Studies of Serum Albumin
In Wound Healing and
Endothelial Apoptosis

By
Christine Bolitho

A thesis submitted in fulfillment of the requirements for the degree
of Doctor of Philosophy

Department of Oral Medicine and Oral Pathology
Faculty of Dentistry
The University of Sydney
March, 2006

© Christine Bolitho
Declaration

This thesis describes work done in the Department of Oral Pathology and Oral Medicine at Westmead Hospital, the Faculty of Dentistry in the University of Sydney between January 2001 and March 2006. This research is entirely my own and has not been submitted in whole or in part for a degree at this or any other university. Nor to my best knowledge does it contain any material published or written by another person except as acknowledged in the text.

Signature

[Signature]

Christine Bolitho
Acknowledgements

I would firstly like to thank my supervisor, Associate Professor Hans Zoellner, for his assistance, advice, encouragement and support throughout this PhD. His extensive knowledge, humour and passion for science have been very inspiring and have made the PhD degree an enjoyable experience.

I would also like to thank the staff in the Department of Oral Medicine and Oral Pathology, The University of Sydney at Westmead Hospital, especially Professor D. M. Walker, Janice Matthews, Carolyn Bruce and Penelope Bayl. Thank you also to my fellow students in the Department, especially Ms. Catherine Emmanuel and Ms. Minh Huyhn for encouragement, support and friendship throughout this PhD.

A special thank you to all the staff at the Animal Research Holding at Westmead Hospital and the Dental X-ray Department, for allowing me to complete my work. In addition, thank you to Dr. Derek Harty from The Institute for Dental Research for assistance with fluorometry and advice with separation of CNBr HSA fragments.

I would also like to thank the many funding bodies that allowed this research to progress such as the Australian Dental Research Foundation, The Tara Old Girls Association Joan Waugh Scholarship and the Australian Post-graduate Award Scheme.

Finally, I would like thank all of my family and friends, especially my wonderful husband Ben, for all their love, support, encouragement and assistance throughout this PhD.
Table of Contents

Title Page
Declaration
Acknowledgements
Table of Contents
List of Figures
List of Abbreviations
Summary

Chapter I: General Introduction

I.1. Overview of this Literature Review

I.2. The Phases of Wound Healing
   I.2.i. The Inflammatory Phase of Wound Healing
   I.2.ii. The Proliferative Phase of Wound Healing
      I.2.ii.a. Angiogenesis
      I.2.ii.b. Proteinases in Wounds
   I.2.iii. The Maturation Phase of Wound Healing

I.3. Apoptosis
   I.3.i. Mechanisms for Cell Death
   I.3.ii. Characteristics of Apoptosis
   I.3.iii. Mechanisms of Apoptosis
I.3. iii.a. Receptor Mediated Apoptosis  I.14
I.3. iii.b. Caspases  I.15
I.3. iii.c. The Bcl-2 Family of Proteins  I.16

I.4. Apoptosis in Endothelial Cells  I.17
I.4.i. The Anti-apoptotic Activity of Adhesion for Endothelium  I.18
I.4.ii. The Anti-apoptotic Activity of Shear Stress for Endothelium  I.19
I.4.iii. Chemical Factors Controlling Endothelial Cell Apoptosis  I.21

I.5. Questions Arising From the Literature and Addressed in This  I.22

Thesis

Chapter II: Establishment of an Experimental Scarring Wound Healing Model in Mice

II.1. Introduction  II.1

II.2. Materials and Methods  II.7

II.2.i. Materials  II.7
II.2.ii. Establishment of Scarring Wounds by Polyvinyl Sponge  II.7

Implantation
II.2.iii. Tissue Harvesting  II.8
II.2.iv. Processing for Paraffin Histology  II.8
II.2.v. Quantitation of Tissue Vascularity  II.9
II.2.vi. Statistical Analysis  II.9

II.3. Results  II.12
II.3.i. Sponge Implants Were Infiltrated by Granulation Tissue Which Matured to Scar Tissue

II.3.ii. Quantitation of Vascularity During Wound Healing

II.4. Discussion

Chapter III: Fragmentation of Plasma Proteins During Wound Healing

III.1. Introduction

III.1.i. Injury Results in Inflammation and Wound Healing

III.1.ii. The Acute Phase Response

III.1.ii.a. Positive Acute Phase Proteins

III.1.ii.b. Negative Acute Phase Proteins

III.1.ii.c. Wound Healing Represents a Challenge to the Host

III.1.iii. The Fate of Acute Phase Proteins in Wounds

III.2. Materials and Methods

III.2.i. Materials

III.2.ii. Preparation of Experimental Wounds in Mice

III.2.iii. Numbers of Animals Used in Experiments

III.2.iv. Tissue Harvesting for Analysis of Proteins

III.2.v. Assay for Determination of Total Protein Concentration

III.2.vi. SDS-PAGE and Silver Staining

III.2.vii. Western Blotting
III.2. viii. Densitometry and Analysis

III.2. ix. Statistical Analysis

III.3. Results

III.3.i. Western Blots of Mouse Serum Confirmed That the Antibodies Used Recognised Proteins of Expected Sizes

III.3. ii. Ceruloplasmin and Transferrin Were Not Fragmented During Wound Healing in Mice

III.3. iii. Apparent Dissociation of Haptoglobin and IgG During Wound Healing and in Control Tissues

III.3. iv. Fragmentation of Albumin Was Observed During Wound Healing

III.3. v. Fragmentation of Albumin Correlated With Tissue Vascularity During Wound Healing

III.3. vi. Albumin Fragmentation Was Observed in Some Tissues Separate From Wound Healing Sites

III.4. Discussion

Chapter IV: Effect of Fragmentation of Serum Albumin Upon the Anti-Apoptotic Activity for Endothelium

IV.1. Introduction

IV.1. i. General Characteristics of Albumin

IV.1. ii. Fragmentation of Albumin

IV.1. iii. Functions of Albumin
IV.1.iii.a. Ligand Binding

IV.1.iii.b. The Effect of Albumin Upon Cells

IV.1.iii.c. Albumin Acts as an Antioxidant

IV.1.iii.d. Albumin as a Predictor of Clinical Outcome

IV.1.iii.e. Albumin Promotes Cell Survival

IV.1.iv. Albumin Inhibits Endothelial Cell Apoptosis

IV.1.v. Proposal of a Cryptic Active Anti-apoptotic Site in Albumin

### IV.2. Materials and Methods

IV.2.i. Materials

IV.2.ii. Isolation of Human Umbilical Vein Endothelial Cells

IV.2.iii. Culture of HUVEC

IV.2.iv. Verification of Cultured Cells as HUVEC

IV.2.v. Experimental Conditions for Studying Apoptosis

IV.2.vi. Confirmation of Apoptosis

IV.2.vi.a. FACS Analysis

IV.2.vi.b. DNA Gel Electrophoresis

IV.2.vi.c. Inhibition of Caspases by zVAD-FMK

IV.2.vii. Fragmentation of Albumin by CNBr

IV.2.viii. SDS-PAGE

IV.2.ix. Separation of CNBr HSA by Protein-Dye Affinity Chromatography

IV.2.x. Statistical Analysis
Chapter V: Exclusion of Radical Scavenging and Contaminant Lipids as Possible Mechanisms for the Anti-apoptotic Activity of Albumin for Endothelium

V.1. Introduction

V.1.i. Free Radicals and Antioxidants

V.1.i.a Free Radicals

V.1.i.b. Antioxidants

V.1.ii. Albumin as an Antioxidant

V.1.iii. Proliferative and Anti-apoptotic Effects of Lipids

V.2. Materials and Methods

V.2.i. Materials
V.2.ii. Alkylation of Albumin

V.2.iii. Determination of Free Sulphydryl Content of Native and Alkylated Albumin

V.2.iv. Delipidation of Albumin

V.2.v. Fluorometric Analysis of Delipidated Albumin

V.2.vi. Culture and Conditions for Studying HUVEC Apoptosis

V.2.vii. Generation of Free Radicals Using Xanthine and Xanthine Oxidase

V.2.viii. Confirmation of HUVEC Apoptosis

V.2.ix. Statistical Analysis

V.3. Results

V.3.i. HSA Inhibited Free Radical Induced HUVEC Apoptosis

V.3.ii. Free Radicals Reduced Endothelial Survival but Alkylation of the Free Thiol Did Not Affect the Anti-apoptotic Activity of HSA for Endothelium

V.3.iii. Delipidation of Albumin Did Not Affect the Anti-apoptotic Activity for Endothelium

V.4. Discussion

Chapter VI: Investigations into Mechanisms Involved in the Anti-apoptotic Activity of Albumin for Endothelium

VI.1. Introduction

VI.1.i. The Mitogen-Activated Protein Kinase Signalling Pathways
VI.1.i.a. The Extracellular Signal-Related Kinase 1/2 Pathway

VI.1.i.b. The p38 MAP Kinase Pathway

VI.1.ii. The Phosphatidylinositol 3-Kinase/Akt Pathway

VI.1.iii. Possible Signalling Pathways Involved in the Anti-apoptotic Activity of Albumin for Endothelium

VI.2. Materials and Methods

VI.2.i. Materials

VI.2.ii. Culture Conditions for Studying HUVEC Apoptosis

VI.2.iii. Western Blotting

VI.2.iv. Statistical Analysis

VI.3. Results

VI.3.i. MAP Kinase Signalling Pathways Were Not Involved in the Anti-Apoptotic Activity of Albumin for Endothelium

VI.3.ii. The PI3K/Akt Pathway Was Involved in the Anti-apoptotic Activity of Albumin

VI.3.iii. The Anti-Apoptotic Activity of Albumin Was Not Mediated Through a Tyrosine Kinase Dependant Mechanism

VI.4. Discussion

Chapter VII: Release of FGF-2 During Serum Deprivation Induced Endothelial Apoptosis

VII. Introduction
VII.1.i. Serum Deprivation as a Model for Endothelial Apoptosis

VII.1.ii. Fibroblast Growth Factor-2

VII.1.ii.a. General Properties and Functions of FGF-2

VII.1.ii.b. Release of FGF-2 by Endothelial Cells

VII.2. Materials and Methods

VII.2.i. Materials

VII.2.ii. Conditions for Studying HUVEC Apoptosis

VII.2.iii. Determination of FGF-2 Release by HUVEC

VII.2.iii.a. ELISA for FGF-2

VII.2.iii.b. Neutralising FGF-2 Antibodies

VII.2.iv. Determination of Bax Levels by Western Blotting

VII.2.v. Statistical Analysis

VII.3. Results

VII.3.i. A Protective Factor Was Released by Serum Deprived HUVEC

VII.3.ii. Release of FGF-2 During Serum Deprivation by Apoptotic Endothelial Cells

VII.3.iii. Antibodies Blocking FGF-2 Activity Reduced HUVEC Survival in Serum Free Culture

VII.3.iv. The Tyrosine Kinase Inhibitor Genistein Decreased HUVEC Survival in Serum Free Conditions

VII.3.v. Bax Expression Was Reduced in HUVEC During Serum Deprivation
Chapter VIII: General Discussion

VIII.1. The Contribution of Work Described in This Thesis

VIII.2. The Direction of Future Work

Appendix

A.1. Unsuccessful Methods Used in Attempts to Separate and Purify CNBr HSA Fragments

References
### List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Graph showing changes over time in overall tissue vascularity as expressed in terms of the mean number of vessel profiles in graticule microscope fields during adipogenic healing.</td>
<td>II.5</td>
</tr>
<tr>
<td>2.2</td>
<td>Photomicrograph of a Gomori Trichrome stained paraffin section of scarring tissue 3 weeks post-implantation used as a reference slide for assessing vessel size.</td>
<td>II.10</td>
</tr>
<tr>
<td>2.3</td>
<td>Photomicrographs of Gomori Trichrome stained paraffin sections of scarring tissues from post-implantation weeks 1, 2, 3, 4, 8 and 12.</td>
<td>II.14</td>
</tr>
<tr>
<td>2.4</td>
<td>Histogram demonstrating the number and distribution of vessels according to size as well as a graph showing the mean total vessel number over time in scarring wounds.</td>
<td>II.16</td>
</tr>
<tr>
<td>3.1</td>
<td>Western blot analysis of mouse serum for ceruloplasmin, transferrin, haptoglobin, IgG and albumin.</td>
<td>III.22</td>
</tr>
<tr>
<td>3.2</td>
<td>Western blots for ceruloplasmin in granulation tissue extracts from adipogenic and scarring wounds from 1 to 12 weeks post-implantation, as well as control tissues.</td>
<td>III.24</td>
</tr>
<tr>
<td>3.3</td>
<td>Western blots for transferrin in granulation tissue extracts from adipogenic and scarring wounds from 1 to 12 weeks post-implantation, as well as control tissues.</td>
<td>III.26</td>
</tr>
</tbody>
</table>
3.4 Western blots for haptoglobin in granulation tissue extracts from adipogenic and scarring wounds from 1 to 12 weeks post-implantation, as well as control tissues.

3.5 Western blots for IgG in granulation tissue extracts from adipogenic and scarring wounds from 1 to 12 weeks post-implantation, as well as control tissues.

3.6 Western blots for albumin in granulation tissue extracts from scarring and adipogenic wounds from 1 to 12 weeks post-implantation demonstrating fragmentation of albumin, as seen in brief and prolonged ECL film exposures.

3.7 Tissue vascularity in scarring and adipogenic wounds over time as expressed in terms of vessel profile number as well as a Western blot for albumin in granulation tissue extracts at 1, 4 and 12 weeks from both scarring and adipogenic wounds.

3.8 A Western blot for albumin in granulation tissue extracts from scarring tissues from multiple animals harvested at 1 and 12 weeks post-implantation with each lane having material from a separate and individual mouse as seen in brief and prolonged ECL film exposures as well as a silver stained parallel SDS-PAGE of these samples.

3.9 Densitometric analysis comparing native and dimer albumin as well as the 49 kDa, 39 kDa, 27.5 kDa and 17.5 kDa albumin fragments identified in Western blots of scarring wounds from 1 and 12 weeks after implantation.
3.10 A Western blot for albumin in granulation tissue extracts from adipogenic tissues from multiple animals harvested 4 and 12 weeks post-implantation with each lane having material from a separate and individual mouse in brief and prolonged ECL film exposures, as well as silver stained parallel SDS-PAGE.

3.11 Densitometric analysis comparing native and dimer albumin, as well as the 49 kDa, 39 kDa, 27.5 kDa and 17.5 kDa fragments identified in Western blots of adipogenic wounds from 4 and 12 weeks post-implantation.

3.12 Western blot for albumin in muscle, liver, peritoneum, spleen, heart and kidney extracts from 2 to 12 weeks after establishment of scarring lesions.

3.13 Western blot for albumin in muscle, liver, peritoneum, spleen, heart and kidney extracts from 1 to 12 weeks after establishment of adipogenic lesions.

4.1 Amino acid sequence of HSA derived from the cDNA sequence, demonstrating the secondary structure of the protein and position of the 17 disulfide bonds.

4.2 Photomicrograph of cultured HUVEC.

4.3 Photomicrographs of cultured HUVEC stained by histochemistry for *Ulex europaeus* lectin I binding or Factor VIII associated antigen with or without application of primary label.
4.4 Confirmation of apoptosis in serum deprived HUVEC by FACS analysis and DNA gel electrophoresis.

4.5 Histogram of cultured HUVEC treated for 24 hours with either BCS, HSA or M199 alone either in the presence or absence of the general caspase inhibitor, zVAD-FMK.

4.6 Silver stained SDS-PAGE gel of untreated HSA and CNBr fragmented HSA.

4.7 Dose response of protection from apoptosis in cultures of serum deprived HUVEC treated for 24 hours with increasing concentrations of either native HSA or CNBr HSA.

4.8 Partial separation of CNBr fragments achieved by protein-dye affinity chromatography and demonstrated by SDS-PAGE.

4.9 Dose response of protection from apoptosis in cultures of serum deprived HUVEC treated for 24 hours with increasing concentrations of native HSA, 14 kDa CNBr HSA fragment or combined 20 kDa and 32.5 kDa CNBr HSA fragments.

4.10 Silver stained SDS-PAGE gel of native BSA and CNBr fragmented BSA.

4.11 Dose response of protection of from apoptosis in cultures of serum deprived HUVEC for 24 hours with increasing concentrations of either BSA or CNBr BSA.

5.1 Graph demonstrating activity of xanthine oxidase by the production of uric acid over time.
5.2 Dose response of protection of serum deprived HUVEC from apoptosis after 24 hours of culture with xanthine and increasing concentrations of xanthine oxidase in the presence or absence of HSA.

5.3 Confirmation of apoptosis in HUVEC by FACS analysis and DNA gel electrophoresis in the presence or absence of HSA with or without xanthine/xanthine oxidase.

5.4 Time course of protection from apoptosis in serum deprived HUVEC for up to 24 hours of culture with BCS, HSA in the presence or absence of xanthine/xanthine oxidase generated free radicals, or M199 in the presence or absence of free radicals.

5.5 Photomicrographs of serum deprived HUVEC treated for 24 hours with either HSA, alkylated HSA or M199 alone either in the absence or presence of free radicals generated using the xanthine/xanthine oxidase system.

5.6 Histogram demonstrating relative protection from apoptosis for serum deprived HUVEC cultured either with HSA, alkylated HSA or M199 alone for 24 hours either in the presence or absence of free radicals generated using the xanthine/xanthine oxidase system.

5.7 Dose response of relative protection from apoptosis for serum deprived HUVEC cultured either with HSA in the presence or absence of xanthine/xanthine oxidase generated free radicals, or alkylated HSA in the presence or absence of similarly generated free radicals.
5.8 Emission fluorometry for HSA and delipidated HSA comparing Trp fluorescence, bis-ANS fluorescence and acrylamide quenching as well as the effect of these preparations upon HUVEC survival over 24 hours.

6.1 Dose response of protection from apoptosis of HUVEC cultured for 24 hours in BCS, HSA or M199 alone with increasing concentrations of PD98059.

6.2 Dose response of protection from apoptosis of HUVEC cultured for 24 hours in BCS, HSA or M199 alone with increasing concentrations of SB203580.

6.3 Dose response of protection from apoptosis of HUVEC cultured for 24 hours in BCS, HSA or M199 alone with increasing concentrations of wortmannin.

6.4 Histogram demonstrating relative protection from apoptosis for HUVEC cultured for 24 hours with BCS, HSA or M199 alone in the presence or absence of LY294002.

6.5 Representative Western blots for Akt, Phospho-Ser473 Akt and Phospho-Thr308 Akt in HUVEC cell lysates treated with HSA for 15, 30 or 60 minutes after 1 hour of serum deprivation.

6.6 Histogram demonstrating the relative protection from apoptosis for HUVEC cultured with BCS, HSA or M199 alone for up to 24 hours either in the presence or absence of genistein.
7.1 Time course of cell survival for cultured HUVEC over 24 hours treated with BCS, M199 alone, washing and application of fresh M199 or harvesting of HUVEC conditioned medium from wells treated with M199 alone before washing and return of conditioned medium to wells.

7.2 Graph of FGF-2 levels as detected by ELISA in supernatants and cell lysates of HUVEC treated over 24 hours with M199 without or with washing and application of fresh medium.

7.3 Photomicrographs of cultured HUVEC stimulated for 24 hours with either FGF-2 or M199 alone either in the presence or absence of neutralising anti-FGF-2 antibodies.

7.4 Histogram showing the effect of exogenously added FGF-2 upon HUVEC survival in serum free conditions for 24 hours in either the presence or absence of neutralising anti-FGF-2 antibodies.

7.5 Histogram showing cell survival of cultured HUVEC treated for 24 hours with either BCS, FGF-2 or M199 alone in the presence or absence of genistein.

7.6 Representative Western blot and densitometric analysis for Bax expression in HUVEC at increasing times of serum deprivation.
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APP</td>
<td>Acute Phase Proteins</td>
</tr>
<tr>
<td>APR</td>
<td>Acute Phase Response</td>
</tr>
<tr>
<td>BCS</td>
<td>Bovine Calf Serum</td>
</tr>
<tr>
<td>BH</td>
<td>Bcl-2 Homology</td>
</tr>
<tr>
<td>Bis-ANS</td>
<td>4-4'-bis (1-Anilinonaphthalene-8-sulfonate)</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>bp</td>
<td>Base Pairs</td>
</tr>
<tr>
<td>CAD</td>
<td>Caspase-Activated DNase</td>
</tr>
<tr>
<td>CM</td>
<td>Complete Medium</td>
</tr>
<tr>
<td>CNBr</td>
<td>Cyanogen Bromide</td>
</tr>
<tr>
<td>DAB</td>
<td>3-3'-Diaminobenzidine Tetrachloride</td>
</tr>
<tr>
<td>DISC</td>
<td>Death-Inducing Signalling Complex</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DTNB</td>
<td>5-5'-Dithio-bis (2-Nitrobenzoic acid)</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial Cells</td>
</tr>
<tr>
<td>ECGS</td>
<td>Endothelial Cell Growth Supplement</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminotetraacetic Acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-Related Kinases</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Scanning</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Fibroblast Growth Factor-2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's Balanced Salt Solution</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse Radish Peroxidase</td>
</tr>
<tr>
<td>HSA</td>
<td>Human Serum Albumin</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial Cells</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitors of Apoptosis</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun amino-Terminal Kinases</td>
</tr>
<tr>
<td>LPA</td>
<td>Lysophosphatidic Acid</td>
</tr>
<tr>
<td>M199</td>
<td>Medium 199</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen Activated Protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>MAPKK</td>
<td>Mitogen Activated Protein Kinase Kinase</td>
</tr>
<tr>
<td>MAPKKK</td>
<td>Mitogen Activated Protein Kinase Kinase Kinase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositols</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-Kinase</td>
</tr>
<tr>
<td>PIP-2</td>
<td>Phosphatidylinositol 4,5 diphosphate</td>
</tr>
<tr>
<td>PIP-2</td>
<td>Phosphatidylinositol 3, 4, 5 triphosphate</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenyl Methyl Sulfonyl Fluoride</td>
</tr>
<tr>
<td>SBTI</td>
<td>Soybean Trypsin Inhibitor</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth Muscle Cells</td>
</tr>
</tbody>
</table>
TAME  N-α-p-Tosyl-L-Arginine Methyl Ester
TBS   Tris Buffered Saline
TE buffer Tris/EDTA buffer
TGF-β Transforming Growth Factor-β
TNF-α Tumor Necrosis Factor-α
t-PA Tissue Type Plasminogen Activator
TUNEL Terminal Transferase Mediated DNA Nick End Labelling
UEA-1 *Ulex europaeus* Lectin 1
u-PA Urokinase Plasminogen Activator
VEGF Vascular Endothelial Cell Growth Factor
X/XO Xanthine/Xanthine Oxidase
Summary

During wound healing, blood clot is replaced with highly vascular reparative granulation tissue which usually remolds to form scar tissue. The formation of reparative granulation tissue involves deposition of a new extracellular matrix by fibroblasts and development of new blood vessels by proliferating and migrating endothelial cells. Remodelling of the extracellular matrix is mediated by various proteinases, while microvascular remodelling occurs through activation of endothelial cell apoptosis. It is unclear how apoptosis is controlled in specific microvascular segments, although endothelial cells in well perfused blood vessels survive while endothelium becomes apoptotic when blood flow is poor.

Blood vessels in early granulation tissue are highly permeable to plasma proteins, while it is known that protein function may be altered by proteolytic cleavage to generate fragments with biologically different activities to those of parent proteins. As wound healing by definition involves a response to injury and acute phase proteins are important in the response to injury, it was considered possible that some acute phase plasma proteins may be fragmented during wound healing and that this might correlate with events in healing wounds.

To examine this possibility, two experimental mouse wound healing models which display vascularity changes characteristic of wound healing were exploited. A scarring wound healing model was established in the earliest stages of work described in this thesis, in which vascularity was maximal at 1 to 2 weeks after establishment of wounds, while in earlier established adipogenic wound healing model, maximal
vascularity occurred 3 to 4 weeks after wounding. In both wound healing models studied, vascularity reduced after these peak times, consistent with expectations from the literature. Important acute phase proteins, ceruloplasmin, haptoglobin, transferrin and serum albumin, as well as IgG, an important immune molecule, were investigated for possible fragmentation during wound healing. No significant fragmentation of ceruloplasmin or transferrin was seen in wounds, while apparent dissociation of both haptoglobin and IgG into subunits did occur but without any clear relationship to the stage of wound healing.

