secondary antibody, an Alkaline Phosphatase conjugated Rabbit anti-mouse Fc-specific monoclonal antibody diluted 1:1000 in Incubation Buffer. The membranes were incubated with the secondary antibody for 1 hour at room temperature on the orbital shaker before washing 3 times as above. The presence of alpha smooth muscle actin was visualised by use of the Vector Red® Alkaline Phosphatase Substrate Kit I prepared according to the manufacturers instructions: adding 2 drops of Reagents 1, 2 and 3 to 5 ml of 200 mM Tris-HCl pH 8.2 buffer, mixing well between reagents. The membranes were incubated in this substrate for 5-10 minutes or until a strong signal was apparent. Following development, the membranes were rinsed thoroughly in tap water to terminate the colour reaction. Images of the developed membranes were captured at sub-saturated intensities using the UVP Epi Chemi II Darkroom and Labworks software to allow semi-quantitative analysis. Membranes were dried between 2 sheets of filter paper for storage.

Staining for total protein

Following staining for alpha smooth muscle actin, the membranes were washed 3 times in TTBS before being incubated in PBS containing 1 µl per ml India Ink (Windsor&Newton) for 30 minutes. The membranes were destained in multiple changes of PBS until bands were resolved.

2.3.3 Crystal Violet Proliferation Assay

Initiation and routine care

Fibroblasts were harvested from monolayer cell culture (Section 2.2.2), and counted (Section 2.2.5). 6000 viable cells were seeded in 200 µl of medium per well of a 96-well plate. One lane on each 96-well plate was left free of cells, acting as a background to correct for any differences due to staining between plates. To those wells that were not seeded with fibroblasts, 200 µl PBS was added to reduce evaporation from the test wells. The 96-well plates were incubated overnight to allow cell adhesion and then all media aspirated carefully. Wells were washed once with 200 µl of PBS before adding 200 µl of the appropriate test medium. Wells were washed and or media changed in sets of 3 to avoid the wells drying out.

For the routine care of cultures, media was carefully aspirated from each well and replaced with 200 µl of the required test medium. This process was repeated every 3 days until completion of the experiment.

In addition to the test plates, a control plate was initiated containing fibroblasts seeded at differing known densities. The fibroblasts were allowed to adhere in 200 µl of NGM overnight and the plate stained the following day to confirm increasing Optical Density (OD) with increasing fibroblast number. This permitted the development of a standard curve of fibroblast number vs optical density. In addition, each experiment set up using the crystal violet assay was repeated and the cell numbers assessed by trypsinisation and counting at least once in order to validate the results of the crystal violet assay.

Staining and reading

Plates were stained with Crystal Violet stain (Appendix 1) and the optical density measured (Biorad Model 550, Microplate Manager 4.0) at 595 nm. Briefly, the media was aspirated from each well and 100 µl of crystal violet stain added. The plates were incubated at room temperature for 10 minutes to allow for fixation of the cells and "uptake" of the stain. After the incubation period, the crystal violet stain was removed and the plates washed twice with PBS to remove any unbound stain and the plates were drained free of excess PBS. 100 µl of 33% acetic acid was then added to each well and the plates incubated at room temperature for a further 10 minutes in order to resuspend the remaining crystal violet. 50 µl aliquots of this final solution were transferred from each well to a clean 96-well plate for determination of optical density at 595nm.

Validation of assay

Fig 2.3 shows a "standard curve" obtained by seeding dermal fibroblasts in triplicate wells of the 96 well plate at densities ranging from 1500 cells per well to 12000 cells per well. This figure illustrates the linear relationship (R^2=0.9935) between increasing cell density and increasing OD over the cell number range tested.

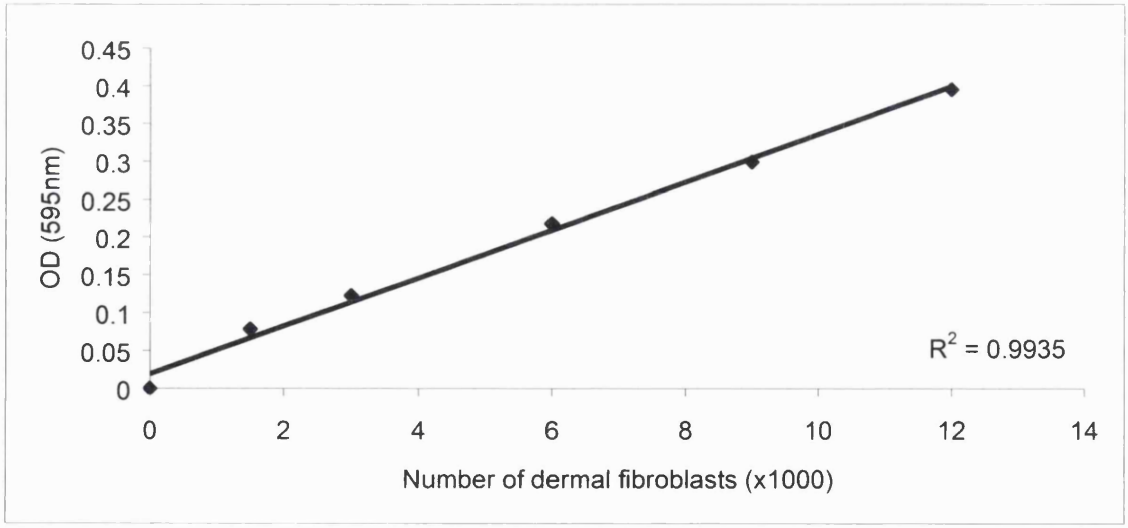


Fig 2.3 Graph showing the relationship between cell density and optical density (measured at 595 nm) following staining of cultures with crystal violet stain.

Changes in optical density relate to changes in dye uptake by the cell, and can be therefore increased by either increasing cell number or by increasing mean cell size. The occurrence of the latter process was discounted by culture of dermal fibroblasts in NGM, GFD and GFD with insulin. Medium was refreshed twice weekly. After 14 days, the number of cells present in each treatment was determined after trypsinisation of the monolayer and counting with a haemocytometer (Section 2.2.5). Counting the number of cells present with each treatment showed increases in cell number of a magnitude similar to that determined with the crystal violet assay.

2.3.4 Protein Assay

The total amount of protein present in samples was determined through use of the Pierce protein assay kit, following the manufacturer's instructions. The assay substrate was initially diluted 1:4 with distilled water and filtered using Whatman #1 filter paper. A series of Bovine Serum Albumin fraction (BSA) protein standards were made from serial dilutions of a 1 mg/ml solution: 0.5, 0.25, 0.125, 0.0625 mg BSA/ml distilled water. 10 µl of the standards and samples were added in triplicate to individual wells in a 96-well plate. Aliquots of the samples were diluted 1:1 with distilled water and 10 µl of this solution added to clean wells. 100 µl of the diluted assay substrate was added to each well before incubating the plate at room temperature for 5 minutes. Plates were then read in a BioRad M550 Micro-Plate Reader at 595 nm. The standard curve was plotted and was then used to calculate the protein concentration of the various test samples.

Test samples were derived from conditioned medium from fibroblast cultures. Three fibroblast cell lines, each initiated from a different patient, were cultured in NGM, GFD or GFD with the addition of insulin (Insulatard at 1 IU/ml). Each cell line was cultured in quadruplicate for each medium tested. Medium was refreshed twice weekly until confluence was achieved (as assessed using an inverted phase contrast microscope). Thereafter, the monolayers were washed with PBS (Phosphate Buffered Saline) prior to addition of "incubation medium" (Table 2.1). Incubation medium differs in composition from the normal medium used for culture of fibroblasts in containing lower levels of FCS (either normal or dialysed). The conditioned cultured medium collected in the

course of this experiment was to be used for both protein and collagen assays. For the purposes of the collagen assay, the presence of FCS in concentrations greater than 5% is reported to cause inaccuracies with measurement (Section 4.2.3) and therefore incubation medium was used as described.

Group	Pre-incubation medium	Incubation medium
A "NGM"	DMEM, Benzyl Penicillin Streptomycin, l-glutamine 10% FCS	DMEM, Benzyl Penicillin Streptomycin, l-glutamine 5% FCS Ascorbic acid
B "GFD"	DMEM, Benzyl Penicillin Streptomycin, l-glutamine 10% FCS – Dial (Dialysed FCS)	DMEM, Benzyl Penicillin Streptomycin, l-glutamine 5% FCS – Dial (Dialysed FCS) Ascorbic acid
C "GFD + Insulin"	DMEM, Benzyl Penicillin Streptomycin, l-glutamine 10% FCS – Dial Insulatard 1 IU/ml	DMEM, Benzyl Penicillin Streptomycin, l-glutamine 5% FCS – Dial Insulatard 1 IU/ml Ascorbic acid

Table 2.1 Composition of dermal fibroblast Pre-incubation and Incubation media for the experiments to determine protein and collagen production.

Fibroblasts were cultured in Incubation medium for a further 48 hours before media was collected from each well. Cell number was determined for each well and used to compensate for differences in proliferation between treatments. The amount of soluble protein present in the supernatant from each well was measured in duplicate using the Pierce Protein Assay, as described above previously. In addition, fresh culture medium was assayed and the amount of protein present subtracted from the results obtained from assays of conditioned media.

2.3.5 Collagen Assay

Initiation of cultures

Fibroblasts were harvested via routine trypsinisation from tissue culture flasks (Section 2.2.2), counted and resuspended in Normal Growth Medium to yield 40000 cells per ml. Five hundred microlitres of cell suspension was added to each well in a 24-well plate. After allowing the cells to adhere overnight, the wells were washed twice with PBS and 500 µl of pre-incubation medium added (See Table 2.1). Thereafter medium was changed twice weekly until confluence had been achieved in all wells. Following a further day to ensure confluence, the monolayers were washed twice with PBS and then 500µl of incubation medium added to each well. After a 48 hour incubation under standard conditions, the medium from each well was collected in labelled eppendorfs. The remaining monolayer was washed twice with PBS, and the cells trypsinised and counted. Collected medium was stored at –20°C until required for the collagen assay.

Collagen assay

Collagen assays were performed using the Biocolor Sircol Collagen Assay kit. This assay relies on the selective binding of a coloured dye ("Sircol Dye Reagent") to the [Gly-X-Y]n tripeptide sequence found within the triple helix sequence of mammalian collagens.

To perform the assay, 1 ml of Sircol Dye Reagent was added to the supplied micro-centrifuge tubes. Collected medium was allowed to defrost at room temperature and 200 µl added to the Sircol Dye Reagent. A collagen dilution series was made from the supplied 1 mg/ml collagen standard. 1 ml of Sircol Dye Reagent was added to 100 µl of each dilution. Each sample and standard was assessed in duplicate. The samples were mixed and placed on a mechanical shaker, incubating at room temperature for 30 minutes. This incubation phase allows the collagen in the medium to bind to and thus precipitate with the dye reagent. The tubes were then transferred to a micro-centrifuge (Sanyo) and spun at 10000 x g for 5 minutes to collect the precipitate at the bottom of the tubes. The supernatant was poured off and any remaining supernatant removed by carefully wiping the top half of the tube. To reduce background measurements, 1 ml of 99% ethanol was added to each tube, the contents mixed and then centrifuged again at 10000 x g for a further 5 minutes. Again the supernatant was poured off and the

precipitate saved. 1 ml of Alkali Reagent was added to each tube and mixed using a vortex mixer. This step releases the bound dye into solution. Once all the precipitates had been dissolved, 100 µl samples were transferred to a clean 96-well plate for measurement of optical density at 540 nm. All measurements were performed in duplicate.

2.3.6 3D Collagen gels

Extraction of Rat Tail Collagen

Rat tail collagen was extracted according to the protocol provided with the Sircol collagen assay kit. Briefly, rat tails were harvested and stored at –20°C until required. Prior to use, a number of rat tails were removed and allowed to defrost overnight at 4°C. Once defrosted, using sterile instruments and in a Class I tissue culture hood, the skin was removed from the rat tails exposing the collagen fibrils of the rat tail tendons. The fibrils from 2-3 tails were removed and placed into 200 ml of ice cold 0.5 M sodium chloride (NaCl). This was incubated at 4°C for 2 hours with stirring and the NaCl changed. After overnight washes in 0.5 M NaCl to remove traces of blood that might have been present on the fibrils, the tails were incubated for a further 48 hrs in 0.5 M acetic acid, again at 4°C with stirring.

Once the collagen had dissolved, the solution was filtered with a fine wire mesh to remove any remaining particulates. A small aliquot of the sample was taken for analysis to determine the collagen content (as described in Section 2.3.5). An appropriate amount of 0.5 M acetic acid was added to give a final collagen concentration of 1 mg/ml. This final solution was then aliquoted and stored at 4°C until required.

Preparation of 3D collagen gels

Fibroblasts were harvested via routine trypsinisation (Section 2.2.2), the number of viable cells present per ml calculated (Section 2.2.5) and sufficient cells to yield 30000 cells / ml of collagen gel were then transferred to a clean 50ml falcon tube. Cells were pelleted at 172 x g for 5 minutes. During centrifugation of the cell pellet, collagen gels were made by mixing 90% collagen mix (1 mg/ml) and 10% 10x MEM. All of the

components of the collagen mix were kept on ice at all times to prevent premature setting of the collagen.

Addition of MEM to the collagen mix results in a change of pH and thus a colour change (due to the indicators present in MEM). In order to encourage cell survival, the pH of the solution must approach neutral, and thus neutralisation of the acetic acid within the collagen gel mix is undertaken via addition of NaOH. Sodium hydroxide is added dropwise with mixing until the solution attains a red / orange colour.

As soon as the cells were pelleted and the pH of the collagen mix corrected, the cell pellet was disaggregated. The collagen gel was then mixed with the dispersed pellet, taking care not to introduce bubbles into the solution and then aliquoted into 3cm diameter petri dishes (bacterial grade). The dishes were transferred to the incubator to allow the collagen mix to set before 1 ml of fibroblast culture medium (either NGM or GFD) was added. Following addition of the fibroblast medium, the gels were loosened from the sides of the petri dishes with a scalpel. This allows the fibroblasts to contract the collagen gels.

Analysis of contraction

Images of the fibroblast populated collagen gels were captured using the UVP Labworks digital imaging system at 12-hour intervals. Measurements from each time point included perimeter, area and gel diameter and were made using the ScionImage image analysis software. To allow for comparison between gels, the diameter of the petri-dish container was also measured and used as a reference measurement.

2.3.7 Detection of Apoptosis

Detection of apoptosis was undertaken using the APOPercentage® kit (Biocolor) according to the manufacturer's instructions. This kit includes a dye that is selectively and specifically imported by cells committed to apoptosis (necrotic cells are not dye labelled). In apoptotic committed cells, flippase regulation (required to maintain the asymmetric composition of the phospholipid cell membrane) is either overwhelmed or inactivated by the activity of scramblase (floppase). Flipping of phospholipid can be

detected by the binding of annexin V to exposed phophatidylserine on the outer leaflet of an apoptotic cell. However, alternatively, the transfer of phosphatidylserine to the outer surface of the cell permits unidirectional transport of APOPercentage Dye into the cell (Manufacturer's information).

Prior to experimentation, each well of a sterile 96 well plate was coated with 100 µl of sterile 0.2% gelatin (provided in Apopercentage kit) for 48 hours at 4°C. The presence of this layer is thought to enhance the anchorage of fibroblasts (Apopercentage kit manual). Excess gelatin was removed, and 5000 fibroblasts added to each well. Cells were cultured under standard conditions until cultures had attained greater than 90% confluence in all wells.

To identify apoptosis in cultures, APOPercentage Dye Label was diluted 1:20 in fresh culture medium and 100 µl added to each well. Medium under investigation included NGM and GFD, both with and without addition of Insulatard® at 1 IU/ml. The 96-well plate was returned to the 37°C incubator for 1 hour before draining medium from each well and washing twice in PBS. Plates were then examined using an inverted microscope, with apoptotic cells staining purple-red and viable cells unlabelled. The number of apoptotic cells per field was calculated.

To measure the number of apoptotic cells in each well, 100 µl of Dye Release Reagent was added to each well for 10 minutes. The resulting coloured solution was transferred to a clean 96-well plate and read in a plate reader at 550 nm.

2.4 Methods - Murine Scarring Model

2.4.1 Husbandry

All animals used within this study were housed at the Northwick Park Institute of Medical Research. Experimental procedures were undertaken under license from the Home Office (Animals Scientific Procedures Act 1986); PIL 70/15696 and PPL 70/4780. Following purchase, mice were acclimatized to their surroundings for a period of not less than one week, with a maximum of 10 mice per cage. All animals were kept

in climate controlled rooms 22-24°C and 45-55% humidity with 12 hours light and 12 darkness as specified in current Home Office guidelines. Water and feed were supplied *ad libatum*. Following surgical procedures, mice were housed individually to prevent them from disturbing each other's wounds.

2.4.2 Anaesthesia

All operative procedures and wound infiltrations with test substances were performed under inhalational general anaesthesia, using the equipment shown in Fig 2.4. No mouse had more than one general anaesthetic per day. Animals were transferred to an anaesthetic chamber and anaesthesia induced by 5% Halothane / 2 litres oxygen. Anaesthesia was maintained with 1.5% halothane / 1 litres oxygen via a "face mask". Mice were recovered individually in a clean cage following completion of the operative procedure / injection and returned to their housing once normal activities had been resumed. Euthanasia was performed by cervical dislocation after anaesthesia. All wounds were harvested following euthanasia.

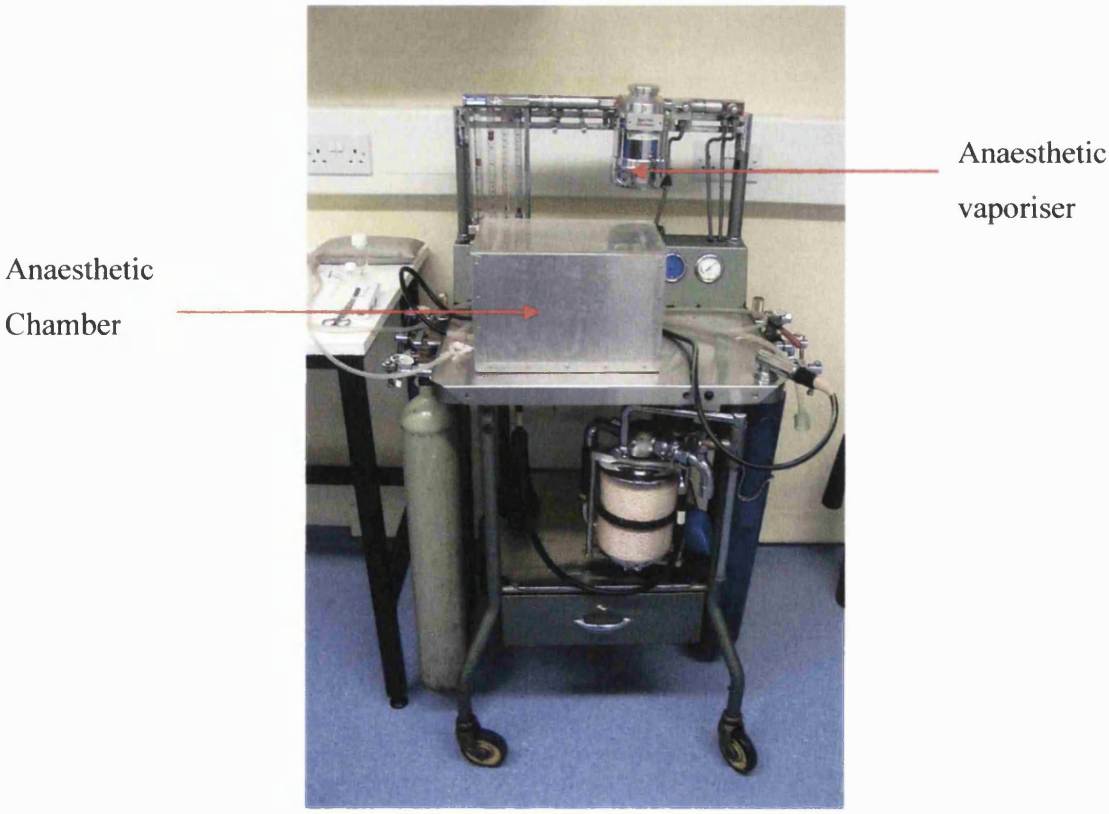


Fig 2.4: Anaesthetic machine showing vaporiser and anaesthetic chamber used for mouse anaesthesia.

2.4.3 Operative Procedures

Procedures performed included creation of incisional wounds, creation of excisional wounds and local infiltration of wounds with test substances as follows.

Incisional Wound

After general anaesthesia, both posterior flanks on male BALB/c mice were shaved and cleaned with chlorhexidine in spirit. Two 1.5 cm wounds were marked (one in each flank) parallel to the spine with permanent marker. Full thickness incisions were made along these marks down to the level of the chest wall. Both lateral skin flaps were dissected from the underlying chest wall and the wound assessed for haemorrhage. The wound edges were then infiltrated with test substance or vehicle only control as necessary. Wounds were closed with interrupted 5/0 Prolene® sutures without further wound dressing. Mice were recovered from the anaesthetic individually and housed in groups of four.

Excisional Wound

Both posterior flanks of the male BALB/c mice were shaved and cleaned with chlorhexidine in spirit. Using a 5 mm punch biopsy, full thickness cutaneous wounds were created, one in each flank. Wounds were assessed for haemorrhage, applying pressure until cessation of the bleeding. Wound edges were dissected from the underlying tissue. The skin flaps were infiltrated with test substance as necessary. Wounds were dressed with Jelonet® (Smith and Nephew Medical), followed by saline gauze and secured with elastoplast tape. Mice were recovered from the anaesthetic and housed individually.

Local Infiltration

Infiltration with test substance was performed at the time of wounding, by infiltration into the wound edges. 10 IU Insulatard® was diluted in 10 ml normal saline (0.9%) to give a final concentration of 1 IU/ml. Each mouse received a maximum dose of 0.15 ml of this insulin solution (0.15 IU). Further infiltrations were performed every 3 days until the required number of doses had been given. Injections were given using the same

amount of insulin and the same volume. Substances were injected intradermally along the centre of the wound using a 25G needle. For the excisional wounds, using the same concentration and same volume, test substance was applied both to the centre of the wound and the circumference, ensuring complete infiltration of the entire wound with the test substance.

2.4.4 Wound Evaluation

Animal well-being was evaluated everyday by the animal technicians at NPIMR. Animal wounds were examined every 3 days and any untoward findings noted. At 2 weeks the animals were anaesthetised and the wounds shaved to allow for closer inspection. Remaining sutures were removed and scar dimensions noted. The surface of the scar was marked with permanent marker to allow for easier identification during tissue processing.

2.4.5 Wound Harvest

At 2 weeks (or when determined by experimental protocol) animals were anaesthetised and euthanised by cervical dislocation. The precise method for wound harvest was dependent upon the further processing required and is detailed below.

Cell culture

To obtain tissue for cell culture, following euthanasia, the area of interest was shaved and then swabbed with Chlorhexidine in spirit. The mouse was then doused in Chlorhexidine and left to air dry for 5 minutes. The mouse was then transferred to a saline-soaked gauze swab and placed within a suitably sized universal container, which was immediately transported to the tissue culture laboratory on ice for processing (section 2.2.2). These steps were undertaken in order to reduce the occurrence of infection (both fungal and bacterial) within the cultures without the need for addition of anti-fungal or antibiotic drugs, that can influence cell proliferation and differentiation.

Histology

For histological analysis of murine tissue, the mouse was euthanised and the skin overlying the wounds shaved. The extent of the wound was marked with permanent marker and any remaining sutures removed. Each wound was removed separately, excising a 5-10 mm cuff of normal tissue around the scar. Each scar was excised as a full thickness excision down to the chest wall musculature. The tissue was placed into 10% formal-saline following harvest and transported to the histology laboratory for further processing.

Protein Preparation

The mouse was euthanised and shaved as above. The wound was identified and marked and any remaining sutures removed. All wounds were removed separately by full thickness excision down to the chest wall. A 1 mm cuff of normal tissue was excised around each scar. Following harvest, wounds were placed in cryovials and transported to the laboratory on ice. All wounds were snap frozen in liquid nitrogen and stored at –80°C until required.

Tenisometry

Wounds were harvested as described previously. However, the tissue was not placed into formal saline but rather wrapped in a saline soaked gauze swab and then placed into a sterile universal for transport to the laboratory for tensiometric testing (Section 2.4.10). Tensiometry was performed on each sample within 1.5 hours of tissue harvest. Specimens were mounted at a fixed distance from each of the two clamps in a materials testing machine (University of Westminster) and stretched at a constant speed of 10 mm per minute until failure, with continuous load (force) measurement. Stress values were calculated by normalising the load values to the cross-sectional area of each specimen.

2.4.6 Murine Fibroblast Culture

The skin was prepared, washed in sterile PBS 3 times before briefly immersing in 70% IMS (Industrial Methylated Spirit) to prevent the risk of microbial contamination of the cultures. The skin was then allowed to stand in a sterile petri dish for up to 1 minute before being minced finely. Small portions of murine skin were placed in 25 cm^2 tissue

culture flasks and allowed to adhere to the flask before addition of normal fibroblast growth medium (NGM). The flasks were inspected daily and those showing evidence of contamination discarded. Murine cultures were then treated in the same manner as human derived fibroblast cultures (Section 2.2.2). Medium was changed twice per week and cultures split 1:3 as required to a maximum of passage 6. Excess cells were cryopreserved using the same protocol as for human fibroblast cultures.

2.4.7 Histological Evaluation of Fixed Tissue

All wounds that were to be evaluated histologically were fixed in 10% buffered formalin in normal saline. To prevent "curling up" of the tissue (and hence distortion of the architecture), freshly harvested wounds were sandwiched between 2 layers of formal-saline soaked sponges within histology cassettes. After soaking in formal-saline for a further 24 hours to allow for complete fixation of the murine tissue, the wounds were removed from between the sponges and cut as shown in Fig 7.4) to yield tissue of a maximal thickness of 2 mm. The tissue fragments were again sandwiched between formal saline soaked sponges and fixed for a further 1 day before being dehydrated in graded alcohol, cleared in toluene and embedded in paraffin wax. Four μm sections were taken to show the scar in cross-section and transferred to glass slides. Sections were then stained with haematoxylin and eosin (H&E) and Masson's Trichrome. Sections for immunohistochemical analysis were cut at 4 μm thickness and mounted onto aminopropyltriethoxy-silane (APES) coated slides before being air-dried in an oven at 37°C overnight.

2.4.8 Immunohistochemistry

As the available anti-alpha smooth muscle actin antibody (Sigma) was raised in mouse, use of this antibody to stain murine tissue would be complicated by the quantity of background staining. In order to reduce the amount of background, a number of blocking steps were undertaken. After dewaxing the sections serially through xylene and alcohol to water, the sections were microwaved in citric acid buffer (ph 6.0) for 10 minutes to retrieve antigens lost during the fixation procedure. Sections were quickly transferred to cold tap water and then washed in 20% acetic acid to block intrinsic

alkaline phosphatase activity (allowing the use of an alkaline phosphatase detection kit to visualise the antibody). Sections were washed well in TTBS before adding the Vector® Avidin-biotin block. One drop of each block was added to each section for 15 minutes before washing well in TTBS. The M.O.M. Immunodetection Kit (Vector®) protein block was reconstituted as per kit instructions and added to each section, incubating for 1 hour.

Sections were then washed in TTBS and then incubated for 5 minute in M.O.M. Immunodetection Kit diluent. Anti-alpha smooth muscle actin antibody was diluted 1:1000 in M.O.M. Immunodetection Kit diluent and added to the sections, incubating for 30 minutes. After 3 further washes in TTBS, sections were incubated in M.O.M. Immunodetection Kit Biotinylated Anti-Mouse IgG Reagent. Sections were again washed with TTBS and then incubated in Alkaline Phosphatase conjugated anti-Streptavidin antibody diluted 1:200 in M.O.M. diluent for 30 minutes.

Following further washes, the Vector Red® Alkaline Phosphatase Substrate Kit I was made up according to manufacturers instructions and added to each section, incubating for 20 minutes at room temperature. Sections were then washed in TTBS, counterstained with Harris haematoxylin and washed well in tap water, before being dehydrated, cleared and mounted with DPX. Sections were viewed using a Zeiss Axioskop microscope and the intensity of alpha smooth muscle actin staining assessed and graded for each scar, as described in the following section. Images were captured as per Section 2.1.6

2.4.9 Analysis of murine wounds

To score the αSMA staining of the murine sections, for each scar, the wound was graded. A score was allocated for both the superficial and deep components of the scar according to the table overleaf. Following examination of the scar under x400 magnification, if no αSMA staining was detected the section was allocated a "negative" grade. If a few scattered foci of stain could be seen, this was graded "weak". Roughly equal areas of positive and negative staining were graded "moderate" whereas scars that contained more stained cells than unstained were awarded a "Strong" grade. The grade

was then converted into a numerical score (see Table 2.2). All scars were assessed at a minimum of 3 random fields along their length and the scores averaged. Representative images of murine wounds stained for alpha smooth muscle actin, and their scores, can be seen in Table 2.2. The mean score was used for comparison between treatments.

2.4.10 Tensiometry

All tensiometry was performed at the University of Westminster. The specimen was removed from the saline soaked swab and processed for tensiometry. Placing the specimen on top of an array of blades, separated by 2 mm gaps, a downwards force applied via a rolling block of wax cut the specimen. Each section of specimen was 2 mm. The precise dimensions of each piece of tissue were ascertained by use of a Nikon Shadowmeter and the tissue section mounted on the tensiometer. Care was taken to ensure that the scar was a uniform distance away from the clamps: In these experiments a distance of 6 mm.

The machine was initiated and each section of the scar tested to destruction, with the results recorded on a plotter (Hewlett-Packard). After completion of testing, each specimen was examined macroscopically to identify the point at which the integrity of the specimen failed.

2.5 Statistics

Categorical data (myofibroblast grading of *in vivo* experiments) were compared between two groups using chi-squared with Yates' continuity correction for small numbers. Ordinal data (myofibroblast proportions, optical density recordings) were subjected to the normality testing. If data was normally distributed, parametric comparative tests were performed and if the data failed the normality test, non-parametric comparative tests were performed. To compare 2 groups, either a Student's t test (parametric) or a Mann-Whitney rank sum test (non-parametric) was used. For comparison of several groups, one-way ANOVA (parametric) or Kruskall-Wallis (non-parametric) tests were used. Analysis was performed using SigmaStat™ statistics software, version 2.03 (Jandel Corporation) or Microcal Origin 6.0.

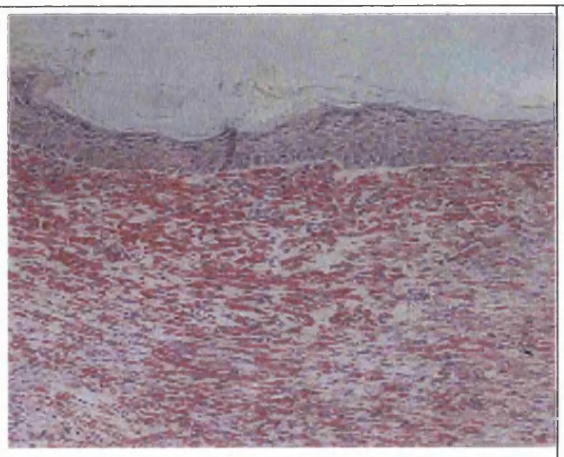
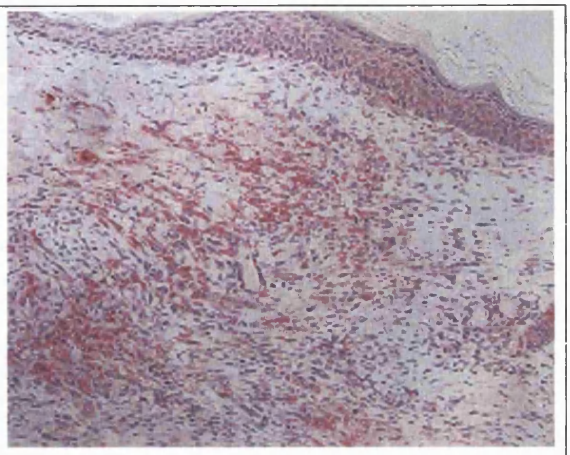
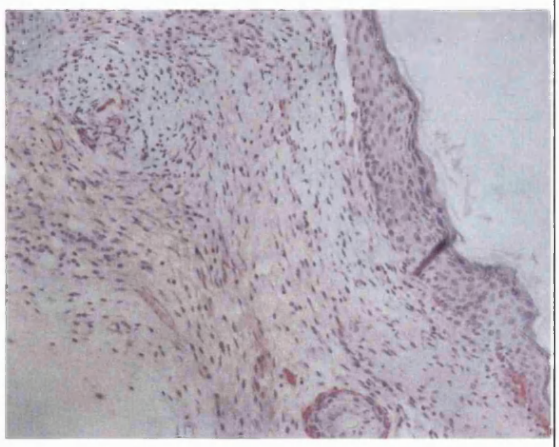
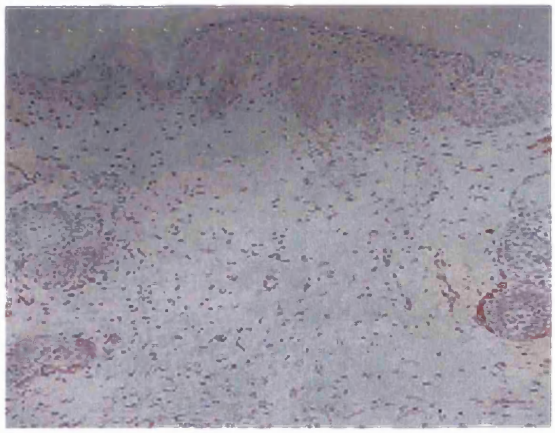
	
Strong (3)	Moderate (2)
	
Weak (1)	Negative (0)

Table 2.2 Table showing the grading system used for the analysis of alpha smooth muscle actin staining in murine scars (200 x).

Chapter 3

Determination of the

effect of insulin on fibroblast differentiation *in vitro*

3.1 Introduction

The aim of this thesis is to build upon the work previously performed by Linge et al. (2004) in order to develop a novel therapy for the reduction of cutaneous scarring. This work highlighted the role that insulin may play in fibroblast - myofibroblast differentiation suggesting that administration of insulin may reduce the formation of the myofibroblast phenotype, a cell thought to be of importance in the aetiology of pathological scarring (Lee & Vijayasingam, 1995), (Chipev, Simman, et al., 2000). However, all of the experiments performed by this group used laboratory grade bovine insulin, a substance that is not typically used in clinical practice. Although the use of bovine insulin is possible in man, owing to the existence of zoonoses such as Bovine Spongiform Encephalopathy (BSE), the use of human insulin would be preferable. In addition, as bovine insulin differs from human insulin by 3 amino acids, whether the reduction in myofibroblast numbers may be achieved using human recombinant insulin, or whether this effect is specific to bovine insulin must be investigated.

If recombinant human insulin proves to be effective in reducing fibroblast - myofibroblast differentiation, further questions need to be answered. Insulin is a commonly used drug, available as many different formulations, each offering different pharmacokinetic and pharmacodynamic properties. Such insulin preparations usually vary with respect to their onset of activity and duration of action (British National Formulary, 2001). Both of these factors may be important considerations for maximal reduction in myofibroblast numbers. This chapter aims to identify whether such preparations reduce fibroblast - myofibroblast differentiation *in vitro*, and to establish the most effective preparation. In order to accomplish this task, three of the commonly used clinical preparations with differing half-lives were chosen for testing (Novo Nordisk) (Table 3.1):

Name	Description	Time of onset	Maximum effect	Duration of action
Actrapid®	Short acting	30 minutes	1-3 hours	8 hours
Mixtard 30®	Intermediate acting	30 minutes	2-8 hours	24 hours
Insulatard®	Long acting	1.5 hours	4-12 hours	24 hours

Table 3.1: Table showing characteristics of insulin preparations chosen for investigation (Manufacturers data – Novo Nordisk)

Aims

- To determine whether the results of Linge et al. can be reproduced in the authors hands.
- To determine if recombinant human insulin can reduce fibroblast - myofibroblast differentiation as effectively as bovine.
- To establish the effects of 3 common clinical preparations of insulin on fibroblast - myofibroblast differentiation.

3.2 Results

3.2.1 Bovine Insulin

As previously performed by Linge et al., fibroblasts from normal human dermis were grown on sterile coverslips and treated for 14 days in NGM (Normal Growth Medium - containing 10% foetal calf serum and therefore insulin activity), or GFD (Growth Factor Dialysed Medium) alone or in GFD together with a titrated dose of bovine insulin (Sigma), from 6.25 μg/ml to 200 μg/ml. Each condition was replicated in triplicate and media refreshed twice weekly. Fig 3.1 shows the appearance of unstained fibroblasts in culture. The cells were harvested for immunohistochemical staining for alpha smooth muscle actin (αSMA) and the proportion of myofibroblasts assessed (See Fig 3.2 and Section 2.3.1). Each coverslip was assessed at 3 random fields at 200x magnification and the number of myofibroblasts and total cells in each field counted.

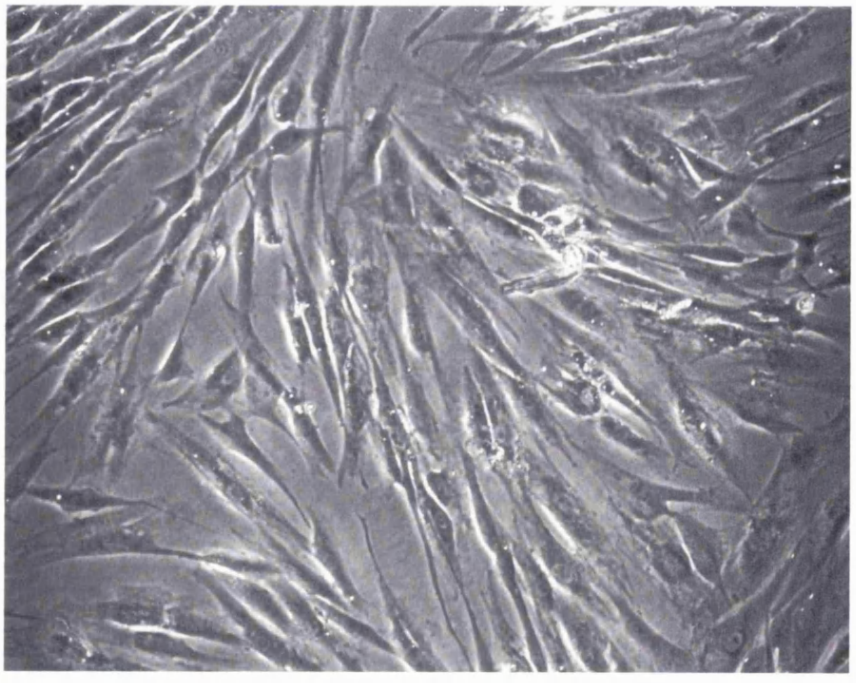
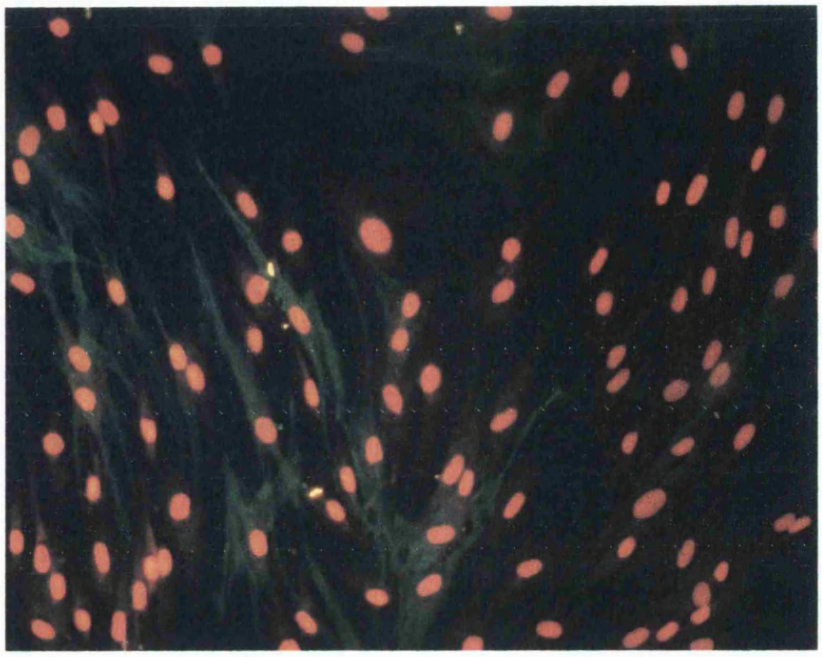


Fig 3.1 Unstained fibroblasts growing on glass coverslips as viewed using an inverted microscope (200 x).

A



B

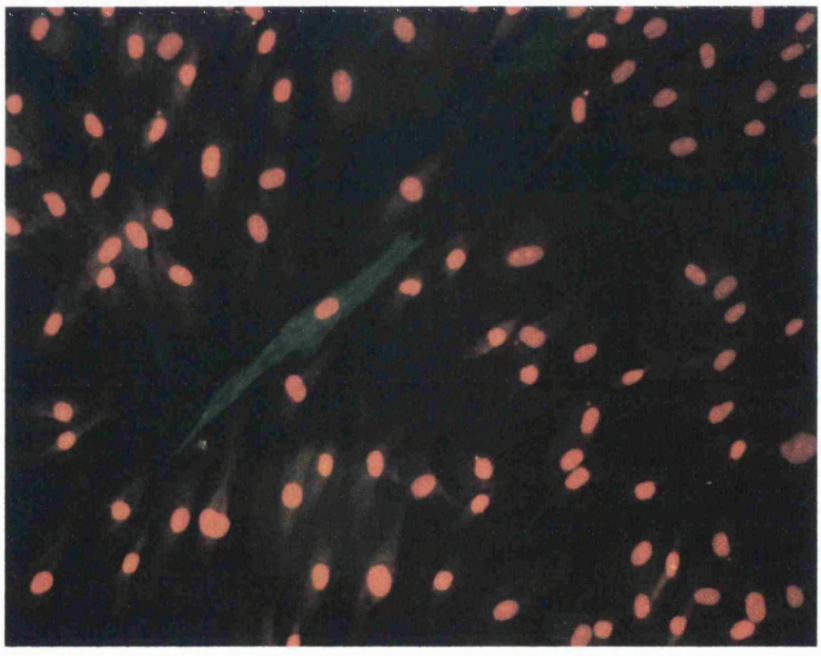


Fig 3.2: Immunohistochemical staining of dermal fibroblasts cultured in GFD medium ***(A)*** *and GFD medium supplemented with insulin* ***(B)*** *demonstrating alpha smooth muscle actin. Green stress fibres may be observed in positive cells (myofibroblasts). Propidium Iodide has been used to counterstain nuclei in order to identify all cells present and therefore calculate myofibroblast proportion (200 x).*

The experiment was repeated with 3 different fibroblast cell lines, and although the exact percentage of myofibroblasts varied between cell lines (0-5% for NGM and 9-27% for GFD) a similar pattern was observed. First, the proportion of myofibroblasts was always significantly increased in GFD compared to NGM. Also, the addition of insulin to GFD always decreased myofibroblast numbers down to that seen in NGM in a dose dependent manner. A representative example of this can be seen in Fig 3.3 where the proportion of myofibroblasts is plotted as a percentage of the total cells present. Here only 2.3% (± 1.6) myofibroblasts were detected in NGM compared to 9.4% (± 3.8) in GFD. This difference is significant ($P<0.05$). Addition of bovine insulin (Sigma) to GFD medium reduces the number of myofibroblasts in a concentration dependent manner, from 6.4% (± 1.5) if 12.5 μg of insulin is added to each ml of medium, to 1.7% (± 1.9) if this is increased to 200 μg/ml. The reduction in myofibroblast number from that seen in GFD alone is significant ($P<0.05$) for insulin concentrations of 25 μg/ml or greater.

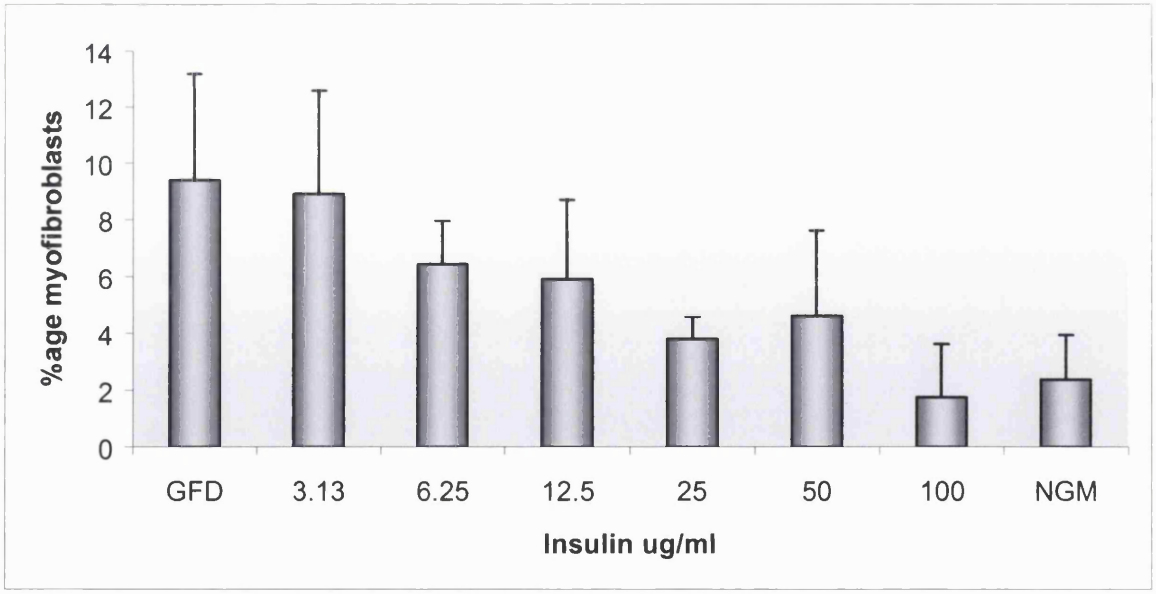


Fig 3.3: Graph showing the mean proportion of myofibroblasts in triplicate dermal fibroblast cultures, expressed as a percentage of the total cells, after 14 days treatment in NGM, GFD or GFD supplemented with a titration of bovine insulin (laboratory grade). Data shown is from a representative dermal fibroblast cell line. Error bars represent the standard deviation from the mean of triplicate coverslips.

Although absolute numbers of myofibroblasts varied for all treatments between the 3 cell lines tested, the proportional reduction seen with the addition of insulin was remarkably similar. Fig 3.4 shows the mean data from all three dermal fibroblast cell lines tested where the results are presented as the reduction in myofibroblast proportion from that seen in GFD media alone, expressed as a percentage of that seen in GFD media. Titration of 12.5 μg/ml insulin reduces myofibroblast proportion 22.0% (±21.5) from that seen in GFD. The reduction in myofibroblast proportion is greater for higher concentrations of insulin, reaching an 81.0% (±5.4) reduction for the addition of 200 μg/ml. In comparison culture of dermal fibroblasts in NGM results in a mean reduction of 87.4% (± 11.7).

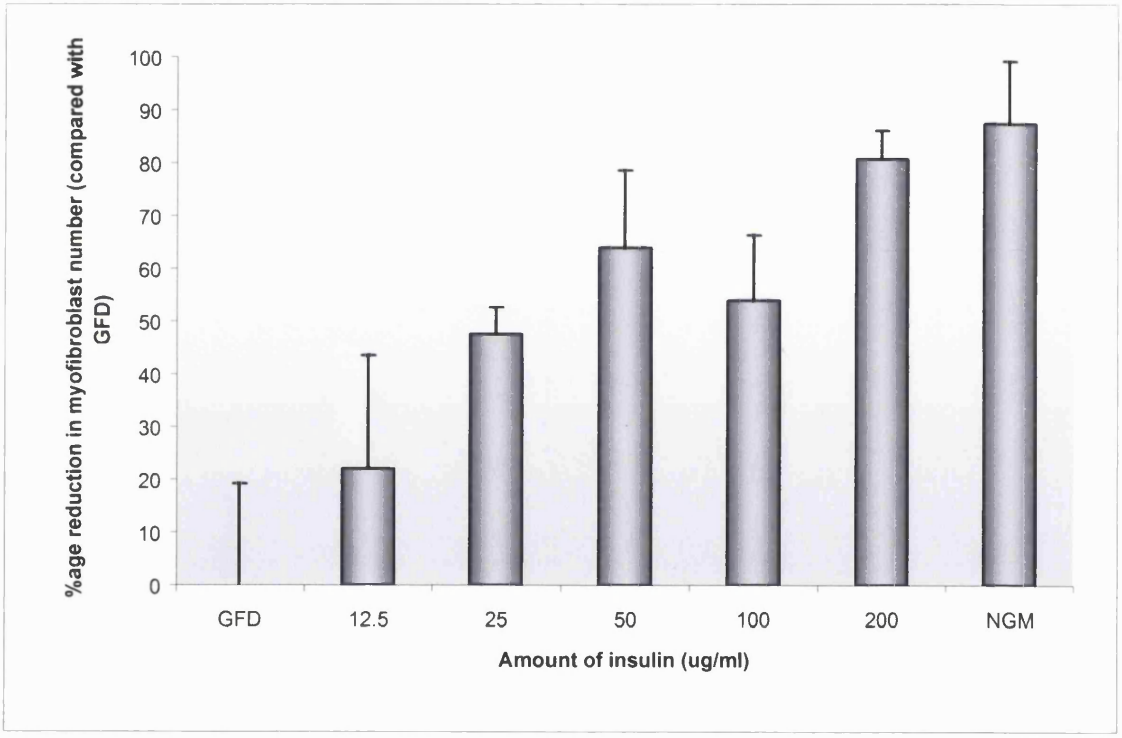


Fig 3.4: Graph showing the percentage change in myofibroblast proportion from that seen in GFD alone for all three dermal fibroblast cell lines tested. Error bars are standard deviations from the mean of 3 different cell lines.

Due to the variability in myofibroblast proportion for the same treatments between cell lines, to present data from more than one cell line, all further data in this chapter is presented as the percentage change in myofibroblast proportion from that seen in GFD medium (as seen in Fig 3.4).

3.2.2 Human Insulin

Immunohistochemistry

The ability of three pharmaceutical formulations of human insulin that differed with regard to their onset and duration of action (Table 3.1) were tested for their ability to reduce fibroblast - myofibroblast differentiation. Six dermal fibroblast cell lines (each initiated from a different patient) were cultured on sterile glass coverslips in triplicate in NGM, GFD or GFD supplemented with insulin. A titration range was derived from the conversion of the amount of bovine insulin used in the previous experiments into the more commonly used clinical dosage, the International Unit (IU). The provision of human insulin at 1 IU/ml would be equivalent to supplementing medium with bovine insulin at 50 μg/ml, a level that reduced myofibroblast proportion in the previous experiments by greater than 50%. A range of titrations was derived from this for all 3 preparations of insulin. After 14 days treatment the proportion of myofibroblasts present was assessed following immunohistochemical staining to demonstrate the presence of αSMA (Section 2.3.1).

Short acting formulation – Actrapid®

The results of immunohistochemical staining of normal dermal fibroblasts cultured for 14 days in NGM, GFD or GFD supplemented with a titration of Actrapid (a short acting formulation of human recombinant insulin, Table 3.1) can be seen in Fig 3.5. Here addition of Actrapid at 0.01 IU/ml results in a reduction in myofibroblast proportion (compared with that seen in GFD) of 19.4% (±14.3). Increasing the amount of Actrapid results in reduction in myofibroblast number of 42.9% (±3.8), 28.1% (±7.7), 51.5% (±5.8) and 29.6% (±13.8) respectively for solutions containing 0.05, 0.1, 0.5 and 1 IU/ml. Dermal fibroblasts cultured in NGM contained 71.6% (±5.7) less myofibroblasts than those cultured in GFD medium alone. The reduction in myofibroblast number was significant ($P<0.005$) for those fibroblasts cultured in NGM and GFD containing 0.05, 0.1 and 0.5 IU/ml Actrapid. Interestingly, the reduction achieved through the addition of Actrapid at 1 IU/ml did not reach significance on testing ($P=0.094$).

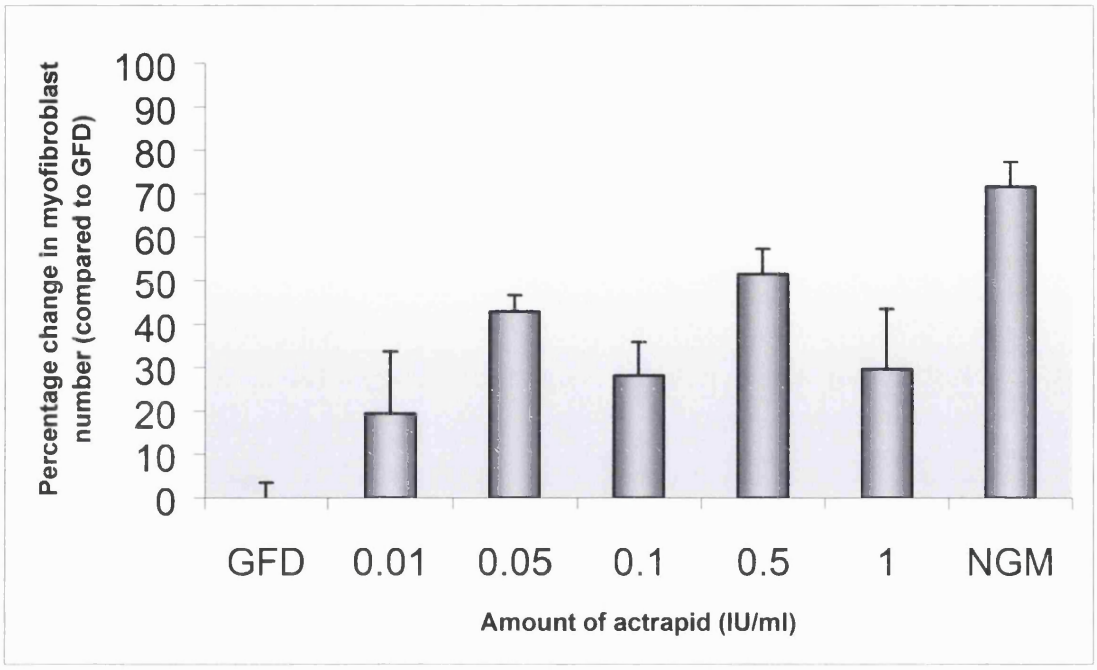


Fig 3.5: Graph showing the percentage change in myofibroblast proportion from that seen in GFD alone for all six dermal fibroblast cell lines tested. Error bars are the standard deviations from the mean of 6 different cell lines.

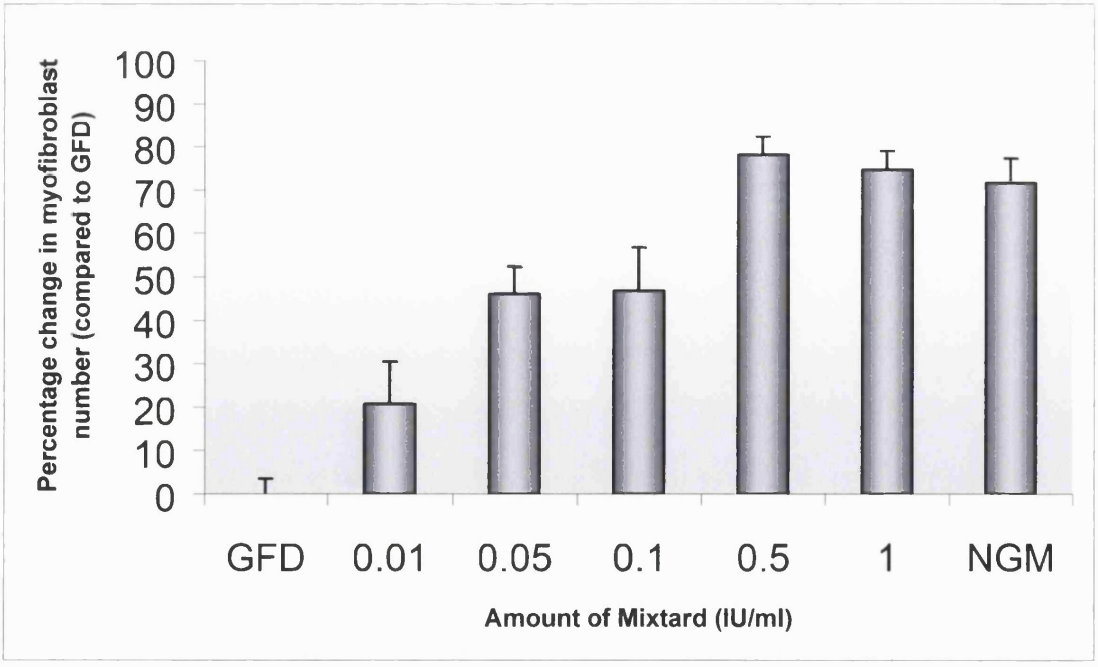


Fig 3.6: Graph showing the percentage change in myofibroblast proportion from that seen in GFD alone for all six dermal fibroblast cell lines tested. Error bars are the standard deviations from the mean of 6 different cell lines.

Intermediate acting formulation – Mixtard 30®

Supplementing GFD with a titration of Mixtard 30 (an intermediate acting formulation of human recombinant insulin, Table 3.1) also caused a reduction in myofibroblast numbers when compared to GFD (Fig 3.6). Addition of Mixtard at 0.01 IU/ml results in a reduction in myofibroblast number of 20.6% (±9.7). Increasing the amount of Mixtard results in reductions in myofibroblast number of 46.1% (±6.3), 46.7% (±9.9), 78.1% (±4.2) and 74.6% (±4.5) respectively for solutions containing 0.05, 0.1, 0.5 and 1 IU/ml. The reduction in myofibroblast number was significant ($P<0.001$) for those fibroblasts cultured in solutions containing greater than 0.05 IU/ml Mixtard. The reduction obtained through addition of Mixtard at 0.01 was not significant, $P=0.081$.

Long acting formulation – Insulatard®

Supplementing GFD with a titration of Insulatard (a long acting formulation of human recombinant insulin, Table 3.1) also caused a reduction in myofibroblast numbers when compared to GFD (Fig 3.7). 0.01 IU/ml Insulatard reduces myofibroblast number by 14.4% (±10.9) whereas increasing the concentration of Insulatard reduces the myofibroblast proportion by 37.9% (±11.9), 49.7% (±6.3), 72.7% (±6.0) and 78.0% (±3.9) respectively for solutions containing 0.05, 0.1, 0.5 and 1 IU/ml. This reduction is significant ($P<0.005$) for those fibroblasts cultured in solutions containing greater than 0.05 IU/ml Insulatard. The reduction obtained with 0.01 IU/ml Insulatard was not significant, $P=0.247$.

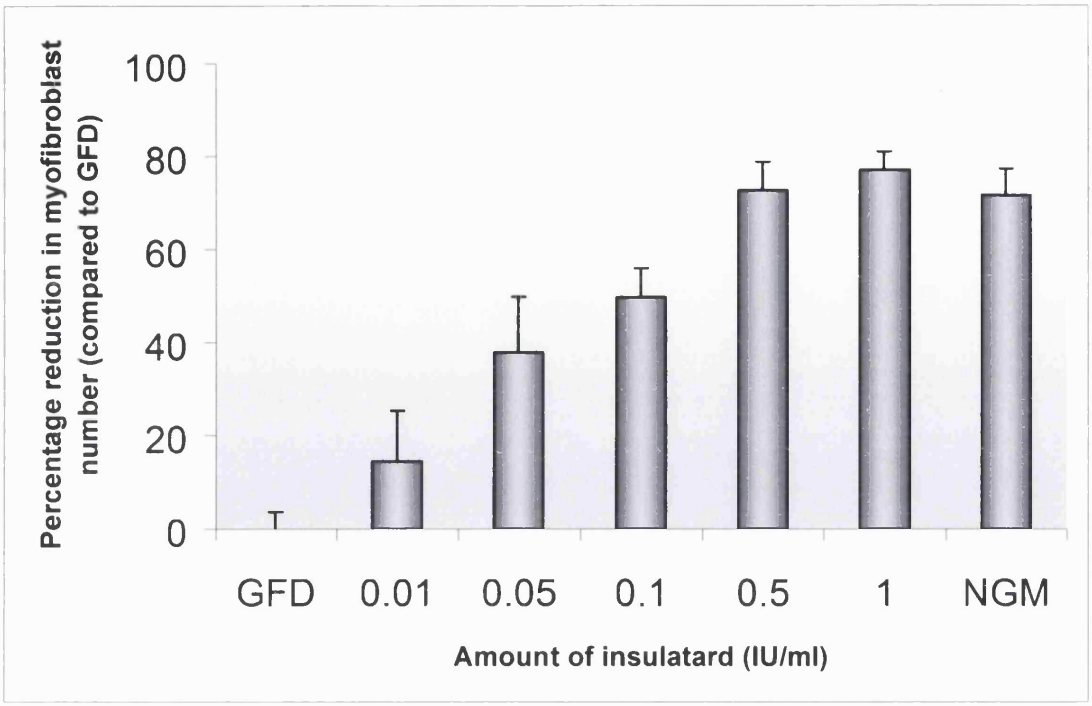


Fig 3.7: Graph showing the percentage change in myofibroblast proportion from that seen in GFD alone for all six dermal fibroblast cell lines tested. Error bars are the standard deviations from the mean of 6 different cell lines.

Western Blotting

Semi-quantitative analysis of western blotting for αSMA was performed to substantiate the immunohistochemical studies. Dermal fibroblast cultures from 4 patients were cultured in NGM, GFD and GFD supplemented with each of the three insulin preparations. As with the immunohistochemical experiments, each preparation of insulin was tested over a range of concentrations. For the purposes of these experiments, concentrations tested were 1 IU, 0.1 and 0.01 IU/ml. After culture for 14 days in test media, protein preparations were derived as described in Section 2.3.2 and analysed for αSMA expression by western blot analysis.

NGM 1 IU/ml 0.1 IU/ml 0.01 IU/ml GFD

Fig 3.8: Representative Western blot showing expression of αSMA in a single dermal fibroblast cell line cultured in either NGM, GFD or GFD supplemented with Insulatard®. Insulatard was added at concentrations of 1 IU/ml, 0.1 IU/ml and 0.01 IU/ml. The amount of protein sample loaded was varied to represent a standardized cell number for each sample ($2x10^5$ cells).

The expression of αSMA in a representative cell line in response to treatment in Insulatard can be seen in Fig 3.8. Each lane was loaded with sufficient protein to give equal cell number ($2x10^5$ cells). The optical density (OD) for each band was measured using UVP Labworks software in order to allow comparison between treatments, and between dermal fibroblasts initiated from different patients. Results were expressed as the change in OD of each band as compared to the OD for dermal fibroblasts cultured in GFD.

Taking the mean change in αSMA expression for dermal fibroblasts from 4 patients, levels of αSMA in those fibroblasts cultured in NGM were reduced to 40.5% of that seen in GFD treated cultures. The reduction in αSMA expression is significant ($P<0.05$) and equivalent to that obtained with immunohistochemical staining.

Considerable variation was evident in the expression of αSMA between fibroblast cultures obtained from different patients. Fig 3.9 shows the mean reduction in percentage expression of αSMA after addition of Actrapid to dermal fibroblast cultures. Addition of Actrapid to fibroblasts cultured in GFD reduced the expression of αSMA by 44.9% (±43.3) of that seen in GFD, if 1 IU/ml is added. The use of Actrapid at concentrations less than this results in little change to the expression of αSMA expression, increasing by 17.2% (±47.5) for 0.1 IU/ml and reducing minimally by 5.5% (±47.5) for 0.01 IU/ml. The reduction in αSMA expression seen with Actrapid when added at 1 IU/ml is not significant (P>0.05) upon testing.

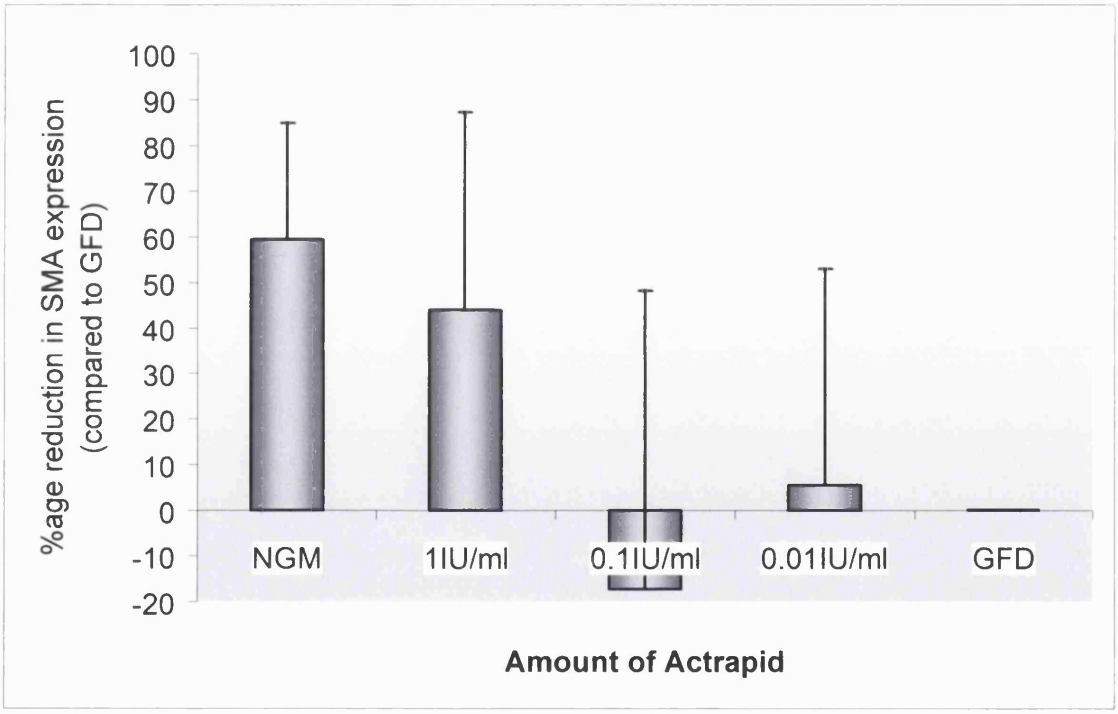


Fig 3.9 Mean percentage reduction in αSMA expression in dermal fibroblast cultures initiated from 4 patients cultured in NGM, GFD or GFD supplemented with Actrapid. The graph displays the percentage expression of αSMA as compared to that measured in GFD medium.

Those dermal fibroblast cultures supplemented with Mixtard expressed lower levels of αSMA than those supplemented with Actrapid. The expression of αSMA was reduced by 56.7% (±25.9) of that seen in GFD with the addition of Mixtard at 1 IU/ml. Similar reductions in αSMA expression were seen when Mixtard was added at 0.1 IU/ml and

0.01 IU/ml, falling by 47.3% (±8.5) and 40.2% (±21.5) respectively. The reduction seen with the addition of Mixtard was significant ($P<0.05$) for all concentrations of Mixtard investigated. These results are shown in Fig 3.10 and are equivalent to the figures derived from immunohistochemical staining of fibroblast cultures.

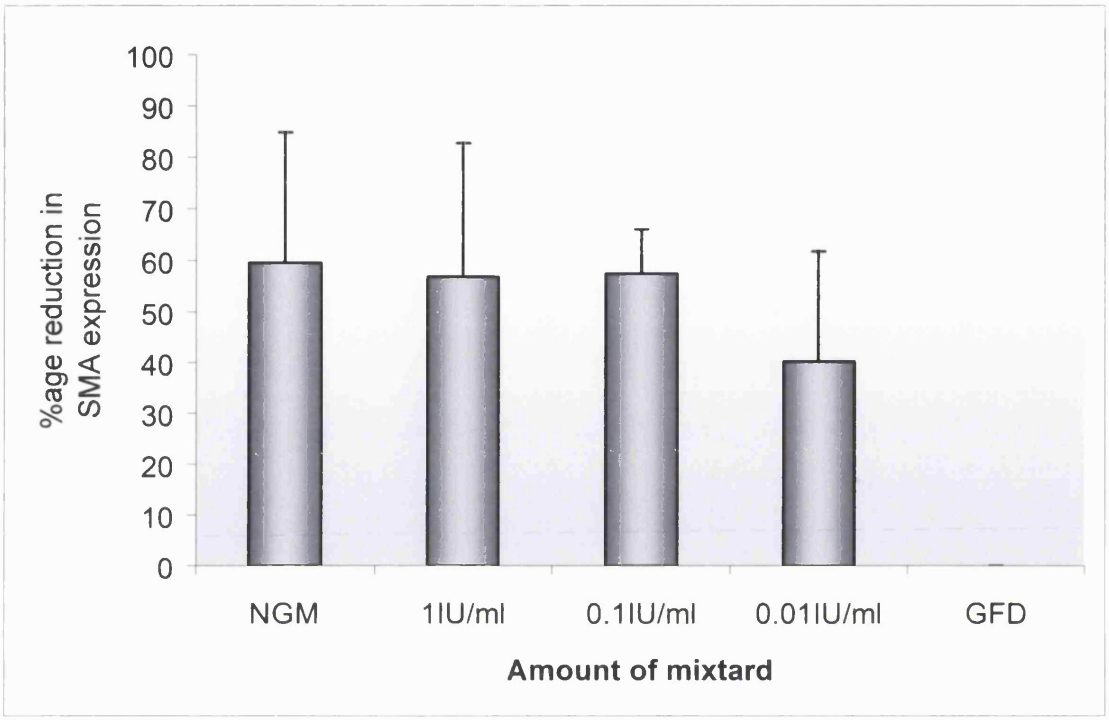


Fig 3.10 Mean percentage reduction in αSMA expression (compared with that of GFD treated cultures) in dermal fibroblast cultures initiated from 4 patients cultured in NGM, GFD or GFD supplemented with Mixtard. The graph displays the percentage reduction as compared to the expression in GFD medium.

Dermal fibroblast cultures supplemented with Insulatard also exhibited a reduction in the expression of αSMA when compared to GFD. Adding Insulatard at 1 IU/ml reduces the expression of αSMA by 67.9% (±19.7) of that seen in GFD medium. A reduction by 39.3% (±22.4) is seen when Insulatard is added at 0.1 IU/ml and by 24.0%±35.6 when 0.01 IU/ml of Insulatard is added. The change in αSMA expression with Insulatard treatment (as compared to GFD) is significant ($P<0.05$) when 1 IU/ml is added to GFD. These results are shown in Fig 3.11 and agree remarkably with the figures derived from the immunohistochemical data.

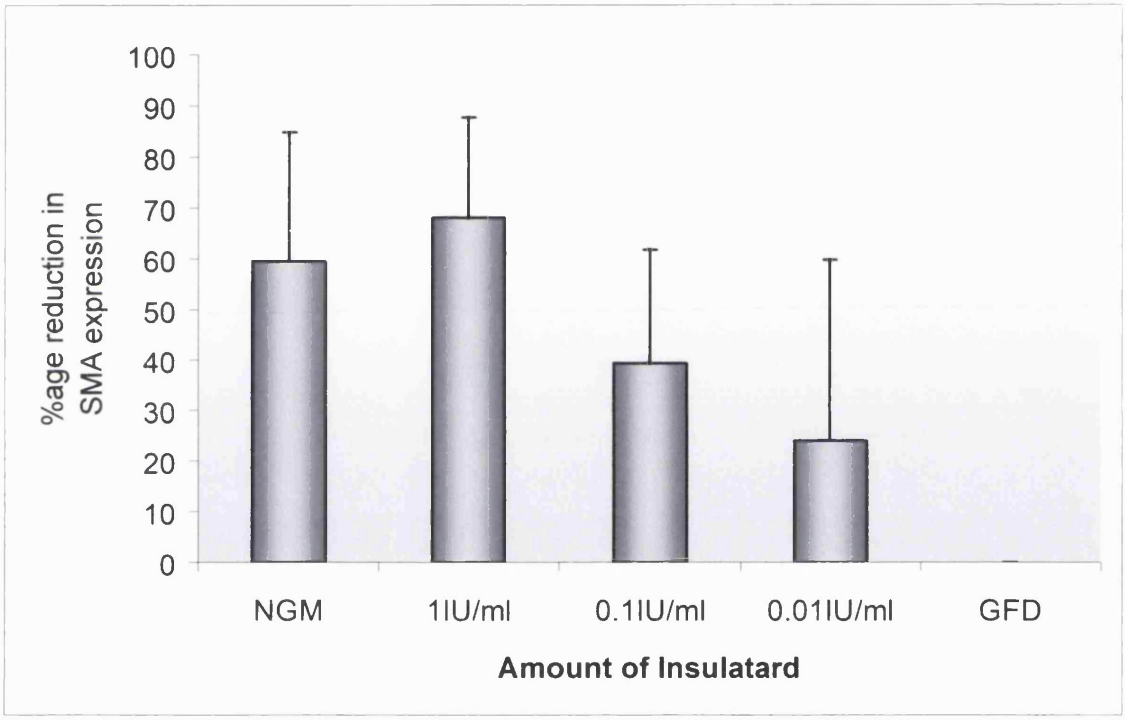


Fig 3.11 Mean percentage reduction in αSMA expression (as compared to that seen in GFD treated cultures) in dermal fibroblast cultures initiated from 4 patients cultured in NGM, GFD or GFD supplemented with Insulatard. The graph displays the expression of αSMA as a percentage of that seen in cultures grown in GFD medium.

3.3 Discussion

The initial work contained within this chapter served to establish the techniques to be used for the thesis and also to reproduce the results obtained by Linge et al. (2004) using bovine insulin to reduce fibroblast - myofibroblast differentiation *in vitro*. Once such conditions had been established, work was undertaken to assess the effect of other insulin preparations on fibroblast differentiation. Insulin is a commonly used medicine, and may be derived from a number of sources. Traditionally, insulin was obtained from animal pancreas extracts, but with improvements in biotechnology and manufacturing processes, it has been possible to obtain recombinant human insulin. It is these products that are more frequently used in clinical practice today.

The work included in Chapter 3 investigated whether human recombinant insulin preparations, considered preferable for use in man, could also prevent the formation of myofibroblasts *in vitro*. Although the structure of human insulin differs slightly from the bovine insulin used in preliminary studies by Linge et al. (2004), and may therefore have different biological effects, supplementing GFD culture medium with human insulin was shown to be equally effective in reducing myofibroblast numbers in human dermal fibroblast cultures. In all three of the insulin preparations tested, the reduction in myofibroblast numbers was found to be dependent on the concentration of insulin present. However, even when insulin was added in amounts well in excess of physiological levels, myofibroblasts were still present albeit in small numbers. Through the addition of insulin alone, it proved impossible to completely remove the myofibroblast phenotype from dermal fibroblast cultures. At best, myofibroblast numbers were equivalent, seldom lower, than that observed in those fibroblast cultures grown in NGM. Whether this intransigence is due to the presence of a different cell type within the culture system or simply due to a small proportion of fibroblasts being refractive to insulin treatment is still unknown.

Growth Factor Dialysed medium was used for the investigation of the effects of insulin as it comprised a better defined medium than Normal Growth Medium, which contains 10% foetal calf serum (FCS). FCS by virtue of its origin contains many growth factors, including insulin, which may influence cellular responses and potentially mask the

effects of added factors. Many investigators avoid this problem by assessing the effect of growth factors in a serum free environment over a relatively short period. However, fibroblasts tend not to proliferate and can even begin to undergo apoptosis in serum free culture systems over the time necessary for the experiments described within this chapter. Alternative methods to deplete serum of much of its growth factor activity have therefore been employed.

Dialysis of FCS differentially removes soluble substances and in the case of GFD, those substances less than 12-14 kDa are thought to have been removed (Section 2.2.1). This has resulted in the removal of much of the growth factor activity, in particular insulin activity, from culture medium whilst maintaining fibroblast proliferation. Whilst it has been assumed that GFD contains less growth factor activity than NGM, specific growth factor assays have not been undertaken during the course of this thesis. It remains a possibility that significant amounts of growth factors are bound to proteins within the foetal calf serum and are therefore not free to move along the dialysis gradient. However, for the purposes of these studies it has been assumed that repeated dialysis against multiple changes of excess PBS gradually depletes the FCS contained within the dialysis tubing of a variety of substances, examples of which are shown in the following table (See Table 3.1).

Although many of the soluble factors thought removed during dialysis of FCS are mitogens (See Table 3.1), dermal fibroblast cultures may be maintained and indeed proliferate in GFD to 14 days and beyond. Analysis of the fibroblasts cultured in such medium demonstrates that many of the cells express αSMA, the marker for myofibroblasts. Addition of insulin to GFD, as shown by Linge et al. (2004) reduces fibroblast - myofibroblast differentiation, suggesting that insulin may play a role in myofibroblast formation.

Growth Factor	Major role in wound healing
EGF	Mitogenic for most epithelial tissues, fibroblasts, endothelial cells
Some TGFα	Potent angiogenenic factor
IGF-I	Mitogenic for fibroblasts, bone cells, neural tissues, haematopoietic cells, endothelial cells
IGF-II	Mitogenic for fibroblasts
Insulin	Mitogenic for many cell types. Major hormone of glucose homeostasis

Table 3.1 Table showing the major growth factors thought to have been removed during preparation of Growth Factor Dialysed medium that are present in Normal Growth Medium.