In contrast, extensive albumin fragmentation was observed in both experimental wound healing models studied, with albumin fragments having approximate molecular masses of 49 kDa, 39 kDa, 27.5 kDa and 17.5 kDa. Importantly, albumin fragmentation correlated with tissue vascularity and was most prevalent at times of maximal vascularity being 1 and 4 weeks in scarring and adipogenic wounds respectively. Examination of other tissues harvested from animals at increasing times following wounding suggested possible metabolic processing of albumin fragments.

Serum albumin is reported as having a specific anti-apoptotic activity for endothelium in serum free conditions. It was considered possible that albumin fragments generated early during wound healing may be responsible for maintaining the high vascularity of early reparative granulation tissue, and for this reason the effect of fragmentation upon the anti-apoptotic activity of both human and bovine albumin was determined. Fragmentation of human albumin with CNBr significantly increased the anti-apoptotic activity, suggesting the presence of a cryptic active site. This was consistent with previous data demonstrating reduced intra-molecular movement together
with reduced anti-apoptotic activity following non-enzymatic glycosylation. The anti-apoptotic activity of human albumin was separable into specific CNBr generated fragments, with the activity residing in either the 20 kDa or 32.5 kDa fragments, but not in the Cys34 containing 14 kDa fragment. Fragmentation of bovine albumin with CNBr partially reduced the anti-apoptotic activity, and the presence of an additional cleavage site in loop 4 not represented in the human protein may account for this.

Free radical scavenging by Cys34 is important in the anti-apoptotic activity of albumin in a number of cell types other than endothelium. To investigate this possibility in endothelium as well as to determine the effect of possible lipid contaminants in protecting endothelium, the effect of both alkylation of the free Cys34 and delipidation upon anti-apoptotic activity was investigated. No effect of these treatments was seen, supporting a specific anti-apoptotic activity of albumin independent of free radical scavenging or delipidation.

Some important intracellular signalling pathways potentially responsible for the anti-apoptotic activity of albumin for endothelium were studied and data indicated that the MAP kinase pathways are not involved. However, it was found that the anti-apoptotic activity was mediated through activation of the PI3 kinase/Akt signalling pathway by a tyrosine kinase independent mechanism.

In the course of performing these experiments, release of a survival factor for endothelium was discovered from apoptotic endothelium. This was determined to be Fibroblast Growth Factor-2, and appears to represent a previously unrecognised negative feedback mechanism for endothelial apoptosis that may have physiological relevance.
It is concluded that the anti-apoptotic activity of serum albumin for endothelium is specific and not mediated through radical scavenging or dependant upon bound lipids, but is instead mediated through activation of the PI3 kinase/Akt pathway by a tyrosine kinase independent mechanism. The anti-apoptotic activity appears to reside in a cryptic site within the molecule, which is exposed by transient conformational change in the native protein, or by fragmentation. Generation of albumin fragments during wound healing may represent a mechanism for maintaining high vascularity early during wound healing, although the potential biological relevance of this remains to be determined. In addition, release of Fibroblast Growth Factor-2 by apoptotic endothelial cells during serum deprivation is proposed as a new physiological level of control of microvascular remodelling.
Chapter I

General Introduction
I.1. Overview of this Literature Review

Each experimental chapter in this thesis contains a literature review of material relevant to the specific experiments described. Despite this, however, it is necessary to provide some general background to put the content of different chapters into context. For this reason, Chapter 1 is a literature review of material important for understanding subsequent chapters.

Experimental work in this thesis commences with the establishment of a wound healing model and the subsequent exploitation of this model to study proteins important for the acute phase response and wound healing. It is for this reason that the current introduction contains a review of wound healing.

A relationship between serum albumin fragmentation and tissue vascularity is established in Chapters 2 and 3, suggestive of a possible role of albumin fragmentation in control of endothelial cell (EC) apoptosis. Chapters 4 to 7 investigate this using a range of methodologies. From this, it becomes important to review what is known about apoptosis in general and EC apoptosis in particular and so these subjects are also reviewed in Chapter 1.

I.2. The Phases of Wound Healing

Following injury, blood clot and or necrotic damaged tissues are replaced by reparative granulation tissue which is subsequently remodelled to form mature scar, adipose or bony tissue. These events involve the migration of several cell types into the
wound and activation of their differentiated function (Bruder et al., 1994; Xaymardan et al., 2002) (for reviews see (Clark and Henson, 1996; Linares, 1996; Mutsaers et al., 1997; Greenhalgh, 1998)). An important step during wound maturation is a significant decrease in cellularity and vascularity through apoptosis of both fibroblasts and EC (Darby et al., 1990; Desmouliere et al., 1995). It has been suggested that reduced cellular apoptosis may contribute to impaired wound healing and cause excessive pathological scarring, so that new approaches to improving healing may arise through better understanding of mechanisms regulating apoptosis (Clark and Henson, 1996; Greenhalgh, 1998).

Three major overlapping phases of wound healing are described; namely the inflammatory, proliferative and maturation phases. The inflammatory phase represents an immediate response to injury and involves blood clot formation and removal of any contaminating bacteria by inflammatory cells. The proliferative phase involves cellular migration into the fibrinous matrix of the clot and extracellular matrix (ECM) deposition to form highly vascular reparative granulation tissue. This is followed by the maturation phase, which involves removal of excess cells by apoptosis and is usually accompanied by increased collagen deposition and wound contraction to form the final scar (Darby et al., 1990; Desmouliere et al., 1995) (for reviews see (Clark and Henson, 1996; Linares, 1996; Martin, 1997; Mutsaers et al., 1997; Greenhalgh, 1998)). In some instances, reparative granulation tissue is populated by either bony progenitor cells (Bruder et al., 1994), or lipoblasts (Xaymardan et al., 2002), in which case bone or adipose tissues are formed respectively during wound maturation.
I.2.1. The Inflammatory Phase of Wound Healing

The inflammatory phase begins immediately after initial tissue damage. Initial blood loss is reduced by vasoconstriction of surrounding blood vessels and platelet aggregation (for reviews see (Clark and Henson, 1996; Linares, 1996; Mutsaers et al., 1997; Esmon, 2005)). Activation of the clotting cascade results in formation of a fibrin clot which fills the wound space, and together with adhesive glycoproteins such as fibronectin and thrombospondin (Bonnefoy and Legrand, 2000), provide a provisional matrix for the recruitment of inflammatory cells, fibroblasts and EC. Degranulating platelets release many cytokines and growth factors, such as Platelet Derived Growth Factor (PDGF), which leads to the recruitment of neutrophils and monocytes into the wound from the surrounding blood vessels (Midwood et al., 2004; Esmon, 2005). Mast cells also play a role in initial recruitment of neutrophils into the wound, but do not appear to have a major role later during tissue repair (Linares, 1996; Egozi et al., 2003). Neutrophils are the most abundant leukocyte cell type within the first 12 to 72 hours of tissue repair, and are central to the removal of invading microorganisms, although these cells are later replaced by macrophages which eventually predominate (for reviews see (Clark and Henson, 1996; Linares, 1996; Martin, 1997; Mutsaers et al., 1997; Greenhalgh, 1998; Barrick et al., 1999)).

Macrophages are essential for wound repair and act to phagocytose both cellular and matrix debris. In an experimental wound healing model in guinea pigs, macrophage levels are maximal after three days following wounding, and are the most prevalent cell type until around the fifth day. Depletion of macrophages in this model using anti-macrophage serum impairs wound healing, indicating a major role for these cells
General Introduction
Chapter I Page 4

(Leibovich and Ross, 1975). Macrophages release inflammatory cytokines such as Transforming Growth Factor β (TGF-β); PDGF; Tumour Necrosis Factor α (TNF-α); Interleukin-1 and Epidermal Growth Factor. These cytokines act as chemoattractants for many cells such as fibroblasts, epithelial cells and EC, and also stimulate angiogenesis and the formation of reparative granulation tissue (Thakral et al., 1979; Hunt et al., 1984; Linares, 1996).

Once wounds have been sealed by blood clot, the provisional fibrin matrix is removed and remodelled by proteinases (Schafer et al., 1994; Barrick et al., 1999). The main proteinase involved in fibrin degradation is plasmin, which is formed by cleavage of plasminogen by either tissue type-plasminogen activator (t-PA) or urokinase-type plasminogen activator (u-PA), while these activators are produced by a variety of cell types (Vassalli et al., 1991). A specific inhibitor of plasminogen activation, plasminogen activator inhibitor-1 binds to the ECM and limits matrix degradation (Clark, 2001). Other proteinases released by leukocytes are involved in this process, including elastase and cathepsin G, although plasmin is more effective in clot dissolution (Francis and Marder, 1986). Inadequate removal of the fibrin clot may impair the wound healing process (Clark, 2001).

Re-epithelialisation occurs by the migration of epithelial cells from the wound margins to cover the wound surface (for reviews see (Clark and Henson, 1996; Linares, 1996; Martin, 1997)). Wound keratinocytes express integrins for some ECM proteins, which allows them to migrate across the wound (Clark, 2001). The wound surface is initially covered by a monolayer of keratinocytes and new epidermis is re-established by proliferation and differentiation of the epithelial cells (Martin, 1997).
L2.ii. The Proliferative Phase of Wound Healing

Reparative granulation tissue develops from the connective tissue surrounding the wound. The main components are fibroblasts, small blood vessels and inflammatory cells in newly deposited ECM. Cytokines, synthesised by macrophages, attract fibroblasts and EC into the wound (Clark and Henson, 1996; Xu and Clark, 1996; Greenhalgh, 1998; Clark, 2001; Chavakis and Dimmeler, 2002). Fibronectin is important in aiding migration of cells into the wound, as demonstrated by the effect of fibronectin removal in decreasing the number of migrating cells into the matrix (Greiling and Clark, 1997). Fibronectin also has a role in modulating EC shape and promoting endothelial survival during angiogenesis (Ingber, 1990). The ECM acts to provide support for migrating and proliferating cells. Fibroblasts synthesise and deposit large amounts of collagen as well as glycosaminoglycans and proteoglycans, with the effect of increasing the tensile strength of wounds. The main type of collagen synthesised by fibroblasts in the early stage of wound repair is type III collagen, while with time, type I collagen becomes predominant (Linares, 1996; Mutsaers et al., 1997; Stadelmann et al., 1998; Latha et al., 1999).

Because of the initial absence of blood vessels, early wound tissue is potentially hypoxic, while fibroblasts and other cells have a requirement for oxygen. Macrophages can tolerate low oxygen levels and release cytokines to stimulate the growth of new blood vessels, and this angiogenic response corrects the initial hypoxia (Clark and Henson, 1996; Linares, 1996; Greenhalgh, 1998).
General Introduction  
Chapter I Page 6

I.2.ii.a. Angiogenesis

Angiogenesis is essential for development, reproduction and wound healing and involves the proliferation and migration of EC from pre-existing vessels to form new microvessels (Hunt et al., 1984; Folkman and Shing, 1992). Macrophages are a major source of soluble angiogenic factors which include: Vascular Endothelial Cell Growth Factor (VEGF); angiopoietin-1; PDGF (Chavakis and Dimmeler, 2002; Li et al., 2003); Fibroblast Growth Factor-2 (FGF-2); and TGF-β (Pepper et al., 1990). VEGF is a key mediator of angiogenesis and there are at least six isoforms of the protein, while it exerts its angiogenic effect by binding to tyrosine kinase dependant receptors such as VEGFR1 and VEGFR2 (Chavakis and Dimmeler, 2002; Li et al., 2003). VEGF induces EC migration and proliferation, while this protein also increases vascular permeability (Kevil et al., 1998; Chang et al., 2000; Chavakis and Dimmeler, 2002). Consistent with the angiogenic role of VEGF is that VEGF is also a survival factor for EC, and this effect is mediated by expression of the anti-apoptotic proteins Bcl-2 and survivin, as well as activation of the PI3K/Akt signalling pathway (Gerber et al., 1998a; Gerber et al., 1998b). Angiopoietin-1 is an EC specific growth factor that mediates its effect through Tie-2, a tyrosine kinase dependant receptor. Angiopoietin-1 also mediates EC survival through activation of the PI3K/Akt signalling pathway (Fujikawa et al., 1999; Kwak et al., 1999; Harfouche et al., 2002), and has a role in pericyte recruitment to stabilise the new vasculature (Li et al., 2003). FGF-2 also protects EC from apoptosis and promotes both EC proliferation and differentiation (Karsan et al., 1997; Satake et al., 1998; Kanda et al., 1999).
During angiogenesis, EC morphology changes so that the usual tubular structures are replaced with flat and elongated forms which eventually revert to a more tubular shape once the new capillaries are established (Folkman and Shing, 1992). Proliferation and migration of EC requires reorganisation of inter-endothelial cell junction proteins to permit EC detachment from neighbouring cells, and migration into the wound, while tyrosine phosphorylation of VE-cadherin is an example of this (Dejana, 1996). Degradation of the basement membrane and surrounding connective tissues is also important for angiogenesis, and this is, at least in part, mediated by matrix metalloproteinases secreted by EC which degrade basement membrane collagen to allow migration and proliferation (Kalebic et al., 1983; Bischoff, 1997; Jackson and Minh, 1997).

Adhesion of EC to the ECM plays an important role in permitting migration of cells into wounds. Apart from acting as a structural support for migrating EC, the ECM can also affect EC shape during angiogenesis (Ingber, 1990). Specific adhesion molecules, such as integrins, mediate adhesion between cells and ECM proteins (Mutsaers et al., 1997; Ruoslahti and Engvall, 1997). Integrins are heterodimer transmembrane proteins consisting of $\alpha$- and $\beta$- chains, and are the major cell surface receptors for the ECM (Ruoslahti and Engvall, 1997; Li et al., 2003). The $\alpha\nu\beta3$ integrin is important during angiogenesis, and is detected on the tips of growing but not quiescent blood vessels (Brooks et al., 1994; Clark et al., 1996; Feng et al., 1999). $\alpha\nu\beta3$ can be upregulated by VEGF (Spyridopoulos et al., 1997; Gupta and Qui, 2003; Li et al., 2003) and is the EC receptor for ECM proteins such as fibrin, fibronectin and vitronectin (Brooks et al., 1994; Clark et al., 1996).
Migration and proliferation of EC into wounds is followed by alignment of cells into new vessels and lumen formation. EC apoptosis can contribute to both lumen and network formation during angiogenesis (Peters et al., 2002; Segura et al., 2002). After lumen formation, new vessels are stabilised by pericyte recruitment and deposition of basement membrane material (Li et al., 2003). Fibroblasts also contribute to structural support and stabilisation of new microvessels by depositing ECM proteins (Villaschi and Nicosia, 1994).

During inflammation capillaries become more permeable so that proteins escape into the extravascular compartment to increase osmotic pressure and mediate oedema formation, while a similar process occurs early in wound healing due to the high permeability of newly formed vessels (Florey, 1962; Nyman et al., 1971). Increased vascular permeability is stimulated by many agents such as histamine, serotonin (Majno and Palade, 1961), bradykinin (Saulpaw and Joyner, 1997), amyloid β peptide (Blanc et al., 1997) and platelet activating factor (Klabunde and Anderson, 2002). This seems largely due to reorganisation of the cytoskeleton and the appearance of gaps between EC (Dejana et al., 1995). Plasma leakage is associated with the distribution of interendothelial gaps in the microvasculature (McDonald et al., 1999), while death of EC also enhances vascular permeability (Lin et al., 1990). Newly formed vessels are especially leaky and it is interesting that VEGF acts to increase blood flow, vasodilation and vascular permeability (Bates and Harper, 2002) through rearrangement of EC junction proteins via the activation of the MAPK pathway (Kevil et al., 1998). This contrasts with the action of angiopoietin-1, which causes reduced vascular leakage.
(Thurston et al., 1999; Thurston et al., 2000; Thurston, 2002), suggesting that control of vascular leakage during wound healing represents a balance between these signals.

I.2.ii.b. Proteinases in Wounds

Proteinases are responsible for removal of the provisional matrix during wound healing, and also remodel ECM while the migration of cells such as fibroblasts and EC is also dependent on proteinase activity. The balance between proteinases and their inhibitors in wound healing contributes to control of matrix degradation and synthesis. All four of the major classes of proteinases are important in wound healing, being the cysteine, aspartic, serine and matrix metalloproteinases. Cysteine and aspartic proteinases have maximal activities in conditions of low pH, and often degrade intracellular proteins within lysosomes. Serine and matrix metalloproteinases, on the other hand, have optimal activity at neutral pH, and play a major role in the degradation of ECM proteins (Barrick et al., 1999; Yager and Nwomeh, 1999; Gerber et al., 2001).

Serine proteinases, such as elastase and cathepsin G, are secreted by leukocytes and degrade fibronectin, laminin, elastin and proteoglycans. They also cleave some plasma proteins, such as clotting factors and complement proteins as well as some cytokines, such as TNF-α and TGF-β (Barrick et al., 1999). Plasminogen is converted to active plasmin by either t-PA or u-PA, while the main role of plasmin appears to be fibrin degradation. This activity is inhibited by plasminogen activator inhibitors, so that the balance between plasminogen activators and inhibitors determines the removal or otherwise of fibrin (Vassalli et al., 1991; Barrick et al., 1999).
General Introduction
Chapter I Page 10

Over 20 different matrix metalloproteinases that degrade ECM components have been identified, and these share a common domain structure. Matrix metalloproteinases are secreted by leukocytes and connective tissue cells as pro-enzymes, and require proteolytic cleavage for activation while these enzymes are specifically inhibited by tissue inhibitors of metalloproteinases (Baker and Leaper, 2000; Visse and Nagase, 2003).

I.2.iii. The Maturation Phase of Wound Healing

In the maturation phase, the balance between synthesis and degradation of collagen is shifted towards deposition. Type I collagen becomes predominant, while this together with collagen remodelling, increases wound strength (for reviews see (Clark and Henson, 1996; Mutsaers et al., 1997; Greenhalgh, 1998)). Some granulation tissue fibroblasts differentiate to form myofibroblasts, which have features of both fibroblasts and smooth muscle cells (SMC). The fibroblast features noted as expressed by myofibroblasts are the presence of large amounts of rough endoplasmic reticulum, as well as the prominence of mitochondria (Ryan et al., 1974; Gabbiani and Badonnel, 1976; Gabbiani et al., 1976; Gabbiani et al., 1978; Gabbiani, 1979; Skalli et al., 1989; Tomasek et al., 2002). In addition, similar to SMC, myofibroblasts have longitudinal bundles of microfilaments and also express α-smooth muscle actin (Darby et al., 1990; Desmouliere, 1995; Lorena et al., 2002). Myofibroblasts are interconnected by gap junctions and bind the ECM through the fibronexus (Singer et al., 1984; Eyden et al., 1992; Eyden, 1993; Eyden et al., 1994; Desmouliere, 1995; Eyden, 2001; Eyden, 2003). There is rapid wound contraction due to the contractile activity of myofibroblasts...
(Charlton et al., 1961; Higton and James, 1964; Gabbiani and Badonnel, 1976; Gabbiani, 1979; Desmouliere, 1995; Tomasek et al., 2002), which is accompanied by decreased cellularity and vascularity of wounds through apoptosis (Darby et al., 1990; Desmouliere et al., 1995). The resulting dense fibrous tissue is defined as scar tissue and there is progressive maturation and remodelling of scar tissue over time. Excessive scarring may occur if mechanisms involved in normal scar maturation, such as apoptosis or collagenolysis do not occur or are reduced (Linares, 1996; Mutsaers et al., 1997). Hypertrophic scars can be treated by application of pressure, which induces scar remodelling and apoptosis (Costa et al., 1999), while covering the wound with a skin flap induces massive apoptosis and enhances healing (Garbin et al., 1996; Darby et al., 2002).

In addition, chronic wounds which fail to heal have increased inflammatory response and persistent cellularity (Yager and Nwomeh, 1999). There are also elevated levels of proteinases, such as MMP-2 and -9 associated with chronic wounds (Wysocki, 1992; Wysocki et al., 1993; Wysocki, 1996; Chen et al., 1999; Yager and Nwomeh, 1999), with degradation of some proteins in chronic wounds also observed (Grinnell et al., 1992; Trengove et al., 1999; Lauer et al., 2000).

I.3. Apoptosis

I.3.1. Mechanisms for Cell Death

There are two main mechanism through which cells may die: apoptosis or necrosis. Apoptosis, also known as programmed cell death, is a highly regulated process
for the removal of cells that are no longer functioning properly or are excessive in number, so that apoptosis plays a fundamental role in the growth and development of tissues (Kerr et al., 1972). In contrast, necrosis is cell death due to cellular damage caused by physical, chemical or osmotic injury with loss of homeostatic activity. Morphologically, necrosis is characterised by irreversible swelling of the cytoplasm and organelles, with membrane disruption and a consequent inability to regulate intracellular osmotic pressure to cause rupture and release of cellular contents. This may stimulate a local inflammatory response, which if severe, can damage surrounding cells and tissues to result in further necrosis. Apoptosis does not typically induce inflammation, as cytoplasmic contents are preserved within intact plasma membrane enclosed particles, known as apoptotic bodies. Generally, groups of cells are involved in necrosis, while in apoptosis, individual cells are affected. A further difference between these two modes of cell death is that cellular adhesion is lost early in apoptosis, while this is a late event in necrosis (Gerschenson and Rotello, 1992; Kroemer et al., 1995; Fadeel and Orrenius, 2005).

I.3.ii. Characteristics of Apoptosis

Although apoptosis occurs in many cell types and is initiated by a wide variety of physiological and pathological stimuli, there are common and characteristic morphological, ultrastructural and biochemical features (Saraste and Pulkki, 2000; Debatin, 2004). The onset of apoptosis is characterised by cellular shrinkage as well as condensation and fragmentation of the nuclear chromatin (Falcieri et al., 1994). Also, there is cellular detachment followed by fragmentation of cells into apoptotic bodies
which contain compacted and preserved cellular components and intact organelles (Kerr et al., 1972; Saraste and Pulkki, 2000). Apoptotic bodies are rapidly phagocytosed by surrounding cells, while exposure of phosphatidylserine on the outer surface of apoptotic bodies allows recognition by phagocytic cells (Orrenius et al., 2003). Secondary necrosis may occur if apoptotic bodies are not removed by phagocytosis, and this commonly occurs in cultures of isolated cells but is not usually seen in vivo (Saraste and Pulkki, 2000).

Apoptosis is also characterised by internucleosomal DNA fragmentation, which occurs early in this process and is the most readily identifiable biochemical feature. This differs markedly from the random DNA fragmentation seen in necrosis, so that internucleosomal DNA fragmentation is widely accepted as a specific marker for apoptosis. During apoptosis, double stranded DNA is cleaved by a Ca\(^{2+}\)-dependant endonuclease at internucleosomal domains to produce DNA fragments of 180 to 200 base pairs (bp) in length (Wyllie et al., 1984; Arends et al., 1990; Gershenson and Rotello, 1992; Kroemer et al., 1995). The specific endonuclease responsible for DNA fragmentation is caspase-activated DNase (CAD), which becomes active after cleavage of its inhibitor, ICAD by caspase-3 (Nagata, 2000).

Many methods are commonly used to detect apoptosis in cultured cells and tissues. Transmission electron microscopy is widely used to demonstrate the characteristic morphological and ultrastructural changes (Falcieri et al., 1994; Zoellner et al., 1996a; Xu et al., 2005). Internucleosomal DNA fragmentation is usually detected by DNA gel electrophoresis, and is recognised by the presence of a 180 bp "ladder", contrasting with the smear seen in material from necrotic cells due to random DNA
fragmentation (Gerschenson and Rotello, 1992; Kroemer et al., 1995; Zoellner et al., 1996b; Emmanuel et al., 2002). Fragmented DNA can also be visualised by light microscopy using the terminal transferase mediated DNA nick end labelling (TUNEL) assay (Saraste and Pulkki, 2000), while fluorescence activated cell scanning (FACS) analysis detects reduced levels of DNA in apoptotic populations of cells (Darzynkiewicz et al., 1992; Falcieri et al., 1994; Emmanuel et al., 2002).

I.3.iii. Mechanisms of Apoptosis

Apoptosis is initiated by two main pathways being the extrinsic, or receptor mediated pathways, and the intrinsic, or mitochondria dependant pathways.