Differences have been highlighted between the preparations of insulin investigated within this chapter. Both immunohistochemical staining and Western blotting revealed subtle differences between the efficacies of the three formulations of insulin tested. Although all three preparations are from the same manufacturer and use the same basic "type" of insulin, differences exist between the other constituents of the formulation that seem to be beneficial in modifying their activities. The longer acting formulations, such as Mixtard and Insulatard, proved to be more reliably effective in reducing myofibroblast number than Actrapid for the equivalent dose. These preparations differ mainly through the addition of zinc, used to stabilize insulin in solution, and thought by some investigators to also be beneficial in wound healing (Udupa & Chansouria, 1971). The trend for longer acting formulations to perform better than Actrapid suggests that these preparations should be used for further investigations. In view of this, Insulatard was chosen as the preparation of choice for the remainder of this thesis, in particular for the *in vivo* experiments described in Chapter 7.

Closer examination of the proportional change in myofibroblast numbers as assessed by immunohistochemistry and western blotting reveals subtle differences between the results obtained from the 2 experimental techniques. Although both techniques confirm

that insulin reduces the expression of αSMA in a dose dependent manner, immunohistochemical analysis would suggest that for a given concentration of insulin, a slightly greater decrease in myofibroblast number is obtained from that observed with western blotting. Using immunohistochemical staining of coverslips it is easy to underestimate the numbers of myofibroblasts if the αSMA is still expressed in cells, but at lower and barely detectable levels. Such cells would still contribute to the αSMA identified using Western blotting and would result in more of an apparent reduction in myofibroblasts seen in immunohistochemistry as compared to the western blotting technique.

Examination of fibroblasts stained to demonstrate αSMA shows variation in the intensity of staining. In a single culture, there may be both "strong" and "weak-moderate" positive cells. Interestingly, this heterogeneity of staining has not been reported previously. Within the media employed for the purposes of this chapter, "strong" staining myofibroblasts are usually seen in either NGM or GFD and insulin, whilst both "strong" and "weak-moderate" myofibroblasts may be seen in fibroblasts cultured in GFD. Such differences in staining may explain the differences in the results obtained from both immunohistochemistry and Western blotting in that "αSMA strong" cells would appear to be the subpopulation that is refractive to insulin treatment. Their persistence in both NGM and GFD supplemented with insulin may explain the reduction in αSMA expression as determined by Western blotting appearing less than the reduction seen with immunohistochemistry. This theory lends credence to a distinct subpopulation of αSMA positive cells being refractive to insulin.

Further comparison of the effect of bovine and human recombinant insulin suggests that the latter preparations are more effective in reducing myofibroblast numbers in human dermal fibroblast cultures. Using bovine insulin at 50ug/ml it was possible to reduce myofibroblast proportions by 50% of that seen in GFD. In comparison, the use of equivalent titrations of human insulin reduces myofibroblast numbers by 71-78% depending on the preparation.

Although the data presented within this chapter has shown that the removal of insulin from fibroblast culture medium can increase the numbers of myofibroblasts present,

proof that insulin alone is responsible for this change has not been provided. Such confirmation could most easily be provided by the removal of the effect of insulin alone from the culture medium, possibly using an anti-insulin antibody that could bind to and specifically and effectively block insulin's action or remove it from solution. However, to the knowledge of the author, such antibodies are not yet available.

Precisely how insulin reduces myofibroblast numbers in dermal fibroblast cell cultures is unknown. Insulin's reported role as a "survival factor" (Prisco, Romano et al., 1999) may suggest a role for apoptosis in this phenomenon, however it is clear that the reduction in myofibroblast number in the response to insulin could not be due to an increased apoptosis rate. This would suggest that insulin has an effect on the initial formation of the myofibroblast cell phenotype. However, no evidence has been provided within this chapter to support this statement. Furthermore, insulin seems to have a maximal effect at very high concentrations. The ability of insulin to also act at the IGF-1 receptor (Chen & Feng, 2001) at high concentrations and its relative lack of effect at low concentrations may suggest that insulin does not achieve the reduction in fibroblast – myofibroblast differentiation solely through its own receptor.

The effect of insulin on myofibroblast formation may be of considerable clinical importance. The myofibroblast is thought to contribute greatly to the aetiology of the pathological scar (Nedelec, Ghahary, Scott, & Tredget, 2000). Although myofibroblasts disappear from normal wounds approximately 30 days after injury in man (Gabbiani, 1996) following completion of the proliferative phase of healing, in pathological scars they may be identified many years after injury (Chipev, Simman, et al., 2000). Proposed therapies for the treatment of pathological cutaneous scars have thus concentrated on addressing the presence of the myofibroblast phenotype and its persistence.

Clearance of myofibroblasts from the wound may be achieved either by preventing their formation or by increasing their removal (by apoptosis). As the TGFβ isoforms are known to be important in the differentiation of the myofibroblast, therapies aimed at removing the effects of the profibrotic forms of this growth factor have been suggested. Chief amongst such therapies are those that remove or reduce the effects of $TGF\beta_1$ (Shah, Foreman, & Ferguson, 1995). However, TGFβ is known to play many roles in

wound healing, causing proliferation and increasing ECM production. The removal of these beneficial effects may, in addition to removing myofibroblasts, prolong the healing process. An alternative mechanism by which myofibroblasts may be removed during wound healing is to hasten their removal by apoptosis, or by ensuring the apoptotic process is more complete. Whilst the targeting of such therapies towards the myofibroblast seems to be simple, in practice the similarity between myofibroblast and fibroblast makes the development of "magic bullet" therapies impractical. The discovery that insulin may play a significant role in the formation and control of the myofibroblast phenotype may suggest a clinical role for this substance in the control of cutaneous scarring. However, prior to making such claims a greater understanding of the effects of this factor during wound healing is required.

The following chapters will assess insulin's suitability as a means of reducing myofibroblast numbers *in vivo* by assessing its effect on other processes important in wound healing (Chapter 4). In order to confirm that insulin is unique in reducing myofibroblast number, the effects of many of the other growth factors that were removed during the production of GFD will be assessed in Chapter 5.

3.4 Conclusions

- A small proportion of fibroblasts cultured in NGM stain positive for αSMA, and can thus be considered to be myofibroblasts.
- Fibroblasts cultured in GFD contain significantly more myofibroblasts than cultures grown in NGM.
- Addition of insulin, either from bovine or human sources, to cells cultured in growth factor deficient medium reduces the number of myofibroblasts present, as assessed both immunohistochemically and by western blot analysis of αSMA expression.
- Short acting preparations of insulin are less effective in reducing myofibroblast numbers than longer acting formulations.

Chapter 4

The effect of insulin on aspects of fibroblast behaviour important during wound healing

4.1 Introduction

Reduction in myofibroblast numbers, either through prevention of differentiation or through facilitation of apoptosis may be considered a goal for the development of novel anti-scarring therapies. The results discussed in Chapter 3 demonstrate that insulin is capable of reducing myofibroblast numbers when added to dermal fibroblast cultures *in vitro*. As the myofibroblast phenotype has been linked with pathological scarring conditions (Lee, Green et al., 1995), (Chipev, Simman, et al., 2000), such as keloid and hypertrophic scars, the addition of insulin during wound healing may represent a novel means for the reduction or prevention of such scars. However, in addition to being effective, "anti-scarring" therapies must be shown to be safe and cause no detriment to other processes important in wound healing.

The term "wound healing" refers to the complex collection of processes initiated following injury (Section 1.3). Of these, cellular differentiation plays only a minor role with expansion of cell populations via proliferation of both keratinocytes and fibroblasts to replace lost tissue being required (Grinnell, 1994). In addition, the repair of defects in the integument requires the replacement of extracellular matrix, necessitating increased production of extracellular matrix proteins, in particular the collagens (Aston, Beasley, and Thorne, 1997). Once the missing tissue has been replaced, wounds undergo remodelling and contraction.

Wound contraction was thought to be dependent upon the presence of cells that have the ability to contract themselves, and thus originally the myofibroblast was thought to be the cell responsible for the production of contractile forces within the healing wound (Koopmann, 1995). Removal of the myofibroblast phenotype may have significant impact on the healing of wounds that depend on contraction, for example those wounds left to close by secondary intention.

Many of the cellular or tissue responses that are initiated following injury may be simply modelled *in vitro*. This chapter aims to assess a number of those aspects of cellular activity that are important in dermal wound healing and that may be influenced through the addition of insulin either directly or via its effect on myofibroblast differentiation. Such activities investigated here include the effect of insulin on

fibroblast proliferation, total soluble protein production and collagen production. In addition, the effect of insulin on the contraction of 3D collagen gels has been evaluated.

Hypothesis:

The addition of insulin will not be detrimental to the cellular processes involved in dermal wound healing.

Aims:

To assess whether the addition of insulin impairs the following processes important in wound healing:

- Fibroblast proliferation.
- Protein production by fibroblasts.
- The production of soluble collagens.
- Contraction of 3D collagen gels

4.2 Results

4.2.1 Proliferation

Investigation of the effects of insulin on dermal fibroblast proliferation was undertaken using a standard colorimetric assay, the Crystal Violet Assay (described and validated in Section 2.3.3). Fibroblasts were cultured over a time course of 14 days in NGM (Normal Growth Medium), GFD (Growth Factor Dialysed) or GFD supplemented with different insulin preparations (Actrapid, Mixtard and Insulatard as used in Chapter 3.2). Each preparation of insulin was tested at titrations ranging from 0.01 IU/ml to 1 IU/ml, as used in the experiments in Chapter 3 to determine the effect of insulin on fibroblast - myofibroblast differentiation *in vitro*. Each experimental variable was tested in triplicate for each of the 3 dermal fibroblast cell lines (each initiated from a different patient). On the 3^{rd}, 7^{th}, 10^{th} and 14^{th} day, one complete set of experimental variables (in triplicate) for each cell line was harvested for colorimetric assay with the bound stain measured at OD 595 nm (Optical Density), and being directly proportional to cell number (See Fig 2.3).

The data from all 3 dermal fibroblast lines was corrected for background (Section 2.3.3) and was then averaged. These results are plotted in Fig 4.1 as the mean corrected OD versus time. Here typical growth curves can be seen over the 14 day time period. The growth curves consisted of an initial lag phase up to day 3 (particularly evident in GFD medium) followed by a phase of exponential growth (day 3 to day 10) then plateau, owing to density inhibition at confluence.

Culture of dermal fibroblasts in both NGM and GFD results in similar growth profiles with the measured OD increasing until day 10, reaching OD 1.2, whereupon no further increase was observed. The cell number at day 3 in NGM was significantly higher than that observed in GFD ($P=0.02$). Fibroblast number at all other time points did not vary significantly between treatments.

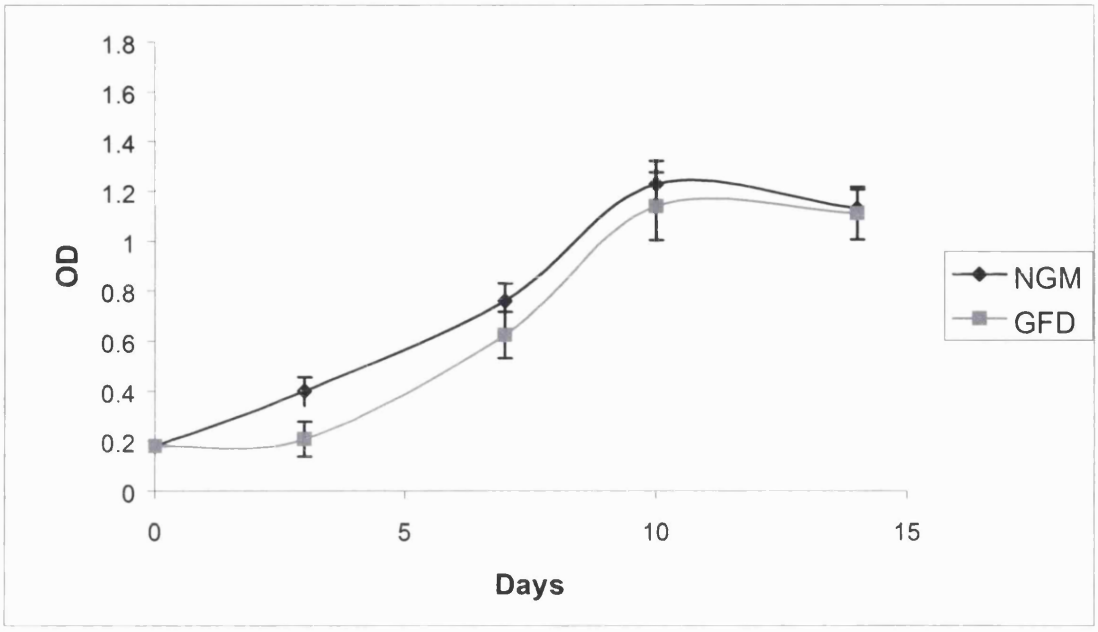


Fig 4.1 Graph showing the change in mean cell number (directly proportional to OD) for 3 dermal fibroblast cell lines cultured in either NGM or GFD media over a 14 day period as assessed using the crystal violet assay. Error bars represent the standard deviation from the mean.

Addition of insulin to GFD, either as Insulatard (Fig 4.2A), Mixtard (Fig 4.2B) or Actrapid (Fig 4.2C), increased the apparent proliferation of fibroblasts above that seen in NGM or in GFD. Error bars have been omitted from the graphs for reasons of clarity, however, the maximal standard deviation from the mean was no more than 10% of the mean throughout. The increase in cell number over basal levels was least in the lowest concentration of insulin used for both Insulatard and Mixtard (0.01 IU/ml), with the lowest proliferation occurring in Actrapid at 0.05 IU/ml. All other concentrations of insulin yielded levels of proliferation greater than that seen in NGM. The increase in cellular proliferation seen at day 14 was significantly greater ($P<0.05$) than that seen in either NGM or GFD only for those fibroblasts grown in medium supplemented with 0.05 – 0.5 IU/ml Insulatard or those grown in 0.05 and 0.5 IU/ml Mixtard. Interestingly, assessing those values obtained at day 10, significant increases ($P<0.05$) in cellular number were seen in only in those fibroblasts cultured in media supplemented with 0.05 – 0.5 IU/ml Insulatard.

Differences existed between the preparations in terms of "plateau" level (the final OD attained), maximal response (the highest OD achieved through the course of the experiment) and the dosage of insulin that resulted in maximal proliferation after 14 days (most effective concentration). Maximal cell number was observed in GFD supplemented with 0.05 IU of Insulatard, 0.05 IU of Mixtard or 0.1 IU of Actrapid. Addition of Insulatard at 0.05 IU/ml resulted in a cell number significantly higher than that seen with addition of Actrapid at 0.1 IU ($P<0.05$). There was no significant difference between proliferation in media supplemented with the most effective dose of Insulatard and Mixtard ($P>0.05$). Maximum cell number occurred in medium supplemented with Insulatard, reaching OD 1.7. This was significantly higher than OD 1.4 achieved in the most effective concentrations of insulin for both Mixtard and Actrapid ($P<0.05$). For all concentrations of Insulatard and Mixtard used, cell number was significantly higher than in GFD alone over the majority of the time points ($P<0.05$). However, only concentrations of Actrapid greater than 0.05 IU/ml showed a significant increase in cell number over the time course from that seen in GFD treated cultures.

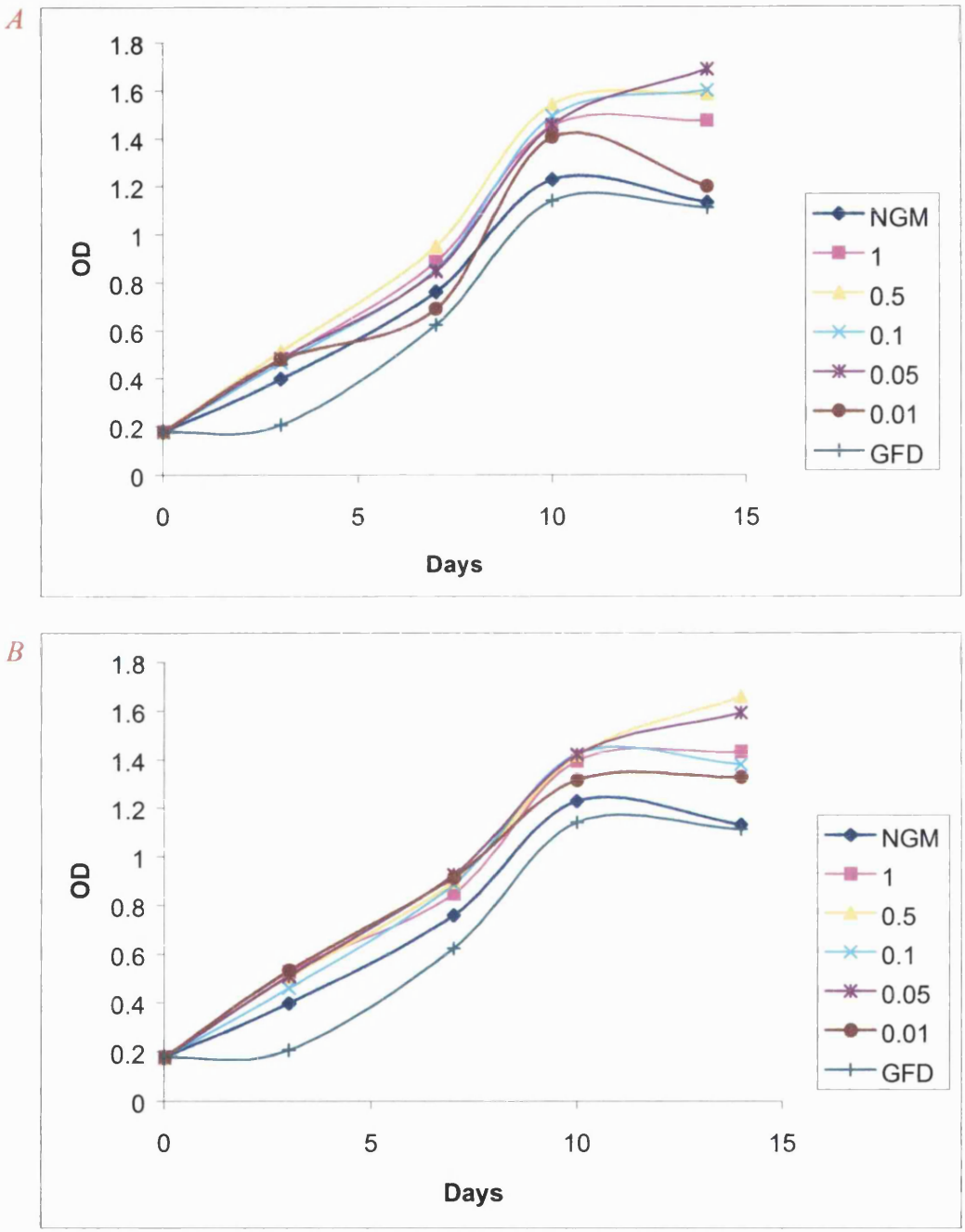


*Fig 4.2 Three graphs showing the change in mean cell number for 3 dermal fibroblast cell lines cultured over a 14 day period as assessed using the crystal violet assay. Graphs represent results of culture in media supplemented with Insulatard (**A**), Mixtard (**B**) and Actrapid (**C**). Error bars have not been included for reasons of clarity; however, the maximal standard deviation from the mean was no more than 10% of the mean throughout*

C

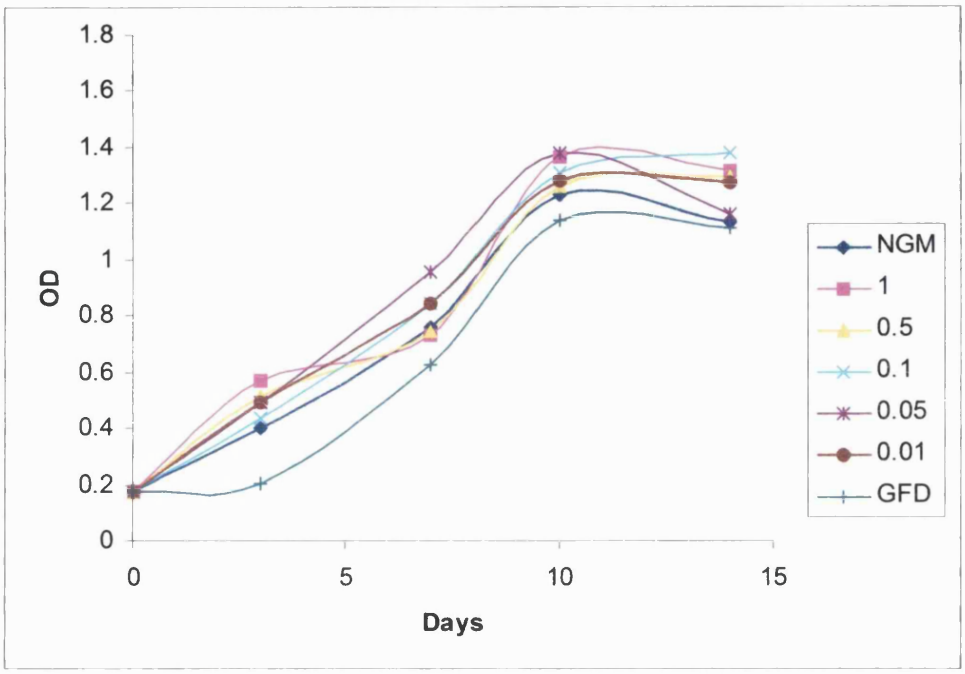


*Fig 4.2 (continued) Graphs showing the change in mean cell number for 3 dermal fibroblast cell lines cultured over a 14 day period as assessed using the crystal violet assay. The results of culture in media supplemented with Actrapid (**C**) is shown. Error bars have not been included for reasons of clarity; however, the maximal standard deviation from the mean was no more than 10% of the mean throughout*

It is clear from these studies that the insulin type and dose determined in Chapter 3 to be both the most effective and representative with regard to its effect on fibroblast - myofibroblast differentiation (i.e. that of 1 IU/ml Insulatard), is not deleterious to the proliferation of fibroblasts and indeed significantly enhances it when compared to GFD medium alone. In view of this, all future experiments that call for investigation of the effects of insulin have been performed using this formulation and this dosage.

4.2.2 Protein Production

The amount of total secreted soluble protein produced by dermal fibroblasts was measured using the Pierce Protein Assay, as described in Section 2.3.5. Fibroblast cell lines initiated from three separate patients were cultured in NGM, GFD or GFD with the addition of Insulin (Insulatard at 1 IU/ml). Each cell line was cultured in quadruplicate

for each medium tested. After achieving confluence, fibroblasts were cultured for 48 hours in "incubation medium" (Table 2.5) before media was collected from each well. Cell number was determined for each well and used to correct for differences in proliferation between treatments. The amount of soluble protein present in the supernatant from each replicate well was measured in duplicate using the Pierce Protein Assay (Section 2.3.5). Fresh incubation medium was also assayed and the amount of protein present subtracted from the results obtained from assays of conditioned media. These results were then normalized with regard to cell number. The average protein production for all 3 cell lines is illustrated in Fig 4.3 shown as the mean amount of protein produced per 10000 cells ± standard deviation from the mean.

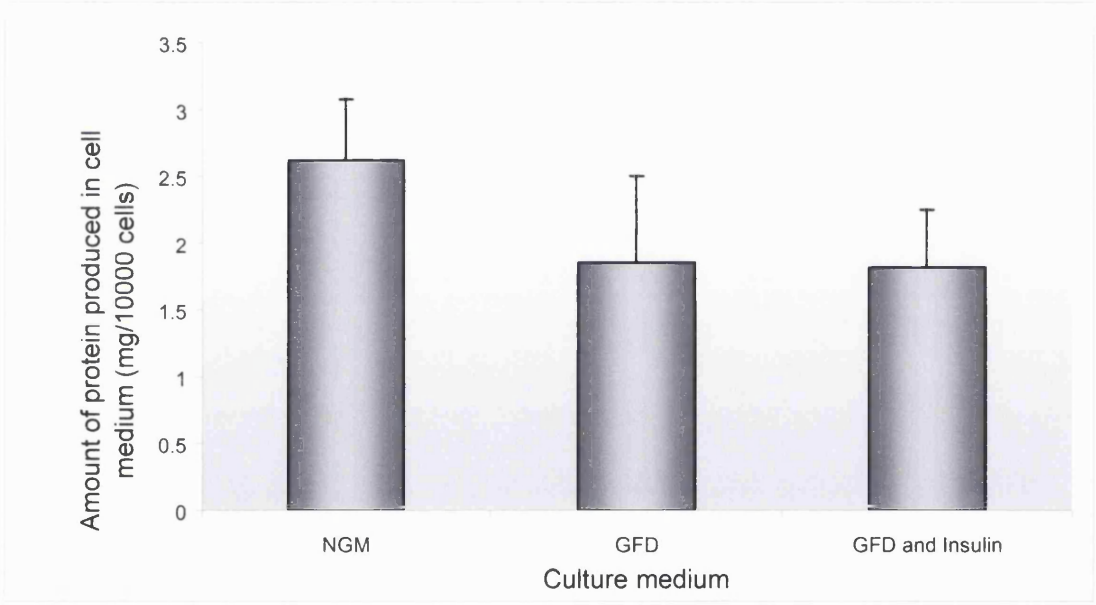


Fig 4.3 Graph showing the amount of secreted soluble protein produced per 10000 dermal fibroblasts. Cells were cultured in either NGM, GFD or GFD supplemented with 1 IU/ml Insulatard. Mean protein production for 3 cell lines is displayed with error bars showing standard deviations.

The culture of dermal fibroblasts in NGM medium (containing FCS) resulted in a supernatant containing 2.625 mg protein (± 0.45) per 10000 fibroblasts. Culture in GFD medium (GFD-FCS) resulted in a reduced production of protein, with the medium containing 1.856 mg (± 0.647) protein. The difference in protein production between NGM and GFD is not significant (P=0.100). Addition of 1 IU/ml Insulatard to GFD had

little effect on the amount of protein present in the culture supernatant, decreasing minimally to 1.821 mg (±0.429) per 10000 cells. This decrease was not significantly different from that obtained from GFD alone (P=0.931), but significantly different from NGM alone (P=0.042).

4.2.3 Collagen Production

Collagen production was determined for the same 3 dermal fibroblast cell lines used in Section 4.2.2. Using the same supernatant as collected for the measurement of protein production, collagen production was assessed in quadruplicate using the Sircol Collagen Assay (Biocolor). Each assay was performed twice as described in Section 2.3.5. This assay measures the amount of soluble collagen present in the supernatant, the results of which can be seen in Fig 4.4. Here the mean amount of collagen for all 4 dermal fibroblast cell lines and standard deviations from the mean are shown corrected for cell number. Assay of the fresh culture medium alone showed undetectable levels of collagen for each medium used. Culture of dermal fibroblasts in NGM yielded 2.40 mg (±0.41) of soluble collagen in the cell culture supernatant per 10000 cells. Culture in GFD medium yielded less collagen, 1.38 mg (±1.24). This was not significantly less than that obtained with culture of dermal fibroblasts in NGM, P=0.250. Addition of insulin (at 1 IU/ml) to GFD increased the amount of soluble collagen produced slightly to 1.65 mg (±1.01) per 10000 cells. This increase was not significant from that seen with GFD (P=0.785) nor was it significantly different from that measured in cells cultured in NGM (P=0.301).

Although mean results for all 3 cell lines tested show a high degree of variability, and the variation between quadruplicates performed on the same cell line was less than 30%, variation between replicate analyses performed on the same samples was less than 10%.

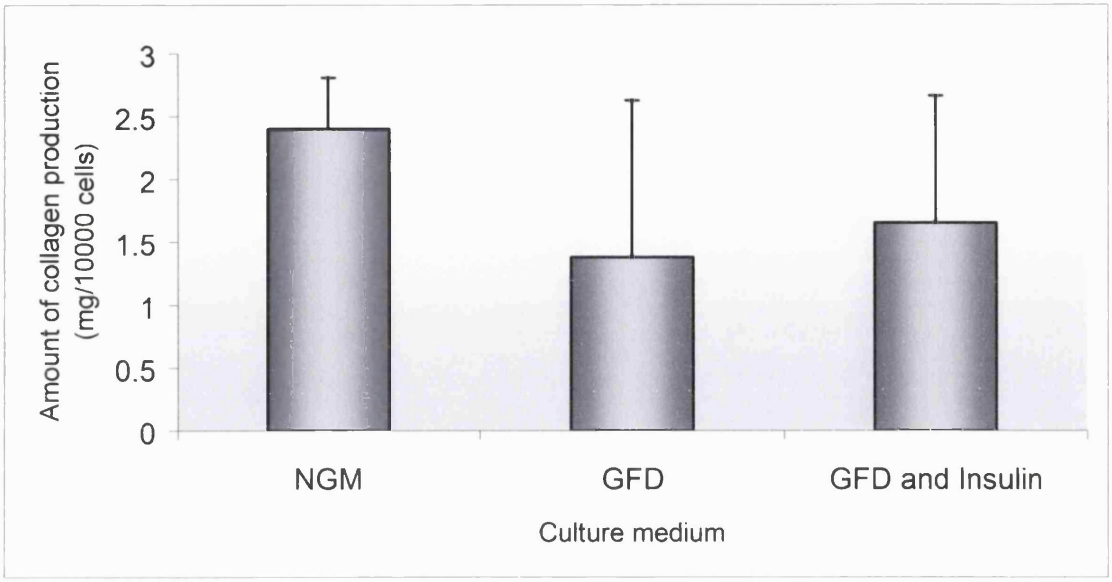


Fig 4.4 Graph showing amount of collagen production per 10000 dermal fibroblasts. Cells were cultured in either NGM, GFD or GFD supplemented with 1 IU/ml Insulatard®. The mean collagen production for 3 cell lines is displayed, error bars represent the standard deviation from the mean.

In order to determine whether the variability of results was simply due to variations between cell lines (some cells being generally high expressors) the results were normalized by expressing collagen production for each cell line in terms of the percentage collagen produced by fibroblasts cultured in NGM (for that cell line). These results are shown in Fig 4.5. Fibroblasts cultured in GFD produce 51.8% (±34.5) of the amount produced when cultured in NGM. In comparison, addition of insulin increases the amount of collagen production to 65.6% (±34.7). This increase is not significant upon testing (P=0.360). Despite normalizing the data, high variation in collagen production is still evident between the different culture conditions for the cell lines tested.

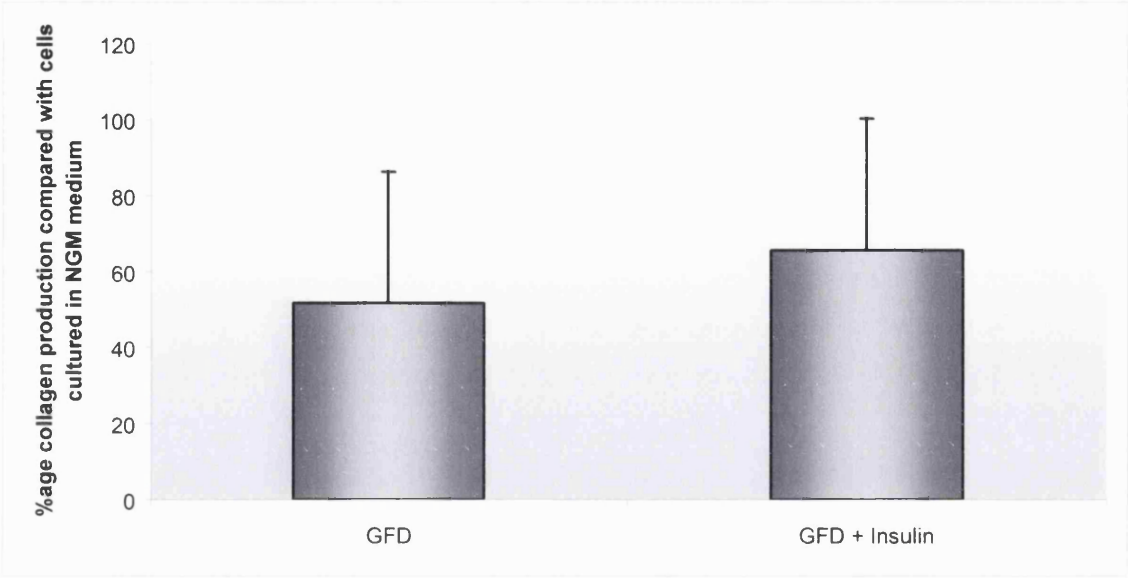


Fig 4.5 Graph showing the amount of collagen production by 3 dermal fibroblast cell lines when cultured in GFD or GFD supplemented with insulin, expressed as a percentage of the amount of collagen produced when cultured in NGM. Error bars represent the standard deviations from the mean.

4.2.4 Collagen Production as a ratio of total protein production

A more traditional manner in which to present the cellular production of both protein and collagen is to calculate the ratio of collagen: protein production. These results are shown in Fig 4.6, where the amount of collagen present in the conditioned medium is calculated as a ratio of the total soluble protein produced in the medium for each cell line and the mean and standard deviation of the mean plotted.

The ratio of collagen to protein production in dermal fibroblasts cultured in NGM medium was 0.99:1 (±0.26). Culture in GFD reduced the ratio to 0.66:1 (±0.42). This decrease was not significant upon testing ($P>0.1$). Addition of insulin to GFD increased the amount of collagen production from that seen in GFD to give a ratio of 0.95:1 (±0.43). This change was not significantly different from that seen in either NGM or GFD media ($P>0.1$).

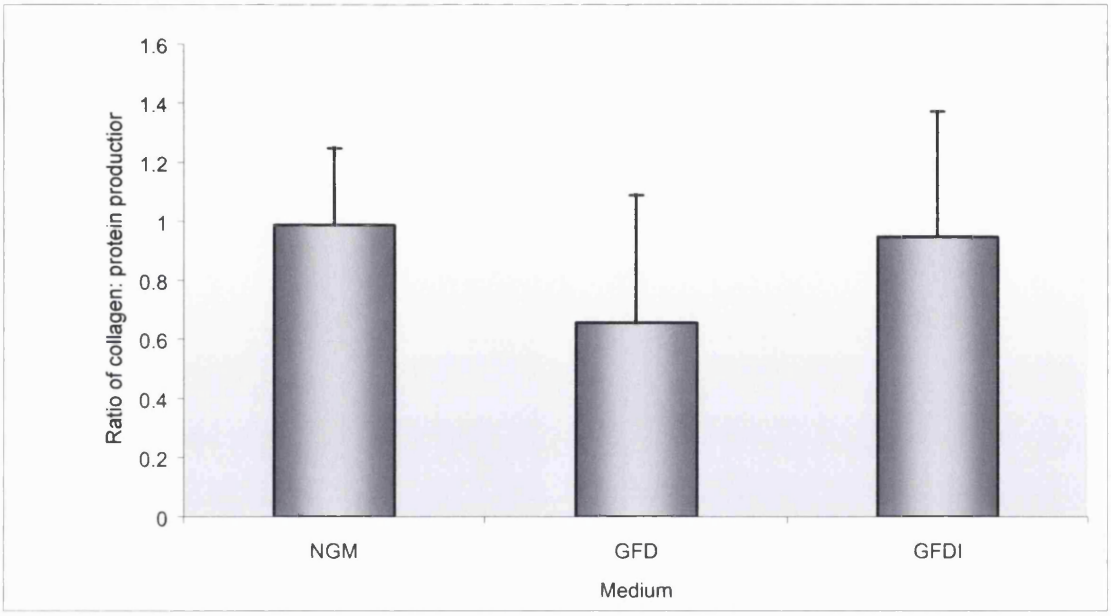


Fig 4.6 Graph showing the mean and standard deviations for the ratio of collagen / protein production for 3 dermal fibroblast cell lines. Fibroblasts were cultured in either NGM, GFD or GFD supplemented with 1 IU/ml Insulatard. Error bars represent standard deviations from the mean.

4.2.5 Contraction of collagen gels

The effect of insulin on the contraction of 3D collagen lattices was determined in triplicate for 3 dermal fibroblast cell lines (each initiated from a different patient), as described in Section 2.3.6. Gels seeded with $1x10^6$ fibroblasts were incubated in NGM, GFD or GFD supplemented with Insulin (Insulatard 1 IU/ml). In addition, fibroblasts were seeded into collagen gels and incubated in Serum Free Medium. Gels were loosened after 2 hours incubation to allow contraction and visualised after 24, 48 and 72 hours. The surface area of each gel was measured using SigmaScan Pro 5. A representative plate showing gel contraction at 72 hours can be seen in Fig 4.7a. A graph illustrating the mean contraction of 3D collagen gels from all 3 cell lines over the 72 hour period is shown in Fig 4.7b.

a

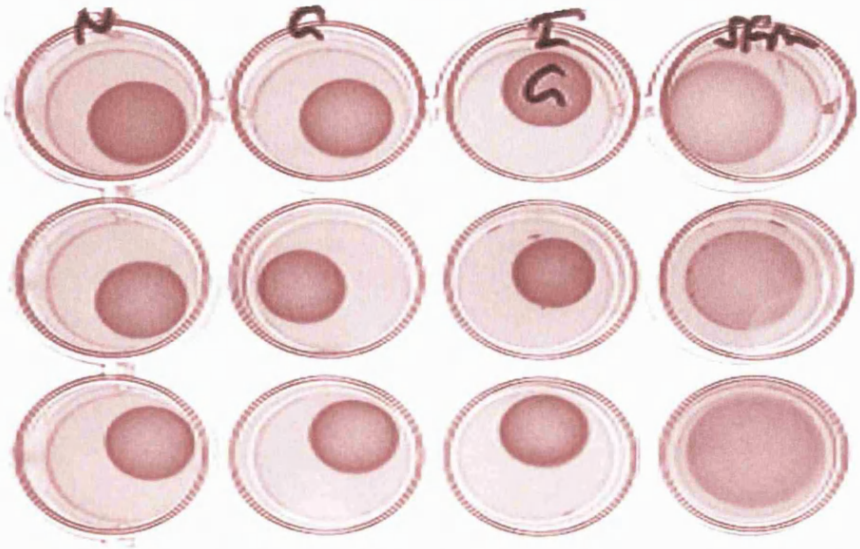


Fig 4.7a Representative dermal fibroblast cell line seeded in collagen gels, visualised after 72 hours. All wells were pre coated with 2% BSA. Media added to each well was NGM (N), GFD (G), Insulatard 1 IU/ml in GFD (IG) or Serum Free Medium (SFM).

b

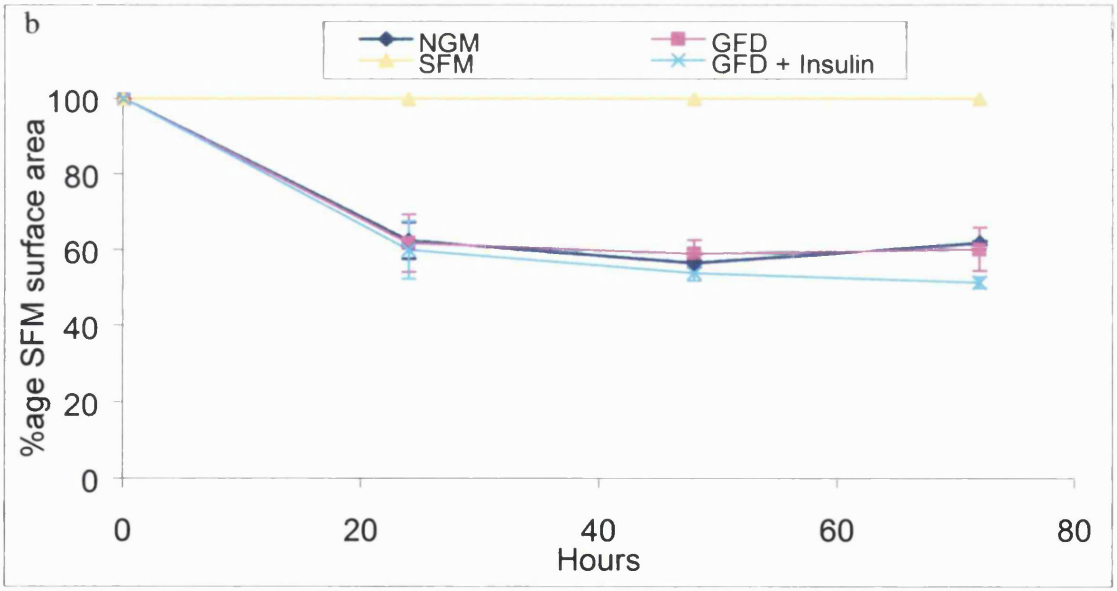


Fig 4.7b Graph showing the mean contraction of 3D collagen gels for 3 dermal fibroblast cell lines over a 72 hour period expressed as a percentage of the surface area of those gels cultured in SFM. Gels were cultured in SFM, NGM, GFD or GFD supplemented with Insulin (Insulatard 1 IU/ml).

Different degrees of contraction were evident between different cell lines in all media investigated although the variability that occurred between triplicate cultures (for the same cell line under the same conditions) was less than 10%. In all cell lines, those cultured in Serum Free Medium demonstrated minimal contraction over the time course of this experiment. Therefore, to allow comparison between cell lines, and to compensate for differences in the production of the collagen gels, the amount of contraction of each collagen gel is expressed as the percentage change in surface area for that culture condition compared to that observed with Serum Free Medium.

Dermal fibroblasts cultured in 3D collagen lattices cultured in NGM (containing 10% FCS) contracted the gels to 61.7% (± 0.4%) of the surface area of SFM gels. Gels incubated with 10% GFD medium contracted to 60% (± 5.6%) of the surface area of those in SFM. Addition of insulin to GFD similarly resulted in a contraction to 51% (± 1.5%) SFM. The contraction profiles for NGM, GFD and GFD and Insulin did not differ significantly for any of the time points investigated.

4.3 Discussion

Systemic insulin replacement has previously been shown to be beneficial in wound healing in diabetic mice (Weringer, Kelso et al., 1982). Here, diabetic mice normally unable to heal wounds rapidly and efficiently, exhibited a normal wound healing response with the addition of insulin. In addition to maintaining euglycaemia, addition of insulin has been found to ameliorate defects in fibroblast proliferation and also in collagen synthesis (Pierre, Barrow et al., 1998). In addition, insulin supplementation of wounds in both diabetic and non-diabetic subjects has been shown to be of benefit where the speed of wound healing is concerned (Greenway, Filler et al., 1999). Work contained within the previous chapter has highlighted that insulin may reduce the formation of the myofibroblast phenotype. As such, the administration of this substance to healing wounds may be indicated in order to reduce the severity of scarring. This would entail the administration of this compound to non-diabetic subjects. Accordingly, this chapter has concentrated on the *in vitro* assessment of insulin's effects on several processes important in dermal wound healing using non-diabetic human derived fibroblasts.

4.3.1 Proliferation

Although fibroblasts can proliferate within a basal medium, such as GFD where many growth factors have been removed, a significant increase in proliferation may be seen with the addition of insulin. These results are in keeping with work published by other authors where insulin has long been known to act as a mitogenic factor (Krupsky, Fine et al., 1996). Its efficacy has been demonstrated in a number of fibroblast cell lines (Selgas, Lopez-Rivas et al., 1989), (Khil, Kim et al., 1997), (Gonzalez-Hernandez, Monreal et al., 1993).

Of considerable importance if insulin is to be used as an anti-scarring therapy, and may therefore be applied in high concentrations, is the response of fibroblasts within medium containing supra-physiological quantities of this growth factor. Although physiological levels of insulin vary from 6-26 μIU/ml in man (Entwistle, 1990), even at levels significantly higher than peak physiological dosages, no impairment in fibroblast proliferation was evident.

Fibroblasts are not the only cell type active during wound healing, as injury will also require repair to the epidermis, necessitating keratinocyte migration and proliferation. Although not assessed in this thesis, addition of insulin to cultured keratinocyte is known to improve the health of cultured keratinocytes and to increase their proliferation (Formanek, Millesi et al., 1996) *in vitro.* Addition of insulin to healing wounds may therefore prove beneficial to the cellular populations of both the healing epidermis and dermis.

Clinically, studies have been performed assessing the effects insulin may have on wound healing. Even when administered to small incisional wounds, insulin has been demonstrated to accelerate healing (Greenway, Filler, & Greenway, 1999), although no data exists as to the quality of the resultant scar. Work performed in burns patients (Pierre, Barrow, et al., 1998) has similarly shown that systemic administration of high levels of insulin can significantly decrease the healing time in partial thickness injuries. This work demonstrated that even in vastly supra-physiological doses, insulin was able

to improve healing by shortening the time to re-harvesting of split thickness skin graft donor sites. However, in the healing of both burn wounds and partial thickness wounds most interest is directed at the keratinocyte, and little mention is made of the effect of insulin administration on either fibroblasts or dermal healing.

Within the *in vitro* data presented in this chapter, insulin has been shown to increase fibroblast proliferation, although the majority of time points show that cell number increases with time in culture, for each formulation of insulin one or two of the points show a decrease in cell number between days 10 and 14. Closer examination of the data relating to these time points does not reveal any particular reason why this decrease should be the case. In all cases, the standard deviations from the mean were less than 10% suggesting that these readings are real and not artefactual.

4.3.2 Synthesis of Extracellular Matrix Components

In addition to requiring cellular proliferation and differentiation, repair of wounds necessitates the reconstruction of the extracellular matrix. As this structure is comprised mainly of collagens, the effect of insulin on collagen production warranted investigation. Although it is accepted that insulin can stimulate collagen synthesis in the presence of serum (Verhofstad, Bisseling et al., 1998), the experiments presented in this chapter assessed whether this effect was evident when insulin was added to dialysed culture medium, as used within this thesis.

Evaluating the effect of addition of insulin to embryonic lung fibroblasts, Goldstein et al (Goldstein, Poliks et al., 1989) detected a 2-fold increase in the production of collagen. Similarly, earlier work by Sato et al. (Sato, Ignotz et al., 1981), found that addition of insulin to chick embryo fibroblasts resulted in a 1.5 fold increase in protein synthesis. In contrast, within the experiments described within this chapter, the change in collagen production proved only marginal and did not prove significant upon statistical testing or approach the magnitude reported by either Goldstein or Sato. This may be explained by the considerable differences between the experimental systems used. The experiments by Goldstein and Sato were performed in a 10% foetal bovine serum environment supplemented with amino acids, and thus contain many factors that may increase

collagen production but have been removed from the dialysed media as used in this thesis. In addition, the embryonic and foetal fibroblasts used as described in the literature may respond differently to the adult dermal fibroblasts used within this thesis. Finally, differences exist between the assay systems employed by previous authors and that used for the work presented within this thesis. Collagen production as determined by both Goldstein and Sato was measured using a radioassay that determines not only the amount of total soluble protein (as measured using the Sircol method used within this thesis) but also the amount of protein laid down by the cells in the extracellular matrix and also that bound within the cells themselves.

Although a trend to increased collagen production was observed in GFD medium supplemented with insulin, the high variability noted resulted in no significant changes overall over the production of collagen in fibroblasts cultured in GFD alone. The apparent reduced efficacy of insulin in the system used within this thesis may be due to a number of reasons. Firstly, differences exist between the assay techniques used in this chapter and much of the published literature, with radioassays historically being the favoured technique. The colorimetric collagen assay utilised within this thesis is relatively new, although is accepted as reliable to quantify changes in collagen production both *in vitro* (Shimizu, Mizobuchi et al., 1999) and *in vivo* (Jimenez & Rampy, 1999).

Secondly, many differences can be seen in the culture conditions used, with the majority of investigators using either 10% foetal calf serum containing medium or serum free conditions. Culture of fibroblasts in serum free conditions may be considered preferential for the investigation of the roles of defined growth factors, however, work performed by Zebrowski et al. (1987) has demonstrated some of the limitations that such conditions have, with some fibroblast cell lines displaying a greater sensitivity to the effects of insulin. The addition of bovine serum seems to reduce the effects of some growth factors. This might suggest the presence of factors in normal serum that modulate the activities of growth factors. Dialysis, as undertaken to produce GFD medium, may have removed some of the factors that stimulate fibroblast metabolism and left behind those that are inhibitory, explaining in some part the effects noted within the experiments described in this chapter.

The relative decrease in both collagen and protein production in fibroblasts cultured in GFD as compared to those in NGM can be explained by analysis of the components of each medium. Cellular activities *in vitro* are dependent upon the "soup" of growth factors, bioactive polypeptides and other molecules present. The extra processing (dialysis) required to make GFD medium will affect the balance of factors present, and in turn alter the cellular behaviour in culture. An imbalance in the interplay between the actions of insulin and other growth factors on protein, and to some extent on collagen synthesis may have been manufactured. Certainly, the insulin-like growth factors and TGFβ, removed during the production of GFD, are known to stimulate both collagen and protein synthesis (Davidson, Zoia et al., 1993). An additional explanation for the relatively poor effects of insulin in the culture systems employed within this chapter may relate to the presence of serum proteins greater than 14 kDa in GFD. The actions of insulin on stimulating protein production, although established, are diminished in the presence of serum (Zebrowski, Singh, et al., 1987). However, precisely what components of serum are responsible for this effect are currently unknown.

In addition to replacement of collagen, reconstruction of the damaged or missing extracellular matrix will require the production of other proteins. The experiments presented within this chapter differ from that in the literature in that total protein production was not assayed. Rather, analysis was undertaken to measure the amount of soluble protein present in the conditioned medium. This technique does not take into account synthesized protein retained by the cell, or that laid down as extracellular matrix. Although the level of protein production was reduced in GFD when compared to NGM, possibly because of the removal of growth factors present within NGM, addition of insulin did not result in a significant change in soluble protein production.

4.3.3 Contraction of collagen gels

Investigation of the effect of insulin upon the contraction of collagen gels showed that addition of this growth factor to GFD medium had little impact on contraction. Fibroblast behaviour in 3D collagen lattices is known to differ from that observed in 2D routine culture, with the 3D culture system thought to resemble the *in vivo* milieu more closely (Tomasek & Hay, 1984). The 3D system used within this thesis shares many

similarities with the collagen lattice model as originally described by Tomasek et al. with fibroblasts being able to interact with their surrounding matrix in 3 dimensions. As this 3D matrix is secured to the underlying substratum, tension can initially be developed. Following release of the collagen from the underlying dish, tension is lost allowing the fibroblasts to respond as though in a contracting wound (Lijnen, Petrov et al., 2001). This results in contraction of the collagen gel, which can be easily assessed by measuring the change in gel surface area.

Contraction of collagen gels has long been thought to be an indicator of myofibroblast function. However, increasing the number of myofibroblasts present within the collagen gels through cellular differentiation, using TGFβ, does not necessarily increase gel contraction (Grinnell, 1994). In experiments assessing the effect of mast cell extracts on fibroblast – myofibroblast differentiation and function, Gailit found that maximal contraction of collagen gels could be achieved, not with TGFβ, but rather with the addition of PDGF-BB at 5ng/ml (Gailit, Marchese, Kew, & Gruber, 2001). Interestingly, medium containing 10% FCS achieved 90% of the maximal contraction achieved using PDGF whilst the addition of TGFβ, known to cause myofibroblast differentiation, resulted in only 50% of this maximal contraction.

Previous work by Hinz (Hinz, Celetta et al., 2001) had already demonstrated that TGFβ treated collagen gels expressed more αSMA than untreated gels. This protein is the marker of myofibroblasts and therefore thought to be of importance in wound contraction, with its presence being correlated with the production of isometric tension (Harris, Stopak et al., 1981). However, the increase in myofibroblast number does not equate to an increase in contraction, suggesting that other processes may be required for the contraction of collagen gels. Similarly, if increases in αSMA do not equate to more contraction then it may be that decreases in αSMA expression may also not necessarily affect collagen gel contraction. The results provided in this chapter seem to support this hypothesis with insulin, shown to reduce myofibroblast numbers in Chapter 3, not significantly affecting collagen gel contraction.

The work evaluating αSMA and contraction in collagen gels might suggest that other mechanisms are required for gel contraction. Work performed by Ghahary (Ghahary,

Shen et al., 1995), (Nedelec, Ghahary, Scott, & Tredget, 2000) suggested alternative mechanisms by which contraction of collagen gels may be achieved. Migration of fibroblasts into and through the extracellular matrix during the initial phases of wound healing was found to be a fundamental component of wound contraction. During this migration, the pulling of collagen fibrils into a streamlined pattern, and the associated production of collagenase, may facilitate a more normal arrangement of collagen. Contraction of 3D collagen gels may therefore be partly reliant on cellular migration and collagenase production.

Other authors have assessed the effect of insulin on collagen gel contraction, although not using dermal fibroblasts. Lijnen et al. (Lijnen, Petrov, & Fagard, 2001) discovered that supplementing serum free medium with insulin-transferrin-selenium mixtures caused a pronounced stimulation of the collagen gel contraction by rat cardiac fibroblasts. Similar experiments in 1993 by Chen (Chen, Li et al., 1993) showed that insulin promoted collagen lattice contraction by arterial smooth muscle cells and fibroblasts again in serum free culture conditions. Interestingly, Chen et al. describe that contraction of collagen lattices occurred with the addition of either acidic fibroblast growth factor (aFGF) or TGFβ. Using inhibitors of cyclic AMP (cAMP), Chen concluded that insulin increases the contraction of collagen gels via a cAMP-independent mechanism.

The similarity of contraction evident in gels incubated with GFD to that seen in NGM may be due to growth factors remaining in GFD. Work performed by Gailit investigating the effects of growth factors on fibroblast contraction rates (Gailit, Marchese, Kew, & Gruber, 2001), demonstrated that one of the most potent stimulators of contraction is PDGF-BB. As the molecular weight of this compound is greater than 14 kDa, it is still present in the GFD used within this thesis, and therefore still able to stimulate collagen gel contraction.

In addition to the measured effect on contraction of collagen gels, within the 3D culture system other cellular processes will be occurring. Mio et al. (1996) evaluated the effects of insulin on fibroblast proliferation, identifying that in 3D culture systems insulin had little effect on fibroblast proliferation. Unfortunately, all experiments were performed in

stressed (fixed) collagen lattices and therefore the authors could not comment on the effect of insulin on contraction.

Although insulin has been shown to have no obvious effect on collagen lattice contraction (when compared to NGM and GFD) under the conditions described in this chapter (although shown to decrease αSMA expression in Chapter 3), it remains uncertain what effect, if any, insulin might have on wound contraction *in vivo*. However, other compounds that are known to decrease the expression of SMA do not affect resulting contraction. Ehrlich et al. (1999) found that, after addition of vanadate to the drinking water of rats that had undergone excisional wounding, SMA expression in both wounds and granulation tissue in implanted PVA sponges was reduced whilst wound contraction was unaffected. In addition to *in vivo* studies, vanadate has been evaluated in human fibroblast culture (Ehrlich, Keefer, Myers, & Passaniti, 1999). Here it prevented the appearance of cytoplasmic stress fibres. This is thought to be due to either its inhibition of phosphatases that remove phosphate groups from selected phosphorylated tyrosine residues in proteins or due to down-regulation of p125 focal adhesion kinase. Vanadate can act as an insulin sensitizer and has been suggested as a diabetes therapy for use in conjunction with insulin (Woo, Yuen et al., 1999). Insulin's effect in reducing αSMA expression without altering contraction may be through a similar mechanism to that seen with vanadate as described by Ehrlich et al. (1999).

4.4 Conclusions

- Insulin caused no discernable adverse effects *in vitro* on some of the processes important in dermal wound healing.
- Insulin increases fibroblast proliferation when added to growth factor dialysed medium, and is still effective when applied at supra-physiological levels. The longer acting preparations are most effective at increasing proliferation.
- Soluble collagen production does not significantly change with the addition of insulin.
- Soluble protein production does not significantly alter with the addition of insulin.

- Contraction of collagen gels is not significantly altered by the addition of insulin.

Chapter 5

Do polypeptides that share certain biological properties with insulin affect fibroblast - myofibroblast differentiation?

5.1 Introduction

The data presented in Chapter 3 has shown insulin to be effective in reducing myofibroblast numbers *in vitro*. On the basis of this finding, it may also be expected that other compounds exist that will also reduce fibroblast - myofibroblast differentiation. Elucidation of the precise mechanism by which insulin is exerting an effect may allow for the identification of these substances, and possibly allow the identification of substances that are even more effective than insulin itself. However, the mechanism by which insulin reduces fibroblast - myofibroblast differentiation is, at present, unknown.

The physiological effects of insulin are well described (Berne and Levy, 1996). In particular, it has a well defined role as a mitogenic factor (Ostlund, Jr. & Yang, 1985), with its effects on dermal fibroblast proliferation being demonstrated in Chapter 4. Furthermore, insulin has other well documented effects, including an ability to reduce apoptosis in many cell culture systems (Bertrand, Atfi et al., 1998; Hermann, Assmus et al., 2000; Jonassen, Brar et al., 2000). The effects upon proliferation and apoptosis may suggest a means by which insulin reduces fibroblast - myofibroblast differentiation. Selective increases in proliferation of non-myofibroblastic dermal fibroblasts, over and above that of myofibroblasts, would result in an apparent reduction in the percentage of myofibroblasts. Likewise, the selective apoptosis of the myofibroblast phenotype, or the inhibition of apoptosis in non-myofibroblastic fibroblasts would have a similar effect. However, if the reduction in myofibroblast numbers is purely because of changes in proliferation or apoptosis, addition of other factors that are known to affect these processes may also be expected to be effective in reducing fibroblast - myofibroblast differentiation. Factors that are known to affect proliferation and apoptosis include platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) (Stewart & Rotwein, 1996), (Hill, Tumber et al., 1997).

Analysis of the process used to produce GFD medium may suggest a list of candidate growth factors that also reduce fibroblast - myofibroblast differentiation. During the preparation of GFD medium (principally during dialysis), a number of polypeptides of molecular weight less than 14 kDa are removed. These include factors such as the

insulin like growth factors (IGF-I and IGF-II) (Linge, Shelton, Mackie, & Sanders, 2004). These factors are known to stimulate proliferation in fibroblast cell lines and have also been shown to reduce apoptosis in a number of cells (Staiger & Loffler, 1998). In addition to the effects on cell proliferation, both insulin like growth factor-I and -II are known to have many functional overlaps with insulin (Greenhalgh, 1996).

Indeed, IGF-I belongs to the same molecular superfamily as insulin, and is known to bind to a very similar receptor (Chen & Feng, 2001). Furthermore, comparison of the 3D structures of these two compounds has shown that the insulin A and B domains are almost identical to the IGF-I A and B domains. Such similarities in secondary structure may be expected to result in functional overlap. Indeed, insulin has been shown to be able to bind to the IGF-I receptor (Chen & Feng, 2001), and significant overlap between the activities of insulin and IGF-I has been reported by many observers (Dupont & LeRoith, 2001).

Insulin has been reported to inhibit apoptosis (Hill, Tumber, & Meikle, 1997). This may be linked to insulin's actions in preventing fibroblast - myofibroblast differentiation. The process of fibroblast differentiation into myofibroblasts has been considered part of a terminal differentiation process since *in vivo* myofibroblast differentiation is normally followed by apoptosis (Clarke, 1996). The effect of insulin upon apoptosis may therefore reflect its actions on fibroblast - myofibroblast differentiation. Although the other growth factors mentioned within this chapter have not been credited with being able to prevent fibroblast - myofibroblast differentiation, it is conceivable that if theoretical mechanism of action is correct, then the factors that are known to reduce apoptosis may also decrease fibroblast - myofibroblast differentiation.

This chapter aims to evaluate the ability of some of these growth factors to affect fibroblast - myofibroblast differentiation. If insulin is reducing fibroblast - myofibroblast differentiation either by affecting proliferation or apoptosis, then the addition of these other polypeptide growth factors will have similar effects.

Aims:

- To evaluate the basal apoptosis rates in NGM and GFD medium and to assess the effect of insulin on these rates.
- To evaluate whether other compounds known to effect either proliferation or apoptosis of fibroblasts may cause a decrease in myofibroblast number in GFD medium.
- To evaluate the ability of insulin-related compounds, which share many of insulin's biological properties, to reduce myofibroblast numbers in GFD medium.

5.2 Results

5.2.1 Basal apoptosis rates in fibroblast culture medium

The rate of apoptosis in 3 dermal fibroblast cell lines was evaluated using the ApoPercentage apoptosis detection kit (Biocolor), according to the manufacturer's instructions (Chapter 2.3.7). Briefly, 5000 dermal fibroblasts were seeded into each well of a 96-well plate, pre-coated with 2% gelatin. Cultures were maintained in either NGM or GFD both with and without the addition of Insulatard (1 IU/ml). Media was refreshed twice weekly until fibroblasts had reached confluence. The cells were then incubated with APOPercentage Dye and washed prior to examination. Microscopic examination was undertaken to determine the presence of apoptosing cells. In addition, each well was incubated with Dye Release Reagent and the OD measured at 540 nm.

Colorimetry

Colorimetric analysis of the above cultures after release of the bound dye (using the supplied Dye Release Agent) yielded the following results (shown in Fig 5.1). Those cultures incubated in NGM displayed a mean OD of 0.041 (±0.0015). Addition of insulin increased the OD to 0.044±0.0013. This increase was not significant. Culture of fibroblasts in GFD yielded an OD of 0.041 (±0.0016), whilst addition of insulin again increased this reading to 0.056 (±0.004). The increase seen with addition of insulin was significant, $P<0.001$. These results were comparable to those from microscope examination of cultured cells. Statistical analysis was undertaken using the ANOVA test.

Microscopy

Examination of dermal fibroblasts cultured in NGM demonstrated that few cells that uptake dye, signifying a low level of apoptosis in this culture medium. Addition of insulin yielded approximately 1-2 cells per well (of a 96 well plate) that had dye uptake. A lack of dye uptake was observed in dermal fibroblasts cultured in both GFD and GFD and insulin.

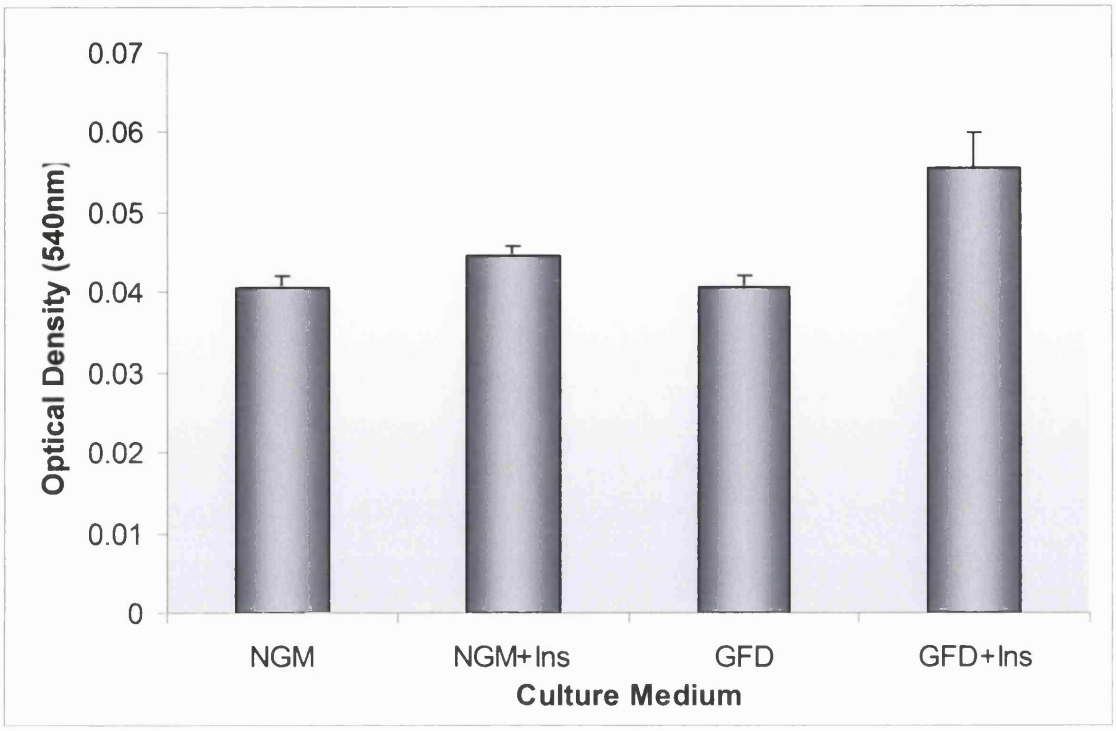


Fig 5.1 Graph showing the mean and standard deviation for apoptosis in 3 dermal fibroblast cell lines. Apoptosis was determined using the Apopercentage Apoptosis assay (Biocolor). Increasing OD correlates with increasing numbers of apoptosing cells.

5.2.2 The Addition of Factors Known To Reduce Apoptosis

The following investigations were undertaken in triplicate for each of the three different dermal fibroblast cell lines following the methodology described in Chapter 2.3.7. For each cell line, fibroblasts were seeded onto sterile coverslips in triplicate in NGM, GFD, GFD and Insulin (Insulatard 1 IU/ml) and GFD supplemented with other growth factors as necessary. Media was refreshed twice weekly. After 14 days, coverslips were stained to demonstrate αSMA and the number of myofibroblasts present in 3 random fields calculated. All results are expressed using the same format as the preceding chapters, with the reduction in myofibroblast numbers seen with treatment expressed as a percentage of that present in GFD medium.

Insulin Like Growth Factor 1 (IGF-I)

Before investigation of the effect of IGF-I on fibroblast - myofibroblast differentiation could be performed, it was necessary to evaluate the concentration range within which this growth factor is effective in dermal fibroblast cultures. The effects of various concentrations of IGF-I on dermal fibroblast proliferation were determined using the crystal violet assay as described in Section 2.3.3. Dermal fibroblasts from 6 cell lines were each evaluated in control medium and control medium supplemented with IGF-I at concentrations ranging from 0 to 200 ng/ml in replicates of 6. Control medium for these experiments contained 0.4% FCS in order to maintain the fibroblasts in a quiescent state but not induce apoptosis (personal communication C. Linge, RAFT). The data presented in Fig 5.2 is the mean and standard deviation of that obtained from 6 cell lines and replicates, and is expressed as the percentage increase in OD (650 nm- crystal violet assay) from the negative control (0.4% FCS only).

Briefly, addition of IGF-I to dermal fibroblast cultures results in an increase in proliferation above that seen in control medium. The extent of proliferation increased in a dose dependent manner, reaching a plateau level of a 40% increase in proliferation when levels of IGF-I exceeded 100 ng/ml.

The effect of addition of IGF-I on fibroblast - myofibroblast differentiation was investigated by supplementation of GFD medium at 10 ng/ml and 100 ng/ml. Although the manufacturer's data describes an ED_{50} of 1-3ng/ml (Sigma), during the preceding experiments, the ED_{50} was found to correlate to 10 ng/ml.

Culture of fibroblasts in normal growth medium yielded a reduction in myofibroblast number of 78.2% (±10.3) when compared to those cultured in GFD. Addition of Insulatard® at 1 IU / ml to GFD, significantly ($P<0.05$) reduced myofibroblast numbers by 75.1% (±14.2). Supplementing GFD with IGF-I reduced myofibroblast numbers by only 27.9% (±36.0) or 29.6% (±30.8) when added at 10 ng/ml or 100 ng/ml respectively. The reduction observed on treatment with IGF-I was not significant upon testing $P>0.05$. These results are shown in Fig 5.3, where the mean and standard deviation of the reduction in myofibroblast number when compared to GFD cultures are plotted for each treatment.

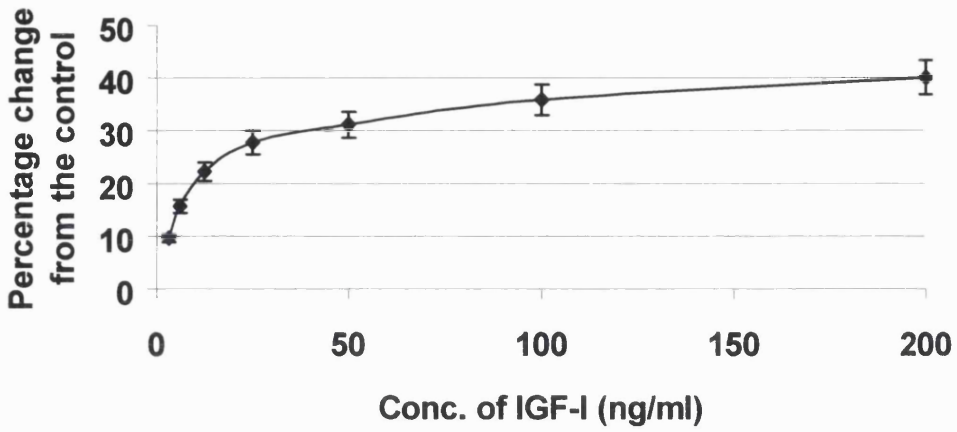


Fig 5.2 Graph showing the change in proliferation rate when dermal fibroblasts are cultured in medium containing IGF-I. Control medium was minimal % of FCS (0.4%) to maintain the cells in a quiescent state but not induce apoptosis (personal communication C.Linge, RAFT). The graph represents data from 6 replicates of 6 cell lines, expressed as the percentage increase in OD (650 nm, crystal violet assay) from the negative control (0.4% FCS only)

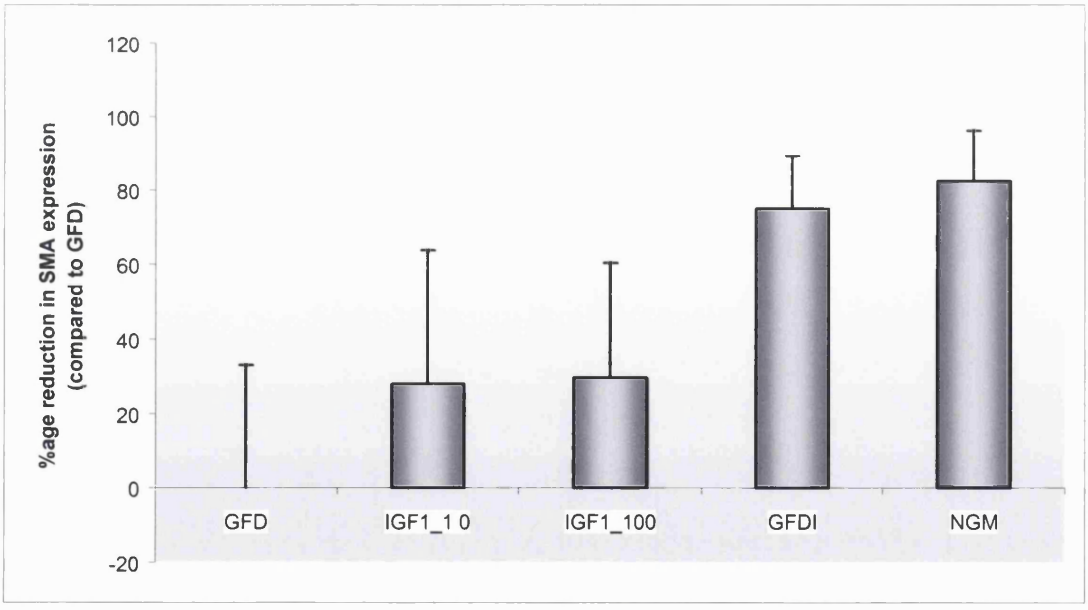


Fig 5.3 Graph showing mean and standard deviation for the percentage reduction in myofibroblast numbers (when compared to GFD). Dermal fibroblast cultures were maintained for 14 days in NGM, GFD or GFD supplemented with either 10 ng/ml or 100 ng/ml IGF-I, or insulin 1 IU/ml.

Insulin Like Growth Factor 2 (IGF-II)

Prior to investigating the effects of IGF-II on fibroblast - myofibroblast differentiation, evaluation of the concentration range within which this growth factor is effective in dermal fibroblast cultures was undertaken. The effects of various concentrations of IGF-II on dermal fibroblast proliferation were determined using the crystal violet assay as described in Section 2.3.3. Dermal fibroblasts from 6 cell lines were each evaluated in control medium and control medium supplemented with IGF-II at concentrations ranging from 0 to 200 ng/ml in replicates of 6. Control medium for these experiments contained 0.4% FCS to maintain the cells in a quiescent state but not induce apoptosis (personal communication C.Linge, RAFT). The data is presented in Fig 5.4 as the mean and standard deviation of that obtained from all 6 cell lines and replicates, and expressed as the percentage increase in OD (650 nm - crystal violet assay) from the negative control (0.4% FCS only).

Briefly, addition of IGF-II to dermal fibroblast cultures results in an increase in proliferation above that seen in control medium. Proliferation increased in a dose dependent manner, ultimately seeming to plateau at a level of a 31% increase in amount of proliferation (200 ng/ml IGF-II).

Although the ED_{50} for IGF-II is quoted at ranging between 0.5-25ng/ml (Sigma, manufacturers data), in the experiments undertaken to evaluate the effects of IGF-II on dermal fibroblasts described above, the ED_{50} was estimated at approximately 40 ng/ml. In order to evaluate IGF-II across its biologically active range, the effect of addition of IGF-II was investigated at both 10 ng/ml and 100 ng/ml.

As shown in Fig 5.5, where the mean and standard deviation for the percentage reduction in myofibroblast numbers are shown, addition of IGF-II resulted in a reduction in myofibroblast number much like that seen with addition of IGF-I. Supplementation of GFD with 10 ng/ml or 100 ng/ml IGF-II reduced myofibroblast numbers from that seen in GFD by 18.8% (±25.9) and 19.6% (±30.4) respectively. This reduction in myofibroblast numbers was not significant from that observed in GFD upon testing ($P>0.05$) whereas insulin once again significantly reduced fibroblast - myofibroblast differentiation ($P<0.05$).

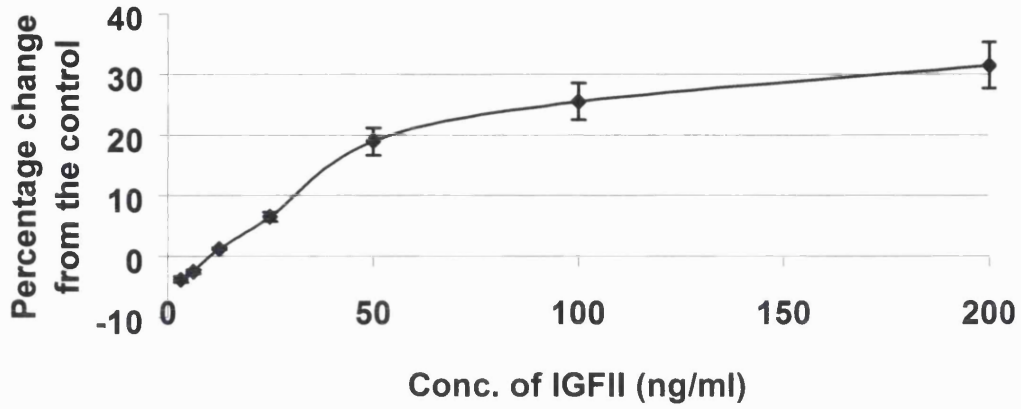


Fig 5.4 Graph showing the change in proliferation rate when dermal fibroblasts are cultured in medium containing IGF-II. Control medium was minimal % of FCS (0.4%) to maintain the cells in a quiescent state but not induce apoptosis (personal communication C.Linge, RAFT). The graph represents data from 6 replicates of 6 cell lines, expressed as the percentage increase in OD (650 nm, crystal violet assay) from the negative control (0.4% FCS only)

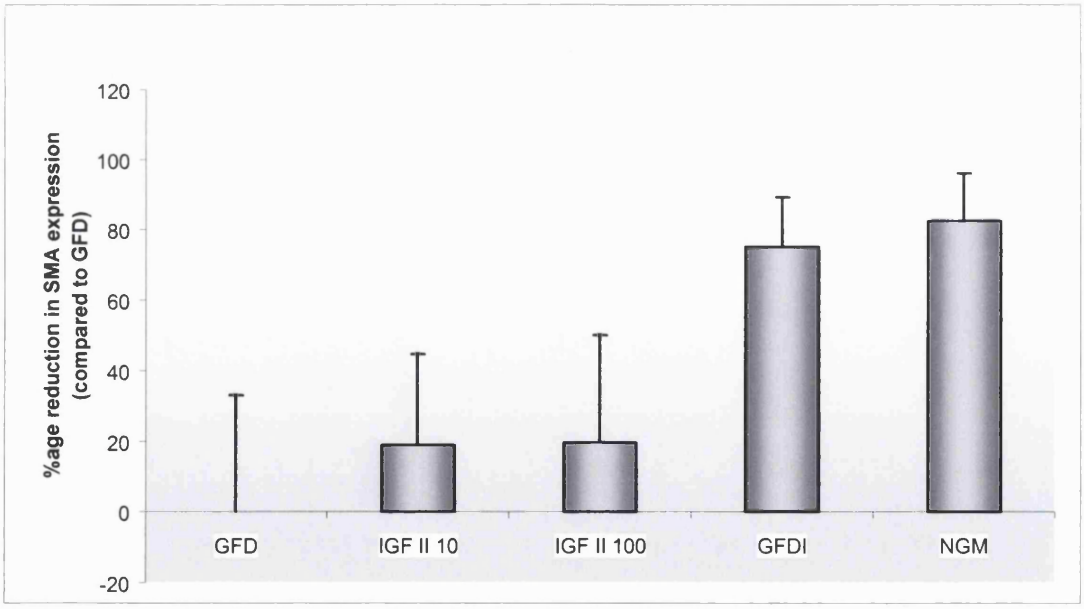


Fig 5.5 Graph showing mean and standard deviation for the percentage reduction in myofibroblast numbers (when compared to GFD). Dermal fibroblast cultures were maintained for 14 days in NGM, GFD or GFD supplemented with either 10 ng/ml or 100 ng/ml IGF-II or insulin 1 IU/ml.

Basic Fibroblast Growth Factor (bFGF)

The effect of bFGF on dermal fibroblast proliferation was determined using the crystal violet assay as described in Section 2.3.3. Dermal fibroblasts from 6 cell lines were each evaluated in control medium and control medium supplemented with bFGF at concentrations ranging from 0 to 200 ng/ml in replicates of 6. Control medium for these experiments contained 0.4% FCS so as to maintain the cells in a quiescent state but not induce apoptosis (personal communication C.Linge, RAFT). The data is presented in Fig 5.6 as the mean and standard deviation of that obtained from all 6 cell lines and replicates, and expressed as the percentage increase in OD (650 nm - crystal violet assay) from the negative control (0.4% FCS only).