I.3.iii.a. Receptor Mediated Apoptosis

Receptor mediated apoptosis involves the activation of receptors bearing so called “death domains” by ligand binding. These death receptors are part of the TNF receptor family and have TNFR1 and Fas as important members. Activation of death receptors results in receptor trimerisation and recruitment of adaptor proteins, such as FADD or TRADD, as well as pro-caspases-8 or 10, to form the death-inducing signalling complex (DISC). This results in the activation of caspases-8 or -10 which then initiates the caspase cascade to effect degradation of intracellular components and cell death (Dragovich et al., 1998; Bratton et al., 2000).
I.3.iii.b. Caspases

Caspases were originally implicated in apoptosis following recognition of sequence homology between mammalian interleukin 1β-converting enzyme (caspase-1), and CED-3 involved in apoptosis in the nematode Caenorhabditis elegans (Nunez et al., 1998; Saraste and Pulkki, 2000; Zimmermann et al., 2001; Zimmermann and Green, 2001). Caspases are cysteine proteases that cleave their substrate at aspartate residues. At least 11 mammalian caspases have been identified and they are all formed as pro-enzymes which must undergo proteolytic cleavage for activation (Zimmermann et al., 2001).

Initiator, or upstream caspases such as caspases -2, -8, -9 and -10 are thought to undergo autocatalytic activation, while effector, or downstream caspases such as caspases -3, -6 and -7 require activation by initiator caspases (Saraste and Pulkki, 2000). Effector caspases cleave vital cellular proteins and this is central to the apoptotic degradation of the cell and production of the characteristic morphological and biochemical features of apoptosis (Dragovich et al., 1998; Zimmermann et al., 2001; Zimmermann and Green, 2001).

Cells contain caspase inhibitors, known as inhibitors of apoptosis proteins (IAP), of which XIAP and survivin are prominent members. Caspases can also be inhibited by artificial inhibitors, such as zVAD-FMK and these inhibitors are widely used for in vitro studies with cultured cells (Zimmermann et al., 2001; Zimmermann and Green, 2001; Jiang and Wang, 2004).
I.3.iii.c. The Bcl-2 Family of Proteins

The intrinsic, mitochondria dependant pathway of apoptosis is generally activated following withdrawal of survival factors, and is primarily controlled by the Bcl-2 family of proteins which contains both pro-and anti-apoptotic members. The overall amino acid sequence homology between members is low, however, sequence homology within four conserved regions, known as Bcl-2 homology (BH) domains, is shared between members. Anti-apoptotic members of the Bcl-2 family contain all four BH domains and include Bcl-2, Bcl-xL, Mcl-2 and A1. Some pro-apoptotic members of the Bcl-2 family, including Bax, Bak and Bok, contain BH domains 1 to 3 while other pro-apoptotic members, such as Bid, Bad and Bik, only share homology with the BH3 domain. Some members of the Bcl-2 family are membrane bound, whilst others such as Bax, are cytosolic but are translocated to the mitochondrial membrane upon activation (Antonsson and Martinou, 2000; Tsujimoto, 2003; Fadeel and Orrenius, 2005).

Bax has a membrane channel forming ability and upon activation, Bax homodimerises to form a mitochondrial membrane permeability transition pore to release cytochrome C into the cytoplasm. Bcl-2 can inhibit Bax by either inhibiting the interaction of Bax with the mitochondrial membrane or alternatively directly inhibiting Bax by forming stable heterodimers (Dragovich et al., 1998; Antonsson and Martinou, 2000; Tsujimoto, 2003; Debatin, 2004). Other pro-apoptotic proteins, such as Bad, exert their pro-apoptotic function by binding and inactivating anti-apoptotic members, such as Bcl-2 or Bcl-xL (Dragovich et al., 1998). Bad can be inactivated by phosphorylation following growth factor activation of the PI3K/Akt survival signalling pathway (Franke et al., 1997; Franke et al., 2003; Osaki et al., 2004; Vara et al., 2004).
Release of cytochrome C by mitochondria allows formation of the apoptosome, which is comprised of cytochrome C, Apaf-1 and procaspase-9. This activates caspase-9, which subsequently activates effector caspases to induce apoptosis. Crosstalk between the extrinsic and intrinsic pathways of apoptosis occurs by activation of caspase-8 by DISC to then activate Bid, which results in release of mitochondrial cytochrome C (Sprick and Walczak, 2004).

Apoptosis can be initiated in ways other than the extrinsic and intrinsic pathways. For example, intracellular calcium levels can initiate apoptosis by activating calpains and promoting externalisation of phosphatidylserine on the plasma membrane for recognition by phagocytic cells. Calcium is also essential for CAD activity during internucleosomal DNA fragmentation (Choy et al., 2001; Orrenius et al., 2003). Ceramide, which is a product of sphingomyelin metabolism, can also activate caspases to initiate apoptosis, while over expression of Bcl-2 inhibits this (Zhang et al., 1996; Harada-Shiba et al., 1998). p53 is a tumour suppressor protein and has roles in cell cycle regulation as well as apoptosis, while the transcription factor NFκB upregulates anti-apoptotic proteins and is associated with survival activities (Vermeulen et al., 2003).

I4. Apoptosis in Endothelial Cells

EC form the inner lining of blood vessels and establish a critical barrier controlling exchange of nutrients and wastes between blood and interstitial fluid. The EC monolayer controls vascular permeability to both plasma proteins and circulating cells (Florey, 1962; Del Vecchio et al., 1987; Choy et al., 2001). During normal
development, reproduction and wound healing, the formation of new blood vessels and vascular regression represents a balance between EC proliferation and apoptosis (Mallat and Tedgui, 2000).

Unique to apoptosis in endothelium is the formation of vesicle-like canalicular structures. These structures are confluent with the plasma membrane and form an extensive interconnecting network throughout the apoptotic cell. Apoptotic EC still display the morphological features characteristic of apoptosis, such as cellular detachment, nuclear condensation and maintained organellar integrity within apoptotic bodies. However, apoptotic EC with these canalicular structures have increased mechanical fragility and this facilitates the fragmentation of apoptotic cells into small particles and thought to reduce the micro-embolic potential of apoptotic EC. One consequence of this “canalicular fragmentation” is that unlike apoptosis in other cell types, there is some spillage of cytoplasmic contents into the extracellular environment during endothelial apoptosis (Zoellner et al., 1996a; Xu et al., 2005).

EC apoptosis and survival is controlled by various physiological and pathological stimuli such as cellular and matrix adhesion, shear stress and chemical plasma factors (Mallat and Tedgui, 2000; Choy et al., 2001) Zoellner, 1996b #51; Chavakis, 2002 #631; Dimmeler, 1996 #12; Dimmeler, 2000 #238; Kaiser, 1997 #10; Freyberg, 2001 #823).

I.4.i. The Anti-apoptotic Activity of Adhesion for Endothelium

Intercellular EC contacts, as well as matrix adhesion inhibit EC apoptosis. This seems biologically relevant, as EC not integrated into an endothelial monolayer or
detached from the vessel wall are non-functional, and might be expected to become 
apoptotic (Pollman et al., 1999). This is supported by observations in vitro, where EC 
cultured under conditions that prevent cellular adhesion and or cell spreading, fail to 
grow, lose viability and become apoptotic (Meredith et al., 1993; Re et al., 1994; 
Zoellner et al., 1996b; Pollman et al., 1999). Contrasting with this is that upregulation of 
integrins and inter-endothelial junction proteins, such as VE cadherin, facilitates cell to 
cell and cell to ECM interactions and promotes EC survival (Dejana, 1996; Mallat and 
Teguid, 2000). Chemical plasma factors are unable to inhibit apoptosis in EC deprived 
of matrix adhesion (Meredith et al., 1993; Zoellner et al., 1996b), indicating that EC 
require the combined effects of both chemical plasma factors and matrix adhesion to 
circumvent apoptosis.

Interestingly, human umbilical vein endothelial cells (HUVEC) deprived of 
adhesion may form clusters of viable EC in which apoptosis is delayed. Cells in the 
peripheral zones of these clusters flatten out and develop features characteristic of 
endothelial monolayers, and this is followed by apoptosis of EC in the cluster centers 
together with lumen formation. Nonetheless, such clustered non-adherent HUVEC do 
not retain viability for long periods of time, indicating that inter-EC adhesion is not 
sufficient to maintain cell survival, and that matrix adhesion is also required 
(Xaymardan and Zoellner, 2006).

I.4.ii. The Anti-apoptotic Activity of Shear Stress for Endothelium

Blood flow generates several important mechanical forces which act on vascular 
EC, including shear stress, pressure and tension (Takahashi et al., 1997). Variations in
General Introduction
Chapter I Page 20

blood flow may play a role in vessel growth and regression as well as in the focal
development of atherosclerotic lesions (Mallat and Tedgui, 2000).

Shear stress stimulates the production of t-PA (Diamond et al., 1989), nitric
oxide (Dimmeler and Zeiher, 1997; Hermann et al., 1997; Lopez-Ferre et al., 1998;
Shen et al., 1998; Dimmeler et al., 1999) and FGF-2 (Malek et al., 1993; Gloe et al.,
2002) by EC. Laminar shear stress also upregulates integrin expression and promotes EC
adhesion (Urbich et al., 2000).

Laminar shear stress reduces EC turnover in vivo and inhibits EC apoptosis
induced by exposed to pro-apoptotic agents such as TNF-α, or by withdrawal of survival
factors such as serum or FGF-2 (Dimmeler et al., 1996; Kaiser et al., 1997). Laminar
shear stress mediates its anti-apoptotic effect upon EC by activation of cellular survival
pathways including the ERK 1/2 MAP kinase and PI3K/Akt pathways (Dimmeler et al.,
1998; Traub and Berk, 1998; Yoshizumi et al., 2003).

In static conditions, EC adopt a rounded, cobblestone morphology, while EC
exposed to shear stress become elongated and align themselves in the direction of flow
(Kaiser et al., 1997; Masuda et al., 2003). There is a baseline rate of apoptosis for EC
cultured in the absence of shear stress (Kaiser et al., 1997), although since EC can be
cultured without flow, it has been argued that chemical plasma factors have a more
potent and predominant role compared with shear stress in regulating EC apoptosis
(Zoellner et al., 1996b).
I.4.3. Chemical Factors Controlling Endothelial Cell Apoptosis

HUVEC become apoptotic when deprived of serum (Araki et al., 1990b), so that serum deprivation of cultured HUVEC is commonly used as a model to study EC apoptosis (Meredith et al., 1993; Hase et al., 1994; Zoellner et al., 1996b; Relou et al., 1998).

Some pro-apoptotic stimuli for EC include oxidized low density lipoprotein (Harada-Shiba et al., 1998), glycated high density lipoprotein (Matsunaga et al., 2001), lipopolysaccharide (Haimovitz-Friedman et al., 1997; Bannerman et al., 1998; Bannerman and Goldblum, 2003), tissue factor pathway inhibitor (Hamuro et al., 1998), extracellular ATP (von Albertini et al., 1998), haemorrhagic snake venom (Araki et al., 1993) and high glucose concentrations (Risso et al., 2001). Both TNF-α and TGF-β have roles in angiogenesis and inflammation but also induce EC apoptosis in vitro (Robaye et al., 1991; Tsukada et al., 1995), while these cytokines are synergistic for induction of HUVEC apoptosis (Emmanuel et al., 2002).

FGF-2 inhibits EC apoptosis in serum free conditions (Araki et al., 1990a; Meredith et al., 1993; Hase et al., 1994; Karsan et al., 1997) through a protein kinase C dependant mechanism (Araki et al., 1990b) while this growth factor also enhances Bcl-2 expression in HUVEC and activates the PI3K/Akt pathway (Karsan et al., 1997; Gu et al., 2004). VEGF and angiopoietin-1 are further anti-apoptotic factors for EC, through activation of the PI3K/Akt pathway and upregulation of the anti-apoptotic proteins Bcl-2 and survivin. (Spyridopoulos et al., 1997; Watanabe and Dvorak, 1997; Gerber et al., 1998a; Gerber et al., 1998b; Fujikawa et al., 1999; Kwak et al., 1999; Harfouche et al., 2002). Supporting the need to regulate apoptosis during angiogenesis and as described
above (I.2.ii.a), VEGF, FGF-2 and angiopoetin-1 are also important angiogenic molecules.

Other inhibitors of EC apoptosis include sphingosine-1 phosphate (Kwon et al., 2001), hepatocyte growth factor (Yamamoto et al., 2001), ouabain (Trevisi et al., 2004), 17β-estradiol (Alvarez et al., 1997) and serum albumin (Zoellner et al., 1996b; Zoellner et al., 1999). Please note that a more detailed description of the anti-apoptotic activity of serum albumin will be provided in Chapter 4.

I.5. Questions Arising From the Literature and Addressed in This Thesis

As discussed above (I.2), during wound healing highly vascular reparative granulation tissue forms and matures to scar tissue which contains very few vessels. Excess blood vessels are removed through EC apoptosis (Darby et al., 1990; Desmouliere et al., 1995) while microvascular remodelling during the maturation phase of wound healing reflects a mechanism for optimising microvascular form. Since the main role of blood vessels is to deliver blood to the tissues, it has been suggested that vessels that are well perfused survive, while those that are poorly perfused are removed by apoptosis (Zoellner et al., 1996b). This is reflected by degeneration of poorly perfused microvessels during vascular remodelling in vivo (Sandison, 1928; Meeson et al., 1996), while occlusion of microvessels by EC detachment results in apoptosis of affected vessels with reduced or poor blood flow during microvascular remodelling in the corpus luteum and corneal capillary regression (Ausprunk et al., 1978; Azmi and O'Shea, 1984; Modlich et al., 1996). The default status of endothelium appears to be apoptosis so that poorly perfused vessels deprived of functional signals, including
chemical plasma factors and laminar shear stress, become apoptotic (Zoellner et al., 1996b).

If it is assumed that intravascular functional signals are required for EC survival, it is difficult to understand how an apparently excessive number of vessels could be established early in wound healing before vascular regression and wound maturation. It seems likely that extravascular factors independent of blood flow account for this inconsistency. This thesis will in part address this possibility, with an initial investigation of the possible contribution of proteinase activated plasma factors entering the extravascular compartment via leaky newly formed vessels. As will be seen in Chapter 3, serum albumin was found to be significantly fragmented in healing wounds, and this correlated with tissue vascularity.

Serum albumin has a potent anti-apoptotic activity for EC both in vitro and in tissue explant cultures (Zoellner et al., 1996b; Zoellner et al., 1999), and increased anti-apoptotic activity by fragmented albumin may account for the high vascularity of early reparative granulation tissue. Much of the remainder of this thesis addresses mechanisms through which albumin may mediate its anti-apoptotic activity for endothelium. Separately, most physiological processes involve negative feedback mechanisms which reverse the processes that activate them, and so drive systems to a homeostatic norm. Although many pro- and anti-apoptotic signals are identified for EC, no clear negative feedback mechanisms are established for EC apoptosis and this thesis addresses this gap in the literature with a study of FGF-2 release by apoptotic EC.
Chapter II

Establishment of an Experimental Scarring Wound Healing Model in Mice
II.1. Introduction

During wound healing, the wound space is filled with a fibrinous blood clot which provides a matrix for cells to migrate upon and forms highly vascular reparative granulation tissue, comprised primarily of fibroblasts and EC (for reviews see (Clark and Henson, 1996; Linares, 1996; Mutsaers et al., 1997; Greenhalgh, 1998)). Over time, this tissue usually matures to scar tissue and undergoes extensive remodelling with deposition and maturation of collagen fibres as well as a significant reduction in vascularity and overall cellularity by apoptosis of both endothelium and fibroblasts (Darby et al., 1990; Desmouliere et al., 1995). Please note that this process is described in more detail in Chapter I.2.

Several in vivo models have been developed to study wound healing. For example, full thickness skin wounds have been used to study cutaneous healing (Higton and James, 1964; Cuthbertson and Tilstone, 1967; Gabbiani et al., 1978; Rigal et al., 1991; Chen et al., 1999; Kane and Greenhalgh, 2000), while angiogenesis during wound healing has been studied using dorsal skinfold chambers, cranial windows (Dellian et al., 1996) and rabbit ear chambers (Sandison, 1928). In addition, implanted devices such as hollow cylinders or sponges allow study of infiltrating cells during formation of reparative granulation tissue (Schilling et al., 1959). There has been extensive use of sponge implantation because granulation tissue contained within such implants is readily collected at increasing time points, while both wound tissues and fluid are easily studied in sponges (Bailey, 1988). The structure, type of material and also position of implants can affect the inflammatory response as well as the formation of granulation tissue.
(Bailey, 1988; Pajulo et al., 1996; Xaymardan et al., 2002). Sponges have been made out of different materials, including collagen (Honma and Hamasaki, 1998), cellulose (Pajulo et al., 1996) and polyvinyl (Schilling et al., 1959; Woessner and Boucek, 1961a, b; Bailey, 1988). Inflammatory stimuli have also been added to sponge implants prior to implantation to modify the host response (Bailey, 1988).

Sponge implants are rapidly infiltrated with cells such as fibroblasts, monocytes and lymphocytes (Bailey, 1988), and a fibrous capsule also often forms around sponges by the end of the first week of implantation, while new reparative granulation tissue forms within implants (Schilling et al., 1959; Woessner and Boucek, 1961a, b). Over time, production of insoluble proteins such as collagen increases (Woessner and Boucek, 1961a, b), while multinucleated foreign body giant cells are also seen associated with the foreign material (Schilling et al., 1959). These cells appear by the fusion of circulating monocytes after stimulation with interleukin-4 (Postlethwaite et al., 1982; McNally and Anderson, 1995, 2003), and are a common feature of granulomas. Multinucleated foreign body giant cells form a barrier separating foreign material from the surrounding tissues and are characteristic of the foreign body response, representing poor host tolerance to the implant material (Smetana, 1987). Derived from and in some ways similar to monocytes, giant cells are phagocytic, although this is decreased relative to monocytes, seemingly due to a reduction in surface receptors (Papadimitriou et al., 1975).

Despite the presence of these giant cells, normal wound healing occurs within sponge implants with continued production of reparative granulation tissue, collagen deposition (Schilling et al., 1959), and increased tissue vascularity (Honma and
Hamasaki, 1998). Sponge implants undergo compression and contraction as the granulation tissue matures to scar tissue due to contraction of myofibroblasts, while this is accompanied by a reduction in vascularity and cellularity by fibroblast and EC apoptosis (Darby et al., 1990; Desmouliere, 1995; Desmouliere et al., 1995; Honma and Hamasaki, 1998).

The host response differs however in each wound healing model. For example, cutaneous wounds differ from those produced by sponge implantation in that the epithelium must proliferate to cover cutaneous wounds, while interestingly, TGF-β expression reduces scarring in cutaneous wounds and in sponge wound healing models, TGF-β has the opposite effect and increases both cellular infiltration and ECM deposition (Shah et al., 1999). Granulation tissue grows into stainless steel wire mesh cylinder implants in a similar manner to events in sponge implants. However, despite similar polysaccharide levels, there is little if any foreign body response in stainless steel implants (Schilling et al., 1959).

Earlier work in this laboratory demonstrated an unusual pattern of healing in which adipose tissue rather than scar tissue appeared during maturation of reparative granulation tissue. This unusual response was seen only when nylon mesh or other space occupying implants were placed into the muscle tissue of mice (Xaymardan et al., 2002). Vascularity in these implants was maximal at 3 to 4 weeks post-implantation, and as wounds matured, there was a reduction in vascularity by 12 weeks, similar to that reported for other model systems and illustrated in Figure 2.1. Nonetheless, during wound maturation, lipoblasts appeared and matured to form adipose tissue, rather than the scar tissue reported in other wound healing models. This defined a new pattern of
wound maturation, and in recognition of the appearance of mature fat tissues was termed “adipogenic healing” (Xaymardan et al., 2002).

Although adipogenic healing displayed vascular changes characteristic of healing by scarring described by others, for the purposes of work described in this thesis, it became important to establish a wound healing model system in this laboratory which produced scar tissue, similar to model systems described by others. This Chapter describes work done in establishing a scarring model system. For the purpose of this thesis, tissues from this model are termed “scarring wounds” to differentiate them from the previously established “adipogenic wound healing” model.
Figure 2.1: Graph showing changes over time in overall tissue vascularity as expressed in terms of the mean number of vessel profiles in graticule microscope fields during adipogenic healing. Vessel profile number peaked by the third week after implantation (p < 0.05), and reduced up to week 12 (p < 0.05). The initial increase in vascularity and reduction over time are consistent with vascular changes reported for wound healing (Xaymardan, 2001).
II.2. Materials and Methods

II.2.i. Materials

Balb/C mice were obtained from the Animal Care Facility, Westmead Hospital. Ketamine was purchased from Parnell Laboratories (Aust) Pty Ltd (Alexandria, NSW, Australia) while Illium xyazil-20 (Rompom) was purchased from Troy Laboratories Pty Limited (Smithfield, Australia). Water for Injections was purchased from Delta West Pty Ltd (Bentley, Western Australia) while Buprenorphine hydrochloride (Temgesic) was purchased from Reckitt & Colman (West Ryde, NSW, Australia). 5% Providone-iodine solution was purchased from Professional Disposables Inc (USA), while silk sutures (braided silk 4/0) were purchased from Ethnor Pty, Ltd. (Sydney, Australia). Polyvinyl sponge material was obtained from Becton Dickinson Acute Care (New Jersey, USA). Phosphate buffered saline (PBS) was from Oxoid (Basingstoke, United Kingdom). Histolene was purchased from Fronine Pty Ltd (Riverstone, NSW, Australia), while Paraplast Tissue Embedding medium was from Oxford Larwine (St Louis, USA). All other reagents were purchased from Sigma (St Louis, USA).

II.2.ii. Establishment of Scarring Wounds by Polyvinyl Sponge Implantation

Implantation procedures were approved by the Westmead Hospital Animal Ethics Committee, protocols 173 and 133. Female Balb/C mice, aged between 6 and 10 weeks, were used for all experiments. Mice were anaesthetised by intraperitoneal injection of Ketamine (8.5 mg/kg) and Rompom (2.0 mg/kg). Once anaesthetised, surgical areas were shaved and disinfected using providone-iodine solution (5 %)
followed by alcohol (70 %). Vertical incisions, approximately 15 mm in length, were made over the base of the tail area to expose the underlying muscle. Scarring wounds were then established by underlying skin lateral to incisions and placing subcutaneous implants, each comprising of a piece of polyvinyl sponge material measuring approximately 3 x 3 x 2 mm. Skin wounds were then closed with continuous silk sutures. Mice regained consciousness beneath a heat lamp, at which time the analgesic, Temgesic (0.05 mg/kg) was administered subcutaneously in the neck region to alleviate post-operative pain before animals were returned to their cages. Mice were monitored closely for any signs of discomfort after surgical procedures. The mice were fed ad libitum with standard laboratory chow and the well-being of each mouse was monitored daily. Mice recovered quickly from this procedure; gaining weight, feeding, drinking, moving and socialising normally after surgery.

II.2.iii. Tissue Harvesting

Mice were sacrificed by CO₂ asphyxiation 1, 2, 3, 4, 6, 8, 10 and 12 weeks after surgery. Implants were excised en bloc and fixed with 10 % neutral buffered formalin before processing for paraffin histology.

II.2.iv. Processing for Paraffin Histology

Tissues within implants, fixed in neutral buffered formalin (10 %), were washed with PBS before dehydration in graded alcohols (70 %, 95 % and 100 %) and clearing of tissues with histolene. Tissues were then infiltrated with paraffin under vacuum, using at least four changes of paraffin before embedding of tissues into paraffin blocks. 4 μm
paraffin sections were cut using a Leica Microsystems microtome (Wetzlar, Germany) and mounted onto poly-L-lysine coated glass slides.

II.2.v. Quantitation of Tissue Vascularity

Preliminary experiments using widely accepted markers for endothelium such as Banderia simplicifolia lectin, Factor VIII associated antigen, PECAM-1 and CD34, revealed unacceptable levels of background staining, seemingly related to the sponge material. For this reason, the Gomori Trichrome stain was used to identify vessels and other structures in tissues. Four specimens from each time point were examined determining changes in vascularity over time. Histometric counts were performed on eight randomly selected fields of view using a 100 square graticule and a 40 X objective. Vessels were classified according to size using vessels in a standard reference section (Figure 2.2) as a guide. Vessels were classified as either small (0-10 μm), medium (10-25 μm), large (25-50 μm) or very large vessels (> 50 μm).

II.2.vi. Statistical Analysis

Data was expressed as means ± standard error. A two tailed Student’s T-test was used to assess the statistical significance of changes seen with $P$ values < 0.05 considered significant.
Figure 2.2: Photomicrograph of a Gomori Trichrome stained paraffin section of scarring tissue 3 weeks post-implantation used as a reference slide for assessing vessel size. Small (arrows), medium (arrowheads) and large (L) vessels are seen as well as multinucleated foreign body giant cells (blue arrows) surrounding polyvinyl sponge material (PV), characteristic of the foreign body response. Deposition of collagen fibres is observed (blue stain), while spaces occupied by polyvinyl sponge which have fallen out of the section (S) were also seen as was muscle (M) supporting the sponge implant (Bar = 50 µm).
II.3. Results

II.3.i. Sponge Implants Were Infiltrated by Granulation Tissue Which Matured to Scar Tissue

A fibrinous matrix was seen impregnating sponges within the first week of implantation (Figure 2.3). Granulation tissue was noted at the periphery of the sponges, with large central areas occupied by fibrinous material. By week 2, granulation tissue had penetrated further into the sponges and some collagen deposits were observed in the peripheral areas. By weeks 3 and 4, spaces in the centers of implants had almost been obliterated by newly formed granulation tissue and collagen deposition appeared greater. Granulation tissue was also seen surrounding implants and a foreign body giant cell response was apparent. By 8 weeks post-implantation, reparative granulation tissue filled the entire volume of implants, while staining for collagen appeared further increased, and this continued together with contraction of implants up to the 12 week time point.

II.3.ii. Quantitation of Vascularity During Wound Healing

As seen in Figure 2.4A, there was a rapid increase in small and medium sized vessels within the first three weeks of implantation (p < 0.05). At week 3, there was a reduction in the number of both small and medium sized vessels (p < 0.05). Also by week 4, some very large vessels were observed within specimens and these persisted throughout the remaining experimental period, with no clear change in the proportion of these larger vessels over time. Most striking in Figure 2.4B was an initial increase in the
total vessel number followed by a rapid reduction after week 2 (p < 0.05), with no change in total vessel number seen after week 4. These observations are consistent with those expected during normal wound healing.
Figure 2.3: Photomicrographs of Gomori Trichrome stained paraffin sections of scarring tissues from post-implantation weeks 1, 2, 3, 4, 8 and 12. Scarring lesions were excised en bloc and infiltration of granulation tissue (Gt) into the space (S) between the epidermis (Ep) and muscle (M) can be seen. Sub-cuticular muscle (arrows) is observed while increased deposition of collagen fibres (blue stain) and granulation tissue infiltration throughout the polyvinyl sponge (arrowheads) was seen with increasing postsurgical time (Bar = 200 μm).
Figure 2.4: Histogram demonstrating the number and distribution of vessels according to size (A) as well as a graph showing the mean total vessel number (B) over time in scarring wounds. (A) Small (□-) (0-10 μm), medium (■-) (10-25 μm), large (○-) (25-50 μm) and very large (□-) (> 50 μm) vessels were quantitated over eight random fields of view per tissue section in four specimens per time point and means calculated. There was a higher prevalence of small vessels during the early time points (p < 0.05) and these reduced by week 4 (p < 0.05). Few very large vessels were seen during the first three post-implantation weeks, but were seen in all sections at later time points. (B) Total vascularity peaked at 2 weeks (p < 0.05) and then reduced to a stable level by week 4.
II.4. Discussion

Wound healing involves the replacement of fibrinous blood clot with reparative granulation tissue and subsequent maturation to scar, adipose or bone tissue (Bruder et al., 1994; Clark and Henson, 1996; Linares, 1996; Mutsaers et al., 1997; Xaymardan et al., 2002). The aim of this study was to develop a simple and reproducible scarring wound healing model that could be used for comparison with adipogenic healing, for which an experimental model system was already established in this laboratory at the time the current project commenced.

Implantation of hollow devices to study granulation tissue formation and maturation is a well established strategy (Schilling et al., 1959; Hunt et al., 1984; Xaymardan et al., 2002) while polyvinyl sponge implants have been used by others (Bailey, 1988; Kyriakides et al., 2001). Sponge implants provide the particular advantage that granulation tissue can be easily removed at various time points for analysis (Bailey, 1988).

Small animals and mice in particular are frequently used to study wound healing because large numbers of animals may be used and animal handling is also easier compared with larger species. Despite these benefits, it is important to note that mice differ significantly from humans in a number of potentially relevant ways, including the presence of fur, a thin epidermis and dermis and a tendency to experience extreme wound contraction (Sullivan et al., 2001). Nonetheless, mouse wound healing models seem reasonable for study of some aspects of healing relevant to humans, and are a sensible prelude to experimentation in larger animals such as pigs.
In the current study, polyvinyl sponge implants were examined as a potential wound healing model. Replacement of fibrin and blood clot with reparative granulation tissue was observed early after implantation. Maturation of reparative granulation tissue to scar tissue with increased collagen deposition, reduced tissue vascularity and wound contraction together with a foreign body giant cell response correlated with changes in similar wound healing models developed by others (Schilling et al., 1959).

Tissue vascularity was characterised by histometric counts of paraffin sections of scarring lesions. Unfortunately, due to unacceptable levels of non-specific labelling in preliminary experiments using immunohistochemistry to identify vascular endothelium, specific EC markers could not be used for identification of blood vessels in tissue sections. However, use of the Gomori Trichrome stain permitted unambiguous identification of vessels and had the further advantage of visualising increased collagen deposition over time. A reference slide was used for defining vessel size categories for vessel profile quantitation. The initial increase in vascularity, followed by reduction over time is consistent with vascular changes expected during normal wound healing, while changes in vessel size are reminiscent for those reported for adipogenic healing (Linares, 1996; Mutsaers et al., 1997; Greenhalgh, 1998; Honma and Hamasaki, 1998; Brown et al., 2002; Xaymardan et al., 2002).

There was a difference in the peak time of vascularity in the here established scarring wound healing model as compared with the previously developed adipogenic wound healing model, such that scarring lesions achieved maximum vascularity at 1 to 2 weeks as compared to 3 to 4 weeks in adipogenic lesions. This difference may reflect some aspect of the adipogenic as compared with scarring processes seen in these two
model systems (Xaymardan et al., 2002). It is possible that the latter time of maximum vascularity in adipogenic wound healing may result from angiogenic leptin production by the adipogenic tissue (Cao et al., 2001; Marikovsky et al., 2002). As will be seen in the following Chapter, the different time course of vascular changes in these two model systems can be exploited to study the relationship between vascularity and plasma proteins during wound healing.
Chapter III

Fragmentation of Plasma Proteins During Wound Healing
III.1. Introduction

III.1.i. Injury Results in Inflammation and Wound Healing

Following major injury, blood loss is reduced by vasoconstriction and platelet aggregation to form a haemostatic plug. Stimulation of the clotting cascade results in the cleavage of fibrinogen by thrombin to form fibrin, which together with fibronectin, forms a provisional matrix. Inflammation is an immediate pre-programmed response to injury which is essentially identical regardless of the nature of the injurious agent. Platelets, activated in the haemostatic plug, release cytokines such as interleukin-6 that stimulate the inflammatory response and induce migration of inflammatory leukocytes such as neutrophils and monocytes into wounds. Neutrophils act against local bacterial contamination, while monocytes differentiate into macrophages and also phagocytose debris. Macrophages also release growth factors, such as PDGF, TGF-β and TNF-α, which stimulate migration of EC and fibroblasts into wounds to form highly vascular reparative granulation tissue. Re-epithelialisation by keratinocytes occurs in cutaneous wounds, while fibroblasts differentiate into myofibroblasts that mediate wound contraction. Scar tissue is formed by maturation of reparative granulation tissue, and this is through the combined effect of increased collagen deposition, and decreased vascularity and cellularity via apoptosis of fibroblasts and EC (Darby et al., 1990; Desmouliere et al., 1995) (reviewed in (Clark and Henson, 1996; Linares, 1996; Mutsaers et al., 1997; Greenhalgh, 1998)).
III.1.ii. The Acute Phase Response

The acute phase response (APR) is a systemic response to otherwise localised inflammation. This involves substantial changes in plasma levels of specific proteins and usually occurs with the onset of inflammation or injury to persist for up to 21 days, at which time base-line levels of proteins may be regained (Gabay and Kushner, 1999). The APR includes a wide range of neuroendocrine and metabolic changes, such as fever and increased protein catabolism. Importantly for this thesis, one of the main features of the APR is a dramatic change in the concentrations of many plasma proteins by at least 25%, and in the case of some proteins, by several hundred fold. Most acute phase proteins (APP) are termed as “positive” APP because they increase in concentration during inflammation, while “negative” APP decrease in concentration (Gabay and Kushner, 1999; Cecilian et al., 2002; Gruys et al., 2005). Inflammatory molecules, produced by macrophages, neutrophils and other cells during inflammation, control expression of APP by the liver, with interleukin-6 playing a major role in regulating APP expression at the transcriptional level (Gabay and Kushner, 1999).

III.1.ii.a. Positive Acute Phase Proteins

Some important positive APP include C-reactive protein, serum amyloid A, complement system proteins, some coagulation and fibrinolytic system proteins, such as fibrinogen and plasminogen, metal transport proteins such as ceruloplasmin and haptoglobin, and also proteinase inhibitors (Cecilian et al., 2002). The functions of APP are varied and some of these relevant to the current thesis are outlined below.
Plasma Proteins in Wounds
Chapter III Page 3

It has been argued that positive APP help to protect the host from foreign organisms and limit both haemorrhage and excessive proteolysis. APP are used as markers of inflammatory status, and an example of this is C-reactive protein which increases in concentration up to 1000 fold in some circumstances. The biological relevance of increased C-reactive protein levels appears to relate to its ability to activate complement proteins and also to induce expression of inflammatory cytokines in monocytes (Gabay and Kushner, 1999). Some APP can be thought of as providing enhanced inflammatory capacity, with the increase in Complement proteins providing a good example, while other positive APP can be thought of as limiting the potential injury of this enhanced inflammatory capacity, by protecting against free radicals, for example (Gabay and Kushner, 1999; Cecilian et al., 2002).

Ceruloplasmin is a 120 kDa glycoprotein which contains more than 95 % of the copper found in plasma. The protein thus functions in copper transport but also appears to contribute to iron metabolism and has antioxidant activity (Hellman and Gitlin, 2002). Haptoglobin is a glycoprotein consisting of a tetramer of two α- and two β-chains. The human protein has a molecular mass of approximately 86 kDa (Lim et al., 2001), although the protein differs in molecular mass across species with mouse haptoglobin having an approximate molecular mass of 73 kDa (SwissProt Entry Q61646). There is also a form of haptoglobin containing an extra free thiol group, permitting the formation of polymers (Lim et al., 2001). The major role of haptoglobin is to bind free haemoglobin through strong non-covalent bonds on the β- subunit of haptoglobin. This prevents iron loss and also reduces oxidative tissue damage to the kidneys during intravascular haemolysis (Van Vlierberghe et al., 2004). Circulating
haptoglobin/haemoglobin complexes bind to specific scavenger receptors, such as CD163, so that the half life of this complex is approximately 20 minutes (Kristiansen et al., 2001). Because of the close association between haptoglobin and haemoglobin, levels of haptoglobin correlate with those of free plasma haemoglobin (Lim et al., 2001).

**III.1.ii.b. Negative Acute Phase Proteins**

Negative APP include: transthyretin, Insulin-like growth factor-1, transferrin and serum albumin. It is unclear what the evolutionary advantages of down-regulating negative APP may be, however, it has been suggested that they may act as a source of amino acids for the production of positive APP (Ceciliani et al., 2002). Some functions of negative APP studied in this thesis are summarised below.

Transferrin is a glycoprotein with an approximate molecular mass of 75 to 80 kDa. This protein acts as a transporter of iron and is involved with control of iron metabolism (Baker et al., 2003). Transferrin is described as having two similar lobes connected by a short peptide. A conformational change occurs during iron binding to permit recognition by the transferrin receptor, with subsequent receptor mediated endocytosis and release of iron (Li and Qian, 2002). Reduced plasma transferrin levels may aid in host defense by limiting access to iron by infecting organisms (Gruys et al., 2005).

Serum albumin is synthesised by the liver as a 66 kDa protein, and is the most plentiful of plasma proteins with a half life of approximately 19 days (Peters, 1996). It is interesting that serum albumin concentration is a clinical predictor in cardiovascular patients, such that low serum albumin levels correlate with poor outcomes (James et al.,
2000; Schillinger et al., 2004). Although some albumin is lost from the circulation into the extravascular space, most of this is recovered via the lymphatics (Peters, 1996; Nicholson et al., 2000). The main known functions of albumin are the transport of metal ion, drugs and fatty acids (Peters, 1996). In addition, because albumin is so prevalent, it contributes greatly to the colloid osmotic pressure of blood so that it plays a role in movement of fluid between vascular and extravascular compartments. Also, albumin is capable of free radical scavenging, while a further function investigated later in this thesis is inhibition of EC apoptosis (Zoellner et al., 1996b; Zoellner et al., 1999). Please note that a more detailed discussion of albumin will be presented in Chapter 4.

III.1.iic. Wound Healing Represents a Challenge to the Host

Wound healing and acute inflammation are complex processes and disruption of these may lead to a variety of pathological states, such as abnormal healing or chronic inflammation (Clark and Henson, 1996; Linares, 1996). An interesting aspect of the APR is over-expression of proteinase inhibitors which balance the actions of activated proteinases, such as clotting cascade proteins and plasmin, and because proteinase activity is important during wound healing, it can be imagined that the APR would help regulate this process (Gabay and Kushner, 1999; Ceciliani et al., 2002; Gruys et al., 2005).

As stated above, the APR is an important systemic response following localised injury and inflammation. Changes in APP levels appear to be mostly beneficial to the host. However, if the systemic response is prolonged with acute inflammation progressing to a chronic inflammatory state, the continuing APR may have severe
pathological consequences including anaemia, hypoalbuminanaemia, amyloidosis and metabolic disturbances (Gabay and Kushner, 1999).

III.1.iii. The Fate of Acute Phase Proteins in Wounds

The plasma concentration of albumin is reduced in inflammation by decreased synthesis and increased turnover of the protein. In addition, increased vascular permeability results in escape of albumin from the blood into the extravascular space, and this may also contribute to reduced plasma albumin levels (Mouridsen, 1968; Mouridsen and Wallevik, 1968; Mouridsen, 1969; Don and Kaysen, 2004).

In normal circumstances, the endothelium is impermeable to macromolecules such as proteins (Florey, 1962; Rippe et al., 2002), while transport of albumin across the endothelium is via albondin, a high affinity albumin binding receptor specific to EC which undergoes receptor-mediated transcytosis (Schnitzer et al., 1988; Schnitzer, 1992; Schnitzer et al., 1992; Schnitzer and Bravo, 1993; Schnitzer and Oh, 1994). During inflammation, there is an increase in the permeability of the microvasculature, so that plasma proteins escape into the extravascular compartment to alter the osmotic balance and mediate oedema fluid formation (Florey, 1962). Separately, newly formed vessels in early reparative granulation tissues also have high vascular permeability, so that there is a similar production of oedematous fluid early during wound healing (Nyman et al., 1971). Inflammatory mediators including histamine, serotonin (Majno and Palade, 1961), bradykinin (Saulpaw and Joyner, 1997), amyloid β-peptide (Blanc et al., 1997), platelet activating factor (Klabunde and Anderson, 2002) and thrombin increase vascular permeability in acute inflammatory lesions by stimulating redistribution of inter-
Plasma Proteins in Wounds
Chapter III Page 7

endothelial cell junction proteins (Dejana et al., 1995), and this is associated with plasma leakage (McDonald et al., 1999). In the case of wound healing, angiogenesis and subsequent remodelling of blood vessels in reparative granulation tissue is accompanied by increased vascular leakage (Kevil et al., 1998; Ezaki et al., 2001; Bates and Harper, 2002).

Protein function can be profoundly altered by proteolytic cleavage, which may activate, inactivate or perhaps generate fragments with biologically different activities to those of parent proteins. Because wound healing involves both acute inflammation and significant protein turnover by proteinases, it is interesting to speculate that some APP may be fragmented during wound healing. In this Chapter, two positive APP, ceruloplasmin and haptoglobin, and two negative APP, transferrin and serum albumin, were studied with regard to possible fragmentation during wound healing. IgG was also studied as an important immune molecule. Albumin was found to undergo fragmentation in healing wounds, and this correlated with times of high tissue vascularity.
III.2. Materials and Methods

III.2.i. Materials

Materials for the preparation of scarring wounds by sponge implantation were as previously described in II.2.i. Nylon mesh for the preparation of adipogenic wounds was cut from netwells purchased from Corning Costar (NY, USA). Protein Assay Reagents, pre-stained SDS-PAGE broad range standards as well as 8-16 % gradient SDS-PAGE Criterion gels were all from BioRad (Hercules, USA). Silver stain reagent kits were from Novex Experimental Technology (San Diego, USA), while nitrocellulose membranes and Enhanced Chemiluminescence (ECL) kits were both from Perkin-Elmer (Boston, USA). Rabbit anti-human ceruloplasmin antiserum was purchased from DAKO Corporation (CA, USA), while goat anti-human haptoglobin and goat anti-mouse transferrin antiserums were both from ICN Cappel (Ohio, USA). Rabbit anti-mouse albumin was from ICN Biomedical (Ohio, USA), while both horseradish peroxidase (HRP) conjugated rabbit anti-goat IgG and HRP conjugated goat anti-mouse IgG were from Biosource International (Camarillo, USA). HRP conjugated donkey anti-rabbit IgG was from Silenus laboratories (Boronia, Victoria, Australia). The proteinase inhibitors phenylmethylsulfonyl fluoride (PMSF), aprtinin, pepstatin A, iodoacetamide, leupeptin, soybean trypsin inhibitor (SBTI) and Nα-p-tosyl-L-arginine methyl ester (TAME) as well as all other reagents were from Sigma (St Louis, USA).
III.2.ii. Preparation of Experimental Wounds in Mice

All implantation procedures were approved by the Westmead Hospital Animal Ethics Committee, protocols 173 and 133, and experiments were performed on female Balb/C mice aged between 6 and 10 weeks. Scarring wounds were established by subcutaneous implantation of polyvinyl sponge material as previously described in II.2.ii. Adipogenic wounds were established by intramuscular implantation of nylon mesh tubes, as previously described (Xaymardan et al., 2002). Briefly, these tubes were formed by rolling a single layer of nylon mesh, with implants measuring approximately 2.5 mm x 7.5 mm. The edges were secured with superglue and tubes sterilised by autoclaving. Earlier work confirmed that the adipogenic response was independent of the material used (Xaymardan et al., 2002). Mice were anaesthetised and surgical areas prepared in the same way as described for animals receiving sponge implants in II.2.ii.

Vertical incisions of approximately 15 mm in length were made over the base of the tail to expose the underlying musculature. Muscle fibres approximately 4 mm to one side of the midline were separated by blunt dissection to a depth of 3 mm to create space for the implants. Muscle was closed over single mesh tubes using two interrupted sutures and skin wounds were then closed with continuous sutures. Mice regained consciousness before administration of the analgesic, Temgesic (0.05 mg/kg) in the neck region. Mice were fed ad libitum with standard laboratory chow and were monitored as described in II.2.ii.
III.2.iii. Numbers of Animals Used in Experiments

Preliminary experiments with only 8 mice with adipogenic lesions were conducted in the context of an Honours project by the current PhD candidate, and this earlier was data included for statistical analysis in the current thesis. In this earlier work, tissues were collected at 2, 3, 4, 5, 6, 8, 10 and 12 weeks post-implantation. In the current work performed within the current PhD project, tissues from two further animals per time point were collected at post-surgical weeks 1, 2, 3, 4, 6, 8, 9 and 12. To further confirm observations at post-surgical weeks 4 and 12, tissues were collected from an additional 6 animals at these time points. To confirm observations in aged animals, further experiments were performed in which adipogenic wounds were established in 8 mice aged 8 months.

In experiments with scarring lesions, granulation tissues were collected at post-surgical weeks 1, 2, 3, 4, 6, 8, 10 and 12, with tissues collected from 3 animals per time point. To confirm observations in scarring tissues at 1 and 12 week time points, 7 further animals were used at each of these times.

III.2.iv. Tissue Harvesting for Analysis of Proteins

Mice were sacrificed by CO₂ asphyxiation and sponge or tube implants were dissected free of surrounding tissues. Sponge implants containing granulation tissue, were transferred into Eppendorf tubes while nylon tubes were cut open and granulation tissue scraped into Eppendorf tubes. Samples were stored on ice while control tissues of muscle, peritoneum, spleen, heart, kidney and liver were collected. 250 μl aliquots of a lysis buffer were added to tissues and this consisted of Tris (50 mM pH 7.4), SDS (0.5
% and the proteinase inhibitors: aprotinin (10 μg/ml), leupeptin (10 μg/ml), pepstatin A (10 μg/ml), PMSF (1 mM), SBTI (100 μg/ml) and TAME (10 mM). Tissues were macerated in lysis buffer and then stored at -80°C until required, at which time samples were thawed and centrifuged at 13,000 rpm for 15 minutes at 4°C to separate solid material from tissue extract. Supernatants were then collected, transferred into fresh tubes and stored, if necessary, at -80°C for further analysis. Mouse blood was collected from the inferior vena cava using a 1 mL syringe with a 29 G needle and allowed to clot in an Eppendorf tube before centrifugation at 13000 rpm for 15 minutes at 4°C and collection of serum which was stored at -80 °C until required.

III.2.v. Assay for Determination of Total Protein Concentration

Total protein concentrations of tissue extracts were determined using the Bradford Assay (Bradford, 1976). Briefly, standard curves were established using BSA at concentrations ranging from 0.2 to 2.0 mg/ml in Tris (50 mM pH 7.4). Tissue extracts were sampled undiluted as well as at dilutions of 1:2, 1:4, 1:5 and 1:10 in Tris (50 mM, pH 7.4). 10 μl volumes of samples or standards were then added to 500 μl of the commercially available Protein Assay Reagent, diluted 1:4 in water. After blanking, the optical densities at 595 nm were determined using a Beckman DU 640 spectrophotometer (Beckmann Instruments, USA) and sample protein concentrations determined from the standard curves.
Plasma Proteins in Wounds
Chapter III Page 12

III.2.vi. SDS-PAGE and Silver Staining

Commercially available pre-cast polyacrylamide electrophoretic gels (8-16 %) were used to separate proteins according to size. Samples were equilibrated with regard to protein concentration to ensure each lane received identical loading. Samples were prepared by combining 5 μl of equilibrated tissue extract with 10 μl of SDS (10 %) boiling for 3 minutes, and then addition of loading buffer (15 μl) consisting of bromophenol blue (0.005 %) in glycerol (20 %). Samples (25 μl) were then loaded onto gels while pre-stained SDS-PAGE standards were prepared and loaded in a similar way. The running buffer consisted of Tris (25 mM), glycine (192 mM) and SDS (1 %) at pH 8.3. Electrophoresis was at constant voltage (200 V) until the dye front reached the end of the gel.

Protein bands were visualised by silver staining gels. Gels were fixed in methanol (50 %) with glacial acetic acid (10 %) in water before staining using reagents from the Novex Xpress Silver Stain kit according to the manufacturer’s instructions. Results were digitally recorded using the GeneGenius gel documentation system (Syngene, Cambridge, UK).

III.2.vii. Western Blotting

Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes using a constant current at 50 mA for 18 hours and an electroblotting apparatus (BioRad, Hercules, USA). Transfer solution consisted of Tris (25 mM), glycine (192 mM) at pH 8.3 with methanol (20 %) and SDS (0.00025 %). After transfer, non-specific binding was blocked by incubating membranes in 5 % non-fat milk in Tris buffered saline (Tris,
20 mM and NaCl, 15 mM, pH 7.5) (TBS) with Tween 20 (0.5%). However, in blots probing for albumin, non-specific binding was blocked by incubation of membranes with casein (5% in PBS, pH 7.5). Primary and secondary antibodies were prepared by dilution in blocking buffer at concentrations dependent upon the specific antibody. Primary antibodies of rabbit anti-mouse albumin, rabbit anti-ceruloplasmin, goat anti-haptoglobin, goat anti-transferrin and goat anti-mouse IgG were all diluted 1:1000 in blocking buffer, while HRP conjugated donkey anti-rabbit and HRP conjugated rabbit anti-goat were diluted (1:10,000) in blocking buffer.