The addition of bFGF to dermal fibroblast cultures resulted in an increase in proliferation above that seen in control medium. Proliferation again increased in a dose dependent manner, with a short plateau at 40% (bFGF: 25-100 ng/ml). Thereafter, proliferation increased further within the concentration range of bFGF tested. The ED_{50} was estimated to be 6 ng/ml.

The effect of addition of bFGF on fibroblast - myofibroblast differentiation was investigated using the growth factor at 20 ng/ml. Previous work by Maltseva et al. (2001) identified that addition of bFGF at this concentration was most effective in reducing myofibroblast numbers in corneal fibroblast cultures. Within the experiments undertaken to evaluate the effects of bFGF on dermal fibroblast proliferation, a value of 20 ng/ml was found to approximate the plateau of activity.

The mean percentage reduction in myofibroblast number as compared to levels seen in GFD is illustrated in Fig 5.7. For comparison, the reduction seen with addition of insulin and with culture in NGM area also displayed. Addition of bFGF at 20 ng/ml yields a 29.1% (±32.0) reduction in myofibroblast number. This change is not significantly different from the levels observed in GFD alone ($P>0.05$).

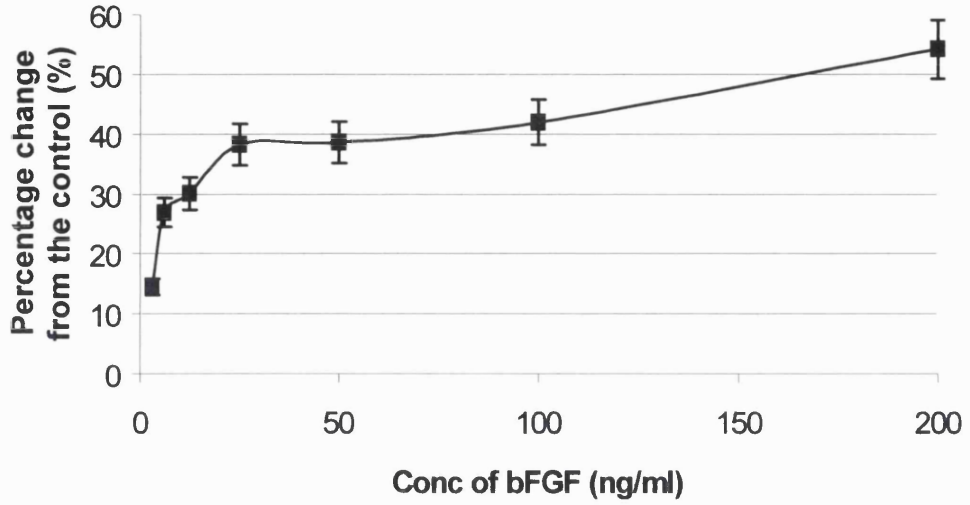


Fig 5.6 Graph showing the change in proliferation rate when dermal fibroblasts are cultured in medium containing bFGF. Control medium was minimal % of FCS (0.4%) so as to maintain the cells in a quiescent state but not induce apoptosis (personal communication C.Linge, RAFT). The graph represents data from 6 replicates of 6 cell lines, expressed as the percentage increase in OD (650 nm, crystal violet assay) from the negative control (0.4% FCS only)

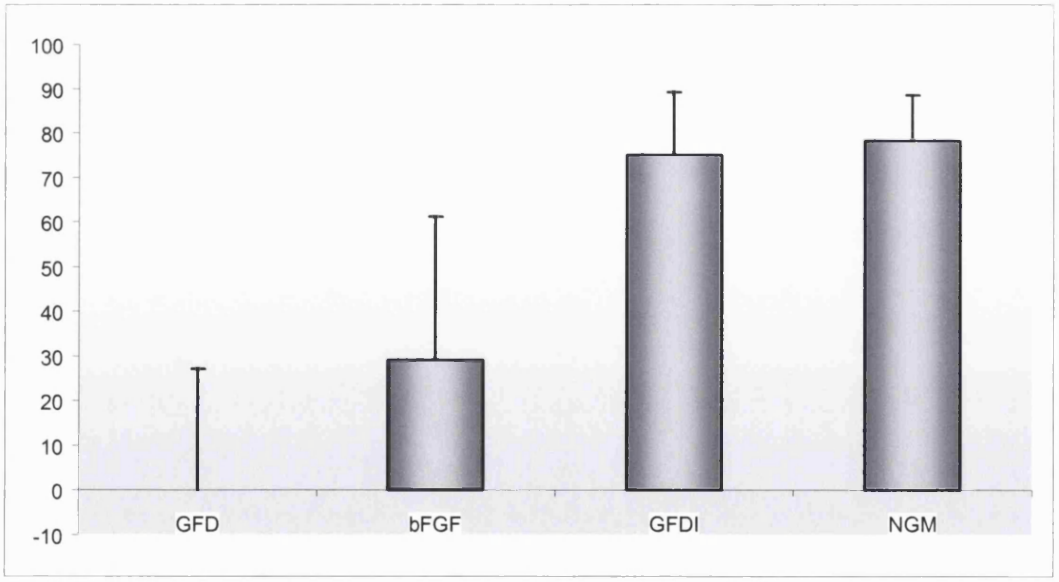


Fig 5.7 Graph showing mean and standard deviation for the percentage reduction in myofibroblast numbers (when compared to GFD). Dermal fibroblast cultures were maintained for 14 days in NGM, GFD or GFD supplemented with 20 ng/ml bFGF or insulin 1 IU/ml.

Platelet Derived Growth Factor (PDGF)

The effect of various concentrations of PDGF on dermal fibroblast proliferation was determined using the crystal violet assay as described in Section 2.3.3. Dermal fibroblasts from 6 cell lines were each evaluated in control medium and control medium supplemented with PDGF at concentrations ranging from 0 to 200 ng/ml in replicates of 6. Control medium for these experiments contained 0.4% FCS so as to maintain the cells in a quiescent state but to not induce apoptosis (personal communication C.Linge, RAFT). The data is presented in Fig 5.8 as the mean and standard deviation of that obtained from all 6 cell lines and replicates, and expressed as the percentage increase in OD (650 nm - crystal violet assay) from the negative control (0.4% FCS only).

Addition of PDGF to minimal medium results in an increase in dermal fibroblast proliferation (as measured using the crystal violet assay). The extent of increase in proliferation reached a plateau at 150% of that seen in control medium for concentrations of PDGF greater than 25 ng/ml.

To assess the effects of PDGF on fibroblast - myofibroblast differentiation, three dermal fibroblast cell lines (each initiated from a different patient) were cultured on glass coverslips. Fibroblasts were cultured in NGM, GFD or GFD supplemented with either Insulatard 1 IU/ml or PDGF at 10 ng/ml or 100 ng/ml concentrations. Interestingly, the ED_{50} estimated from the proliferation data shown in Fig 5.8 is approximately 10 ng/ml, which is within the range quoted by the manufacturers (Sigma). The concentrations used within these experiments therefore represented a dose equivalent to the manufacturers quoted ED_{50} (10 ng/ml), although the experiments performed on fibroblast proliferation might suggest a more effective dose. The dose of 100 ng/ml was used in order to evaluate a possible maximal response to this growth factor.

Addition of PDGF to fibroblasts cultured in GFD resulted in a reduction in the numbers of myofibroblasts. An optimal reduction of 66.3% (±29.1) from that seen in GFD was observed with addition of 100 ng/ml PDGF. This reduction was significant ($P<0.001$). Addition of PDGF at 10 ng/ml also significantly ($P<0.001$) reduced myofibroblast numbers by 32.0% (±27.5). For comparison, addition of insulin results in a reduction in fibroblast numbers, 75.0% (±14.2). The reduction seen with addition of PDGF at 100

ng/ml was not significant when compared with that seen with addition of insulin at 1 IU/ml. The results of addition of PDGF to GFD can be seen in Fig 5.9, where the mean and standard deviation reduction in myofibroblast number (when compared to GFD) can be seen.

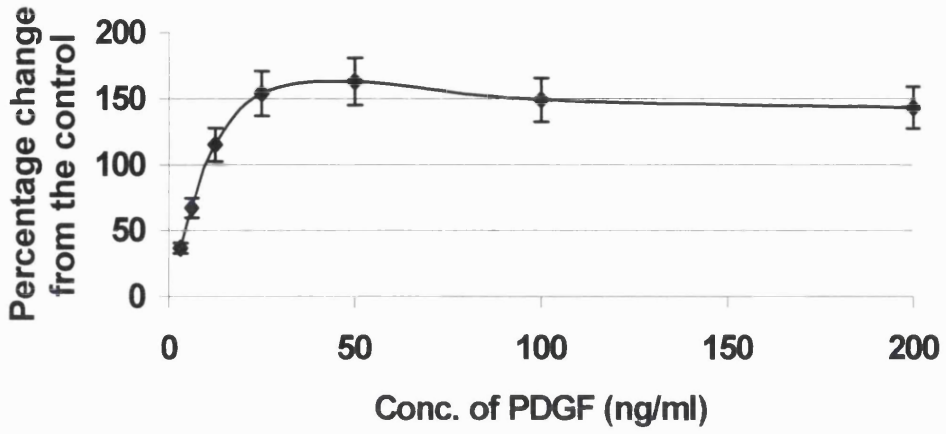


Fig 5.8 Graph showing the change in proliferation rate when dermal fibroblasts are cultured in medium containing PDGF. Control medium was minimal % of FCS (0.4%) so as to maintain the cells in a quiescent state but not induce apoptosis (personal communication C.Linge, RAFT). The graph represents data from 6 replicates of 6 cell lines, expressed as the percentage increase in OD (650 nm, crystal violet assay) from the negative control (0.4% FCS only)

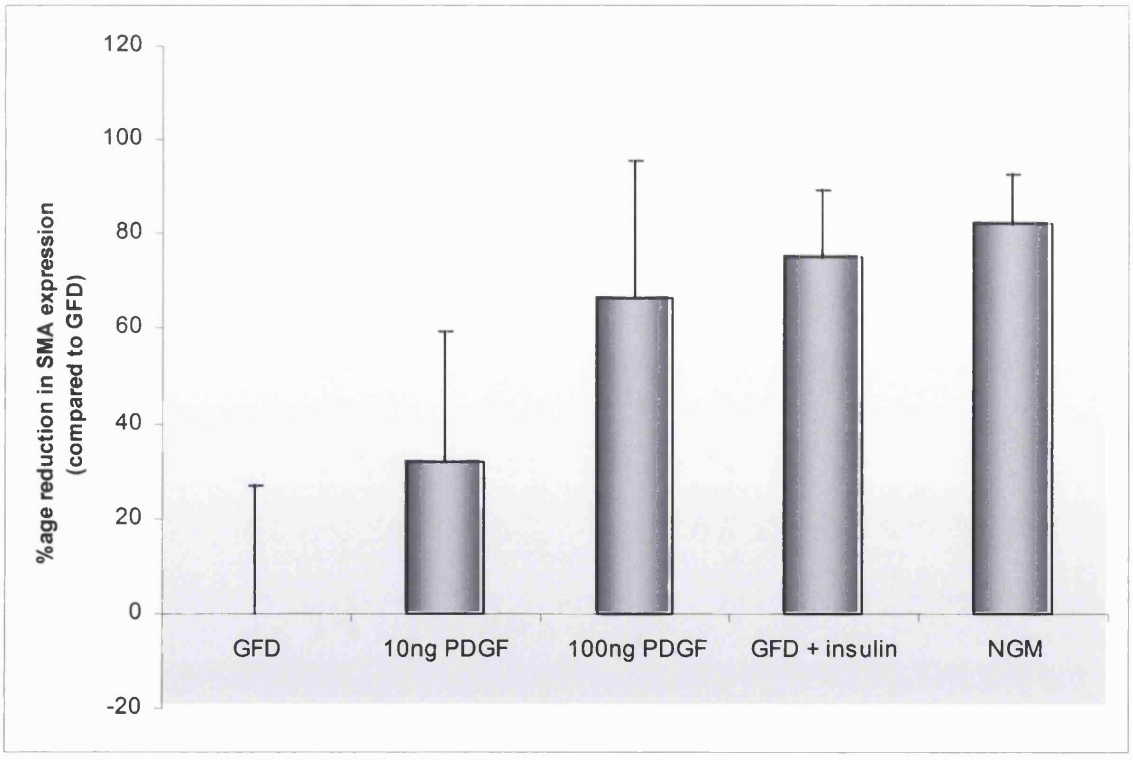


Fig 5.9 Graph showing mean and standard deviation for the percentage reduction in myofibroblast numbers (when compared to GFD). Dermal fibroblast cultures were maintained for 14 days in either NGM, GFD or GFD supplemented with either 10 ng/ml or 100 ng/ml PDGF or insulin 1 IU/ml.

Wortmannin, an Inhibitor of PI 3-kinase

Following the finding that addition of PDGF reduced fibroblast-myofibroblast differentiation to a similar extent as that seen with insulin, the internal signalling mechanisms triggered by both of these molecules following receptor binding were examined. Although few common physiological actions can be attributed to both of these compounds, a number of common signalling pathways were identified.

Both insulin and PDGF exert their effects upon the cell via intracellular signalling through the PI 3-kinase molecule (Hooshmand-Rad, Hajkova et al., 2000), (Whitehead, Clark et al., 2000). The evaluation of these common pathways may allow for the development of a therapy for the prevention of fibroblast myofibroblast differentiation. Although time constraints precluded an in depth analysis of analysis of these common pathways, the role of PI 3-kinase in myofibroblast differentiation was evaluated.

PI 3-kinase is a key molecule in the intracellular signalling of a number of substances (Whitehead, Clark, Urso, & James, 2000). However, this molecule can be easily and specifically inhibited *in vitro* by the addition of wortmannin (Park, Lee et al., 1997). The effect of wortmannin on fibroblast - myofibroblast differentiation at its recommended effective concentration (Sigma – manufacturer's data) was thus undertaken.

Dermal fibroblast cell lines were derived from 3 patients and seeded individually onto sterile coverslips. Each cell line was tested in triplicate for each of the following compounds. Fibroblasts were cultured in NGM, GFD or GFD supplemented with the substance under investigation. Medium was refreshed twice weekly and cultures stained for αSMA after 14 days. The concentration of each compound used to determine the effects on fibroblast - myofibroblast differentiation was either derived from manufacturer's data or from literature depicting their effects on other cell lines.

As absolute numbers of myofibroblasts varied considerably between cell lines, the following results are expressed in the manner used earlier in this thesis. Results are expressed as the mean percentage reduction in myofibroblast number for each treatment

as compared to the proportion of myofibroblasts identified in GFD medium alone for that cell line.

Wortmannin was added to GFD medium at concentrations of 2 nM and 20 nM. These doses were chosen due to their approximation to the EC_{50} for wortmannin in inhibiting PI 3-kinase (10 nM (Vemuri, Zhang et al., 1996)).

As can be seen in Fig 5.10, where the mean change in myofibroblast number is shown, addition of wortmannin to dermal fibroblast cultures increases the numbers of myofibroblasts present. A 2 nM solution of wortmannin increases myofibroblast numbers significantly ($P<0.05$) over that seen in those fibroblasts cultured in GFD by 60.1% (±50.5). Increasing the concentration of wortmannin 10 fold, to 20 nM, results in little further increase in myofibroblast number, remaining at 68.5% (±62.2). Interestingly, addition of insulin to medium containing 20 nM wortmannin had little effect on fibroblast - myofibroblast differentiation. Myofibroblast number is only reduced to 38.1% of that seen in GFD alone. This change in myofibroblast proportion seen with insulin in the presence of wortmannin is not significant from that seen in dermal fibroblasts cultured in GFD alone ($P>0.05$).

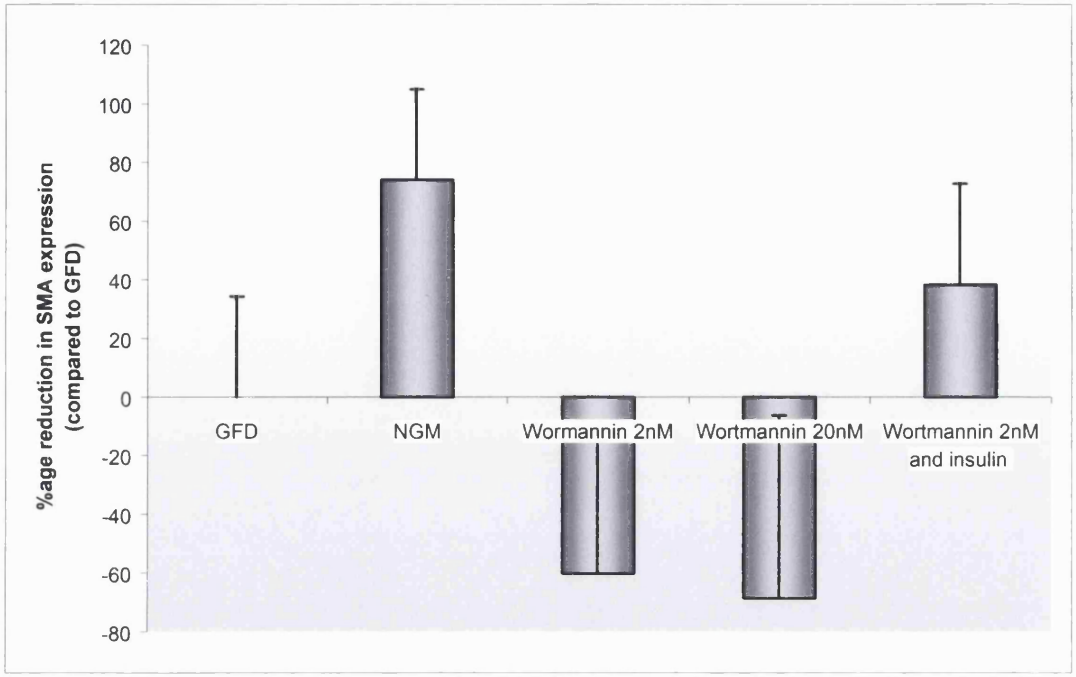


Fig 5.10 Graph illustrating the mean percentage change in myofibroblast number (and standard deviation) for 3 dermal fibroblast cell lines, from that seen when cultured in GFD alone, with addition of wortmannin to GFD medium.

5.3 Discussion

Elucidation of the precise mechanism by which insulin reduces fibroblast - myofibroblast differentiation may allow for the development of an effective therapy for the prevention or reduction of cutaneous scarring. Within the *in vitro* experiments performed in this chapter, efforts have been made to determine insulin's effect on dermal fibroblast apoptosis as a means to discovering its mode of action.

Although a reduction in fibroblast - myofibroblast differentiation can theoretically be achieved by selective proliferation of αSMA negative fibroblasts, the data presented in the previous chapter assessing the effect of insulin on proliferation might suggest that this mechanism is unlikely. Evaluation of insulin's effect on dermal fibroblast proliferation showed a minimal increase over a 14-day period. It may therefore seem unlikely that insulin reduces myofibroblast numbers through its action as a growth factor. If insulin were to be acting in this manner, then one might expect its proliferative effects to be greater than that measured to account for the significant decreases in fibroblast - myofibroblast differentiation seen. In addition, if insulin were reducing fibroblast - myofibroblast differentiation via its role as a growth factor, then the addition of other factors known to stimulate fibroblast proliferation should yield comparable reductions. As seen within this chapter, although high concentrations of PDGF do indeed reduce fibroblast - myofibroblast differentiation, another potent fibroblast mitogen (bFGF) does not appear to affect fibroblast differentiation at the concentration tested.

The analysis of apoptosis rates in dermal fibroblast cultures suggests that insulin does not reduce myofibroblast numbers through an inhibition of this process. Indeed, the results described within this chapter show insulin to increase apoptosis rates in dermal fibroblast cultures. These findings are in contrast to many authors who have shown that insulin reduces apoptosis rates either by its own action or behaving as a potentiating factor for many other anti-apoptotic factors (Bertrand, Atfi, et al., 1998). This difference may be explained either by a variation in the tissues investigated or in the differences between the culture systems employed, in particular in the culture medium used.

When comparing the work contained within this thesis to that in the literature, allowances must be made for the culture medium used. In addition to NGM (containing 10% FCS) a novel medium has been employed. Although GFD is lacking much of the growth factor activity seen in NGM, it is able to support cell proliferation (as seen in Chapter 4). However, the partial growth factor activity is in contrast to that seen in the medium usually employed in many studies. Within such studies, culture medium either contains FCS (similar to NGM used within this thesis), or is serum free. Both of these media seem less than ideal when evaluating the effects of substances upon cellular behaviour. In the case of FCS containing media, the numerous growth factors present may have significant implications on both the behaviour of the cell, and also upon the activity of the substance under investigation. Similarly, the lack of growth factors in serum free cultures may also influence cellular behaviour.

Despite the surfeit of data published that suggests insulin as a survival factor, there are some published studies which are congruent with the apoptosis findings presented in this chapter. Work by Godbout et al. has suggested that insulin can activate Caspase-3 (a molecule important during apoptosis) (Godbout, Cengel et al., 1999). It is possible that insulin decreases myofibroblast numbers by a number of mechanisms and not by simply causing a preferential increase in apoptosis in myofibroblasts, but also by acting as a cell-survival factor in normal dermal fibroblasts. Although studies to confirm or refute this have not been performed during the course of this thesis, future investigation into such a mechanism seems warranted.

Insulin like Growth Factors

The results described within this chapter suggest both IGF-I and IGF-II have little effect in reducing the expression of alpha smooth muscle actin. Indeed, much interest has recently been paid to the role of the insulin like growth factors during wound healing (Ghahary, Shen et al., 1995). Analysis of normal and pathological scar has suggested a role for IGF in the development of fibrosis.

Many of the activities of insulin, particularly those related to growth-promotion, are considered mediated through activation of its receptor and / or activation of the IGF receptors. Previous studies have shown that insulin is capable of exhibiting 3% of the

growth promoting activity of IGF-I; but its receptor-binding to the IGF-I receptor is only 1.5% that of IGF-I itself (Chen & Feng, 2001). This suggests that insulin is capable of growth promotion through binding to its own receptor. The lack of response seen with IGF-I in our work suggests that this molecule plays a small part in the control of fibroblast-myofibroblast differentiation. Indeed, even at supra-physiological levels, little reduction in myofibroblasts numbers can be detected. In contrast, the work presented in the previous chapters depicts the effect of insulin. As insulin binds so weakly to the IGF-I receptor, the reduction in myofibroblast numbers is likely due to the action of insulin binding to its own receptor. However, insulin's relative lack of effect at physiological concentrations might suggest that its effect on fibroblast – myofibroblast differentiation is not mediated solely through its actions on its own receptor.

Basic Fibroblast Growth Factor

bFGF has previously been found to reduce SMA expression (Desmouliere & Gabbiani, 1996), (Khouw, van Wachem et al., 1999). Work performed by Velez et al. has also demonstrated the ability of this growth factor to reduce αSMA expression within cardiomyocytes, showing that it to be less effective than PDGF (Velez, Aranega et al., 1995). Even allowing for differences in culture conditions, as well as differing cell/tissue types, the work of Velez et al. still yields results comparable to that achieved within the dermal fibroblast culture system employed in this thesis. However, the magnitude of the reduction in αSMA expression is substantially lower than that observed by Khouw et al. (1999), although such differences may either be a consequence of differing species (porcine vs human) or the unique culture conditions employed within this thesis.

Interestingly, the ability of bFGF to reduce fibroblast - myofibroblast differentiation may be manipulated by the addition of heparin, with Maltseva et al. showing a greater reduction in myofibroblast phenotype corneal scarring with the addition of FGF and heparin (Maltseva, Folger, et al., 2001).

Platelet Derived Growth Factor

The most effective compound amongst those tested here, other than insulin, was PDGF. The ability of this growth factor to decrease αSMA has been described by Corjay et al.

(1990) assessing the effects of PDGF on rat vascular smooth muscle cells. Similarly, reductions in αSMA expression have been noted in many different mesenchymal tissues. Zaleskas et al. (2001) studied the effects of PDGF on chondrocytes in a collagen-GAG matrix, whereas Blank et al. (1990) achieved similar results to Corjay et al. using rat aortic smooth muscle cells. Shirakawa reported that PDGF (and also EGF and NGF to some extent) caused a reduction in the expression of αSMA in human retinal epithelial cells, whereas insulin and FGF did not (Shirakawa, Yoshimura et al., 1986).

Interestingly, in their work on Chinese Hamster Ovary cells, Knight et al. (1995) reported that whereas insulin transiently reduced αSMA expression (over a 60 minute experiment), PDGF transiently increased its expression. This might suggest that insulin is the superior compound for the reduction of myofibroblast numbers although the difference between the two treatments at optimal doses is not significant. Although a body of evidence has confirmed that PDGF reduces αSMA expression, contradictory results have also been reported. Tredget et al. suggested, in a study performed on human dermal fibroblasts, that PDGF may actually increase the expression of α-smooth muscle actin (Tredget, 1994). The work presented within this chapter agrees with that supporting PDGF as a compound that reduces αSMA expression.

Common signalling pathways

Both insulin and PDGF can, in the dermal fibroblasts used within this thesis, reduce expression of αSMA. This might suggest a common mechanism of action and analysis of their signalling pathways has revealed similarities. One common signalling pathway is the use of phosphatidylinositol 3-kinase (PI 3-kinase) (Hooshmand-Rad, Hajkova, et al., 2000). PI 3-kinase is composed of a p110 catalytic subunit and a p85 regulatory subunit that possesses 2 SH2 domains that interact with the IRS proteins (Myers, 1992). Although many factors activate this molecule, the downstream signalling pathways can therefore differ. In the case of PDGF, its effects can be negated via the inhibition of p110(alpha) whereas inhibition of p110(beta) is required for a similar effect with insulin (Hooshmand-Rad, Hajkova, et al., 2000). PI 3-kinase is not only implicated in the signalling of PDGF and insulin but also has a pivotal role in the metabolic and mitogenic actions of both insulin and IGF-I (Tsakiridis, Tsiani et al., 2001).

PI 3-kinase is a family of multiple members, which are grouped into three classes (Class I, II and III) depending upon their lipid substrate specificity, the subunit structure and regulation mode of the enzymatic activity (Oka, Nagai et al., 2000). Investigation of the signalling pathway of insulin has shown type I PI 3-kinase to be essential (Pessin & Saltiel, 2000). The effect of this family of PI 3-kinase can be blocked with the use of the inhibitor wortmannin at relatively low concentrations (Oka, Nagai, et al., 2000). The experiments performed in the course of this chapter showed wortmannin to increase myofibroblast number when added to GFD medium. This may reflect the inhibition of residual PI 3-kinase activity intrinsic to the medium, suggesting that even this medium has limited "insulin-like activity". Alternatively, the addition of wortmannin may have increased myofibroblast numbers by a mechanism independent of insulin. As PDGF is still present in GFD at biologically active concentrations, it is possible that this growth factor is exerting an effect on fibroblast - myofibroblast differentiation. The addition of wortmannin may increase αSMA expression through inhibition of the PDGF effect on fibroblast - myofibroblast differentiation. Additional pathways that may be affected through the addition of wortmannin include not only the inhibition of PI 3-kinase but also mitogen-activated protein kinase and myosin light-chain kinase (manufacturers data sheet). Both of these proteins have no published data on their ability to reduce fibroblast - myofibroblast differentiation to date.

Conclusions

- Apoptosis rate in insulin treated cultures is increased from that seen in non-insulin treated controls.
- Fibroblast - myofibroblast differentiation can be achieved within the tissue culture system employed within this thesis using growth factors other than insulin.
- PDGF is able to produce a similar reduction in fibroblast - myofibroblast differentiation to insulin
- Insulin achieves the reduction in myofibroblast numbers via its own receptor, as both IGF – 1 and –2 do not reduce fibroblast - myofibroblast differentiation to the same extent as insulin

Chapter 6

The effect of substances with physiologically antagonistic roles to insulin on insulin's efficacy in reducing fibroblast - myofibroblast differentiation

6.1 Introduction

The *in vitro* work presented in the preceding chapters suggests that the administration of insulin during wound healing represents a novel means by which cutaneous scarring may be prevented or reduced. However, in order to develop this laboratory finding into a clinical therapy, examination of its effects in an *in vivo* system is necessary. *In vitro* work is undertaken in carefully regulated, controlled and predictable conditions in contrast to the situation *in vivo*. As some of the physiological effects that administration of insulin would initiate are predictable, it seems prudent to evaluate the efficacy of insulin in reducing fibroblast - myofibroblast differentiation under such conditions *in vitro*.

Insulin is one of many hormones that control glucose homeostasis *in vivo*. Following insulin administration, glucose levels are reduced by stimulation of glucose uptake by hepatocytes (and subsequent conversion to glycogen by glycogenogenesis), and adipocytes (with storage as fat) (Berne and Levy, 1996). The uptake of glucose will therefore lead to significant hypoglycaemia if unopposed and thus compensatory physiological responses are necessary. Typically, reduction in blood glucose levels lead to the stimulation of glucagon secretion from the pancreas, and both steroid (such as cortisol) and catecholamine release (principally adrenaline) from the adrenal glands. Any wound that is supplemented with exogenous insulin may therefore be subjected to increased concentrations of not only insulin but also, if insulin has systemic and not just local effects, increases in glucagon, dexamethasone and catecholamines.

Although catecholamines are released in response to hypoglycaemia, many of the vasoconstrictive properties of these molecules are desirable during surgery. Thus, many commonly used local anaesthetic preparations contain adrenaline. Furthermore, nor-adrenaline and adrenaline also act as neurotransmitters and are contained within the walls of blood vessels. Tissue injury itself results in the release of these compounds, thus increasing the concentration of these substances within the local wound environment (Koopmann, 1995).

The actions of physiological insulin antagonists on fibroblast – myofibroblast differentiation, both in isolation and in combination with insulin, have been assessed within this chapter.

Aims:

- To evaluate the effect of physiological insulin antagonists on fibroblast - myofibroblast differentiation
  - Glucagon
  - Adrenaline
  - Noradrenaline
  - Dexamethasone
- To assess whether insulin reduces myofibroblast number in the presence of physiological insulin antagonists

6.2 Results

The proportion of myofibroblasts present in dermal fibroblast cultures varies considerably between different cell lines, as does the apparent differentiation response to different culture conditions. However, as described in Chapter 3, the relative change in myofibroblast proportion from that seen in basal medium (GFD) is similar for all dermal fibroblast cell lines. The results in this chapter are therefore expressed as the percentage reduction in myofibroblast number for a given treatment when compared to the number of myofibroblasts present in GFD treated cultures.

6.2.1 Glucagon

In isolation

The effective range for glucagon *in vitro* (from manufacturer's data) is 0.03 to 10 μg/ml. Although the levels of glucagon present both in the systemic circulation and wound environments are unknown, it is anticipated that they would be raised above normal physiological levels. The effects of glucagon on fibroblast - myofibroblast differentiation were therefore evaluated at the upper end of the known effective scale (at 10 μg/ml and 1 μg/ml) in order to maximise any physiological response seen, even though the actual levels of glucagon present *in vivo* after the administration of insulin are not known. The results are plotted in Fig 6.1.

The culture of dermal fibroblasts in GFD supplemented with glucagon at a concentration of 10 μg/ml decreases the proportion of myofibroblasts present from the levels seen in GFD alone by 28% (±6.88). This change is significant upon testing ($P<0.001$). Reducing the concentration of glucagon 10 fold to 1 μg/ml results in a similar decrease in the proportion of myofibroblasts from that seen in GFD by 24.4% (±18.3). This change just reaches significance upon testing ($P=0.052$), although the 1 μg/ml results are not significantly different from that obtained with 10 μg/ml glucagon ($P>0.1$). The reduction in myofibroblast proportion (from that seen in GFD) with the addition of glucagon is significantly lower than the reduction seen with the addition of insulin, $P=0.013$ (1 μg/ml glucagon) and $P=0.003$ (10 μg/ml glucagon).

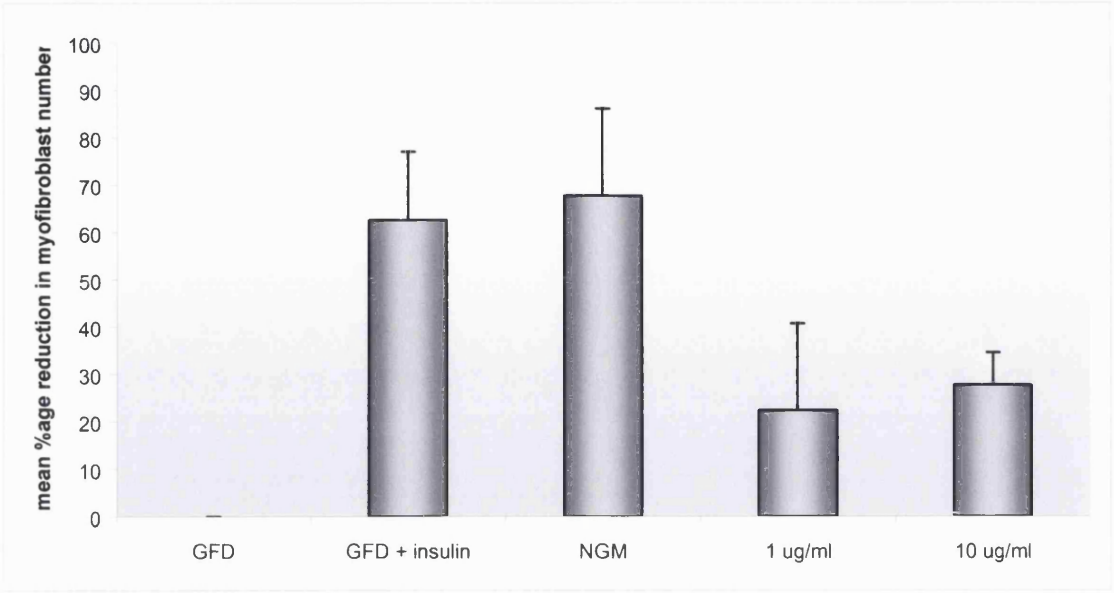


Fig 6.1 Graph showing the mean percentage reduction (and standard deviation) in myofibroblast number for 3 dermal fibroblast cell lines as compared to that seen in GFD medium. Fibroblasts were cultured in GFD or GFD supplemented with glucagon at 10μg/ml or 1μg/ml.

Effect on insulin action

If insulin is to be added to wounds *in vivo*, its actions will be modified by the presence of physiological insulin antagonists. In order to assess the effect of insulin in a more "physiological situation" the effect of insulin on fibroblast - myofibroblast differentiation was assessed in the presence of glucagon at the concentrations used above and the results plotted in Figure 6.2. The therapy of dermal fibroblast cultures with both glucagon and insulin (at 1 IU/ml) reduces the efficacy of insulin in reducing fibroblast - myofibroblast differentiation. The addition of both insulin and glucagon at 10μg/ml and 1μg/ml reduces myofibroblast proportion from that seen in GFD medium alone by 47.4%±16.7 ($P=0.002$) and 38.9% ±28.6 ($P<0.001$) respectively. This reduction is not significant ($P>0.1$ for both concentrations of glucagon evaluated) when compared to that seen when fibroblasts are cultured in medium containing insulin alone. For comparison, culture in GFD medium supplemented with insulin alone reduces myofibroblasts by 62.6±14.4% from that seen in GFD medium alone ($P<0.001$).

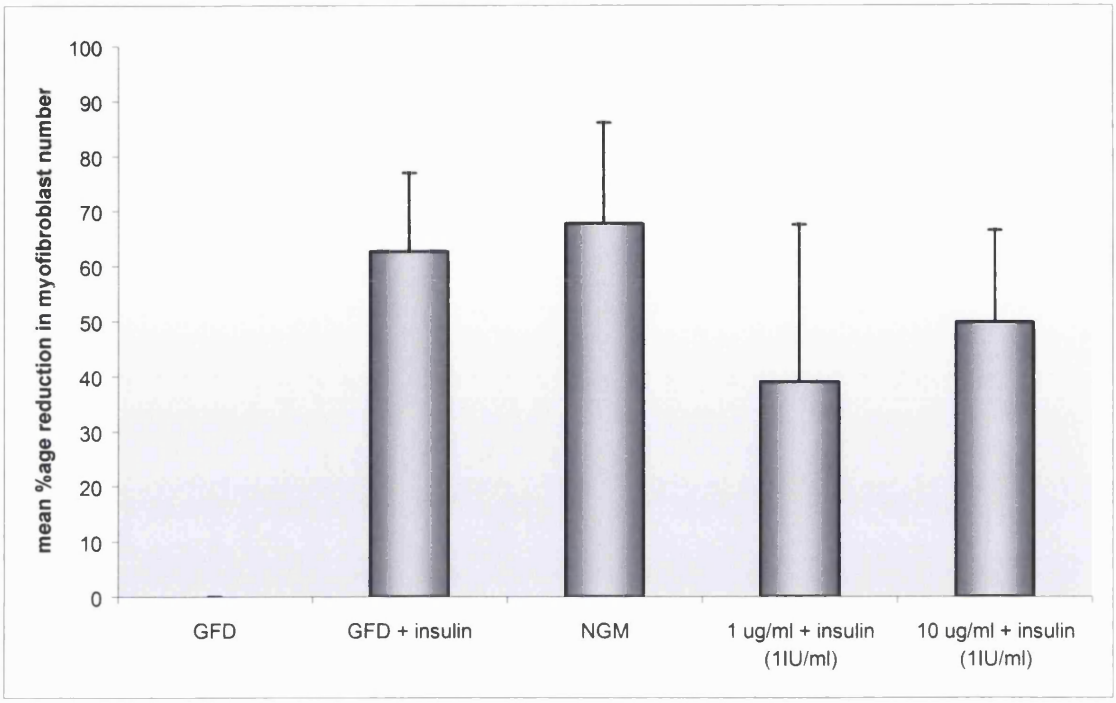


Fig 6.2 Graph showing the mean percentage reduction (and standard deviation) in myofibroblast number for 3 dermal fibroblast cell lines as compared to that seen in GFD medium. Fibroblasts were cultured in GFD or GFD supplemented with glucagon at 10μg/ml and 1μg/ml both with the addition of Insulatard at 1 IU/ml

6.2.2 Dexamethasone

In isolation

The manufacturer's data for the preparation of dexamethasone used (Sigma) quotes an effective range in cell culture of between 4-500 ng/ml. As with the other compounds with physiological antagonistic effects to insulin explored within this chapter, it is anticipated that the *in vivo* addition of insulin will result in high systemic concentrations. Its effect on dermal fibroblast - myofibroblast differentiation was therefore evaluated at the higher end of the known effective range, at concentrations of 100 ng/ml and 10 ng/ml. These results are shown in Fig 6.3, where both the mean and standard deviation for the percentage reduction in myofibroblast number (as compared to fibroblasts cultured in GFD) are shown.