Primary antibodies were incubated with membranes for 1 hour at room temperature on a laboratory rocker after which membranes were washed with TBS with Tween 20 (0.5%) for another hour at room temperature changing wash buffer every 5 minutes. Secondary antibodies were then applied to membranes for 1 hour at room temperature, followed by a further hour of washing in TBS with Tween 20 (0.5%). Bound secondary antibodies were then detected by ECL. Briefly, equal volumes of enhanced luminal and oxidising reagents were combined and incubated with membranes for 1 minute at room temperature. Excess reagent was drained from membranes before placing membranes in protective plastic sleeves and exposing to radiographic films for varying amounts of time as determined by signal to noise ratios. Films were developed using an automatic developer. Controls for non-specific binding of secondary antibodies consisted of parallel blots probed in an identical manner with the difference that primary antibodies were omitted.
III.2.viii. Densitometry and Analysis

Western blots were digitally recorded using the GeneGenius gel documentation system (SynGene, Cambridge, England). Molecular mass determination of protein bands was performed using GeneTools analysis software (Syngene, Cambridge, England) with reference to standard curves determined from molecular mass markers. Quantitation of specific protein bands by densitometry was performed using GeneTools analysis software. To facilitate comparison of results for albumin across separate blots and experiments, densitometric readings were expressed as relative percentages with reference to the mean densitometric reading of the native protein.

III.2.ix. Statistical Analysis

Statistical significance of densitometric data was determined using the two tailed Mann Whitney U test with $P$ values of $< 0.05$ considered significant.
III.3. Results

III.3.i. Western Blots of Mouse Serum Confirmed That the Antibodies Used Recognised Proteins of Expected Sizes

To confirm that the primary antibodies raised from mostly human antigens were capable of detecting mouse proteins, mouse serum was probed by Western blotting for each protein of interest in this study (Figure 3.1). Bands were seen consistent with each of the proteins probed for. In the case of haptoglobin and albumin, dimer forms were detected in serum, while apparently unreported higher molecular mass bands were present in the case of ceruloplasmin. Also lower molecular mass bands were observed for haptoglobin at 10 kDa and 27 kDa, consistent with reported molecular masses of α- and β- subunits of the protein. A 50 kDa lower molecular mass band was also observed for IgG and interpreted as heavy chain dissociation from the larger molecular complex. Importantly for later work in this Chapter, no fragmentation of albumin was observed in serum.

III.3.ii. Ceruloplasmin and Transferrin Were Not Fragmented During Wound Healing in Mice

Granulation tissues and control tissue extracts from both scarring and adipogenic wounds were analysed for the presence and possible fragmentation of positive and negative APP as well as IgG during wound healing. Ceruloplasmin was present during both adipogenic and scarring healing across all time points studied, and was also present in all control tissues (Figure 3.2). Apparent fragmentation of ceruloplasmin was seen
only at weeks 3 and 4 in scarring tissues with the appearance of one minor band (75 kDa). This band was not seen at any other time point or during adipogenic healing and it was difficult to be convinced that such transient and faintly represented bands would be biologically significant. Similar to serum, higher molecular mass bands representing apparently unreported large protein complexes were seen in most samples. The levels of ceruloplasmin appeared maximal between weeks 2 and 4 in both adipogenic and scarring lesions, suggestive of an APR to the implants. Control blots in the absence of primary antibodies were negative.

Transferrin was also present in granulation tissue from both adipogenic and scarring lesions as well as in control tissue extracts (Figure 3.3). No significant fragmentation was observed in most samples, with the exception of one minor band (65 kDa) in scarring lesions from 2 to 10 weeks which was maximal at 3 and 4 weeks.

III.3.iii. Apparent Dissociation of Haptoglobin and IgG During Wound Healing and in Control Tissues

Figure 3.4 shows the result of Western blots for haptoglobin in adipogenic and scarring wounds as well as a range of control tissues. Native haptoglobin and its dimer were present in all healing tissue samples, while there appeared to be a slight reduction in both these haptoglobin species over time. The protein was also found in most control tissues, although at much lower levels compared with wound tissues. Minor bands were noted in healing tissues at 10 kDa and 27 kDa, particularly before 8 weeks post-implantation. These were consistent with the α- and β-chains respectively, suggestive of some degree of dissociation of haptoglobin in wounds, although the origin of these
smaller bands from fragmentation events could not be excluded. In addition, several minor bands were noted with slightly higher molecular mass to the native protein as well as much larger complexes and the origin of these is unknown. A gradual reduction in haptoglobin with time, particularly in adipogenic lesions suggested an early and resolving APR.

In the case of IgG (Figure 3.5), no clear change in antigen level was noted over time and native protein was apparent. Minor bands of 50 kDa and 100 kDa were interpreted as heavy chain dissociation from the larger molecular complex (150 kDa) and an unreported higher molecular mass complex, possibly an aggregate comprised for two heavy chains (100 kDa). These bands were also seen in control tissues.

From this, it was not possible to be certain of unambiguous fragmentation of either haptoglobin or IgG during wound healing.

III.3.iv. Fragmentation of Albumin Was Observed During Wound Healing

Western blots for albumin of granulation tissue extracts from scarring and adipogenic wounds (Figure 3.6) revealed extensive fragmentation of albumin in both wound healing models. As seen by brief (i) and prolonged (ii) ECL exposures of radiographic film, fragments of approximate molecular mass 27.5 kDa, 39 kDa and 49 kDa were observed in both scarring and adipogenic wounds, while an additional fragment of 17.5 kDa was also seen in adipogenic lesions. These fragments were more prominent early after wounding, while levels of these fragments appeared to decrease by 12 weeks in both models studied. The pattern of albumin fragmentation over time differed in adipogenic lesions from that observed in scarring wounds, such that there
was no clear change in the level of the 49 kDa fragment in adipogenic lesions, while the 49 kDa fragment became less prominent with time in scarring lesions. Native albumin (66 kDa) and its dimer (130 kDa) were also observed. Minor bands of 90 kDa and 250 kDa were also observed, seemingly representing high molecular mass complexes.

Reduction by treatment with mercaptoethanol of samples where albumin fragmentation was extensive prior to SDS-PAGE did not affect the size of bands observed by Western Blot, suggesting the absence of additional cleavage sites within the protein fragments seen.

III.3.v. Fragmentation of Albumin Correlated With Tissue Vascularity During Wound Healing

Figure 3.7 shows changes in the vascularity of both scarring and adipogenic wounds over time (From Figure 2.4B and (Xaymardan, 2001)), as well as a Western blot for albumin fragmentation at times of maximal vascularity and at the conclusion of experiments for both wound healing models studied. It was clear that albumin fragmentation was extensive at times of maximal vascularity being 1 and 4 weeks in scarring and adipogenic wounds respectively.

To confirm these observations, additional experiments were performed with granulation tissues from multiple animals at relevant time points. Figure 3.8 shows Western blots for albumin in granulation tissue extracts from 8 separate animals with scarring lesions harvested at each time point being weeks 1 and 12, representing times of high and low tissue vascularity respectively. Brief (Figure 3.8A) and prolonged (Figure 3.8B) ECL film exposures demonstrated prominent albumin fragments at week 1, with
molecular masses of 27.5 kDa, 39 kDa and 49 kDa, while in addition the 17.5 kDa fragment, previously seen only in adipogenic lesions, was also observed. Further minor bands were also seen in prolonged ECL exposures, with approximate molecular masses slightly larger than the 27.5 kDa and 17.5 kDa fragments and these may have been conformational variants, while other minor bands at 19 kDa, 21 kDa and 25 kDa were also seen. Less albumin fragmentation was present at week 12 compared with week 1, despite similar total protein loading across all samples, as confirmed in parallel silver stained SDS-PAGE (Figure 3.8C). Levels of native (66 kDa) and dimer (130 kDa) albumin appeared to be slightly reduced in extracts from week 12, suggesting that although total protein levels were similar over time, there was reduced total albumin present in granulation tissue at the latter time and this may have contributed to the reduced albumin fragmentation at 12 weeks.

Densitometric analysis was performed to further characterise these changes (Figure 3.9) and revealed a reduction in both native (Figure 3.9A) and dimer (Figure 3.9B) albumin, however, only the reduction in dimer albumin was statistically significant (p < 0.01). Significant reductions in levels of the 49 kDa, 39 kDa and 27.5 kDa fragments (Figures 3.9C, D and E) were observed (p < 0.002) while the 17.5 kDa fragment (Figure 3.9F) and minor bands of 19 kDa, 21 kDa, 25 kDa were only detected at week 1 (p < 0.001). These quantitative data confirm significant albumin fragmentation at the time of maximal tissue vascularity in scarring wounds.

A similar Western blot and densitometric analysis was performed for adipogenic wounds, and is shown in Figures 3.10 and 3.11, comparing tissues from the 4 week time point with those collected 12 weeks after surgery. A slight reduction in intensity of
Plasma Proteins in Wounds
Chapter III Page 20

bands for native and dimer forms of albumin was noted, however, this was not statistically significant. Although the 49 kDa band did not change in intensity over time, the 39 kDa, 27.5 kDa and 17.5 kDa bands were substantially reduced at 12 weeks compared with the 4 week time point (p < 0.05). Data indicate an association between albumin fragmentation and maximum vascularity during wound healing.

III.3.vi. Albumin Fragmentation Was Observed in Some Tissues Separate From Wound Healing Sites

A number of tissues separate from wound healing sites from animals with either scarring (Figure 3.12) or adipogenic lesions (Figure 3.13) were examined for albumin fragmentation. In the case of animals with scarring lesions (Figure 3.12), the 49 kDa albumin fragment was present in muscle, liver, peritoneum, spleen, heart and kidney, while levels of this fragment did not appear to change over time. Other albumin fragments, with molecular masses of 39 kDa and 27.5 kDa, were also observed in all control tissues. The 39 kDa fragment was present at all time points in peritoneum, spleen and kidney, while this fragment was only present at weeks 2, 3 and 12 in muscle samples and at weeks 3 and 4 in heart samples, but was not observed in liver extracts. The 27.5 kDa albumin fragment was most prominent at weeks 3 and 4 in liver, peritoneum, heart and kidney and was also present at week 2 in muscle and spleen samples. The 17.5 kDa albumin fragment was detected mainly at weeks 3 and 4 in most control tissues.

When tissues from mice with adipogenic lesions were examined (Figure 3.13), the 49 kDa albumin fragment was present in all control tissues at each of the time points
Plasma Proteins in Wounds
Chapter III Page 21

investigated. The 27.5 kDa albumin fragment was also prominent at most times in peritoneum, spleen and heart, while this was not detected in muscle or liver samples. The 39 kDa fragment was not detected in muscle or liver samples, while it was faintly present in peritoneum and heart extracts from all time points studied. The 39 kDa fragment was also strongly detected in spleen samples at all time points. In addition, both the 27.5 kDa and 39 kDa albumin fragments were only observed in kidney samples from weeks 3 and 4.

In general, there appeared to be increased albumin fragmentation at 2 to 4 weeks post-implantation, and this suggests a possible mechanism of removal of albumin fragments generated during wound healing from the body. However, these findings were unexpected, and unfortunately increased numbers of control tissue samples were not collected so that these observations could not be further confirmed.
Figure 3.1: Western blot analysis of mouse serum for ceruloplasmin, transferrin, haptoglobin, IgG and albumin. Bands consistent with native ceruloplasmin (120 kDa), transferrin (75 kDa), haptoglobin (73 kDa) and IgG (150 kDa) were seen (N). Large molecular mass forms of haptoglobin (150 kDa) and albumin (130 kDa) were noted and interpreted as dimer forms (Dim), while an additional high molecular mass band in ceruloplasmin was observed and interpreted as an apparently unreported larger protein complex (C). Low molecular mass bands were seen for haptoglobin (10 kDa and 27 kDa) and IgG (50 kDa) and interpreted as disassociation of the haptoglobin α- and β-chains and IgG heavy chains respectively (Dis).
Figure 3.2: Western blots for ceruloplasmin in granulation tissue extracts from adipogenic and scarring wounds from 1 to 12 weeks post-implantation, as well as control tissues. Bands consistent with ceruloplasmin (120 kDa) were present in all samples, as well as the presence of high molecular mass complexes (C). Faint minor bands were present at 3 and 4 weeks in scarring lesions only, having a molecular mass of 75 kDa. Ceruloplasmin appeared most prevalent in wound tissues between 2 and 4 weeks after implantation, suggestive of an acute phase response to the wound inducing implants.
Figure 3.3: Western blots for transferrin in granulation tissue extracts from adipogenic and scarring wounds from 1 to 12 weeks post-implantation, as well as control tissues. Bands consistent with transferrin (75 kDa) were present in all samples. A minor band was present at weeks 2 to 10 scarring lesions only, having a molecular mass of 65 kDa and being most strongly expressed at weeks 3 and 4. Levels of transferrin seemed higher in wound tissues than in control tissues.
<table>
<thead>
<tr>
<th>Weeks</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipogenic Wounds</td>
<td>75</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scarring Wounds</td>
<td>65</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Tissues</td>
<td>75</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Muscle
- Peritoneum
- Spleen
- Heart
- Kidney
- Liver

KDa

- 250
- 150
- 100
- 75
- 50
- 37
- 25
- 15
- 10
Figure 3.4: Western blots for haptoglobin in granulation tissue extracts from adipogenic and scarring wounds from 1 to 12 weeks post-implantation, as well as control tissues. A band consistent with mouse haptoglobin (73 kDa) was present in all samples, as well as apparent dimer forms of the protein (Ddim). Minor bands, having a molecular mass of 10 kDa and 27 kDa, were noted in most tissues, particularly before 8 weeks post-implantation, consistent with the α- and β- chains respectively, suggestive of some degree of dissociation of haptoglobin. There appeared to be a modest and gradual reduction in total haptoglobin antigen over time, particularly in adipogenic wounds, consistent with an early and resolving APR. Also minor bands slightly higher than the native 73 kDa protein, as well as larger molecular mass complexes (C) were noted. A single band having molecular mass of 45 kDa was present in heart extracts, while the origin of this band is unknown. Haptoglobin also appeared to be at much lower levels in control tissues compared with wound tissues.
Figure 3.5: Western blots for IgG in granulation tissue extracts from adipogenic and scarring wounds from 1 to 12 weeks post-implantation, as well as control tissues. A band of approximate molecular mass of 150 kDa was present in all samples, consistent with IgG. Minor bands of molecular mass 50 kDa and 100 kDa were also noted in all samples and interpreted as heavy chain dissociation (50 kDa) and an unreported higher molecular mass complex, possibly a heavy chain aggregate (100 kDa). No differences in intensity of these bands were noted over time, however, levels of IgG appeared to be higher in scarring lesions compared with adipogenic lesions. Less IgG antigen was found in control tissues compared with wound tissues.
Figure 3.6: Western blots for albumin in granulation tissue extracts from scarring and adipogenic wounds from 1 to 12 weeks post-implantation demonstrating fragmentation of albumin, as seen in brief (i) and prolonged (ii) ECL film exposures. Bands representing native protein (66 kDa) and dimer (130 kDa) were present as were larger bands of 90 kDa and 250 kDa. Fragmentation of albumin was seen in both wound healing models with fragments having approximate molecular masses of 27.5 kDa, 39 kDa and 49 kDa in tissues from both models, with an additional fragment of 17.5 kDa observed in adipogenic wounds. These fragments were more prominent early after wounding and appeared to decrease in amount by 12 weeks post-implantation.
Figure 3.7: Tissue vascularity in scarring (■) and adipogenic (□) wounds over time as expressed in terms of vessel profile number (A) as well as a Western blot for albumin in granulation tissue extracts at 1, 4 and 12 weeks from both scarring and adipogenic wounds (B). (A) Tissue vascularity was maximal at 1 to 2 weeks in scarring lesions (p < 0.05) and 3 to 4 weeks in adipogenic lesions (p < 0.05) with these peaks followed by a significant reduction in vascularity accompanying wound maturation (p < 0.05). (Chapter II.3.ii and (Xaymardan, 2001)). (B) Bands consistent with native protein (66 kDa) and dimer (130 kDa) were present. Albumin fragments, with approximate molecular masses of 27.5 kDa, 39 kDa and 49 kDa, were observed in both scarring and adipogenic wounds, while an additional fragment of 17.5 kDa was seen in the week 4 adipogenic lesion only. In scarring wounds, albumin fragments were most prominent at week 1 being considerably reduced at later times. In adipogenic wounds, albumin fragments were prominent at week 1 and maximal at week 4. Also in adipogenic lesions, no clear change in the 49 kDa fragment was observed over time despite changes in smaller fragments. Overall albumin fragmentation correlated with tissue vascularity in both wound healing models studied.
Figure 3.8: A Western blot for albumin in granulation tissue extracts from scarring tissues from multiple animals harvested at 1 and 12 weeks post-implantation with each lane having material from a separate and individual mouse as seen in brief (A) and prolonged (B) ECL film exposures, as well as a silver stained parallel SDS-PAGE of these samples (C). Bands consistent with native protein (66 kDa) and dimer (130 kDa) were present, as were larger bands of 90 kDa and 250 kDa. Albumin fragments, with approximate molecular masses of 27.5 kDa, 39 kDa and 49 kDa, were seen in all samples. A 17.5 kDa fragment was only observed at week 1 in prolonged ECL exposures while additional minor “doublet” bands with molecular masses slightly higher than the major 27.5 kDa and 17.5 kDa bands were seen, as well as at 19 kDa, 21 kDa and 25 kDa. Fragmentation of albumin was more prominent and extensive in granulation tissue extracts from 1 week as compared with those from 12 weeks. There appeared to be less native and dimer albumin at week 12, compared with week 1, despite similar total protein loading as confirmed by parallel silver stained SDS-PAGE (C).
Figure 3.9: Densitometric analysis comparing native (A) and dimer (B) albumin as well as the 49 kDa (C), 39 kDa (D), 27.5 kDa (E) and 17.5 kDa (F) albumin fragments identified in Western blots of scarring wounds from 1 and 12 weeks after implantation (Figure 3.8). Significant reduction in levels of albumin fragments between weeks 1 and 12 were observed for 49 kDa, 39 kDa and 27.5 kDa (p < 0.002), while the 17.5 kDa fragment was only observed at week 1 (p < 0.001). Minor bands of 19 kDa, 21 kDa and 25 kDa were also only detected in granulation tissue extracts from week 1 (p < 0.001). There appeared to be reductions in both native (66 kDa) and dimer (130 kDa) forms of the protein, while only the reduction in dimer albumin was statistically significant (p < 0.01).
Figure 3.10: A Western blot for albumin in granulation tissue extracts from adipogenic tissues from multiple animals harvested 4 and 12 weeks post-implantation with each lane having material from a separate and individual mouse in brief (A) and prolonged (B) ECL film exposures, as well as silver stained parallel SDS-PAGE (C). Bands consistent with native protein (66 kDa) and dimer (130 kDa) were observed as were larger bands of 90 kDa and 250 kDa and there seemed to be a modest although not statistically significant reduction in intensity of these bands over time. The 49 kDa albumin fragment did not change over time, while the smaller albumin fragments (39 kDa, 27.5 kDa and 17.5 kDa) were more prominent at week 4 as compared with week 12, despite similar levels of protein loading revealed in a silver stained parallel SDS-PAGE gel (C).
Figure 3.11: Densitometric analysis comparing native (A) and dimer (B) albumin, as well as the 49 kDa (C), 39 kDa (D), 27.5 kDa (E) and 17.5 kDa (F) fragments identified in Western blots of adipogenic wounds from 4 and 12 weeks post-implantation (Figure 3.10). No significant difference was seen for the 49 kDa fragment (A) between weeks 4 and 12. A significant reduction in levels of the 39 kDa, 27.5 kDa and 17.5 kDa fragments was observed over time (p < 0.05). No statistically significant reduction in either the native or dimer albumin was observed.
Figure 3.12: Western blot for albumin in muscle, liver, peritoneum, spleen, heart and kidney extracts from 2 to 12 weeks after establishment of scarring lesions. Bands consistent with native protein (66 kDa) and dimer (130 kDa) were seen in all tissues as was a larger band of 90 kDa, while an additional band at 250 kDa was observed in muscle extracts. The 49 kDa albumin fragment was observed in all tissues at all time points studied. In addition, the 39 kDa and 27.5 kDa albumin fragments were observed in most tissues, with levels appearing maximal at 3 to 4 weeks after establishment of scarring wounds. An additional albumin fragment having a molecular mass of 17.5 kDa was also observed at weeks 3 to 4 in all tissues, with the exception of liver and spleen extracts. Demonstration of albumin fragments in these tissues may represent a mechanism for removal of these fragments from the body.
Figure 3.13: Western blot for albumin in muscle, liver, peritoneum, spleen, heart and kidney extracts from 1 to 12 weeks after establishment of adipogenic lesions. Bands consistent with native protein (66 kDa) and dimer (130 kDa) were seen in all control tissues, while larger bands of 90 kDa and 250 kDa were observed in most tissues. The 49 kDa albumin fragment was observed in all tissues at each time point studied. In addition, the 39 kDa and 27.5 kDa albumin fragments were observed in all tissues, with the exception of muscle and liver. Levels of the 27.5 kDa fragment were constant over all early time points in peritoneum, spleen and heart extracts, with levels reducing by 12 weeks, and levels of this fragment appearing maximal early after establishment of adipogenic lesions. The 39 kDa fragment was most intense in spleen extracts, while both the 39 kDa and 27.5 kDa fragments were seen in kidney samples only at weeks 2 to 4. Demonstration of albumin fragments in these tissues may represent a mechanism for removal of these fragments from the body.
III.4: Discussion

Wound healing involves both acute inflammation and significant protein turnover by proteinases. Protein function may be altered by proteolytic cleavage and for this reason, the question of whether some important APP are fragmented in wounds was investigated. Two positive APP, ceruloplasmin and haptoglobin and two negative APP, transferrin and albumin were studied with regard to this question, whilst IgG was also examined as an important immune molecule. Since not all APP could be investigated in this study, it seemed reasonable to investigate these specific APP as each protein may function as a transport protein.

Fragmentation of APP was investigated by Western blotting. Western blot analysis provides a highly sensitive and specific method for detection of proteins and possible fragments in complex tissue extracts. Monoclonal antibodies were not appropriate in this study as epitope specificity would restrict recognition to only one fragment in Western blots, while use of polyclonal antibodies permitted recognition of multiple epitopes on internal and external surfaces of proteins, and allowed detection of multiple fragments. However, it is possible, due to the multiplicity of specific antibody species generated in polyclonal sera, that the antibodies used may have had cross-reactivity with other proteins. Nonetheless, if this were the case such cross-reactive antibodies would likely only represent a small proportion of antibodies present, so that any cross-reactive bands would be expected to be very faint.

In addition, it was also possible to test for non-specific binding of both primary and secondary antibodies in Western blots by using an irrelevant primary antibody
preparation or by elimination of primary antibodies from Western blots. In the current study, such control blots using an irrelevant antibody or excluding the primary antibody were negative, indicating specific binding in Western blots. Some advantages of Western blotting include the capacity for quantitation and analysis by densitometry, as well as the potential to use antibody preparations for immunoprecipitation and characterisation of protein fragments.

Two experimental models were used to investigate APP in wound healing. The adipogenic (Xaymardan et al., 2002) and scarring (Chapter 2) wound healing models are useful as both models display increasing tissue vascularity after implantation, followed by reduction in vascularity by 12 weeks. However, in adipogenic lesions, there is no collagen deposition, myofibroblasts or wound contraction, and adipose tissue is formed rather than scar tissue. Scarring lesions, by comparison, have no lipoblasts or adipocytes, while granulation tissue matures to scar tissue over time. In addition, despite both models displaying changing vascularity over time, the time of peak tissue vascularity differs, with maximal vascularity at 3 to 4 weeks in adipogenic lesions and at 1 to 2 weeks in scarring lesions.

It was important to avoid artifactual protein fragmentation in granulation tissue extracts. Firstly proteinase inhibitors, specific for the four major classes of proteinases, were used in the lysis buffer. However, it was possible that some proteinases may have been insensitive to, or perhaps overcome the effect of these inhibitors. Nonetheless, this seems unlikely as both granulation tissue and control tissue extracts were treated in a similar fashion, while albumin fragmentation was extensive only at early time points. In addition, samples were re-analysed up to 3 years after collection with similar results.
obtained. Albumin fragmentation was also independent of the age of animals. Most experiments were performed with mice aged 6 to 10 weeks, while similar patterns of albumin fragmentation during wound healing were observed in animals aged 8 months. However, experiments were only performed on female mice, so that confirmatory experiments with some male animals may have been warranted if time permitted.

There was no significant fragmentation of either ceruloplasmin or transferrin, although levels of these two proteins appeared to be higher early during wound healing, indicative of an APR. There was apparent dissociation of both haptoglobin and IgG during wound healing and in control tissues. However, levels of this dissociation did not change significantly over time or between control tissues, so that a clear correlation between dissociation of haptoglobin or IgG with events in tissues was not established.