The culture of dermal fibroblasts in GFD with dexamethasone at a concentration of 100 ng/ml decreases the proportion of myofibroblasts present from the levels seen in GFD by 32.5% (±28.7). This change approaches significance upon testing (P=0.059). Decreasing the concentration of dexamethasone 10 fold to 10 ng/ml reduces the proportion of myofibroblasts from that seen in GFD by 26.8% (±19.6), a significant change upon statistical testing (P=0.036) although not significantly different from the reduction seen when fibroblasts are cultured in medium containing 100 ng/ml dexamethasone (P>0.8). The magnitude of the reduction in myofibroblast proportions with the addition of dexamethasone is significantly lower than that seen with the addition of insulin alone for the addition of 10 ng/ml dexamethasone only (P=0.024, Fig 6.3).

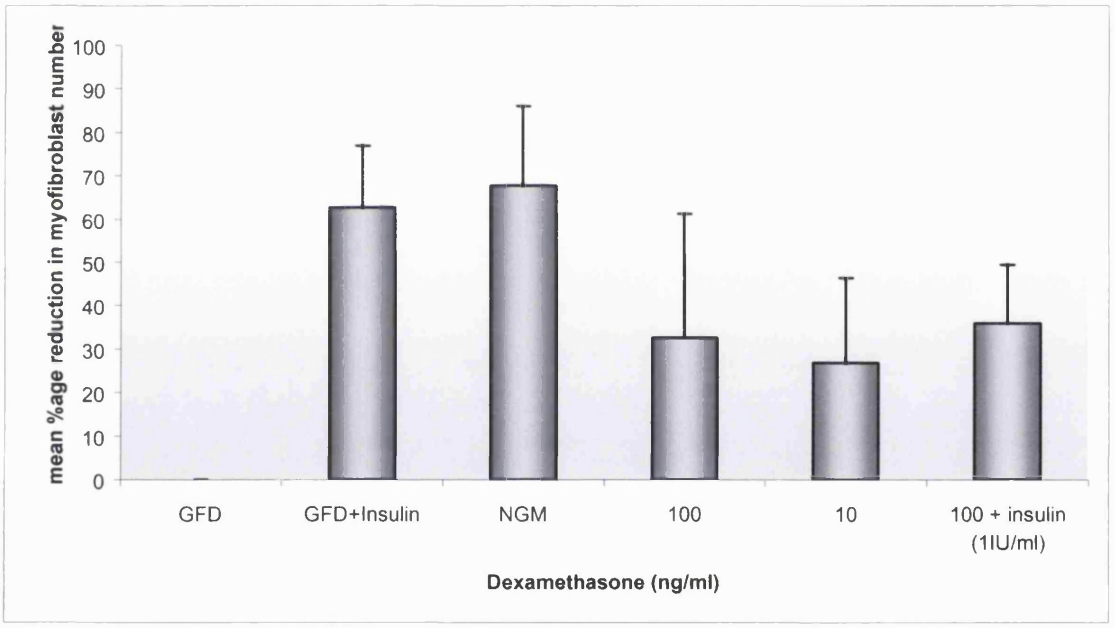


Fig 6.3 Graph showing the mean percentage reduction (and standard deviation) in myofibroblast number for 3 dermal fibroblast cell lines as compared to that observed in GFD medium. Dermal fibroblasts were cultured in GFD or GFD supplemented with dexamethasone at 100 ng/ml and 10 ng/ml.

Effect on insulin action

Interestingly, insulin's effect in reducing myofibroblast proportion is diminished when administered in combination with dexamethasone at 100 ng/ml, with the reduction of

myofibroblasts being only 36% (±13.6), as compared to a reduction of 62.6%±14.4 with the addition of insulin alone. This reduction is however still significantly different when compared to the proportion of myofibroblasts present in GFD treated cultures ($P<0.001$) but not significantly different from that seen when fibroblasts are cultured in GFD medium containing insulin alone ($P>0.1$).

6.2.3 Adrenaline

In addition to being a physiological antagonist of insulin, adrenaline is a common constituent of local anaesthetic preparations and therefore likely to be administered to surgical wounds. As the concentration of adrenaline present in local anaesthetic preparations is many times greater than that found at physiological levels, its effects were assessed at concentrations found in local anaesthetic preparations (approximately 1 ng/ml) and thus likely to be added to surgically created wounds. In addition, the effects of adrenaline were assessed at concentrations 10-fold higher (10 ng/ml) and 10-fold lower (0.1 ng/ml). Interestingly, increasing the concentration of adrenaline beyond 10 ng/ml resulted in the death of fibroblasts in culture and thus no data could be derived from such conditions.

In isolation

Culture of dermal fibroblasts in GFD supplemented with adrenaline at 10 ng/ml increases the proportion of myofibroblasts present over that seen in GFD by 12.6% (±18.4). This increase is not significant upon testing ($P=0.542$). Decreasing the concentration of adrenaline 10 fold to 1 ng/ml again increases the proportion of myofibroblasts present over that seen in GFD but only by 1.3% (±13.9). Again, this change in myofibroblast proportion is not significant upon testing ($P=0.906$). Further reductions in the concentration of adrenaline, to 0.1 ng/ml, also increases the proportion of myofibroblasts present over that seen in GFD alone by 15.42% (±12.79). This increase is also not significant upon testing ($P=0.318$). The change in myofibroblast number with the addition of adrenaline is significantly different from that seen with the addition of insulin, $P<0.05$ for all concentrations of adrenaline tested and shown in Fig 6.4.

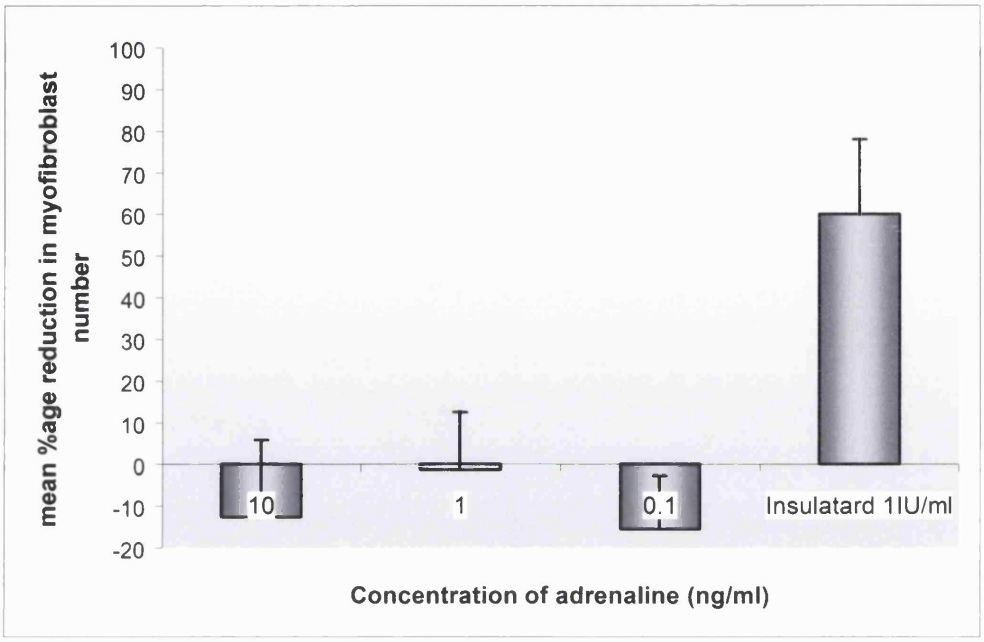


Fig 6.4 Graph showing mean percentage reduction (and standard deviation) in myofibroblast number for 3 dermal fibroblast cell lines. Fibroblasts were cultured in GFD, GFD supplemented with adrenaline at 10, 1, 0.1 ng/ml and in GFD supplemented with 1 IU/ml Insulatard.

Effect on insulin action

The mean and standard deviation of the mean percentage reduction in myofibroblast number as compared with fibroblasts cultures in GFD medium is shown in Figure 6.5. As adrenaline may be present if insulin is to be applied to the wound as a prophylactic agent to prevent or reduce the formation of the myofibroblast phenotype, the effect of insulin in the presence of adrenaline was also examined.

The culture of dermal fibroblasts in medium containing both insulin (Insulatard 1 IU/ml) and adrenaline results in a reduction in myofibroblast proportions from that seen in GFD (1 ng/ml = 26.99±15.22, 0.1 ng/ml = 38.54±10.13). This reduction is not significantly different from the reduction in myofibroblast number seen when fibroblasts are cultured in GFD medium containing insulin alone ($P>0.1$, $P>0.2$ respectively). Therefore, in the presence of adrenaline, the efficacy of insulin is not significantly reduced.

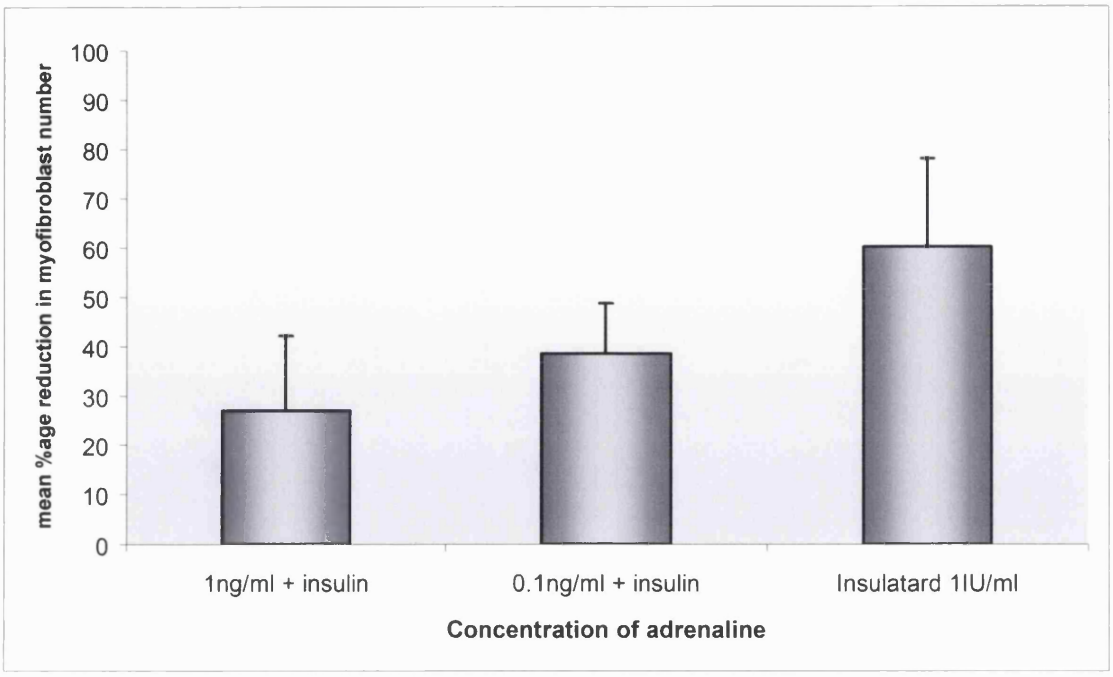


Fig 6.5 Graph showing mean percentage reduction (and standard deviation) in myofibroblast number for 3 dermal fibroblast cell lines. Fibroblasts were cultured in GFD or GFD supplemented with adrenaline at 1 ng/ml and 0.1 ng/ml with the addition of insulin (Insulatard 1 IU/ml).

6.2.4 Noradrenaline

In isolation

The effects of noradrenaline were assessed at concentrations of 10 ng/ml and 1 ng/ml, equivalent to the concentrations of adrenaline used in local anaesthetic preparations. Increasing the concentration of noradrenaline beyond this range resulted in the death of fibroblasts. The culture of dermal fibroblasts in GFD containing noradrenaline at a concentration of 10 ng/ml decreases the number of myofibroblasts present from that seen in GFD by 16% (±20.4). This change is not significant upon testing from the number seen in GFD alone (P=0.447). Reducing the concentration of noradrenaline 10 fold to 1 ng/ml decreases the number of myofibroblasts further over that seen in GFD by 26.4% (±19.3). This increase is again not significant upon testing (P=0.226). The change in myofibroblast number seen with the addition of noradrenaline from that seen with the addition of insulin approaches significance, P<0.10. These results are shown in Fig 6.6.

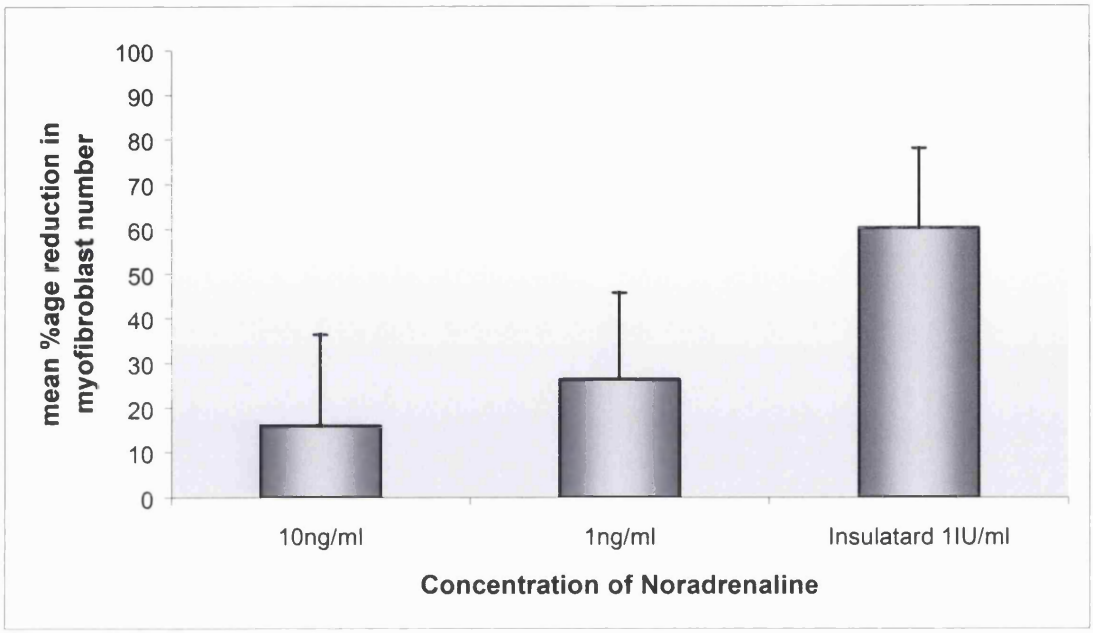


Fig 6.6 Graph showing mean percentage reduction (and standard deviation) in myofibroblast number for 3 dermal fibroblast cell lines. Fibroblasts were cultured in GFD or GFD supplemented with noradrenaline at 10 ng/ml and 1 ng/ml.

Effect on insulin action

Noradrenaline is also a beta-symphatomimetic compound, and could therefore have similarly desirable effects as adrenaline if added to local anaesthetic compounds. Although such preparations are not commonly available, this compound was evaluated in order to determine whether it could offer any advantage to the use of adrenaline. Furthermore, the administration of insulin results in an increase in the levels of this catelcholamine in addition to adrenaline. The effect of addition of noradrenaline to GFD medium containing insulin on myofibroblast numbers is also shown in Fig 6.7. Addition of noradrenaline at 10 ng/ml to GFD containing insulin (Insulatard 1 IU/ml) results in a reduction in myofibroblast number (from that seen in GFD) by 20.7% (±24.3). This is not a significant change from the reduction in myofibroblast numbers seen with addition of noradrenaline alone (P=0.825), or indeed from the number of myofibroblasts present in GFD treated cultures (P=0.429). In addition, the change in myofibroblast number is not significant from that seen when fibroblasts are cultured in GFD medium supplemented with Insulin (P=0.589).

Addition of noradrenaline at 1 ng/ml to GFD containing insulin results in a decrease in myofibroblast numbers by 55.3% (±7) from that seen in GFD treated cultures. This reduction is significant (P=0.023). Interestingly, the reduction in myofibroblast numbers achieved with culture of fibroblasts in medium containing both noradrenaline at 1 ng/ml and insulin (Insulatard 1 IU/ml) is not significantly different from that seen with the addition of insulin alone (P=0.687).

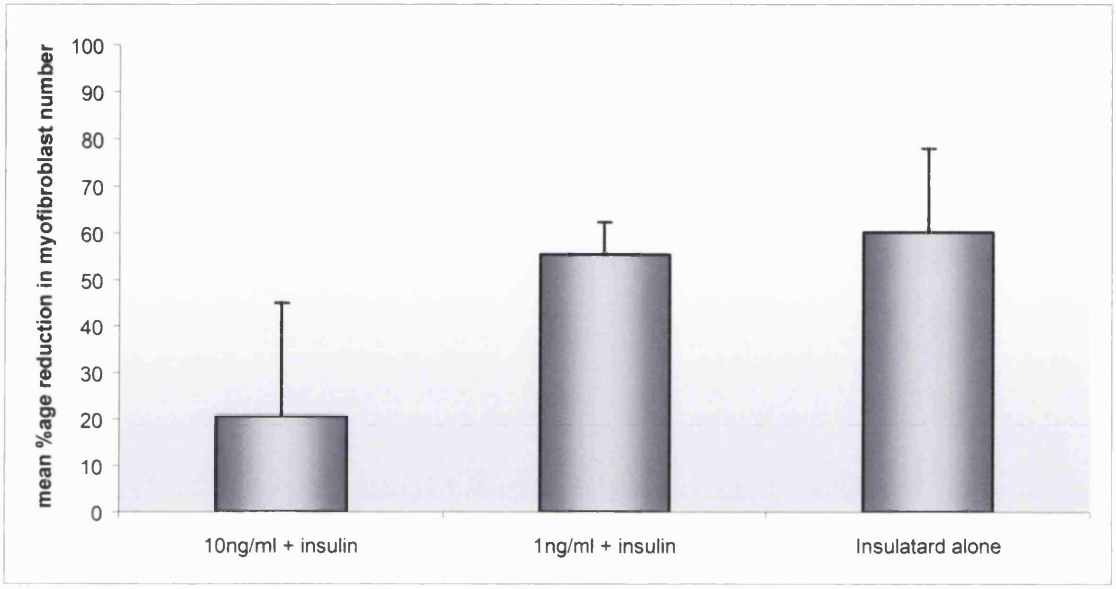


Fig 6.7 Graph showing mean percentage reduction (and standard deviation) in myofibroblast number for 3 dermal fibroblast cell lines. Fibroblasts were cultured in GFD, GFD supplemented with insulin alone (Insulatard 1 IU/ml) or GFD supplemented with both insulin (Insulatard 1 IU/ml) and noradrenaline at either 10 ng/ml or 1 ng/ml.

6.3 Discussion

Administration of insulin to an *in vivo* model should result in the initiation of a physiological response. This would aim to counteract the systemic effects of the exogenous insulin in order to avoid hypoglycaemia. Such a response would typically involve an increase in the systemic levels of glucagon, catecholamines (adrenaline and noradrenaline) and corticosteroids (evaluated within this chapter as dexamethasone) (Berne and Levy, 1996). As no data exists describing the effects of insulin antagonists on fibroblast - myofibroblast differentiation, prior to evaluating the effect of these substances on insulin's inhibition of fibroblast - myofibroblast differentiation, evaluation of their effects in isolation was undertaken.

6.3.1 Glucagon

Glucagon is produced within the pancreas, with insulin and glucagon being secreted reciprocally. Physiologically, when there is need for one hormone, the other is not required (Berne & Levy, 1996). It is commonly held that the overall systemic concentration of insulin is not important physiologically, but rather the relative amounts of both insulin and glucagon are critical. This is important, as "stressful situations" such as trauma and surgery are well known to augment the secretion of glucagon (Berne & Levy, 1996). This is thought to occur through the stimulation of α cells within the pancreas via the sympathetic nervous system's effects on α-adrenergic receptors. Such an increase in glucagon levels during stress may have significant implications on fibroblast behaviour.

Within the experiments performed during the course of this chapter, the administration of glucagon to GFD medium reduced the numbers of myofibroblasts present in dermal fibroblast cultures, with the magnitude of reduction being statistically significant at higher concentrations. Such results are compatible with previous work performed by Chen et al. in 1998 (Chen & Masters, 1988). During the course of Chen's work, both insulin and glucagon were noted to reduce glycolytic enzyme release from porcine kidney cells, with concurrent decreases in actin expression. However, no effort was made to evaluate the effects of insulin and glucagon on the different sub-types of actin, in particular α-smooth muscle actin. The work performed by Chen et al. confirmed

previous work by Rao et al. in 1985 on the effects of glucagon on actin expression (Rao, Betschart et al., 1985).

During a "stressful situation", it is unlikely that insulin levels will rise. In fact, it is quite likely that they will actually fall as such circumstances are frequently associated with fasting, and therefore reducing the systemic concentrations of glucose. This in turn leads to decreased insulin requirements and a relative increase in glucagon levels. This may potentially increase the apparent effects of glucagon on fibroblast behaviour.

Within the work contained in this chapter, a significant reduction in fibroblast - myofibroblast differentiation was noted when dermal fibroblasts were cultured in medium containing both insulin and glucagon as compared to those fibroblasts cultured in GFD medium. However, comparing the reduction in myofibroblast numbers achieved with glucagon with that achieved when insulin is added alone, the effectiveness of insulin in reducing fibroblast - myofibroblast differentiation was reduced in the presence of glucagon. This reduction in efficacy was not significant upon statistical testing.

These results seem contradictory: with glucagon alone significantly inhibiting myofibroblast differentiation and yet its combination with insulin not significantly increasing the inhibition illicited by insulin alone. Indeed it might be argued that the addition of glucagon to insulin shows a trend towards reducing insulin's inhibitory effect. If such a trend proved significant on testing of greater numbers of cell lines, it could be rationalised that a putative mechanism for the reduction of myofibroblast numbers by insulin may be via insulin stimulated glucose transport. Such a mechanism is known to be inhibited by a number of compounds such as glucagon and noradrenaline (Hollenberg and Cuatrecasas, 1975) and thus the relative decrease in the efficacy of insulin may suggest that its effects upon differentiation are via a mechanism associated with glucose transport.

6.3.2 Dexamethasone

Corticosteroids have been shown to reduce neovascularization (Hashimoto, Nakanishi et al., 2002) in addition to their well established effects upon the inflammatory and immune responses (British National Formulary, 2001). However, processes such as inflammation and neovascularisation play considerable roles in wound healing and the use of any substance that negates these important activities may be considered deleterious, both theoretically and in practice (Pollack, 1982).

Fortunately, the effect of corticosteroids during wound healing *in vivo* is dependent upon the type of steroid used, the dosage and the timing of administration. If corticosteroids are applied to wounds after the inflammatory phase of wound healing is complete, healing as measured by tensile strength is unaffected (Saragas, Arffa et al., 1985). Dexamethasone, as used within this chapter, has itself has been demonstrated to improve wound healing in rodents, and reduces fibrous tissue formation in implanted wound chambers (Hinton, Warejcka et al., 1995). In addition, dexamethasone has long been used *in vitro* as a non-specific differentiation agent, which when added to a population of myocytes result in cultures containing osteoblasts, chondrocytes and adipocytes (Williams, Southerland et al., 1999).

Within this chapter, the addition of corticosteroids (dexamethasone) to dermal fibroblast cultures resulted in a decrease in myofibroblast numbers, although significant reductions were only observed for GFD supplemented with 10 ng/ml. Such a reduction is comparable to the results obtained by Miki et al. (Miki, Mio et al., 2000), when using equivalent concentrations of steroid, during their investigations on the effect of corticosteroids on F-actin and contractility. Interestingly, although the addition of corticosteroids to GFD medium reduces myofibroblast numbers, the addition of both insulin and dexamethasone to fibroblast cultures results in a trend towards a reduction in fibroblast - myofibroblast differentiation that is less than that seen with insulin alone. This reduction is however not significant upon testing. Whilst the reasons for this contradiction are not apparent, it may reflect differences in respective mechanisms of action.

6.3.3 Adrenaline and Noradrenaline

Catecholamines such as adrenaline and noradrenaline have been considered to be among the major physiological antagonists of insulin *in vivo* (Berne & Levy, 1996) and therefore with their addition, an antagonistic response (i.e. an increase in myofibroblast numbers) was expected. However, within the work performed in this chapter, no significant increase in fibroblast - myofibroblast differentiation was noted with the addition of either adrenaline or noradrenaline. Addition of these compounds to fibroblasts cultured in GFD was similarly expected to reduce the efficacy of insulin in reducing fibroblast - myofibroblast differentiation. This anticipated effect was noted for both compounds evaluated. Although not significant upon testing, the addition of lower concentrations of antagonists seemed to result in a lesser effect on the inhibition of insulin.

Whilst the effect of catecholamines on dermal fibroblast differentiation has not previously been published, their effects upon other cell lines are known. In a study utilising cardiac myoblasts, Bhambi et al. (Bhambi & Eghbali, 1991) assessed the effects of short term administration of noradrenaline, discovering that addition of this compound increased αSMA expression 4-fold after 1 hour. Although the results of Bhambi et al. differ to those presented within this chapter, the different time scale and cell lines used may explain the differences. Similar increases in the expression of αSMA were discovered in prostatic tissue after the administration of noradrenaline, (Smith, Rhodes et al., 1999). The differences in culture media between the studies performed by both Smith and Bhambi and those presented here may explain the opposing results. Additionally, cardiac cells display different catecholamine receptors to many other tissues and have been shown to act differently to stimulation (Berne and Levy, 1996).

6.3.4 The effect of insulin in combination with its physiological antagonists

The results of the *in vitro* experiments described within this chapter suggest that insulin is less effective if administered with at least one of its physiological antagonists than when used alone. Thus, the physiological response to insulin administration (increasing production of insulin antagonists in order to reduce the effect of the administered

substance on glucose homeostasis) may reduce the efficacy of insulin on scar prevention. Additionally, the reduced efficacy of insulin when added to its physiological antagonists suggests that its evaluation *in vivo* should be performed in the absence of solutions containing these substances. To this end, the work assessing insulin's effects on fibroblast – myofibroblast differentiation *in vivo* (as described in Chapter 7) is performed on wounds treated with insulin alone.

Following the completion of many surgical procedures, it is common practice to provide analgesia through the administration of a local anaesthetic solution around the wound edges. It would be tempting to add insulin to such a solution, and to apply the substance as a wound infiltration. However, many local anaesthetic preparations are available that contain adrenaline. The effectiveness of insulin within such a cocktail requires evaluation before such a technique can be recommended although the results within this chapter suggest that this would not be a significant problem.

6.4 Conclusions:

Although insulin is effective in reducing myofibroblast numbers *in vitro*, its efficacy is reduced when at least one of its physiological antagonists is added to culture conditions. Physiological insulin antagonists do not significantly increase the numbers of myofibroblasts in the culture system employed within this thesis.

Chapter 7

The effectiveness of insulin on fibroblast - myofibroblast differentiation during wound healing *in vivo*

7.1 Introduction

During wound healing the presence, or rather the persistence, of the myofibroblast has been linked to the development of pathological scarring (Lee, Green, & Amiel, 1995), (Chipev, Simman, et al., 2000). These myofibroblasts are thought to derive from the dermal fibroblasts that surround the wound (Desmouliere, Badid, et al., 1997). The results described in the preceding chapters have shown that insulin can reduce fibroblast - myofibroblast differentiation *in vitro* and hence suggest that insulin may prove to be a novel therapy for the reduction or prevention of cutaneous scarring. However, prior to making such claims, the effectiveness of insulin *in vivo* must be demonstrated. Due to the differences between the *in vitro* and *in vivo* environments, such as the presence of many proteases that affect growth factor activity in the latter (Robertson, Belford, & Ballard, 1999), the results from the *in vitro* experiments must first be validated *in vivo*.

The choice of wound healing model was dictated by a number of factors, including the number of animals required for the planned studies together with economic cost, which ruled out the use of larger animals. In spite of the closer similarity of porcine skin to human skin (Gottrup, Agren, & Karlsmark, 2000), and the pig being accepted as one of the best models of human dermal repair (Wang, Olson et al., 2000), the high costs associated with the use of pigs in the laboratory ruled out their use for the purposes of this thesis. In view of this, an alternative, more economical animal model was evaluated. The mouse has long been used in wound healing studies, and whilst all species of mouse are appropriate to study the effects of substances on wound healing, BALB/c mice have been used extensively (Stallmeyer, Kampfer et al., 2001), (Beer, Longaker et al., 1997), (Brown, Dubin et al., 1993), (Frank, Madlener et al., 1996).

A relatively simple wound model was chosen for evaluation of the effects of insulin, using male BALB/c mice. Prior to the commencement of *in vivo* experiments, the suitability of this murine model was assessed by evaluating the effects of insulin on murine dermal fibroblast cultures *in vitro*. Following confirmation of the suitability of the proposed model, *in vivo* experiments were conducted using both standardised incisional and excisional wounds. Insulin was administered to the wound environment

using a local infiltration technique which directly delivers known amounts of the drug to the necessary site of action (the wound edge).

Following completion of the surgical procedures, the wounds were not dressed. Rodents groom their coats compulsively, and it is accepted that mice will remove or gnaw at anything adherent to their fur, making the use of wound dressings difficult. Although the lack of wound dressings may be detrimental to wound healing, many other authors use a similar wound healing model without dressings in mice e.g. Beer et al. (2000), Stallmeyer et al. (2001). The lack of wound dressings may in fact be considered an advantage as inspection of the wound is facilitated.

Insulin has been applied to wounds in many previous studies (Udupa & Chansouria, 1971), (Greenway, Filler, & Greenway, 1999), and is accepted to be necessary for wound healing to proceed normally (Andreassen & Oxlund, 1987) but to date, the quality of the resulting scar has not been assessed. Aside from assessing insulin's effects as a growth factor, few investigators have attempted to understand how insulin might affect the wound healing process. Irrespective of its effects on fibroblast behaviour, insulin will have other physiological actions *in vivo*.

The primary physiological effect of insulin is to reduce blood glucose levels, mainly by promoting uptake by muscle and liver and then conversion of hexose sugars to the more inert starch glycogen for storage. As glucose is required for cell respiration (exclusively so in some organs e.g. brain), low serum levels may prove problematical. In cases of insulin overdose the resulting hypoglycaemia may prove fatal. In view of this, the amount of insulin that can be administered to animals is limited. Udupa et al. (1971) during their work on wound healing in the rat, state that increasing the dosage of insulin above 0.6 IU / 100g body weight results in unacceptable levels of mortality. Although these experiments were performed in rats, accounting for the difference in body weight it is possible to predict an upper limit for the amount of insulin that can safely be given to mice. As the average weight of the BALB/c mice used within this thesis was 25g, 0.15 IU of insulin was administered to each mouse when necessary.

In addition to assessing insulin's effects on myofibroblast numbers, it is important to evaluate the effect that administration of this drug might have upon the strength of repair. Many therapies have been proposed to combat pathological scarring in the past, such as the use of anti-TGFβ_1 antibodies (Shah, Foreman, & Ferguson, 1995), but the importance of TGFβ during wound healing (Abe, Donnelly et al., 2001) has suggested that such therapies may be deleterious to cutaneous repair. Within this chapter, tensiometry is used to determine the breaking strain of wounds following insulin therapy.

Finally, insulin may be administered during wound healing via a number of routes, some of which may be more practical for use in different wounds. The surgical wound may be treated simply using a local infiltration but for larger wounds that are known to be prone to pathological scarring (such as burns), it may be more efficient to apply substances topically or even systemically. Such administration regimens are also evaluated in this chapter.

Hypothesis:
Insulin reduces the number of myofibroblasts in a healing murine wound

Aims

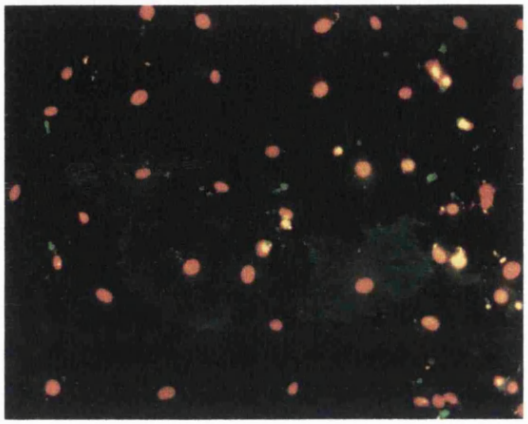
- To determine whether insulin reduces fibroblast - myofibroblast differentiation in murine fibroblasts *in vitro*
- To evaluate the effect of insulin on *in vivo* wound healing models (both incisional and excisional)
- To determine the effect of insulin on wound strength
- To evaluate the optimum route of administration to prevent myofibroblast formation

7.2 Results

7.2.1 Confirmation of the effectiveness of insulin on murine derived fibroblasts

Immunohistochemistry

Three murine fibroblast cell lines were derived from different BALB/c mice and cultured in Normal Growth Medium (NGM, consisting of DMEM and 10% Foetal Calf Serum), Growth Factor Dialysed medium (GFD, DMEM and 10% dialysed FCS) and GFD medium supplemented with 1 IU per ml Insulatard®, Mixtard® or Actrapid® on glass coverslips in 6-well plates. For comparison, laboratory grade bovine insulin (Sigma) was used at 100μg/ml, a level approximately equivalent to 1 IU/ml. These concentrations of insulin were chosen having previously shown to be optimum in cultures of human dermal fibroblasts. Culture medium was refreshed twice weekly. After 14 days, the coverslips were fixed and stained immunohistochemically for alpha smooth muscle actin. Fig 7.1 shows typical images of murine fibroblasts after staining immunohistochemically to demonstrate the presence of α SMA. The mean (of three cell lines) and standard deviation of the percentage reduction in myofibroblast number from GFD for each treatment is shown in Fig 7.2.



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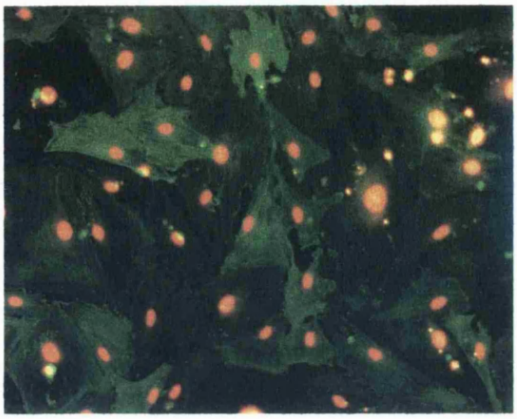

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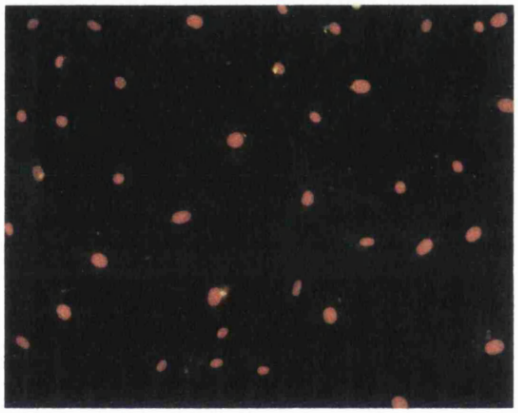


C.

Fig 7.1 Photographs showing murine fibroblasts in culture. Fibroblasts have been stained immunohistochemically to demonstrate αSMA, and are hence myofibroblasts (Green). A. Murine fibroblasts cultured with NGM, B. Murine fibroblasts cultured with GFD, C. Murine fibroblasts cultured with 1 IU/ml Insulatard (200 x).

In NGM, the number of fibroblasts expressing αSMA (i.e. myofibroblasts) is significantly reduced from that seen in GFD by 48.7% (±9.8), $P<0.05$. Addition of insulin to GFD (as Insulatard®) significantly ($P<0.01$) reduced the numbers of myofibroblasts by 53.1% (±18.11). A reduction in myofibroblast number was also seen with Mixtard (16.3±25.2, $P=0.406$), Actrapid (37.9±24.2, $P<0.01$) and laboratory grade insulin (20.0±18.1, $P=0.483$), of these the reduction was only significant with Actrapid. The difference in reduction between Insulatard and Actrapid was not significant ($P=0.245$).

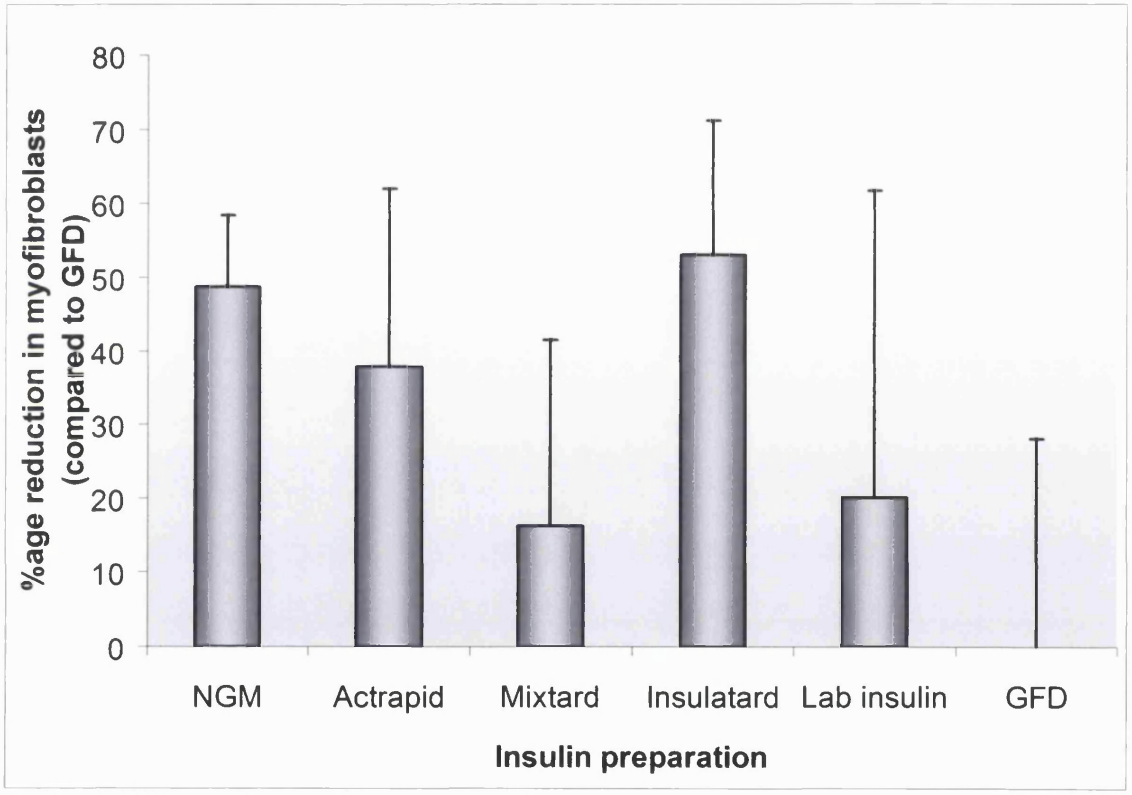


Fig 7.2 Graph showing, for murine dermal fibroblast cultures, the mean percentage reduction in the number of myofibroblasts present in various media (as compared to those of dermal fibroblasts cultured in GFD medium). Dermal fibroblasts from 3 mice were cultured in either NGM, GFD or GFD supplemented with 1 IU/ml insulin (either Laboratory Grade insulin (Sigma), Actrapid, Mixtard 30 or Insulatard) for 14 days. Error bars represent the standard deviation.