Extensive fragmentation of albumin was observed in granulation tissue extracts from both wound healing models, with increased albumin fragmentation during times of high vascularity, and reduced albumin fragmentation associated with decreased vascularity. Importantly, the different time course of vascularity between scarring and adipogenic wounds corresponded to the changing levels of albumin fragmentation in these two model systems. Four major albumin fragments were identified of approximate molecular masses of 49 kDa, 39 kDa, 27.5 kDa and 17.5 kDa. From these sizes, it seems likely that the bands identified represent the products of two discrete cleavage sites in albumin, producing either 49 kDa and 17.5 kDa fragments, or 39 kDa and 27.5 kDa fragments.

Although overall albumin fragmentation reduced over time in both wound healing models, production of the 49 kDa fragment appeared independent of this in
Plasma Proteins in Wounds
Chapter III Page 51

adipogenic wounds, suggesting that this fragment, and or its possible 17.5 kDa cleavage counterpart, may not have an important role during wound healing. On balance, this data supports a strong correlation between albumin fragmentation and tissue vascularity over time during wound healing.

There was also varied albumin fragmentation in tissue extracts from sites separate to wound healing, and once again the 49 kDa fragment appeared independent of events during healing. The smaller albumin fragments were, however, in general more prevalent at weeks 2 to 4 in both wound healing models studied, suggesting the possible role of these tissues in metabolic processing and removal of these albumin fragments from the body. Unfortunately, these observations were unexpected and because collection of larger numbers of control tissues was not performed, this possibility could not be further probed in the current study. However, it would be interesting to further investigate the possible metabolic processing of albumin fragments by different tissues in further experiments using radioactively labelled albumin.

Injury increases catabolism of proteins (Powanda and Moyer, 1981; Biolo et al., 1997) while fragmentation of VEGF (Lauer et al., 2000) and insulin (Shearer et al., 1997) has been detected in wounds. Catabolism of proteins during wound healing has been thought to provide a source of amino acids for the synthesis of new proteins (Jeffay, 1960; Powanda and Moyer, 1981) and this could be related to data presented in this Chapter demonstrating extensive fragmentation of albumin during wound healing. It has been thought that the mechanism of albumin degradation involves uptake of the protein into lysosomes after initial binding to EC scavenger receptors such as gp18 and gp31 (Schnitzer and Oh, 1994). Albumin catabolism in rat kidney cortical lysosomes has
been described and been attributed to the actions of aspartic and cysteine proteinases, perhaps accounting for some of the albumin fragmentation observed in kidney tissues in the current study (Baricos et al., 1987), while chymase, secreted by mast cells, is also able to degrade albumin (Raymond et al., 2003). Fibroblasts have also been found to be capable of albumin catabolism in skin and muscle (Strobel et al., 1986) and it is possible that fibroblasts present in wounds may have a role in albumin fragmentation. Also, there are numerous proteinases present during wound healing (Barrick et al., 1999) which may generate albumin fragments and it would be interesting to determine the identity of the proteinase(s) involved in albumin fragmentation. Reduced vascular leakage during maturation of reparative granulation tissue, accompanied by decreased proteinases may contribute to reduced albumin fragmentation observed later in wound healing.

Albumin fragments have been detected in other tissues and circumstances, such as in brain oedema following trauma (Bodsch and Hossmann, 1983; Liu and Sturner, 1988), supporting a role for albumin fragmentation during wound healing. Interestingly with regard to the current work, albumin fragments have also been detected in serum and urine with molecular masses similar to the albumin fragments detected in this study (Kshirsagar et al., 1984; Wiggins et al., 1985). It seems possible that these fragments reported as present in urine, and perhaps identical to those seen in kidney tissues in the current study, represent a mechanism for removal of albumin fragments leaking into the circulation from sites of wound healing. Also, because the albumin fragments reported in serum have been determined as comprising less than 2 % of the total plasma albumin, it seems likely that under normal circumstances there is only a low level of albumin fragmentation occurring in tissues.
Plasma Proteins in Wounds
Chapter III Page 53

The importance of serum albumin as an indicator of good clinical outcome has been extensively reported (Margarson and Soni, 1998; Don and Kaysen, 2004; Prinsen and de Sain-van der Velden, 2004), especially for cardiovascular disease (Schilling et al., 2004) while beneficial effects of albumin therapy have been described (Liu et al., 2001; Belayev et al., 2002). Of relevance to the current study, albumin concentration has been used to predict clinical outcome for healing and non-healing wounds, with poor outcomes correlating with low serum albumin concentrations (James et al., 2000).

Albumin is a specific anti-apoptotic factor for endothelium (Zoellner et al., 1996b; Zoellner et al., 1999) and the correlation between vascularity during wound healing and albumin fragmentation suggests a possible role for albumin fragments in the control of tissue vascularity. As will be discussed in more detail in Chapter 4, it is possible that albumin fragmentation exposes a cryptic site with potent anti-apoptotic activity for endothelium, and that this is the biological significance of the albumin fragmentation seen in the current study. If this were the case, it is possible that albumin leaking out of newly formed vessels in early reparative granulation tissue is cleaved by one or more of the many proteinases present during wound healing to produce anti-apoptotic fragments for endothelium. These fragments would have a role in maintaining the high vascularity of early reparative granulation tissue, while with time, maturation of newly formed leaky vessels to vessels with the normally low permeability to protein would greatly reduce levels of the native albumin substrate in the extravascular compartment. Simultaneous to this, reduced proteinase levels would also be expected in maturing granulation tissue and the combined effect of reduced extravascular albumin substrate and reduced proteinase enzyme would reduce levels of anti-apoptotic albumin
Plasma Proteins in Wounds
Chapter III Page 54

fragment products with the effect of increased endothelial apoptosis and reduced vascularity in maturing wounds. In this way, it is possible that the fragmentation of albumin seen in the current study is a fundamental mechanism controlling levels of vascularity during wound healing.

An unfortunate limitation of the current study is the absence of data confirming the identity of bands detected by Western blotting as representing albumin fragments. Attempts were made in a series of preliminary experiments to separate native albumin from smaller molecular mass proteins by dialysis across centicon membranes, but unfortunately this was ineffective and resulted in depletion of the bands of interest. Once having separated native protein from smaller molecular mass forms, it was intended to use an immuno-precipitation or an immuno-affinity column chromatography approach to purify fragments and further characterise these by mass spectrometry and sequencing. This would have permitted both confirmation of the identity of fragments as well as provided information regarding possible proteolytic mechanisms responsible for formation of the fragments in wounds. It is unfortunate that time limitations in the current thesis prevented sufficient progress of this aspect of the work using an alternative approach of native gel electrophoresis to first separate native from fragmented material before further analysis. With regard to the possible role of albumin fragments in maintaining tissue vascularity by inhibiting endothelial apoptosis discussed in Chapter 4, it would be interesting to examine purified wound albumin fragments for anti-apoptotic activity in endothelium.
Chapter IV

Effect of Fragmentation of Serum Albumin Upon the Anti-Apoptotic Activity for Endothelium
IV.1. Introduction

Observations in Chapter 3 of this thesis revealed extensive fragmentation of albumin in healing wounds, with levels of fragmentation correlating with tissue vascularity. This Chapter investigates the possible relationship between albumin fragmentation and endothelial apoptosis.

IV.1.i. General Characteristics of Albumin

Serum albumin has a molecular mass 66.5 kDa and is the most abundant plasma protein with a mean concentration of approximately 600 µM and levels ranging from 470 µM to 750 µM. The albumin gene is located on chromosome 4 while the protein is synthesised in the liver. The protein consists of a single polypeptide chain of 585 amino acids and has a remarkably low tryptophan content such that HSA contains only one tryptophan residue, while BSA contains two. Albumin also has many cysteine residues so that of the 35 cysteines, 17 form disulfide bonds and the remaining cysteine (Cys34) is free and available as a reactive sulfhydryl group. In circulating albumin, approximately 30% of Cys34 is oxidised by glutathione and cysteine, or occupied in forming albumin dimers, while albumin containing free Cys34 is termed “mecaptalbumin” (Carter and Ho, 1994; Peters, 1996).

The secondary structure of HSA has 9 double loops, and these are grouped into three homologous domains numbered I, II, III. Each domain contains two long loops, separated by a short loop, and is further divided into two subdomains (Ia, Ib, IIA, IIB, IIIA, IIIB) (Figure 4.1) (Peters, 1996). The three dimensional structure of HSA,
determined by X-ray crystallography, is heart shaped (He and Carter, 1992), and the majority of the protein structure is α-helical (67%) while the remainder is comprised of β-turns and extended chain (He and Carter, 1992; Carter and Ho, 1994).

HSA is very flexible so that it displays a high level of intramolecular movement. Conformational variants emerge with changing pH (Carter and Ho, 1994; Peters, 1996; Margarson and Soni, 1998), so that at neutral pH, an N form of the protein is defined and appears the most stable (Hvidt and Wallevik, 1972) with a structure likely to resemble that described from crystallographic studies (Ferrer et al., 2001). At low pH, reversible unfolding of the protein occurs to obtain the conformations designated F and E, which have reduced α-helicity and are associated with increased viscosity of protein solutions (Carter and Ho, 1994; Muzammil et al., 1999; Dockal et al., 2000). At high pH, the B and A conformations of the protein occur and these conformational forms are thought to more readily bind some ligands (Peters, 1996).

Despite these structural changes, the protein is very stable so that denaturation only occurs in dramatically non-physiological environments such as urea or guanidinium chloride (Peters, 1996). Reduction of disulfide bonds and denaturation results in loss of α-helical structure and ligand binding ability (Johanson et al., 1981). However, albumin may refold into its native state after reduction in the presence of salts (Teale and Benjamin, 1976), while fragments of albumin are able to regain native configuration much more quickly than native albumin, due to the reduced number of possible disulfide bond recombinations (Johanson et al., 1981).
Albumin Fragmentation and Anti-apoptotic Activity
Chapter IV Page 3

IV.1.ii. Fragmentation of Albumin

Fragmentation of albumin has been used to study the amino acid sequence, pattern of disulfide bonds, ligand binding and other functional properties of the protein (Peters, 1996). Fragmentation may be achieved using many enzymes and chemicals, so that trypsin at high pH, for example, yields two fragments of molecular masses 51 kDa and 18.5 kDa (Markus et al., 1967) and other enzymes that digest albumin include pepsin, chymotrypsin and pronase (Liu et al., 1967; Das et al., 1970; Heaney-Kieras and King, 1977; Sagisaka et al., 1978; Ledden et al., 1982; Bos et al., 1988). Chymase, secreted by mast cells, can also fragment albumin (Raymond et al., 2003) while lysosomal proteinases, such as cathepsin D, fragment albumin at low pH and may mediate albumin catabolism in vivo (Mego, 1984).

Cyanogen bromide (CNBr) oxidises methionine residues to cleave proteins, and in the case of unreduced HSA, generates three fragments having molecular masses of 14 kDa (residues 1-123), 20 kDa (residues 124-298) and 32.5 kDa (residues 299-585) (McMenamy et al., 1971). Partial cleavage of Met123 results in a 34 kDa fragment, comprised of the 14 kDa and 20 kDa fragments combined (Doyen et al., 1982). Reduction of disulfide bonds reveals further internal CNBr cleavage sites in the 14 kDa and 32.5 kDa fragments to yield a total of seven CNBr HSA fragments (McMenamy et al., 1971). CNBr fragmentation of HSA leaves the secondary structure of the protein relatively unchanged as assessed by measuring changes in α-helicity by circular dichroism (Sjoholm and Ljungstedt, 1973; Compagnini et al., 1993).
IV.1.iii. Functions of Albumin

IV.1.iii.a. Ligand Binding

Albumin is thought to mainly function in ligand binding and transport. The most strongly bound ligands are long chain fatty acids, hematin and bilirubin. Other compounds that bind to albumin include; tryptophan, ascorbic acid, phospholipids, bile acids, steroids and metal ions such as copper and zinc. The primary long chain fatty acid binding site is in loop 8 in Domain III, while secondary long chain fatty acid binding sites are found in loop 6 in Domain II and in loops 2 and 3 in Domain I. The primary binding site for bilirubin is within loop 4 in subdomain IIA (Reed, 1986; Hamilton et al., 1991; Peters, 1996), while medium chain fatty acids bind to albumin in subdomain IIIA (Lee and McMenamy, 1980). Metal ions, such as copper, bind at the N-terminus of the protein (Peters, 1996), and many drugs bind at either Sudlow Site I, in subdomain IIA or at Sudlow Site II, located in subdomain IIIA (Sudlow et al., 1975).

IV.1.iii.b. The Effect of Albumin Upon Cells

Albumin is responsible for 80 % of the colloid osmotic pressure of plasma and also acts as a pH buffer in serum (Peters, 1996; Nicholson et al., 2000). Interestingly, albumin has some direct effect upon cell function. For example, HSA enhances mRNA expression for VEGF in cultured human luteinizing granulosa cells (Doldi et al., 1999). Physiological concentrations of BSA inhibit TNF-α induced upregulation of VCAM-1 expression by human aortic EC and this reduces monocyte adhesion (Zhang and Frei, 2002). Separately, albumin affects vascular smooth muscle metabolism by stimulating
glucose oxidase and oxygen consumption (Barron et al., 2000). HSA can increase cholesterol efflux in certain EC cell lines (Ha et al., 2003) and also increases IL-8 production by renal proximal tubular epithelial cells (Tang et al., 2003). Albumin can stimulate proliferation of opossum kidney cells (Dixon and Brunskill, 2000) and astrocytes (Nadal et al., 1995).

IV.1.iii.c. Albumin Acts as an Antioxidant

Albumin has potent antioxidant activity through its free thiol group, Cys34 (Halliwell, 1988; Halliwell and Gutteridge, 1990; Peters, 1996). The antioxidant activity of albumin has been demonstrated to protect many cells types from free radicals and oxidative stress, including chronic lymphocytic leukaemia cells (Moran et al., 2002), macrophages and renal tubular epithelial cells (Iglesias et al., 1999). Albumin can scavenge free radicals either by; interacting with them directly through Cys34 (Soriani et al., 1994; Iglesias et al., 1999; Kouoh et al., 1999; Bourdon et al., 2005), increasing cellular glutathione (Cha and Kim, 1996; Cantin et al., 2000), binding metal ions to make them less available to participate in radical-generating reactions (Halliwell and Gutteridge, 1990; Bourdon et al., 1999; Gabaldon, 2002; Gryzunov et al., 2003), or acting as a sacrificial antioxidant (Halliwell, 1988). Please note that the antioxidant activity of albumin will be discussed in more detail in Chapter 5.

IV.1.iii.d. Albumin as a Predictor of Clinical Outcome

Albumin concentration is a predictor of clinical outcome in patients with subclinical atherosclerosis or peripheral artery disease (Schillinger et al., 2004),
supporting a possible vasculo-protective activity for the protein. Also, albumin concentration correlates with improved healing outcomes in patients with chronic wounds, with poor wound healing occurring when albumin concentrations in wound exudate are less than 20 g/l (James et al., 2000). A beneficial effect of albumin therapy has also been described, with good clinical outcomes observed with albumin administration in patients after subarachnoid haemorrhage (Suarez et al., 2004), trauma (Osband et al., 2004b) and acute ischemic stroke (Liu et al., 2001; Belayev et al., 2002).

IV.1.iii.e. Albumin Promotes Cell Survival

Albumin appears protective for many cell types. Recombinant albumin promotes survival for bovine oocytes during fertilisation and development (Lane et al., 2003) and protects against gut-induced lung injury (Osband et al., 2004a), while albumin increases synthesis and release of glutamate and promotes neuronal survival (Tabernero et al., 2002). Albumin also acts as a survival factor for human lymphocytes (Spieker-Polet and Polet, 1976) and chronic lymphocytic leukaemia cells through activation of the Akt pathway (Jones et al., 2003), and also protects human hepatoma HEP3B cells from retinoic acid induced apoptosis (Hsu et al., 1998). Importantly for this thesis, albumin inhibits endothelial apoptosis in isolated culture as well as in tissue explants (Zoellner et al., 1996b; Zoellner et al., 1999).

IV.1.iv. Albumin Inhibits Endothelial Cell Apoptosis

Serum deprivation induces apoptosis in cultured human EC, and albumin strongly inhibits this (Zoellner et al., 1996b). Maximal activity is observed at
physiological concentrations and native conformation of the protein is essential as
denaturation of the protein with dithiothreitol or mercaptoethanol destroys activity. This
activity is identical in albumin preparations from many sources as well as albumin from
bovine and human species and is not due to bound contaminants as recombinant human
albumin, raised in yeast, has identical activity. Also, the anti-apoptotic activity of
albumin is not due to a non-specific protein effect as an unrelated protein, ovalbumin, is
not protective (Zoellner et al., 1996b).

Fragmentation of HSA by CNBr has no reported effect upon activity at maximal
concentrations, while CNBr fragmentation of BSA partially reduces the anti-apoptotic
activity (Zoellner et al., 1996b). The difference between the activities of CNBr cleaved
HSA and BSA may be due to differing cleavage sites between the two species of
albumin, as BSA contains an additional cleavage site for CNBr at Met184 that is not
represented in the human protein (Peters, 1996). The anti-apoptotic activity of albumin
for EC has also been demonstrated in rat skin and human gingival tissue explants with
maximal protection observed at physiological concentrations (Zoellner et al., 1999).
This data supports a protective role of albumin for maintaining EC in vivo and is also
consistent with the apparent clinically vasculo-protective activity of albumin (Schillinger
et al., 2004).

IV.1.v. Proposal of a Cryptic Active Anti-apoptotic Site in Albumin

An interesting difficulty in understanding the specificity of albumin in protecting
endothelium from apoptosis is that the high protein levels required to achieve maximal
protection (Zoellner et al., 1996b; Zoellner et al., 1999) seem inconsistent with the
presence of a specific high-affinity binding site in the protein, and thus argue for a non-specific protective mechanism. At an earlier time, preliminary data was outlined suggesting that the active site in albumin may be cryptic and exposed only by transient conformational change, such that very high concentrations of native protein would be required in order to achieve low concentrations of the transient conformational molecular sub-population with the active high-affinity binding site exposed (Zoellner et al., 2000).

One prediction from this model is that reduced intramolecular movement in albumin should inhibit anti-apoptotic activity, and this appears to be the case as HSA which has been subjected to high levels of non-enzymatic glycosylation is inactive and also has greatly reduced intra-molecular movement as demonstrated in earlier published work (Zoellner et al., 2001). A second prediction is that fragmentation of the protein should expose the anti-apoptotic site and this idea is supported in this Chapter where experiments are described demonstrating that CNBr fragmentation of HSA significantly increases anti-apoptotic activity.

Since albumin fragmentation results in greatly increased anti-apoptotic activity, the presence of albumin fragments in granulation tissues at times when tissue vascularity is exceptionally high (Chapter 3) raises the possibility that these fragments may be important in maintaining the high vascularity of early granulation tissue, and also supports a vasculo-protective role for albumin during wound healing and inflammation.
Figure 4.1: Amino acid sequence of HSA derived from the cDNA sequence, demonstrating the secondary structure of the protein and position of the 17 disulfide bonds. The secondary structure has 9 double loops and these are grouped into three homologous domains, with each domain containing two long loops, separated by a short loop (Peters, 1996).
IV.2. Materials and Methods

IV.2.i. Materials

Materials for SDS-PAGE and silver staining were as previously described in III.2.i. Cell culture plasticware was from Costar (Cambridge, USA), while culture medium 199 (M199), bovine calf serum and L-glutamine were all from JRH Biosciences (Lenexa, USA). Penicillin and streptomycin were purchased from CSL Biosciences (Parkville, USA) and amphotericin B, Thermax cell culture coverslips and BSA were all from ICN Biomedicals (Ohio, USA). Endothelial cell growth supplement (ECGS) was purchased from Upstate (Charlottesville, USA), while heparin was from Pharmacia and UpJohn (Perth, Western Australia, Australia). RNAase A, proteinase K, DNA molecular mass markers (MW XI) and zVAD-FMK were all purchased from Roche (Basel, Switzerland). HSA was purchased from Calbiochem (San Diego, USA) and also from Sigma (St Louis, USA). Phenol was obtained from Ajax Chemicals (Auburn, NSW, Australia), while analytical grade ethanol and methanol were from Biolab (Clayton, Vic, Australia). HiTrap™ Blue Column (5 ml) and PD10 columns were from Amersham Biosciences (Piscataway, USA). Goat anti-rabbit HRP antiserum was from DAKO (Carpinteria, USA) while Streptavidin-HRP was purchased from Biosource (Camarillo, USA). Spectra/Por dialysis tubing of molecular mass cut-off of 3.5 kDa was purchased from Spectrum Laboratories (Rancho Dominguez, USA), while Diaflow ultrafiltration membranes for pressure dialysis (molecular mass cut-off 3 kDa) were from Amicon, Inc. (Beverly, USA). GelCode Blue Stain Reagent was purchased from Pierce (Rockford, USA), while aquamount was from Fronine Pty Ltd (Riverstone, NSW,
Albumin Fragmentation and Anti-apoptotic Activity
Chapter IV Page 12

Australia. CNBr, 3-3’-Diaminobenzidine Tetrachloride (DAB), collagenase, ethidium bromide, Hanks Balanced Salt Solution (HBSS), and biotin labelled *Ulex europaeus* Lectin 1 (UEA-1) as well as all other reagents were from Sigma (St Louis, USA).

IV.2.ii. Isolation of Human Umbilical Vein Endothelial Cells

Ethical approval for collection and use of human tissues was obtained by the Westmead Hospital Human Research Ethics Committee (HREC 95/6/4.14). HUVEC were isolated by collagenase perfusion of umbilical cords, obtained with informed consent from the Westmead Hospital Maternity Unit, Westmead Hospital, Westmead, Australia as previously described (Jaffe *et al.*, 1973; Zoellner *et al.*, 1996a; Zoellner *et al.*, 1996b). Briefly, umbilical cords were collected in HBSS with penicillin (100 μg/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ml) as antibiotics. Cords were washed with HBSS before veins were cannulated and flushed with warmed HBSS (100 ml). 10 ml volumes of filtered collagenase (1 mg/ml in HBSS) were then incubated within veins for 30 to 40 minutes at room temperature to allow detachment of HUVEC from the vessel lining. Veins were then flushed with HBSS (100 ml) and detached cells collected into centrifuge tubes containing BCS (10 ml). Cells were then pelleted by centrifugation at 1000 rpm for 6 minutes at 4°C. Cell pellets were then resuspended in filtered complete medium (CM) comprising of M199 with BCS (20 %), heparin (30 U/ml) and ECGS (45 μg/ml). Penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ml) were used as antibiotics. Cells were cultured on gelatin-coated (0.1 % in PBS) tissue culture flasks and maintained in a humidified incubator at 37°C under 5 % CO₂.
IV.2.iii. Culture of HUVEC

HUVEC were grown to confluence before being passaged at a split ratio of 1:3. For passaging, cells were washed with M199 before detachment by trypsin/EDTA. Detached cells were then transferred to BCS (20 %) to neutralise trypsin and flasks were washed with M199 to collect remaining cells which were then added to those earlier collected. HUVEC were pelleted by centrifugation at 1000 rpm for 6 minutes at 4°C before resuspension in filtered CM. Cells were cultured in fresh gelatin-coated (0.1 % in PBS) tissue culture flasks under conditions described in IV.2.ii and were grown to fourth passage before freezing cells in CM with DMSO (10 %) and storage in liquid nitrogen. Experiments were performed with HUVEC in fifth passage.

IV.2.iv. Verification of Cultured Cells as HUVEC

The identity of cells was confirmed on the basis of morphology as well as by immunohistochemistry for UEA-1 binding and Factor VIII associated antigen as previously described (Jaffe et al., 1973; Holthofer et al., 1982; Zoellner et al., 1996a; Zoellner et al., 1996b).

For immunohistochemistry, HUVEC were grown to confluence before detachment by trypsinisation and seeding onto gelatin-coated (0.1 % in PBS) tissue culture coverslips in 24 well tissue culture plates. After attaching overnight, media was removed and cells were washed with M199 before fixation of HUVEC with ice-cold methanol at -20°C for 5 minutes. Cells were then air dried for storage until labelling, at which time cells were rehydrated in PBS for 10 minutes before endogenous peroxidase was blocked with H₂O₂ (3 %) for 10 minutes. Cells were washed with PBS before
incubation with vehicle comprising PBS with BCS (10 %), and Tween-20 (0.5 %) for 20 minutes, after which primary biotinylated lectin (UEA-1) or antibody (Factor VIII associated antigen) were applied (1:100 in vehicle) for 45 minutes. Cells were then washed in PBS with Tween-20 (0.5 %), three times for 10 minutes each. Streptavidin-HRP (1:100 in vehicle) was used to detected biotinylated primary lectin, while goat anti-rabbit HRP antiserum (1:100 in vehicle) was used to detected primary antibodies for Factor VIII associated antigen for 45 minutes. Cells were washed again in PBS with Tween-20 (0.5 %), three times for 10 minutes each before detection of bound HRP using DAB solution (PBS with DAB (220 μg/ml) and 3 % H₂O₂) from 5 minutes to 1 hour until development of signal. Cells were washed in PBS before counterstaining with haematoxylin. Coverslips were mounted onto glass slides using Aquamount for microscopy. Negative controls were performed in the absence of primary antibodies or lectin.