Western Blotting

BALB/c dermal fibroblast cultures were seeded in duplicate tissue culture flasks in the following media: NGM, GFD or GFD and 1 IU/ml insulin (either Actrapid, Mixtard 30 or Insulatard) and maintained for 14 days. Cell number was assessed using one set of experimental flasks, whilst the other was used for cell lysates. Expression of αSMA in the cell lysates was assessed by western blotting, using the method described in Section 2.3.2, loading sufficient sample to give equivalent cell number loaded per well. Western Blots can be seen in Fig 7.3.

	NGM	GFD	Insulatard	Mixtard	Actrapid
Optical Density	59.684	77.372	50.458	83.611	60.476

Fig 7.3 Western blot showing expression of alpha smooth muscle actin in murine dermal fibroblasts cultured in NGM, GFD or GFD supplemented with insulin (Insulatard, Mixtard or Actrapid). Optical density readings are given for each well. Each lane was loaded with adequate protein equivalent to equal cell number in each well.

αSMA expression in murine dermal fibroblasts cultured in GFD is reduced in the presence of insulin. Densitometric comparison of the gels revealed that addition of Actrapid reduced αSMA expression by 21.7%. In comparison, addition of Mixtard increased SMA expression by only 8.2%. Insulatard, however, reduced αSMA expression by 34.7%. For comparison, murine dermal fibroblasts cultured in NGM expressed 22.8 % less αSMA than those cultures in GFD.

Murine dermal fibroblasts were shown to behave in a similar manner to human derived dermal fibroblasts. In addition, supplementing GFD with any of the three preparations

of insulin tested here resulted in a reduction in αSMA expression. Either method of evaluating myofibroblast number demonstrated that Insulatard was most effective at reducing myofibroblast numbers. In view of this, Insulatard was chosen for evaluation in a murine wound healing model.

7.2.2 Incisional wounds

Two 1.5 cm full thickness incisional wounds were created on the posterior flanks of 32 male BALB/c mice after anaesthesia (See Section 2.4). Mice were then divided into 4 groups of 8 as shown in Table 7.1:

Group	Treatment	Number of mice
A	Single dose administration (Day 0)	8
B	Two dose administration (Day 0 and day 3)	8
C	Four dose administration (Days 0,3,7,10)	8
D	Delayed single dose administration (Day 7)	8

Table 7.1 Table showing treatment groups for the investigation of the effect of insulin on incisional wound healing. The figures in parentheses represent the days on which substances were administered to murine wounds.

One wound per mouse was arbitrarily selected for treatment with 0.15 ml insulin (Insulatard 0.15 IU) representing approximately 50% of the predicted LD50 (lethal dose) for mice. Equal numbers of left and right wounds received similar treatments. The remaining wounds received 0.15 ml saline as vehicle control. The initial administration of substances for groups A, B and C was by local infiltration of the wound edges at the time of injury. All doses for Group D and all further doses for Groups A, B and C was administered by local infiltration of the scar. Fourteen days after injury, all mice were culled and their scars harvested as described in Section 2.4.5. This time point was

selected for analysis of scars, as it was an equivalent length of time to that used for *in vitro* experiments.

Following harvest, the scars were processed as shown in Fig 7.4, sectioned and stained immunohistochemically to show expression of αSMA. Throughout the course of the experiment, no animals died either during the anaesthetic or in the post-operative or post-injection periods. No complications were noted relating to either the wounds or infections e.g. wound infection or wound dehiscence. All murine wounds were healed by 14 days with no obvious difference in time required for healing between the different groups. Each wound was created as a 15 mm incision and resulted in a scar of length 10.5 mm (±2.1). All wounds healed leaving a fine linear scar, with no obvious differences in either prominence or width.

Immunohistochemical staining to demonstrate αSMA expression was undertaken as described in Section 2.4.8, using a monoclonal mouse anti-human anti-alpha smooth muscle actin antibody (Sigma) and a Vector Mouse on Mouse Blocking kit. Visualisation of the antibody was undertaken by a 3-layer "sandwich" technique as shown in Fig 7.5.

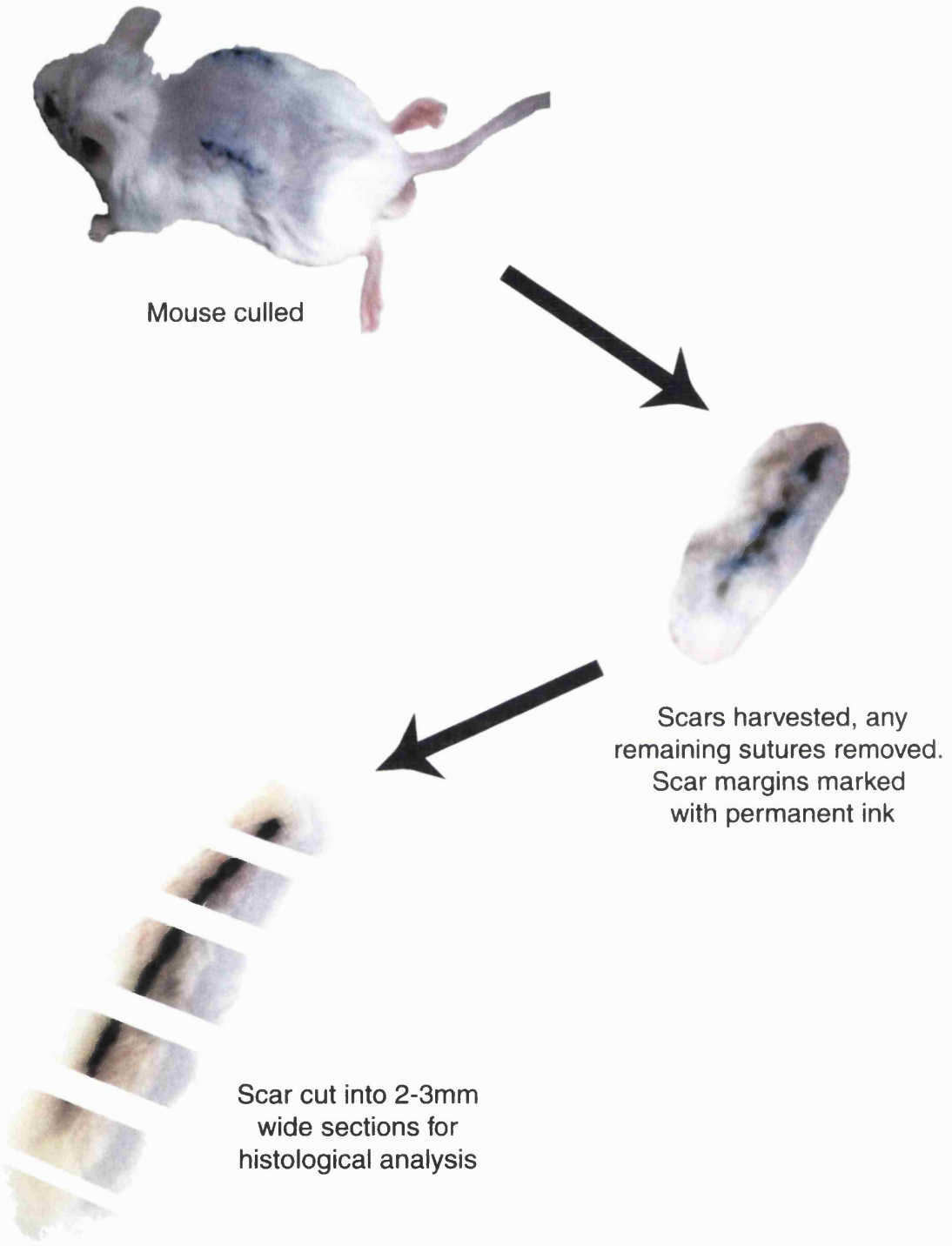
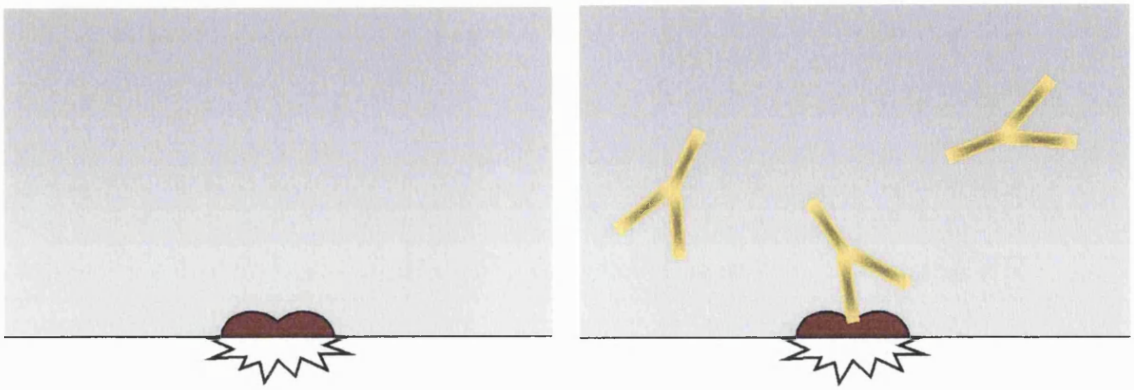
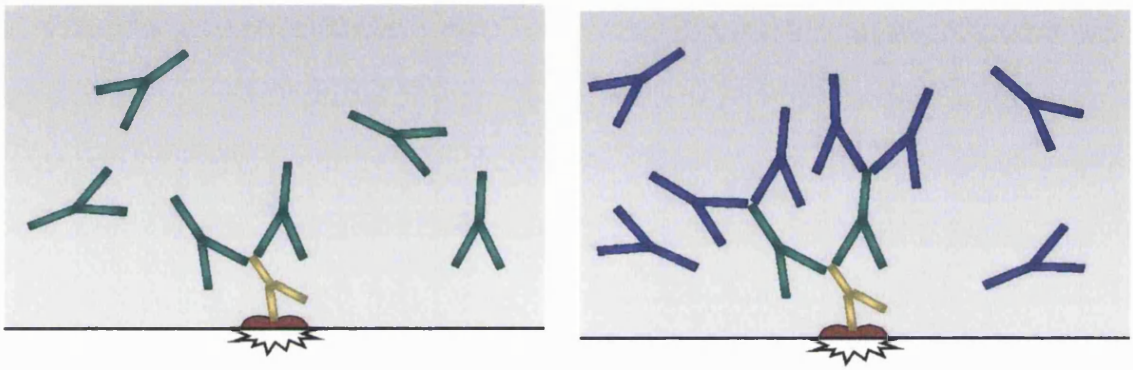


Fig 7.4 Diagram showing method by which murine scars were processed prior to staining to demonstrate the presence of myofibroblasts.

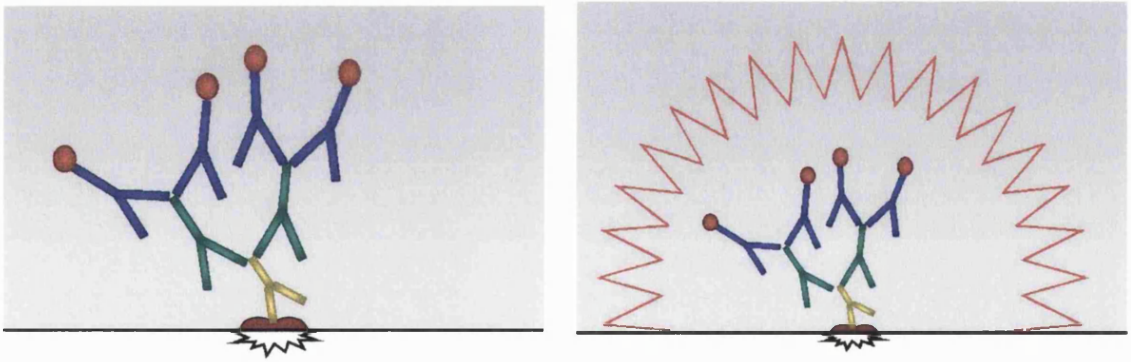


Addition of primary antibody.



Addition of secondary biotinylated antibody

Addition of Avidin / Biotinylated Enzyme complex



Addition of enzyme substrate

Detection of substrate

Fig 7.5 Diagram showing the immunohistochemical method used to detect the presence of αSMA in murine tissue sections

Following immunohistochemical staining, each scar was examined and scored (as detailed in Table 2.3) at three random points. Briefly, if no staining was present, the scar portion of the section was scored "0". If a few scattered foci of stain could be seen, this was scored "1". Roughly equal areas of positive and negative staining were scored "2" and those scar areas that contained more stained areas than unstained were awarded a "3" score. Scores were averaged for each section, and then for each treatment. The results of analysis of αSMA expression can be seen in Fig 7.6, where the mean score and standard deviation for each group of 8 mice are plotted.

Single dose administration of 0.15 IU Insulatard at the time of wounding results in a significant diminution in alpha smooth muscle actin expression from 1.25±0.45 for saline treated controls to 0.49±0.25 ($P<0.01$). Similar reductions can also be seen with two applications of insulin, one at the time of wounding and one after 3 days. This diminution from 1.94±0.60 to 0.71±0.67 is also significant ($P<0.05$) when compared to two applications of saline; however, the amount of αSMA seen after this insulin treatment regime is slightly greater than that seen with single dose administration of insulin although this was not significant upon testing. Unexpectedly, administration of insulin to healing full thickness incisional wounds 4 times (on day 0, 3, 7 and 10) actually resulted in an increase in the expression of αSMA when compared to control treated wounds (receiving equal applications of control substance) from 1.75±0.35 to 2.25±1.06 although this was not significant on testing ($P=0.226$). The myofibroblast scores for single and double administrations of insulin are not significantly different from each other upon testing ($P=0.468$). However, the score seen with single and double administration of insulin is significantly lower than the score obtained after 4-dose administration ($P<0.02$). Administration of insulin 1 week after injury, by local infiltration of the scar, does not significantly alter the myofibroblast score from that seen in similarly healed saline administered wounds, changing minimally from 1.25±0.35 to 1.63±0.17, again not statistically significant. This score is, however, significantly higher than the reduction seen with single dose administration of insulin at the time of wounding ($P=0.001$)

Representative immunohistochemical images showing myofibroblasts at day 14 for each treatment group are shown in Fig 7.7.

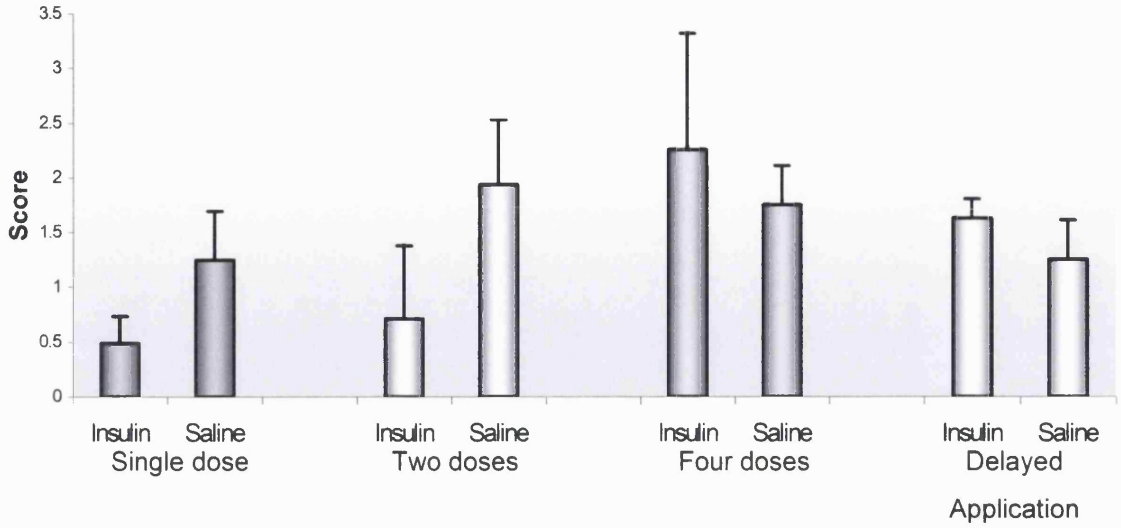
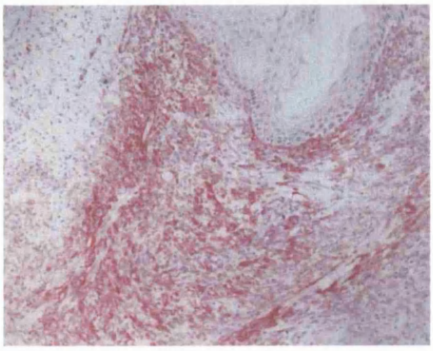
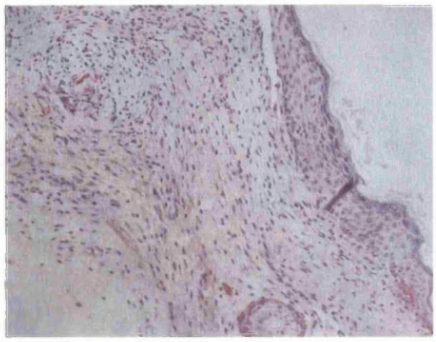


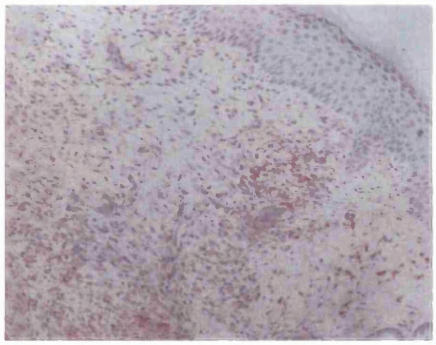
Fig 7.6 Graph showing mean "myofibroblast score" and standard deviation for each treatment applied to a murine incisional wound healing model. Scores were derived from the analysis of 8 wounds per treatment group with each scar examined at 3 random points along its length.



A. Saline



B. Single dose insulin



C. Two doses of insulin

D. Four doses of insulin

Fig 7.7 Immunohistochemical staining of murine incisional wound scars to demonstrate the expression of αSMA. Presence of myofibroblasts (αSMA) identified by red stain. Scar having received (A) single administration of saline (control), (B) single dose administration of insulin (0.15 IU) (C) two doses of insulin (D) four doses of insulin (200 x).

7.2.3 Excisional model

To assess the effect of insulin on larger area wounds, two 0.5 cm diameter full thickness excisional wounds were created on the posterior flanks of 12 male BALB/c mice following anaesthesia (See Section 2.4). Mice were then randomised into 3 groups of 4 as shown in Table 7.2:

Group	Treatment	Number of mice
A	Single dose administration (Day 0)	4
B	Two dose administration (Day 0 and day 3)	4
C	Four dose administration (Days 0,3,7,10)	4

Table 7.2 Table showing treatment groups for the investigation of the effect of insulin on excisional wound healing. The figures in parentheses represent the days on which substances were administered to murine wounds.

One wound per mouse was selected for treatment with 0.15 ml insulin (Insulatard 0.15 IU), with equal numbers of left and right wounds treated. The remaining wounds received 0.15 ml saline as vehicle control. Substances were administered by local infiltration of the wound edges at the time of injury (Day 0) and the wounds left to close by secondary intention. Subsequent doses were also administered as a wound infiltration into the scar on the days indicated in Table 7.2. Fourteen days after injury, all mice were culled, their scars harvested and processed as for incisional wound investigations. Following immunohistochemical staining for αSMA (as described in Section 7.2.2 and Section 2.4.8), wounds were graded and allocated a "myofibroblast score". No complications were noted relating to either the wounds or infections, with no incidence of wound infection or wound dehiscence recorded.

Analysis of excisional wounds proved difficult. In the mouse, excisional wounds heal mainly by contraction and therefore any residual scar is significantly reduced in size

when compared to the initial injury. This results in a small area of scar that has to be evaluated for the presence of myofibroblasts. Unlike the incisional wound healing model (used in Section 7.2.2) where the scar may be assessed at 3-4 points along its length, excisional wounds can be assessed at only one or two points because of the degree of wound contraction. In many sections, it proved impossible to identify the scar. This is in contrast to the incisional wound healing model where scars were always located and then assessed at multiple points along their length. The data concerning the numbers of myofibroblasts present in the excisional model is considered by the author to be both unreliable and incomplete with many treatment groups only being able to be assessed in one wound.

Excisional wounds treated with single dose saline scored 0.75±0.35 (n=2), using the same scoring system used for the incisional wounds. Application of a single dose of Insulatard (0.15 IU at the time of wounding) increased this score to 1.75±0.35 (n=2). Treatment of wounds with multiple doses of insulin reduced the myofibroblast score when compared to saline treated controls. For those that received 2 applications of insulin, the score reduced from 1 (n=1) to 0.25±0.35 (n=2) with insulin. Those wounds treated with 4 doses of insulin reduced myofibroblast score from 0.17±0.24 (n=2) to 0 (n=1).

Comparison of the ease of interpretation of both the full thickness incisional wound and excisional wound healing models suggested that the incisional wound healing model was more reliable for interpretation. Scars were more readily identified both macroscopically and microscopically, allowing for a more reliable analysis. All further *in vivo* work was therefore performed using the incisional wound healing model as described above and in Section 2.4.3.

7.2.4 Time course

To determine whether insulin was preventing myofibroblast formation in the first instance or resulting in an increase in the clearance of this cell phenotype from the wound, an incisional wound healing time course was undertaken over a 28-day period. Briefly, the incisional model was initiated as described previously, with two 1.5 cm full

thickness incisional wounds created on the posterior flanks of 48 male BALB/c mice after anaesthesia (See Section 2.4). One wound per mouse was selected for treatment with 0.15 ml insulin (Insulatard 0.15 IU), with equal numbers of left and right wounds treated. The remaining wounds received 0.15 ml saline as vehicle control, administered at the same time points. Both substances were administered as a local infiltration of the wound edges as described in Section 7.2.2.

The mice were culled at time points over the 28-day period, and their tissue processed for either immunohistochemical analysis to determine myofibroblast number, or for Western blotting to determine the expression of αSMA. Table 7.3 shows the number of mice culled for each treatment group and time point.

Days from injury	Number of mice culled for immunohistochemical analysis of wounds
3	4
7	4
10	4
14	4
17	4
21	4
24	4
28	4

Table 7.3: Table showing number of mice culled at each time point during the 28 day incisional wound healing time course.

Immunohistochemistry

Incisional wounds were stained for αSMA and scored at a minimum of 3 points along their length using the scoring system outlined in Table 2.3. Myofibroblast scores for all wounds increased over the first 7 days following injury to reach a maximum of 1.96 (saline treatment group) and 1.92 (insulin treatment). Thereafter, myofibroblast scores fell gradually in the saline treatment group. However, in those wounds treated with insulin, although the myofibroblast score decreases from the peak seen at day 7, there is a rapid decrease in myofibroblast score between days 10 and 14, resulting in the myofibroblast score for the insulin treated group being less than that of the saline treated group. Statistical tests show no significant difference between the insulin-treated group and control throughout the duration of the experiment. Although the most obvious difference between the two experimental groups occurs at 14 days, this just fails to reach significance ($P>0.05$) within this experiment. Comparison of these results with those obtained using larger numbers at this time point ($n=8$, Section 7.2.2) show that the absolute values of myofibroblast score agree nicely between studies, but that doubling the number of mice allowed the difference to become statistically significant. The change in myofibroblast score over the time course is illustrated in Figure 7.8.

Although the number of animals used to determine the change in myofibroblast numbers is not of sufficient magnitude to give statistically significant differences for each of the time points and treatments, a trend regarding the change in myofibroblast numbers with time and treatment is evident. Shortly after injury, the numbers of myofibroblasts present increases, reaching a peak at approximately day 7. Thereafter, the number of myofibroblasts present gradually decreases.

The myofibroblast score attained 28 days following injury is significantly less than the peak achieved at day 7 for all treatments ($P<0.05$).

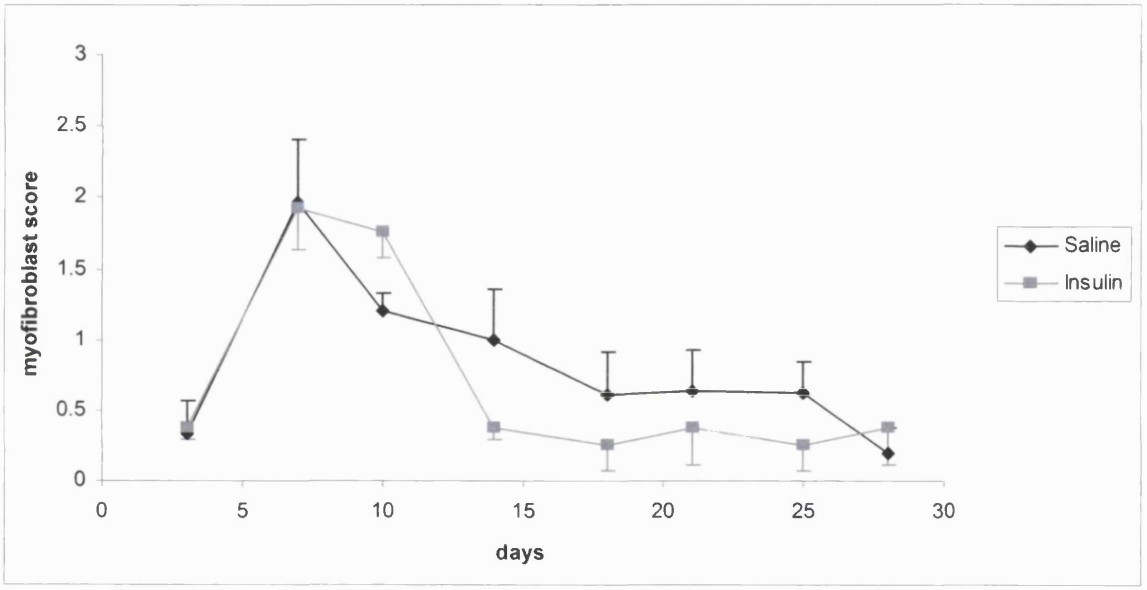


Fig 7.8 Graph showing mean "myofibroblast score" and standard error over a 28 day time course for each treatment applied to a murine incisional wound healing model. Scores were derived from the analysis of 4 wounds per treatment group with each scar examined at 3 random points along its length.

Western Blotting

Using western blotting (Chapter 2), analysis of αSMA expression in healing incisional murine wound time course was determined. Wounds from 16 mice were harvested and processed to produce protein extracts; 4 mice were culled at each time point on days 7, 14, 21 and 28, as shown in Table 7.4. Each mouse having received an incisional wound as described above, and treated with a single dose of insulin or saline, administered using the same methods used for the immunohistochemical studies. Equal numbers of

left and right wounds were treated with insulin. The mean αSMA expression between insulin treated wounds and saline treated wounds over the 28 day period is shown in Fig. 7.8. Sufficient volume of protein preparation (derived from the excised scars) was loaded into each lane to give equal "dry weight" of scar per well.

Days from injury	Number of mice culled for western blot analysis of wounds
7	4
14	4
21	4
28	4

Table 7.4: Table showing number of mice culled at each time point during the 28 day incisional wound healing time course.

Western blot analysis of saline (control) treated murine incisional wounds over a 28 day time course shows that OD rises from 0.04 (±0.01) at day 7 to 0.17 (±0.16) at day 14 and 0.28 (±0.09) at day 21. Thereafter levels of αSMA decrease slightly to 0.27 (±0.09) at day 28. These results are illustrated in Fig 7.9. In contrast, analysis of insulin treated incisional wounds shows that αSMA at day 7 is 0.14 (±0.04), significantly higher than that seen in saline treated wounds ($P<0.05$). Expression of αSMA then rises slightly to 0.19 (±0.06) at day 14 before decreasing to 0.15 (±0.10) at day 21 and 0.13 (±0.04) at day 28. The decreased expression of αSMA at day 28 in insulin treated wounds vs. saline treated wounds approaches significance upon testing ($P=0.088$).

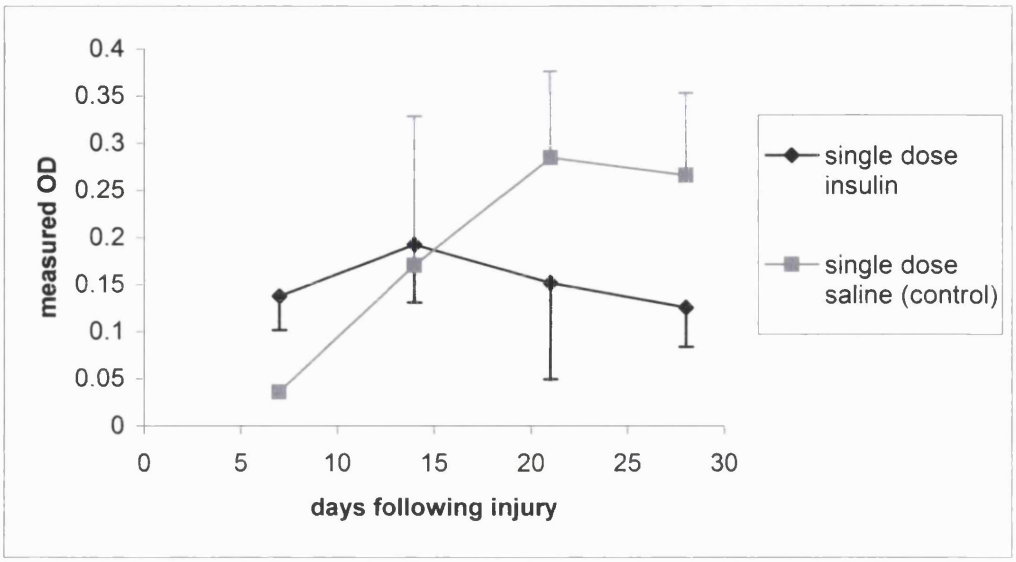


Fig 7.9 Graph showing mean (and standard error) expression of alpha smooth muscle actin in single dose insulin treated vs. single dose saline treated full thickness incisional wounds. All treatments were applied at the time of injury and scars were harvested at days 7, 14, 21 and 28. The expression of αSMA was determined by western blotting.

7.2.5 Tensiometry

To assess the effect administration of insulin may have on wound breaking strength, incisional wounds that had been treated with insulin or saline were harvested after 14 days (n=4 for each group) and the wound breaking stress assessed using tensiometry. Scars were wrapped in saline soaked swabs and transported immediately to the laboratory for testing. Each scar was bisected to yield 2 strips, each of which was measured using a Nikon Shadowmeter. The strip of tissue was mounted on the tensiometer (constructed in house, University of Westminster), with the scar mounted at 6mm from the jaws of the clamps. Traction was applied across the scar at a constant rate of 10 mm/minute, and the force required for disruption of the scar measured. Stress values were calculated by normalising load values to cross-sectional area of each specimen and are given in units of MPa (mega-pascal). One pascal is equivalent to a force of 1 Newton per square metre.

Each of the samples tested disrupted through the line of the scar. Fig 7.10 shows both the mean and standard deviation for each group of scars. Treatment of incisional

wounds with saline resulted in a median wound breaking strength of 1.71MPa (±0.29) 2 weeks following wounding. Treatment of the wounds with either 1 dose of insulin at the time of wounding or 4 doses at the time of wounding and every 3 days thereafter resulted in median wound breaking strengths of 1.62MPa (±0.31) and 1.80MPa (±0.19) respectively. These differences were not significantly different from the control.

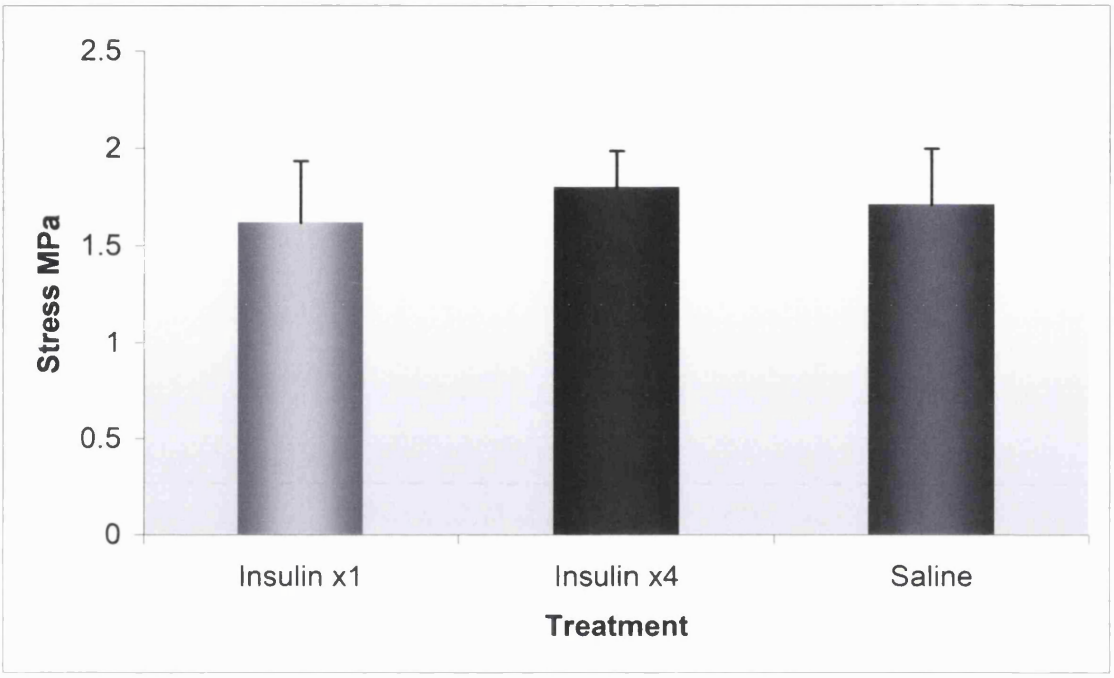


Fig 7.10 Graph showing mean and standard deviation for the stress (in MPa) required to disrupt murine incisional scars. Scars had previously been treated with a single dose or multiple dose administrations of insulin or saline (control).

7.2.6 Alternative methods of administration

Using the incisional wound healing model described above (see section 7.2.2) insulin was applied to the healing wound systemically and also via topical irrigation, both methods being more practical for the treatment of large area wounds in man. Insulin was administered systemically via a sub-cutaneous injection of 0.15 IU into the skin of the anterior right iliac fossa (a site considered remote from the wound), with the wounds again created on the posterior flank skin. Topical administration of insulin was achieved by bathing the wounds with insulin solution (containing 0.15 IU) for 5 minutes. The wounds were then washed with saline prior to wound closure. Administration of insulin

via the topical route used for the purposes of this thesis could therefore only be performed prior to wound closure and thus the effects of multiple topical administrations of insulin have not been evaluated. Myofibroblast scores were obtained as described previously. Mean score and standard deviation for each administration method (each undertaken in 8 animals) are shown in Figure 7.11.

Of concern following the previous *in vivo* experiments was that the wounds in the control groups may be responding in some way to the administration of saline. Therefore, a "sham" group was evaluated in which wounds were created and then sutured with no administration of either insulin or saline. Evaluating the wounds of this sham group resulted in a myofibroblast score of 1.32±0.84, whereas the score for a group of mice treated with a single dose administration of saline was 1.25±0.45. These scores are not significantly different (P=0.861) and suggest that the administration of saline during wound healing does not affect the myofibroblast numbers.

Administration of insulin via alternative routes (systemic and topical irrigation) resulted in a reduction in the efficacy of insulin in reducing fibroblast - myofibroblast differentiation. Systemic administration of insulin as a single subcutaneous injection given remotely to the wound resulted in a myofibroblast score of 0.96±0.73, compared to a score of 0.49±0.25 for local infiltration of insulin into the wound. This difference was not significant (P=0.102). The reduction seen with systemic insulin was also not significant from that observed in saline treated controls (P=0.382). However, multiple systemic administration of insulin resulted in a myofibroblast score of 1.37±0.70, significantly different from the single local infiltration and similar to the score derived from both sham and saline treated controls. Topical administration of insulin resulted in a myofibroblast score of 1.04±0.64, but this was not significantly different from local infiltration, topical administration or indeed saline treated controls (P>0.05).

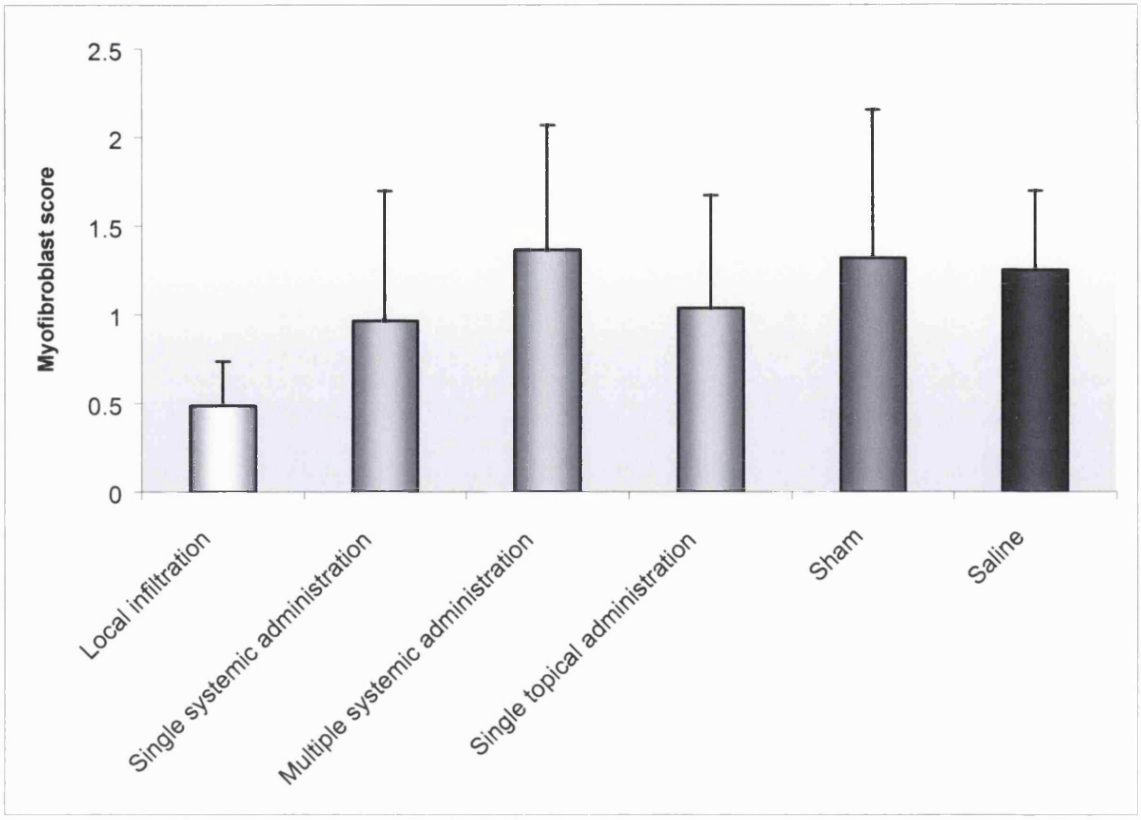


Fig 7.11 Graph showing mean "myofibroblast score" and standard deviation for each treatment when applied to a murine incisional wound healing model. Insulin (0.15 IU Insulatard) was applied either as a single application systemically, topically (onto the wound), as a local infiltration into the wound edges or as a multiple application applied systemically. Scores were derived from the analysis of 8 wounds per treatment group with each scar examined at 3 random points along its length.