IV.2.v. Experimental Conditions for Studying Apoptosis

HUVEC were grown to confluence before detachment by trypsinisation and seeding into gelatin-coated 24 or 96 well tissue culture plates. Cells were stimulated in quadruplicate for up to 24 hours with BCS (20 %), albumin (HSA, CNBr HSA, BSA or CNBr BSA) or M199 alone. ECGS and heparin were excluded from experiments as these agents affect cell survival (Zoellner et al., 1996b). HUVEC rapidly detached during apoptosis, permitting quantitation of apoptosis by determination of remaining adherent cell number (Zoellner et al., 1996b). Apoptosis was quantitated indirectly by haemocytometer counts of remaining adherent cells, harvested by trypsin/EDTA
treatment, as a measure of cell death. Cell counts were expressed using the Apoptosis Protection Index as defined by Zoellner et al., (1996b), where the number of remaining adherent cells after 24 hours in each condition is expressed as a percentage of surviving cells at 24 hours maintained with BCS (20 %) (Zoellner et al., 1996b; Emmanuel et al., 2002).

IV.2.vi. Confirmation of Apoptosis

Apoptosis was confirmed by the presence of a sub-diploid population typical of apoptosis seen by FACS analysis (Darzynkiewicz et al., 1992), and the characteristic internucleosomal DNA fragmentation as seen by DNA gel electrophoresis (Nagata, 2000) using a modification of the method described by Smith et al., (1989) (Smith et al., 1989; Zoellner et al., 1996b; Emmanuel et al., 2002). In addition, involvement of caspases during apoptosis was confirmed by using the general caspase inhibitor zVAD-FMK (Granville et al., 1999; King et al., 2003).

IV.2.vi.a. FACS Analysis

Confluent HUVEC in 25 cm² flasks were deprived of serum by culture in M199 alone for 16 hours. Floating cells were harvested by washing while adherent cells were released with trypsin/EDTA and combined with the floating population. Cells were pelleted by centrifugation at 2000 rpm for 6 minutes at 4°C and then resuspended in HBSS (1 ml). Cells were then fixed by adding the cell suspension drop wise to 7 ml of ice-cold ethanol (70 %) with constant mixing to obtain a single cell suspension. Fixed cells were stored at -20°C overnight before collection by centrifugation at 2000 rpm for
10 minutes at 4°C. Supernatants were discarded and pellets air dried for at least 30 minutes before resuspension in HBSS (500 µl) with propidium iodide (50 µg/ml), RNAase A (0.1 mg/ml), sodium citrate (0.1 % w/v) and Triton-X 100 (0.1 %). Samples were stored in the dark for between 20 and 24 hours before analysis using a Becton Dickinson FACScan Device machine and Cell Quest software (version 3.2.1 f1). Data was then further analysed using Windows Multiple Document Interface (WinMDI) Version 2.8 (http://facs.scripps.edu/software.html).

IV.2.vi.b. DNA Gel Electrophoresis

Confluent HUVEC in 225 cm² flasks were deprived of serum for 8 hours by culture in M199 alone. Floating cells were harvested by washing and kept separate from adherent cells collected after release with trypsin/EDTA. Both populations of cells were pelleted by centrifugation at 2000 rpm for 6 minutes at 4°C and cell pellets resuspended in sterile TBS (1 ml) before transfer to sterile 1.5 ml Eppendorf tubes. Cells were then centrifuged at 2000 rpm for 5 minutes at 4°C and pellets resuspended in a lysis buffer consisting of Tris-HCl (50 mM), EDTA (10 mM), SDS (0.5 %) and RNAase A (0.1 mg/ml) with 50 µl of lysis buffer added to floating cells, while 500 µl of lysis buffer was added to released adherent cells. Samples were then incubated for 1 hour at 37°C before addition of proteinase K (0.5 mg/ml) which was followed by further incubation at 55°C for at least 3 hours. The volume of samples was made up to 500 µl with TE buffer (Tris-HCl 10 mM, EDTA 1 mM), before phenol extraction of DNA. Sodium acetate (0.3 M) was added to samples before DNA was precipitated by ice-cold ethanol (100 %). Samples were then mixed by inversion and incubated at -80°C for at least 30 minutes.
after which DNA was pelleted by centrifugation at 13,000 rpm for 20 minutes at 4°C. 100 µl of ice-cold ethanol (70 %) was added to pellets before pelleting at 13,000 rpm for 15 minutes at 4°C. Pellets were allowed to air dry for 30 minutes at room temperature before DNA was resuspended with TE buffer (15 µl) and loading buffer (15 µl) consisting of glycerol (30 %) and bromophenol blue (0.25 %). Molecular mass DNA standards (MW XI) were also suspended in TE and loading buffer. DNA was then subjected to electrophoresis in agarose gels (2 % in TAE with 0.5 % ethidium bromide) at 75 V for 30 to 45 minutes and DNA bands visualised by UV transillumination using the GeneGenius gel documentation system.

IV.2.vi.c. Inhibition of Caspases by zVAD-FMK

Confluent HUVEC in 96 well tissue culture plates were cultured in triplicate for up to 24 hours with BCS (20 %), HSA (600 µM) or M199 either in the presence or absence of zVAD-FMK (150 µM). Quantitation of apoptosis was by haemocytometer counts of remaining adherent cells and expressed using the Apoptosis Protection Index (IV.2.v. and (Zoellner et al., 1996b)).

IV.2.vii. Fragmentation of Albumin by CNBr

Both HSA and BSA were fragmented by CNBr as previously described (McMenamy et al., 1971). Briefly, albumin (1 g) was dissolved in 70 % formic acid (30 ml) before addition of CNBr (0.5 g). Air was displaced from the reaction tubes with N₂ gas before sealing tubes with parafilm and reactions were allowed to proceed for 18 to 24 hours in the dark. CNBr albumin was then dialysed into PBS using Spectra/Por
dialysis membranes with a molecular mass cut off of 3.5 kDa and using at least six changes of 400 ml. CNBr albumin solution was then kept in PBS for chromatography or dialysed into M199 with antibiotics for cell culture before concentration by pressure dialysis with an Amersham pressure dialysis cell (Amersham Biosciences, Piscataway, USA) using 3 kDa molecular mass cut-off pressure dialysis membranes. The concentration of CNBr albumin in M199 was determined by measuring the optical density at 280 nm and calculation of protein concentration based on OD$_{280}$ HSA (1 mg/ml = 0.531) and BSA (1 mg/ml = 0.667) (Peters, 1996).

IV.2.viii. SDS-PAGE

Fragmentation of albumin by CNBr was confirmed by SDS-PAGE and silver staining as described in III.2.vi. Coomassie staining was performed using GelCode Blue Stain Reagent.

IV.2.ix. Separation of CNBr HSA by Protein-Dye Affinity Chromatography

Partial separation of CNBr HSA fragments was achieved by protein-dye affinity chromatography using a HiTrap™ Blue column (5 ml). This column contains the dye ligand Cibacron Blue F3GA covalently attached to Sepharose high performance matrix, and chromatography was performed using a Biologic HR medium pressure liquid chromatography system (BioRad, Hercules, USA).

CNBr HSA was exchanged into KH$_2$PO$_4$ (50 mM, pH 7.0) using a PD10 column. The HiTrap™ Blue column was equilibrated with KH$_2$PO$_4$ (50 mM, pH 7.0) at a flow rate of 2 ml/min before application of sample. This was followed by isocratic flow of
KH₂PO₄ (50 mM, pH 7.0) for 10 ml before application of a linear gradient ranging from 0 to 4 M KCl in KH₂PO₄ (50 mM, pH 7.0) over 80 ml, after which an isocratic flow of KCl (4 M) in KH₂PO₄ (50 mM, pH 7.0) was applied for 25 ml. Fractions (1 ml) were collected during the entire run, and protein was detected at 214 nm. Please note that the 214 nm wavelength was used rather than the more usual 280 nm as only one CNBr HSA fragment contains Trp and is thus able to absorb significantly at 280 nm. Fractions representing separate peaks were pooled and concentrated by pressure dialysis before further dialysis into M199 with antibiotics for cell culture experiments. Concentrations of fragments in M199 were determined using the Bradford assay as previously described in III.2.v.

IV.2.x. Statistical Analysis

Data was expressed as means ± standard deviation. Statistical significance within experiments was determined using the two-tailed Student’s T-Test, while data over multiple experiments was analysed using the Wilcoxon’s Ranked Signed Test, with $P$ values of $< 0.05$ considered significant.
IV.3. Results

IV.3.i. Cells Isolated from Umbilical Veins Were Endothelial in Origin

Cells isolated from umbilical veins formed a monolayer with cobblestone morphology, characteristic of endothelium (Figure 4.2). Cells also displayed UEA-1 binding and the presence of Factor VIII associated antigen, characteristic of cultured HUVEC (Figure 4.3), confirming the endothelial identity of cultured cells.

IV.3.ii. HUVEC Death Was Due to Apoptosis

HUVEC cultured in M199 in the absence of serum rapidly detached to form gaps in the monolayer. It was important to confirm that this was due to apoptosis and not necrosis or some other process. Apoptosis was confirmed by assessment of the sub-diploid peak by FACS analysis, intermucleosomal DNA fragmentation by DNA gel electrophoresis and also dependence upon caspase activation by use of the general caspase inhibitor zVAD-FMK.

As seen in Figure 4.4A, FACS analysis on combined adherent and floating populations of serum deprived HUVEC demonstrated the presence of the subdiploid population, typical of apoptosis. Figure 4.4.B shows a DNA gel electrophoretic preparation which revealed the presence of a 180 bp DNA ladder in material collected from the floating population as well as the almost exclusive presence of only high molecular mass DNA in adherent cells. Figure 4.5 demonstrated the effect of HSA in protecting HUVEC in serum free conditions (p < 0.0001). In addition, the caspase inhibitor zVAD-FMK clearly increased HUVEC survival in the absence of serum or
Albumin Fragmentation and Anti-apoptotic Activity
Chapter IV Page 21

HSA (p < 0.01), supporting the conclusion that HUVEC death in conditions of serum deprivation is by apoptosis. This, together with the presence of the sub-diploid peak and internucleosomal DNA fragmentation, confirm HUVEC apoptosis in response to serum deprivation as well as inhibition of this by HSA.

IV.3.iii. CNBr Fragmentation of HSA Increased the Anti-apoptotic Activity for Endothelium

To investigate the possibility that the active site of albumin is cryptic and exposed by fragmentation of the protein, experiments were performed in which HSA was fragmented by CNBr and the effect of this upon HUVEC survival determined in dose response experiments. Figure 4.6 shows a silver stained SDS-PAGE gel of HSA and CNBr treated HSA. Native (66 kDa) and dimer (130 kDa) forms of the protein were observed prior to fragmentation while CNBr treatment produced three fragments of the expected molecular masses of 32.5 kDa, 20 kDa and 14 kDa. Successful near complete fragmentation of HSA was demonstrated by the presence of only extremely weak native and dimer bands of albumin. A minor band at 28 kDa was noted, suggestive of the dimer form of the 14 kDa fragment held together by Cys-Cys disulfide formation, as well as a band at 34 kDa, consistent with incomplete separation of the 14 kDa and 20 kDa fragments.

Figure 4.7 demonstrates the effect of CNBr fragmentation upon the anti-apoptotic activity of HSA for endothelium. A clear increase in activity was demonstrated by a significant left-shift in the dose response of CNBr fragmented HSA compared with native HSA, consistent with exposure of a cryptic anti-apoptotic site (p < 0.01). The
magnitude of this shift indicated an approximate 100 fold increase in activity, and similar results was obtained using several different preparations of CNBr HSA, as well as CNBr fragmented recombinant HSA. However the increase in anti-apoptotic activity varied between these preparations and ranged from 16 to 100 fold.

IV.3.iv. Partial Separation of CNBr HSA Fragments Using Cibacron Blue Affinity Chromatography

In order to identify which CNBr HSA fragment contains the active anti-apoptotic site, separation of CNBr HSA was attempted using various methods (Appendix A.1). Amongst the approaches used, protein-dye affinity chromatography proved to be the most successful. As seen in Figure 4.8A, application of CNBr HSA onto a HiTrapTM Blue column containing Cibacron Blue F3GA dye resulted in binding of most of the protein with only a small amount of protein failing to bind and eluting in Peak 1. The remainder of the protein was eluted using a linear gradient of 0 to 4 M KCl, as two overlapping peaks 2 and 3. Analysis of the eluted peaks by SDS-PAGE (Figure 4.8B) revealed Peak 1 to be primarily the 14 kDa fragment while Peaks 2 and 3 were a combination of the 20 kDa and 32.5 kDa fragments. An additional minor band at 28 kDa was also present in Peak 1 and was interpreted as the dimerised form of the 14 kDa fragment through Cys34. In Peaks 2 and 3, an additional band of 52 kDa was also observed, and is suggested as incomplete cleavage product. The 20 kDa and 32.5 kDa fragments were not separated so that it was only possible to determine the biological activities of these two fragments combined. It was interesting to note that the 32.5 kDa
fragment appeared as triplicate band in SDS-PAGE, suggestive of the emergence of at least 3 conformational variants.

The biological activity of the partially separated fragments was determined in dose response experiments with cultured HUVEC as seen in Figure 4.9. The 14 kDa fragment was not protective against HUVEC apoptosis at any of the concentrations used, while the combined 20 kDa and 32.5 kDa fragments retained anti-apoptotic activity and were more active than native HSA as demonstrated by a left shift in the dose response.

Data indicate that the anti-apoptotic activity of HSA for endothelium is separable into specific fragments and resides in either the 20 kDa or 32.5 kDa CNBr HSA fragments, but not in the Cys34 bearing 14 kDa fragment.

IV.3.v. Effect of CNBr Fragmentation Upon the Anti-apoptotic Activity of BSA

CNBr fragmentation of BSA produced the two fragments with expected molecular masses of 45 kDa and 21 kDa (Figure 4.10). CNBr fragmentation of BSA partially decreased the anti-apoptotic activity for HUVEC at maximal concentrations (600 to 900 μM) (p < 0.01) (Figure 4.11).
Figure 4.2: Photomicrograph of cultured HUVEC. A monolayer of cells with cobblestone morphology is seen, typical of endothelium (Bar = 100 µm).
Figure 4.3: Photomicrographs of cultured HUVEC stained by histochemistry for UEA-1 binding (A) or Factor VIII associated antigen (B) with (i) or without (ii) application of primary label. Nuclei (arrowheads) were counterstained with haematoxylin. Widespread labelling for UEA-1 was seen (arrows) (Ai), while the punctate labelling typical of Factor VIII associated antigen was also seen (arrows) (Bi) and controls were clearly negative for these labels. These data confirmed identity of cultured cells as HUVEC (Bar = 100 μm).
Figure 4.4: Confirmation of apoptosis in serum deprived HUVEC by (A) FACS analysis and (B) DNA gel electrophoresis. (A) FACS analysis on combined adherent and floating populations of serum deprived HUVEC demonstrated the presence of the sub-diploid peak, typical of apoptosis (Apop), as well as the expected G0/G1, S and G2/M populations. In this particular experiment, 16 % of the events recorded were in the sub-diploid population. (B) The ethidium bromide stained agarose DNA electrophoretic gel of floating and adherent cells demonstrated the presence of high molecular mass DNA in adherent cells, indicative of viable cells. This contrasted with a 180 bp DNA ladder (arrows) typical of apoptosis in material from the floating population, while there was little high molecular mass DNA remaining in these cells. These data indicate that cell death induced by serum deprivation was due to apoptosis.
Figure 4.5: Histogram of cultured HUVEC treated for 24 hours with either BCS (20 %), HSA (600 μM) or M199 alone either in the presence (■-) or absence (□-) of the general caspase inhibitor, zVAD-FMK (150 μM). Each data point represents the mean ± standard error from twelve separate replicate experiments using cells from six HUVEC donors. Serum deprivation greatly reduced cell survival in the absence of HSA (p < 0.0001), while HSA abrogated this effect. When zVAD-FMK was present, there was a significant improvement in cell survival in serum free conditions and in the absence of HSA (p < 0.01). Addition of zVAD-FMK to cells in the presence of BCS resulted in a slight increase in cell survival (p < 0.05), while the inhibitor had no detectable effect upon HUVEC in the presence of HSA. These data indicate that HUVEC death in serum free conditions is dependant on caspase activity and also confirm the published observations that HSA inhibits HUVEC apoptosis.
Figure 4.6: Silver stained SDS-PAGE gel of untreated HSA and CNBr fragmented HSA. Bands consistent with native protein (66 kDa) and dimer (130 kDa) were present prior to fragmentation. After fragmentation of HSA with CNBr, both native protein and dimer forms were only faintly visible, however CNBr fragmentation produced three major fragments of the expected molecular masses of 32.5 kDa, 20 kDa and 14 kDa. In addition, an apparent dimer form of the 14 kDa fragment was noted at 28 kDa, while incomplete cleavage and separation of the 14 kDa and 20 kDa fragments may account for a further minor band at 34 kDa.
Figure 4.7: Dose response of protection from apoptosis in cultures of serum free HUVEC treated for up to 24 hours with increasing concentrations of either native HSA (●) or CNBr HSA (□). Maximal protection from apoptosis by native HSA was seen at physiological concentrations (600 – 900 µM). Fragmentation of HSA by CNBr resulted in an approximate 100 fold increase in activity as seen by the left shift in the dose response, with maximal activity of CNBr HSA seen at 2.3 µM (p < 0.01). Similar results were seen in seven separate replicate experiments using cells from five HUVEC donors as well as recombinant HSA.
Figure 4.8: Partial separation of CNBr fragments achieved by protein-dye affinity chromatography (A) and demonstrated by SDS-PAGE (B). (A) Chromatograph of CNBr HSA fragments on a 5 ml HiTrap™ Blue HP column. The column was equilibrated with KH₂PO₄ buffer (50 mM, pH 7.0) at a flow rate of 2 ml/min before application of the sample. Some of the protein did not bind to the column and was eluted in Peak 1. The remainder of the protein eluted as Peaks 2 and 3 with application of a linear gradient of 0 to 4 M KCl in KH₂PO₄ (50 mM, pH 7.0) over 80 ml, followed by isocratic flow with KCl (4 M in KH₂PO₄ 50 mM, pH 7.0) for 25 ml. (B) Coomassie stained SDS-PAGE gel of starting material (HSA), CNBr HSA and eluted protein peaks from protein dye-affinity chromatography. Bands consistent with native protein (66 kDa) and dimer (130 kDa) were present in the HSA starting material sample, while CNBr fragmentation of HSA produced three major fragments of 32.5 kDa, 20 kDa and 14 kDa. Peak 1 from the affinity dye column was mainly the 14 kDa fragment, with an additional band at 28 kDa also seen, representing a dimerised form of the fragment. Peaks 2 and 3 were enriched for the 32.5 kDa and 20 kDa fragments, while some minor bands were also seen (52 kDa).
Figure 4.9: Dose response of protection from apoptosis in cultures of serum deprived HUVEC treated for 24 hours with increasing concentrations of native HSA (○), 14 kDa CNBr HSA fragment (△) or combined 20 kDa and 32.5 kDa CNBr HSA fragments (□). Maximal protection against apoptosis by native HSA was seen at physiological concentrations (600-900 μM), while the 14 kDa fragment was not protective. However, combined 20 kDa and 32.5 kDa fragments retained anti-apoptotic activity and were more active than native material as demonstrated by a left shift in the dose response (p < 0.05). Similar results were seen in five separate replicate experiments using cells from three HUVEC donors. These results reveal that the anti-apoptotic activity is separable into specific fragments and resides in either the 20 kDa or 32.5 kDa fragments but not in the Cys34 containing 14 kDa fragment.
Figure 4.10: Silver stained SDS-PAGE gel of native BSA and CNBr fragmented BSA. Bands consistent with native protein (66 kDa) and dimer (130 kDa) were seen, while fragmentation of BSA by CNBr resulted in two major bands of approximate molecular masses of 45 and 21 kDa. Also present were some higher molecular mass bands which could represent uncleaved material or dimerisation of fragments.
Figure 4.11: Dose response of protection from apoptosis in cultures of serum deprived HUVEC for 24 hours with increasing concentrations of either BSA (○-) or CNBr BSA (□-). Maximal protection from apoptosis by BSA was observed at physiological concentrations (600-900 μM), similar to the dose response for HSA (Figure 4.7). Fragmentation of BSA by CNBr resulted in a partial decrease in activity (p < 0.01). Similar results were seen in seven separate replicate experiments using cells from four HUVEC donors.
Albumin Fragmentation and Anti-apoptotic Activity
Chapter IV Page 44

IV.4. Discussion

This Chapter investigated the possibility that the active anti-apoptotic site of albumin for endothelium is cryptic and exposed by fragmentation. Cultured HUVEC were used as a convenient and widely accepted model to study endothelial function. Isolated cultured HUVEC have the advantage that responses can be studied without interference by other cell types, although it is accepted that cultured HUVEC may lose some specific functions or develop new metabolic characteristics not seen in vivo, (Bachetti and Morbidelli, 2000), while EC of different origins differ in some of their behaviour in culture (Kvietys and Granger, 1997). HUVEC are widely used for study of EC function (Kvietys and Granger, 1997) and are readily recognised by their cobblestone morphology and positive labelling for UEA-1 binding and Factor VIII associated antigen (Jaffe et al., 1973; Holthofer et al., 1982). Importantly, published work on endothelial apoptosis and albumin has primarily involved HUVEC (Zoellner et al., 1996b). For this reason, it seemed reasonable to use HUVEC as the culture model of choice in the current study.

It is widely accepted that serum deprivation of cultured HUVEC results in apoptosis (Araki et al., 1990a, 1990b; Meredith et al., 1993; Hase et al., 1994; Zoellner et al., 1996a; Zoellner et al., 1996b; Emmanuel et al., 2002). In the current study, confirmation of apoptosis was by FACS analysis, DNA gel electrophoresis and involvement of caspase activity in cell death. The sub-diploid DNA peak seen by FACS analysis (Darzynkiewicz et al., 1992) is the result of extensive DNA fragmentation during apoptosis, although this may also occur in necrotic cells (Gerschenson and
Rotello, 1992). During apoptosis, DNA fragmentation occurs at internucleosomal sites while in necrosis DNA is randomly fragmented so that a smear is seen in DNA electrophoretic gels rather than the "ladder" characteristic of apoptosis (Gerschenson and Rotello, 1992). The 180 bp DNA ladder demonstrated by DNA gel electrophoresis of material from serum deprived HUVEC confirmed the apoptotic death of HUVEC in these experiments.

Activation of caspases is thought important during apoptosis (Gerschenson and Rotello, 1992; Zimmermann et al., 2001; Zimmermann and Green, 2001) so that inhibition of caspase activity using the general caspase inhibitor, zVAD-FMK would be expected to protect cultured cells from death if apoptosis is responsible for reduced cell number, and this was seen in the case of serum deprived HUVEC in the current study (Granville et al., 1999; Raymond et al., 2002). Interestingly, in static cultured HUVEC, there is a baseline rate of apoptosis (Araki et al., 1990a; Zoellner et al., 1996b; Kaiser et al., 1997) while in the current study, inhibition of caspases reduced the baseline rate of apoptosis in BCS stimulated cells, but not in HSA stimulated cells. This may indicate that the baseline rate of HUVEC apoptosis observed in the presence of HSA may be mediated through caspase independent mechanisms, although this possibility was not further studied in the current thesis. The presence of a sub-diploid peak in FACS analysis, together with internucleosomal DNA fragmentation, and an effect of caspase inhibition on HUVEC survival confirm that HUVEC death induced by serum deprivation was due to apoptosis and not necrosis.