7.3 Discussion

7.3.1 The effect of insulin on murine derived fibroblasts

The assessment of the effects of human insulin on cultured murine fibroblasts was necessary in order to demonstrate whether the proposed *in vivo* model was appropriate for the evaluation of insulin's action on fibroblast - myofibroblast differentiation. Although mice have been used to model wound healing for many years (Gottrup & Andreassen, 1981) and insulin found to accelerate healing in diabetic murine wounds (Weringer, Kelso, Tamai, & Arquilla, 1982) the effect of insulin on murine dermal fibroblast - myofibroblast differentiation or indeed on scar severity has yet to be described. In addition, the effects of insulin preparations formulated for use in man have yet to be evaluated on murine wounds.

Application of two of the three formulations of human insulin tested within this chapter, to murine fibroblasts cultured in GFD medium, resulted in a significant reduction in the number of myofibroblasts (αSMA expressing fibroblasts) in comparison to the number seen in un-supplemented GFD medium. This demonstrated that not only do murine fibroblasts readily form myofibroblasts *in vitro* in the absence of serum derived inhibitory factors but also, like the human derived fibroblasts, they can be prevented from differentiating into this phenotype through the addition of insulin. In addition, as with human fibroblasts, of the insulin preparations tested, the long acting formulation Insulatard was most effective in preventing this differentiation. From these results and those of the previous chapters, this particular insulin preparation seemed to be most likely formulation to be of use both in clinical practice and for further evaluation *in vivo*.

Interestingly, neither the Mixtard formulation of human insulin nor the laboratory grade bovine insulin resulted in a significant reduction of myofibroblast numbers. The results with laboratory grade insulin might be expected by species incompatibility with bovine insulin having cross-activity in humans but not in mice. The lack of response seen with the addition of Mixtard cannot be explained easily. All three preparations of human insulin evaluated within this thesis are from the same manufacturer and differ only in formulation in order to yield compounds with different pharmacokinetic and

pharmacodynamic properties. The differences between Insulatard and Mixtard are minimal, and therefore one would expect similar results in human and murine derived dermal fibroblast cultures. The reasons for the lack of activity with Mixtard when compared to the other preparations of insulin used in this chapter are therefore unclear.

7.3.2 *In vivo* models

Determination of the effects of insulin on myofibroblast numbers during wound healing *in vivo* was undertaken in both murine incisional and excisional wound healing models. In view of the relative ease of use of the incisional model, and its reproducibility, this model was chosen for further examination. The linear incisions accurately represent most surgical wounds, are simple to produce and allow for easy injection and administration of substances in a controlled fashion. This also allows for reproducible analysis in a non-subjective, highly controlled manner (Mustoe, Pierce, et al., 1987).

The excisional wound model was found to be more difficult to interpret, its main difficulties concerning the identification of scars prior to wound harvest and during histological processing. Common laboratory stock male BALB/c mice were used for the experiments described in this thesis and are known to heal normally after injury. In the case of the wounds used within this chapter, after 2 weeks all had healed, contracting to such an extent that scarring was minimal making their location difficult. Where identification of the scar was possible, this was only achieved microscopically by the lack of skin appendages in a portion of the histological section. Whilst a simple solution to this problem may have been to shorten the duration of the experiment, 14 days was chosen to be equivalent to the duration of the *in vitro* experiments.

In the excisional wound model, the difficulty locating the scar macroscopically made histological processing and sectioning difficult if one is to guarantee the presence of a scar in the centre of a section. These problems have been overcome by other authors (Stallmeyer, Kampfer, et al., 2001), (Gottrup & Andreassen, 1981), demonstrating that the problems posed by this model are surmountable, through the use of better marking of the incision sites. However, the most effective means of achieving this is by tattooing of the scar area. This may have entailed marking the centre of the scar, possibly

disrupting the architecture if applied following harvesting of tissue or even causing an inflammatory reaction or myofibroblastic response if performed at the time of injury. This in turn may have affected the results obtained following staining to identify the presence of myofibroblasts.

Although every effort was made to ensure no variation between surgical procedures, subtle differences are bound to have occurred between animals. As described by Saragas et al. (1985), "even the most careful of surgeons cannot control precisely the angle and width of incision, the sharpness of the blade, the tension of the sutures or even the overall trauma of the procedures". By using one operator, and a simple mode, the majority of variability between animals has been reduced. Although some variation is sure to exist this should be equally distributed amongst all of the animals used for these experiments, and not skew results.

7.3.3 The effect of insulin on *in vivo* fibroblast - myofibroblast differentiation

Application of insulin as a single local infiltration to full thickness incisional cutaneous wounds reduced the numbers of myofibroblasts as compared to control (saline) treated wounds. The reduction in fibroblast - myofibroblast differentiation was comparable to that seen in the *in vitro* studies (See Section 3, also Section 7.2.1).

The effect of insulin of multiple doses of insulin

A deficiency of the work as described within this chapter is that multiple dose administration of insulin as an infiltration into the scar will have resulted in repeated local micro-trauma of the scar area. During the local injection, entry of both needle and fluid may have caused injury, disrupting the extracellular matrix or breaking cell-matrix or cell-cell interactions. These seemingly minor injuries may themselves have required repair, effectively "resetting" the healing process. In effect, although wounds were harvested 14 days after initial injury, for those wounds that received 4 doses of insulin, wound harvest occurred only 3 days after the final administration (and therefore only 3 days following the last episode of microtrauma). As the myofibroblast phenotype is known to be more prevalent early during wound healing (Gabbiani, 1996), the increased

numbers of myofibroblasts seen with multiple administrations of insulin may be a direct result of these repetitive injuries.

Alternatively, the higher myofibroblast scores seen in the multiple local infiltration insulin treated group may have occurred due to immune response. Multiple applications of human insulin may have initiated a delayed hypersensitivity response, resulting in inflammation. The inflammatory response has itself been linked with myofibroblast formation (Badid, Mounier, Costa, & Desmouliere, 2000) and thus any such reaction is likely to increase the number of myofibroblasts present.

The credibility of controls

Given the myofibroblast activity of insulin *in vivo,* it may be that the myofibroblast score for saline treated controls was falsely low. Minimal differences were noted between single and multiple dose applications of saline. These differences were not significant upon testing, and may have been due to natural variation between animals or to variation in surgical techniques. However, the method of administration of saline may have played some part, either affecting the integrity of the wounds physically or having a pharmacological effect. Administration of insulin by local infiltration of the test wound may have influenced both the intended wound and the control due to their proximity, as there is no way of preventing either the systemic absorption of insulin or of limiting its effect to a single wound. Even though insulin was applied locally to each wound in minute amounts, systemic absorption will have occurred, resulting in an albeit minor extent of artificial "hyper-insulinaemia". Such raised systemic levels of insulin may have affected the "control" wound, and possibly reduced the numbers of myofibroblasts present within these wounds. Even allowing for the systemic absorption of insulin, after dilution by the circulation, the levels of insulin that the control wounds would experience would be far less than the test wounds.

Another argument against accidental absorption affecting control wounds is that deliberate application of insulin systemically had no significant effect on myofibroblast scores. Furthermore, the administration of saline may itself have had some effect on myofibroblast numbers. To answer this question, one of the treatment groups used to determine the most effective means of applying insulin (Section 7.2.6) assessed the

effect of sham treatment, with both wounds on the mouse being treated without injection with either insulin or control. Within these wounds, a myofibroblast score similar to and not significantly different from that observed with saline treatment was observed, suggesting that saline does not influence myofibroblast differentiation.

A time course of myofibroblast events *in vivo*

Although the cellular time course of wound healing is well established in a number of animals (Grinnell, 1994) there are no published time courses available for the appearance of myofibroblasts in a murine model. However, in all models evaluated to date, myofibroblasts appear early during wound healing and disappear once the proliferating phase of healing is complete (Desmouliere, Badid, Bochaton-Piallat, & Gabbiani, 1997), (Clarke, 1996). In the closest rodent model available (the rat), maximal myofibroblast numbers are identified 12 days following injury, with a decline in myofibroblast numbers seen after this point. Counting the number of apoptotic myofibroblasts, these increase gradually following cutaneous injury, reaching a peak at 20 days (Desmouliere, Badid, et al., 1997).

In the studies on murine wounds presented in this chapter, the myofibroblast score in control wounds reached a peak at 7 days and declined steadily thereafter. Although the difference to the results obtained on infiltration of the wounds with a single dose of insulin only approaches significance at day 14 (presumably simply due to the lower numbers of wounds assessed compared with the single time point studies), it is clear that the differentiation into the myofibroblast phenotype is not prevented by administration of insulin. These results suggest that the effects of insulin treatment are delayed in some way; either that its effects are on late rather than initial induction of myofibroblast differentiation or an increase in the clearance of myofibroblasts via apoptosis. Nevertheless, it has been clearly demonstrated within this chapter that the high numbers of the myofibroblast phenotype still occur in the healing wound shortly after injury, irrespective of whether or not they have been treated with insulin or not. This is important as the myofibroblasts are thought to be responsible in part for wound contraction and extracellular matrix formation as well as playing some role in the control of the subsequent processes of wound healing (Desmouliere & Gabbiani, 1996), (Gailit, Marchese, Kew, & Gruber, 2001). Addition of insulin to *in vivo* cutaneous

wounds appears to cause a more rapid decline in the number of myofibroblasts present which is then maintained at a generally lower steady level than that seen in saline treated wounds. This appears to be in contrast to the effects reported *in vitro* (Linge, Shelton, et al., 2004) where there is a steady increase in myofibroblast numbers from day 4 to day 14 when dermal fibroblasts are cultured in GFD medium, whereas addition of insulin abrogates this increase completely.

The effects reported *in vitro* may reflect the presence of multiple stimuli that cause fibroblast - myofibroblast differentiation. The first stimulus causing fibroblast - myofibroblast differentiation may be the release of TGFβ from platelets shortly after injury. This initial stimulus may not be affected by the presence or indeed the addition of insulin. The production of autologous TGF-β by fibroblasts following wounding may act as a subsequent stimulus to the myofibroblast phenotype. This later stimulus may be inhibited by the addition of insulin, reducing the sustained auto-stimulation of myofibroblasts in the wound. Experiments supporting this hypothesis have been performed by Linge et al. (2004) where addition of mannose-6-phosphate blocks the autologous production of TGFβ and results in reduced numbers of myofibroblasts *in vitro*.

It is possible that the myofibroblast is being cleared from the insulin treated wound more rapidly than in the control wound. However, if this is indeed the case, the processes involved are unknown. Evidence exists that suggests that the clearance of the myofibroblast during wound healing is undertaken through apoptosis (Clarke, 1996), (Desmouliere, 1995), and although this may be occurring in the experiments described within this chapter, no efforts have been made to investigate this phenomenon. Certainly, work by Godbout et al. (1999) has highlighted that insulin can cause apoptosis through stimulation of the Caspase-3 cascade, but prior to making these assumptions for this model, further work would be required to specifically investigate this process.

Immunohistochemistry versus western blotting

Interestingly, the expression of αSMA as determined by western blotting and immunohistochemical staining and subsequent scoring yields differing results. For

example, during the time course, immunohistochemical staining for αSMA shows similar numbers of myofibroblasts for both saline and insulin treated groups at day 7. However, the levels of αSMA as determined by Western blotting are significantly different. This difference was also evident in the earlier *in vitro* work presented in Chapter 3. In vitro differences were thought due to the presence of αSMA in the fibroblasts at levels too low to be detectable by immunohistochemical analysis. The same mechanism may account for the differences seen in the *in vivo* model. Another factor that may further confound these results is that samples were loaded so that they represented equal dry weight of scar tissue. It is possible that insulin causes alterations in total protein production (not just soluble protein as assayed in Chapter 4) which would bias the comparison of αSMA expression between controls and insulin treated groups.

7.3.4 Tensiometry

The effects of insulin on wound breaking strength have been studied previously, although not in isolation. Saragas et al. discovered that application of insulin to healing corneal wounds reversed the decrease in breaking strength seen with application of corticosteroids (Saragas, Arffa, et al., 1985). In cutaneous wound healing, the effects of insulin in normalising the biomechanical properties of wounds in diabetic animals are well described (Andreassen & Oxlund, 1987). Despite this, little research has been performed assessing the effects of addition of insulin alone in non-diabetic animals. The work presented in this chapter suggests that using insulin during wound healing in non-diabetic animals does not significantly change the strength of the wound and may therefore be used in clinical trials in man without concern for the resulting wound strength. Indeed work by other groups suggests that the use of insulin might be remedial in wounds that would otherwise heal with sub-optimal wound breaking strength.

7.3.5 Alternative methods of insulin administration

Insulin appears to reduce myofibroblast formation most effectively when applied as a local, subcutaneous infiltration into the wound edges. The results seen with alternative administration techniques might reflect a reduced concentration of active insulin

reaching the wounds. Although administration of a depot of insulin is advantageous in that the amount of insulin absorbed can be easily determined, its subsequent dilution by the circulation means that the effective increase in insulin levels around the wound is minimal. Topical irrigation poses other problems given the high levels of proteases present in healing wound matrix (Robertson, Belford, & Ballard, 1999) and relating to the amount of active substance that is actually absorbed by the animal model. Insulin is only in contact with the wound for a relatively short time, during which the amount of insulin that has been absorbed is variable. Although the concentrations of insulin were identical throughout all three methods of administration, differences between absorption may have meant that some methods (in particular the topical administration) received a significantly lower dosage whereas local infiltration gets active insulin straight to the important area i.e. the wound edge from which the fibroblasts that eventually populate the granulation tissue originate. Further work assessing the effects of higher concentration insulin solutions on fibroblast - myofibroblast differentiation *in vivo* may be able to better define the beneficial role of topical insulin in wound healing.

For many patients, this optimum mode of applying insulin as an infiltration would not be practicable. For example, in burns patients, the large and diffuse nature of the injury and the lack of defined wound edges make this method of administration impossible. In these patients, alternative methods of administration would be best employed, two examples of which were investigated within this chapter. Although the two examples of alternative application techniques investigated within this chapter did not prove significantly effective, higher doses or improved topical vehicles that reduce protease-induced breakdown of insulin might improve their efficacy to some extent. As severe or pathological scarring is very common in burns, and its therapy very difficult, any proposed therapy that is proven not to adversely affect healing and that may reduce myofibroblast numbers would be worth considering. Both patient and clinician would welcome even a very small reduction in either the incidence or extent of pathological scarring in this situation.

7.4 Conclusions

- Insulin reduces fibroblast - myofibroblast differentiation in murine dermal fibroblast cultures, with Insulatard being the most effective preparation evaluated
- Insulatard reduces myofibroblast numbers during wound healing *in vivo* most effectively when administered as a single dose local infiltration shortly after injury
- Myofibroblasts are not prevented from forming during healing with the addition of insulin, but rather are reduced during the later stages of wound healing (>10 days) when compared to control wounds
- Insulin does not effect the strength of the wound repair or delay healing

Chapter 8

General Discussion

This thesis set out to build upon the work performed by Linge et al. (2004) and to investigate whether insulin would be beneficial in reducing cutaneous scarring when added to healing wounds. Many substances have shown promise in the laboratory, but to date there are no effective and guaranteed mechanisms for scar prevention. The work presented herein has demonstrated that human formulations of insulin can prevent myofibroblast formation in both *in vitro* and *in vivo* systems. It has also shown that insulin can be safely used *in vivo* at low concentrations, and that there are no ill effects upon either the host animal or, importantly, on the wound healing process.

Despite advances in surgical technique, dressings and patient care, cutaneous scarring is difficult to manage quickly and successfully. Many groups are striving to develop a therapy that will either prevent or reduce both normal and pathological scars. To date, much interest has focussed upon the role TGFβ isoforms play during wound healing and scar formation (Shah, Foreman, & Ferguson, 1995), (Shah, Revis et al., 1999) in an effort to produce the ideal anti-scarring therapy. As described in the introduction to this thesis, this approach may be unsuccessful and may even prevent or delay healing. There is therefore scope for an alternative approach to the prevention of cutaneous scarring, and through an examination of the cell biology of wound healing and fibroblast culture, the role of insulin during wound healing has been investigated (Linge, Shelton, Mackie, & Sanders, 2004).

The addition of insulin to healing wounds is not a new concept, numerous authors having applied this substance historically with mixed success (Greenway, Filler, & Greenway, 1999), (Pierre, Barrow, et al., 1998), (Weringer, Kelso, Tamai, & Arquilla, 1982). In the diabetic subject, there is little doubt that the addition of insulin can be beneficial, with systemic insulin therapy shown to reverse the impaired wound healing associated with diabetes mellitus (Andreassen & Oxlund, 1987), (Goodson & Hunt, 1978). However, such systemic therapy is only effective if started before injury or during the first week after injury. Greenway et al. (1999) showed that standard wounds treated with insulin healed 2.4 ± 0.8 days faster than similar wounds treated with saline. This study was performed using a crystalline form of insulin (similar to Insulatard used in this trial) in non-diabetic subjects. The work of Pierre et al. (1998) demonstrated that

systemic insulin administration accelerated large area burn wound healing (and donor site regeneration), and improved wound matrix formation.

The work of Linge et al. (2004) established that insulin may be beneficial if added to cutaneous wounds through the inhibition of the myofibroblast phenotype. However, the preparation of insulin chosen for use in that work was not licensed for use in man. To further develop the findings of Linge et al. into a possible therapy therefore required the evaluation of forms of insulin licensed for use in man. This determined whether this finding was a characteristic of insulin or merely a feature of the preparation previously assessed.

8.1 Proof or refutation of hypotheses

8.1.1 Determination of the effect of insulin on fibroblast differentiation *in vitro*

The work described in Chapter 3 demonstrated that the inhibition of fibroblast - myofibroblast differentiation shown by Linge et al. (2004) is independent of the formulation of insulin used *in vitro*. Although only 3 preparations of insulin have been evaluated within this thesis, they represent the spectrum of insulin preparations in common clinical use today. Significant differences have been demonstrated between these compounds with respect to their efficacy, which seem to be in keeping with the predicted half life of the preparations *in vitro*.

The increased efficacy of the long acting formulation (Insulatard) may be due to the persistence of active insulin *in vitro* but similarly may also reflect the nature of the preparation itself and any adjuvant compounds therein. Although such concerns have not been addressed within this thesis, the adjuvant compounds present in both Insulatard and Mixtard are similar (manufacturer's data) and may suggest that the apparent differences are not related to the composition of the formulation but rather a reflection of the active half-life of insulin within each formulation.

8.1.2 The effect of insulin on aspects of fibroblast behaviour important during wound healing

Although insulin has been shown to reduce fibroblast - myofibroblast differentiation, its use as a therapy to reduce or prevent scarring would be hindered if it were to prevent or impede wound healing. The work contained within Chapter 4 served to assess the effect of addition of insulin upon some of the processes known to be of importance during wound healing. Although the processes evaluated within this chapter were by no means the only important processes occurring during wound healing, insulin was demonstrated to have no detrimental effect.

Previous studies have shown insulin to be beneficial in promoting fibroblast proliferation *in vitro*, (Selgas, Lopez-Rivas, et al., 1989), (Krupsky, Fine, et al., 1996). Similarly, insulin has previously been shown to increase protein and collagen production (Sato, Ignotz, et al., 1981), (Goldstein, Poliks, et al., 1989). Although the results obtained within this thesis show a similar trend, the magnitude of the responses observed does not compare with that documented by the above authors. As discussed within Chapter 4, this may reflect the novel nature of the medium employed within this thesis, or may simply reflect the characteristics of the human dermal fibroblasts used.

The assessment of collagen gel contraction was undertaken in order to estimate whether insulin may affect wound contraction *in vivo*. Whilst contraction of collagen gels has been thought to reflect myofibroblast activity (Grinnell, 1994), the addition of insulin (known to reduce myofibroblast numbers) had little effect on overall contraction. This disparity may be explained by the differing durations of the experiments. Those undertaken to show myofibroblast numbers *in vitro* showed a decrease in myofibroblast proportion at 14 days, whilst those demonstrating collagen contraction ran for only 3 days. It is possible that the reduction in myofibroblast numbers occurs after a number of days. This is supported by the *in vivo* time course experiments performed in Chapter 7.

8.1.3 Do polypeptides that share certain biological properties with insulin affect fibroblast - myofibroblast differentiation?

It is conceivable that some of the other growth factors removed during the preparation of GFD medium may share similar activities to insulin and also be capable of reducing fibroblast - myofibroblast differentiation. Although, in contrast to insulin, neither insulin like factors (IGF-I and IGF-II) was shown to have a significant effect on fibroblast - myofibroblast differentiation, previous work has demonstrated that these factors have little effect in isolation but act synergistically when combined with other growth factors (Greenhalgh, 1996). The evaluation of these factors in combination with insulin may yield a more effective therapy.

During the course of this chapter, the ability to reduce fibroblast - myofibroblast differentiation has been shown to be shared by PDGF, although the magnitude of the reduction falls short of that seen with insulin. Such results suggests a similar mechanism of action, with preliminary studies showing that both insulin and PDGF (the next most effective growth factor) signal through the intracellular messenger PI 3-kinase (Hooshmand-Rad, Hajkova, et al., 2000). Preliminary studies evaluating the role of PI 3-kinase suggest that this compound may play a significant part in fibroblast - myofibroblast differentiation. The continuation of such mechanistic work may identify a means by which the myofibroblast phenotype can be prevented or reduced without significantly affecting other physiological processes.

8.1.4 The effect of substances with physiologically antagonistic roles to insulin on insulin's efficacy in reducing fibroblast - myofibroblast differentiation

Originally, the ability of insulin to reduce fibroblast - myofibroblast differentiation was thought to be linked to its major physiological function, glucose homeostasis. The purpose of chapter 6 was therefore to evaluate whether insulin would still be effective in an environment rich in its physiological antagonists. Such an environment was predicted to be present during *in vivo* studies following insulin administration.

The combination of insulin and antagonist had a lesser effect on fibroblast - myofibroblast differentiation than insulin alone although the reduction noted proved not to be significant upon statistical testing. Unexpected however, was the reduction in myofibroblast number seen with glucagon and dexamethasone. It is conceivable that these compounds may activate similar pathways as insulin to reduce fibroblast - myofibroblast differentiation. Further studies may wish to explore the mechanisms underlying this response to further develop an "anti-scarring" therapy.

8.1.5 The effectiveness of insulin on fibroblast - myofibroblast differentiation during wound healing *in vivo*

Whilst the "gold standard" for the evaluation of novel therapies *in vivo* seems to be the porcine model, financial considerations precluded the use of this model within this thesis. Despite this, the validity of the BALB/c mouse has been demonstrated, with both a tendency to the formation of αSMA containing cells during wound healing confirmed and a corresponding decrease in their number after administration of insulin. Although the *in vivo* experiments were performed using only one preparation of insulin, Insulatard, this had been shown to be the most effective *in vitro* on both human and BALB/c derived fibroblasts.

The *in vivo* experiments have confirmed that insulin, despite its effects on myofibroblasts, does not impede wound healing nor reduce the strength of the repair. The experimental time course has also demonstrated that insulin does not prevent the initial formation of the myofibroblast (at 7 days) after injury, but rather reduces the presence of myofibroblasts seen longer term (>14 days) via reducing their formation or hastening their removal. This may prove to be useful in man, where the beneficial effects of the myofibroblast may be utilised prior to their removal.

8.2 Critique

Although every effort has been made to provide a logical and comprehensive account of the effects insulin may have during wound healing, the work contained within this thesis has some obvious deficiencies that could not be readily addressed.

8.2.1 General considerations

This thesis has been built on the finding that insulin causes a reduction in myofibroblast numbers. This has been investigated throughout by either manually counting the numbers of αSMA positive cells present, or by evaluating the cellular expression of this protein by western blotting. However, it is feasible that insulin merely prevents or masks the expression of this protein. The cells may still be capable of other roles that myofibroblasts perform. In view of the results obtained when investigating contraction, of collagen gels this remains a possibility. To date, the classification of cells as myofibroblasts relies on the cell achieving a number of criteria (Eyden, 2001),(Sappino, Schurch, & Gabbiani, 1990). Like many workers studying these cells (Ma, Zarbo et al., 1992), (Foo, Naylor et al., 1992), (Powell, Cranor et al., 1995), (McParland, Taylor et al., 2000), the spindle shaped "fibroblast-like" cells that express αSMA has been accepted as a myofibroblast. Through not investigating the complete phenotype of these αSMA expressing cells, by the use of electron microscopy (looking for abundant rough endoplasmic reticulum and the presence of a fibronexus), it is possible that we may be investigating cells that are differentiating towards becoming myofibroblasts (Eyden, 2001) rather than true myofibroblasts.

A further flaw may be through the assumption that a decrease in αSMA (or myofibroblasts) leads to improved scarring. The reduction in αSMA positive cells both *in vitro* and *in vivo* may be a cosmetic phenomenon, with such cells continuing to display other aspects of the myofibroblast phenotype that contribute to the formation of pathological scarring. This issue may only be resolved by the evaluation of insulin during wound healing in a model known to form cutaneous scars, such as man himself.

Until other facets of myofibroblast biology have been determined more precisely, it can only be assumed that insulin does indeed prevent their formation. When a more exact means of identification becomes available, further work should be performed to assess the true effect of insulin on the myofibroblast.

8.2.2 *In vitro* experiments

Aspects of the *in vitro* work open to criticism include the reliance on a previously uncharacterised medium. Although this medium was used by Linge et al. (personal communication) during their investigations, to date no publications are available that document its use and effects. It is conceivable that some of the results seen within the *in vitro* work are artefactual. However, the similar results obtained during the *in vivo* experiments (confirming the ability of insulin to reduce fibroblast - myofibroblast differentiation) give credence to the *in vitro* findings, and thus support the use of such a medium.

The validity of the methods used to assess fibroblast - myofibroblast differentiation must also be questioned. Whilst the counting of cells would appear to be a simple technique for the determination of αSMA expression levels, the variation in the intensity of staining can lead to considerable inter-observer differences. Nevertheless the same pattern of changes in myofibroblast differentiation were demonstrable by different observers, such as the significant increase in myofibroblast differentiation seen in GFD over NGM medium and its subsequent reduction on supplementation of the GFD medium with insulin (Linge et al., 2004). Although both observation and calculation of myofibroblast proportion within this thesis were performed by the author, leading to the possibility of observer bias, the fact that all quantification of myofibroblast proportion was assessed blindly along with the similarity of the results obtained with western blotting (a more objective technique) suggests that this technique is valid.

The assessment of myofibroblast number by immunohistochemistry is a limiting factor for the number of experiments that can realistically be performed. For many of the experiments performed within this thesis, approximately 600 –1000 cells were counted for each cell line for each substance tested. Counting was always performed by hand, as using numerous software packages, and image manipulation techniques, an accurate and consistent estimation of the number of cells present was not possible. Using western blotting can speed up the process, but issues regarding differing cell number between treatment groups make the use of this technique also subject to error.

Future "screening" of substances to determine compounds that are more effective in reducing myofibroblast numbers could use a system as described by Tanaka et al. (Tanaka, Sano et al., 2001). Here a novel cell-capture enzyme immunoassay allows for the rapid measurement of expression of α-smooth muscle actin. However, this system also has its flaws in that differences in fibroblast proliferation between treatments cannot be compensated for. An ideal solution would be to combine immunoassay with the colorimetric assay used earlier in this thesis to correct for differences in cell number.

8.2.3 *In vivo* experiments

Insulin may have a role as a potential anti-scarring therapy. However, the lack of data concerning the long term outcomes of wounds treated with insulin, as well as whether pathological scars may be reduced or prevented with its application, would check our enthusiasm in proclaiming this substance as a definitive anti-scarring therapy. In order to explore insulin's role in scarring more thoroughly, work will have to proceed in a model in which the characteristics of the scar may be examined. Although many workers have tried to draw conclusions from rodent wound healing, and the effect of substances upon these wounds, the fact that pathological scars do not occur within these models precludes such conclusions. Further work on the anti-scarring potential of insulin must therefore be performed in a model in which pathological scar occurs, this model possibly being man himself.

The wounds chosen for investigation in this thesis were simple incisional wounds, and the model chosen for its ease and reproducibility. Administration by injection and local infiltration is appropriate for such wounds, but difficulties can be expected if this mode of application is to be applied to larger wounds e.g. burns. Large burn wounds pose unique problems. Here, surgical closure of the wound is rare and the wounds usually close due to keratinocyte proliferation and migration. Application of insulin by injection to such wounds would be both time-consuming and impractical. Alternative methods of insulin administration are therefore required.

In a burn wound, the topical administration of insulin may behave in a different manner to that seen with the incisional wound. The wound remains open and so prolonged

contact with the insulin solution may be achieved. This may be better controlled if a wound dressing could be impregnated with this compound.

8.3 Proposals for future studies

The work contained within this thesis by no means confirms insulin to be the optimum therapy for the prevention or reduction of cutaneous scarring. The data contained herein has demonstrated that insulin can reduce myofibroblast formation both *in vitro* and *in vivo* without detriment to wound healing. However, whilst some may feel that this compound may progress to human studies, its use in man should be carefully monitored in order to avoid or prevent significant morbidity. Such morbidity would conceivably be linked with insulin's role in glucose homeostasis.

The author considers that further work on insulin as an anti-scarring therapy should continue, but suggests that efforts are made to dissect the mechanism by which insulin reduces fibroblast - myofibroblast differentiation to allow its dissociation from insulin's homeostatic roles. In addition, it would be prudent to evaluate many other compounds known to have insulin-like actions *in vivo*, to determine whether such compounds could confer an advantage over insulin. Such compounds could include oral hypoglycaemics and also substances such as vanadate (Shechter & Shisheva, 1993).

Further studies should also aim to assess insulin in a scarring model. Whilst this may be performed in laboratory animals (such as the rabbit ear model or the porcine excisional wound model) even these models do not accurately reflect the wound healing situation in man. It may therefore be argued that an appropriate next step for the further progression of this work may be to evaluate the effect of insulin in control wounds in human volunteers. The nature of such an experiment and the need for clinical follow up for a prolonged period (approaching 2 years) makes such a study infeasible for MD studies and more appropriate for research within a clinical department.

Returning to *in vitro* work, the effect of insulin has so far been assessed on dermal fibroblasts isolated from normal dermis. Whilst the results obtained from these experiments should give an indication of the effects of insulin upon wound healing in

"virgin tissue", they cannot be extrapolated to provide an indication of the behaviour of established scar, whether normal or pathological, or indeed of the result of addition of insulin to new wounds created in scar tissue (e.g. scar revisions). These tissues may respond differently to insulin administration, and although preliminary data obtained by the author and not included within this thesis might suggest that insulin reduces fibroblast - myofibroblast differentiation even in scar (both normal and pathological), this work must be completed prior to the use of this compound in such clinical situations.

In conclusion, insulin has been demonstrated to reduce fibroblast - myofibroblast differentiation *in vitro* and *in vivo*. Some of the processes known to be of importance during wound healing have not been shown to be adversely affected by the addition of insulin. In addition, it has been shown that insulin may be administered safely to *in vivo* wounds without detriment to healing.

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Appendices

Appendix I Composition of Solutions

1.1 Tissue culture solutions

Normal Growth Medium (NGM)

For culture of fibroblasts:

	[final]	
DMEM	500 ml	90%
Benzyl Penicillin	2.5 ml	100 units / ml
Streptomycin	2.5 ml	100 μg / ml
L-glutamine	5 ml	2 mM
HEPES	3.5 ml	
FCS	50 ml	10%

Growth Factor Dialysed Medium (GFD)

DMEM	500 ml
Pen strep	5 ml
l-glutamine	5 ml
Dialysed FCS (*see below*)	30 ml

Dialysed FCS

Foetal Calf Serum was dialysed against twice daily changes of Phosphate Buffered Saline (with calcium and magnesium) for 7 days using Medicell dialysis tubing (MWCO 12-14 kDa). The product was filter sterilized, aliquoted and stored at –20°C until required.

1.2 Immunohistochemistry Solutions

Phosphate Buffered Saline (PBS) Without Calcium and Magnesium

		[final]
KCl	0.2 g	2.685 mM
KH_2PO_4	0.2 g	1.470 mM
NaCl	8 g	0.130 M
$Na_2HPO_4.2H_2O$	1.435 g	8.060 mM
ddH_2O	1000 ml	-

Calcium / Magnesium Supplement (x1000)

	For x1000 stock	[final]
$CaCl_2$	19.72 g	0.9 mM
$MgCl_2$	10.16 g	0.5 mM
ddH_2O	100 ml	-

Tween, Tris Buffered Saline (TTBS)

	For 500 ml	[final]
Tris pH 7.5		10 mM
Sodium Chloride		100 mM
Tween-20		0.1%
ddH_2O		-

14-Diazo dicyclo 2,2,2 octane (DABCO)

	For 40 ml
14-Diazo dicyclo 2,2,2 octane	1 g
PBS	4 ml
Glycerol	36 ml

Store at 4°C wrapped in silver foil

1.3 Western Blotting Gel Formulations

Linear Separating Gel

For a 10% gel:

	For 15 ml
30% Acrylamide	5.000 ml
1M Tris-HCl pH8.8	5.600 ml
ddH_2O	4.350 ml
10% SDS	0.150 ml
10% APS	0.050 ml
TEMED	0.010 ml

Linear Stacking Gel

For a 5% gel:

	For 5 ml
30% Acrylamide	0.835 ml
1M Tris-HCl pH6.8	0.625 ml
ddH_2O	3.515 ml
10% SDS	0.050 ml
10% APS	0.025 ml
TEMED	0.005 ml

For a 2.5% gel:

	For 5 ml
30% Acrylamide	0.417 ml
1M Tris-HCl pH6.8	0.625 ml
ddH_2O	3.930 ml
10% SDS	0.050 ml
10% APS	0.025 ml
TEMED	0.005 ml

1.4 Western Blotting Solutions

Lysis Buffer

	For 5 ml	[final]
SDS	50 µl	0.1%
PMSF	50 µl	1 mM
1M Tris-HCl pH6.8	625 µl	125 mM
ddH_2O	5.27 ml	-

2x Laemelli Sample Buffer – Non-reducing

	For 5 ml	[final]
1M Tris pH6.8	600 µl	60 mM
10% SDS	2 ml	2%
(0.1%) Bromophenol Blue	500 µl	0.005%
Glycerol	500 µl	5%
ddH_2O	4 ml	-

Sample Buffer – Reducing

This was made as for 2x Laemelli Sample Buffer – Non reducing, but with the addition of 30.86 mg of DTT to each ml (equivalent to 100 mm final concentration)

Running Buffer

For 10x stock	For 1 l	[final]
Tris Base	30.28 g	0.025 M
Glycine	144.2 g	0.192 M
SDS	10 g	0.1%

Make up to 1 litre with ddH_2O

Transfer Buffer – Semi Dry Transfer

For 1 litre of 10x buffer for Semi-dry Blotting (Antibodies by Harlow and Lane, p488)

	For 1 l	[final]
Tris Base	58 g	48 mM
Glycine	29 g	39 mM
SDS	3.7 g	0.037%

To one volume of concentrated stock, add 7 volumes of ddH_2O and 2 volumes of methanol.

Transfer Buffer – Wet Blotting Transfer

	For 1 l	[final]
Tris Base	58 g	48 mM
Glycine	29 g	39 mM
SDS	10 g	0.1%

To one volume of concentrated stock, add 7 volumes of ddH_2O and 2 volumes of methanol.

Blocking Solution

	For 500ml	[final]
PBS	495 ml	-
Powdered Milk	15 g	3%
10% NaN_3	2.5 ml	0.05%
10%Tween-20	2.5 ml	0.05%

Total Protein Stain

PBS	50 ml
India Ink	50 µl
Tween-20	150 µl

The membrane was incubated in total protein stain solution overnight, and destained with multiple changes of PBS until the bands were apparent.

1.5 Proliferation Assay Solutions

"Crystal Violet" Stain

Crystal Violet	0.5%
Formol saline	5%
Ethanol	50%
NaCl	0.85%
ddH_2O	-

"Crystal Violet" Destain

Acetic Acid	33%
ddH_2O	-

1.6 Collagen Assay Solutions

0.5M acetic acid

Glacial Acetic Acid	6%
ddH_2O	-

Appendix II - Suppliers

Adams Healthcare Ltd, Leeds, UK.

Agar Scientific Ltd, Stansted, Essex, UK.

Aldridge Chemical Company Ltd, Gillingham, Dorset, UK.

Amersham Biosciences, Buckinghamshire, UK

Astra Pharmaceuticals Ltd, Kings Langley, UK.

Bayer UK Ltd, St. Edmunds, Suffolk, UK.

BDH, R & L Slaughter, Upminster, Essex, UK.

Biogenesis Ltd, Poole, Dorset, UK.

Biorex Laboratories, London, UK.

BOC, Letchworth, Hertfordshire, UK.

Bright Instrument Company Ltd, Huntingdon, Cambridgeshire, UK.

Civa Ltd, Watford, Hertfordshire, UK.

DAKO Ltd, High Wycombe, Buckinghamshire, UK.

Ethicon Ltd, Edinburgh, UK.

Euro-Path, Bude, Cornwall, UK.

Genta Medical, Leeds, Yorkshire, UK.

Gibco Ltd, Life Technologies, Paisley, UK.

Griener Labortechnik, Stonehouse, Gloucester, UK.

Janssen Animal Health,

Jessops Photographic, Watford, Hertfordshire, UK.

Johnson & Johnson, Ascot, Berkshire, UK.

Koch-Light Ltd, Haverhill, Suffolk, UK.

M*lnlycke, M*lnlycke, Sweden

MSD Agent, Division of Merck Sharp & Dome Ltd, Hoddesdon, Hertfordshire, UK.

Medicell International Limited, Liverpool, UK

Mycofarm UK Ltd, Uxbridge, Middlesex, UK.

Park-Davies Vetinary Ltd, Pontypool, Gwent, UK.

Renner GMBH, Dannstadt, Germany.

Roche Diagnostics Ltd, Lewes, East Sussex, UK.

Rexel Office Suppliers, Watford, UK.

Serotec Ltd, Kidlington, Oxford, UK.

Seton Healthcare Group plc, Oldham, UK.

Sigma Ltd, Poole, Dorset, UK.

Smallwares Ltd, Manchester, Lancashire, UK.

Smith & Nephew, Chessington, Surrey, UK.

Steripak Ltd, Runcorn, Cheshire, UK.

Stiefel Laboratories, Wooburn Green, Buckinghamshire, UK.

Veron-Carus Ltd, Preston, Lancashire, UK.

W. M. Lillico, Dorking, Surrey, UK.