To investigate the possibility that the active anti-apoptotic site of HSA is cryptic and exposed by fragmentation, HSA was fragmented by CNBr, generating three
fragments with molecular masses of 14 kDa, 20 kDa and 32.5 kDa consistent with previous studies (McMenamy et al., 1971; Doyen et al., 1982; Compagnini et al., 1993). A clear increase in the anti-apoptotic activity of CNBr HSA compared with the native protein was observed, consistent with the presence of a cryptic active anti-apoptotic site. This increase in activity varied between CNBr HSA preparations and ranged from 16 to 100 fold. The reason for variations in activity between CNBr HSA preparations is unclear; however this may be due to variations in the extent of fragmentation between preparations, or perhaps in the relative proportions of different fragments produced.

Partial separation of CNBr HSA fragments was achieved by Cibacron Blue F3GA dye affinity chromatography. Cibacron Blue F3GA dye has a high affinity for HSA and is routinely used to isolate HSA from serum. The binding sites for the dye in HSA have been identified in Domains IIA, IIIA (Compagnini et al., 1996) and in the C-terminal portion of Domain I (Dockal et al., 1999), while no binding sites for Cibacron Blue are present on the 14 kDa fragment (Compagnini et al., 1993). This permitted separation of the 14 kDa fragments from the two larger fragments, while unfortunately a further convincing separation of the 20 kDa and 32.5 kDa fragments was not achieved using this approach. The anti-apoptotic activity of albumin was retained in the 20 kDa and 32.5 kDa fragments, while the 14 kDa fragment was not protective; demonstrating the anti-apoptotic activity of HSA is separable into specific fragments.

CNBr fragmentation of BSA partially reduced the anti-apoptotic activity for endothelium, consistent with previous studies (Zoellner et al., 1996b). Comparison of the amino acid sequences of human and bovine albumin reveals an additional CNBr cleavage site in BSA at Met184 which is not represented in the human protein. It seems
reasonable to suggest that the partial reduction in anti-apoptotic activity of CNBr BSA is
due to disruption of the active site by fragmentation at this residue. Met184 is located in
loop 4 of albumin and corresponds to a region of the protein in the 20 kDa CNBr HSA
fragment. In this way, data are consistent with the active site being in the 20 kDa
fragment and involving loop 4 of the protein.

Further separation of the 20 kDa and 32.5 kDa CNBr HSA fragments with
determination of biological activity is necessary to more certainly identify the active
anti-apoptotic CNBr fragment, while discovery of the specific active site requires further
structural analysis. Albumin binding proteins have been identified in EC (Ghinea et al.,
1988; Schnitzer et al., 1988; Tiruppathi et al., 1996), with the receptors gp18 and gp31
involved in endocytosis and degradation of albumin, and albondin (gp60) mediating
transcytosis of albumin through the endothelium (Schnitzer and Oh, 1994). As the
known receptors have high affinity for native albumin, dose response experiments for
the anti-apoptotic activity in endothelium are inconsistent with such a high affinity site
and appear to exclude a role for albondin and the other known receptors.

Other data from this laboratory indicate loss of anti-apoptotic activity with high
levels of non-enzymatic glycosylation (Zoellner, unpublished data), while published
work reveals greatly reduced intramolecular movement in HSA with equivalent levels of
non-enzymatic glycosylation (Zoellner et al., 2001). Taken together, the data presented
in this Chapter, as well as observations with non-enzymatic glycosylated albumin are
consistent with the idea that the active anti-apoptotic site in albumin is cryptic and
exposed by either transient intramolecular movement or fragmentation.
Chapter V

Exclusion of Radical Scavenging and Contaminant Lipids as Possible Mechanisms for the Anti-apoptotic Activity of Albumin for Endothelium
Non-specific Protein Effects Do Not Account for the Anti-apoptotic Activity
Chapter V Page 1

V.1. Introduction

Previous studies (Zoellner et al., 1996b; Zoellner et al., 1999), as well as data presented in this thesis (Chapter 4), demonstrate the anti-apoptotic activity of serum albumin for endothelium. Other investigations have demonstrated that serum albumin protects chronic lymphocytic leukaemia cells (Moran et al., 2002), macrophages and renal tubular epithelial cells (Iglesias et al., 1999) from apoptosis by scavenging free radicals. Also, some lipids mediate protection of cells from apoptosis (Levine et al., 1997; Koh et al., 1998; Welters et al., 2004; Katsuma et al., 2005), so that it is possible that albumin bound lipids may account for the protective activity for EC. This Chapter investigates these possible non-specific mechanisms for the anti-apoptotic activity of albumin for EC, and the data shown suggests that such mechanisms are not responsible.

V.1.i. Free Radicals and Antioxidants

V.1.i.a Free Radicals

Free radicals are produced as by-products during some metabolic reactions, especially in the mitochondria during respiration (Fernandez-Checa, 2003), while respiratory bursts by neutrophils and macrophages during intracellular killing also generate radicals (Forman and Torres, 2002). Free radicals can act as secondary messengers to initiate pro-inflammatory and growth stimulatory signals (Hensley et al., 2000), although metabolic systems require protection by a number of antioxidant defences to prevent damage to cells and tissues (Fernandez-Checa, 2003). Apoptosis can
Non-specific Protein Effects Do Not Account for the Anti-apoptotic Activity
Chapter V Page 2

also be induced by free radicals (Cantara et al., 2004), while free radicals have been implicated in the development of atherosclerotic lesions (Dimmeler and Zeiher, 2000).

Reactive oxygen species include superoxide (O$_2^{-}$), hydrogen peroxide (H$_2$O$_2$), hypochlorous acid (HOCl) and hydroxyl radicals (OH·) (Hancock et al., 2001). Some enzymes that produce reactive oxygen species include NADPH oxidase and xanthine oxidase (Hancock et al., 2001). Xanthine oxidase catalyses the oxidation of hypoxanthine and xanthine to uric acid, releasing O$_2^{-}$ and H$_2$O$_2$ as by-products (Meneshian and Bulkley, 2002). The xanthine/xanthine oxidase system of free radical generation has been used extensively in many studies to produce exogenous free radicals to investigate the antioxidant properties of proteins (Radi et al., 1991; Dimmeler et al., 1999; Iglesias et al., 1999; Kouoh et al., 1999; Dimmeler and Zeiher, 2000; Moran et al., 2002; Fukuzawa et al., 2005).

V.1.1.6. Antioxidants

Antioxidants are substances that can significantly delay or inhibit the oxidation of other substances or scavenge free radicals directly (Halliwell and Gutteridge, 1990). Important intracellular antioxidants include superoxide dismutase, catalase and glutathione peroxidase. There are also several extracellular antioxidants, including proteins such as lactoferrin, ceruloplasmin which binds metal ions, and haptoglobin which binds free haemoglobin and prevents it from participating in free radical generating reactions (Halliwell and Gutteridge, 1990). Non-protein antioxidants include ascorbate (vitamin C) and α-tocopherol (vitamin E) (Carr et al., 2000).
V.1.ii. Albumin as an Antioxidant

Albumin is an important extracellular antioxidant (Halliwell, 1988; Halliwell and Gutteridge, 1990) and this activity is mainly due to the presence of a free thiol group at Cys34 (Peters, 1996), comprising most of the reactive free thiols in plasma (Carballal et al., 2003). Albumin scavenges reactive oxygen, nitrogen and carbon species and prevents these free radicals from inducing oxidative damage (Soriani et al., 1994; Cha and Kim, 1996; Kouoh et al., 1999; Bourdon et al., 2005). The antioxidant role of Cys34 is demonstrated by the loss of antioxidant activity after blocking of Cys34 by either alkylation or oxidation (Soriani et al., 1994; Iglesias et al., 1999; Bourdon et al., 2005).

Metal ions, such as copper and iron accelerate free radical generating reactions (Halliwell, 1988) and this is inhibited by albumin binding (Halliwell, 1988; Bourdon et al., 1999; Gabaldon, 2002; Gryzunov et al., 2003). Albumin has a strong binding site for copper at the N-terminus (Peters, 1996) and copper bound albumin has reduced redox-cycling activity (Gryzunov et al., 2003). Interestingly, Cys34 plays a role in this because free Cys34 inhibits the redox-cycling activity of bound copper, while blockage of Cys34 by either oxidation or alkylation permits copper to participate in radical generating reactions, despite being bound to albumin, and therefore converts albumin from an antioxidant to a pro-oxidant copper-protein complex (Gryzunov et al., 2003). Other mechanisms by which albumin may act as an antioxidant include increasing intracellular glutathione (Cha and Kim, 1996; Cantin et al., 2000), or acting as a sacrificial antioxidant by site directing radical damage (Halliwell, 1988).

Since the antioxidant activity of albumin has been well described, and radical scavenging by albumin is known to protect a number of non-endothelial types from
apoptosis (Iglesias et al., 1999; Moran et al., 2002), it seems reasonable to consider whether the anti-apoptotic activity of albumin for endothelium is also mediated through these antioxidant activities. Experiments in this Chapter investigate this question.

V.1.iii. Proliferative and Anti-apoptotic Effects of Lipids

Saturated long chain fatty acids induce apoptosis in many cell types, such as EC (Yamagishi et al., 2002; Artwohl et al., 2004) and human mesangial cells (Mishra and Simonson, 2005), while monounsaturated fatty acids are anti-apoptotic for human mesangial cells (Mishra and Simonson, 2005) and pancreatic β-cells (Welters et al., 2004). Other studies show that fatty acids enhance survival of STC-1 cells by inhibition of caspase-3 and also by activation of the MAPK/ERK and PI3K/Akt signalling pathways (Katsuma et al., 2005). Separately, the ability of albumin bound lipids to affect cells is demonstrated by the fact that fatty acids bound to albumin can modulate production of fibronectin by proximal tubular epithelial cells (Arici et al., 2002), while such cells are also protected from apoptosis.

Lysophosphatidic acid (LPA) (1-acryl-glycerol-3-phosphate) is the simplest of all glycerophospholipids and is formed as a precursor to phospholipid biosynthesis (Moolenaar, 1995). Thrombin activation of platelets during blood clotting results in the rapid release of LPA, which then binds to albumin (Eichholtz et al., 1993). LPA stimulates proliferation of many cell types including fibroblasts (Fang et al., 2000), murine renal proximal tubular cells (Levine et al., 1997), and HUVEC (Lee et al., 2000) and is a reported survival factor of macrophages and proximal tubular epithelial cells through the activation of the PI3K/Akt pathway (Levine et al., 1997; Koh et al., 1998).
Non-specific Protein Effects Do Not Account for the Anti-apoptotic Activity
Chapter V Page 5

LPA can also activate the MAPK/ERK pathway to promote proliferation and survival in fibroblasts (Fang et al., 2000) and has been implicated in decreasing EC permeability (Minnear et al., 2001). LPA mediates the majority of its activity through pertussis toxin-sensitive G protein coupled receptors (van Corven et al., 1992).

As LPA binds albumin and has well described proliferative and survival activities for many cell types (Moolenaar, 1995), while fatty acids also affect cell survival and apoptosis, it is important to consider the possible role of bound lipids in the anti-apoptotic activity of albumin for EC. In this Chapter, the effect of delipidation upon the anti-apoptotic activity for endothelium is determined by treating albumin with activated charcoal and confirming delipidation by assessment of conformational changes by fluorometry. In addition, the activity of commercially delipidated albumin was studied.
V.2. Materials and Methods

V.2.i. Materials

Materials for cell culture and confirmation of apoptosis were previously described in IV.2.i. Xanthine, xanthine oxidase, iodoacetamide, 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), acrylamide, fatty acid free HSA as well as all other reagents were obtained from Sigma (St Louis, USA).

V.2.ii. Alkylation of Albumin

Alkylation of the free HSA sulphydryl group Cys34, was performed by treatment of HSA (50 mg/ml) in Tris (1 M, pH 8.5) with iodoacetamide (50 mg/ml) in the dark overnight at 37°C, after which excess iodoacetamide was removed by dialysis (Iglesias et al., 1999). Alkylated HSA was dialysed into M199 with antibiotics by pressure dialysis and total protein concentration was determined by spectrophotometry at 280 nm using a Beckmann DU 640 spectrophotometer (Peters, 1996). Three separate preparations of alkylated HSA were prepared in this way and used in cell culture experiments with similar results.

V.2.iii. Determination of Free Sulphydryl Content of Native and Alkylated Albumin

The free sulphydryl content of HSA before and after alkylation was determined by the reaction of DTNB. 60 μl of native HSA (600 μM) or alkylated HSA (600 μM) was mixed with 60 μl of DTNB solution (10 mM) before incubation at room temperature for 15 minutes. Absorbance of this solution at 412 nm was determined using
Non-specific Protein Effects Do Not Account for the Anti-apoptotic Activity
Chapter V Page 7

a Beckman DU640 Spectrophotometer and free sulphydryl content determined ($e_M = 13,600 \text{ M}^{-1}\text{cm}^{-1}$) (Ellman, 1959).

V.2.iv. Delipidation of Albumin

Albumin was delipidated using activated charcoal as previously described (Chen, 1967). Briefly, HSA (1 g) was dissolved in water (10 ml) before addition of activated charcoal (0.5 g). The pH was lowered to 3.0 by addition of HCl (0.2 M) and the resulting slurry was stirred on ice for 1 hour after which the protein solution was separated from charcoal by centrifugation at 13,000 rpm for 20 minutes at 4°C. The supernatant was collected and filtered before the pH was raised to 7.0 by addition of NaOH (0.2 M). The protein solution was then dialysed into either PBS for fluorometric analysis or M199 with antibiotics for cell culture.

V.2.v. Fluorometric Analysis of Delipidated Albumin

Stock preparations of HSA and delipidated HSA were dialysed into PBS, filter sterilised and adjusted to equivalent protein concentration by dilution with PBS to achieve an identical optical density at 280 nm of 0.763 ± 0.002. Fluorometry was performed using a Perkin Elmer LS 50B luminescence spectrophotometer (Perkin Elmer, Wellesley, USA) at 25°C with a 10 mm x 10 mm quartz cuvette. The slit width for both excitation and emission wavelengths was 5 nm with a scan speed of 1500 nm/min, while 10 fluorometric scans were averaged to obtain data with PBS as a blank.

Protein solution (75 µl) was added to PBS (3 ml) in the quartz cuvette and after mixing, Trp fluorescence was determined by emission scans from 300 to 450 nm using
Non-specific Protein Effects Do Not Account for the Anti-apoptotic Activity
Chapter V Page 8

an excitation wavelength of 295 nm. This wavelength was selected to minimise the contribution of Phe residues to readings (Zoellner et al., 2001). Trp fluorescence quenching was studied by measuring the change in maximum Trp fluorescence after addition of progressive 10 µl aliquots of acrylamide (6 M in PBS). Correction factors were calculated to eliminate the effect of dilution and internal filter effects (Zoellner et al., 2001) for the construction of Stern-Volmer plots, in which the ratio of the fluorescence in the absence of acrylamide relative to the fluorescence in the presence of acrylamide was plotted against acrylamide concentration (Eftink and Ghiron, 1981; Zoellner et al., 2001).

Accessibility to hydrophobic domains was determined using 4-4′-bis (1-anilinonaphthalene-8-sulfonate) (bis-ANS), a small organic compound which fluoresces only when associated with hydrophobic materials (Rosen and Weber, 1969). Protein solutions were added to PBS as above, with bis-ANS (1 µl, 0.125 mM in PBS) and fluorescence determined by emission scans from 400 to 550 nm using an excitation wavelength of 385 nm.

V.2.vi. Culture and Conditions for Studying HUVEC Apoptosis

HUVEC were isolated and cultured as described in IV.2.ii and IV.2.iii, while an approach similar to that described in IV.2.v was used for quantitation and analysis of apoptosis. Briefly, HUVEC at confluence were seeded into gelatin-coated (0.1 % in PBS) 24 well tissue culture plates before treatment for 24 hours with BCS (20 %), M199 alone, HSA, delipidated HSA or alkylated HSA at increasing concentrations ranging from 0.6 µM to 900 µM. Surviving adherent cells at 24 hours were quantitated by
release with trypsin/EDTA and counting using a haemocytometer and in most cases data expressed using the apoptosis protection index (IV.2.v), although in time course experiments, data were expressed as the percentage of cells surviving relative to 0 hour.

V.2.vii. Generation of Free Radicals Using Xanthine and Xanthine Oxidase

Xanthine oxidase catalyses the oxidation of xanthine and hypoxanthine to uric acid producing free radicals as by-products, including superoxide and hydrogen peroxide (Meneshian and Bulkley, 2002). This system has been used extensively in other cellular systems (Radi et al., 1991; Dimmel er et al., 1999; Iglesias et al., 1999; Kouoh et al., 1999; Dimmel er and Zeiher, 2000; Moran et al., 2002; Fukuzawa et al., 2005) and was used in the current study to probe the antioxidant activity of albumin. The activity of xanthine oxidase was confirmed by assessment of uric acid production (Radi et al., 1991). Briefly, stock concentrations of xanthine (287 mM in 1 M NaOH) and xanthine oxidase (5 U/ml) were prepared. Xanthine was diluted in PBS to obtain a final concentration of 0.8 mM before incubation either in the presence or absence of xanthine oxidase (10 mU/ml in PBS) for up to 60 minutes and production of uric acid measured by optical density at 308.5 nm ($\varepsilon_M = 2.750 \text{ M}^{-1}\text{cm}^{-1}$).

The optimum concentration of xanthine oxidase for cell culture was determined in dose response experiments in which cultured HUVEC were treated with increasing concentrations of xanthine oxidase (0.016 to 40 mU/ml) at a constant level of xanthine (0.8 mM) for 24 hours in the presence or absence of HSA (600 μM). The optimum level of xanthine oxidase of 10 mU/ml was chosen on the basis that HSA was demonstrably protective relative to culture in the absence of the protein.
V.2.viii. Confirmation of HUVEC Apoptosis

Apoptosis of HUVEC stimulated with HSA (600 μM) or M199 either in the presence or absence of free radicals was confirmed on the basis of a sub-diploid apoptotic peak as assessed by FACS analysis, while internucleosomal DNA fragmentation was demonstrated by DNA gel electrophoresis as previously described (IV.2.vi).

V.2.ix. Statistical Analysis

Data was expressed as means ± standard deviation. Statistical significance from observations within individual experiments was assessed using the two tailed Students T-Test, while data over at least six replicate experiments were assessed by Wilcoxon’s matched pair signed rank test. $P$ values of $<0.05$ were considered significant.
V.3. Results

V.3.i. HSA Inhibited Free Radical Induced HUVEC Apoptosis

Xanthine oxidase activity was confirmed by measuring the production of uric acid, as seen in Figure 5.1. Uric acid production was catalysed by xanthine oxidase with xanthine, demonstrating activity of the enzyme, while $O_2^-$ and $H_2O_2$ are known products of this reaction.

Dose response experiments with cultured HUVEC were performed to determine the optimum concentration of xanthine oxidase for use in further experiments (Figure 5.2). Concentrations of xanthine oxidase up to 2.5 mU/ml did not have any effect upon cell survival, regardless of the presence or absence of HSA (600 μM). However, at concentrations of 10 mU/ml there was a significant reduction in cell survival in the absence of HSA, while HSA inhibited this ($p < 0.0001$). The ability of HSA to protect HUVEC from free radical induced death was partially decreased when concentrations of xanthine oxidase were increased to 40 mU/ml ($p < 0.0001$), while no surviving cells were detected in the absence of HSA at this concentration ($p < 0.001$). This data demonstrated the ability of HSA to act as a protective antioxidant for HUVEC and also identified the optimum concentration of xanthine oxidase (10 mU/ml) for further experiments.

It was important to confirm that cell death induced by exogenously generated free radicals was due to apoptosis and not necrosis. The subdiploid peak expected for apoptotic cells was observed by FACS analysis in all conditions studied (Figure 5.3A), although this was greatly increased in cells deprived of both HSA and serum (bi). Free
Non-specific Protein Effects Do Not Account for the Anti-apoptotic Activity
Chapter V Page 12

deadicals increased the magnitude of the sub-diploid peak significantly in the absence of HSA (bii), but had no clear effect when HSA was included (aii). As seen in Figure 5.3B, adherent cells from all conditions displayed high molecular mass DNA, indicative of non-apoptotic cells. Detached floating cells, however, had minimal high molecular mass DNA and instead a 180 bp DNA ladder was seen, characteristic of apoptotic internucleosomal DNA fragmentation. These data confirm that death in the absence of HSA, or in the presence of free radicals was apoptotic rather than due to necrosis.

Further characterisation of the antioxidant activity of HSA was performed by investigating the time course of apoptosis over 24 hours (Figure 5.4). A baseline rate of apoptosis was observed for each of the protective media BCS (20 %), and HSA (600 μM), both in the presence and absence of free radicals. HUVEC treated with culture media M199 alone in the absence of free radicals had significantly reduced cell survival by 24 hours, as compared with BCS and HSA (p < 0.001), with the majority of HUVEC lost in the first 4 hours of the experiment. The presence of free radicals resulted in a further reduction in cell survival, compared with M199 alone in the absence of free radicals (p < 0.0001).

V.3.ii. Free Radicals Reduced Endothelial Survival but Alkylation of the Free Thiol Did Not Affect the Anti-apoptotic Activity of HSA for Endothelium

Free sulfhydryl content of native HSA was determined as 3.96 ± 0.33 μM/g HSA, while alkylation of HSA resulted in a 62 % reduction in free sulfhydryl content, as 1.51 ± 0.24 μM/g HSA was detected after alkylation. Figure 5.5 shows phase-contrast photomicrographs of HUVEC monolayers stimulated with HSA, alkylated HSA or
Non-specific Protein Effects Do Not Account for the Anti-apoptotic Activity
Chapter V Page 13

M199 in the presence or absence of xanthine/xanthine oxidase generated free radicals. HSA (Ai) and alkylated HSA (Bi) (600 μM) were protective compared with M199 alone (Ci), however when free radicals were included, monolayer integrity was greatly reduced in the absence of albumin (Cii), while HSA was comparatively protective (Aii), however, alkylated HSA was ineffective in protecting from free radical induced cell death (Bii).

Quantitation of cell survival (Figure 5.6) confirmed the impression from morphological observations, in that there was significantly reduced HUVEC survival after exposure to free radicals in albumin free medium (p < 0.02). Nonetheless, inclusion of HSA (600 μM) protected HUVEC from the damaging effect of free radicals generated by xanthine/xanthine oxidase (p < 0.02). When alkylated HSA was present in the absence of free radicals, no significant difference was observed in protection of HUVEC as compared with native HSA. However, when free radicals were included, there was a significant reduction in protective activity of alkylated HSA as compared with HSA alone (p < 0.05). These data indicate a critical role of Cys34 in protecting HUVEC from exogenously generated radicals, but also reveal that Cys34 does not contribute to protection of HUVEC in the absence of exogenously added radicals.

To further characterise these changes, dose response experiments were performed as shown in Figure 5.7. Alkylation of the free thiol group of HSA did not change the anti-apoptotic activity for HUVEC in the absence of free radicals. However, upon exposure to free radicals, there was partial reduction in the protective activity of alkylated HSA at maximal concentrations (600 to 900 μM) (p < 0.05), with complete loss of activity at concentrations below 150 μM. For HUVEC treated with native HSA,
maximal protection from free radical damage was observed at 600 to 900 μM, while when concentrations of HSA were reduced four-fold to 150 μM, there was a partial reduction in protective activity (p < 0.05), with complete loss of protective activity by 37.5 μM.

These data indicate that there are at least two separate protective activities in HSA for endothelium, one which is mediated by the Cys34 dependant scavenging of radicals, and another which is independent of radical scavenging.

V.3.iii. Delipidation of Albumin Did Not Affect the Anti-apoptotic Activity for Endothelium

Fluorometric studies were performed to detect likely conformational change after delipidation. Emission fluorescence scans of HSA and delipidated HSA demonstrated increased Trp fluorescence after delipidation (Figure 5.8A), indicating increased access to Trp by activating light. There was also increased accessibility to hydrophobic domains after delipidation, as assessed by bis-ANS fluorescence (Figure 5.8B), indicating the expected increase in accessibility of hydrophobic domains following the removal of previously bound lipids. However, Stern-Volmer plots for acrylamide quenching (Figure 5.8C) demonstrated no effect upon the accessibility of acrylamide to Trp residues following delipidation, consistent with the suggestion that lipid binding has little effect upon internal mobility of the protein. Figure 5.8D demonstrates that despite the minor effect of delipidation upon conformation of HSA, there was no effect upon the anti-apoptotic activity for endothelium. Similar results were observed in further experiments using commercially delipidated HSA, confirming these observations (data
Non-specific Protein Effects Do Not Account for the Anti-apoptotic Activity
Chapter V Page 15

not shown). These data indicate that although some conformational changes occurred with delipidation, the anti-apoptotic activity of albumin for endothelium is independent of bound lipids.